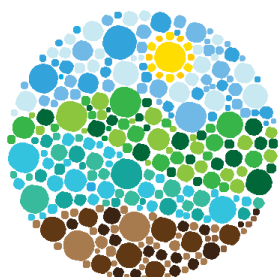


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**JUNE 5th – WORLD
ENVIRONMENT DAY**

BOOK OF PROCEEDINGS

05 - 07 June, 2024, Bihać, Bosnia and Herzegovina

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Eighth International Scientific Conference
"June 5th - World Environment Day"

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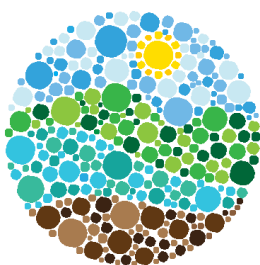
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Bihać, June 2024.

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"June 5th - World Environment Day"

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CONTENTS:

ECOSYSTEM PROTECTION

1. IMPORTANCE OF WETLANDS – RAMSAR SITE BARDAČA, CASE STUDY 1 - 6
Vera Nikolić, Rajko Roljić, Dragana Šnjegota
2. ECO - FRIENDLY PRACTICES IN FURNITURE 7 - 15
Selma Mujanić, Dejlja Ramić, Nejra Alibašić
3. MAJOR BIOTIC & ABIOTIC STRESSES IN PLANTS = AN ENVIRONMENTAL
PERSPECTIVE 16 - 36
Elif Çatıkkaş, Hakan Ulukan
4. CONTEMPORARY APPROACHES TO COMBAT INVASIVE ALIEN SPECIES 37 - 44
Mirjana Bartula, Ivana Šekler, Slobodan Stefanović, Slobodanka Pavlović
5. PREVENTING BIODIVERSITY LOSS: RESTORING WETLANDS WITH THE HELP OF
'EGG BANK' - THE CASE OF THE RAMSAR SITE "BARDAČA WETLAND" (BOSNIA
AND HERZEGOVINA) 45 - 51
Dragana Miličić, Dejan Dmitrović, Goran Šukalo
6. BIOAVAILABILITY OF LEAD IN SOIL SOLUTION 52 - 58
Husejin Keran, Amra Odobašić, Indira Šestan, Sead Ćatić, Melisa Ahmetović
7. THE AESTHETIC POTENTIAL OF THE LEDENICA CAVE AS A FACTOR IN THE
PROTECTION AND VALUATION OF THE KARST ECOSYSTEM 59 - 70
Svjetlana Kodžo, Slobodanka Pavlović

ENVIRONMENT, NUTRITION AND HEALTH

1. THE IMPACT OF GENETIC FACTOR ON INDIVIDUAL NUTRITIONAL NEEDS 72 - 81
Huska Jukić, Amina Seferagić, Azira Hrnjica, Sara Redžić, Samira Dedić
2. HEAVY METALS CONTAMINATION IN URBAN SOIL OF LUKAVAC AND NON-
CARCINOGENIC AND CARCINOGENIC HUMAN HEALTH RISK ASSESSMENT ... 82 - 93
Abdel Đozić, Hana Alihodžić, Indira Šestan, Halid Junuzović, Melisa Ahmetović
3. PHYTOACCUMULATION OF CADMIUM AND LEAD BY MINT (lat. *Mentha piperita* L.)
Minela Čejvan, Vedran Stuhli, Jasmina Ibrahimpašić, Ekrem Pehlić 94 - 104
4. CATEGORIZATION OF PATIENTS AND IDENTIFICATION OF HEALTH CARE NEEDS
Sena Našić, Sulejman Kendić, Alen Lonić 105 - 111
5. BENEFITS OF USING FRESH CHOKEBERRY AND CHOKEBERRY PRODUCTS 112 - 129
Aleksandra Šupljeglav Jukić, Jasna Hasanbegović, Lejla Škaljić, Semina Hadžibulić

Eighth International Scientific Conference
"June 5th - World Environment Day"

6. INHIBITION OF CHOLINESTERASE AND α -GLUCOSIDASE ENZYMES BY WATER EXTRACTS OF FIVE *CENTAUREA* SPECIES 130 - 139
Olivera Politeo, Kristina Kardum, Mirko Ruščić, Mejra Bektašević
7. COMPARATIVE ANALYSIS OF ANTIBACTERIAL EFFECT OF CRANBERRY TEA VS CRANBERRY FRUIT ON GRAM-NEGATIVE BACTERIA USING AGAR WELL METHOD
Ena Konjalić, Elida Avdić, Amna Moro, Aja Borić and Irma Mahmutović- Dizdarević 14 - 145
8. MORPHOLOGICAL CHARACTERISTICS OF CORNELIAN CHERRY GENOTYPES (*Cornus mas* L.) FROM THE KONJIC AREA 146 - 158
Semina Hadžiabulić, Jasna Hasanbegović, Jasmina Aliman, Azra Skender, Dinko Bećirspahić
9. EFFECT OF FOSFOMYCIN ON THE RESISTANCE PATTERNS OF GRAM-NEGATIVE BACTERIA TO DIFFERENT CLASSES OF ANTIBIOTICS USING THE BAUER-KIRBY DISK DIFFUSION METHOD 159 - 166
Ilderina Jusufović, Elida Avdić, Lamija Vrtić, Samra Međedović
10. AN OVERVIEW OF VARIATIONS IN SYNTHETIC APPROACHES FOR THE PREPARATION OF (BENZ)IMIDAZOLIUM SALTS: BIOACTIVITY AND GREEN SOLVENT PERSPECTIVES 167 - 176
Enis Šuta, Sead Ljubijankić, Nevzeta Ljubijankić
11. METAL CHELATING CAPACITY OF MELATONIN PRE-TREATED LEMON BALM AND VALERIAN PLANTS 177 - 185
Hodžić Elvisa, Rekanović Sebila, Vuković Anita, Bećirspahić Dinko
12. AIR QUALITY COMPARISON IN UNA-SANA, ZENICA-DOBOJ AND TUZLA CANTON FROM 2020 TO 2023 186 - 195
Minela Čejvan, Majda Huskić, Selma Prošić, Zlata Ibrišimović-Subašić
13. DANGERS FROM CONSTRUCTION A RADIOACTIVE WASTE STORAGE CENTRE AT THE ČERKEZOVAC SITE, TRGOVSKA GORA, THE REPUBLIC OF CROATIA 196 - 211
Draženko Bjelić, Borislav N. Malinović, Tijana Đuričić
14. EFFECT OF FOSFOMYCIN ON BIOFILM FORMATION IN GRAM-NEGATIVE BACTERIA USING MICROBROTH DILUTION AND TISSUE CULTURE PLATE METHODS 212 - 221
Emina Pramenković, Hata Džino, Samra Međedović
15. EFFECT OF FOSFOMYCIN ON THE RESISTANCE PATTERNS OF GRAM-POSITIVE BACTERIA TO DIFFERENT CLASSES OF ANTIBIOTICS USING THE BAUER-KIRBY DISK DIFFUSION METHOD 222 - 229
Ilderina Jusufović, Elida Avdić, Lamija Delić, Samra Međedović
16. EFFECT OF FOSFOMYCIN ON BIOFILM FORMATION IN GRAM-POSITIVE BACTERIA USING MICROBROTH DILUTION AND TISSUE CULTURE PLATE METHODS 230 - 240
Aida Lavić, Hana Brekalo, Irma Mahmutović-Dizdarević

Eighth International Scientific Conference
"June 5th - World Environment Day"

17. MALACHITE GREEN ADSORPTION BY *AMORPHA FRUTICOSA* L. ACTIVATED CARBON 241 - 246
Ines Cindrić, Lidija Jašić, Elizabeta Zandona, Marijana Blažić
18. MONITORING OF CLIMATE ELEMENTS AND AIR QUALITY INDEX IN THE CITY OF BIHAC IN 2023 247 - 252
Asmir Aldžić, Aldina Baltić, Jasmin Toromanović, Emra Toromanović

SUSTAINABLE ANIMAL AND PLANT PRODUCTION

1. INFLUENCE OF CULTIVATION SYSTEM ON STRAWBERRY QUALITY AND YIELD .. 254 - 263
Dinko Bećirspahić, Azra Skender, Zineta Vuković, Semina Hadžiabulić, Jasna Hasanbegović
2. LECULAR DETECTION AND IDENTIFICATION OF SELECTED ISOLATES *ERNARIA* SPP. Mehira Perviz, Vojislav Trkulja, Osman Perviz 264 - 271
3. A CONTRIBUTION TO THE KNOWLEDGE OF THE REPRODUCTIVE BEHAVIOR OF CATTLE AND SHEEP AS A PARAMETER OF SUSTAINABILITY IN ANIMAL PRODUCTION 272 - 285
Husein Vilić, Refik Šahinović
4. SUSTAINABLE FOOD SYSTEMS AS PART OF THE GREEN DEAL 286 - 295
Huska Jukić, Azira Hrnjica, Suad Habeš, Aida Džaferović
5. EFFECT OF VERMICOMPOST ON ROOTING OF *NERIUM OLEANDER* L. CUTTINGS Alisa Hadžiabulić, Elma Temim, Semina Hadžiabulić, Jasmina Aliman, Dženan Vukotić 296 -302
6. INFLUENCE OF LAYER HEN FEEDING ON EGG QUALITY CHARACTERISTICS 303 - 316
Suzana Jahić, Sebila Rekanović, Samira Hotić
7. CONCENTRATION OF VITAMIN C IN LEAVES OF LIMEIRA LETTUCE DEPENDING ON DEVELOPMENT PHASE 317 - 325
Vildana Jogic., Jelena Nikitović., Merima Toromanović., Subha Avdić
8. POWDERY MILDEW OF BULBOUS WILD BARLEY (*Hordeum bulbosum*) IN BİNGÖL UNIVERSITY CAMPUS AREA, TÜRKİYE 326 - 330
İşıl Saraç Sivrikaya, Arzu Çelik Oğuz, Aziz Karakaya

FOREST MANAGEMENT

1. COMPARATIVE ANALYSIS OF SOIL QUALITY FOR BEECH GROWTH IN THE AREAS OF NATIONAL PARKS KOZARA AND SUTJESKA, BASED ON TAKSATION DATA 332-341
Mirsad Ičanović, Đorđe Topić, Mihajlo Marković, Husnija Kudić
2. ENVIRONMENTAL IMPLICATIONS OF WOOD MODIFICATION PROCESSES .. 342 - 353
Redžo Hasanagić, Selma Mujanić, Asmir Suljadžić, Damir Hodžić

Eighth International Scientific Conference
"June 5th - World Environment Day"

3. COMPARATIVE PHYSICAL CHARACTERISTICS OF WOOD SPECIES FROM ZAGROS FORESTS 354 - 357
Redžo Hasangić, Leila Fathi, Selma Mujanić, Mostafa Sefidrouh, Mohsen Bahmani
4. THE INFLUENCE OF NATURAL DISTURBANCES ON THE HARVEST PLAN AND TIMBER PRODUCTION FROM MOUNTAINOUS FORESTS 358 - 369
Igor Stankić, Zlatan Lonić, Sara Bašanović, Vanja Jurišić

WASTE MANAGEMENT

1. ANALYSIS OF SOIL COMPOSITION NEAR THE CITY WASTE LANDFILL 371 - 378
Ajla Japić, Merima Toromanović
2. THE ROLE OF DIGESTATE IN THE CIRCULAR ECONOMY 379 - 391
Mihaela Grubišić Šeba, Marin Miletić, Igor Stankić
3. QUANTIFICATION OF THE ENVIRONMENTAL BENEFITS OF THE INTRODUCTION AND IMPLEMENTATION OF THE ISO 14001 STANDARD IN THE MEAT INDUSTRY SHOWN BY MONITORING THE CONCENTRATIONS OF PHOSPHORUS AND SULFUR COMPOUNDS IN WASTEWATER 392 - 398
Faris Muminović, Halid Makić, Jasmina Ibrahimpašić, Husejin Keran, Amela Semić, Toni Babić
4. SPATIAL-PLANNING ASPECT OF WASTE MANAGEMENT IN VARAŽDIN COUNTY
Melita Srpak, Silvija Zeman, Vladimir Križaić, Darko Pavlović 399 - 411
5. OPTIMIZATION OF THE PROBLEM OF THE LOCATION OF TRANSFER STATIONS IN THE SYSTEM OF INTEGRAL WASTE MANAGEMENT 412 - 423
Fatka Kulenović

ENERGY EFFICIENCY AND RENEWABLE ENERGY SOURCES

1. A CONSTRUCTED WETLAND FOR WASTEWATER TREATMENT AS A PRODUCT
Sabina Bišćević 425 - 431
2. ASSESSMENT OF *ARUNDO DONAX* IN A CONTINUOUS BIOGAS PRODUCTION SYSTEM 432 - 439
Jurišić Vanja, Matin Ana, Brandić Ivan, Tomić Ivana, Stankić Igor, Špelić Karlo

LEGAL AND ECONOMIC REGULATIONS IN ENVIRONMENTAL PROTECTION

1. THE MECHANISM OF CROSS-BORDER SETTLEMENT OF CARBON EMISSIONS AND ITS IMPORTANCE IN THE EUROPEAN GREEN DEAL 441 - 449
Ermin Bajramović, Emir Bajramović
2. COUNCIL OF EUROPE AND ENVIRONMENTAL PROTECTION 450 - 458
Nikola Findrik, Suan Islamović

Eighth International Scientific Conference
"June 5th - World Environment Day"

3. CRIMINAL OFFENSES AGAINST THE ENVIRONMENT IN THE REPUBLIC OF SERBIA
Ivana Šekler, Mirjana Bartula, Slobodan Stefanović, Nikola Petrić 459 - 469
4. THE IMPACT OF TRAFFIC NOISE IN THE UNA-SANA CANTON 470 - 477
Medina Mešić
5. UNDERSTANDING SOUVENIRS IN TOURIST CONSUMPTION OF EU TOURISTS IN
BOSNIA AND HERZEGOVINA 478 - 489
Ćatić Berina, Berberović Denis, Makić Ikbala

ECOSYSTEM PROTECTION



IMPORTANCE OF WETLANDS – RAMSAR SITE BARDAČA, CASE STUDY

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Key words: Ramsar site, Bardača, degradation, wetland

ABSTRACT:

Wetlands are among the most threatened habitats in the world. Wetlands are referred to as “biological supersystems” because they produce large amounts of food that support a remarkable level of biodiversity. In terms of the number and variety of species they harbor, they are as rich as rainforests and coral reefs.

Wetlands are being degraded and destroyed faster than any other ecosystem on earth. Many important functions and values that wetlands provide have already been lost. Since the Ramsar Convention on Wetlands, more and more countries around the world have recognized the critical benefits of restoring and creating wetlands.

Bosnia and Herzegovina ratified the Convention on Wetlands of International Importance, especially as Waterfowl Habitat, better known as the Ramsar Convention, in 1992. Since then, three wetlands have been designated in Bosnia and Herzegovina under this convention: Bardača, Hutovo blato and Livanjsko polje, of which only Hutovo blato Nature Park – has the status of a protected area in Bosnia and Herzegovina.

Bardaca Wetland (Bardača-močvarni kompleks). 02/02/07; 3,500 ha; 45°06'N 017°27'E, important bird sanctuary. The area is located in the floodplain of the Sava River near the border with Croatia. In addition to the natural features mentioned above, it should be emphasized that in the last 50 years, anthropogenic influence has ensured the survival and development of the ecosystem on the one hand and worsened it on the other. This conflict between the various spatial influences is still present today. After privatization in 2012, nine out of 11 lakes were drained and fish farming was replaced by corn cultivation. If something does not change soon, Bardača could permanently lose its natural and cultural values as well as its status as an

internationally important wetland. This shows that the responsible institutions do not have the power and influence to preserve our only international wetland.

1. INTRODUCTION

Reports by the Intergovernmental Panel on Climate Change (IPCC) indicate that climate change is progressing even faster than expected. Climate change is directly affecting wetlands of international importance, both through a net loss of wetlands within areas due to drying (Xi et al. 2021), desertification and coastal erosion, and through multiple impacts on associated biodiversity and ecosystem services (Harrison et al. 2018). Wetlands are among the most threatened habitats in the world. Wetlands are referred to as “biological supersystems” because they produce large amounts of food that support a remarkable level of biodiversity. In terms of the number and variety of species they harbor, they are as rich as rainforests and coral reefs. (Davidson et al, 2020).

1.1 Wetland Characteristics

As defined by the U.S. Fish and Wildlife Service, wetlands are “areas in transition between terrestrial and aquatic systems where the water table is normally at or near the surface or the land is covered by shallow water” (Suloway and Hubbell, 1994). Basically, it is an area that is both land and water. A wetland does not necessarily have to be “wet” all year round, but can also be characterized by periodic flooding of a particular area (Marble, 1992). wetlands can be fed at the surface by flowing water, such as rivers or streams, or by rainwater runoff. They can also be fed from below by a high groundwater table. As the problem of climate change has intensified in recent years, the function of wetlands in controlling carbon dioxide is emerging as a new solution to climate change (Mitsch et al., 2013). Wetlands are unique terrestrial and aquatic ecosystems (Cowardin, 1979) that provide important habitats and resources for animals and plants.

1.2 Ramsar Convention and Bardača Ramsar site

The Bardača wetland complex itself covers about 3,500m². This is a unique wetland ecosystem in Bosnia and Herzegovina. Complex of the fishpond Bardača is one of two IBAs (Important Birds Areas) areas in Bosnia and Herzegovina. Thanks to the registered large diversity of the birds, this area was designated in 1969 as a protected area and finally on the 2nd February 2007, World Wetland Day, Bardača was officially designated as a Ramsar site, no. 1658. The location and influence of the Sava and Vrbas rivers had a direct impact on the formation of the geological structure and were also reflected in the pedological features that favoured the development of flora and fauna in this area. The complex terrain structure also determines the hydrological characteristics, which determine the hydrological regime important for the development of the wetland habitat. According to the data collected, more than 200 bird species have been recorded

Eighth International Scientific Conference "June 5th - World Environment Day"

in Bardača in the previous ornithofauna research period. The fish fauna is very important as a food source for birds, whether they are migrating or permanently presented. The Bardača wetland consists of 10 ha of natural marshes and bogs and 657 ha of natural and artificial fish ponds. It was a natural wetland, an important habitat for wildlife (including endemic species) and a stopover for migratory birds. The restoration of this sensitive ecosystem is urgently needed to restore biodiversity, attract the interest of scientists and tourists and to promote the economic development of the area.

The Bardača area is a unique ecosystem characterized by the interaction of many natural and anthropogenic factors. The location and influence of the Sava and Vrbas rivers had a direct impact on the formation of the geological structure and are also reflected in the pedological features that favored the development of flora and fauna in this area. The complex terrain structure also dictates the hydrological characteristics that have determined the hydrological regime important for the development of the marsh habitat. In addition to the natural features mentioned above, it must be emphasized that the anthropogenic influence over the last 50 years has ensured the survival and development of the ecosystem on the one hand, but has also degraded it on the other. This conflict between the various influences of outer space is still present today. The construction of protective dams on the Sava and Vrbas rivers, the largest rivers associated with the formation of this area, prevented natural flooding in the area, resulting in a lack of water or moisture. Floods were necessary for the development of the wetland ecosystem. For the benefit of other parts of Lijevo polje, a whole network of canals was built to drain the entire area and gravity drain the water into the Bardača, the lowest part of Lijevo polje. The Convention on Wetlands is an intergovernmental treaty adopted in the Iranian city of Ramsar on February 2, 1971 (hence the common name "Ramsar Convention"). It is the first of the modern global intergovernmental treaties on the conservation and sustainable use of natural resources. As of May 2015, 169 countries from around the world have ratified the treaty. Although the treaty was developed in a very different time in world history, the decisions of the triennial Conference of the Parties have succeeded in further developing and interpreting the general guidelines of the treaty and making the Ramsar Convention a pioneer in all matters relating to wetlands.

An important instrument of the Convention is the List of Wetlands of International Importance (the "Ramsar List"). All Parties to the Convention are obliged to include at least one site on the list that meets the criteria set by the Conference of the Parties. There are currently over 2,400 Ramsar sites around the world.

Bosnia and Herzegovina ratified the Convention on Wetlands of International Importance, especially as Waterfowl Habitat, better known as the Ramsar Convention, in 1992. Since then, three wetlands have been designated in Bosnia and Herzegovina under this convention: Bardača,

Hutovo blato and Livanjsko polje, of which only Hutovo blato Nature Park – has the status of a protected area in Bosnia and Herzegovina.

1.3 Ecosystem of Bardača and proposal of the protection measures

The central part of Bardača was transformed into a pond for cyprinids at the beginning of the last century due to anthropogenic activities. Today it comprises 11 basins and additional fish ponds (canal net, cassettes for rearing juvenile fish, cassettes for overwintering juvenile fish, etc.), which are necessary for its functioning. This ecosystem has been affected by the construction of fish ponds in the area, as well as by the implementation of numerous agrotechnical measures, drainage, etc. Despite the constant anthropogenic activities, Bardača has largely preserved its authentic appearance and the composition of flora and fauna typical of this area. After the ownership of the fishpond was privatized, a new era began for Bardača. With the aim of improving fish production, unplanned and unreasonable measures are being carried out throughout the area, leading to the destruction of different types of habitats and the natural balance of ecosystems. By cutting down trees, clearing scrub and bushes, destroying macrophyte vegetation and killing birds, for whose richness this area was known not only among scientists but also among the public, the entire area began to lose its natural appearance and ecological importance not so long ago.

Human actions and climate change have severely degraded this Ramsar site, risking to lose its global significance and status. Our project aims to assess biodiversity, water quality, and climate effects, and raise local community awareness for ecosystem restoration. Furthermore, it aims to create an action plan proposing the concrete actions for the same purpose. Despite the challenges, there is hope.

2. MATERIAL AND METHODS

Exploring biodiversity, water, and soil quality, and climate impact – will be conducted using different model organisms and by comparing newly produced results with existing literature to discern the current state and the impacts of human activity and climate fluctuations. Employing the different model organisms like fish, birds, and mammals, and diverse methodologies such as photo-traps, drones, and transect methods will allow to assess the biodiversity richness in this devastated ecosystem.

3. RESULTS

The DaWetRest Horizon project aims to conserve and restore wetland ecosystems by employing various activities such as biodiversity assessments, habitat restoration, community engagement, and collaboration with local stakeholders and institutions and offers an excellent opportunity to

Eighth International Scientific Conference "June 5th - World Environment Day"

help in Bardača rehabilitation, by simply following and accomplishing its main objectives. By implementing all these objectives, we may do a lot for the long-term sustainability of Bardača wetland. By participating in the DaWetRest project we are in position to preserve natural habitats and biodiversity and to contribute to the conservation of wetland ecosystems worldwide; include and motivate local residents to participate and play an active and important role in protecting and restoring their natural environment, fostering a sense of responsibility in their community; learn and additionally develop skills on wetland ecology, biodiversity assessment techniques, restoration practices, and community engagement strategies; create a platform for individuals from diverse backgrounds, including scientists, researchers, local residents, and NGOs, to collaborate and network, fostering relationships and partnerships for future conservation efforts; make a personal positive impact on the environment; enable the long-term sustainability of wetland ecosystems, ensuring that future generations can continue to benefit from these valuable natural resources.

4. DISCUSSION

Wetlands are the living space for many (protected) species as well as migratory birds and are crucial in their role of providing habitats and water-related ecosystem services. Erosion control, sediment transport, water filtration and regulation are a few of the many valuable services delivered by this ecosystem. In recent times, the role of healthy wetlands in tackling climate change (mitigation, adaptation, resilience) has been emphasized, namely their capacity to capture and store carbon and so reduce atmospheric greenhouse gases, and provide better resilience to hazards such as flooding, storm surges and coastal inundation. The degraded condition of wetlands in Europe is influenced by several factors related mainly to a) the lack of a comprehensive European policy framework targeting these ecosystems in a consistent manner, b) the misdefinition and mis-representation of wetlands in different classification systems, and c) the low socio-cultural valuation of these ecosystems, facilitating changes in land uses. At a socio-cultural and economic level, European wetlands have been historically considered as low productive land that should be subject to dryland forms of cultivation. This profit-driven approach to wetland resources (for example peat extraction, or dryland agriculture) has overlooked the biological, hydro-ecological and socio-economic values and status of wetlands, which as a consequence has degraded this ecosystem, altering its functioning and other undervalued services it provides to people. In view of the major problems facing the Bardaca Ramsar site, it is necessary to revitalize it as soon as possible. As project participants, we hope to gain knowledge, experience and the necessary financial resources for the planned activities that would lead to the improvement of the quality of this area in all respects, the restoration of biodiversity, opportunities for tourism and raising awareness of the importance of the Ramsar site, the only site in Bosnia and Herzegovina located on the Sava River.

5. CONCLUSIONS

Human actions and climate change have severely degraded this Ramsar site, risking to lose its global significance and status. Our effort should be to assess biodiversity, water quality, and climate effects, and raise local community awareness for ecosystem restoration. Furthermore, it is necessary to create an action plan proposing the concrete actions for the same purpose. Despite the challenges, there is hope. Local non-governmental organizations have already initiated restoration initiatives aimed at rejuvenating Bardača's wetland ecosystem.

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ECO - FRIENDLY PRACTICES IN FURNITURE

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Key words: eco-friendly, natural resources, innovative materials

ABSTRACT:

Environmentally friendly furniture practices have become a major concern in modern times, due to their profound implications for environmental sustainability. The term "environmentally friendly" means products or practices that are not harmful to the environment and aim to minimize their impact on the environment. Given the growing demand for furniture production around the world, facilitated by fashion trends and a culture of single-use goods and fast consumption, it is vital to rethink the relevance of traditional manufacturing techniques and look for more sustainable alternatives. In this regard, this paper explores various environmental practices adopted in the furniture manufacturing industry to promote sustainability and environmental protection. The use of recycled materials, non-toxic finishes, energy-efficient production processes and innovative design strategies are discussed. When it comes to sustainable design, choosing the right materials is key to reducing the impact on the environment and promoting a healthier planet. Environmentally friendly materials commonly used in sustainable design are wool, cork, bamboo, hemp, recycled paper composite, mycelium, recycled ocean plastics, algae-based bioplastics and others. The research examines the use of innovative materials embedded in atypical sources such as avocados, mycelium and pineapple for textiles. In addition to providing identical, if not higher, power indices and meeting the visual requirements set for textiles, unusual materials reduce the need for petroleum products and combat pollution and environmental degradation caused by traditional textile production. The use of these materials in the manufacture of furniture is increasingly integrated. Indeed, environmental practices are essential to combat global warming and reduce the growth of waste that, at least indirectly, becomes every piece or object made. Future generations will undoubtedly depend on natural resources, which the human generation must not exhaust.

Eighth International Scientific Conference "June 5th - World Environment Day"

Inherent in the success and sustainability of most human industries, they require compliance with green and sustainable standards and, if possible, have a positive effect.

1. INTRODUCTION

Sustainability can be defined as meeting our own needs without compromising the needs of future generations, which requires the optimization of environmental, economic, and social aspects (Kloepffer, 2008). This principle is essential in the creation and maintenance of the built environment, where human-made structures and products fulfill specific purposes. Among these products, furniture plays a critical role as the first component of the built environment with which humans interact daily. The life cycle of housing and furniture are closely linked, as increased household income often leads to greater living space and, consequently, a higher demand for furniture. In the European Union alone, 10 million tonnes of furniture are discarded annually, with most of it destined for landfills or incineration (Li et al., 2011). Research indicates that furniture contributes to 10% of the environmental impact in a building, highlighting the importance of considering the entire life cycle of furniture, from raw material acquisition to disposal, to understand its environmental footprint.

Eco-design, which incorporates environmental considerations into all stages of product development, can significantly reduce these impacts. Although initially focused on energy-related products, the principles of eco-design can also be applied to non-energy-related products like furniture, offering substantial benefits in terms of material efficiency and reduced environmental impact. Eco-design is a practice that incorporates environmental aspects into all stages of the product development process with the intent to reduce the environmental impacts throughout the lifecycle of a product (Mattias L. and Sara E., 2013). This study aims to elaborate on eco-design strategies in the development of sustainable furniture. This paper is based on published research on eco-design, eco-design in furniture, and sustainable furniture. A study of European product policy regarding eco-design is also included. A combination of eco-design qualitative and quantitative tools offers a practical way of eco-design implementation. Eco-design practice adapted from integrating Design for Environment (DFE) and Life Cycle Assessment (LCA) is presented. With the presented case study, a more comprehensive design decision can be made if the eco-design approach is used (Zhang, Zhang and Wang, 2008). The environmental aspect of the product may influence how products are designed, selected, and manufactured in comparison to using the traditional design method. A specific aspect of eco-design in furniture manufacturing involves the use of environmentally friendly adhesives that reduce the emission of harmful chemicals and improve product recyclability. The use of these adhesives contributes to a healthier indoor environment and reduces the overall environmental footprint of the furniture. Another innovative approach in sustainable furniture design is the use of fabrics made from biological waste, such as fruits and vegetables. These fabrics not only reduce waste but also decrease the need for conventional textile materials, which often require

significant resources to produce. The use of these eco-friendly materials further supports sustainability in furniture manufacturing. This paper aims to explore how the eco-design approach can be integrated into the development of furniture, particularly during the early product phases, to minimize environmental impacts.

2. METHODS OF WORK AND MATERIALS

Life cycle Assessment (LCA) is an internationally standardized environmental assessment methodology of a product or service over its whole life with the ISO 14040. LCA carried out environmental assessment to assign potential environmental impacts according to indicators processes from the product's life cycle (Abele, Anderl and Birkhofer, 2005). LCA methods can be used to show the sustainable performance of products (Wiloso et al., 2019). In the context of eco-friendly furniture, LCA is particularly valuable as it provides a comprehensive framework for assessing the environmental impacts of furniture throughout its entire life cycle. By evaluating each stage—from raw material extraction, manufacturing, and transportation to use and end-of-life disposal—LCA helps identify critical areas where environmental improvements can be made. For example, eco-design strategies can be implemented to use sustainable materials, such as fabrics made from biological waste like fruits and vegetables, or environmentally friendly adhesives that reduce harmful emissions.



Figure 1: The Concept method of traditional and eco-design processes in the development of furniture product (Bumgardner&Nicholls, 2020)

Using LCA, manufacturers can quantify the benefits of adopting eco-friendly practices and materials, providing a clear comparison to traditional methods. This holistic approach ensures that all potential environmental impacts are considered, leading to more informed and sustainable design decisions. In this study, LCA is applied to evaluate the environmental performance of eco-designed furniture, illustrating how these practices contribute to reducing the overall environmental footprint and supporting sustainability goals in the furniture industry. In the process of furniture production too many types of materials such as wood and it's by products, plastics, glass and metals are used. However, in producing these materials those substance like melamine, formaldehyde, landform and resin have detrimental effects on human health. Whereas, ecological materials can be produced by the less energy consumption, and they are easily recyclable and environment friendly that satisfies the needs of today's society. Beside

Eighth International Scientific Conference "June 5th - World Environment Day"

these, the period of recycling and renewability of ecological materials are shorter than other materials. However, as to sustainability of production of ecological materials are concern, the following conditions need to be met.

- a) A material must be produced as a substance,
- b) It should be recyclable,
- c) It must be renewable.

And, ecological materials can be classified as wooden rooted, biological, timber fiber and plaque. Woods an indispensable material in furniture design, because of its natural beauty. Those materials containing varnish and glue have some detrimental effects on human health, and they are banned in developed countries, especially for e kid furniture. Taking measures like forbidding some materials, to protect the public interest, resulted in increasing demand for wooden materials (Zhang, Zhang & Wang, 2008). The production process of ecological materials b, palm trees, and wooden stocks is also preferred. However, these materials are subject to some to face improvements. Some other wooden materials such as oak, balsa and pine trees are also under consideration in producing ecological materials. Among them bambu is the one that can be easily put into production process. Ecological materials made of bambu are provided from Far East countries and China. These imported materials from the Eastern countries are very suitable to the garden furniture.



Figure 2: Furniture Samples made of wood (img.freepik.com/premium-photo/discover-beautiful-elegant-wood-furniture-stunning-furniture-images-included_629704-1400.jpg)



Figure 3: Furniture made from 2000 pounds of recycled cardboard (Thukral, 2020)

Biomaterials have become an increasingly popular choice in furniture production due to their ecological friendliness and sustainability. One such biomaterial is wool, which is often used as a textile in furniture manufacturing. However, if the process of obtaining wool is not executed properly, it can have detrimental effects on the environment. For example, conventional wool production often involves the use of pesticides on sheep farms to prevent diseases and parasites (Jie and Zhang-Feng, 2014). These pesticides can pollute the soil, water, and air, and have a negative impact on surrounding ecosystems.

In addition to wool, there is growing interest in using biomaterials such as pineapple and avocado in furniture fabric production. For instance, pineapple fibers can be used to create fabrics known as "pineapple leather" or "Piñatex". This fabric is derived from pineapple leaves that would otherwise go unused after harvesting. The process involves manually or mechanically extracting fibers from the leaves, which are then processed to obtain fibers similar in texture to leather. On the other hand, avocado skin is also being researched as a potential source of biomaterials for fabric production (Anonimno, 2016). To create fabric from avocado skin, the pulp of the fruit must first be separated, and then the skin is processed to obtain fibers that can be turned into textile materials. These innovative approaches to biomaterial fabric production demonstrate the potential for creating more sustainable options in the furniture industry.



Figure 4: Pinatex fabric (Anonimno, 2015)

Today, pineapple has become an important eco-fashion player. Especially considering the toxic environmental effects of leather production, it can be said that Piñatex, an innovative sustainable, natural fabric made from pineapple leaves and very similar to leather, is an important fabric alternative. In addition, it is a very remarkable aspect that it is a fabric that provides positive social and economic impact and also maintains a low environmental footprint throughout its life cycle. No waste is removed during the Piñatex production process. Later in the process, the fabric is divided into many parts, which are used by designers to produce shoes, accessories, clothing, bags, automobile upholstery, and furniture. MycoTEX is a 100% biodegradable material based on mycelium (fungus root). Using this material, NEFFA has created a biodegradable fabric with a 3D modeling technique (Martinko, 2020). Emerging from the imagination of Dutch-born designer Aniela Hoitink, MycoTEX takes shape as a three-dimensional fabric. Thanks to its flexible structure, it adapts to the shape and has a form that can be easily repaired and extended/shortened. Depending on the structure of mycelium, this threadless fabric can take on various textures and shapes. When the clothing comes to the end of its life, it returns to nature as a compost additive. When furniture made from these fabrics no longer suits or becomes old, it is buried in the ground instead of being thrown away. It is possible to say that MycoTEX, the first wearable garment based on mycelium, will find a wide place in the furniture industry with environmentally friendly designs in the future. Coconut is an extremely nutritious and fortifying fruit called the tree of life. Coconut water and milk, as well as fiber, is very valuable. In the ready-to-wear industry, many brands use a type of fabric obtained from coconut shells in their products (UN Environment Programme, 2020). For example, sports brands such as North Face use a fabric obtained from coconut shells and volcanic materials in their products. This fabric also reduces the dependence on synthetic materials.



Figure 5: Coconut shell & teak wood dining room set
(<https://i.pinimg.com/564x/e8/05/20/e8052055abd943557670d490eb24e5fa.jpg>)

It is seen that this fabric, which is used especially in sportswear, has high performance and also has many advantages. In addition to being biodegradable, recyclable, sustainable and environmentally friendly, it can be used in many different areas due to its functional properties such as rigid structure, high strength and moisture resistance.

3. CONCLUSION

It is important to emphasize the role of environmentally friendly materials in furniture design and their significance for the sustainability of our environment. Furniture designers recognize that using ecologically friendly materials enhances the quality of life and reduces the exploitation of natural resources, as they are produced economically. They also understand that material sustainability requires recyclable and renewable characteristics. Furniture designers aim to use more environmentally friendly materials that will not harm nature. Functionality and aesthetic characteristics are the most important features of furniture design, which require materials to be produced at lower production costs, sustainable, and renewable. On the other hand, the interconnectedness of art, industry, and technology must be considered. In line with these developments, I support using environmentally friendly ecological materials in European countries. In conclusion, environmentally friendly materials enable better utilization of limited natural resources and meet the needs of today's consumer society. Therefore, it is important to develop these types of ecological materials and encourage their usage. Unfortunately,

Eighth International Scientific Conference "June 5th - World Environment Day"

environmentally friendly materials are not widely used in European countries compared to Western developed countries. For this reason, research institutions in this field should be established, and furniture designers should be supported in developing new production techniques in ecological materials. Despite being considered a new perspective, designers have previously considered environmentally friendly material design. Therefore, in terms of environmental friendliness, improving features and using resources economically has had a significant impact. Designs created through original works or unconventional approaches have positively supported our environment, so using recyclable materials will yield more positive results. The goal in environmentally friendly designs is clear: to use less energy, less material, and minimize harm to the environment. Compared to the history of furniture design, its significance for today and the past is difficult to express. However, designers have been aware of this issue and have created examples. In furniture design, functionality and aesthetics were valued. In fact, it was synonymous with utility, high quality, and affordability. In today's world, individual designs are mostly noticeable. For this reason, art, industry, and technology are integrated, and ideas have advanced. In recent years, environmentally friendly design has gained significance. This study supports the environmentally friendly approach made through industrial waste, unconventional forms, ready-made, and recyclable designs. In European countries where environmentally unfriendly destruction occurs, we can conclude that our designers need to take precautions for using environmentally friendly materials and they need to be encouraged.

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Eighth International Scientific Conference
"June 5th - World Environment Day"

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MAJOR BIOTIC & ABIOTIC STRESSES IN PLANTS = AN ENVIRONMENTAL PERSPECTIVE

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Key words: Stress factors, ecophysiological response(s), growth and development

ABSTRACT:

Biotic and abiotic factors reflect and specify the plants' morphology. They act generally biochemical and physiologically are called "stress". These factors hurt growth, development, quality, and quantity and can reduce average plant productivity by 65 to 87%, depending on the plants. They give various permanent or temporary damage(s) according to the length of exposed period, violence/density, developmental stage, age, etc. This review revealed that despite the technology level with modern scientific knowledge up to now, due to their complex structure, firstly taken response(s) have/have not yet fully understood and secondly any "resistant" or "tolerant" cultivar(s) has/have not been released/created up to now. This is clear that with the help of multi-disciplinary approaches, it is possible to be able to get any promising result(s) in the near future.

1. INTRODUCTION

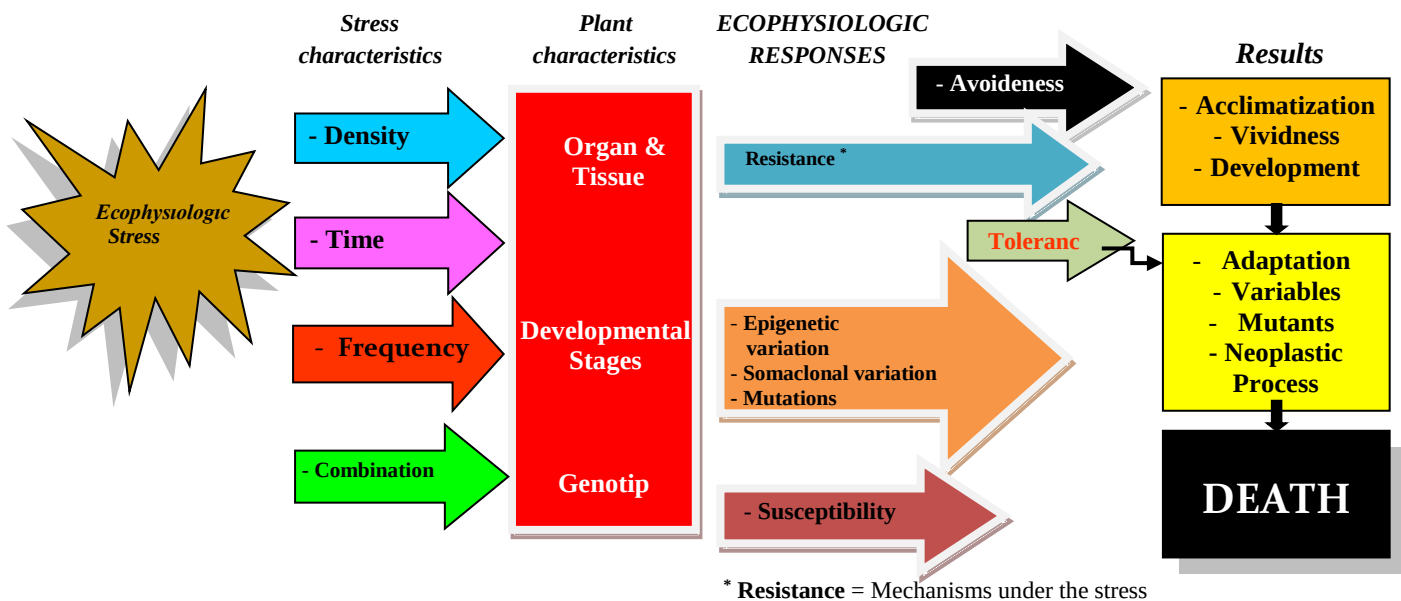
The world population is growing persistently and is expected to reach about (8.0-8.5) billion by the end of this century biotic and abiotic stresses (Shanker and Venkateshvarlu 2011). As known, plants show very wide ecophysiological, functional diversity, growth rates, productivity, population, and community dynamics at local, regional and global scales (Ackerly et al. 2000). So, these factors cause stress and they are the main constraints for crop productivity the stresses can come in many forms depending on the crop production systems (Leung 2008). When they

Eighth International Scientific Conference "June 5th - World Environment Day"

(plants) are subjected to less-than-ideal growing conditions, considered to be under stress and these stresses directly affect production and yield levels, etc. (Table 1 and Figure 1).

Table 1: Some stress factors and their ecophysiological interactions.
(Mahajan ve Tuteja 2005, Ashraf ve Foolad 2007, Gençtan et al. 2012)

Abiotic		Biotic
Physical	Chemical	
Drought, Salinity, Temperature, Chilling, Radiation, Plant nutrients, Light, Flood, Freezing, Mechanical effects, Ice sheet, Wind, etc.	Air pollution, Plant nutrients, Pesticides, Toxic compounds, Salts, Soil pH, Heavy metals, etc.	Pathogens, Wild plants, Bugs, Microorganisms, Animals, Fungi, Bacteria, Nematodes, Viruses, Parasitic Plants and Animals, Antagonism, Allelopathy, Rodentia, etc.



	Drought	Salinity	Temp.	Chilling	Frozen	Ozone	Pathogen	UV	Nutrient
Drought									
Salinity									
Temperature									
Chilling									
Frozen									
Ozone									
Pathogen									

Eighth International Scientific Conference "June 5th - World Environment Day"

UV									
Nutrient									
	No Interact.	Unknown							
	(+) Interact.	(-) Interact.							

Figure 1: Some agricultural stresses and their ecophysiological interactions (Mahajan and Tuteja 2005, Mittler 2006, Gençtan 2012).

Determining the growth characteristics and ecophysiological etc. limitations under stress conditions. For example, the effects of different amounts (s) of herbicides and pesticides on plant health and growth are very valuable and can be used to reduce off. Due to sessile organisms; plants are always open to low/high temperature, salinity, drought, flood, oxidative (stress), air pollution/heavy metal poisoning, etc., solely or in combinations (Figure 1 and 3) and their consequences (Figure 2) (Ashraf and Foolad 2007; Peleg and Blumwald 2011).

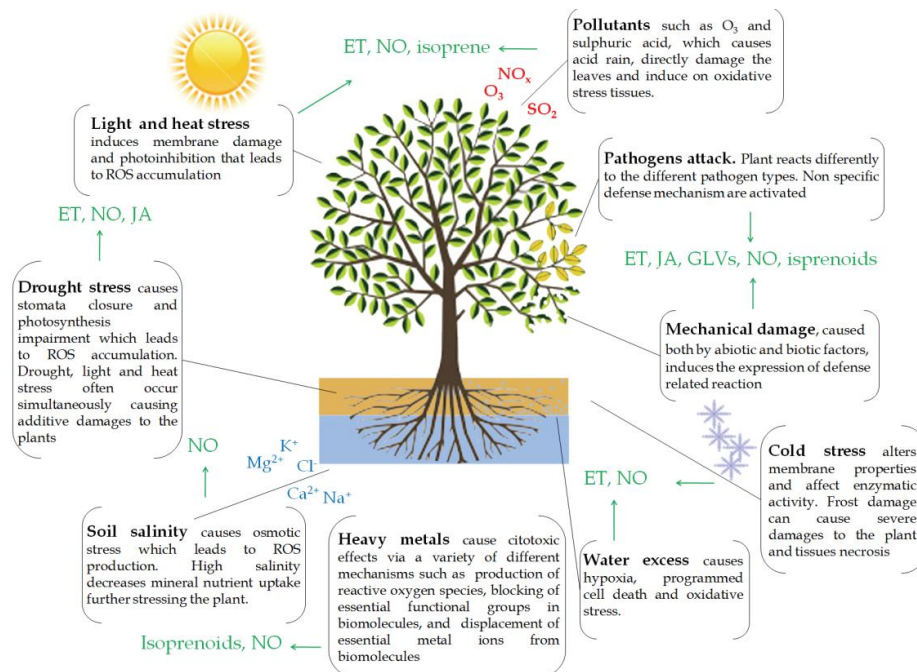


Figure 2: Some abiotic stresses in crops, ecophysiological results, and their responses (general) (Blum and Jordan 1985, Witcombe et al., 2008, Shanker and Venkateswarlu 2011, Gençtan 2012).
ET: Etilen gas; JA: Jasmonic acid, NO: Nitric oxide, ROS: Reactive oxygen species, O₃: Ozone gas

In general, one may categorize stresses as biotic, those caused by biological agents such as biotic and abiotic, and those brought about by the physical environments, such as too much or too little water, and inadequate or excess minerals in the soil (Leung 2008). Plants are unable to dislocate in their environment therefore they have evolved to solve abiotic and biotic stress with internal

Eighth International Scientific Conference "June 5th - World Environment Day"

mechanism(s) of tolerance and/or resistance. So, they have developed various adaptations (such as hairiness, waxiness, and thorniness) mechanism(s) (to provide resistance or tolerance) against them. As a physical term, this “stress” is an applied mechanical force to an object in a unit area; but, as a biological, “stress” is varying responses that depend on the size of force (Chinnusamy et al. 2007). According to Ashraf and Harris (2004), an environmental factor that limits crop productivity or destroys biomass is referred to as stress or disturbance.

These responses appear anywhere, at any time and at any stage of the plant’s life cycle as water capacity, osmotic/turgor pressure, ion capacity balance, etc. Plant stress response(s) to stress factors (Fig. 2) have/has been widely studied and were founded on global transcript profiling data that have shown that environmental stresses trigger simultaneous up-and down-regulation of a large number of genes through tight control of gene transcription (Ambrosone et al. 2011), and different chemical compounds. For example, the order of the Dicotyledoneae, Cactaceae, Chenopodiaceae and Aizoaceae families are rich in plants for adapted habitat tolerance (and also to harsh climatic conditions).

The C4 and “*Crassulacean acid metabolism*” (CAM) plants are common in places where water is scarce. The most beautiful example of this is “*Craterostigma panchaginum*”. This plant is also known to be one of the most tolerant plants to excessive dryness conditions within the 100 cultivated plants (Bartels and Nelson 1994; Bohnert et al. 1995) and it exploits the carbohydrates’ (CHs) under the stresses (Chinnusamy et al. 2007). Many findings showed that stress damage in plants is also influenced by environment or stress factors and the agronomic—genetic adaptation mechanisms for the internal and external parameters (Ulukan 2008) of ecophysiological responses.

Biotic & abiotic stresses cause changes in soil-plant-atmosphere relations and are responsible for reduced yield in several major crops (Miguel et al. 2006; Ahmad and Prasad 2012). Under abiotic stress (they are believed to cause major problems in agriculture by reducing crop growth and productivity because of their sessile nature, plants must endure adverse environmental conditions) and consequently evolve a variety of responses to acclimatize to environmental stresses (Gao et al. 2017).

Plants are generally more sensitive to insects, diseases, pests, and weeds, and yield level decreasing happens much more clearly at this stage (Reddy et al. 2004). On the other hand, due to the complexity of the affected factors, (metabolism x factor(s)), the presence of the environmental, ecophysiological, origin, etc. to be able to determine the results successfully/properly is not easy and possible (Gregorio and Cabuslay 2005). Particularly, it has not developed/released any tolerate or resistant cultivar(s) for the salinity stress in rice (*Oryza sativa* L.), common wheat (*Triticum aestivum* (L.) Em Thell) and other cultivated plants up to now (IRRI 1998; CSSRI 1999; Senadhira et al. 2002; Mithofer et al. 2004; Taulavuori et al. 2005; Shao et al. 2007; Sunkar 2010; Farooq et al. 2011). This is only possible either by using some preventive function(s) or using certain biochemical mechanism(s) (Mittler 2006; Chinnusamy et al. 2007). As known, stress factor(s) reduce(s) agricultural plant yield(s) by

approximately 65.0% or more, and plants are usually affected adversely (Munne-Bosch 2005; Chinnusamy et al. 2007; Razmjoo et al. 2008; Jalel et al. 2009; Doubnerova and Ryslava 2011). When plants look sick or appear to be under attack by insects, etc., the symptoms that are seen are a sign that the plant is being (or maybe) stressed by environmental factors and related ecophysiological responses. Here are some common symptoms of stress and the conditions that cause them: Wilting; (indicates insect pest or disease problems, but the main reason for them is lack of soil moisture). Bleached/colorless/discolored or black areas (frost damage); ragged or thorn the leaves, plants (heavy winds, rain, or hail); pale/colorless or light yellow of the leaves (nutrient deficiency); reddish or purplish cast; stunted growth; dried leaf margins (fertilizer/wind burn); burned on the leaves (herbicide overspray or animal urine). At the same time, all of these signs can accept the consequences of the ecophysiological responses of the plants against related stress factors (Figure 2). In this paper, ecophysiological responses of some cultivated plants' under biotic and abiotic stress factors will be evaluated generally, and some relevant results will be drawn.

1.1 Water/Drought Stress



Figure 3: Water/drought stress symptoms.

When water loss occurs in the plant, immediately turgor pressure drops. It directly has negative effects on cell growth, causes separation of membrane proteins, decreases chlorophyll content and losses of germination capability (Jaleel et al. 2009). Water or drought stress in the plants has not been known properly and clearly (Gutschick 1999), but, it is widely thought that the “escape” or “tolerance” (Witcombe et al. 2011). Reduction of the leaf morphology (size reducing, rolling), weakness in grain shapes and insufficient size development in different parts of the plant such as stem, leave, ear, head, etc. (in cereals), severe plant height reduction (in potatoes (*Solanum tuberosa* L.), in soybean (*Glycine max* Merr.), in okra (*Abelmoschus esculentus* (L.) Moench), in cowpea (*Vigna unguiculata* (L.) Walp.), in parsley (*Petroselinum crispum* (Mill.) Nyman and AW Hill), in sunflower (*Helianthus annuus* L.), in citrus (*Citrus* spp.) are typical symptoms (Specht et al. 2001; Zhang et al. 2004; Manivannan et al. 2007; Wu et al. 2008; Kadioğlu et al. 2011). As known, the water shortage effect brings important limitations for all agricultural production(s), especially in legume production (Dita et al. 2006).

Eighth International Scientific Conference
"June 5th - World Environment Day"

Plant hormones such as brassinosteroids (BLs), auxin, and gibberellin (GA) promote growth, while ethylene and abscisic acid (ABA) suppress the growth of the plants (McSteen and Zhao 2008). ABA is closely related to water stress. Ehsanpour and Razavizadeh (2005) found out that in plants as a response; the main task is to carry out and keep the balance between organism and water stress tolerance; and, to provide tolerance against the osmotic pressure (Zhu et al. 1997; Chinnusamy and Zhu 2007) and closing the stomata during transpiration (Blum and Jordan 1985). By doing this, ABA prevents water loss, slows down the development of an apical organ(s), allows an increase in the water usage by roots and gives more chance to find water in the soil (Anonymous 2011).

This stress reduces the number of grains at the pre-anthesis stage and largeness (especially for the *Q* gene) of numerous morphological and domestication-related traits such as plant height, flowering time, leaf morphology, structural deformation(s) at the spike in wheat (*Triticum* spp.) (Iqbal et al. 1999; Yin-Shan 2007; Farooq et al. 2011); pod number and related yield components of soybean (*Glycine max* Merrill); 100—seed weight reduces in sunflower (*Helianthus annuus* L.), yield level per plant decreases in corn (*Zea mays* L.); damage(s) between the grain filling and anthesis stage in beans (*Phaseolus vulgaris* L.), in chickpea (*Cicer arietinum* L.) and parsley (*Petroselinum* spp.) (Webber et al. 2007). Under these circumstances, nearly all cultivated plants developed important protective and resistant strategies against this stress in both topsoil and subsoil horizons. Especially, pre-drought and/or after rains, rapid maturation, and water loss reduction an important topics so the plants formed “a deep root system” to save or keep the water available for their development and growth period(s).

The structures that are *protective* and *xerophytic* such as hairness, acanthaceous, waxiness, etc. in plants have an important place in terms of the stress resistance strategy (Shepherd and Griffiths 2006). Under this stress, the plants excrete ABA, amino acid(s), proline and prolamins, quaternary amino acids, glycine betaine, mannitol, sorbitol, raffinose, trehalose, H₂O₂, ascorbate, α —tocopherols, etc. (Lichtenthaler 1996; Yordanov et al. 2000; Mittler 2006; Ashraf and Foolad 2007; Hussain et al. 2011). Similarly, with brief exposure of plants to high temperatures during the seed filling stage, senescence can accelerate, seed set and seed weight diminish, and (finally) yield level reduces (Porporato et al. 2003). The number of photosynthetic pigments in the plants and reducing the photosynthetic efficiency in leaves under drought stress are decreased yield levels and imbalances between available CO₂ and turgor pressure (Bohnert et al. 1995; Yordanov et al. 2000; Mittler 2006). It was found that there is a close correlation between “drought resistance” and “dehydrated accumulation” in the wheat (*Triticum* spp.) and poplar (*Populus* spp.) (Yordanov et al. 2000). A severe drought period accelerates the moisture loss from the plant and environment, blocks the transmission to vascular bundles, and negatively affects protein structure(s), oil and enzyme activities in the cell walls of leaves (Anonymous 2011). This reduction was recorded as (3–12) times faster than in birdsfoot trefoil (*Lotus corniculatus* L.) (Yordanov et al. 2001), (*starch/sucrose*) ratio was decreased in bean (*Vicia faba* L.), and it happens that the CO₂ imbalance in the form of *sucrose* in sugar beet (*Saccharum* spp.) leaves

(Mittler 2006). Plants intake O_2 , CO_2 and water vapor from the air through their leaves. If they (= stomata) close, the transpiration event immediately interrupts, and finally, it stops. This process happens faster in drought-resistant plants and this formation is an important barrier to water and gas losses (Mittler 2006). But, the early closure of stomata is mostly related to “Soil dryness” (Porporato et al. 2003). It was understood that this is a helper mechanism for the water balance, depending on the leaf transpiration rate (Gutschick 1999; Aina et al. 2007). Similarly, coating the leaf surface with a cuticle or a wax layer prevents water loss and reduces CO_2 intake (Yordanov et al. 2000). Many cultivars are tolerant to the aforementioned abiotic stress factors (especially in wilds (Massarelli et al. 2006) and were discovered that some nitrogenous chemical compound(s) (= NCC), such as proline, polyamines, sucrose, dehydrated, etc., accumulate(s) under the stress conditions, and, was found that many genes responsible from this processes (Galinski 1993; Potts 1994; Mittler 2006; Chinnusamy et al. 2007; Hussain et al. 2011). Waterlogged soils adversely affect the growth of plants not adapted to wetland conditions, also salinity can affect the growth and water content of some plant species (Grabner 2003). So, the strategies for survival and growth of the plants under water stress, are divided into two main groups “*The morphologies*” and “*The physiologies*”.

The morphologies are: Descending deeper root system than those of the morphological forms as narrowing of the leaf and stem surface; curling or rolling of the leaves, hairness in the stem; increase in thickness of the cuticle/wax layer, the deep embedding of the stomata, the leaf shedding, to do photosynthesis of stem or other parts (Lichtenthaler 1996; Mittler 2006; Chinnusamy et al. 2007; Farooq et al. 2011); and, the physiologies are: stomatal originated, photosynthetic regulations, osmotic adjustments, protective solutions in the leaves, etc. (Zhu et al. 1997; Yordanov et al. 2000; Chinnusamy et al. 2007).

1.2 Salt stress



Figure 4: Salt stress symptoms.

There are approximately 397 million ha under salinity; 5,762.9 million ha under drought in the World (Ashraf and Foolad 2007); and 434 million ha are also under alkalinity. Alkaline soils are usually categorized by low availability of plant nutrients, high concentrations of HCO_3^{-1} , CO_3^{-2}

and high pH and bicarbonate (HCO_3^{-1}) and carbonate (CO_3^{-2}) are principal contributors to the alkalinity, whereas hydroxide, borate, ammonia, organic bases, phosphates, and silicates are considered minor contributors, hence plant/crop growth is mainly inhabited by the HCO_3^{-1} and CO_3^{-2} ions rather than OH^{-1} in the alkaline soils (Shanker and Venkateswarlu 2011).

In the regions, where the air temperature is quite high but rainfall/precipitation amount is rather low, either water losses by evaporation or the salt significantly accumulate in the different soil layers as a white thin layer (especially at the surface) and it limits agricultural production level. Chlorides (NaCl , CaCl_2 , and MgCl_2), sulfates (Na_2SO_4 and MgSO_4), nitrates (NaNO_3 KNO_3), carbonates and bicarbonates (Na_2CO_3 and NaHCO_3) are the main reason of soil salinity, and (Fe), (Zn) and (Mn) become less available to plants under the alkaline stress conditions (Shanker and Venkateswarlu 2011). Under salt stress, the increasing amount and density of dissolved salt at the plant root zone, hampers the water uptake, on account of ion concentration increasing, and finally, it poisons the plant (Zhu et al. 1997; Zhu 2011). High salinity affects the plants in different ways such as water stress, ion toxicity, nutritional disorders, oxidative stress, alteration of metabolic processes, membrane disorganization, reduction of cell division and expansion, and genotoxicity (Shanker and Venkateswarlu 2011), and all of them reduce plant growth, development and survival.

When the plant is under excessive salt stress (Zhu et al. 1997), its height and root growth, bud formation, root, stem, and shoot length, fresh dry weight, and leaf area are decreased; emerged plant number reduces, chlorophyll content decreases and yield level falls, flavor and color of the fruits degenerate, ion balance of the cells breaks down and “oxidative” stress happens, all the leaves remain small, the root system, bud(s), leaf edge(s) have yellow spots (necrosis) (especially they appear at the growth points), water amount and enzyme(s) activity(ies) decrease(s), protein synthesis slows down, cell membrane permeability decreases, whole organelles (first *chloroplasts*) are damaged (Gzik 1996; Chinnusamy and Zhu 2007); if the plant(s) remain(s) under the long–termed salt stress; ion toxicity and lack of water appear, CHs deficiency and related symptom(s) are seen in the leaves (Zhu et al. 1997; Zhu 2011). Plants allow using and taking of the development of K^{+1} or Ca^{+2} instead of Na^{+1} and the highness of the (K/Na) or (Ca/Na) ratios increases under saline conditions. Studies have shown that all cereals are more durable than legumes due to salt stress (Zhu et al. 1997; Lopez et al. 2009; Sunkar 2010).

1.3 Heat stress



Figure 5: Heat stress effect symptoms.

Temperature easily breaks the bonds of molecules, causing the structure of cell membranes to convert into softening but when it (temp.) turns to low temperature makes the cell membranes hard. In temperate regions, short the grain-filling period is (Murchie et al. 2008), and closely related to the plant's biology, reproduction ecophysiology, used growing techniques, ecology, etc. (Frei and Wang 2011). Depending on the growing period, its violence, and metabolic activity regulating the growth and development, with geographical limitations, almost all plants produce ABA, α -tocopherol, cytokinin, salicylic acid, etc. (Blum and Jordan 1985; Munne-Bosch 2005; Kotak et al. 2007). It was determined that all cultivars normally develop between (15–45)°C; however out of this range, depending on the environmental conditions, ecophysiological responses, quality and quantity components are urgently and negatively affected (Frei and Wang 2011). Whereas, high temperature accelerates and encourages growth, causes the reduction of enzyme activity digests the proteins, provokes the cell structure and changes their functions; its genetic pathway and mechanism are still unknown and mysterious. However, it was discovered that there are some special proteins (such as heat shock proteins, etc. (Volk 2010) that are produced and play an important role in the plants (Mittler 2006; Chinnusamy et al. 2007). Similarly, seeds and dormant buds can earn an “*endogenous tolerance to desiccation*” and “*cryogenic temperature stresses*” against very low heat stress (Volk 2010). In cultivated plants, heat stress is crucial. According to research, in wheat and other cool season cereals, during the reproductive and grain filling stages, due to performing of pollination, partially heading and seed setting events, should not be “*heat stress*”. The optimum temperature value varies (12–22)°C for the wheat plant at anthesis and grain filling stages and if exposure to temperatures out above, heat stress can easily and significantly reduce grain yield (Farooq et al. 2011). And, this finding is being added that during the acclimation period to heat stress is reversible, but if the stress conditions(s)/are too great, irreversible changes occur and these formations lead to plant death (Amal et al. 2007).

1.4 Cold/Freezing stress:



Figure 6: Cold/Freezing stress symptoms.

Cold treatments cause instant or abrupt changes in the structure of cellular lipids and cytoplasm; ice crystals form in hydrated cells and undesirable plasma membrane change(s) happen with the temperature dropping. This extracellular ice formation triggers or invokes “*a desiccation stress*”

(Frei and Wang 2011). In general, (0–15)°C values are optimum, but it is desirable under the 15°C for tropical and semi-tropical plants not to be cold stress. Low or cold stress damage depends on mainly density, water viscosity, duration, environmental temperature(s), ecophysiological responses, time, age, and ecology. Although cold climate plants easily adapt to low temperatures; citrus fruits, cotton, rice, sugar cane (*Saccharum officinarum* L.), soybean, potato (*Solanum* spp.) are known as (very) sensitive plants to the cold. When they are exposed to cold stress; the root cell membranes have lost or reduced their permeability and/or the water viscosity has increased so the plants can not water intake; The stomatal aperture is full or little, then, if the stress continues before all the leaves color turns into the pale, and plants die (Taulavuori et al. 2005).

Die of tropical plants (such as in banana (*Musa* spp.) plant) happens below 13°C within (1.0–1.5) hours (Chinnusamy et al. 2007). If the environmental temperature falls gradually, a progressive reduction of the cell water happens, so the plants are not easily damaged by the frost, and they become “cold tolerant” (Thakur et al. 2010). Freezing stress arises when the environmental temperature is below 0°C, but the main responsible for this stress is “cell water freezing”. At this temperature, nearly whole metabolic events slow down and all vital functions come to a stopping point in the cells. The cell water goes to intracellular apertures and begins to freeze, osmotic pressure balance disorders, and organism loss of its water (Zhu et al. 1997). If the freezing stress just started, do not freeze all water in the cells, and it does not give heavy damage; but if it continues, the whole water freezes and this time ice crystals happen. After this event, these formations tear the surrounding cell membranes, blast them, and cause plant death (Burke et al. 1976; Taulavuori et al. 2005; Sunkar 2010).

However, in some plants, due to unknown (for now but it should be detailed research) reasons, the ice crystal formations do not happen because these plants carry some “sugar molecule(s)” instead of water in their cell walls and these chemicals increasing the producing of “antifreeze” or “water freezing retarder” compounds which are called (stress proteins such as simple sugars, glycol, proline, etc.) (Burdon 1993; Patra and Panda 1998; Chinnusamy et al. 2007; Anonymous 2011).

1.5 Light stress



Figure 7: Light stress symptoms.

Eighth International Scientific Conference "June 5th - World Environment Day"

Light plays a critical role in regulating plant growth and development through the modulation of expression levels of light-responsive genes that regulate developmental and seed germination, seedling photomorphogenesis, chloroplast development and orientation, photokinesis, stem growth, pigment biosynthesis, flowering, senescence, etc. (Shanker and Venkateshvarlu 2011). Plants utilize light as their primary source of energy converting light to usable chemical energy through photosynthesis and it is an essential prerequisite for chlorophyll biosynthesis and chloroplast development; events that do not take place in darkness (Shanker and Venkateswarlu 2011). The amount of chlorophyll grown under plenty of light is less than the plants grown under low light/shadow; and, when they are left to low light/shadow, they expand their leaf areas and reduce the production of CHs (Anonymous 2011). But, this time respiration process slows down and less amount of nutrient(s) go(es) to the root system. However, in these plants (grown under plenty of light), the leaves are thick (esp. under extreme lights), the photosynthesis rate increases, respiration velocity or ratio accelerated and the leaves are covered with a waxy or a cuticle layer(s). If the shadow plants are put under high light density, their photosynthetic activities accelerate, but, due to oxygen radicals not remove from the leaves (for example H_2O_2), other free radicals could easily react with them and emerge some “*toxic compounds*”, and they caused the death (Kotak et al. 2007). Similarly, elevated UV radiation has pleiotropic effects on plant development, morphology and ecophysiology. Generally, the morphological consequences of UV-supplemented white-light treatment in plants are growth reduction, leaves thickening and covering the cuticular wax layers (Amal et al. 2007). Rice (*Oryza sativa* L.) showed a significant decrease in total biomass with increased (UV—B) radiation; chlorophyll a and b contents of the *Phaseolus vulgaris* L. leaves dropped after (UV—B) stress (Amal et al. 2007); and (UV—AB) rays break the proteins’ disulfide bonds of the DNAs (Anonymous 2011) in organisms.

1.6 Pest and Disease stress



Figure 8: Pest and Disease stress symptoms.

Disease factor(s) enter(s) from the leaves, stomata and scratch(es) that caused “*infection*”. If the plant is susceptible and the pathogenicity level is high, when the other conditions (host-pathogen interactions, light, temperature, humidity, etc.) are optimum, can easily happen “*epidemy/ies*” at local, national, or international scale(s). It was reported that there are 0 to 80% dry matter losses occur each year in wheat plant (*Triticum* spp.) production due to wheat rust (*Puccinia* spp.) diseases (Ulukan 1998). Under the disease stress, pustules on the leaves, chlorosis, necrosis,

wilting, crusting in the stem, rot in the root system, and fruits (= a disease product) are some of the important symptoms (Ulukan 1998). When the pathogen enters, each plant takes some preventive measurement(s) against it such as thickening of the protective tissue, some chemical compound secretion—tannins, alkaloids, etc. (Liu and Ekramoddoullah 2006).

1.7 Flooding and submergence (Water logging) stress



Figure 9: Flooding and submergence (Water logging) stress symptoms.

They are major abiotic stresses and rank alongside water shortage, salinity and extreme temperatures. Due to the overflow of rivers or excessive rain, “water” does not enter the soil when the O_2 infiltration to the soil is impossible to stop, then, cultivated plants do not respire and die. However, rice is usually thought of as being highly tolerant of flooded conditions (Visser et al. 2003). Their growth is adversely affected and the end product of photosynthesis’ amount (yield level) decreases. O_2 levels run off into these types of soils between (1.0–2.0) hours, and then anaerobic respiration begins. In the cell, (Fe), (Mn), (H_2S), sulfides, lactic and butyric acid(s) are activated; and, they reach to toxic level; protein synthesis significantly decreases, mitochondria are damaged; division and elongation (of the cells slow down, ion transmission negatively adversely affects; stomata closes, the leaves lose their turgor pressure than the plant(s) die(s)) (Sunkar 2010).

1.8 Oxidative stress



Figure 10: Oxidative stress symptoms.

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Oxidative stress is known to be generated by some environmental factors including light, temperature, water, mineral deficiency, toxic metals, air pollutants, such as O₃, SO₂, CO₂ and NO, and plants after reacting with oxygen can exhibit a broad range of physiological responses including changes in gene expression (Patra and Panda 1998). It results from conditions promoting the formation of active oxygen species that damage or kill cells. Most of the abiotic stresses induce plant oxidative damage of the cell structure and consequently a loss in cellular activities (Shanker and Venkateswarlu 2011). Reactive oxygen species (ROS) have important deleterious effects under saline conditions due to the ROS stress effect, which causes oxidative stress as one of the most general stress types (Shanker and Venkateswarlu 2011). These environmental factors that cause oxidative stress are air pollution, oxidant herbicides, heavy metals, drought, heat and cold stress, wounding, UV light, and dense daylight (sunlight). Among them, particularly ozone (O₃) is one of the most important factors (Anonymous 2011). This gas is located in the upper layers of the atmosphere, and, protects from the world's influence of (UV—AB) rays, and is also very dangerous because it is a very reactive oxidant (Iglesias et al. 2006). O₃ has been considered to be the most harmful agent to plants as reduces photosynthesis and speeds up senescence.

Findings showed that it could break the cycle of glycolysis and pentose phosphate cycle in *Pinus halepensis*, *Acer saccharum*, *Betula pendula*, *Picea abies*, *Fagus sylvatica* genotypes; plays an important role in the Ribulose-1,5-biphosphate (RuBisCO) enzyme formation (Burdon 1993; Jordan 2007; Sunkar 2010; Doubnerova and Ryslava 2011); and, it is reported that *oxidative stress* is being increased with the aging in plants before anthesis (Munne-Bosch and Alegre 2002); decreased rates of photosynthesis, leaf injury, reduced growth of shoots and roots; accelerated flowering and reducing yield. Alters ion transport increases membrane permeability, inhibits H⁺—pump activity, collapses the membrane potential and activity, increases Ca₂⁺ uptake from apoplast, and oxidative damage(s) can be mentioned.

1.9 Air pollution and/or heavy metal stresses



Figure 11: Air pollution and heavy metal stress symptoms.

Another stress is air pollution and/or heavy metal stress. Researchers reported that various pollutant effects' could be changed on the same plant (Bernardo et al. 1996). Some plants contain some toxic elements such as Zn, Pb, Ni, Co, Cr, Cu, Mn, Cd, Se, Sb, As and Al, and start

over their specific/lethal dose(s). Interestingly, heavy metals have biological importance in plant development and growth, where they play key roles affecting cellular processes such as homeostasis and photosynthesis (Depres and Goloubinffi 2007; Shanker and Venkateswarlu 2011), and they inhibit root development and cell division in plants. Particularly, Al^{+3} could easily react with the plant cell wall, nucleus, etc. in acid soils (Ahmed and Prasad 2012). In general, their damages happen at the electron transfer in photosynthesis; they block the vital enzyme(s) processing in the organism to a certain extent, inconvenience(s) emerge(s) in the meeting of energy requirement(s), and reduce(s) the quality components (Volk 2010).

2. RESULTS AND DISCUSSION

Today, due to limited knowledge of biotic and abiotic stress, a better understanding of plants' environmental and ecophysiological behaviors/responses basic(s) is/are rather complex and very important. Especially to be able to do this evaluation in the light of related factors such as salinity, drought, flooding, heavy metal, temperature extremes, high-light intensities, (UV-AB) radiation, herbicides, ozone gas, etc. is a very problematic and difficult occupation especially at the problem areas (Hasanuzzaman et al. 2010). Similarly, their integration with global warming and "*climate change*," leads to an inextricable and deeply troubling outcome for humanity (Ahmad and Prasad 2012). Nowadays, stress studies in plants have major difficulties (their mechanisms are very complex and they can give generally unexpected results due to interactions; the lack of resistant or tolerant and stable genotypes against stress factors, etc. are very important handicaps (Roy et al. 2011; Anonymous 2012) will happen now or in future.

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CONTEMPORARY APPROACHES TO COMBAT INVASIVE ALIEN SPECIES

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Key words: Invasive alien species, biodiversity, IAS management

ABSTRACT:

Invasive alien species (IAS) are animal and plants species that are introduced accidentally or deliberately into a natural environment where they are not native. They often possess high reproductive rates, enabling them to rapidly establish populations and outcompete native species for resources. This competitive advantage results in significant adverse impacts on local biodiversity, exacerbating the vulnerability of native threatened species. IAS are considered to be one of the greatest threats to biodiversity, particularly through their interactions with other drivers of change. They hinder conservation efforts and the sustainable use of biodiversity and also impact the goods and services provided by ecosystems. IAS also harm human health and affect various economic sectors like agriculture, forestry, and fisheries by reducing productivity and obstructing waterways, which can hinder navigation. They may also reduce the recreational and aesthetic value of affected areas.

In response to the pressing need for comprehensive management strategies, a range of tools, spanning from legislative frameworks to practical field interventions, have been developed to combat IAS. This manuscript aims to furnish an overview of contemporary IAS management approaches implemented globally, with the intention of informing and guiding future efforts in this critical domain.

1. INTRODUCTION

Invasive alien species (IAS) are species introduced into environments beyond their native range, encompassing all taxonomic groups including animals, plants, fungi, and microorganisms. These

Eighth International Scientific Conference "June 5th - World Environment Day"

species pose significant threats to ecosystems, biodiversity, and agriculture, with profound ecological and economic impacts (Olson and Roy, 2010; Simberloff et al., 2013). IAS are characterized by high reproductive rates, adaptability, aggressive competition, lack of natural predators, and efficient dispersal mechanisms.

Globally, more than 37,000 established alien species have been recorded, including over 3,500 with documented negative impacts. The number of established alien species is rising at unprecedented rates, with approximately 200 new species recorded annually. Human activities, especially global trade and travel, primarily drive the spread of IAS. Climate change and biodiversity loss further exacerbate their establishment and spread (Roy et al., 2023).

IAS cause dramatic changes to biodiversity and ecosystems, leading to local and global species extinctions (IPBES, 2023). They compete for essential resources, disrupting ecological systems and threatening endangered species (Epanchin-Niell and Hastings, 2010). IAS are a primary driver of biodiversity loss worldwide (MAE, 2005; CBD, 2007). The economic damages from IAS are substantial, including management costs, reduced crop yields, job losses, infrastructure damage, and impacts on international trade (ISC, 2019). In the United States, approximately 50,000 non-native species cause \$115 billion in damages and losses annually, with \$21 billion spent on control (Pimentel et al., 2000). In the EU, the economic impact is estimated at EUR 12 billion per year (EC, 2024).

IAS also have significant implications for human health. They can serve as vectors for pathogens, causing diseases such as dengue, Zika, and chikungunya (Benedict et al., 2007). Invasive aquatic species can alter water quality, increasing waterborne diseases, while certain invasive plants exacerbate allergies and respiratory problems (Vanderploeg et al., 2002). Due to accelerating globalization, the number of IAS and their impacts are expected to continue increasing (IPBES, 2023). This trend highlights the need for effective and sustainable IAS management that balances environmental, social, and economic factors (Larson et al., 2011). As IAS are one of the major drivers of biodiversity loss, comprehensive management strategies are crucial.

The purpose of this article is to provide an overview of contemporary approaches to combat IAS, emphasizing integrated and sustainable practices to mitigate their negative impacts on ecosystems, economies, and human health.

2. INVASIVE ALIEN SPECIES MANAGEMENT

The key elements of invasive species management, that effectively protect native ecosystems, economies, and human health, include (i) prevention, (ii) early detection and rapid response, and (iii) control and management (Figure 1).

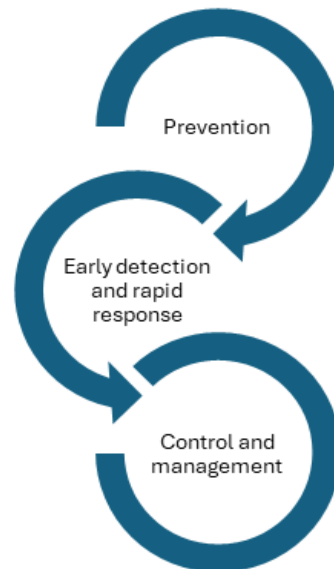


Figure 1: Key elements of the invasive species management

2.1 Prevention

Having in mind that the spread of invasive alien species (IAS) is likely to continue, prevention remains one of the most effective approaches to IAS management. Preventing the introduction and establishment of IAS is crucial for sustainable management strategies, necessitating a combination of regulatory measures, monitoring systems, public awareness, and international cooperation.

International agreements like the Convention on Biological Diversity (CBD) and the International Plant Protection Convention (IPPC) provide frameworks for coordinated action and information exchange on IAS management. The Kunming-Montreal Global Biodiversity Framework, a significant agreement under the CBD, sets targets to be achieved by 2030, including actions to control IAS.

The EU Biodiversity Strategy for 2030 aims to manage established IAS and reduce their threat to Red List species by 50% by 2030 (EC, 2024). The EU Invasive Alien Species Regulation (EU

Eighth International Scientific Conference "June 5th - World Environment Day"

1143/2014) outlines measures for dealing with IAS, including restrictions on their importation, sale, and release into the environment.

Preventing the arrival of IAS is a top priority in managing biological invasions. Information on IAS is available in major databases like the IUCN's Global Invasive Species Database (GISD) and the European Invasive Alien Species Gateway (DAISIE), using a new categorization scheme adopted by the CBD.

IAS risk assessment is an effective management tool, aligned with targets in the Kunming-Montreal Global Biodiversity Framework to mitigate their impacts (Trueman, 2023).

International cooperation through initiatives like the European Alien Species Information Network (EASIN) and the European and Mediterranean Plant Protection Organization (EPPO) is essential for IAS prevention and management.

Collaboration with neighbouring countries is crucial to address transboundary IAS issues through joint surveillance and rapid response efforts. These efforts highlight the importance of proactive and coordinated approaches to prevent and manage the impacts of IAS on ecosystems and economies.

2.2 Early detection and rapid response

Early Detection and Rapid Response (EDRR) is a crucial component in the management of invasive alien species (IAS) in the European Union (EU). The EDRR system aims to detect and eradicate IAS before they can spread and cause significant harm to biodiversity and ecosystem services.

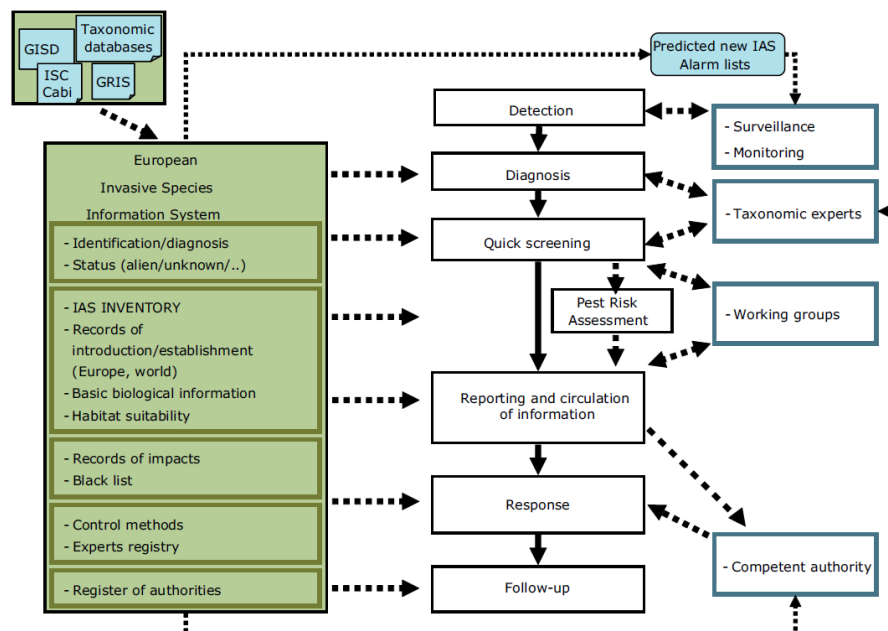


Figure 2: Structure of a pan-European early warning and rapid response framework (Source: EEA Technical report No 5/2010)

Comprising six interconnected components, the EDRR framework encompasses detection (surveillance and monitoring), diagnosis, data processing, risk assessment (or quick screening), reporting to competent authorities and information dissemination, response action (e.g., eradication, control), and subsequent follow-up, as presented in Figure 1 (EEA, 2010).

Monitoring and surveillance activities are essential to collect the information needed to guarantee rapid response actions and implement measures to prevent newly-introduced IAS establishing. Identifying a species correctly is the first step in an early warning and rapid response process aimed at preventing its establishment in a newly invaded region. Quick and reliable identification of the species and its status in the concerned area will allow, in the event that the taxa is considered alien, a prompt start of the entire process for an early warning and rapid response.

A pivotal component of an early warning and information system is risk assessment. Evaluating the risks associated with the actual or potential introduction of an alien species is imperative and builds on the collected information. It leads to a decision on the actual measures (e.g. eradication, control, regulation of trade) that should be undertaken so as to prevent a target species being introduced or permanently established.

Upon detecting a new incursion, swift screening of potential risks (informed by invasiveness records from other areas, ecological characteristics, etc.) is imperative to determine the appropriate response. After collating and meticulously analyzing all available information and

knowledge concerning the target species, disseminating the results of the risk assessment and recommended response actions to competent national and local authorities is imperative.

Once a new incursion is detected, and associated risks are preliminarily screened, it is crucial to decide promptly what measures have to be implemented (either eradication, control, containment or no action), what techniques have to be applied and who should enforce them. Reporting by the authorities overseeing response actions serves as a final yet indispensable element of EDRR. This reporting tracks the progress of management measures and assesses their impact upon task completion.

2.3 Control and Management

There are three options on how to control the spread of IAS: eradication, containment, or control (ERA, 2024).

Eradication efforts often involve targeted removal of invasive species using methods such as manual removal, mechanical control (e.g., cutting or mowing), chemical treatments (e.g., herbicides), or biological control using natural enemies (e.g., predators or parasites). While eradication is the preferred strategy, its feasibility largely depends on the early stage of invasion, characterized by small and isolated populations.

In contrast, both control, which diminishes the presence of the invasive species, and containment, which restricts its further spread, play vital roles in managing larger invasions. However, these approaches require continual investments of time, effort, and financial resources to effectively restrain the IAS expansion.

These global strategies, programs, and policy documents provide essential frameworks and guidelines for countries to prevent, control, and manage IAS effectively. Through international collaboration and adherence to these standards, the global community can better address the challenges posed by IAS.

3. CONCLUSION

Invasive Alien Species (IAS) pose a significant threat to ecosystems, biodiversity, and economies worldwide, resulting in severe ecological and economic consequences such as biodiversity loss, habitat degradation, reduced crop yields, infrastructure damage, and disruptions to international trade. Furthermore, they can have substantial implications for human health, acting as disease vectors and exacerbating respiratory issues and allergies.

Addressing the challenges posed by IAS requires a multifaceted approach that includes prevention, early detection and rapid response, and effective control and management strategies.

Eighth International Scientific Conference "June 5th - World Environment Day"

Prioritizing these measures is crucial for mitigating the negative impacts of IAS on ecosystems, economies, and human health.

In conclusion, comprehensive and integrated management strategies are essential for combating IAS effectively. By investing in prevention and early detection efforts, we can significantly reduce the economic costs associated with managing invasive species. Once an invasive species establishes and spreads, management becomes exponentially more expensive and less efficient. Therefore, proactive measures are essential for achieving sustainable solutions to the problems posed by invasive alien species.

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**PREVENTING BIODIVERSITY LOSS: RESTORING WETLANDS WITH THE HELP
OF 'EGG BANK' - THE CASE OF THE RAMSAR SITE
"BARDAČA WETLAND" (BOSNIA AND HERZEGOVINA)**

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Key words: Branchiopoda, 'egg bank', "Bardača Wetland", biodiversity protection

ABSTRACT:

The „Bardača Wetland“ is one of the three Ramsar sites in Bosnia and Herzegovina. This area has a large number of water bodies with different hydrological systems that harbour diverse, locally adapted flora and fauna. So far, a total of nine large branchiopod taxa have been recorded. In spring 2021 and 2022, several samples of dried soil were taken from the already known biotopes in the village of Bajinci, both from the sites in the seasonally flooded zone and outside the flooded zone. In all trials, hatchlings and adults of five taxa (one from the order Anostraca and four from the order Spinicaudata) were collected after approximately one month. The successful attempts to recover specimens from the 'egg banks' in the „Bardača Wetland“ offer a good perspective for the future restoration of natural processes, habitat protection and species recovery. This could contribute to a better recognition of this area as a natural area of great importance in Bosnia and Herzegovina and help in its management and conservation.

1. INTRODUCTION

The large branchiopod crustaceans (Branchiopoda) are considered the flagship group for wetland ecosystems. The species have a limited and fragmented distribution pattern worldwide and are seasonally highly dependent on humidity. The species exhibit considerable ecological plasticity and can spend the unfavourable conditions as encapsulated embryos (the 'egg banks') in the soil (Brendonck et al., 2008). In the "Bardača" area, a total of nine large branchiopod taxa have been recorded so far and several species occurred sympatrically (Miličić et al., 2023). The species show different seasonal preferences for temperature and other physicochemical water characteristics and hatch and reach sexual maturity at different times (Eder et al. 1997). Many of the species favour open habitats and can survive in furrows on agricultural soils.

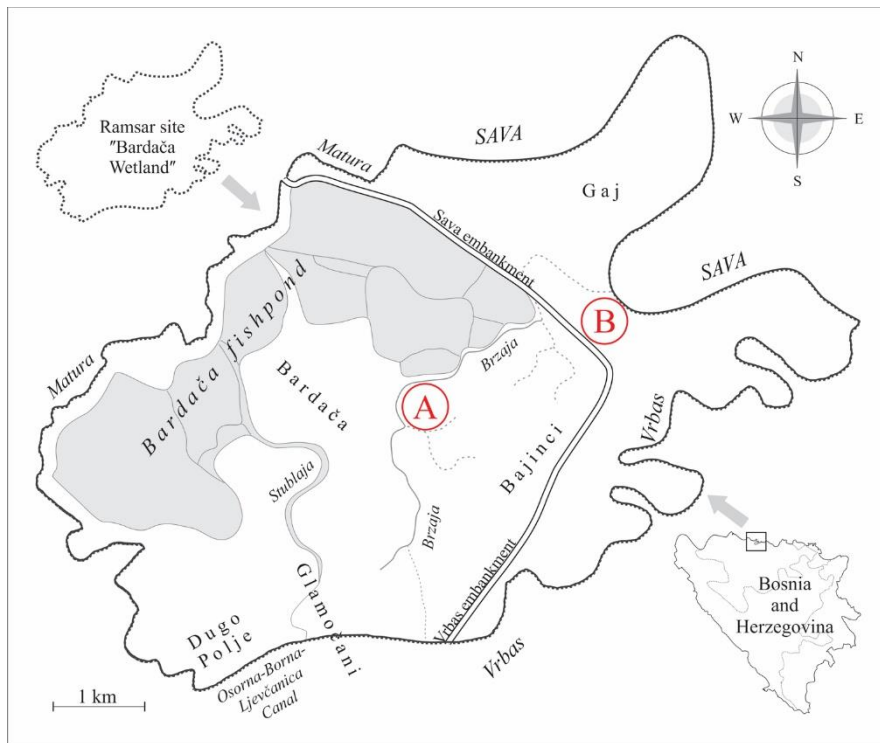


Figure 1: Map of the Ramsar site "Bardača Wetland" with experimental sites (A and B).

The „Bardača Wetland“ is one of the three Ramsar sites in Bosnia and Herzegovina and the only Ramsar site on the territory of the Republic of Srpska. In this area there are several ephemeral habitats that are filled by precipitation, but also depend on the groundwater level. In this area, there are a variety of water bodies with different hydrological systems that harbour diverse, locally adapted flora and fauna (Miličić et al., 2022). However, the floodplain, permanent water bodies and ephemeral water bodies in the area are severely threatened by agricultural and urban

expansion. In recent decades, the Bardača area has been heavily modified by structures such as a embankment near the village of Bajinci, which was built to protect it from flooding (Figure 1). A transformation of the natural landscape into a fish pond, canal-like depressions or agricultural land can also be observed. In this ecological context, we wanted to find out if it is possible to recover the large branchiopods from the 'egg banks' in the „Bardača Wetland“, which would provide a good perspective for the future restoration of natural processes in this area of obvious high natural and scientific value.

2. MATERIALS AND METHODS

In 2021 and 2022, several samples of the dried soil were taken from sites already known as large branchiopod biotopes near the village of Bajinci. The soil samples were collected both from the sites outside the flooded zone and in the seasonally inundated zone (labelled A and B, respectively, in Figure 1). Samples from each habitat were doused with rainwater and incubated in plastic containers in a sunny place (Figure 2). The locations with the experimental sets were visited at regular intervals, video-recorded and photographed. The hatching time for each taxon was recorded. Taxon identification was carried out when the individuals had reached the adult stage.



Figure 2: The outdoor experimental sets.

In 2021 and 2022, there were some unsuccessful hatchings from soil samples treated with rainwater from the surrounding area. In other trials, the animals hatched and the adults were collected after about a month. The hatching trials from soil samples from site A were repeated three times during the entire trial period (A1-A3) (Table 1).

Eighth International Scientific Conference "June 5th - World Environment Day"

Table 1: Time of the successful experimental hatchings of large branchiopods from soil samples: A - site in the flood defence zone; B - site in the outside the flood defence zone.

Experimental sites	The beginning of the experiment	Completion of experimental hatching
A1	23.04.2021.	27.05.2021.
A2	10.06.2021.	30.06.2021.
A3	16.04.2022.	25.05.2022.
B	16.04.2022.	22.05.2022.

3. RESULTS

Hatching took place and the adults were collected both in 2021 and 2022. The adults belonging to five taxa, one from the order Anostraca and four from the order Spinicaudata were collected after about one month (Table 2).

Table 2: Results of experimental hatchings from soil samples:
A - site in the flood defence zone and B - site outside the flood defence zone.

Taxa	A1	A2	A3	B
Anostraca				
<i>Branchipus schaefferi</i> Fischer, 1834		+		
Spinicaudata				
<i>Cyzicus</i> Audouin, 1837				+
<i>Leptestheria dahalacensis</i> (Rüppell, 1837)		+		+
<i>Eoleptestheria ticinensis</i> (Balsamo-Crivelli, 1859)	+	+	+	
<i>Limnadia lenticularis</i> (Linnaeus, 1761)		+	+	

Cyzicus sp. Audouin, 1837 and *Leptestheria dahalacensis* (Rüppell, 1837) were collected in May from the dry samples from the seasonally flooded area. *Eoleptestheria ticinensis* (Balsamo-Crivelli, 1859) and *Limnadia lenticularis* (Linnaeus, 1761) were also collected in May, but from drought samples from outside the flooded area. Several individuals hatched and were collected in June: *E. ticinensis*, *L. lenticularis*, *L. dahalacensis* and *Branchipus schaefferi* Fischer, 1834. Some individuals hatched from soil samples can be seen in Figure 3.

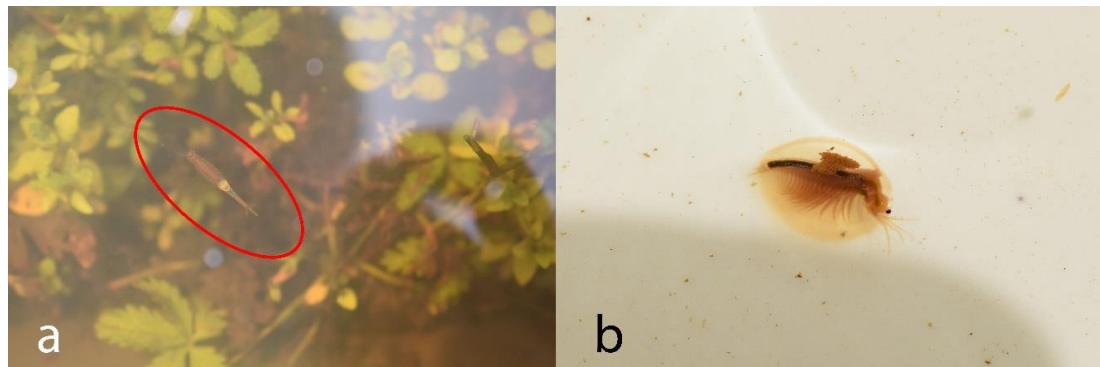


Figure 3: Adults of the hatched large branchiopods: *B. schaefferi* (a) and *L. lenticularis* (b).

4. DISCUSSION

Large branchiopod crustaceans generally favour small and eutrophic ponds, especially those with complex macrophyte growth. The spatial and temporal distribution of this group is not only influenced by the conditions in each individual habitat, but rather by the combination of a number of hydrological, climatic and biotic factors in a larger area. The survival rate of large branchiopods under such conditions depends on the ability to hatch the eggs in a timely manner. A certain percentage of dormant eggs are always waiting for the next watering, creating a kind of reserve ('egg banks'). This can allow the population to recover after a long dry period or a series of unsuccessful hatching runs (Mura, 2004). This phenomenon is interpreted as a genetically determined, stabilised adaptation to the unpredictability of ephemeral habitats, which reduces the likelihood of all offspring dying before reaching sexual maturity (Beladjal and Mertens, 2017). Although the large branchiopods are the least known of all inland water macroinvertebrates, they are considered the flagship group for these ecosystems in general (Colburn, 2004). They can also be used as bioindicators of environmental conditions to assess the quality and function of temporary wetlands.

The natural water bodies in the "Bardača" region are heavily impacted by agricultural and urban expansion, similar to many wetlands in Europe, which are severely threatened (Boix et al., 2012; Janssen et al., 2016). Although they play an important role in regional biodiversity, these wetlands, like the other areas of high ecological value in Europe, have been mostly undervalued (Downing, 2010). Recently, there have been some attempts to promote little-known habitats in Austria, which are recognised as important areas for a variety of highly interesting species. New temporary water bodies were constructed near Vienna and the 'egg bank' and aquarium-breeding tadpole shrimp *Triops cancriformis* (Bosc, 1801) was released in the area, as the species had disappeared from the region since 1952 (Eder, 1999). The management measures recommended to maintain the reintroduced population in an irregularly flooded meadow were permanent grazing or mowing shortly before flooding with removal of the cuttings. Direct sunlight and thus the necessary warmth and primary production are also seen as the favoured maintenance measures (Eder, 1999). Such similar measures could be prioritised in large parts of the "Bardača Wetland Ramsar site, especially in the areas that have been drained and used as farmland. The open areas of this site favour species associated with sunny habitats, such as *L. dahalacensis*, *E. ticinensis* and *L. lenticularis*.

Together with habitat fragmentation, eutrophication is the most widespread impact in ephemeral aquatic habitats and usually leads to a significant loss of complexity with negative effects on biodiversity (Meerhoff and González-Sagrario, 2022). Recently, there has been an initiative for the conservation actions and measures necessary for the management of endangered habitats and species. According to the Conservation Actions Classification Scheme, various measures are proposed for species recovery, species reintroduction and ex situ conservation, such as artificial propagation, a genome resource bank and other mediations by human activities (IUCN 2025).

Eighth International Scientific Conference "June 5th - World Environment Day"

Although the scientific results presented in our study are rather preliminary, they show that rearing specimens from the 'egg banks' offers good prospects and significant potential for species recovery and reintroduction in the area of the "Bardača Wetland" in Bosnia and Herzegovina.

5. CONCLUSION

Nowadays, the risk of extinction of local habitats and species is increasingly high and requires continuous monitoring and protection, especially because temporary water bodies are still neglected by specialists and landowners (Blaustein and Schwartz, 2001). Several habitats for large branchiopod taxa have been found in the "Bardača Wetland" that are suitable for future restoration of natural processes. This work helps to better recognise the "Bardača" as a natural area that could still recover if better management and conservation measures, such as the use of dried soil with dormant eggs, were implemented in the future. Such conservation measures could improve land use while adequately protecting the large branchiopods and other groups and their habitats.

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BIOAVAILIBILITY OF LEAD IN SOIL SOLUTION

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Key words: lead, bioavailability, soil

ABSTRACT:

The application of different methods could provide the possibility for determination smaller amounts of soil constituents. According to that DPASV (Differential Pulse Anodic Stripping Voltammetry) method has been used to determine lead concentration in soil and other soil parameters, such organic matter, pH value, etc., and using available software to determine the its bioavailability in soil.

The aim of this research was to develop a model that can predict the release of lead (Pb) from soil in solution under the influence of various factors such as pH, organic matter content, soil texture and lead concentration.

The research results show that soil pH and organic matter content have a significant influence on release of lead, while soil texture may act as a moderating factor. Developed model shows high accuracy in predicting lead release under different soil conditions. These findings provide useful information for managing soil lead contamination and for developing strategies environmental protection.

1. INTRODUCTION

Detection of lead after many years showed, it is really dangerous pollutant. The research of other many scientists performed until today, has showed, the soil close to roads, industrial areas is very polluted [14]. Reactions acting on phase soil solution and solid are presumably determined and constrained by soil properties: soil texture, content of organic matter, content and type of clay

minerals and Al, Fe and Mn oxides; and prevailing physicochemical conditions in the soil: soil saturation, soil aeration, pH, and redox potential.

Plants accumulate the lead in non-soluble forms with organic matter, via root, from soil solution, but also from air via leaves. Big concentrations of lead in soil has less influence on its content in edible parts of plants, with exception of roots [13]. A key factor in the solubility of lead is the pH of the soil. The soil acidity is used as indicator of lead intake by plants, but usual level of acidity for agricultural soils, show that there is not significant influence on lead intake by plants [7]. Lead is molecularly sticky in that it forms Pb complexes with organic matter, sorbs on clay and oxide particles, and precipitates as carbonates, hydroxides, and phosphates. Since lead bonds strongly with soil minerals and organic matter, it is difficult for plants to extract it from the soil and into its roots [8].

For calculation of the Pb balance in soil, knowledge of the extent of Pb uptake by plants is required. Extractable Pb in soil is known to correlate with Pb in plant tissue, and a linear relation between Pb in soil and Pb in plants has been reported [1]. Once lead is absorbed by the plant, it complexes with plant nutrients limiting its ability to be translocated to the harvestable shoots. Soil pH is a significant parameter in the uptake of metal contaminants. This is a result of the fact that the soil pH value is one of the principal soil factors controlling metal availability [9]. By maintaining a moderately acidic pH in the soil through the use of lower pHs the lead in soil has a greater potential to translocate from a plant's roots into its shoots. However, it is essential to keep in mind that the ideal pH range for the growth of most plants and grasses is from 5.0-8.0. Thus, a pH around 5.0 seems to be an optimum pH level, since lower values may inhibit plant growth. Increasing Pb solubility increases the chance for it to enter the groundwater. In addition, the increased bioavailability to plants also makes it easier for Pb to enter the food chain by being ingested by animals eating the plants.

From 1990 to 2015, declining background deposition of Pb could be demonstrated by Pb-monitoring in earthworms and in moss species [3].

Organic matter in soil which consists of humic and fulvic acids also influence on Pb availability. Modeling by Visual MINTEQ uses defined parameters such as concentration, ionic conductivity and pH of the sample solutions to analyze the free metal ion and bound-metal distribution as a function of pH.

Heavy metals are more accurately referred to metals and metalloids that are stable and have density greater than 5 g/cm³ such as Cu, Co, Zn, Ni and Pb as discussed by some authors [4], [9] have reported that the different forms i.e. chemical, physical and biological, determines the distribution and fate of heavy metals in the eco-system. Metal ions can form insoluble and soluble complexes at different pH and this in turn determines whether they will be up-taken by plants or not.

The toxicity of heavy metals is known not only to depend on the concentration, but also the physicochemical forms in which metals appear in soil. The different molecular forms of metals

interact with different chemical components of the soil and are redistributed differently in the environment. This relationship determines the mobility, bioavailability, and the toxicity that heavy metals impart on the ecosystem as described [6].

Some of the examples of computer programs for metal speciation modeling include WHAM, PHREEQ and Visual MINTEQ. These programs can accurately predict metal speciation and thus provide important and quick information about the behavior and fate of a metal in a medium. As reported in [3] [12] [13] the models use the metal binding ability of ligands and soil components to calculate metal speciation.

Gustafsson [6] developed Visual MINTEQ is a freeware chemical equilibrium model that can be used to calculate metal speciation, solubility equilibrium, sorption and toxicology, of metals in aqueous sources. The program applies known thermodynamic relationships among the chemical forms to predict equilibrium distribution. In this work, Visual MINTEQ was used to estimate metal speciation of field samples collected from mining area on the Copperbelt. The metal speciation (soluble or insoluble) was determined by finding the total metal concentration, the pH and the ionic strength of the soil solution samples. For this work, lead was chosen because the mining and agricultural activities produce this heavy metal as waste.

2. MATERIALS AND METHODS OTHER CHAPTERS

Preparation of samples, including soil and fresh vegetables was performed by conventional methods of digestion; dry burning and wet mineralization. Lead was determined by simultaneous and individual analyses. Standard solutions for heavy metals determinations used in experiment are suprapure chemicals produced by Merck.

Analyses of lead were performed in various supporting electrolytes, including, KNO₃, KCl with the following instrumental parameters:

Working Electrode (WRK): HMDE; Referent Electrode (REF): Ag/AgCl (1 M KCl), $E = 0.22$ V,
Potential: Initial from -0.65 V to -0.6 V and final from -0.4 V to 0.3 V.

Deposition Time 300 s in samples,

Speed of Scanning 2 mV/s,

Deaeration Time 120 s

Amplitude 0.05 V Equipment:

Electrochemical Cell, Princeton Applied Research (EG & G) model 303A with Working (HMDE), Referent (Ag/AgCl) and Supporting (Pt-wire) Electrode, Potentiostat/Galvanostat, PAR, model 263 A, Computer P II with software Model270/250 Research Electrochemistry Software, version 4.3.

3. RESULTS

In the soil samples sampled from the area of Sprečki polje, the average moisture content was 5.57%, while the average lead content was 3.78 mg/kg of moist soil. The following picture shows an example of a voltammogram that is rejected as a result of lead determination using the DPASV method.

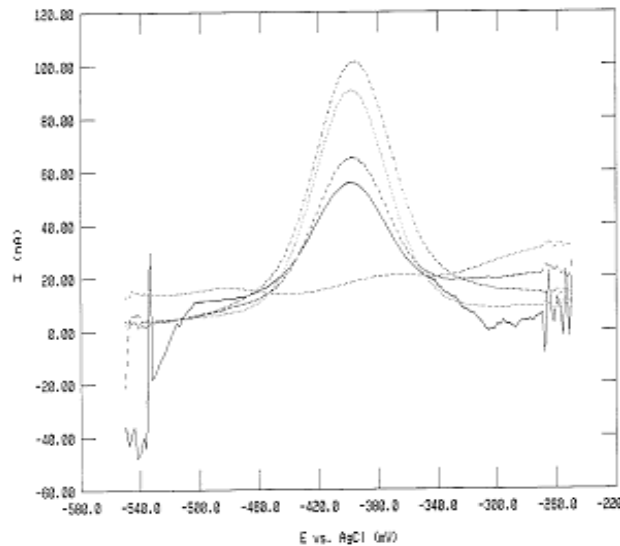


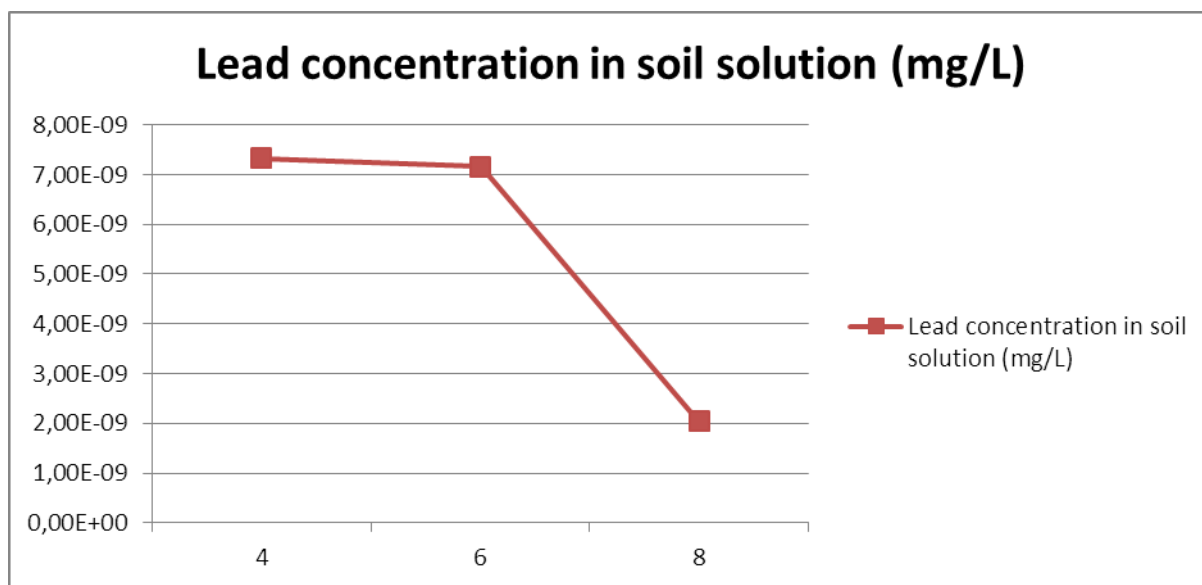
Figure 1: Voltammogram of lead determination in soil samples

The following table shows the data on the concentration of free and bioavailable of lead in soil solutions using the commercial available software e.g. Visual Minteq, version 3, <https://vminteq.com/download>

Table 1: Concentrations of aqueous inorganic species (mg/L), pH=4

Forms of lead	Concentration at pH = 4	Concentration at pH = 6	Concentration at pH = 8
H+1	0,000101	1E-06	1E-08
OH-	1,02E-10	1,01E-08	1,01E-06
Pb(OH)2 (aq)	5,71E-18	5,74E-14	1,63E-10
Pb(OH)3-	5,8E-25	5,79E-19	1,65E-13
Pb+2	7,33E-09	7,16E-09	2,04E-09
Pb2OH+3	2,17E-19	2,05E-17	1,66E-16
Pb3(OH)4+2	4,78E-33	4,71E-25	1,09E-18
Pb4(OH)4+4	3,75E-38	3,39E-30	2,22E-24
PbOH+	1,81E-12	1,81E-10	5,14E-09

Table no. 1. Presents the data of soil solution when there is lead and hydrogen ions in it, and lead is present in ions form and as hydroxide. It has been seen that the concentration of lead is different and it changes with the change of pH soil solution. The highest concentration of Lead ion is at $\text{pH} = 4$, $7,33 \cdot 10^{-9} \text{ mg/L}$, but the lowest at $\text{pH} = 8$, $2,04 \cdot 10^{-9} \text{ mg/L}$. In order to have better view of the dependance of ions of lead in soil solution, it has been presented on the following graph.



Graph 1: The change of Lead concentration in soil solution (mg/L) depending of pH of soil, obtained by Visual Minteq, version

As it has been seen, there is very small change in free Pb^{2+} in soil solution, when pH is changed from 4 to 6, and the concentration of lead is still high and very bioavailable to plants roots. The graph no. 1. Shows that the change of bioavailability of lead in soil solution is much less when pH is changed from 6 to 8. This change indicates that the lead is connected to soil either to organic or inorganic particles.

3. CONCLUSION

Based on results, the following could be concluded

- pH value of soil is very important to lead bioavailability and it is the main factor for the lead intake control;
- when total concentration of lead in soil is higher than it is allowed by valid regulation in some countries, does not mean that soil could not be used for agriculture production;
- in some situation, commercially available software could be used to predict the species of heavy metals and its forms in soil and solutions;
- regulating of pH of soil and its organic matter is oftenly important then other parameters, particularly those which regulate its fertility.

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Eighth International Scientific Conference
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THE AESTHETIC POTENTIAL OF THE LEDENICA CAVE AS A FACTOR IN THE PROTECTION AND VALUATION OF THE KARST ECOSYSTEM

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ABSTRACT:

The Ledenica cave is a well-known underground form of the karst ecosystem, a speleological object, and one of the five Resanovacke caves. Due to its exceptional natural beauty, a wealth of ornaments, other functions, and general importance, it has been investigated through passive (examining the cave's physical characteristics) and active research (implementing interventions in the cave system) for tourist development.

The paper aims to analyze Ledenica's aesthetic potential as an essential factor in protecting and evaluating the wider karst area.

Based on the interpretation of previous research and observations in the field, an analysis of the morphological and aesthetic characteristics of the cave was carried out concerning the existing infrastructure and the experience of the cave environment. The general condition and threat of this protected "natural rarity and natural phenomenon" were discussed. The research covered the determination of the current level of tourism valorization and the perspective of the concept of sustainable use while considering that the primary goal is the protection of expressed specific features and associated biodiversity and habitats.

The research results underscore the crucial role of recognizing the Ledenica cave's aesthetic potential as a valuable resource within the framework of integral management and protection of the karst ecosystem. The adequate evaluation and presentation of this geological form's aesthetic features can contribute to its sustainable use and foster awareness of the importance of

Eighth International Scientific Conference "June 5th - World Environment Day"

preserving karst landscapes, instilling a sense of responsibility and motivation for environmental conservation. It can also bring economic benefits.

1. INTRODUCTION

Caves are among the most visited geological sites in the world, attracting over 70 million people each year to more than 1,200 caves worldwide, with ticket revenue alone of up to €800 million. The global show cave employs approximately 25,000 people directly (management, guides) and at least 100 times more people if we consider related tourism activities (souvenir shops, local transport, travel agencies, restaurants, and bars) (Chiarini et al., 2022).

As a factor of protecting and valuing the karst ecosystem in general and as a potential for rearrangement of the cave for a tourist visit, the paper discusses the aesthetic potential of the Ledenica cave.

Considering that during the sixties of the 20th century, the Ledenica cave was formally and legally protected as a "natural rarity and natural phenomenon," and as one of the most interesting karst objects in this area, it was proposed to use it exclusively sustainably.

The investigated part of the Ledenica cave is part of a more extensive cave system built by the water sinkholes of the Resenovačko polje¹. It consists of one scattered descending cascade channel oriented mainly from south to north and shorter blind channels. In the cave, one encounters elevations and expansions alternately filled with layers of clay and calcite deposits. Calcite formations, in addition to stalactites and stalagmites, are smaller pools filled with clear water distributed along the bottom of the cave. Inside the cave, there are depressions of different depths and diameters.

However, this speleological object's main specificity and outstanding value is the concentration of cave jewelry, especially jewelry of large dimensions, high quality, and huge volumes compared to the total volume of the explored underground.

Therefore, the aesthetics of the Ledenica cave were singled out and primarily analyzed, not excluding the importance of other types of evaluation of the geodiversity of similar karst systems.

2. MATERIAL AND METHODS

For this paper, different research methods were used: the method of collection, analysis, interpretation, and application of previous research, which included theoretical and empirical,

¹ The explored section has a length of 697.5 m.

Eighth International Scientific Conference "June 5th - World Environment Day"

professional and scientific literature from geography, geology, geomorphology, speleology, environmental protection, and tourism.

In addition, field research was carried out, which can be understood as "a formal process of research and study of the geographical space that takes place outside the usual workplace and which uses the geographical space as a source of knowledge" (Vojnović, 2016). Direct field research included methods of observation, field sketching, photography, and interviews.

During the field research, the aspect of the once-valued tourist site was considered with all its spatial and temporal complexity. This includes the natural and social attractiveness of the locality, with particular emphasis on the protected natural area and with the aim of the current level and sustainability of tourist valorization, with the allocation of new measures respecting the concept of sustainable tourism (Vojnović, 2016).

The observational method enabled direct observation of the current situation, which gave the researchers rich primary experience and insight into the topic. The sketching method was used to record relevant indicators within the speleological object in the observed area, confirmed from earlier field research. Existing photos of the cave objects were analyzed and compared with newly created photos, facilitating processing material collected from the field. The interviews relevant to nature conservation and tourism issues were included in the open interview method.

The field research was carried out through three stages. The preparation stage consisted of collecting literature and sources, collecting photographic equipment and equipment for visiting the cave, organizing preparations for the trip, and agreeing with the local guide. The stage of conducting the research included a one-day visit to the site and place and a tour, and the final stage was detailed analytical-synthetic procedures with the purpose of processing data collected during field research to produce scientific and research work, including the production of maps.

3. RESULTS

The diverse concentration and arrangement of cave jewelry and the appearance inside the halls form the essence of the aesthetic impression. The overall aesthetic value includes the position of the cave, its length, width, and height, the floor's structure, the parent rock (substratum), the genesis of the cave, the surrounding space on the surface, and anthropogenic landscaping.

The Ledenica cave abounds in rich cave jewelry that differs in type, dimensions, and color. It is dominated by tall and massive forms. There are saber-shaped and spindle-shaped stalactites, cave columns made of colorful draperies and stalagmites of ornate forms, bell canopies, remains of inter-floor constructions, coral jewelry, rimestone and calcite tubs with coral structures filled with transparent water, blocks fallen from the ceiling covered with slag and calcite, as well as other forms.

The colors of cave jewelry range from black and gray, through shades of ocher, to snow-white. Of particular value and exceptional natural rarity is sparkling jewelry made of crystalline calcite,

regardless of the primary color of the jewelry, so that it has snow-white, dark, and reddish surfaces.

In addition, we found that the essential aesthetics of the Ledenica cave are the names of the cave forms and phenomena related to the social changes that happened over time. Professor Radenko Lazarević, a respected geomorphologist-speleologist, investigated the cave in detail. He named all cave formations using the mystical approach to the nature of the cave. Therefore, this particular karst phenomenon's aesthetic mark and value was essential and crucial to its preservation.

4. DISCUSSION

4.1 Aesthetic characteristics of the Ledenice cave

Ledenica is one of the rare caves for which passive and active research were carried out as early as 1978 and for which a development project was implemented even then. Of the five caves in the area Resanovci, on the northwestern slopes of Stražbenica (Skakića cave, Ledenica, Čadava cave, Water cave, and Struga abyss), Ledenica was the only speleological facility equipped for tourism that still contains anthropogenic infrastructure. Due to the exceptional morphology and beauty of the cave jewelry, the cave's luxuriousness impressed many tourists.

"At Ledenica, the observer is surrounded by jewelry; jewelry is in front, behind, up, down. You begin to feel like a part of a beautiful world, confused, it seems, if you do not move immediately - you will remain there in eternity" (Lazarević, 1979; Vuković et al., 2023).

Although the infrastructure of the cave is damaged and neglected today, and visits are carried out spontaneously and unorganized, we have confirmed that there is a basis for protection and its adequate use.

4.1.1 Morphospeleological units of Ledenice

The morphospeleological units of the cave are rich galleries of natural art created through a long geological history. The forms of karst erosion are part of the karst hydrographic system in limestone terrains, which are exceptional about the entire explored land surface.

The formation of the hydrological-speleological system began about 15 million years ago. It lasted until 2-3 million years ago, during the lake phase in the evolution of the valley of River Unac. Along with the development of river valleys and the bringing of lake sediments into the basins, the karst process develops.

The intensive formation of cave jewelry in Ledenica began after the end of the active hydrographic phase and lasted throughout the entire upper Pliocene and Pleistocene until today.

Eighth International Scientific Conference "June 5th - World Environment Day"

Due to the nature and complexity of its creation, this karst phenomenon must be protected as a comprehensive above-ground and underground system with all channels, bedrock, and the surface through which water is drained.

The explored part of the Ledenice cave represents only a part of a vast cave system, which is supposed to be one of this region's most extended cave systems.

In the cave, the morphospeleological halls with authentic names assigned during the research in the 1980s can be distinguished. Due to the fact that the names themselves represent and describe the aesthetics of the cave, the names of the halls and cave jewelry are presented through original names.

Table 1: Dimensions of the morpho speleological units of the cave

Hall	Length (m)	Length - side channels (m)	Width (m)	Height (m)	Surface (m ²)
1 - Ulazna dvorana	45	-	-	-	646
2 - Tamni vilajet	132	54	45	25	2875
3 - IV krajiške brigade	40	14,5	8 - 20	1 - 7	538
4 - Mramorna dvorana	48,1	17,9	5 - 18	1 - 6	674
5 - Šarena dvorana	32,6	12	6 - 18	1 - 6	538
6 - Dvorana krajiških heroja	29	14		12	486
7 - 27. juli 1941	34	-	6 - 18	2 - 9	450
8 - Dvorana stogova	46,1	7,1	4 - 28	0,8 – 10,5	710
9 - Jezerska dvorana	24	-	16	1,5 – 7,0 (15,0)*	321
10 - Visoka dvorana	28,8	-	2,5 - 14	2 – 5,3 (25)*	281
11 - Mala ponorska dvorana	23	4	4 - 9	1,7 – 7,5	196
12 - Mlada Bosna	36,9	13	17	14	605
13 - Sala stalaktita	23,5	8	10	4	157
Cave Ledenica	564	133,5			8477

* height of one part of the cave; Source: Lazarević, R. (1979)



Map 1: Morphospeleological units of the Ledenice cave and arrangement of cave decorations (made by Kodžo D.);
source: Vuković et al., (2023)

„Ulazna dvorana“ (The Entrance hall) includes the space from the entrance to the iron gate called Uroš's gate. The entrance hall is reached by a concrete path between beech trees and limestone blocks overgrown with moss on the steep slope of a sinkhole, 50 - 60 m in diameter and 23 m deep.

To provide functionality, two paths were built to the cave's entrance: an entrance and an exit. The entrance to Ledenica is 10.9 m wide and 2 m high.

At a distance of some 5 - 6 m from the entrance to the cave, a stone wall with a metal gate was built to prevent direct external influences and air currents between the entrance and the viewing platform in the boiler-shaped extension on the right side of the hall. This reduced the scope of creating ice jewelry, which was formed in winter and is similar to cave jewelry in shape and dimensions.

„Tamni vilajet“ (The Dark Vilayet) is the largest hall in the explored system of Ledenica and is one of the largest halls in caves in this region. It got its name from the dark color of the jewelry and the unusual shapes and dimensions of cave jewelry that evoke the atmosphere from a fairy tale. The walls of this hall are decorated with long multi-colored draperies, the height of which sometimes exceeds 15 - 20 m. Long stalactites appear on the ceiling.

In the middle of the hall stretches an elevation of blocks on which large dark and white jewelry, with an intense shimmer, is formed.

In the hall of the „Tamni vilajet“, the forms called „Baš-čelik“ and „Karavan“ (Caravan) stand out among the individual compositions of the cave jewelry. „Baš-čelik“ is a stalagmite group in the form of a massive head with large ears, natural eyes, raised black eyebrows, and a body immersed in blocks in shackles. The head has a diameter of about 8 m. Here are the most significant forms in this hall and Ledenica.

A striking massive black stalagmite, over 9 m high, located at the entrance to the „Tamni Vilajet“ is called „Stražarska kula“ (the Watchtower). The „Karavan“ is a column of stalagmites of various shapes and dimensions, with a maximum height of 5.5 m. It is at the end of the ridge, stretching in the middle of the hall. Two interesting stalagmites stand out, one of which resembles a camel, and the other resembles its guide, which is why the group of stalagmites is called the Caravan.

The hall is filled with numerous columns and stalagmites, between which there is a veritable labyrinth of corridors and passages. The height of the pillars ranges from 2-6 m. They are made of white and dark calcite that sparkles. This part of the hall is called „Začarana šuma“ (the Enchanted Forest).

Other stalagmites, figures with dominant shapes and dimensions, stand out in the Dark Vilayet hall.



Figure 1: Hall of the Tamni Vilajet with white and dark calcite stalagmites (photo, Kodžo, D.)

At the place where the „Tamni vilajet“ narrows, over the entrance cascades, you reach the section through which you descend to the next hall.

The hall of the „**IV krajiške Brigade**“ (4th Krajina Brigade) is characterized by a striking column of stalagmites and cave columns bearing the names of Krajina heroes. To the right of these stalagmites is an exciting composition of red and ochre bigra called the „Šarena česma“ (Colorful Fountain). In front of the fountain are large tubs, similar in color, which are periodically filled with water.

„**Mramorna dvorana**“ (The Marble Hall) got its name because of white calcite material and the color of marble predominates.

The second part of the hall is much richer in cave jewelry, columns, and stalagmites than the first part, where the jewelry is relatively sparse. Richly carved, thin stalagmite with a symmetrical section, whose height is 3.2 m and diameter of 0.5 m, is very impressive and is called the „Štafetna palica“ (Baton). Near here are several striking groups of connected pillars, built of glittering calcite of different colors.

„**Šarena dvorana**“ (The Colorful Hall) got its name from the colorful cave jewelry. The longitudinal profile of this hall first descends and then rises in a cascade.

Stalagmites of impressive size and attractive shape are natural rarities that adorn the cave.

„**Dvorana krajiških heroja**“ (The Hall of Krajina Heroes 6) has an approximately circular amphitheater shape, with massive columns and a stage in the middle, on which stalagmites stand out as monuments, 2.5 - 2.8 m high. The hall looks monumental, like some ancient building. In this regard, it is intended to symbolize the courage of all Krajina heroes.

„**27. juli 1941.**“ (The 27th July 1941) is a hall where the central part has a composition, a complex cave pillar, which consists of a very dynamic combination of white, sharp, calcite

Eighth International Scientific Conference "June 5th - World Environment Day"

stalagmites and slender, spar-shaped pillars, which are joined with calcite draperies and resemble flags fluttering in the wind. This composition, 6 m high, was chosen as the emblem of the Ledenica cave. The massive column opposite the emblem combines stalagmites and draperies, 7.3 m high.

Massive red cave columns and stalagmites, with calcite material peeking out from under them, towards the hall of „Krajiški heroji, “in an area with a diameter of more than 20 m, fill the hall and leave an impression of dignity, called „Hajduci, uskoki I ustanici. “ Between these pillars are large tubs filled with water up to 1 m deep. The largest bathtub is 10 m long and 4.5 m wide.

Today, Ledenica is characterized only by the dripping water, which has a seasonal regime and differs between individual halls. The drip water in the big tubs only provides water in the distant halls. To the observer, bathtubs surrounded by cave jewelry and filled with clear and calm water create a unique experience.

„Dvorana stogova“ (The Hall of Stacks) got its name from the giant stalagmites in the form of stacks and hay, 3 - 4 m high. The hall has one descending and one ascending low and difficult passage. The walls are mostly bare and rocky, and the floor is clay. Only the descending part of the hall is rich in jewelry.

„Jezerska dvorana“ (The Lake Hall) is the last larger hall in the direction of the central canal. It got its name from the lake at the end of the hall, intended for draining and digging out the submerged channel during the stage of the cave arrangement. The lake hall is poor in jewelry, the walls are rocky, and the floor is covered with a thick layer of clay.



Figure 2: Composition of stalagmites and cave columns in the hall „27. juli 1941“ (Photo, Kodžo, D.)

„Visoka dvorana“ (The High Hall) includes a higher blind canal which is entered from the Jezerska hall. The hall is relatively rich in cave jewelry. Several thin pillars stand out, up to 2 m high. There are many stalactites in the hall.

Eighth International Scientific Conference "June 5th - World Environment Day"

„**Mala ponorska dvorana**“ (The Small Abyssal Hall) is located in the side cave part, which separates from the main channel from the hall of „Krajiški heroji“. It got its name from the abyss, which is 15 m deep. This is a descending, cascading hall.

There are several cave pillars about 5 m high. Between the pillars, there is a narrow corridor which connects these two halls. A return pedestrian path was made through that corridor. The right cave wall is rocky, and at a certain point, it turns into an abyss 1.5 m deep. The left cave wall is lined with jewelry.

The hall "**Mlada Bosna**" is in the extension of the "Mala ponorska dvorana". During the research, it was called the Great Abyssal Hall (Cosmic or Martian Hall) because there are several deep abysses at its bottom.

The symbols of this hall are a broad, massive, red-colored, spherical stalagmite on top of which there are several smaller stalagmites called "1914" and a massive pillar, 8.5 m high and 1.1 m in diameter, named Gavriilo Princip. Around these massive cave forms, there are a large number of bigrene and calcite tubs filled with water.

The eastern wall of the hall is covered with the most beautiful, multi-colored, and luxurious cascading overflow, 10 m high. The front wall of the hall, facing the „**Sala stalaktita**“ (Hall of Stalactites), is characterized by long reddish cascading overflow. This is the smallest cave complex in the Ledenice system. You enter it through a narrow and low passage at a height of 4.7 m. You descend into a 3 m long lake and a low and relatively wide hall. It got its name from the abundance of long, spindle-shaped stalactites, the length of which exceeds 2 m, and the thickness ranges from 3 - 10 cm. They often reach the floor, and in some parts, they previously hid the passage to the hall. In addition to stalactites, there are plenty of cave columns and stalagmites, the height of which reaches 4 m. The floor is covered with bigram, in which bigrain tubs were formed. The hall ends with a sloping channel that has the characteristics of an abyss.

5. CONCLUSIONS

The aesthetic potential is one of the factors in evaluating the speleological site of the Ledenica cave, which had a significant influence on determining its importance and degree of protection. It is equally important as other types of geodiversity evaluation, mainly because it is impossible to restore the destroyed aesthetics of such an object of geological heritage.

In the formal legal framework, the concentration and opulence of the cave jewelry and the morphology of the Ledenice cave have contributed to the fact that it, together with its surroundings, has been protected and valued for many years as a natural rarity and natural phenomenon², a special geological reserve³, an ecozone of particular interest⁴, a locality within the area proposed for protection as forests of high conservation value with the functions of

² Rješenje Republičkog zavoda za zaštitu prirode br. 08-431-1 od 05.06.1959.

³ Zakon o zaštiti prirode (službeni list SR BiH br.4/65) – lista zaštićenih objekata prirode.

⁴ Odluka Skupštine Hercegbosanske županije (Kanton 10) o pećini Ledenici i Šatorskom jezeru.

Eighth International Scientific Conference "June 5th - World Environment Day"

exceptional ecological attributes, ecosystem production and social impacts⁵ and a monument of nature and associated features⁶.

Geological forms such as caves, with high tourist potential, are legally protected to protect expressed specific natural features and associated biodiversity and habitats.

Despite formal protection, the threat to the aesthetics of the Ledenica cave and the karst landscape has not been completely removed. The cave is threatened by mechanical (breaking of cave jewelry and collapse of infrastructure) and chemical damage (ignition of fire), but also by human ignorance of the nature of the cave system. Therefore, adequate arrangement of the Ledenice cave, adapted to a clearly defined way of exploitation, would reduce the danger of such damage and potential pressure from visitors.

Exceptional forms of erosive and accumulative relief, colors, microclimatic conditions, darkness, and gorgeous geological nature justify man's curiosity for mutual relationships and charms of the underground world. Therefore, the tourism valorization that began in the eighties, speleology tourism and aesthetics, are the basis for reactivating the tourist potential and contributing to the local community regarding economy and business.

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⁵ HCVF High Conservation Value Forests, šumsko-gospodarsko područje “Bosansko Grahovo” na teritoriji Kantona 10 u Federaciji BiH

⁶ Zakon o zaštiti prirode (Službene novine FBiH, br. 66/13)

Eighth International Scientific Conference
"June 5th - World Environment Day"

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THE IMPACT OF GENETIC FACTOR ON INDIVIDUAL NUTRITIONAL NEEDS

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Key words: genes, genetic variations, nutritional requirements, nutrigenetics, nutrigenomics, personalized nutrition

ABSTRACT:

The impact of genetic factors on individual nutritional needs is a complex area of research that underscores the significance of genetic variations in shaping an individual's response to nutrition. Genes control the body's ability to absorb, metabolize, and utilize nutrients from the food consumed. The diversity of genetic variations among individuals can result in different nutritional requirements and predispose individuals to specific diseases and dietary issues. Through the development of nutrigenetics and nutrigenomics, scientists have identified numerous genes and genetic variations that play a crucial role in determining how the body absorbs and utilizes nutrients. This knowledge opens the door to personalized nutrition, where dietary regimens are tailored to individual genetic profiles to achieve optimal well-being and disease prevention. The influence of genetic factors on individual nutritional needs also highlights the importance of moving away from the use of universal dietary guidelines. Instead, the approach to nutrition should be customized based on each person's genetic characteristics to achieve the best possible outcomes. Despite the challenges and uncertainties in this research area, the future of nutrition appears promising, as personalized nutrition has the potential to enhance the health and well-being of people worldwide.

The aim of this paper is to explore the impact of genetic factors on individual nutritional needs and provide an overview of how genetics shapes the body's responses to nutrition, with an emphasis on the development of personalized nutrition for better health and disease prevention.

1. INTRODUCTION

Genetic variation is a fundamental characteristic of human biological systems. These variations are responsible for various aspects of human physiology, including appearance, metabolic processes, ability to defend against disease, and the like. Genetic variation can be observed at different levels, including individual genes, entire chromosomes, and entire gene sets. Each of these variations can have different effects on human health and well-being. Genetic variation is a product of evolution and patrimony, and it is the key off understanding why people are different in their responses to the environment, including diet. Genetics and nutritionism are fields that have developed more and more in recent decades, providing a deeper understanding of the bond between genes and nutrition. This interdisciplinary field, known as nutrigenetics and nutrigenomics, investigates how genetic variation in individuals affects their response to diet, metabolic processes, and susceptibility to certain diseases. Understanding how human genes affect diet can be significant from the perspective of chronic disease prevention and treatment, personalized nutrition, and health optimization.

2. THE METHODOLOGY OF WORK CREATION

Relevant literature from the field of research on "The impact of genetic factors on individual nutritional needs" was reviewed. Literature was found in academic databases: ScienceDirect, PubMed, Web of Science. Using methods of analysis and synthesis of relevant data on key chronic non-infectious diseases through the framework of nutrigenetics and nutrigenomics, more significant aspects of the possibility of personalized nutrition were observed.

3. NUTRIGENETICS AND NUTRIGENOMICS

Nutrigenomics is a research science that studies how nutrition affects the genome and gene expression, while nutrigenetics is an applied science that studies how genotype determines an individual's response to nutrition and susceptibility to disease. According to the original conception of the author Brennan, nutrigenetics refers to the interaction between nutritional and genetic factors that may play a role in the etiology of disease. Efforts are aimed at determining increased risks of occurrence and development of chronic non-infectious diseases such as cardiovascular diseases, diabetes or psychiatric disorders, and by applying the genome-wide association studies (GWAS); linking environmental factors and genetic variation (Novaković et al. 2014). Nutrigenetics works on how an individual's genetic makeup affects the phenotypic response to food intake. Nutrigenomics, on the other hand, focuses on gene expression with respect to nutrition. Nutrigenomics aims to investigate the impact of diet and nutrition on gene expression, particularly through epigenomic (e.g. histone methylation), transcriptomic (e.g. RNA transcription), proteomic (e.g. protein synthesis) and metabolomic (e.g. metabolite synthesis) high-throughput analysis (Simopoulos and Milner, 2010).

Through mutual interactions, nutrigenetics and nutrigenomics complement each other as a specialty of nutrigenetics/nutrigenomics to provide an overall picture of an individual's integrated metabolism, with the aim of constructing a personalized diet to avoid diseases and possible health risks and to promote health and longevity as well (Dimitrov, 2011).

3.1 Principles of nutrigenomics and nutrigenetics

The basic principles of nutrigenomics and nutrigenetics refer to the influence of macro and micronutrients on the human genome, causing changes in its structure and gene expression, which may have implications in the occurrence, frequency, progression and severity of the disease. In addition, under certain circumstances and in some individuals, nutrition can be an important risk factor for the development of numerous diseases. The extent to which diet affects on balance between health and disease may depend on an individual's genetic framework. Ultimately, nutritional intervention is based on knowledge of the nutritional status and needs of the individual, as well as the genotype, and can be used for the prevention, mitigation or treatment of chronic diseases (Novaković et al. 2014).

3.2 Personalized nutrition as a goal of nutrigenetics and nutrigenomics

Understanding how human genetic profiles affect nutritional needs enables the creation of personalized nutritional strategies that can improve quality of life and reduce the risk of chronic diseases (Corella, 2018). If the human population were genetically identical and lived in a constant environment, then the human response to diet and medication would be equivalent; however this is not the case. Inter-individual variability in response to dietary interventions is a well-known phenomenon in nutrition research and practice. Thus, the effect of dietary changes on phenotypes such as blood cholesterol level, body weight, and blood pressure can vary considerably between individuals. There are many factors that can influence dietary response, such as age, sex, physical activity, smoking and genetics (Carlberg et al. 2016). Attempts are being made to develop personalized dietary guidelines for individuals and specific subpopulations (Moschonis et al. 2019). The goal of personalized nutrition is to identify individuals who benefit from a specific nutritional intervention (responders), and to find alternatives for those who do not (non-responders). This alternative approach to nutrition is considered more effective compared to unnecessary diets that are considered unpleasant and ineffective. Personalized nutrition can also be useful in the prevention and treatment of chronic diseases by adapting dietary advice to the unique genetic profile of each individual (Kohlmeier, 2012).

3.3 Metabolomics in the field of dietetics

Metabolomics is an area of functional genomics that studies changes in metabolites, and its goal is to isolate and characterize them. The metabolome consists of the set of small primary/secondary metabolites and body fluids of an organism or species.

In the field of nutrition, metabolomics enables the understanding of metabolic changes and disorders that are caused by or susceptible to the influence of nutrition. This contributes to the understanding of how an excess or deficiency of certain nutrients or compounds (secondary metabolites) present in food can affect the health/disease of an individual. These compounds (whether nutritional or not) interact in different ways within the body, changing the metabolic pathways of the metabolome. As an example, peryl alcohol (a monoterpene extracted from strawberries) can act as an anticancer molecule under certain organic stimuli (Weckwerth, 2007). Metabolomics is the study of the total number of endogenous and exogenous metabolites in a cell, organ or body fluids and is a useful tool for generating individual metabolite profiles, such as complete plasma lipid profiles (e.g. cholesterol, triglycerides) and vitamins.

4. INFLUENCE OF GENETIC ERRORS ON NUTRIENT METABOLISM

Metabolic processes such as digestion, absorption, and excretion are selectively affected by many different inborn errors of nutrient metabolism. More than 200 such disorders have already been described: lactose intolerance; malabsorption of glucose-galactose; familial hypercholesterolemia; hypophosphatemic rickets and others. These disorders can lead to a lack of essential nutrients despite adequate food intake; which can result in unfavorable conditions such as chemical toxicity (by blocking the catabolic pathway necessary for the metabolism of the ingested nutrient) or interfering with the formation of the necessary product from the ingested nutrient.

Genetic factors play a key role in the process of absorption of nutrients from the digestive tract. The genes responsible for the synthesis of enzymes and transport proteins in the intestines affect the ability of the body to absorb macro and micronutrients. Genetic variations in these genes can result in different absorption rates in different individuals. Genetic variations can affect the enzymes involved in carbohydrate and fat breakdown, resulting in variations in energy balance and fat storage. Genes also influence the level and function of proteins that have roles in the regulation of glucose and insulin, and also influence the absorption, transport and metabolism of micronutrients (Ross et al. 2014):

- Absorption of iron from food is a complex process. The genes encoding the proteins ferritin and transferrin affect the storage and transport of iron in the body. Genetic variations of these genes can lead to altered levels of iron in the body and the risk of iron deficiency anemia.

- Calcium is a key mineral for bone and muscle health. Genetic variations in the genes responsible for the transport of calcium across cell membranes in the intestines can affect absorption efficiency, which means they can result in variations in calcium levels in the body.
- Genes can affect the enzymes that are responsible for the conversion of inactive forms of vitamins into their active forms. The absorption of vitamin D, which is crucial for bone health, depends on the presence of genes that code for enzymes in the liver and kidneys to convert inactive vitamin D into an active form. Genetic variations of these genes can affect the level of vitamin D in the blood.
- Vitamin B₁₂ is necessary for the normal functioning of the nervous system and the formation of red blood cells. Absorption of vitamin B₁₂ from food requires the presence of certain genes, including a gene known as IF (Intrinsic Factor). Genetic mutations that affect IF can result in disturbances in the absorption of vitamin B₁₂ and the risk of deficiency (Hansen, 2011).

5. NUTRIGENOMICS IN DISEASE ETIOLOGY AND SPECIFICATION OF NUTRITIONAL REQUIREMENTS

5.1 Gene polymorphisms associated with heart diseases with aspect on specific nutritional needs

A major locus on chromosome 9p21 was identified for coronary heart disease. The 9p21 variant is a new risk factor for coronary heart disease and cerebrovascular disease, independent of known risk factors such as diabetes, hypertension, cholesterol and obesity. This information implies a new mechanism for coronary heart disease related to vascular pathology, perhaps from the lipid hypothesis (Willis, 2020). Also, genetic variation in 5-lipoxygenases (5-LO) play a key role in an individual's susceptibility to coronary heart disease, and the interaction between omega-6 and omega-3 fatty acids may be essential to understanding and prevention of this serious heart disease (Artemis and Leslie, 2003). 5-LO is an enzyme that participates in the metabolism of arachidonic acid and the production of inflammatory compounds. Studies have shown that people with certain genetic variants in the 5-LO gene may have an increased sensitivity to increased intake of omega-6 fatty acids, which for these people means a higher risk of inflammation if they consume large amounts of these fatty acids.

The level of omega-6 and omega-3 fatty acids in the body can be determined by genetic variations in the FADS1 and FADS2 gene clusters. Studies have shown that omega-6 fatty acids can stimulate inflammatory processes in the body, which is an important factor in the development of atherosclerosis, the basic pathology in coronary heart disease. On the other hand, omega-3 fatty acids have an anti-inflammatory effect and can reduce inflammatory reactions. Thus, some individuals may be more susceptible to atherosclerosis due to their genetic

predisposition that promotes increased intake of omega-6 fatty acids, while others may have better protection against coronary heart disease due to their ability to exercise better the same omega-3 fatty acids (Artemis and Leslie, 2003).

5.1.1 APOE gene polymorphisms in the development of heart disease and nutritional specifications

The Apolipoprotein E (APOE) gene is the gene that codes for apolipoprotein E, a protein that plays a key role in lipid metabolism and lipoprotein transport in the body. The APOE gene plays a significant role in cholesterol metabolism and affects its level in the blood, and it also plays an important role in the regulation of inflammation in the body and the way the body processes lipids from food.

The APOE gene is located the chromosome 19 and exists in several different variants, the best known of which are APOE ϵ 2, APOE ϵ 3 and APOE ϵ 4. These are gene variants that affect the risk of various diseases, including cardiovascular disease and Alzheimer's disease. Each of these variants has a different effect on lipid metabolism and the way the body manipulates cholesterol. People with the APOE ϵ 2 gene variant have a lower risk of cardiovascular disease, but an increased risk of Alzheimer's disease. APOE ϵ 3 is considered the reference variant of this gene and it is also the most frequently represented variant. Its presence usually does not increase or decrease the risk of disease. People with the APOE ϵ 4 variant have an increased risk of cardiovascular disease and Alzheimer's disease. Thus, people at high risk of Alzheimer's disease can consider specific measures to preserve a healthy brain, while people at high risk of cardiovascular disease can adjust their diet and lifestyle to reduce that risk (Brigelius-Flohé and Joost, 2006).

5.2 Nutritional needs of people with phenylketonuria

Phenylketonuria (PKU) is a disease caused by mutations in the gene for the enzyme Phenylalanine hydroxylase (PAH) which converts phenylalanine into tyrosine. PKU was the first "inborn error of metabolism" to respond to dietary treatment, using a low-phenylalanine diet regimen for nutrigenetic treatment. Genetic variations in phenylalanine hydroxylase can lead to a reduction of its activity or insufficiency of this enzyme, which leads to elevated concentrations of phenylalanine in the blood, which crosses the blood-brain barrier and damages brain function. When phenylalanine is broken down by other metabolic pathways, phenylketones are formed, which are also neurotoxic so rooms with a PKU can develop neurological damage (severe mental retardation and seizures) (Marinović, 2019).

Dietary measures for people with a PKU refer to diet with a low intake of phenylalanine and taking supplements of other amino acids that enable normal development of the child. In this

context, it is necessary to detect PKU by newborn screening in order to start a child with a low phenylalanine content as early as possible.

5.3 Interactions of genes and nutrients in the etiology of cancer

Twin studies show that the probability of identical twins developing the same cancer is less than 10%, suggesting that environment plays an important role in cancer susceptibility. Evidence of genome and epigenome damage as biomarkers, in the absence of exposure to genotoxins, are themselves sensitive indicators of micronutrient deficiencies required as cofactors or components of DNA repair enzymes. Diet is considered a source of carcinogens present in certain foods and their ingredients. Some food components, such as phytoestrogens, are known to be processed by the same metabolic pathways as sex hormones, so their action can be modulated by polymorphisms (George et al. 2011). Molecules present in contaminated food can create toxic metabolites that can interact with DNA, changing its structure and causing mutations. This is the case with aflatoxin B₁, which creates an additional compound capable of binding to the N-7 position of guanine residues, creating a new product. This new molecule then breaks the interaction between the sugar and the nitrogenous base of the nucleotide, and on this basis can cause severe liver damage; necrosis, cirrhosis and cancer.

Previous research has shown that deficiencies in micronutrients such as folic acid, vitamins B₁₂, B₆, C, and E, selenium, niacin, and zinc can cause changes in DNA similar to those seen after exposure to radiation. These changes can lead to DNA double-strand breaks, oxidative lesions or both, and have been shown to be closely related to the development of cancer. However, various minerals act as protectors against the development of cancer. Among them is selenium, which stimulates the production of the enzyme glutathione peroxidase which acts to reduce hydrogen peroxide and maintain the integrity of cell membranes; and an example is zinc which acts on the processes of maintaining genomic stability and genetic expression (George, 2011).

5.4 Implications of the C677T polymorphism of the MTHFR gene in folate deficiency

Polymorphisms in genes such as Methylenetetrahydrofolate-reductase (MTHFR) and Methionine synthase (MTR) can affect the metabolism of folic acid, which can have consequences on the level of folate in the body. Folic acid deficiency can be associated with various diseases, including neural tube defects and birth defects.

The MTHFR gene codes for an enzyme called methylenetetrahydrofolatereductase. This enzyme plays a key role in the metabolism of folic acid, which is important for DNA synthesis and methylation, a process necessary to control gene expression. The MTHFR gene determines the activity of this enzyme, and mutations or polymorphisms in this gene can affect the function of the enzyme and the way the body uses folic acid metabolically. The best known and most studied mutation in the MTHFR gene is known as the C677T polymorphism. This mutation can lead to

a partial deficiency of the MTHFR enzyme, resulting in a reduced ability of the body to convert folic acid into its active form, methylenetetrahydrofolate. This can result in increased levels of homocysteine in the blood, which is a risk factor for certain diseases, including heart disease. Polymorphisms in the MTHFR gene have been investigated in the context of various diseases and reactions to nutrition. Understanding the genotype for this gene can help tailor diet and nutritional supplements to ensure that people with certain polymorphisms get enough folic acid and other important nutrients (Kaput and Rodriguez, 2006).

5.5 FTO gene polymorphisms in increasing the risk of obesity

The Fat Mass and Obesity-Associated (FTO) gene is a gene that has been identified as a key factor associated with obesity. This gene plays an important role in the regulation of body mass and fat metabolism. Polymorphisms in the FTO gene are associated with an increased risk of obesity and an increased body mass index (BMI). One of the most famous polymorphisms in the FTO gene is called rs9939609. People with certain alleles of this polymorphism have a higher risk of obesity and have a harder time maintaining a healthy body weight. Research has shown that the FTO gene affects appetite and satiety, which can lead to greater food consumption in people with certain polymorphisms of this gene. In addition, the FTO gene plays a role in regulating fat metabolism and the way the body stores and uses energy from food. However, it is important to note that the FTO gene is not the only factor influencing body mass and obesity. The interaction between genes, eating habits, level of physical activity and other factors plays a key role in the development of obesity (Kaput and Rodriguez, 2006). Obesity is the main risk factor for cardiovascular diseases, so polymorphic genes involved in the control of energy balance certainly provide a "favorable" or "unfavorable" basis for the development of coronary heart disease.

6. CONCLUSION

Genes are key factors in nutrient metabolism and individual response to nutrition. Nutrigenomics is a relatively new scientific discipline that studies the interaction between genes and nutrition and how diet can affect gene expression. This area of research opens up possibilities for personalized nutrition, which could improve health, help prevent disease, and optimize the nutritional needs of each individual. Future developments in technology and research will provide a better understanding of the genetic and nutritional factors that influence health. It is important that researchers, clinicians and society face together the challenges of nutrigenetics in order to realize its full potential for human well-being (Bouchard and Ordovas, 2012).

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"June 5th - World Environment Day"

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HEAVY METALS CONTAMINATION IN URBAN SOIL OF LUKAVAC AND NON-CARCINOGENIC AND CARCINOGENIC HUMAN HEALTH RISK ASSESSMENT

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Key words: heavy metals, pollution, urban soils, risk assessment

ABSTRACT:

The problem of exposing to heavy metals in soil for a long time is an important aspect of environmental studies. Concentrations of metallic elements (As, Ba, Cd, Co, Cr, Cu, Hg, Mn, Ni and Pb) were determined in soil samples collected in urban area of Lukavac city. Samples were taken at 18 positions to assess the environmental pollution status, non-carcinogenic and carcinogenic human health risk assessment. Pollution by these metals was examined by using several calculation methods: degree of contamination (DC), contamination factor (CF), ecological risk factor (ER), pollution load index (PLI) and geo-accumulation index (I_{geo}). The degree of contamination is the highest for Ni, 77% of the tested soil samples are contaminated with more than 200% of Ni. Enrichment factor revealed extremely contaminated with As, Cd and Hg, significantly enriched with Cr. According to CF, soil was classified as very highly contaminated with Cd and Ni and considerably contaminated with As, Hg and Pb. Index of geo-accumulation shows that study area was extremely contaminated with Cd. The (ER) values for Cd was 9101 indicating that the risk level was very high. Results of (PLI) indicated very highly polluted with As, Cd and Hg. Carcinogenic risk by ingestion (C_{Ring}) consumption of Pb, Ni and Cr in children was 4.5E-02, 5.4E-04 and 9.9E-04, respectively, while the lifetime carcinogenic risk (LCR) for all sampling points exceeded LCR limits for both, adults and children. Hazard quotient (HQ) trend in both adults and children was found in order $HQ_{ing} > HQ_{derm} > HQ_{inh}$ of soil for all metals.

1. INTRODUCTION

Urbanization and industrialization in the area of Tuzla Canton and in particular municipality of Lukavac, have a significant impact on environmental pollution and deterioration of water, air and soil quality. Numerous industrial plants in the municipality of Lukavac discharge into environment large quantities of waste materials in solid, liquid and gaseous form. Heavy metals accumulation in soils, sediments and their subsequent release to ground or surface water poses an environmental threat. The extent of heavy metals contamination in these media is dependent on their sources, redox conditions, microbial activities and the physicochemical properties of solid and aqueous phases (Dousova et al., 2012). Soil contamination by heavy metal such as As, Ba, Cd, Co, Cr, Cu, Hg, Mn, Ni and Pb is a matter of great concern because of possible influence on the food chain. Recent studies, by several researchers, have shown that deposition, in both urban and rural settings, can contribute significantly to soil contamination with heavy metals (Seung-Muk et al., 2006, Zhong et al., 1994, Xiangdong et al., 2004, Wang et al., 2003). Also, heavy metals are a critical threat to soil quality due to their permanence in the soil (Ghrefat et al., 2012). Furthermore, heavy metals in soil can be easily transferred into the human body via the oral route (ingestion), dermal contact absorption, and inhalation (Qu et al., 2012). Exposure to heavy metal contamination soils through hand-to-mouth contact causes adverse human health effects, particularly in children (Saeedi et al., 2012). Toxic heavy metals such as Cd, Pb and As elicit adverse effects such as mental retardation in children, kidney dysfunction, bronchitis, nervous system disorders and immune system damage at relatively low concentrations (Yang and Massey, 2019; Singh et al., 2011). Conversely, the potential ecological risk index in the soil comprehensively considers the concentration of biotoxicity and migration regularity, associates ecological toxicology, and evaluates the pollution (Weissmannov'a and Pavlovsk'y, 2017). Health risk assessment is assigned as a process of estimating the possibility of an occurrence of any adverse health effects in humans throughout the hazard chemical exposure and is usually based on an expression in parts of noncarcinogenic, carcinogenic health risk, and measurement of the risk level (USEPA, 2009). Exposure to carcinogenic or noncarcinogenic metals can result in adverse health consequences including damage to the nervous system, bone fractures, pulmonary adenocarcinomas, cardiovascular disease, kidney and liver dysfunction, and immune systems disorder (Dean et al., 2017, Ghasemidehkordi et al., 2018). According to the classification orders defined by the International Agency for Research on Cancer (IARC), Ni (group 1), Pb (group 2B), Cd (group 1), and Cr (group 1) are appointed as potential carcinogenicity metals. Meanwhile, Mn and Cu are appointed as noncarcinogenic metals (McAloon and Mason, 2003, Adepoyu-Bello et al., 2009). Metals are known to constitute highly persistent environmental pollutants thus; their tendency to remain as an environmental contaminant for a long period and to be magnified through food chains is high. Some of the metals such as calcium, magnesium, potassium and sodium are essential to sustaining life and must be present for normal body functions. While others such as Co, Cu, Fe, Mn, Mo and Zn are needed at low levels as catalyst

for enzyme activities 12. The aim of this study was to focus on the environmental risks and human health posed by these urban soils. Therefore, the first aim of this study was to determine the heavy metal contamination, including As, Ba, Cd, Co, Cr, Cu, Hg, Mn, Ni and Pb in soil in urban area of Lukavac city. The secondary aim was to assess the soil pollution and the potential ecological risks of heavy metal contamination in the soil via oral ingestion, inhalation and dermal exposure. Common approach in all study of environmental contamination is to estimate how much the soil is impacted with metal elements. As a diagnostic tool for determination degree of pollution in the soil we used: degree of contamination (*DC*), contamination factor (*CF*), pollution load index (*PLI*) and geo-accumulation index (*I_{geo}*).

2. MATERIALS AND METHODS

2.1 Sample collection

Soil sampling was conducted at eighteen locations in urban area of Lukavac city (Picture 1). Lukavac is selected as a municipality with developed chemical industry that greatly contributes to environmental pollution. All samples were taken in summer 2018. Soil sampling was carried out according to the Instruction on procedures, practices and conditions for control of soil fertility (Official Gazette FBiH, No. 72/09). Soil samples were taken at a depth of 0-10 cm. For each location an average sample of approximately 2000 g was obtained by homogenization. Soil samples were dried in laboratory at room temperature to remove free moisture and sieved to a sieve hole diameter 0.2 mm.

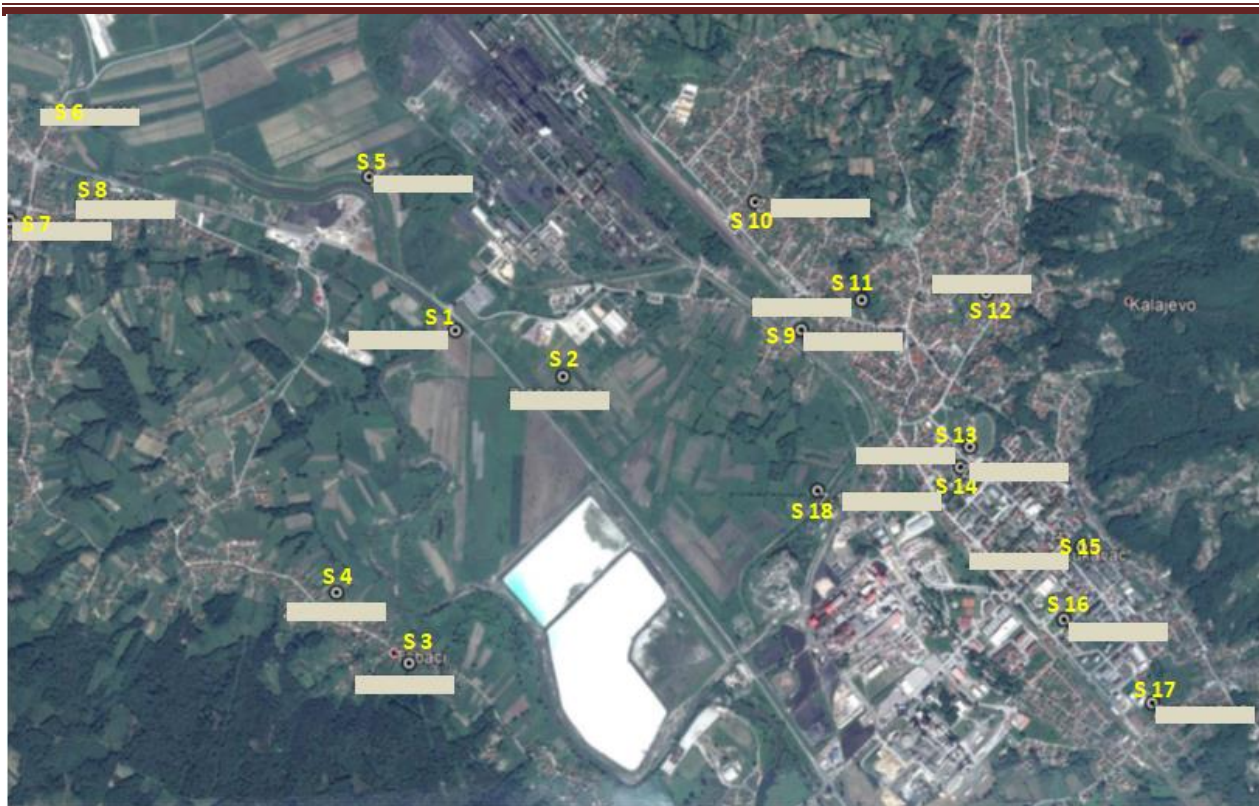


Figure 1: Locations of sampling sites in urban area of Lukavac city

2.2 Analytical methods

All chemicals and reagents were of analytical grade. Heavy metals standard reagents were prepared from reagent grade chemicals (Perkin Elmer Pure – Atomic Spectroscopy Standard). Determination of heavy metals concentration Heavy metals concentration in soil samples was determined according to method BAS ISO 11047:2000 Soil quality - Determination of Cd, Cr, Co, Cu, Pb, Mn, Ni and Zn in soil extract using aqua regia - Method of atomic absorption spectrometry (AAA). Triplicate solid samples of approximately 0.5 g were dried and digested in 12 mL freshly prepared aqua regia (1/3 HNO₃ + 2/3 HCl) for 16 hours at room temperature. After the destruction of samples, extracts were filtered in a 50 mL flask, which were then supplemented by deionized water. Total heavy metal concentrations (As, Ba and Hg,) were measured in soil extracts by Inductively Coupled Plasma-Optical Emission Spectrometer, ICPOES OPTIMA 2100 DV, Perkin-Elmer, with the standard of 100 mg/L for metals. The results were expressed in mg/kg soil.

2.3 Assessment of contamination levels

Assessment of contamination levels, give information about how an individual element and group of elements is concentrated at a site of interest relative to a background were used to

evaluate metal contamination. These include, degree of contamination (DC), contamination factor (CF), pollution load index (PLI) and geo-accumulation index (I_{geo}). The degree of soil contamination with heavy metals is calculated according to the following equation:

$$DC = \frac{C_s}{C_d} \cdot 100 \quad (1)$$

Where DC is degree of soil contamination, C_s is the concentration of metal in soil sample and C_d is the maximum allowed concentration for same metal according to Instruction on procedures, practices and conditions for control of soil fertility (Official Gazette FBiH, No. 72/09). Contamination factor is a ratio of an element in sample to the background site value or an established criterion for that metal. The contamination factor was calculated using the following equation:

$$CF = \frac{C_s}{C_b} \quad (2)$$

Where CF is contamination factor, C_s is the concentration of metal of interest at a site and C_b is the concentration of the same metal in modern preindustrial time (Hakanson, 1980) and shown in Table 1. The pollution load index is an integrated index that describes the overall pollution status of a site and allows for comparison between different locations (Yahaya et al., 2021, Weissmannova et al., 2019). It is estimated as the geometric mean of the contamination factors of heavy metals (Tomlinson et al., 1980). The following equation was used to estimate the PLI for sites:

$$PLI = (CF_1 \cdot CF_2 \cdot CF_3 \cdot CF_4 \cdot CF_n)^{\frac{1}{n}} \quad (3)$$

Where CF refers to the contamination factor of heavy metals in sampling soil and n refers to the number of heavy metals being studied. The geoaccumulation index (I_{geo}) was introduced by (Müller, 1969) and it is used to quantify and define heavy metal pollution in soil and sediment. The I_{geo} was computed by using the following equation:

$$I_{geo} = \log_2 \left[\frac{C_s}{1.5B_n} \right] \quad (4)$$

Where C_s is the concentration of metal found in the sampled soil (mg/kg), B_n is the geochemical background value (average scale) of metal n (mg/kg) as listed in Table 1, and factor 1.5 is the background matrix correlation factor due to lithogenic variation.

2.4 Indices of the Ecological Risk Assessment

Ecological risk factor (ER) quantitatively expresses the potential ecological risk of a given single contaminant which can be calculated for each metal by using the following equation:

$$ER = CF \cdot TR \quad (5)$$

Where CF is contamination factor for each metal and TR is the toxic response coefficients of the metals in the water, sedimentary, and biological phases, as listed in Table 1.

2.5 Human Health Risk Assessment

The average daily intake (ADI) of heavy metal in soils was expressed as units of the contaminated body exposed per unit of body mass and day (USEPA,1997), calculated using the following equations:

$$ADI_{oral} = \frac{C \cdot IR_{ing} \cdot EF \cdot ED}{BW \cdot AT} \cdot 10^{-6} \quad (6)$$

$$ADI_{dermal} = \frac{C \cdot SA \cdot SAF \cdot ABS \cdot EF \cdot ED}{BW \cdot AT} \cdot 10^{-6} \quad (7)$$

$$ADI_{inh} = \frac{C \cdot IR_{inh} \cdot EF \cdot ED}{PEF \cdot BW \cdot AT} \quad (8)$$

Where ADI is the average daily intake (mg/kg/day); C is the concentration of the heavy metals in soil (mg/kg); IR_{ing} is the ingestion rate (mg/day), for adults (100 mg/kg) and children (200 mg/kg); EF is the exposure frequency, 350 days/year; ED is the exposure duration, for adults (30 years) and children (6 years); SA is the exposed dermal area, for adults (5700 cm²) and children (2800 cm²); SAF is the dermal adherence factor for adults (0.07 mg cm⁻²) and children (0.2 mg cm⁻²); ABS is the dermal absorption factor, 0.001 for adults and children; IR_{inh} is inhalation rate, for adults (15 m³/day) and children (5 m³/day); PEF is the particle emission factor (1.36×10⁹ m³/kg); BW is the body weight, for adults (70 kg) and children (20 kg); and AT is the averaging time (day): for noncarcinogens, $ED \cdot 365$ days, and for carcinogens (Ni and Cr) and 70 (lifetime)×365 days (USEPA, 2002; Chen et al., 2017). Noncarcinogenic hazards are typically characterized by the hazard quotient (HQ). The health risk from soil contamination was assessed concerning its chronic as well as carcinogenic effects, based on the calculation of ADI_{oral} , ADI_{dermal} , and ADI_{oral} , plus a reference dose (RfD) that defined the toxicity values for each heavy metal, as shown in Table 1. The noncarcinogenic risk was calculated by the hazard quotient (HQ) as follows:

$$HQ = \frac{ADI}{RfD} \quad (9)$$

Then, the individual (HQ) of each metal was combined for the risk assessment of the hazard index (HI).

$$HI = \sum HQ \quad (10)$$

If the HQ or $HI > 1$, there may be potential noncarcinogenic effects on health, while HQ or $HI \leq 1$ means there is no experience of any health risks for exposure by noncarcinogenic metals (USEPA,1999; USEPA,2011). Carcinogenic risk (CR_i) was estimated as the incremental probability of developing cancer during a lifetime due to exposure to a potential carcinogen (USEPA,2011) as follows:

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"June 5th - World Environment Day"

$$CR_i = ADI_i \cdot SF_i \quad (11)$$

Where SF_i is the cancer slope factor (mg/kg/day) through oral ingestion, dermal contact, and inhalation of each metal, as illustrated in Table 1.

$$LCR = \sum CR_i \quad (12)$$

If the value of CR_i and LCR exceeds $1 \cdot 10^{-4}$, it represents a lifetime carcinogenic risk to the human body (USEPA,1989; EPA,2011).

Table 1: Geochemical background value (B_n), concentration of the same metal in modern preindustrial time (C_b), toxic response coefficients (Tr), reference dose (RfD) and cancer slope factor (SF) for oral, dermal and inhalation in soil of As, Ba, Cd, Co, Cr, Cu, Hg, Mn, Ni and Pb

Heavy metal		As	Ba	Cd	Co	Cr	Cu	Hg	Mn	Ni	Pb
Soil	B_n	2	628	0.1	11.6	35	14.3	$8.5 \cdot 10^{-2}$	527	18.6	17
	C_b	1.8	59	$5.34 \cdot 10^{-2}$	25	66.7	23.1	$4 \cdot 10^{-2}$	600	28.2	25
	TR	10	1	50	5	2	5	40	1	5	5
RfD	Oral	$3 \cdot 10^{-4}$	$7 \cdot 10^{-2}$	$1 \cdot 10^{-4}$	-	$3 \cdot 10^{-3}$	$4 \cdot 10^{-2}$	$3 \cdot 10^{-4}$	$140 \cdot 10^{-3}$	$2 \cdot 10^{-2}$	$3.5 \cdot 10^{-3}$
	Dermal	$1.23 \cdot 10^{-4}$	$4.9 \cdot 10^{-3}$	$1 \cdot 10^{-5}$	-	$6 \cdot 10^{-5}$	$1.2 \cdot 10^{-2}$	$2.1 \cdot 10^{-5}$	$1.8 \cdot 10^{-3}$	-	$5.25 \cdot 10^{-4}$
	Inhalation	$3 \cdot 10^{-4}$	$1.43 \cdot 10^{-4}$	$1 \cdot 10^{-4}$	-	$2.86 \cdot 10^{-5}$	$4.02 \cdot 10^{-2}$	$3 \cdot 10^{-4}$	$140 \cdot 10^{-3}$	$2.4 \cdot 10^{-4}$	$3.25 \cdot 10^{-3}$
SF	Oral	1.5	-	0.38	-	0.5	-	-	-	1.7	$8.5 \cdot 10^{-3}$
	Dermal	1.5	-	-	-	-	-	-	-	20	$4.2 \cdot 10^{-2}$
	Inhalation	1.5	-	6.3	-	42	-	-	-	84	-

3. RESULTS AND DISCUSSION

The metals evaluated in the present study included As, Ba, Cd, Co, Cr, Cu, Hg, Mn, Ni and Pb, the concentrations of all of the heavy metals in soils showed a relatively wide range of values, except for As, Ba and Co. The mean concentration of As (6.98 mg/kg) in soil samples did not exceed the limit recommended of 20 mg/kg. The concentrations of Ni exceeded standard limit (Ni=50 mg/kg) of all sampling sites except S8, the level (343.19 mg/kg) of Ni was highest at site S13. At 94.4% of the locations, the measured Ni concentrations exceeded the prescribed limit value. According to CF , average value of Ni is 6, which indicate very high contamination by this metal. The mean concentration of Cd in soil (4.75 mg/kg) was found above the standard limit set by regulatory agencies like EU (soil: 1–3 mg/kg), US EPA (soil: 1.4 mg/kg), Cd being strongly associated with soil and in this study area, its primary source is the emissions from industrial activities, traffic, and coal combustion in households. The high concentration of Cd has toxic effects on the beneficial microbes, disturb their metabolic process, and inhibit their growth. Cd has a lengthy biological half (10–35 years) in humans, Cd exhibits highly adverse effects on human health and ecological safety. In the study area, the average I_{geo} value of Cd is 10, which

Eighth International Scientific Conference
"June 5th - World Environment Day"

indicates that the overall level of Cd contamination in the study area can be categorized as extremely contaminated. The highest I_{geo} value of Cd is 20. In addition, average I_{geo} for all sites is 2 which indicates that soil is moderate contaminated by heavy metals. Industries activities are main sources of Cd contamination. The mobility of Cd shows that vehicles and coal combustion are also sources of Cd contamination in the study area. Cr has been widely recognized as a heavy metal that causes serious harm to human health and is known to have carcinogenic and teratogenic effects. In the study area, the average CF value of Cr is 2 which indicates that overall level of Cr contamination in the study is moderately contaminated. Cr being strongly associated with soil and in this study area, its primary source from industries facilities. Biological activities of soil are considerably affected by Cr contamination, chernozem's biota is highly affected by toxic effects of Cr and reduce its catalysis activity. The average metal concentrations decreased in the order $Mn > Ni > Cr > Pb > Ba > Co > Cu > As > Cd > Hg$ (Table 2).

Table 2: Concentrations of heavy metals in soil samples

Sample No.	Heavy metals concentration, mg/kg									
	As	Ba	Cd	Co	Cr	Cu	Hg	Mn	Ni	Pb
S1	6.30	79.00	0.99	28.79	176.3 1	29.38	0.28	989.02	248.2 7	55.24
S2	5.60	47.00	0.02	31.78	51.42	12.35	0.05	444.71	102.2 8	8.32
S3	5.00	36.00	0.89	46.47	142.6 0	9.70	0.04	791.53	142.3 4	20.61
S4	3.20	47.00	2.02	26.76	99.63	8.68	0.04	662.14	75.82	35.78
S5	6.20	46.00	0.38	26.60	104.3 3	25.97	1.40	630.74	202.2 4	1.67
S6	4.80	38.00	0.30	40.00	118.8 4	27.45	0.13	804.22	284.3 2	40.54
S7	3.20	51.00	1.03	25.80	69.53	15.37	0.05	628.10	62.13	32.16
S8	4.80	42.00	0.84	19.42	94.28	17.08	0.03	424.61	42.54	64.01
S9	7.50	70.00	5.60	58.20	134.8 9	34.41	0.05	792.30	160.5 9	117.01
S10	5.10	42.00	5.38	48.26	93.80	29.08	0.07	532.24	181.8 4	92.52
S11	8.40	41.00	6.39	54.18	137.7 1	17.08	0.04	357.48	152.3 5	88.13
S12	6.50	51.00	7.12	56.78	129.8 3	34.41	0.06	740.71	151.4 1	91.82
S13	9.00	52.00	8.41	67.02	215.2 2	29.08	0.14	355.22	343.1 9	329.83
S14	9.00	76.00	8.93	71.45	144.6 5	17.69	0.24	1114.3 4	223.4 4	110.49
S15	18.0 0	78.00	9.61	56.49	162.9 0	28.54	0.13	535.12	138.2 9	131.80
S16	11.0 0	83.00	9.31	53.07	82.30	86.30	0.09	296.55	65.03	197.67
S17	4.20	46.00	8.50	59.92	138.2 4	33.03	0.05	321.50	133.9 4	105.80

Eighth International Scientific Conference
"June 5th - World Environment Day"

S18	7.90	65.00	9.72	66.37	150.9 8	48.22	0.09	1077.1 8	205.6 0	115.53
Avg	6.98	55.00	4.75	46.52	124.8 6	27.99	0.17	638.76	161.9 8	91.05
Min	3.20	36.00	0.02	19.42	51.42	8.68	0.03	296.55	42.54	1.67
Max	18.0 0	83.00	9.72	71.45	215.2 2	86.30	1.40	1114.3 4	343.1 9	329.83

The average calculated **CF** of heavy metals also revealed that 55.5 % of sample sites were very high contamination (S9, S10, S11, S12, S13, S14, S15, S16, S17 and S18), considerable contamination of (S1, S3, S4, S5, S6 and S7) and moderate contamination of S2 and S8. The mean concentration of Cu (27.99 mg/kg) in soil samples did not exceed the limit recommended of 80 mg/kg. The **PLI** indicated extreme pollution of heavy metals at 50% of sampling points, the average **PLI** value is 5 which indicates that soil in the study area is highly polluted by heavy metals. The potential ER for the heavy metals in soil in the study area showed an order of Cd > Hg > As > Ni > Pb > Co > Cu > Cr > Ba = Mn. Generally, 88.8 % of the locations had very high ecological risk for the Cd in the soil. Most of the sampling points in the urban region of Lukavac demonstrated a strong potential **ER** for Cd and Hg. Cadmium was the key influencing factor causing the potential **ER**. These single pollution indices shed light on the quality of soil in the study area, and indicate that Cd, Cr, Ni and Pb were very high to extremely polluted regard to calculated levels of contamination. Most of the very high to extremely polluted sites fell within the urban region of Lukavac city. For noncarcinogenic risk assessment, HQ_{oral} , HQ_{dermal} and HQ_{inh} in the soil for both children and adults is ≤ 1 that means there is no experience of any health risks for exposure by noncarcinogenic metals. In terms of carcinogenic risk assessment, the CR_{oral} , CR_{dermal} and CR_{inh} in children were greater than adults, both (0.0044 for adults and 0.0364) exceeded the carcinogenic risk limit at $1 \cdot 10^{-4}$ and indicated that a lifetime carcinogenic posed a risk to both adults and children. In this study has determined that the soil contains extremely high concentrations of nickel which is major carcinogen to humans and up-take is through the respiratory tract, digestive system and skin. The primary target organs to Ni are the kidneys and lungs. Exposure to Ni is intricately linked to an increased risk of human lung and nasal cancer.

4. CONCLUSIONS

In this study the average concentrations of Cd, Cr and Ni exceeded allowed values. The **PLI** indicated extreme pollution of analyzed heavy metals at 50% of sampling points, which indicates that soil in the study area is highly polluted by heavy metals. A great concern is the fact that the soil used for recreational purposes (S13) and the kindergarten (S15) is contaminated with heavy metals, especially Cd, Cr, Ni and Pb. The I_{geo} of Cd was can be categorized as extremely contaminated. According to **CF**, average value of Ni indicate very high contamination by this metal. The **ER** factor showed very high ecological risk for the Cd in the soil, most of the

Eighth International Scientific Conference "June 5th - World Environment Day"

sampling sites in the urban region of Lukavac demonstrated a strong potential *ER* for Cd and Hg. Ultimately, the metals transfer from the soil to human may pose serious health problems to humans. The CR_{oral} , CR_{dermal} and CR_{inh} in the soil for children were greater than adults but both values are greater than the carcinogenic risk limit, which indicates a lifetime carcinogenic risk to children and adults.

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"June 5th - World Environment Day"

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PHYTOACCUMULATION OF CADMIUM AND LEAD BY MINT (lat. *Mentha piperita* L.)

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Key words: mint, contaminated soil, phytoremediation, phytoaccumulation factor, heavy metals, lead, cadmium

ABSTRACT:

Heavy metals are an increasing problem for human health. This is the reason for many researches that are based on the elimination of heavy metals from the soil as a natural recipient of all anthropogenic substances that can also contain heavy metals. This study aimed to evaluate the behavior of mint (lat. *Mentha piperita* L.) under cadmium (Cd) and lead (Pb) soil contamination and their transfer from soil in plants compared with a control sample without metal addition. The experiment was performed in laboratory controlled conditions on 10 mint seedlings that were planted in 2,5 kg of soil and watered with a two metal and salt mixtures (hydrated cadmium sulfate - $3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$ and lead nitrate - $\text{Pb}(\text{NO}_3)_2$) for a period of three months. The results of metal concentration in mint plants showed that Cd and Pb were accumulated with average concentration of Cd 486,5 mg/kg and Pb 1531,6 mg/kg of dry matter of mint. The value of the phytoaccumulation factor for Cd was 8,0 and for Pb was 4,4. The highest value of the phytoaccumulation factor for Cd and Pb were detected in plant material of mint which had been watered with the smallest volume of heavy metal mixtures. In this study, extremely high concentrations of heavy metals were obtained in mint compared to plants grown in a conventional way, due to artificial soil pollution and limitations of space for root development in pots and constant root contact of mints with a mixture of heavy metals in an ionic highly mobile and reactive form of metal. Based on the results, mint can be considered as hyperaccumulator of Cd and Pb despite significant impact on the growth and development that

was observed on mint plants and phytotoxic effects that occurred on plants after three months of exposure.

1. INTRODUCTION

Heavy metals and metalloids in the soil come from the parent material which is a lithogenic source and various anthropogenic sources (Alloway, 2013). Soil pollution with heavy metals has serious consequences in various areas of human activities, because the soil is the ultimate recipient of all pollution, regardless of whether it is released into the air, water or deposited on the ground. One of the most common risks for human health and the environment is soil contaminated with heavy metals. Also, heavy metals are also natural components of the Earth's crust, they are difficult to degrade by microorganisms, they accumulate in living organisms, in polluted areas and eighter can enter the body through air, food and water, causing long-term health consequences (Zhang et al., 2012). Due to their toxicity, long persistence and accumulation in nature, as well as circulation in the biosphere, heavy metals occupy a special place among contaminants (Vidović, 2014). Increased concentrations of the heavy metals lead and cadmium are typical for urban pollution, considering that the largest amounts of cadmium in the soil come from the atmosphere through the emission of exhaust gases. Areas in the zone of influence of highways and other main roads are polluted primarily by lead and cadmium, but also by other heavy metals (Cui et al., 2006 and Shi et al., 2008). In order to rehabilitate long-term pollution and reduce the negative impact of new pollution in recent years more and more attention has been paid to the application of biological, less obstructive technologies and numerous remediation procedures are carried out in the domain of a new interdisciplinary scientific discipline called phytoremediation (Bertović, 2016). Phytoremediation uses plants that have the ability to accumulate and detoxify heavy metals and organic pollutants with the aim of restoring and cleaning polluted habitats by translocating them to their above-ground parts (Baker, et al., 2000). In this paper, the relationship between the concentration of heavy metals in plants and in the corresponding soil is represented by the phytoaccumulation factor for lead and cadmium in the plant material of mint.

LEAD (Pb)

Lead belongs to the 14th group of the periodic system of elements and forms compounds of low solubility. It has a strong affinity for sulfur, so it's found in rocks and sulfide minerals, especially in the mineral galena (Ćipurković, 2016). Lead sometimes also occurs in the form of the minerals cerusite, anglesite and crocoite. Crude lead isn't pure and contains greater or lesser amounts of copper, antimony, arsenic, sulphur, tin and silver. Most of these impurities are removed by melting and mixing the lead. In this way, the entire mass can come into contact with oxygen from the air on the surface (Filipović and Lipanović, 1995). Lead vapors in the air oxidize rapidly, creating aerosols of lead oxide. Pure lead is a heavy and soft metal, it melts at 327°C and evaporation starts at 400°C (Ćatović et al., 2004). When it is freshly cut, it shows a silver-

metallic luster on the cross-section, consisting of oxides and carbonates (Filipović and Lipanović, 1995). In the surface layer of the soil in Europe the average mean concentration of lead is 15 mg/kg and the average mean concentration of cadmium in the world is 25 mg/kg (Kabata-Pendias and Pendias, 2001). Lead most often accumulates in the surface part of the soil, from 3 to 5 cm, while the concentration decreases as the depth increases (Kisić, 2012). There are several ways to reduce the risk of exposure to lead, such as reducing lead emissions into air and water, restricting the use of lead in the production of pesticides and paints, banning the use of lead water pipes, lead foils on wine bottles, reducing the use of lead in gasoline and reducing the use of lead cans. for food packaging (Cipurković, 2016).

Lead toxicity is one of the most dangerous heavy metal toxicities. Lead poisoning is called saturnismus or plumbism and is a widespread health problem. The negative effects of lead in professional exposure have long been observed, and more recently, importance has been given to the study of subclinical effects at relatively low concentrations of lead exposure in the environment. In some parts of the world, as well as in Bosnia and Herzegovina, lead poisoning is rarely or almost never recognized, diagnosed and/or treated (Pranjić, 2007). Symptoms of lead poisoning are an unpleasant sweet taste in the mouth, headache, nausea and a general feeling of weakness. Chronic lead poisoning is characterized by the formation of a "lead rim" on the teeth and the appearance of sudden stomach cramps (Cipurković, 2016).

CADMIUM (Cd)

Cadmium belongs to the 12th group of the periodic system of elements and is classified as a non-essential heavy metal. It doesn't have any essential biological function. It's found in more than 1000 species of flora and fauna. Cadmium is a relatively rare element and it's most often associated with lead and zinc. It is commercially obtained as a by-product in the production of zinc and lead (Vidović et al., 2014, Cipurković, 2016 and Filipović and Lipanović, 1995). In the surface layer of the soil in Europe the average mean concentration of cadmium is 0.145 mg/kg of dry soil, and the average mean concentration of cadmium in the world is 1.1 mg/kg (Alloway et al., 2013 and Kabata-Pendias and Pendias, 2001). All cadmium concentrations higher than 0.5 mg/kg are anthropogenic origin (Kabata-Pendias and Pendias, 2001). In elemental form, cadmium occurs as a soft, silver-white metal that is fairly stable at room temperature. It dissolves in acids, more difficult in non-oxidizing acids, and easily in acids that have a strong oxidizing effect. It doesn't dissolve in bases (Filipović and Lipanović, 1995).

Cadmium is a stable element and retains its toxic properties for a long time. By entering the biosphere, it can cause many adverse effects, especially due similarity to essential metals and the possibility of replacement with them. Presence of cadmium can cause various damage to flora and fauna and negative effects on human health (Vidović, 2014). Absorption of cadmium is particularly high through cigarette smoke. The content of cadmium in the blood is much higher in smokers than in non-smokers. Intensive consumption of cigarettes can introduce relatively

high amounts of cadmium into the body, because tobacco contains 1 to 2 µg of cadmium per gram. Chronic cadmium poisoning can cause changes in the bones and very painful deformations of the skeleton. Symptoms of „Itai-itai“ disease were observed in Japan, where certain elderly women with a predisposition to osteoporosis were affected by water containing cadmium, which was used to irrigate rice fields during the 1950s. Damage to the skeleton was primarily caused by the weakening of renal functions after cadmium poisoning, and the biomineral part of the patient's „Itai-itai“ mostly contained 1% cadmium. The lack of calcium in food aggravated the toxic demineralizing effect and cadmium remains in the skeleton for more than 10 years. Also, it was found that as many as 44% of workers exposed to cadmium for more than 15 years have kidney stones (Cipurković, 2016). Due to its high toxicity, it is important to pay special attention to the prevention of cadmium poisoning.

PHYTOREMEDIATION

Plant remediation or phytoremediation has the potential for accumulation, immobilization and transformation of low concentrations of persistent contaminants. The process is based on the ability of plants to act as solar pumps for water, where they also draw in water-soluble toxic substances. Phytoremediation is applicable for a wide range of substances, numerous metals and radionuclides, and various organic compounds. The advantages of this method are low project costs, and the limitations of this technology are primarily the long duration of the remediation process, difficulties in establishing and maintaining vegetation in locations with high contamination, as well as the possibility of increased mobility of degradation products and bioaccumulation in animals (Beškoski et al., 2012). Phytoremediation is a new green technology which is used to remove pollutants from the environment. The main reason for using this technology is to deposit pollutants and pollutants from the environment and transform them into a form that can be separated (Karami and Shamsuddin, 2010). Plant cultures that are fast-growing, develop deep roots, are easy to harvest, accumulate more than one heavy metal in their aerial parts and tolerate high concentrations of heavy metals can be used for the phytoremediation process (Hem Singh and Jaitly, 2014).

AROMATIC PLANT CULTURES PHYTOREMEDIATION

The latest, safe, economically viable and environmentally friendly approach for phytoremediation involves the use of non-edible aromatic plant cultures. With this approach, heavy metals do not enter the food chain. Wild animals don't consume aromatic plants because of their essence and the resources of aromatic plants are very rich and can be used on a large scale. These plants offer a new option in phytoremediation of heavy metals in contaminated sites. Aromatic plants such as vetiver (lat. *Vetiveria zizanioides* L.), palmarosa (lat. *Cymbopogon martinii* Var.Motia.), lemon grass (lat. *Cymbopogon flexuosus* Nees ex Steud.), citronella (lat. *Cymbopogon winterianus* Jowitt), mint (lat. . *Mentha* L.), basil (lat. *Ocimum basilicum* L.), lavender (lat. *Lavandula* L.) and rosemary (lat. *Rosmarinus* L.) are ecologically acceptable

perennial plants used for the production of essential oils (Hem Singh and Jaitly, 2014, Dinu et al., 2021 and Gupta et al., 2013).

MINT (lat. *Mentha* L.)

Mint (lat. *Mentha piperita* L.) is a perennial, aromatic, herbaceous plant. The height of the plant varies from 40 to 90 cm. The stem is bushy, branched and relatively strong. The root is weakly developed, veined, and its main veins are located 3 to 5 cm below the soil surface. Mint essential oil can be used in the form of a spray against superficial pains, colds, rheumatism and neurosis. Mint is also used in the production of toothpaste, mouthwash, sweets, alcoholic and non-alcoholic drinks, fragrances and the like. Mint has a best progression in warm and humid climates and prefers fertile and deep soils (Kolak et al., 2001).

2. MATERIAL AND METHODS

The research was conducted on artificially polluted soil that was treated with a solution of heavy metals in laboratory conditions where mint plant cultures were grown. The plants were watered every five days for a period of three months. First pot was watered only with water and was determined as a control sample for monitoring the natural concentration of heavy metals in the plant material. In the other pots, from the second to the tenth, the soil was treated with a solution containing the heavy metals lead and cadmium. The soil was watered a total of 18 times.

The soil in the pots was watered with a 0.05 M solution of lead(II) nitrate ($\text{Pb}(\text{NO}_3)_2$) and a 0.02 M solution of cadmium sulfate octahydrate ($3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$). The solutions were prepared by dissolving in distilled water 16.56 g of $\text{Pb}(\text{NO}_3)_2$, molar mass 331.2 g/mol and 15.39 g $3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$, molar mass 769.546 g/mol, and the volume of the solution was increased due to testing the optimal volume and concentrations that can be absorbed by plant crops and that will not interfere with their growth and development.



Figure 1: Mint samples during the period of watering with heavy metal solutions.

Eighth International Scientific Conference
"June 5th - World Environment Day"

During the experiment plant cultures (Figure 1) developed unevenly and some samples of plant material showed signs of disease and visible damage.

After three months samples were prepared using the dry burning technique. Ten samples of mint plant material were homogenized (root, stem and leaves) and ground into a fine powder. Then, samples of the dry substance of the mint plant material were weighed (approx. 1 gram) and burned in a pre-heated porcelain pot on a burner until all the mass was charred and smoke stopped forming. Then the samples were annealed in an annealing furnace (Selecta) at 550°C until the organic substances were completely destroyed and the ash became completely white. After annealing, the samples were cooled in a desiccator. The samples were annealed again for 10 to 30 minutes, cooled in a desiccator and weighed to a constant mass.

After annealing, the contents of the crucible were qualitatively transferred into a measuring flask of 100 cm³ and topped up to the mark with 10% nitric acid (HNO₃) (Somenath Mitra, 2003). In the samples thus prepared, the concentration of the heavy metals lead and cadmium in the plant material were determined by the method of flame atomic absorption spectrophotometry „Analytical Methods“ FP-3 Analysis (2000) (Analytical Methods, 2000) on an atomic absorption spectrophotometer with Zeman correction „Perkin Elmer Analyst-800“.

In order to determine the relationship between the concentration of heavy metals in plant material and the soil, i.e. the ability to phytoaccumulate heavy metals, the phytoaccumulation factor (FAF), which is called and bioaccumulation factor (BF), calculated according to the formula (1):

$$FAF = \frac{\text{heavy metals concentration in the dry matter } (\frac{mg}{kg})}{\text{heavy metals concentration in soil } (\frac{mg}{kg})} \quad (1)$$

The phytoaccumulation factor wasn't calculated for the control samples, and the concentrations of heavy metals in the soil were calculated based on the mass of the solution of lead(II) nitrate (Pb(NO₃)₂) and the solution of cadmium sulfate octahydrate (3CdSO₄ · 8H₂O), which was added to 2.5 kg of soil in which mint was grown.

3. RESULTS AND DISCUSSION

Heavy metal concentrations were determined with an average standard deviation of 0.15% for lead and 0.11% for cadmium in mint plant material.

Table 1: Concentrations of heavy metals lead and cadmium in samples of mint plant culture.

Sample	Pb (mg/kg)	Standard deviation (%)	Cd (mg/kg)	Standard deviation (%)
M-1	355,8	0,055	89,2	0,164
M-2	1370,8	0,109	614,3	0,290
M-3	1130,8	0,394	905,9	0,048
M-4	729,0	0,010	105,6	0,008
M-5	412,6	0,057	96,3	0,012

Eighth International Scientific Conference
"June 5th - World Environment Day"

M-6	1678,1	0,247	676,5	0,043
M-7	3367,3	0,186	762,9	0,070
M-8	4513,3	0,302	1057,9	0,026
M-9	1361,6	0,095	386,0	0,023
M-10	396,7	0,081	170,6	0,369
Average	1531,6	0,15	486,5	0,110

The concentration of the heavy metal lead (Pb) in the control sample of dry matter of the mint plant culture, i.e. sample M-1, which is not artificially contaminated with heavy metals, is 355.8 mg/kg of dry matter. The concentration in the other samples (Table 1.) ranges from 396.7 to 4513.3 mg/kg of dry matter.

The concentration of the heavy metal cadmium (Cd) in the control sample of mint plant culture, i.e. sample 1, which is not artificially contaminated with heavy metals, is 89.2 mg/kg. The ratio of concentrations in the other samples (Table 1.) ranges from 96.3 to 1057.9 mg/kg of dry matter.

The average Pb concentration in mint samples in polluted soil (samples M-2 to M-10) is 1662.2 mg/kg of dry matter and is 4.67 times higher than the Pb concentration in the control sample (sample M-1). and the concentration of Pb in mint is not directly related to the concentration of Pb in the soil where the mint was grown. The average value of the Pb phytoaccumulation factor (Table 2.) is 4.4 for the mint plant material.

Table 2: Phytoaccumulation factor of Pb for mint plant material.

Sample	Pb (mg/kg)	Concentration Pb in soil (mg/kg)	FAF (Pb)
M-1	-	-	-
M-2	1370,8	103,6	13,2
M-3	1130,8	207,2	5,5
M-4	729,0	310,8	2,3
M-5	412,6	419,4	1,0
M-6	1678,1	524,3	3,2
M-7	3367,3	629,1	5,4
M-8	4513,3	713,0	6,3
M-9	1361,6	745,9	1,8
M-10	396,7	838,8	0,5
Average	1662,2	499,1	4,4

The average Cd concentration in mint samples in polluted soil (samples M-2 to M-10) is 530.7 mg/kg of dry matter, which is 5.95 times higher than the Cd concentration in the control sample (sample M-1), and the concentration of Cd in mint is not directly related to the concentration of Cd in the soil where the mint was grown. The average value of the phytoaccumulation factor Cd (Table 3.) is 8.0 for the mint plant material.

Eighth International Scientific Conference
"June 5th - World Environment Day"

Table 3: Phytoaccumulation factor Cd for mint plant material.

Sample	Cd (mg/kg)	Concentration Cd in soil (mg/kg)	FAF (Cd)
M-1	-	-	-
M-2	614,3	22,5	27,3
M-3	905,9	45,0	20,1
M-4	105,6	67,4	1,6
M-5	96,3	89,9	1,1
M-6	676,5	112,4	6,0
M-7	762,9	134,9	5,7
M-8	1057,9	152,9	6,9
M-9	386,0	161,9	2,4
M-10	170,6	179,8	0,9
Average	530,67	107,4	8,0

The mint plant material used in this work has values of phytoaccumulation factors for lead of 4.4 and for cadmium of 8.0. If the value of the phytoaccumulation factor of a certain heavy metal is greater than 1 it indicates that the examined plant culture can be considered a hyperaccumulator of that heavy metal (Stančić et al., 2015). Thus, the mint plant culture can be considered a hyperaccumulator for heavy metals lead and cadmium. Hem Singh et al., 2014 and Memon et al., 2021, state in research that plants grown on polluted soil, which accumulate 100 mg/kg cadmium and 1000 mg/kg lead in dry matter can be considered hyperaccumulators. Considering this information, the plant culture of mint can be considered a hyperaccumulator for the heavy metals lead and cadmium. In 2011, Kumar et al. investigated the accumulation of lead and cadmium in mint (lat. *Mentha spicata* L.), and the results showed that the concentration of lead in mint was on average 7.03 mg/kg and cadmium 1.24 mg/kg. Similar concentrations in the plant culture of another type of mint (lat. *Mentha piperita* L.) were established by Dina et al., in 2015, who investigated the accumulation of lead and cadmium in mint grown in 5 kg of soil, and the average lead concentration was 11.88 mg/kg, and cadmium 1.56 mg/kg in the root of the mint plant culture. These concentrations have lower values than the values obtained during the preparation of this work, because during the implementation of the experiment, mint was grown in 2.5 kg of soil and the root inside the pots was in contact only with polluted soil, which resulted in a significantly higher accumulation of heavy metals by the plant.

4. CONCLUSIONS

The highest concentration of lead (Pb) is 4513.3 mg/kg for sample M-8, and the highest concentration of cadmium (Cd) is 1057.9 mg/kg for sample M-8 in mint.

Heavy metals lead and cadmium affected the growth and development of mint.

Eighth International Scientific Conference "June 5th - World Environment Day"

In this work, extremely high concentrations of heavy metals were obtained, because the plant culture of mint was grown in 2.5 kg of soil and the root inside the pots is in constant contact with the solution of heavy metals, which is in the ionic, highly mobile and reactive form of the metal, which resulted in significant by greater accumulation of heavy metals by the plant.

Plants that were watered with the smallest volume of heavy metal solution have the highest level of accumulation for heavy metals, and the concentration of Pb and Cd in the dry matter of mint is not directly related to the concentration of Pb and Cd in the soil where they were grown.

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CATEGORIZATION OF PATIENTS AND IDENTIFICATION OF HEALTH CARE NEEDS

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Key words: patient categorization, progressive care, nurse, critical factor

ABSTRACT:

Health care is the basic overall part of health care aimed at improving, preserving and restoring health. The organized form represents one of the disciplines because the basic task of health care is to help individuals and groups to perform their physical, mental and social work in different states of health on the best possible way.

The purpose of categorizing patients is to respond to the changing nature of health care requests. Patient's categorization systems are based on a theoretical framework that reflects the concept of health care. Categorization does not necessarily mean that patients in the same category are identical, but that they are similar to each other given certain characteristics in relation to patients in another category. The aim of this paper was to show the real needs for nurses based on the calculation of required hours through categorization of patients and records of working hours. It determined the division of the patients whose care requires more complex procedures and interventions seeking for a higher level of nurses competencies. Therefore, to determine which patients need greater provision of health care and according to which criteria the categorization of such patients is to be made.

Categorization of patients is a relevant data that gives an accurate insight into the number of nurses needed providing the complexity of the interventions to be performed by the nurse.

1. INTRODUCTION

Categorizing patients depending on the amount of health care that needs to be provided to the patient was recognized at the beginning of the XX. A significant development of categorization took place in the 1950s and 1960s with the development of progressive health care.

"Progressive care means complete adjustment to the patient's needs, that is to the pathophysiological needs of the body. It requires receiving the patient in a timely manner, its placing in a bed, undertaking the appropriate treatment and directing care to the treatment" (Čukljek 2005).

When classifying patients in progressive health care, three categories are most often used; intensive care, semi-intensive care, minimal (ordinary, standard) care. (Kendić, 2009)

Determining the severity of the condition of individual patients (categorizing the patient) provides a quick insight into the severity of the patient's condition in the ward, and thus indicates the need for health care to required be provided.

The critical elements when categorizing the patient are assessed namely: the patient's independence in maintaining personal hygiene, dressing, feeding, physical activity - walking and standing, sitting, moving and turning, risk of falling. It also take cares on risk of decubitus, ability to communicate, the patient's need for education, state of consciousness, vital signs, specific procedures in health care (eg wound care, central venous catheter care, drainage, bronchial tree aspiration), diagnostic and therapeutic procedures. For each of the mentioned factors, individual categories on a scale from 1 to 4 were described in order to make categorization easier for nurses but also to achieve as much objectivity as possible. (Čikeš, 2017)

2. HEALTH CARE

Definition of healthcare by F. Nightingale:

"It is the provision of the best possible conditions so that natural healing forces can act on the patient." (Kendić, 2009)

Virginia Henderson, a university-educated nurse, gives a simple definition of the role of a nurse: "The unique role of a nurse is to help an individual, sick or healthy, in performing those activities that contribute to health or knowledge, i.e. to help him become independent as soon as possible." (Fučak, 1995)

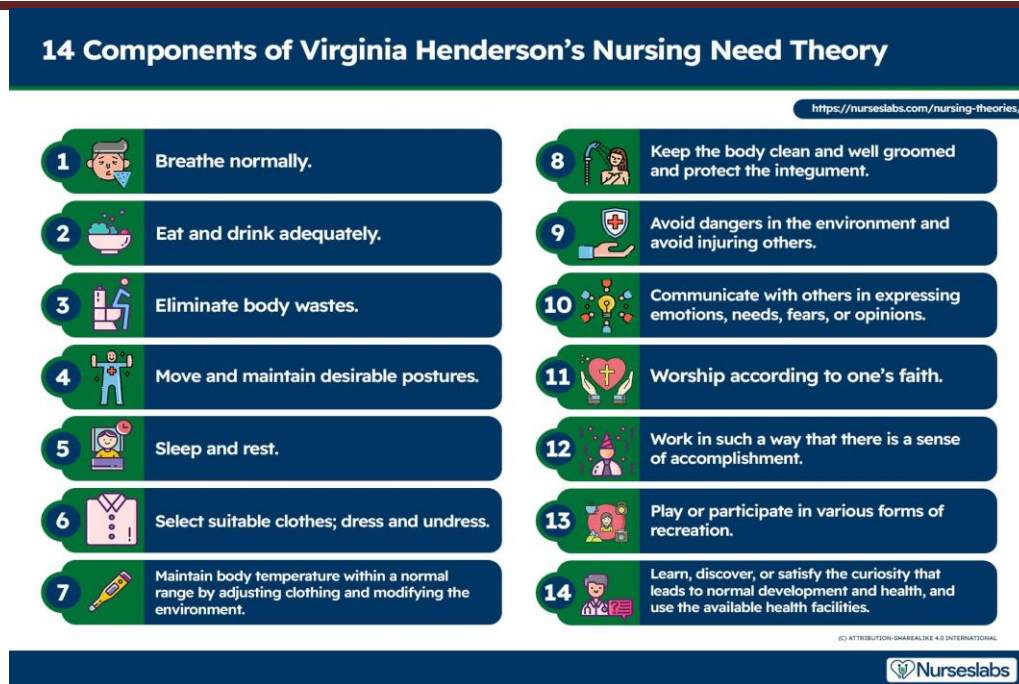


Figure 1: Activities during which the nurse helps patients are aimed at meeting basic human needs according to V. Henderson (<https://nurseslabs.com/virginia-hendersons-need-theory/>)

3. CATEGORIZATION OF PATIENTS

By categorizing patients, one get a quick insight into the real needs of patients for health care and thus enable a fairer distribution of nurses on the wards within the hospital system. Through categorization, patients are classified into four categories depending on the help needed to meet basic human needs, and on the diagnostic and therapeutic procedures performed on the patient. The number and complexity of interventions provided by a nurse or the amount of health care depends on these needs.

In order to categorize the patient based on their needs for health care, it is necessary to classify the patients according to the needs for care based on the critical factors.

3.1 Critical factors of patient categorization

Categorization factors: Hygiene, dressing, feeding, walking, standing, sitting, moving, turning, risk of falling, state of consciousness, risk of decubitus, vital signs, communication, specific procedures in health care, diagnostic or therapeutic procedures and education. (Cikeš, 2017)

The nurse is to assess the patient's condition daily, or as needed, according to factors and depending on its needs, places one in a certain category on a scale from 1 to 4. Each category is to be scored separately (the number of points for each factor is equal to the category in which the patient is classified - each factor of category 1 = 1 point, each factor of category 2 = 2 points,

etc.) and calculates the total number of points. Depending on the total number of points, the category in which the patient will be classified is determined. (Cikes, 2017).

Table 1: Display of points by category (Čikeš 2017.)

CATEGORY	NUMBER OF POINTS
1	16-26
2	27-40
1q3	41-53
4	54-64

The lowest number of points that the patient can achieve is 16, in case the patient's condition in each category had been evaluated with 1 and the highest number of points that the patient can achieve is 64, in the event that the patient's condition had been evaluated with 4 in each category.

3.2 Triage

Triage is the process of classifying or categorizing patients according to their health care needs based on the urgency of the condition. Triage as a process is essential in categorizing patients in order to determine priorities depending on the medical condition. To establish triage, the scales are to be used as a means of ranking urgency and a basic set of data: head complaints, age, comorbidity, severity of symptoms, objective condition, vital signs. (Turkalj, 2018)

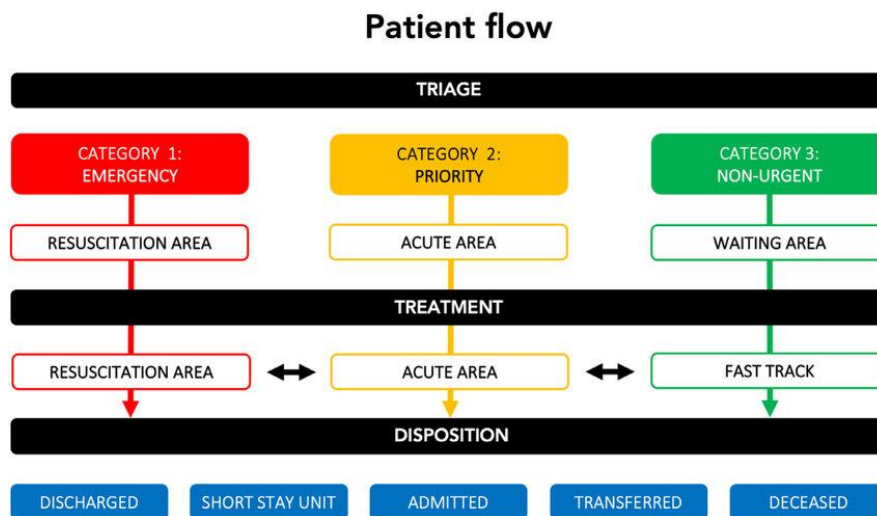


Figure 2: Patient flow process (https://www.researchgate.net/figure/Patient-flow-process-linking-triage-category-with-key-stages-in-the-ED-patient-journey_fig2_347772248)

Categories indicate the degree of urgency of patient treatment. The first category implies the highest level of urgency such as life-threatening conditions and those patients require immediate treatment. The second one includes conditions that require treatment of the patient within 10 minutes, threatening life. The third one must be treated within 30 minutes and the fourth one within 60 minutes. The fifth one includes less urgent conditions and clinical administrative problems with the start of processing within 120 min.

3.3 Motivation in the service of the patients' categorization system

Motivation is a common term for all internal factors consolidating intellectual and physical energy, initiating and organizing individual activities, guiding behaviour and determining its direction, intensity and duration. (Maslow, 1982)

The most frequently cited theory has been the hierarchy of needs theory developed by Abraham Maslow, which has undoubtedly had the greatest influence on thought and research on human behaviour in organizations. The basic assumption for this theory is the relative supremacy of needs organized in a hierarchical structure. Maslow's theory provides important lessons for understanding work motivation and acting on it. It points to the importance and variety of needs that people want to satisfy at work. He warns that organizations should give more space to the continuous development of people, and not only to the satisfaction of existential needs. A person who is developing and self-actualizing has a need for knowledge and experience of all kinds and for confirming and applying its potential in and outside of work. Maslow also said that people cannot always be motivated in the same way and that one should find new motivation strategies following the needs and preferences of own employees. (Katuša, 2015) The importance of Maslow's theory of motivation lies in the fact that theoretically one can have a well-developed and established system of patient categorization, but if in the practice there are not enough motivated people to implement it, the project will never become a living tissue that will be modelled according to the needs.



Figure 3. Maslow's hierarchy of needs(<https://www.thoughtco.com/maslows-hierarchy-of-needs-4582571>)

4. CONCLUSION

Categorization of patients depending on the need for health care will help to ensure a sufficient number of nurses and thus enable quality care of health care for patients and also facilitate the organization of the work of nurses. The progressive care mentioned in the paper is also very important in categorizing the patient ensuring the best possible care. This care as an important factor is further being divided into: intensive, semi-intensive and self-care. Based on this division, one will classify the patient in the appropriate group depending on how much care the patient needs.

The aim of the categorization is to provide a quick insight into the severity of the patient's condition by department, to provide an insight into the changing condition of patients during the day or from day to day, to enable a fair distribution of nurses within the hospital, department and other sectors of the health sector. It also provides each patient the best healthcare care according to its needs, enable the proper distribution of time per patient and according to the needs for health care, enabling reasoned estimation of the needs for nursing staff.

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BENEFITS OF USING FRESH CHOKEBERRY AND CHOKEBERRY PRODUCTS

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Key words: survey, producers, consumers, use of chokeberry

ABSTRACT:

In recent years, chokeberry has become increasingly popular as a berry fruit species that is attracting more and more attention among producers, consumers and medical professionals. The aim of this study was to examine different ways of using chokeberry and chokeberry products: user satisfaction, producer perspective, and the stance of medical experts, as well as the impact of this "super berry" on the human body and health. The research results indicate that chokeberry juice (60%) and syrup form (38.2%) are the most common products offered by producers on the market. The diversity of products allows for different options for consumption and meeting different consumer preferences. The survey also revealed that the majority of producers grow black chokeberry (34.5%), while (32.7%) of producers are dedicated to growing red chokeberry and the same amount to purple chokeberry. This indicates the variety of varieties available on the market, which can provide more opportunities for customization of cultivation according to specific conditions and consumer preferences. Respondents positively rated the effect of chokeberry on improving health, with most agreeing that chokeberry has a significant impact on strengthening the immune system, reducing lipids in the blood and urine, and helping to control glucose levels in the urine and blood. For producers, it is important to continuously monitor customer satisfaction to ensure the quality of products and services and develop strategies for improving offerings. It is particularly important to highlight that there has been an increase in the consumption of chokeberry during and after Covid, which further speaks to the importance of consuming chokeberry for boosting and enhancing the immune system.

Chokeberry has the potential to become an even more important fruit species in the food and pharmaceutical industries, and further research and public education can contribute to a better understanding and application of its health benefits for people.

1. INTRODUCTION

Chokeberry belongs to the rose family (*Rosacea*). Several synonyms are used for *Aronia melanocarpa* (black chokeberry) such as *Aronia nigra*, *Sorbus melanocarpa*, *Pyrus melanocarpa*, and *Mespilus arbutifolia* var. *Melanocarpa*, but in most countries the accepted name is *Aronia melanocarpa*. Chokeberry originates from North America and has spread worldwide. The genus of this fruit species includes three types: *Aronia arbutifolia* [L.] Elliot (red chokeberry), *Aronia melanocarpa* [Michx.] Elliot (black chokeberry), and *Aronia prunifolia* (Marsh.) Rehd. (purple chokeberry). Chokeberry is a lesser-known and highly valued fruit due to its unique taste, texture, and color. The fruits contain a high content of antioxidants, vitamins, minerals, fiber, folic acid, etc. In addition to fresh consumption, it is used in beverages, ice cream, yogurt, jams, jellies, and many other food products (Serce et al., 2010; Ercisli et al., 2012; Cuce & Sokmen, 2017; Alibabic et al., 2018; Skender et al., 2019). Chokeberry is also grown as an ornamental plant and for its high nutritional value, antioxidant properties, and distinctive taste. There are many varieties of chokeberry, with the most important ones in Europe being: Aron (Denmark), Nero (Czech Republic), Viking (Finland), Rubin (Russia via Finland), Kurkumäcki (Finland), Hugin (Sweden), Fertödi (Hungary) (Jeppsson, 1999; Janick & Paull, 2008). By its chemical composition, chokeberry stands out for its high content of anthocyanins, a natural antioxidant highly sought after in the food industry. chokeberry also contains a large amount of vitamin P, which is of great importance for a healthy diet. The content of other vitamins is also high, for example, 100g of fruits is enough to meet the daily requirement for folic acid. In addition, chokeberry contains minerals, primarily iron, and the amount of iodine is also relatively high. In relation to its relatively high sugar content of about 10%, the acidity content is relatively low at 1%. The concentration of pectin is around 0.75%, while the tannin content decreases from 0.6% to 0.35% with ripening.

Chokeberry is considered extremely medicinal because of its specific chemical composition which depends on a large number of factors such as climatic conditions of cultivation, soil, humidity, degree of maturity at the time of harvesting. Therefore, literary data on the quantity of individual components somewhat differ. Like other fruits rich in water, chokeberry has a low energy value. The content of reducing sugars (glucose, fructose) is 13-18%, while sucrose is not detected. Sorbitol, a sugar alcohol used as a sugar substitute, is present. The fat content averages 0.14%, and protein 0.7%. The total organic acid content is low compared to other fruits, ranging from 1 to 1.5%. The presence of dietary fibers has been detected in the amount of 5.62 g/100 g of fresh fruit with a pectin content of 0.3% to 0.6%. The richness of aroma is mostly due to the presence of amygdalin with a bitter taste and a mild almond scent, as well as strong tannins

(Kulling and Rawel, 2008).

It is important to emphasize the wealth of various polyphenolic components, which are attributed with possible pharmacological activity, as well as high levels of vitamins and minerals, which will be further described in the text. Although the *in vivo* bioactivity and bioavailability of polyphenols are not fully understood, the antioxidant and anti-inflammatory effects of polyphenols can reduce the risk of cardiovascular disease, metabolic syndrome, inflammation, and neurodegenerative disease (Jakobek and Seruga, 2012; Bhaswant et al., 2017).

Phenolic acids can participate in the process of healing damaged parts of the plant. From the above, it can be concluded that polyphenols enable the normal growth and development of the plant by providing it with the necessary protection (Kondakova et al., 2009).

Many studies suggest that chokeberry, based on its chemical composition, has the potential to have a positive effect on the human body. Polyphenolic components are highlighted as responsible for numerous biological activities such as antioxidant, antimutagenic, cardioprotective, anticancer, and antihyperglycemic activities (Kokotkiewicz et al., 2010).

Antioxidants act in multiple ways in defense against oxidative stress, such as binding metal ions, i.e. chelation of metals that act as catalysts in oxidation reactions (Jakobek, 2007). Additionally, antioxidants act by capturing free radicals, achieving neutralization and stabilization of radicals by donating electrons. It is believed that flavonoids can participate in the capture of free radicals, forming a flavonoid phenoxide radical that does not promote further chain reactions. This defense mechanism is also known as antiradical activity. The interaction between flavonoids and other molecules such as vitamins results in antioxidant effects. Interaction effects have been observed in interactions between vitamin C and quercetin, as well as α -tocopherol and certain polyphenolic compounds (Kazazić, 2004).

Consumption of chokeberry and chokeberry products is associated with positive effects on the human body. Several studies have been conducted to investigate the anti-cancer, cardioprotective, and anti-diabetic properties of chokeberry (Kulling and Rawel, 2008). chokeberry extract applied in the process of anti-tumor chemotherapy enhances the action of gemcitabine, a cytotoxic preparation used in the treatment of pancreatic cancer (Thani et al., 2014). Due to the association of oxidative stress with the development of breast cancer, a study was conducted to monitor changes in platelet proteins (thiol groups, carbonyl groups). Cells from three groups of patients were studied: those with benign tumors, invasive tumors, and healthy individuals (control group). The results confirmed that protein changes caused by oxidative stress are associated with breast cancer. It was found that chokeberry extract can impact the reduction of protein changes in cells of patients with benign and invasive breast tumors in terms of thiol oxidation, indicating its protective properties (Kedzierska et al., 2010).

Since chokeberry belongs to the group of berry fruits, whose cultivation in our area has been highly underrated in the past period. Due to its medicinal properties, it has become increasingly

popular in recent times, leading to a growing number of producers opting for its cultivation. chokeberry fruits are rarely consumed fresh, but they are an excellent raw material for the production of juices, and as such they currently have significant economic value. The aim of this work is to provide guidelines and recommendations based on precise indicators obtained from this research through conducted surveys and interviews, about the possibilities and prospects for improving the production, consumption, and benefits of using fresh chokeberry and chokeberry products. By surveying the influence of the medicinal properties of chokeberry, as well as consumer habits, attitudes of producers, consumers, and medical staff were determined.

2. MATERIAL AND METHODS

In this study, both quantitative and qualitative research methods were used. Quantitative and qualitative data were collected through a survey. The survey was constructed based on information gathered through desk research, from which secondary information on key aspects of the use of chokeberry and chokeberry products was collected. Since researching this topic requires analyzing the views of different interest groups, including user satisfaction, producers, and medical experts.

An electronic survey was distributed to key interest groups and data was collected. The analysis of the collected data was conducted using the statistical program SPSS. Based on statistical analysis, specific findings and insights into the views of different target groups and the perspectives on the impact of chokeberry on the human body and health, as well as its economic potential, were obtained. Besides statistical methods, an inductive method was used in this scientific study, through which the final view on the use of chokeberry was reached based on individual facts about chokeberry. In addition to the inductive approach, a deductive method and analysis and synthesis methods were also used in this study.

User satisfaction: One of the most important aspects of the project is surveying users who already use chokeberry products. Feedback from users provides insight into their satisfaction with the products, their experiences, and the benefits they have noticed. The percentage of users who repurchase chokeberry and regularly use it can indicate a high level of satisfaction and trust in the products.

Producer perspective: Talking to chokeberry producers gives insight into their goals and reasons for producing chokeberry. Understanding their perspectives and the challenges they face in growing and processing chokeberry can help assess the quality of the products and ways to improve production to achieve the highest possible level of health benefits.

Expertise of medical professionals: The opinion of medical experts plays a key role in understanding the potential health benefits of chokeberry. Surveying medical experts and scientists can help assess the scientific evidence and research supporting claims about the health benefits of chokeberry. Their perspective is particularly important as it contributes to patient education and provides information based on scientific knowledge.

Through all these perspectives, the project of analyzing the comparative advantages of chokeberry can provide a comprehensive insight into the product and its use for improving health. Surveying users, producers, and medical experts allows us to better understand the various aspects of the product, identify opportunities for improvement, and ensure that users receive high-quality products that can contribute to their health and well-being. The combination of these perspectives provides a foundation for informed decisions and encourages further research into the potential benefits of this extraordinary berry.

3. RESULTS

The results of the survey indicate that respondents are distributed across different age groups: under 18 years old: 1.8% of respondents belong to this age group, meaning they are mostly adolescents and younger individuals. Then 18-37 years old: 9.1% of respondents fall into this age group, suggesting that the majority of respondents are young adults. From 38-56 years old: 50.9% of respondents belong to this age group, indicating that they are middle-aged individuals. In the 57-75 age group: 35.5% of respondents fall into this age group, indicating that they are older adults. Over 75 years old: 2.7% of respondents are over 75 years old, representing a smaller percentage of older individuals in the study. The range of age structure of respondents shows diversity and includes various age groups.

Source: processing by the author

The statistical analysis of the collected data shows how often respondents use chokeberry. The data is presented in frequencies and percentages:

"Every day": 4.5% of respondents use chokeberry every day.

"Once a week": 17 respondents (15.3%) use chokeberry once a week.

"Several times a week": 19.8% of respondents use chokeberry several times a week.

"Once a month": 24.3% of respondents use chokeberry once a month.

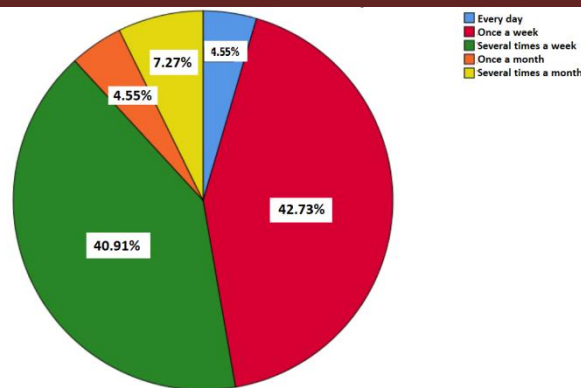
"Several times a month": 35.1% of respondents use chokeberry several times a month.

Additionally, respondents cite several reasons for discontinuing the consumption of chokeberry:

Lack of supply: 10% of respondents stated that the reason for discontinuing the consumption of chokeberry was the lack of availability of the product, which may indicate that chokeberry products are not always easily accessible in the market or in their vicinity.

Personal preferences: 37.3% of all respondents stopped consuming chokeberry due to their own preferences. This may mean that they did not like the taste or texture of chokeberry products or simply stopped consuming chokeberry for personal reasons.

High prices: 52.7% of respondents cited high prices as the reason for discontinuing the consumption of chokeberry.



Graph 1: How often do you use chokeberry. Source: processing by the author

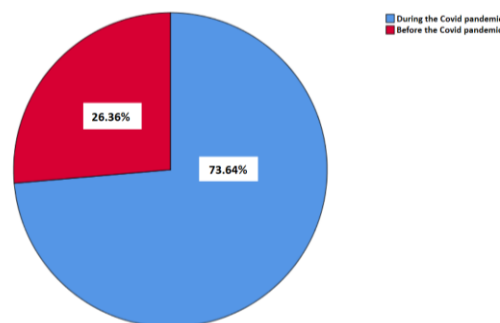
Survey results indicate the importance of providing a variety of accessible chokeberry products to meet different consumer needs and preferences, as well as to ensure continuous demand for these products.

Additionally, producers may consider adjusting prices to make chokeberry products more competitive in the market and attract a larger number of consumers. According to the survey results, 61.8% of respondents believe that chokeberry products are adequately available in the market of Bosnia and Herzegovina, while 38.2% believe they are not. These findings are crucial as they demonstrate how consumers perceive the availability of chokeberry products in the market. A positive response from the majority of respondents may indicate satisfaction with their current product choices, while a negative response may point to the need for improved availability and expanded offerings of chokeberry products to meet consumer needs and expectations. These data can be valuable for producers and retailers to better understand their consumers' perceptions and develop strategies to meet the demand for chokeberry products in the market of Bosnia and Herzegovina. Survey results also show respondents' attitudes towards the availability of chokeberry products in their place of residence: "Minimal": 5.5% stated that chokeberry products are very rarely available in their place of residence. "Hardly noticeable": 10.0% said that chokeberry products are only slightly noticeable in their place of residence. "Average": 23.6% mentioned that chokeberry product availability is average in their place of residence. "Noticeable": 38.2% claimed that chokeberry products are quite noticeable and available in their place of residence. "Maximum": 22.7% believe that chokeberry products are fully available and highly present in their place of residence. According to the survey results, respondents provided the following responses about where they buy chokeberry products: "Directly from the producer": 57.3% purchase chokeberry products directly from the producer, meaning they buy from the fruit producer or their authorized distributors. "Supermarkets": 28.2% buy chokeberry products in supermarkets, large retail chains offering a variety of products to a wide range of consumers. "Specialty stores and pharmacies": 10.9% buy chokeberry products in specialized stores or pharmacies focusing on selling healthy foods and dietary supplements. "I

Eighth International Scientific Conference "June 5th - World Environment Day"

produce it myself": 3.6% of respondents stated they produce chokeberry products themselves, suggesting they likely grow this plant and use it to make their own products. These data provide insight into preferred purchasing channels for chokeberry products among respondents. The majority of respondents prefer direct purchases from producers, indicating trust in the freshness and quality of the products. Additionally, product availability in supermarkets seems to be a relevant option for many, while specialty stores and self-production also play a smaller but significant role in overall chokeberry product distribution. When asked about when they started using chokeberry, 73.6% of respondents answered "During the Covid pandemic," indicating that this period was crucial for the beginning of chokeberry consumption, while 26.4% stated they began using chokeberry "Before the start of the Covid pandemic," meaning they had prior experience with this fruit. The data suggest that the Covid pandemic played a significant role in introducing chokeberry into the dietary habits of most respondents.

This could be related to increased interest in health and immunity strengthening during the pandemic, as chokeberry is promoted as a fruit with high nutritional value and antioxidant properties. On the other hand, there is also a smaller number of respondents who used chokeberry before the pandemic, which may indicate prior familiarity with this fruit or other reasons for incorporating it into their diet.

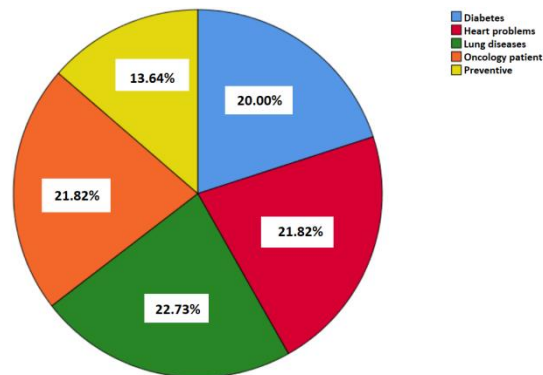


Graph 2: When did you start using chokeberry Source: processing by the author

According to the research results, respondents gave the following reasons for using chokeberry: "Diabetes": 20.0% stated that they use chokeberry because of diabetes, indicating a potential awareness of the benefits of this fruit in controlling blood sugar levels. "Heart problems": 21.8% mentioned using chokeberry for heart problems, suggesting they believe chokeberry may have a positive effect on cardiovascular health. "Respiratory diseases": 22.7% stated using chokeberry for respiratory diseases, indicating a belief in its potential benefits for respiratory health. "Cancer patients": 21.8% said they use chokeberry as cancer patients, which may suggest awareness of its potential role in supporting the immune system and fighting cancer cells. "Preventive": 13.6% of respondents mentioned using chokeberry preventively, meaning they include it in their diet as a general health preservation measure. These data show that respondents use chokeberry for various health reasons, including specific health conditions such as diabetes, heart problems, and respiratory diseases, as well as in prevention and support during cancer treatment. This indicates

Eighth International Scientific Conference "June 5th - World Environment Day"

a growing awareness of the health benefits of chokeberry and its potential as a dietary supplement for improving overall health and preventing disease.



Graph 3: The reason you use chokeberry Source: processing by the author

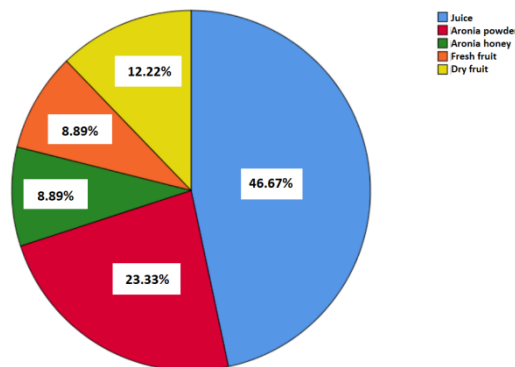
Oxidative stress is a common occurrence in many cancer cells, leading researchers to explore the potential benefits of antioxidants in cancer treatment. Studies have shown that antioxidant extracts from Aronia berries, as well as their natural compounds or modified versions, may have therapeutic effects on a variety of cancer types. These include breast, cervical, colon, glioblastoma, liver, and lung cancer, as well as leukemia cells. (Sidor et al., 2019. Yang et al., 2021. Jurendić 2021).

Aronia berries exhibit promising effects on human colon cancer cells. Extracts from commercially available red (*Aronia arbutifolia*), purple (*Aronia prunifolia*), and black (*Aronia melanocarpa*) chokeberry fruits were analyzed for their levels of total phenols, antioxidant properties, and ability to inhibit the growth of HT-29 colon cancer cells. The findings revealed that only the extract from black chokeberry was effective against HT-29 cells, with its efficacy corresponding to its total phenolic content, antioxidant activity, and presence of caffeic and chlorogenic acids. (Gill et al., 2021) It has been previously documented in studies that Aronia berries have strong inhibitory activity against various infections, believed to be due to their powerful antioxidant components. (Sidor et al., 2019. Yang et al., 2021. Jurendić 2021) For instance, both water and ethanol extracts of Aronia berries have shown antimicrobial effects against strains of *E. coli*, *S. aureus*, and *S. pyogenes* (Daoutidou et al., 2021), with the phenolic compounds in the berries exhibiting varying antimicrobial effects. (Denev et al., 2019).

Which products were most commonly purchased? The results show that 40.0% of respondents most commonly buy "chokeberry juice." Chokeberry juice is often considered one of the more popular products because it is easily accessible and can be suitable for daily consumption. "chokeberry powder" is most commonly purchased by 18.2% of respondents. Chokeberry powder can be a convenient form for consumption, especially for adding to smoothies, yogurts, or other beverages. "chokeberry honey" is most commonly purchased by 20.0% of those surveyed. chokeberry honey can be an interesting choice due to the combination of honey and the medicinal properties of chokeberry. The survey also found that 10.9% of respondents most

Eighth International Scientific Conference "June 5th - World Environment Day"

commonly buy chokeberry in "Fresh fruit form." Consuming fresh fruits can provide the most natural form of this fruit, but may be limited by seasonal availability, while 10.9% also indicated that they most commonly purchase dried chokeberry fruit. Dried fruits can be a practical and long-lasting way to consume chokeberry.



Graph 4: What type of chokeberry products do you most often buy Source: processing by the author

The majority of participants (96.4%) stated that they feel better after using chokeberry. Based on these data, it can be concluded that the vast majority of chokeberry users experience positive effects and benefits after consuming this fruit or chokeberry products. On the other hand, only 3.6% stated that they do not feel better after using chokeberry.

The reasons for this may be different and individual, which may include various reactions of the body, other health factors, or perhaps inadequate or insufficient consumption of chokeberry. It is important to note that these results show the positive experience of most participants using chokeberry, but individual effects may vary from person to person. Also, for a more precise analysis, other aspects such as dosage, frequency of consumption, overall lifestyle, and dietary habits of individuals need to be taken into account.

Analyzing the effects of consuming chokeberry and chokeberry products, different participants have felt positive effects from using chokeberry in different time periods. 16.4% experienced the positive effects of consuming chokeberry in less than seven days after starting chokeberry consumption, 25.5% stated that they felt positive effects after seven days of consumption, 19.1% after a month of using chokeberry, 27.3% noticed positive effects after several months of regular consumption, while only 11.8% of participants felt positive effects after a year of consuming chokeberry.

The data indicates a diversity in the timeframes in which participants feel positive effects after starting to consume chokeberry. Some have experienced quick changes, while others required a longer period of regular consumption to notice the positive effects of consuming chokeberry on their health. The results suggest that individual responses to chokeberry consumption may vary and that it is important to be patient and consistent in consumption in order to achieve desired effects. It is also worth noting that various factors, including individual health conditions, dietary habits, and dosage of consumption, can impact the speed of feeling positive effects.

Eighth International Scientific Conference
"June 5th - World Environment Day"

Looking at the reflection of chokeberry usage from a health perspective, 6.4% of participants highlighted the positive impact of chokeberry on circulation, blood pressure, and cholesterol, while 8.2% noticed a positive effect. Additionally, 8.2% stated that chokeberry helped regulate diabetes, and 10.0% felt a positive effect of consuming chokeberry on strengthening the immune system. Only 1.8% of participants mentioned the positive aspect of chokeberry use on weight regulation, while 3.6% noticed an improvement in skin quality. The majority of participants, 20.9%, reported that the use of chokeberry positively affected lung diseases, allergies, and asthma. Even 40.9% emphasized the positive effect of consuming chokeberry on their overall health. The results suggest that chokeberry can have various positive effects on people's health and that its beneficial effects can be observed in different areas of health, including circulation, blood pressure, cholesterol levels, diabetes, immune system, skin, lung diseases, and general well-being. The results also indicate the importance and breadth of chokeberry application for the purpose of preserving and improving the health of participants. Based on the table, it can be concluded that all respondents are familiar with the benefits of using fresh chokeberry and chokeberry supplements.

Table 1: Who do you recommend the use of chokeberry and chokeberry preparations?

	Frequency	Percent	Valid Percent	Cumulative Percent
Chronic patients	3	3.3	3.3	3.3
Autoimmune patients	18	20.0	20.0	23.3
Oncological patients	27	30.0	30.0	53.3
Covid and post-covid patient	42	46.7	46.7	100.0
Total	90	100.0	100.0	

The survey results show the familiarity of respondents with the benefits of using fresh chokeberry and chokeberry products, as well as recommendations for using chokeberry. 3.3% of respondents recommend the use of chokeberry and chokeberry products for "chronic patients", 20.0% for "patients with autoimmune diseases", and 30.0% for "oncology patients". The use of chokeberry and chokeberry products for "Covid and post-Covid patients" is recommended by 46.7% of respondents.

The majority of respondents rate the use of chokeberry as significant for improving the health of patients. The results show that 55.6% of them responded with "I mostly agree", meaning they believe that the use of chokeberry mostly significantly improves the health of patients, and 44.4% of respondents answered "I completely agree", meaning they believe that the use of chokeberry completely significantly improves the health of patients.

The majority of survey respondents, 35.6%, completely agree that chokeberry is an extremely strong antioxidant, while 34.4% mostly agree, and 30.0% do not have a decisive stance, that is, neither agree nor disagree that chokeberry is an extremely strong antioxidant.

Eighth International Scientific Conference "June 5th - World Environment Day"

Table 2: According to your experience, is chokeberry an extremely strong antioxidant

	Frequency	Percent	Valid Percent	Cumulative Percent
I neither agree nor disagree	27	30.0	30.0	30.0
I mostly agree	31	34.4	34.4	64.4
I completely agree	32	35.5	35.6	100.00
Total	90	100.00	100.00	

Opinions of survey respondents on whether diabetics should use chokeberry vary. 27.8% of respondents do not have a clear stance, while 44.4% agree that diabetics should use chokeberry. 27.8% completely agree that diabetics should use chokeberry.

Table 3: Do you agree with the statement that aronia improves the general state of the immune system?

	Frequency	Percent	Valid Percent	Cumulative Percent
I neither agree nor disagree	5	5.6	5.6	5.6
I mostly agree	9	10.0	10.0	15.6
I completely agree	76	84.4	84.4	100.00
Total	90	100.00	100.00	

The majority of survey respondents (84.4%) completely agree with the statement that chokeberry boosts the overall condition of the immune system. A smaller percentage of respondents mostly agree with this statement (10%), while only 5.6% do not have a decisive stance on it.

Table 4: Do you think chokeberry should be used by cancer patients?

	Frequency	Percent	Valid Percent	Cumulative Percent
I mostly agree	29	32.2	32.2	32.2
I completely agree	61	67.8	100.00	100.00
Total	90	100.00	100.00	

The majority of survey respondents (67.8%) completely agree that oncology patients should use chokeberry. An additional 32.2% of respondents mostly agree with this statement. The majority

Eighth International Scientific Conference "June 5th - World Environment Day"

of survey respondents (55.6%) completely agree that chokeberry helps maintain normal blood pressure levels. An additional 43.3% of respondents mostly agree with this statement.

Table 5: Do you think chokeberry helps maintain normal blood pressure

	Frequency	Percent	Valid Percent	Cumulative Percent
I neither agree nor disagree	1	1.1	1.1	1.1
I mostly agree	39	43.3	43.3	44.4
I completely agree	50	55.6	55.6	100.0
Total	90	100.0	100.0	

According to the survey results, respondents' attitudes differ regarding the use of chokeberry for the prevention and control of cancer development: 1.1% of respondents mostly disagree with the claim that chokeberry should be used, 34.4% do not have a decisive stance, 43.3%

mostly agree with the claim that chokeberry should be used for the prevention and control of cancer development, while 21.1% of respondents completely agree with the claim that chokeberry should be used for this purpose.

Most survey participants (43.3%) fully agree that they recommend the use of chokeberry to patients with lung diseases and allergies tendencies. An additional 40% of participants mostly agree with this statement. Only 16.7% of participants do not have a decisive stance on the matter. According to the results, most survey participants (75.6%) neither agree nor disagree with the recommendation of using chokeberry for pregnant women and children. 20% of participants mostly agree with this statement, while only 4.4% of participants fully agree with the recommendation of using chokeberry for pregnant women and children. Most survey participants (68.9%) fully agree that they recommend the use of chokeberry to Covid and Post Covid patients, while 31.1% of participants mostly agree with this statement. The majority of survey participants (95.6%) agree that patients who use chokeberry alongside prescribed medications experience greater effectiveness and improvement in health compared to those who do not use chokeberry, while 4.4% of participants claim otherwise.

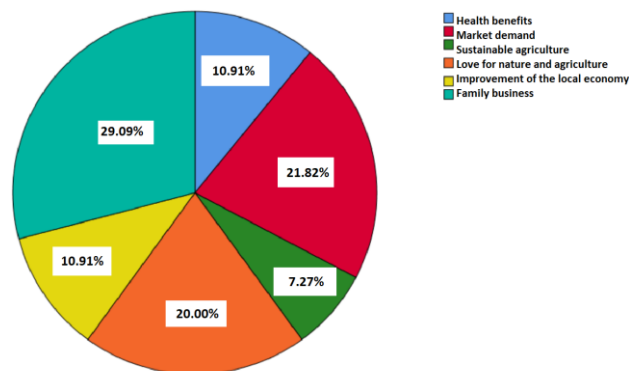
Based on the results, we see different perceptions among survey participants regarding the timeframe in which they observe improvements in the health of patients using chokeberry products: 13.3% claim to notice improvement in less than seven days after using chokeberry products, 21.1% stated improvement within a week of using chokeberry products, 32.2% notice improvement within a month of using chokeberry products, 22.2% see improvement within a few months after using chokeberry products, and 11.1% notice improvement within a year of using chokeberry products. Based on the results, we see which chokeberry products survey participants most commonly recommend: chokeberry juice 46.7% of respondents, 23.3% often recommend chokeberry powder, 8.9% often recommend chokeberry honey, 8.9% often

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recommend fresh chokeberry fruit, and 12.2% of respondents often recommend dried chokeberry fruits. Based on the results, we see the reasons that motivated producers to start growing and processing chokeberry: 10.9% were motivated by health reasons to engage in chokeberry cultivation and production. High demand in the market for chokeberry was the motivation for 21.8% of chokeberry producers to start production, while 7.3% emphasized that sustainable agriculture was an important factor in the decision to engage in chokeberry cultivation.

Love for nature and agriculture was a motivation for 20.0% of surveyed chokeberry producers. 10.9% highlighted that their desire to improve the local economy was a motivation for engaging in chokeberry cultivation and production.

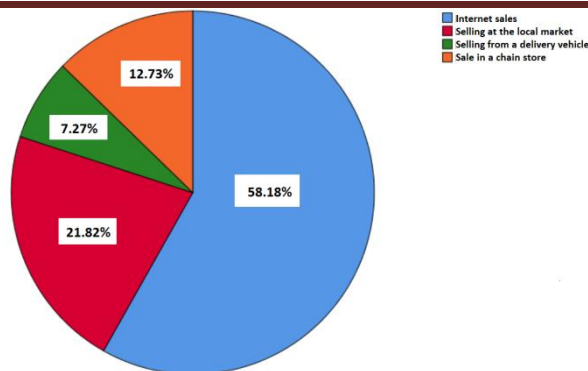
The survey shows that 3.6% of producers have been involved in chokeberry production for less than a year, exactly one year 7.3% of producers, two years 20.0%, three years 65.5% of producers and more than three years: only 3.6% of chokeberry producers. The majority of survey respondents (92.7%) produce chokeberry according to organic production principles, while only a small number of respondents (7.3%) produce chokeberry according to conventional production principles.



Graph 5: What motivates you to start growing and producing chokeberry and products Source: processing by the author

The survey shows that producers cultivate three different varieties of chokeberry: Red chokeberry 32.7% of producers, Black chokeberry - 34.5% of producers. The majority of 60% of producers produce pure chokeberry juice, and 38.2% produce chokeberry juice in the form of syrup. Also, one of the producers (1.8%) produces chokeberry tea.

Chokeberry juice and syrup are popular products due to their convenience and versatility in consumption in various situations. Chokeberry juice retains most of the natural properties and nutrients of chokeberry because it is made from fresh berries. Syrup-form juice may be attractive due to its sweet taste and ease of use in making drinks and various recipes.



Graph 6: How you sell your products Source: processing by the author

Manufacturers use different methods to sell their products. A survey showed that 58.2% of manufacturers sell their products online. This may include online stores, websites, or platforms for selling products. 21.8% of manufacturers sell their products at local markets, which may include sales stands or outdoor stands where products are sold directly to customers. 7.3% use sales through a delivery vehicle, which may include mobile sales at different locations or delivery of products directly to customers. 12.7% of chokeberry producers sell their products in retail chains, which means their products may be available in supermarkets, specialty stores, or retail chains.

These data indicate the diverse sales strategies used by chokeberry producers. Online sales make up the largest share of sales methods, which may be a result of the growing trend of online shopping and wider availability to the market through the internet. Sales at local markets and through delivery vehicles allow for direct interaction with consumers and promote products at a local level. On the other hand, sales in retail chains provide an opportunity to expand distribution and reach a larger number of consumers.

A combination of different sales methods can be beneficial for diverse needs and goals of chokeberry producers, and it is essential to ensure product quality and adapt to market demands to ensure successful product placement. Most subjects (85.5%) use a slogan when promoting their product or company, while a smaller number of subjects (14.5%) do not. Using a slogan can be a useful tool in a marketing strategy for product or company recognition and creating brand awareness and connection with the target market.

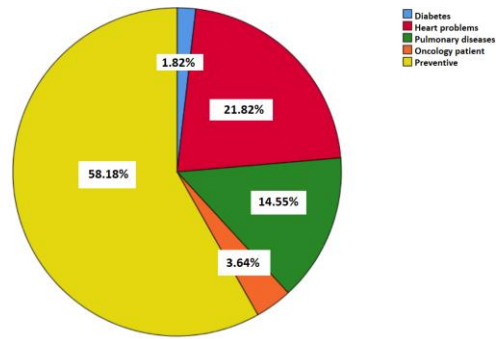
All manufacturers (100%) who participated in the survey reported an increase in the number of customers during the Covid pandemic. This increase in customers may be a result of various factors present during the pandemic. In some cases, changes in consumer habits and preferences may lead to increased demand for products considered healthier or with specific health benefits, such as chokeberry products.

Also, increased awareness of the health benefits of chokeberry and its potential contribution to strengthening the immune system may be a reason for the demand for chokeberry products during the pandemic.

Eighth International Scientific Conference "June 5th - World Environment Day"

The most common reasons customers buy chokeberry are: preventive (58.2%), for heart problems (21.8%), for lung diseases (14.5%), which may be related to research suggesting that chokeberry may have health benefits for lung and respiratory health, for diabetes (1.8%), cancer patients (3.6%), which may be related to research exploring potential antioxidant and anti-inflammatory effects of chokeberry that may be beneficial in cancer.

Aronia berries are rich in bioactive compounds, such as anthocyanins, proanthocyanidins, flavonoids, and phenolic acids, that exhibit antiviral properties against influenza viruses. These compounds have been found to inhibit the replication of viruses both directly and indirectly by interfering with viral surface glycoproteins and enhancing the immune response of the host. This suggests that Aronia berries may have potential benefits in the prevention and treatment of influenza, a serious illness that can lead to complications (Gramza et al., 2017).



Graph 7: What are the most common reasons why customers buy chokeberry Source: processing by the author

Most producers (89.1%) survey customer satisfaction, while a smaller number of subjects (10.9%) do not. Surveying customer satisfaction is an important tool in business management as it allows subjects to receive feedback from their customers about their products and services.

This feedback can help subjects understand what their customers value, which aspects of their business need improvement, and how they can better meet the needs and demands of customers.

Table 6: Do you survey customer satisfaction

	Frequency	Percent	Valid Percent	Cumulative Percent
YES	49	89.1	89.1	89.1
NO	6	10.9	10.9	100.00
Total	55	100.00	100.00	

All customers (100%) who participated in the survey re-use chokeberry products after making a purchase. A high percentage of customers re-using products suggest that they are satisfied with chokeberry products and have had a positive experience using them. When customers re-use products after making a purchase, it may indicate their trust in the quality and effectiveness of the products. Satisfied and loyal customers are key to the success of any business, so it is important to continue providing quality products and services to maintain customer trust and

encourage loyalty to the brand. In addition, loyal customers often become brand ambassadors who recommend products to their friends and family, which can lead to an increase in new customers and business growth.

4. CONCLUSION

In recent years, chokeberry has become an increasingly popular plant that is attracting growing interest among producers, consumers, and medical professionals. This berry stands out for its richness in antioxidants, vitamins, minerals, and other beneficial phytonutrients, making it an excellent addition to the food and health industry. Investigating the comparative benefits of chokeberry plays a key role in providing relevant information and confirming its actual impact on human health. A survey conducted among chokeberry producers, users, and medical professionals revealed that all respondents were familiar with the benefits of using fresh chokeberry and chokeberry products. Participants positively rated the effect of chokeberry on improving health, with the majority agreeing that chokeberry has a significant impact on strengthening the immune system, reducing lipid oxidation in the blood and urine, and helping control glucose levels in the urine and blood. Chokeberry juice (60%) and syrup form (38.2%) are the most common products offered by producers in the market. This variety of products allows for different consumption options and satisfies various consumer preferences. The survey also found that most producers grow black chokeberry (34.5%), while 32.7% are dedicated to growing red chokeberry and the same for purple chokeberry. This indicates the diversity of varieties available in the market, which can provide more opportunities for adapting cultivation to specific conditions and consumer preferences. It is important for producers to continuously monitor customer satisfaction to ensure product and service quality and develop strategies to improve offerings. Chokeberry has the potential to become an even more important fruit plant in the food and health industry, and further research and public education can contribute to a better understanding and application of its comparative advantages for human health. It is particularly important to emphasize that there has been an increase in chokeberry consumption during and after Covid, which further emphasizes the importance of consuming chokeberry for boosting and enhancing the immune system.

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INHIBITION OF CHOLINESTERASE AND α -GLUCOSIDASE ENZYMES BY WATER EXTRACTS OF FIVE *CENTAUREA* SPECIES

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Key words: Asteraceae, *Centaurea*, AChE, BChE, α -glucosidase

ABSTRACT:

Inhibition of important enzymes is one of the fundamental therapies for treating diseases such as Alzheimer's dementia and diabetes mellitus. Genus Centaurea is one of the most important genera of the Asteraceae family, with a large number of species. In Croatia there are around 80 species of this genus, of which 27 are endemic. In this study, the chemical and biological profile of the water extracts of five plants of the genus Centaurea was determined. The chemical profile of the extracts was determined by qualitative determination of secondary metabolites and by quantitative determination of the total phenol content and the total flavonoid content. Biological potential of the extracts were investigated in terms of the inhibition of acetylcholinesterase (AChE), butyrylcholinesterase (BuChE) and α -glucosidase enzymes. Glycosides and saponins have been identified in plant water extracts, while alkaloids, tannins, steroids and terpenoids have not been identified. The results showed that all Centaurea plant water extracts contain a relatively small content of phenolic and flavonoid compounds. The extracts of C. scabiosa (36.6%) and C. rhenana (28.9%) showed a low inhibition of AChE (36.6% and 28.9%), C. triumphetti and C. ragusina showed a very low inhibition of AChE (3.2% and 9.7%), whereas C. alba did not inhibit AChE. C. alba, C. rhenana and C. scabiosa extracts poorly inhibited BuChE (11.1%, 3.1% and 2.9%), while C. triumphetti and C. ragusina did not inhibit this enzyme. The C. scabiosa extract showed a moderate inhibition on α -glucosidase (20.6%), C. rhenana extract showed a low inhibition (6.7%), while other tested extracts did not inhibit this enzyme. No significant

inhibitory potential of extracts isolated from Centaurea plants on the enzymes AChE, BuChE and α -glucosidase has been shown. It is necessary to perform further investigation by using other types of extracts of tested plants species.

1. INTRODUCTION

Research on new biologically active compounds of natural origin still has its importance. People have used medicinal plants throughout history, and long ago, records of their use were kept (Kabera et al., 2014). The primary role of secondary plant metabolites is defensive, primarily the defense of plants against pests (Pagare et al., 2015). In modern times, these compounds are isolated and studied with the goal of discovering and developing medicines (Kabera et al., 2014). Nowadays, a large number of secondary plant metabolites are known to have valuable physiological and pharmacological properties (Kabera et al., 2014; Velu et al., 2018). Therefore, each new research on isolated compounds from nature can be a significant contribution to health (Nisar et al., 2017; Pan et al., 2013).

Inhibition of important enzymes is one of the basic therapies in the treatment of diseases, such as Alzheimer's disease (AD) and diabetes (DB) (Ucar et al., 2018). Cholinesterases (ChE) are enzymes that catalyze the hydrolysis of acetylcholine (ACh) into choline and acetic acid. There are two types of ChE, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). Although these two enzymes are similar to each other, their importance and localization in the body differ (Pohanka, 2011). AChE plays a crucial role in accelerating amyloid deposition in the brain of people with Alzheimer's disease. Therefore, cholinesterase inhibitors are used to improve cognitive functions in patients with AD (Giacobini, 2003). α -glucosidase is a digestive enzyme that directly affects blood glucose levels. In the human digestive system, a number of glucosidase enzymes participate in the process of digesting starch and other polysaccharides. The α -amylase enzymes in the mouth and intestines hydrolyze the α -(1,4)-glycosidic bond and break down polysaccharides into shorter oligosaccharides. Since such sugars cannot be absorbed in the digestive system, they are further hydrolyzed into simpler oligosaccharides or monosaccharides with the help of α -glucosidase enzymes that cleave the α -(1,4)- and α -(1,6)-glycosidic bonds.

Inhibition of this enzyme plays a role in the treatment of type 2 diabetes (Ali et al., 2006; Bhandari et al., 2008). The genus *Centaurea* L. is one of the most important and widely distributed genera in the Asteraceae family, with over 400 species. In Croatia, there are around 80 species of this genus, of which 27 are endemic (Nikolić, 2013). Chemical studies have shown the presence of compounds such as sesquiterpene lactones, flavonoids, and lignans – to which the medicinal properties of some species of the genus *Centaurea* can be attributed (Bessada et al., 2015; Sanchez-Mata and Tardio, 2016; Erel et al., 2011; Radan et al., 2017).

Therefore, in this study, the chemical and biological profile of plant extracts from the species of the genus *Centaurea*: *Centaurea rhenana* Boreau, *Centaurea scabiosa* L., *Centaurea triumfetti* All., *Centaurea ragusina* L., and *Centaurea alba* L. were investigated as part of the research on

isolated volatile and non-volatile compounds with potentially valuable pharmacological effects.

2. MATERIAL AND METHODS

Collection of plant material and preparation of extracts. Plants of the genus *Centaurea* were collected in Croatia and Bosnia and Herzegovina in the spring of 2018: *C. rhenana* (Pasjača, Konavli), *C. scabiosa* (Biokovo, Vošac), *C. triumphetti* (Tijarica, Doci), *C. ragusina* (Marjan, Split) and *C. alba* (Međugorje). Plant samples were herbalized and are stored at the Department of Biochemistry, Faculty of Chemistry and Technology in Split. Aqueous extracts were isolated after hydrodistillation of essential oils (100 g of plant material + 500 mL of water) of the mentioned plant species, using a modified Clevenger-type apparatus. The aqueous extracts were separated from the plant material by filtration and lyophilized for further analysis. From the lyophilized extracts, solutions of known concentrations were prepared by dissolving in distilled water for the purposes of the analyses.

Chemicals. Gallic Acid; Folin-Ciocalteu's phenol reagent; AlCl_3 ; Quercetin $\geq 95\%$ solid (HPLC); DTNB (5,5'-Dithiobis(2-nitrobenzoic acid) $\geq 98\%$; Acetylcholinesterase from *Electrophorus electricus* – electric eel, type V-S; Butyrylcholinesterase from equine serum; Acetylthiocholine iodide; Butyrylthiocholine iodide; α -Glucosidase from *Saccharomyces cerevisiae*; 4-Nitrophenyl α -D-galactopyranoside, Acarbose $\geq 95\%$ (Sigma Aldrich GmbH, Steinheim, Germany).

Qualitative proof of certain classes of compounds of extracts. The isolated extracts of plants of the genus *Centaurea* were tested for the presence of alkaloids, tannins, steroids, terpenes, glycosides, and saponins. The reactions of proving the mentioned compounds are based on simple precipitation and staining reactions (Guettaf et al., 2016).

Quantitative determination of phenols and flavonoids in extract samples. In the isolated aqueous extracts of plants of the genus *Centaurea*, the content of total phenols and total flavonoids was quantitatively determined. The content of total phenols was determined spectrophotometrically using the Folin-Ciocalteu method with gallic acid (GA) as the standard (Singleton and Rossi, 1965; Katalinic et al., 2013). The results are expressed as mg of gallic acid equivalent (GAE) per 1 g of extract. The content of flavonoids was tested using the aluminum (III) chloride method (Chang et al., 2002). The results are expressed as mg of quercetin equivalent (QE) per 1 g of extract.

Determination of ability to inhibit cholinesterase: AChE and BChE. The ability of water extracts of plants to inhibit cholinesterases AChE and BChE was tested spectrophotometrically, using the Ellman method (Ellman et al., 1961). Extracts of the plants *C. rhenana*, *C. scabiosa*, *C. triumphetti*, *C. ragusina* and *C. alba* with a stock solution concentration of 1 mg/mL were tested. Galantamine solution with a concentration of 5 $\mu\text{g/mL}$ was used as a positive control (i.e. a standard that

successfully inhibits cholinesterase). Results are presented as % of enzyme inhibition.

Determination of ability to inhibit α -glucosidase enzyme. The ability of plant extracts to inhibit the α -glucosidase enzyme was tested spectrophotometrically using the method described by Pistia-Brueggeman and Hollingsworth (2001), and Bhatia et al., (2019). Extracts with a stock solution concentration of 1 mg/mL were tested. An acarbose solution of the same concentration was used as a positive control (standard). The results are presented as % of enzyme inhibition.

3. RESULTS

The chemical and biological profile of plants of the genus *Centaurea* (*C. rhenana*, *C. scabiosa*, *C. triumphetti*, *C. ragusina*, *C. alba*) was determined by qualitatively or quantitatively detecting specific classes of compounds in previously isolated aqueous extracts of these plants. The biological profile was determined by testing the ability of these extracts to inhibit enzymes such as acetylcholinesterase, butyrylcholinesterase, and α -glucosidase. Qualitative detection of specific classes of compounds in the extracts was performed according to the protocol by Guettaf et al. (2016). Quantitatively, the total content of phenols was determined using the Folin-Ciocalteu method, and the content of flavonoids was determined using the aluminum (III) chloride method. The ability to inhibit the enzymes cholinesterase, AChE and BChE, was tested using the Ellman method, while the ability to inhibit the enzyme α -glucosidase was tested using the method described by Brueggeman and Hollingsworth (2001). In the extracts of *C. rhenana*, *C. scabiosa*, *C. triumphetti*, *C. ragusina* and *C. alba*, the presence of glycosides and saponins was qualitatively proven, and the presence of alkaloids, tannins, steroids and terpenoids was not proven. All extract samples contain a relatively low content of phenolic compounds (65.0 – 143.5 mg GAE/g extract) and a low content of flavonoid components (34.5 – 61.9 mg QE/g extract). The highest content of phenolic compounds is in the extract of the *C. scabiosa* plant, while the lowest content of phenolic compounds is in the extract of the *C. rhenana* plant. The highest content of flavonoids is in the extract of the *C. alba* plant, while the lowest is in that of the *C. rhenana* plant (Table 1). The results of testing the inhibitory potential of the extracts on the enzymes AChE, BuChE and α -glucosidase (Table 1) showed that the extracts of *C. scabiosa* and *C. rhenana* weakly inhibit the enzyme AChE (36.6 and 28.9%), while the extract of *C. alba* does not inhibit AChE. *C. triumphetti* and *C. ragusina* extracts inhibit AChE very weakly (3.2 and 9.7%). Extract samples of *C. alba*, *C. rhenana* and *C. scabiosa* weakly inhibit BuChE (11.1, 3.1 and 2.9%), while extract samples of *C. triumphetti* and *C. ragusina* do not inhibit this enzyme. Galantamine (positive control for AChE and BuChE) at a concentration of 5 μ g/mL inhibits AChE with 78.6%, while BuChE inhibits with 40.9%. The sample of *C. scabiosa* plant extract, tested in this work, slightly inhibits the enzyme α -glucosidase (20.6%), the sample of *C. rhenana* weakly inhibits (6.7%), while the other tested extracts do not inhibit this enzyme. Acarbose

Eighth International Scientific Conference "June 5th - World Environment Day"

(positive control for α -glucosidase) in the same tested concentration of 1 mg/mL inhibits the enzyme by 44.2% (Table 1).

Table 1: Content of total phenols and total flavonoids in water extracts of *Centaurea* plants and their ability to inhibit the enzymes acetylcholinesterase, butyrylcholinesterase and α -glucosidases.

Plant	Phenols (mg GAE/g)	Flavonoids (mg QE/g)	AChE % inhibition*	BuChE % inhibition*	α -glucosidase % inhibition*
<i>C. rhenana</i>	65.0	34.5	28.9	3.1	6.7
<i>C. scabiosa</i>	143.5	45.5	36.6	2.9	20.6
<i>C. triumfetti</i>	94.2	60.5	3.2	‡	‡
<i>C. ragusina</i>	84.2	54.5	9.7	‡	‡
<i>C. alba</i>	83.5	61.9	‡	11.1	‡
Galantamine*	§	§	78.6	40.9	§
Acarbose**	§	§	§	§	44.2

Legend: * Tested concentration 1 mg/mL. † Tested concentration 5 μ g/mL. ‡ No inhibition. § Not tested.

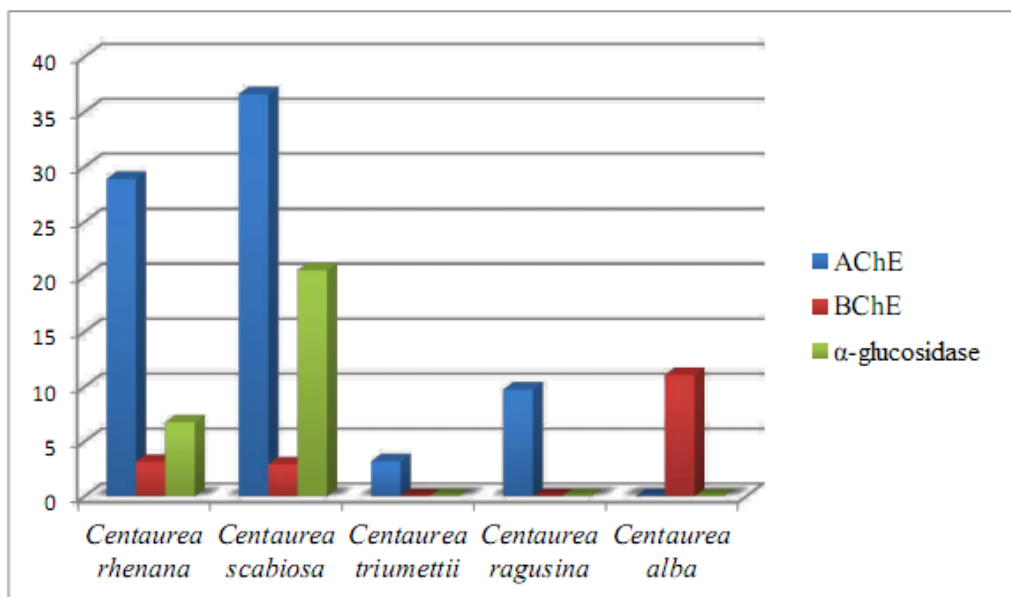


Figure 1: Histogram representation of the ability to inhibit the enzymes AChE, BChE and α -glucosidase

3. DISCUSSION

In the aqueous extracts of the plants *C. rhenana*, *C. scabiosa*, *C. triumfetti*, *C. ragusina* and *C. alba*, the presence of glycosides and saponins was qualitatively proven, but the presence of alkaloids, tannins, steroids and terpenoids was not proven. All extracts contain a relatively low content of phenolic compounds and a low content of flavonoid components. Zengin et al. (2001) determined the content of total phenols and flavonoids in ethyl acetate and chloroform extracts of *C. triumfetti*. The content of total phenols in the ethyl-acetate extract of this plant is twice as high

as that of the chloroform extract and comparable to the results obtained in this research. No flavonoids were recorded in the ethyl acetate extract of that plant, while low flavonoid content was determined in the chloroform extract. The content of phenolic and flavonoid components of the ethanolic, methanolic and ethyl-acetate extract of the flower and stem of the plant *C. triumfetti* was also determined by Acet (2020). The results showed that the extracts from the stem generally have a higher content of both phenols and flavonoids. The ethyl-acetate extract isolated from the stem of this plant contains the highest content of both phenols and flavonoids, and noticeably higher than the extracts tested in this work. The reason for the different extraction of phenolic and flavonoid compounds from plant material is the use of different solvents during the extraction process of these classes of compounds.

The results of testing the inhibitory potential of the extracts on the enzymes AChE, BuChE and α -glucosidase showed weak or no ability to inhibit these enzymes. Ethyl-acetate and chloroform extract of *C. triumfetti* plant were tested by Zengin et al. (2001) on the ability to inhibit the enzymes AChE, BuChE and α -glucosidase. These extracts show a noticeably better ability to inhibit the tested enzymes. The ability to inhibit the enzyme α -glucosidase with extracts (ethanolic, methanolic and ethyl-acetate) obtained from dry flowers and stems of the *C. triumfetti* plant was also tested by Acet (2020). The results showed a better inhibition ability of the extracts isolated from the stem of the plant compared to the inhibition ability of the extracts obtained from the flowers. The author of this study assumes that the ability to inhibit α -glucosidase is related to the content of phenolic components of the extracts (Acet, 2020).

Currently approved medicines for the clinical treatment of Alzheimer's disease are cholinesterase inhibitors such as donepezil, rivastigmine, galantamine, tacrine, and memantine. They alleviate the symptoms of the disease, but they also have numerous side effects (Simunkova et al., 2019). Among the best inhibitors of AChE and BuChE enzymes isolated from nature, alkaloids (e.g. galantamine, huperzine A, solanidine, solanine) and flavonoids (e.g. galangin) stand out (Bosak, 2011). Considering that the tested samples do not contain alkaloids and the flavanoids are contained in low concentrations, the weak inhibition of AChE and BuChE by the extracts of *Centaurea* plants can be attributed to the presence of some of the flavanoid compounds contained in the extracts.

Acarbose, which is used as a very potent inhibitor of the enzyme α -glucosidase, is a compound that was used as a positive control in this work when testing the ability of the extracts to inhibit the aforementioned enzyme as well. Acarbose is a pseudo-tetrasaccharide with a nitrogen atom that, thanks to its modified tetrasaccharide structure, strongly and competitively inhibits α -glucosidase. The history of acarbose begins with a screening program of various compounds isolated from *Actinomycetes* sp. which was initiated by Naik and Kokil (2013). One of the compounds (acarbose) was found to be a strong inhibitor of digestive enzymes such as α -amylase, α -glucosidase, sucrase, and maltase. Among the phenolic and flavanoid compounds there are compounds with a good ability to inhibit the α -glucosidase enzyme. Such compounds

are, for example, gallic acid, methyl gallate, propyl gallate, rosmarinic acid, quercetin, sarcosyl, genistein, apigenin, luteolin, catechins (Yin et al., 2014). The recorded inhibition of this enzyme by two extracts, especially that of the *C. scabiosa* plant, can be attributed to the presence of phenolic compounds.

4. CONCLUSION

In the aqueous extracts of the tested plants, the presence of glycosides and saponins was qualitatively proven, but the presence of alkaloids, tannins, steroids and terpenoids was not proven. All extracts contain a relatively low content of phenolic compounds and a low content of flavonoids. The results of testing the inhibitory potential of the extracts on the enzymes AChE, BuChE and α -glucosidase showed weak or no ability to inhibit these enzymes.

To our knowledge, this is the first paper that investigates the chemical and biological profile of aqueous extracts of these plants. The research that follows should be carried out on other types of extracts of these plants in order to isolate and possibly prove the presence of biologically potent compounds by testing, and thus try to confirm the hypothesis that plants of the genus *Centaurea* are a valuable source of these compounds.

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Eighth International Scientific Conference
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COMPARATIVE ANALYSIS OF ANTIBACTERIAL EFFECT OF CRANBERRY TEA VS CRANBERRY FRUIT ON GRAM-NEGATIVE BACTERIA USING AGAR WELL METHOD

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Keywords: cranberry, agar well, gram-negative bacteria

ABSTRACT:

*The word "cranberry" originated from the Pilgrims, who called it "craneberry" because the small, pink blossoms seen in spring resemble the head and bill of a Sandhill crane. Cranberries come from North America, where they are native to the cooler regions of the continent. They grow naturally in acidic bogs, marshes, and wetlands in areas such as the northeastern United States and eastern Canada. Cranberries are also cultivated in other parts of the world with suitable climates, including parts of Europe and Asia. There are two known species of cranberry those are: the American cranberry (*Vaccinium macrocarpon*) and the European cranberry (*V. oxycoccos*). For centuries, Cranberry (*Vaccinium macrocarpon*) has served dual purposes both as a food and as medicine. Cranberry's efficacy in treating urinary infections is widely acknowledged and supported by empirical evidence. This natural remedy has been consistently utilized to address urinary tract infections, showcasing its therapeutic potential and longstanding reputation as a reliable treatment option. Cranberries contain proanthocyanidins that interfere with the ability of bacteria to grow, specifically Gram-negative bacteria. These compounds may prevent the bacteria from sticking to the urinary tract walls, making it more difficult for them to cause infections such as urinary tract infections (UTIs). Additionally, cranberries contain other bioactive compounds that may have antibacterial properties, further contributing to their potential effects against Gram-negative bacteria. In this experimental study, we employed the agar well technique to investigate the impact of various cranberry formulations on Gram-negative bacteria. Our approach involved examining multiple types of cranberries to distinguish their comparative efficacy in inhibiting the growth of Gram-negative bacterial*

strains. By analyzing the size and extent of inhibition zones produced by different cranberry forms, our aim was to identify the most effective form for combating Gram-negative bacterial infections.

1. INTRODUCTION

1.1 The history of cranberry

The term "cranberry" originates from the Pilgrims, who called the fruit "craneberry" due to its resemblance to the head and bill of a Sandhill crane. Cranberries are indigenous to North America, thriving in cooler regions of the continent. They typically grow in acidic bogs, marshes, and wetlands, primarily in the northeastern United States and eastern Canada. Although native to North America, cranberries are also cultivated in other suitable climates worldwide, including parts of Europe and Asia. There are two recognized species of cranberry: the American cranberry (*Vaccinium macrocarpon*) and the European cranberry (*V. oxycoccos*) (Neto et al., 2011). Folklore recordings by the Native Americans from the 1600s have information about the treatment of urinary infections with cranberry, also cranberries were used to cure blood poisoning. Aside from the cranberry fruit, cranberry leaves were used as well for urinary infections, diarrhea, and diabetes. In the 1980s research on the health benefits of cranberry started, the research took 25 years altogether to find the active substances of cranberry. The research took so long because, in the first trials, there were inconsistent conclusions given, this is due to the experiments not being in a controlled environment. The first conclusive research came in the 1990s by Avron et al. In this research experiment fifty-three female patients from a nursing home, with an average age of 78.5 years old. The study included a large number of patients who had a long period of treatment. This study showed that the occurrence of bacterial urinary infections was lowered by 50 % following daily consumption of cranberry juice. The experiment showed the positive effect of cranberry use on urinary infections. Another study concluded at the same time was the Walker et al. study, which has also had similar positive conclusion as well as the previously mentioned study. The difference in this study is that the test subjects were given two 400 mg capsules of cranberry powder to ingest once a day for three months. In the 2000s research on cranberry continued, Kontiokari et al researched the effect of cranberry juice as well as tablets in the prevention of urinary infections. The tested patients were healthy females ages 21-72 years of age, with a long history of resistant UTIs. Pediatric trials have been conducted throughout the 2010s. The research continued in the 21st century but more focused on other beneficial factors. (Shaomin et al., 2020).

1.2 Antibacterial properties of cranberry

In this era of antibiotic resistance, there is a turn to traditionally used medicine. Traditionally used medician such as cranberry. Cranberries in different forms have generally been used to treat UTIs for centuries (Caceres et al., 2019). Cranberries contain bioactive compounds such as

proanthocyanidins, anthocyanins, phenolic acids, flavonols, and triterpenes. These compounds have antibacterial, antioxidant, anti-inflammatory, and other biological effects. For instance, anthocyanins inhibit bowel inflammation and modulate gut bacteria, while type A proanthocyanidins reduce *E. coli* adhesion in the urinary tract. Triterpenes have anti-inflammatory and anti-cancer properties (Šedbarè et al., 2023). UTIs are primarily caused by uropathogenic *Escherichia coli* (*E.coli*) that adhere to uroepithelial cells through Type-1 and P-fimbriae. UTIs have been a major focus point of prevention studies because of increasing bacterial resistance to antibiotics, which makes treatment of infections more difficult and costly. Recently, there has been increased interest in alternative measures and remedies, including the use of naturally available herbal solutions, which are affordable, cost-effective, and very potent. Cranberry juice, *Vaccinium macrocarpon*, has been used for over 200 years, especially by native Americans to help prevent UTI. Studies show that not the acidic pH of urine but an agent in cranberries prevents *E-coli* from adhering to uroepithelial cells. Cranberries contain fructose, an inhibitor of Type-1 fimbriae, and proanthocyanidins, an inhibitor of P-fimbriae. Proanthocyanidins inhibit the adhesion of both sensitive and trimethoprim-sulfamethoxazole-resistant *E. coli* and asymptomatic bacteriuria in patients with ileal enterocystoplasty. However, the effect of proanthocyanidins against multi-drug-resistant uropathogens has not been thoroughly investigated (Gupta et al., 2011). Condensed tannins (cPAC) from cranberries can inhibit the attachment of bacteria to surfaces, compromise bacterial motility, cause iron limitation, and disrupt quorum sensing. Data also indicate that cranberry consumption prevents bacterial infections, and a trial showed that cranberry derivatives that lack proanthocyanidins combined with β -lactam antibiotics are effective against Gram-positive bacteria. cPAC can also inhibit the formation of biofilm and enhance the efficacy of gentamicin against *P.aeruginosa*. However, the potential of cPAC in preventing antibiotic resistance development or in restoring antibiotic efficacy has yet to be tested in depth. The present study shows that cPAC can prevent the development of resistance to tetracycline in *E. coli* and *P.aeruginosa*. It shows that cPAC has broad-spectrum antibiotic-potentiating activity against a range of Gram-negative bacteria *in vitro* and *in vivo*. It also represses two key mechanisms of antibiotic resistance: selective membrane permeability and the activity of multiple drug efflux pumps (Maisuria et al., 2011).

2. MATERIAL AND METHODS

2.1 Material

2.1.1 Cranberry

For this research project a few forms of cranberries were used, those were cranberry tea, dried cranberry, and fresh cranberries.

2.1.2 Bacterial strains

- *Escherichia coli* ATCC 14169

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- *Escherichia coli* ATCC 25922
- *ESBL Escherichia coli* 35218
- *Pseudomonas aureginosa* ATCC 10145
- *Pseudomonas aureginosa* ATCC 27855

2.1.3 Chemicals

In this research project, we used Tryptic Soy Broth (TSB). That was made by dissolving the TSB in 1 L of distilled water. The broth was stirred on the magnetic stirrer. Miller-Hinton plates were made by dissolving MH in 1L of distilled water.

2.2 Methods

2.2.1 Agar well method

Miller-Hinton agar plates were used, which are made by weighing 38g of MH and dissolving it in 1L of distilled water. In the MH plates wells were made by pressing pipetting tips, that beforehand were autoclaved and scorched with a lighter, and then the excess of the MH plate was removed by using a loop. Into the well 80 μ L of cranberry tea was added, in two concentrations, one tea and two tea bags. The tea was prepared in 300 mL of sterile water that was cooked until boiling. The dry cranberry was weighed in Eppendorf tubes 0,1 g and those 0,1 grams were inserted by tweezers into the wells. Fresh cranberry was processed using a mortar and pestle after 0,1 g was weighed into Eppendorf tubes, and that content was inserted into the wells. Zone of inhibition we read in millimeters after 24 h in the incubator at 37 °C.

3. RESULTS

Table 1: Agar well method results for fresh and dried cranberry.

Bacterial strains	Fresh Cranberry 0.1 g	Dried Cranberry 0.1 g
• <i>Escherichia coli</i> ATCC 14169	17 mm	25,3 mm
• <i>Escherichia coli</i> ATCC 25922	18,6 mm	27 mm
• <i>ESBL Escherichia coli</i> 35218	14,3 mm	29 mm
• <i>Pseudomonas aureginosa</i> ATCC 10145	15,3 mm	R
• <i>Pseudomonas aureginosa</i> ATCC 27855	16,6 mm	R

Table 2: Agar well method results for cranberry tea, one and two tea bags in 300 mL of water.

Bacterial strains	Cranberry tea one bag	Cranberry tea two bags
• <i>Escherichia coli</i> ATCC 14169	R	R
• <i>Escherichia coli</i> ATCC 25922	R	R
• <i>ESBL Escherichia coli</i> 35218	R	R

Eighth International Scientific Conference
"June 5th - World Environment Day"

· <i>Pseudomonas aureginosa</i> ATCC 10145	R	R
· <i>Pseudomonas aureginosa</i> ATCC 27855	R	R

4. DISCUSSION

Cranberry has long been used in folk medicine due to its potent health properties. Research has established that cranberries contain active components that have remarkable antibacterial effects on gram-negative bacteria. In our research experiment, we investigate and quantify the antibacterial properties of cranberry against five specific gram-negative pathogenic bacteria, including *Escherichia coli* ATCC 14169, *Escherichia coli* ATCC 25922, *ESBL Escherichia coli* 35218, *Pseudomonas aeruginosa* ATCC 10145, and *Pseudomonas aeruginosa* ATCC 27855. The agar well method was the technique of choice for conducting this study, considering it a very established method to detect the antibacterial activity of various substances. This technique involves the formation of wells in an agar plate where bacteria are grown and the substance being tested—in this case, different forms of cranberry extract—is introduced into the wells. Different forms of cranberry were tested to determine which type had the best antibacterial effect. These included fresh cranberries, dried cranberries, and cranberry tea. Each form was prepared under standardized protocols for consistency and reliability in the results.

5. CONCLUSION

Our findings revealed that all forms of cranberry showed some degree of antibacterial activity on gram-negative bacteria, although effectiveness varied immensely according to the form of cranberry utilized. More specifically, fresh and dried cranberries provided inhibition zones to the gram-negative bacterium, although their size and presence vary among the various strains tested. Fresh cranberries generated zones of inhibition on all the bacteria tested for, which evidently shows broad-spectrum antibacterial activity. Although the inhibition zones were fairly smaller as compared to the other forms of cranberries, inhibition on all the bacterial strains, including *Escherichia coli* ATCC 14169, *Escherichia coli* ATCC 25922, *ESBL Escherichia coli* 35218, *Pseudomonas aeruginosa* ATCC 10145, and *Pseudomonas aeruginosa* ATCC 27855, shows their potential as a versatile antibacterial agent. Dried cranberries showed more selective antibacterial activity. Their inhibition zones against the *Escherichia coli* strains were bigger. The drying action may concentrate the active compounds in cranberries, increasing their activities against some bacteria. However, it is important to note that dried cranberries did not have any significant effect on *Pseudomonas aeruginosa* strains. This lack of effect, therefore, expresses the fact that while being effective against some bacteria, dried cranberries are not universally effective against all gram-negative pathogens. All tested bacteria strains were resistant to the effect of cranberry tea in both concentrations, implying that either the active antibacterial compounds found in fresh or dried cranberries are lost or highly reduced in making cranberry tea. This may

be because boiling water can partly denature some of the heat-labile bioactive compounds, or it may be that the concentration of these compounds is too low to have a measurable effect. These results demonstrate that cranberries, especially the fresh and dried forms, have significant potential as natural antibacterial agents. Conclusively, our study showed the variant degrees of the antibacterial activity of different forms of cranberries against gram-negative bacteria. Fresh cranberries exert broad-spectrum activities of antibacterial effects, while the dried form demonstrates higher activity against specific strains. No high antibacterial activities are noted with cranberry tea. These insights put focus on the potential role of cranberries in the development of novel, natural antibacterial treatments and stress the further need for optimization of preparation methods to gain full health benefits.

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MORPHOLOGICAL CHARACTERISTICS OF CORNELIAN CHERRY GENOTYPES (*Cornus mas* L.) FROM THE KONJIC AREA

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Key words: cornelian cherry, fruit weight, fruit length, fruit width

ABSTRACT:

The genus Cornus within family Cornaceae includes about 65 species of shrubs and trees, where the fruits and trees are used for decorative purposes.

*This study was carried out on 10 local (*Cornus mas* L.) genotypes grown in Konjic area in Bosnia and Herzegovina. Cornelian cherry fruits from these 10 genotypes were harvested and analyzed during the ripening period in 2021. Some physical characteristics of genotypes (fruit weight, fruit length and fruit width) were determined.*

The representative sample represented 30 fruits of ten selected genotypes of cornelian cherry. The results showed that the average fruit weight ranged from 1.24-2.25 g, the average fruit width from 10.94-13.13 mm and the average fruit length from 13.53-17.73 mm.

The lowest average fruit weight was recorded in genotype G1 (1.24 g), while the lowest average fruit width (10.94 mm). The highest average fruit weight was recorded in genotype G7 (2.25 g), as well as the highest fruit width (13.13 mm). The highest average fruit length of the studied cornelian cherry genotypes had genotype G7 (17.73 mm), while the lowest average value of fruit length had genotype G1 (13.53 mm).

The present study showed that genetic resources even in a small area indicates a great phenotypic diversity among genotypes that may have hidden important genes in them and may be important for breeding better varieties of cornelian cherry in the future as parental material.

1. INTRODUCTION

Within the family *Cornaceae*, the genus *Cornus* includes about 65 species of shrubs and trees, whose fruits have a high nitrifying value. Cornelian cherry (*Cornus mas* L.) is one of the wild forest plants that can be used as food and medicine, and which in folk tradition symbolizes health. Thanks to the high-quality composition of the fruit, cornelian cherry has high standards for use in nutrition, as well as for medicinal purposes. Thanks to its shiny leaves and abundance of flowers, it is also suitable as a horticultural species because it achieves a very interesting effect in parks and gardens. It tolerates winter and dry conditions well, it is resistant to diseases and pests, and it is suitable for ecological production.

The genus *Cornus* is widely distributed, occupying the areas of central and southern Europe (Ball 1968), Asia Minor, the Caucasus, Crimea and southwestern Iran (Šilić 2005). Thus, it covers the territory of Albania, Austria, Belgium, Great Britain, the Czech Republic, Slovakia, Croatia, France, Germany, Greece, Switzerland, Spain, Hungary, Italy, Serbia, Macedonia, Montenegro, Kosovo, Bosnia and Herzegovina, Moldova, Poland, Romania, Slovenia, Turkey, Ukraine, and the southwestern part of the European part of Russia.

In Bosnia and Herzegovina, there are extraordinary conditions for growing cornelian cherry, so the expansion of this neglected fruit culture and the exploitation of its huge potential for organic fruit production would be of great economic importance. (Bijelić et al., 2011).

Bosnia and Herzegovina is well known by its rich gene pool of cornelian cherry genotypes and has good potential for exploitation. Cornelian cherry fruits collected in natural populations are a valuable source of natural bioactive compounds and used as valuable selection material in breeding programs (Skender et al., 2022).

In Bosnia and Herzegovina, the cultivation of cornelian cherry dominates on family farms, where the trees are not protected from disease-causing agents and pests, care measures are less applied, so the fruits produced in such conditions meet the standards of biologically valuable food. This kind of production would have its significant place, especially with the cultivation of selections and varieties of grafted cornelian cherry with favorable phenological and other production characteristics.

Cornelian cherry is one of the rare plant species that gives a full yield without the use of chemical preparations and bears fruit in significantly more modest agrotechnical conditions.

Apart from work on selection and breeding, recently a lot of investment has been made in the presentation and marketing of this species (Rop et al., 2010).

The aim of this work was to contribute to the research of the diversity of genetic resources of *Cornus mas* L. genotypes in northern Herzegovina in terms of morphological fruit characteristics and the selection of prospective genotypes that would be recommended for growing in orchards.

2. MATERIAL AND METHODS

The study was conducted in Konjic area in northern Herzegovina. A total 10 cornelian cherry (*Cornus mas* L.) genotypes were selected in 2021 according to their better yield and fruit characteristics in natural growing places and were assessed in this research. Ten cornelian cherry genotypes G1, G2, G3, G4, G5, G6, G7, G8, G9 and G10 were found, marked and tracked in the field and their GPS coordinates were determined.

Fruits were picked at full maturity stage for each genotype (consumption ripeness for morphological analysis) and transported to the laboratory. Each sample consisted of 500 g of fruits per genotype.

The pomological analyses encompassed 30 random mature fruits per genotypes. Laboratory analysis of fruits was carried out in the chemical laboratory of the Agromediterranean Faculty in Mostar.

Morphological analysis included monitoring and measurement of three agronomically significant traits: fruit width (mm), fruit length (mm) and fruit weight (g). fruit width and fruit length were The length and width of the fruit were measured using a digital fruit caliper. Fruit weight was measured by using a digital balance with a sensitivity of 0.01 g.

The statistical analyses were carried out using SAS (SAS Inst., 2020). Descriptive statistics with basic data were performed: mean value, standard deviation, one-factor analysis of variance and statistical significance test Tukey-Krammer test for significance levels 0.05 and 0.01. The evaluation of morphological characteristics was conducted through principal component analysis (PCA) using a computer program R v. 3.2.3, based on a correlation matrix. The methodology was adapted from (Hotelling, 1936) and implemented in accordance with the guidelines of the (R core team, 2016).

Principal component analysis (PCA) was conducted using mean values of 3 morphological traits following fruit analysis. The spatial distribution of the analyzed corelian cherry genotype based on the first two components of the experimental factors is presented graphically. The principal components succinctly elucidate the data variability and variable interrelations. The graphical illustrations (Figs. 1, 2, 3, 4) were generated using the computer program R version 3.2.3 (R core team, 2016).

Description of cornelian cherry genotypes

Genotype 1.

Location: 43°41'35.1"N 17°59'24.5"E



Figures 1 and 2: Tree and fruit of genotype G1

G1 Description: The bush is in a semi-shady position, lush, with smaller, bright red fruits and smaller seeds.

Genotype 2

Location: 43°41'45.9"N 17°59'26.9"E

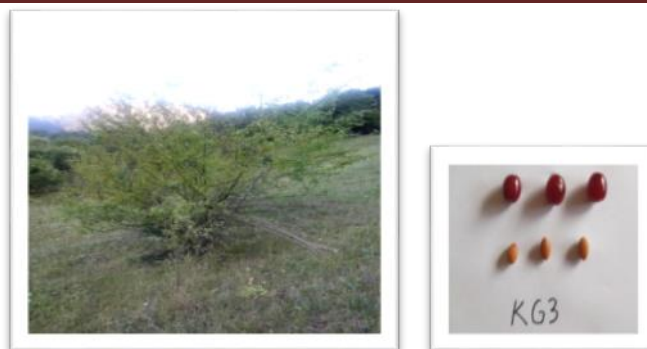


Figures 3 and 4: Tree and fruit of genotype G2

G2 Description: The bush is located in a semi-shady position, of medium growth, the fruits ripen a few days later compared to genotype 1. They are smaller in diameter and bright red in color, and the seeds are smaller in size.

Genotype 3

Location: 43°41'48.3"N 17°59'27.6"E



Figures 5 and 6: Tree and fruit of genotype G3

G3 Description: The bush is located on an open surface, of medium growth. The fruits are beautifully colored and ripen earlier due to their position, they are red in color and the seeds are medium in size.

Genotype 4

Location: 43°41'49.1"N 17°59'30.6"E

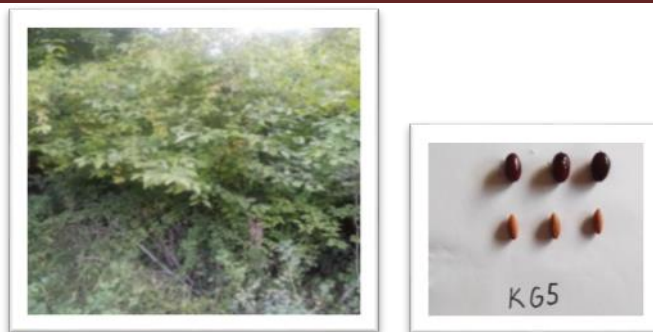


Figures 7 and 8: Tree and fruit of genotype G4

G4 Description: The bush is located on an open surface, of medium growth. The fruits are beautifully colored, medium-sized, red in color, while the seeds are medium-sized.

Genotype 5

Location: 43°41'49.2"N 17°59'31.2"E



Figures 9 and 10: Tree and fruit of genotype G5

G5 Description: The bush is located in a semi-shady position, it is of medium growth. The fruits are smaller in size, blue-red in color with medium-sized seeds.

Genotype 6

Location: 43°41'27.5"N 17°59'44.2"E



Figures 11 and 12: Tree and fruit of genotype G6

G6 Description: The bush is located on an open area, it has a lush growth. The fruits are larger with a bright red color and smaller seeds.

Genotype 7

Location: 43°41'27.2"N 17°59'44.1"E



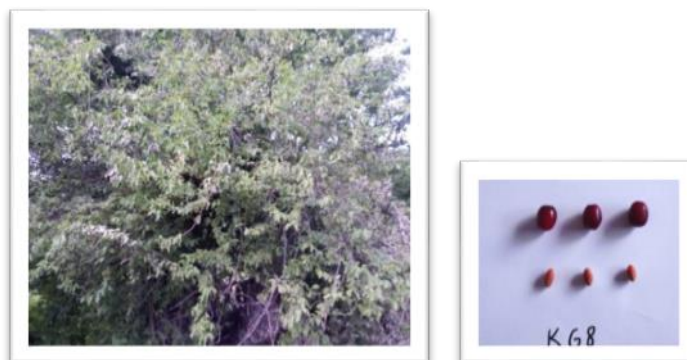
Figures 13 and 14: Tree and fruit of genotype G7

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G7 Description: This genotype is found in the form of a stem, without root shoots. It has a more lush growth, the fruits are large with a red color and smaller seeds.

Genotype 8

Location: 43°41'29.0"N 17°59'49.8" E



Figures 15 and 16: Tree and fruit of genotype G8

G8 Description: The bush is located in a semi-shady position. This bush is extremely old, it is less visible in the picture because of the forest that was felled, so it fell on it a little. It is of medium luxuriance. The fruits are slightly smaller with a red color and smaller seeds.

Genotype 9

Location: 43°41'28.5"N 17°59'51.1"E

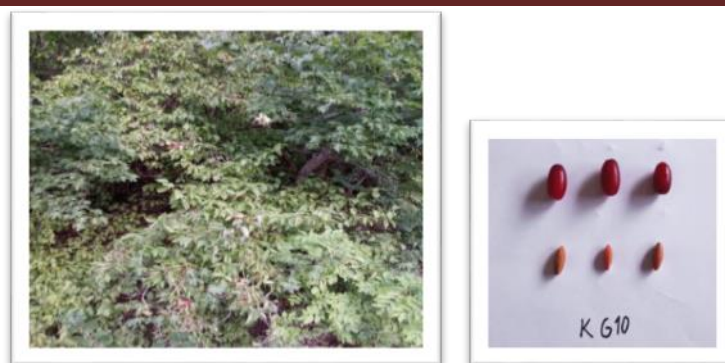


Figures 17 and 18: Tree and fruit of genotype G9

G9 Description: The bush is located in a semi-shady position, it has a lush growth. The fruits are larger, red in color with smaller seeds.

Genotype 10

Location: 43°41'28.9"N 17°59'51.7"E



Figures 19 and 20: Tree and fruit of genotype G10

G10 Description: The bush is in a semi-shady position, with medium growth. The fruits are larger, red in color with smaller seeds.

3. RESULTS

Results for morphological characteristics of cornelian cherry fruit are shown in Table 1.

Table 1: Morphological properties of cornelian cherry fruits.

Genotypes	Fruit weight (g)	Fruit width (mm)	Fruit length (mm)
G1	1.24 ±0.20 c	10.94 ±0.70 c	13.53 ± 0.81 cde
G2	1.86 ±0.44 a	11.76 ±0.66 b	15.45 ±1.12 cd
G3	1.79 ±0.35 b	12.14 ±0.74 b	14.42 ±0.82 cde
G4	1.78 ±0.24 b	12.04 ±0.44 b	13.96 ±0.73 cde
G5	1.91 ±0.27 a	12.23 ±0.52 b	16.84 ±0.99 a
G6	1.83 ±0.32 a	12.19 ±0.69 b	16.08± 1.18 bc
G7	2.25 ±0.31 a	13.13 ±0.54 a	17.73 ±0.57 ba
G8	1.43 ±0.31 c	11.73 ±0.76 b	13.63± 0.79cde
G9	1.71 ±0.29 b	12.20 ±0.61 b	16.03 ±1.04 c
G10	2.08 ±0.26 a	12.21 ±0.55b	17.35± 0.80 ba

In the present study, we found considerable variations in all traits searched among cornelian cherry genotypes at $p < 0.05$ level. The lowest average fruit weight was recorded with genotype G1 (1.24 g), and the highest average fruit weight was recorded with genotype G7 (2.25 g). The average fruit width of the tested cornelian cherry genotypes ranged from 10.94 mm for genotype G1 to 13.13 mm for genotype G7.

The highest average fruit length of the tested cornelian cherry genotypes was recorded in genotype G7 (17.73 mm), while the lowest average value of fruit length was moderate in genotype G1 (13.53 mm). From the above data, it can be concluded that genotype G7 had the

highest values of all measured parameters and can be recommended as superior compared to the other tested genotypes.

Our data about of fruit weight confirm the information previously provided by Drkenda et al. (2014), and Kashirina et al. (2021) but lower than the results of Skender et al. (2022a); Skender et al. (2022b); Bijelić et al. (2012) and Ercisli et al. (2011).

The fruit width (10.94–13.13 mm) were measured similarly to the results of Skender et al. (2022); Guzel (2021) and Cornescu and Cosmulescu (2017) but lower of results of Bijelić et al. (2012).

Our obtained data of the fruit length comparable with results of Skender et al. (2022) (10.94 mm to 19.69 mm), but lower of the other authors (Bijelić et al., 2012; Guzel, 2021).

The lowest average fruit weight was recorded in genotype G1 and was (1.24 g), and the highest was recorded in genotype G7 (2.25 g). Based on the results of the variance analysis for fruit weight, it can be concluded that the genotype factor has a statistically significant effect on the examined statistical feature of fruit weight that was investigated in this paper. Statistically significant differences, shown by the Tukey test for the weight of the fruit of the tested correlian cherry genotypes, were shown by genotype G7 in comparisons with genotypes G9, G3, G4, G8 and G1. Statistically significant differences in the comparisons were shown by genotype G10 in comparisons with G8 and G1, then genotype G5 in comparisons with G8 and G1. Statistically significant differences in comparisons were shown by genotype G6 in comparison with G1, then G9 in comparison with G1, G2 in comparison with G1, G3 in comparison with G1 and G4 in comparison with G1. The results presented in the paper were processed using statistical tools that clearly distinguished the genotypes that stand out due to their characteristics.

The average fruit width of the tested correlian cherry genotypes ranged from (10.94 mm) for genotype G1 to (13.13 mm) for genotype G7. Based on the analysis of variance for the fruit width of the tested correlian cherry genotypes, it can be concluded that the F-test shows that only certain genotypes have a statistically significant influence on the fruit width of the tested correlian cherry genotypes, which is the expected assumption. Tukey's test for the width of the fruit of the tested correlian cherry genotypes showed that the genotype G7 showed statistically significant differences in the comparisons with the genotypes G10, G5, G6, G9, G2, G3, G4, G8 and G1. Statistically significant differences in the comparisons were shown by genotype G10 in comparison with G1, then genotype G5 in comparison with G1. Statistically significant differences in comparisons were shown by genotype G9 in comparison with G1, G6 in comparison with G1, G3 in comparison with G1 and G4 in comparison with G1. The average length of the fruit of the tested correlian cherry genotypes ranged from (17.73 mm) for genotype G7 to (13.53 mm) for genotype G1. By analyzing the variance for the length of the fruits of the tested correlian cherry genotypes, it can be seen that there is a statistically significant difference ($F_{exp} > F_{tab}$) between the tested correlian cherry genotypes.

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In other words, the genotype as a factor had a statistically significant influence on the examined statistical characteristic. Based on the data of the Tukey test, i.e. the comparison of the fruit length of the tested corleian cherry genotypes, it can be concluded that there are certain differences between individual genotypes. Genotype G7 showed statistically significant differences in comparisons with genotypes G6, G9, G2, G3, G4, G8 and G1. Statistically significant differences in comparisons were shown by genotype G10 in comparisons with G9, G2, G3, G4, G8 and G1, then genotype G5 in comparisons with G2, G3, G4, G8 and G1. Genotype G6 showed statistically significant differences in comparisons with G3, G4, G8 and G1, G9 in comparison with G1, G3, G4 and G8, genotype G2 showed statistical significance with G4, G8 and G1.

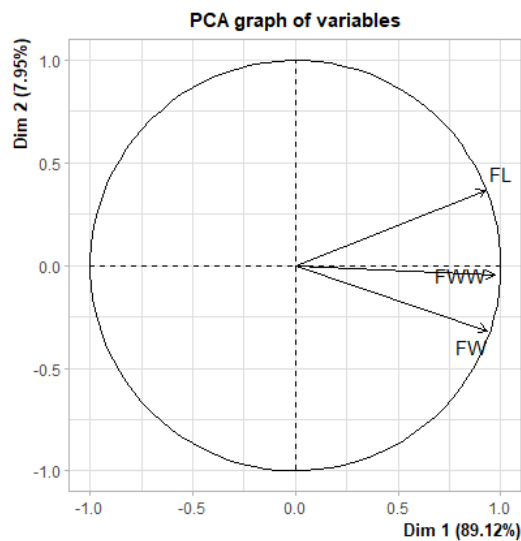


Chart 1: Grouping and interrelationships of 3 variables analyzed on 1 corleian cherry genotypes against the first two main components (PC1 and PC2)

Analyzing the results of the three main components of the PCA analysis shown in Table 1, one can see the contribution of each of the 3 analyzed properties in the total variability present in the analyzed set of corleian cherry genotypes. Each of the 3 observed traits is found with a high value of eigenvectors in one of the first three principal components.

Table 2: Eigenvalues, proportion of variance and cumulative variance associated with the first five principal components (PCA), estimated from the correlation matrix with 3 variables in 10 genotypes of corleian cherry.

Variables	PCA1	PCA2	PCA3
Eigenvalue	2.674	0.239	0.088
Proportion of variance (%)	89.117	7.953	2.930
Cumulative variance (%)	89.117	97.070	100.000

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Table 3: Analysis of 3 quantitative properties of correllian cherry genotypes in the total variability of the experiment
(significant sources of variability in bold)

	PCA1	PCA2	PCA3
Fruit weight - FW	31.961	56.877	11.162
Fruit length - FL	32.811	42.301	24.888
Fruit width - FWW	35.228	0.822	63.949

The variables with the highest eigenvector values in the first three main components are presented:

PCA1 -;

PCA2 – fruit weight and fruit lenght

PCA3 – fruit width

By analyzing the obtained results, it can be concluded that through the dominant eigenvectors in the first principal component (PCA), which accounted for 89.117% of the total variance of the research. The most significant properties of the second component refer to morphometric ones. The highest values of the eigenvectors were the length and width of the fruit (56,877 and 42,301).

The most important characteristic of the third main component is the weight of the fruit. With a total variability of (63,949).

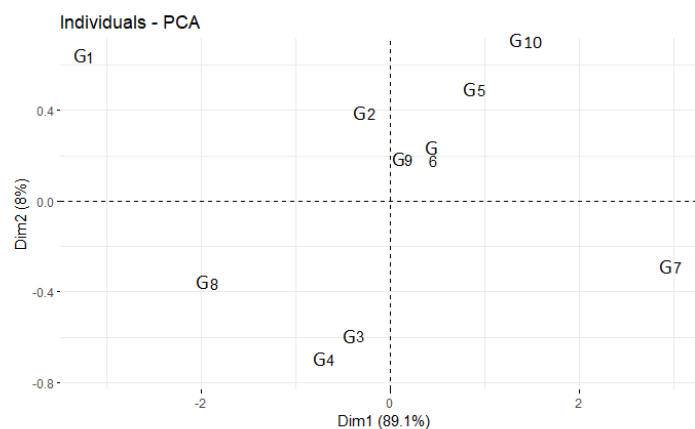


Chart 2: Grouping of 10 correllian cherry genotypes in relation to the first two main components (PCA1 and PCA2), calculated via the correlation matrix for 3 characteristics

On the chart 2. the distribution of 10 genotypes of correllian cherry obtained by using the first two main components, which were calculated via the correlation matrix for the 3 examined morphological characteristics of the fruit, is shown, and it can be concluded that no separation of groups into separate groups was observed.

Analyzing the first two components, which contain 97.070% of the total variability of the experiment, it is noticeable that the studied cornelian cherry genotypes occupied a certain place in the coordinate system. Partial grouping of individual genotypes is also visible, which indicates the fact that their average values of the tested characteristics are in positive correlation with each other.

4. CONCLUSION

Wild fruit species have great genetic potential for selection and breeding work. Their introduction into culture would significantly enrich the existing gene pool of fruit trees. Thanks to numerous studies that have confirmed the high nutritional value of the fruit of cornelian cherry (*Cornus mas* L.), today intensive work is being done on the selection and breeding of cornelian cherry.

Bosnia and Herzegovina is very rich in natural resources of cornelian cherry. This research showed that cornelian cherry fruits from natural populations have good morphological properties.

From the above data, it can be concluded that genotype G7 had the highest values of all measured parameters and can be recommended as superior compared to the other tested genotypes.

The research of cornelian cherry genetic resources even in a small area indicates a great phenotypic diversity among genotypes that may have hidden important genes in them and may be important for breeding better varieties of cornelian cherry in the future as material in the breeding program.

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EFFECT OF FOSFOMYCIN ON THE RESISTANCE PATTERNS OF GRAM-NEGATIVE BACTERIA TO DIFFERENT CLASSES OF ANTIBIOTICS USING THE BAUER-KIRBY DISK DIFFUSION METHOD

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Key words: fosfomycin, gram-negative bacteria, antibiotic

ABSTRACT:

One of the main areas of focus for antimicrobial research is the impact of fosfomycin on the antibiotic resistance patterns of Gram-negative bacteria. Because of its distinct mode of action, fosfomycin is frequently used to treat infections brought on by gram-negative bacteria that are resistant to drugs. The purpose of this work is to use the Bauer-Kirby disk diffusion method to examine how fosfomycin affects the resistance patterns of gram-negative bacteria to various antibiotic classes.

Gram-negative bacteria (Escherichia coli ATCC 14169, 25922, and ESBL) were grown for the investigation and then exposed to fosfomycin. Following therapy, the bacteria's sensitivity to many more antibiotics of different classes was evaluated using the Bauer-Kirby disk diffusion method. We measured and compared the changes in the zones of inhibition surrounding the antibiotic discs to control groups that did not get fosfomycin treatment.

The zone of inhibition decreased from the positive control to the fosfomycin-treated groups, according to the results for all antibiotics. This implies that the bacteria's susceptibility to several antibiotics has been lessened by the presence of fosfomycin. A smaller zone of inhibition usually denotes reduced sensitivity or resistance, which in this instance may be brought about by fosfomycin's impact on the bacteria's pattern of antibiotic resistance. A notable exception was the antibiotic ampicillin, for which a tiny zone of inhibition developed following fosfomycin administration, suggesting that the resistance pattern may have changed.

Overall, these findings suggest a potential antagonistic effect between fosfomycin and the tested antibiotics, which may have implications for their combined use in clinical settings. Further

research is warranted to elucidate the underlying mechanisms and to explore the clinical relevance of these observations.

1. INTRODUCTION

Antimicrobial resistance, often known as antibiotic resistance (AMR), has become a significant and cost-increasing global concern for healthcare systems. Recent research has linked it to notable rates of morbidity, mortality, and higher expenses as a result of longer hospital stays and treatment durations (Ayoub and Hammoud, 2020; Vrancianu et al., 2020). There are three main types of antibiotic resistance mechanisms. First, resistance can be developed by decreasing membrane permeability or raising antibiotic efflux, which will block the antibiotic's ability to reach the target. Finally, antibiotics can be directly rendered inactive by hydrolysis or other alteration. Bacteria can also defend the antibiotic target via genetic mutation or post-translational modification (Blair et al, 2015).

In the field of medicine, antibiotic resistance is a serious problem, especially when it comes to Gram-negative bacteria. Since these bacteria are skilled at creating resistance mechanisms, treating illnesses becomes challenging. A derivative of phosphonic acid called fosfomycin has demonstrated potential against Gram-negative bacteria. Research on the impact of fosfomycin on Gram-negative bacteria's resistance patterns to various antibiotic classes is still ongoing, nevertheless.

According to Falagas et al. (2016), some older medications were not created to modern standards, which implies that suitable trials must be carried out to assess these medications' potential efficacy. Due to its continued effectiveness against extensively drug-resistant (XDR) and multidrug-resistant (MDR) Gram-positive and Gram-negative bacteria, fosfomycin has drawn attention.

One common approach for figuring out a bacteria's antibiotic susceptibility is the Bauer-Kirby disk diffusion method. To ascertain the bacteria's antibiotic susceptibility, antibiotic-impregnated disks are placed on agar plates that have been infected with the test strain of bacteria. The zones of inhibition surrounding the disks are then measured.

Healthcare systems around the world are extremely concerned about the seriousness of infections caused by Gram-negative bacteria, their high potential for environmental transmission and dispersal, the challenges of using empirical treatment (or even carefully targeted treatment), and the lack of new antibiotics to combat them (Curcio, 2014).

The effectiveness of fosfomycin alone and in combination with other antibiotics against Gram-negative bacteria has been the subject of numerous studies. For instance, a 2011 study by Michalopoulos et al. showed that fosfomycin combined with carbapenems effectively combats

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Gram-negative bacteria that are resistant to carbapenems. Falagas et al. (2010) demonstrated the synergistic effects of fosfomycin in combination therapy as a means of combating Gram-negative bacteria that are resistant to many drugs.

Although research studies demonstrate the efficacy of fosfomycin in treating Gram-negative bacterial infections, nothing is known about how fosfomycin, when taken in combination, affects the antibiotic resistance patterns of these bacteria. It is essential to comprehend this impact in order to maximize the use of fosfomycin and create efficient treatment plans for Gram-negative bacteria that are resistant to antibiotics.

In this study, we use the Bauer-Kirby disk diffusion method to examine the impact of fosfomycin on the antibiotic resistance patterns of Gram-negative bacteria. We will specifically look at how pre-treatment with fosfomycin affects Gram-negative bacteria's resistance to different antibiotic classes.

The overall goal of this research is to shed important light on how fosfomycin affects the antibiotic resistance patterns of Gram-negative bacteria. We intend to contribute to the creation of fresh approaches to treating antibiotic resistance by comprehending how fosfomycin influences the emergence of resistance in these bacteria.

2. MATERIALS AND METHODS

2.1 Bacterial Strains and Culture Conditions:

In this study three strains of Gram-negative bacteria were used: *Escherichia coli* ATCC 14169, *Escherichia coli* ATCC 25922, and an *Extended-Spectrum Beta-Lactamase (ESBL)*-producing strain. Bacteria were cultured overnight on Mueller-Hinton (MH) agar at 37°C to reach a logarithmic growth phase.

2.2 Fosfomycin Treatment and MIC:

Minimum inhibitory concentrations (MICs) of fosfomycin were determined for each bacterial strain using the microdilution broth method. The concentration, after the MIC, was used for further antibiotic susceptibility testing. From these wells 0.5 Mc Farland turbidity was prepared. This suspension contains 1.5×10^8 CFU/ml of bacteria. To obtain 5×10^5 CFU/ml, we added 100 μ L of 0.5 McF suspension to 9900 μ L of TSB. The same procedure was done for untreated bacteria. Bacteria were grown on Mueller Hinton agar. After that, 0.5 McF was prepared and 100 μ L of that was added to 9900 μ L of TSB in order to get 5×10^5 CFU/ml.

2.3 Antibiotic Susceptibility Testing:

Antibiotic susceptibility testing was performed using the Bauer-Kirby disk diffusion method. By using a sterile swab treated and untreated bacterial suspension was evenly spread onto Mueller-Hinton agar plates. Then the antibiotic discs were placed onto the agar surface using sterile

forceps, ensuring they are well spaced. It was done in triplicate in order to confirm the results. The plates were then incubated at 37°C for 18-24 hours.

The results are interpreted based on the diameter of the zone of inhibition and the breakpoints established by CLSI or EUCAST for each antibiotic which classify bacteria as susceptible, intermediate, or resistant. The diameter of the zone of inhibition indicates the susceptibility of the bacteria to the antibiotic. A larger zone of inhibition indicates greater susceptibility, while a smaller zone or no zone indicates resistance.

2.4 Antibiotics and Disk Preparation:

Fifteen antibiotics from different classes were selected for this study: Ciprofloxacin (CIP 5µg), Doxycycline (DXT 30µg), Tobramycin (TOB 10µg), Gentamicin (CN 30µg), Levofloxacin (LEV 5µg), Tetracycline (TE 30µg), Vancomycin (VA 30µg), Cefoxitin (FOX 30µg), Erythromycin (E 15µg), Ampicillin (AMP 2µg), Augmentin (AUG 30µg), Rifampicin (RD 30µg), Azithromycin (AZM 15µg), Amikacin (AK 30µg), and Streptomycin (S 300µg).

2.5 Data Analysis:

The diameter of the zones of inhibition around each antibiotic disk was measured using a ruler. The mean zone diameter for each antibiotic was calculated for both the fosfomycin-treated and control groups. The ratio of the mean zone diameter of the treated group to the control group was calculated to determine the effect of fosfomycin on the resistance patterns of the bacteria.

This methodology allows for the evaluation of the impact of fosfomycin on the resistance patterns of Gram-negative bacteria to different classes of antibiotics using the Bauer-Kirby disk diffusion method, particularly after biofilm formation.

3. RESULTS

In this study three strains of Gram-negative bacteria (*Escherichia coli* ATCC 14169, *Escherichia coli* ATCC 25922, and an *Extended-Spectrum Beta-Lactamase (ESBL)*-producing strain) were tested on 15 antibiotics from different classes (Ciprofloxacin (CIP 5µg), Doxycycline (DXT 30µg), Tobramycin (TOB 10µg), Gentamicin (CN 30µg), Levofloxacin (LEV 5µg), Tetracycline (TE 30µg), Vancomycin (VA 30µg), Cefoxitin (FOX 30µg), Erythromycin (E 15µg), Ampicillin (AMP 2µg), Augmentin (AUG 30µg), Rifampicin (RD 30µg), Azithromycin (AZM 15µg), Amikacin (AK 30µg), and Streptomycin (S 300µg)). The initial concentration of fosfomycin for biofilm formation for all tested bacteria was 190 mg/ml. According to the results from biofilm formation, minimum inhibitory concentration (MIC) of fosfomycin for *Escherichia coli* ATCC 14169 was 0.0463 mg/ml, for *Escherichia coli* ATCC 25922 was 0.00580 mg/ml and for *Extended-Spectrum Beta-Lactamase (ESBL)*-producing strain was 0.0463 mg/ml.

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Table 1: The zones of inhibition for all three treated with fosfomycin bacteria and the positive control

Antibiotics	BACTERIA					
	<i>E. COLI ATCC 14169</i>		<i>E. COLI ATCC 25922</i>		<i>ESBL</i>	
	Pos. Control	Fosfomycin	Pos. Control	Fosfomycin	Pos. Control	Fosfomycin
Ciprofloxacin (CIP5)	40 mm	30 mm	44 mm	28 mm	40 mm	31 mm
Doxycycline (DXT30)	21 mm	16 mm	25 mm	19 mm	21 mm	16 mm
Tobramycin (TOB10)	23 mm	18 mm	26 mm	19 mm	23 mm	18 mm
Gentamicin (CN30)	28 mm	23 mm	37 mm	23 mm	27 mm	24 mm
Levofloxacin (LEV5)	35 mm	27 mm	39 mm	27 mm	35 mm	27 mm
Tetracycline (TE30)	20 mm	12 mm	26 mm	14 mm	21 mm	12 mm
Vancomycin (VA30)	R	R	R	R	R	R
Cefoxitin (FOX30)	29 mm	21 mm	29 mm	24 mm	25 mm	21 mm
Erythromycin (E15)	R	R	8 mm	7 mm	R	R
Ampicillin (AMP2)	8 mm	8 mm (DG)	8 mm	7 mm	R	9 mm (DG)
Augmentin (AUG30)	23 mm	20 mm	22 mm	17 mm	21 mm	19 mm
Rifampicin (RD30)	17 mm	13 mm	24 mm	14 mm	16 mm	12 mm
Azithromycin (AZM15)	17 mm	16 mm	27 mm	14 mm	17 mm	15 mm
Amikacin (AK30)	21 mm	19 mm	26 mm	19 mm	22 mm	19 mm
Streptomycin (S300)	29 mm	23 mm	31 mm	21 mm	25 mm	23 mm

In the Table 1. are shown the zones of inhibition for all treated and untreated bacterial strains. According to the results, for all antibiotics, the zone of inhibition decreased for most of the fosfomycin-treated groups in comparison to the positive control. This implies that the bacteria's susceptibility to several antibiotics has been lessened by the presence of fosfomycin. A smaller zone of inhibition usually denotes reduced sensitivity or resistance, which in this instance may be brought about by fosfomycin's impact on the bacteria's pattern of antibiotic resistance.

All three bacteria were resistant to antibiotic vancomycin (VA 30µg) for both positive control and treated bacteria. In the case for antibiotic erythromycin (E 15µg) bacteria *E. coli ATCC 14169* and *ESBL* also stayed resistant after the treatment with fosfomycin. A notable exception was the antibiotic ampicillin (AMP 2µg) for *ESBL* bacteria, where a tiny zone of inhibition developed after being resistant, suggesting that the resistance pattern may have changed. Only in this case fosfomycin exhibited synergistic effects with other antibiotics, including beta-lactams like ampicillin.

4. DISCUSSION

The findings of this research shed important light on how fosfomycin affects Gram-negative bacteria's resistance to various antibiotics. The three examined bacterial strains—two *Escherichia coli* strains and one that produced *Extended-Spectrum Beta-Lactamase (ESBL)*—showed variable reactions to fosfomycin treatment, including noticeable shifts in their sensitivity to a number of antibiotics.

When comparing the fosfomycin-treated groups to the positive control, the observed decrease in the zone of inhibition for the majority of antibiotics suggests that the bacteria are less susceptible to these antibiotics. This implies that the administration of fosfomycin may alter the antibiotic resistance pattern of the bacteria, hence facilitating the emergence of antibiotic resistance in these strains. According to Ramos and Lletí (2019), these results align with earlier research indicating that fosfomycin can alter the patterns of bacterial resistance.

Interestingly, in both the treated and positive control groups, all three bacterial strains exhibited vancomycin resistance. Since vancomycin is a glycopeptide antibiotic that primarily works against Gram-positive bacteria, it is expected that Gram-negative bacteria will become resistant to it, as evidenced by earlier research.

Both the *ESBL*-producing strain and *E. Coli ATCC 14169* retained their resistance to erythromycin following treatment with fosfomycin. This highlights the intricacy of antibiotic resistance mechanisms in Gram-negative bacteria by indicating that the mechanisms of resistance to erythromycin in these strains are not impacted by fosfomycin treatment.

A noteworthy difference was seen with ampicillin in the *ESBL*-producing strain, where initially resistant bacteria developed a small zone of inhibition. This implies that following fosfomycin treatment, the *ESBL*-producing strain's ampicillin resistance pattern may have altered. The observed behavior may be explained by the synergistic interactions between fosfomycin and other antibiotics, such as ampicillin and beta-lactams (Suich et al., 2022). It has been demonstrated that fosfomycin inhibits the formation of bacterial cell walls, which may increase the penetration of beta-lactam antibiotics into bacterial cells and hence increase their activity.

Overall, our findings demonstrate the intricate relationship between fosfomycin and the antibiotic resistance patterns of Gram-negative bacteria. To clarify the precise processes driving these modifications and ascertain the therapeutic ramifications of these discoveries, more investigation is required. Effective treatment options against multidrug-resistant Gram-negative bacteria require an understanding of how fosfomycin affects patterns of antibiotic resistance.

5. CONCLUSION

The results of this study provide insight into the intricate relationship between fosfomycin and the antibiotic resistance patterns of Gram-negative bacteria. The findings imply that fosfomycin may be involved in changing the bacteria's pattern of antibiotic resistance as it can reduce the susceptibility of these bacteria to a number of antibiotics.

Effective treatment options against multidrug-resistant Gram-negative bacteria require an understanding of how fosfomycin affects patterns of antibiotic resistance. Fosfomycin is a useful addition to the collection of antibiotics available for the treatment of diseases since it has been demonstrated to be effective against multidrug-resistant bacteria, particularly those that produce ESBLs.

To conclude, this study's findings advance our knowledge of the intricate relationships that exist between fosfomycin and antibiotic resistance in Gram-negative bacteria. To completely understand these interactions and assess the clinical relevance of these results in the context of antibiotic therapy, more investigation is required.

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AN OVERVIEW OF VARIATIONS IN SYNTHETIC APPROACHES FOR THE PREPARATION OF (BENZ)IMIDAZOLIUM SALTS: BIOACTIVITY AND GREEN SOLVENT PERSPECTIVES

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Key words: Ionic liquids, bioactive (benz)imidazolium salts, green solvents, synthetic approach

ABSTRACT:

In addition to the fact that (benz)imidazolium salts have a significant application due to their ability to act as ionic liquids, in recent decades their biological activity has become more prominent. Imidazolium salts as well as their polymeric materials have been shown to have antitumor, antioxidant, antifibrotic and antimicrobial activity. The growing interest in evaluating this aspect of the application of these salts is based on the long-known toxicity of structurally simpler (benz)imidazolium salts. Ionic liquids are, according to their name, substances of ionic structure with a melting point below 100 °C with practically nonexistent vapor pressure and exceptional ability to dissolve substances of different structures and polarities. The largest portion of ionic liquids are (benz)imidazolium salts with inert anions, which are all properties that nominate them for green solvents in addition to their potential bioactivity. The issue of optimization of the conventional synthetic approach to (benz)imidazolium salts and its innovations will be the topic of this paper, bearing in mind that one of the main obstacles to the application of potentially active substances and their benefits is the profitability of the synthetic process.

1. INTRODUCTION

The interest in finding the relationship between the structure and directed bioactivity of ionic liquids based on (benz)imidazolium salts stems from the long-known toxicity of simpler structural analogues of these substances (Gravel and Schmitzer, 2017). Characterization of a substance as toxic means that it has, although undesirable, strong biological activity, which gave birth to the idea of structural modification as a means of mitigating, directing, and optimizing biological activity, or defining and describing a line dividing this class of compounds as drugs or poisons in the same application dose. If the resulting substance shows biological indifference but retains interesting physicochemical properties that are important for potentially green solvents, then the synthesis is optimized for the application of the resulting substance in this field (Priya and Bassy, 2016). Tetrasubstituted imidazolium salts have been shown to have remarkably high toxicity to the parasites *Trypanosoma cruzi* and *Trypanosoma brucei* accompanied by good selectivity and pharmacokinetic properties, thus, again, emphasizing the potential of these substances as an alternative approach to conventional drugs and therapies. The authors further emphasized that the optimization of activity can be expected by subtle changes in their structure, implying an innovative synthetic approach (Ghashghaei et al., 2018), which is the same principle that manipulates the properties of ionic liquids as solvents of desired polarity, melting point and viscosity (Philippi and Welton, 2021). The issue of optimization of the conventional synthetic approach to (benz)imidazolium salts as bioactive ionic liquids and its innovation is the most important milestone in their wider application, for whatever purpose.

2. SYNTHETIC APPROACH AND APPLICATION OF (BENZ) IMIDAZOLIUM SALTS

2.1 (Benz)imidazolium salts as condensation reactions products

Condensation reactions are usually used when the goal is to obtain symmetric (benz)imidazolium salts, ie. salts in which nitrogen atoms are substituted by identical substituents. These reactions can be carried out in two steps or in one step (one-pot synthesis). Picture 1. [A] shows a two-step condensation reaction via a diimine intermediate formed by condensation of a dicarbonyl compound with a primary amine and subsequent condensation with an aldehyde, in the presence of a hydrogen halide, leading to imidazole ring closure. The identity of the substituent on the C atom between the two N atoms of the said ring will depend on the type of aldehyde so that formaldehyde will give imidazolium and ethanal will give 2-methylimidazolium salt with two N substituents. The reaction can be performed without isolation of the diimine intermediate (Picture 2. [B]) by combining all reagents in one step.

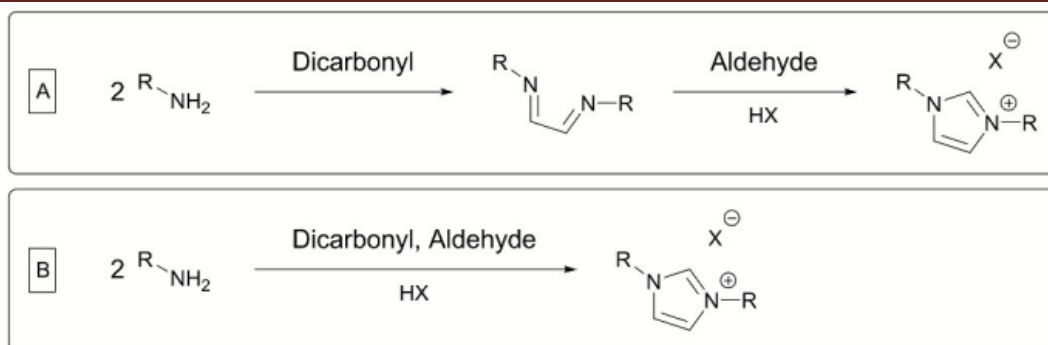


Figure 1: Conventional, condensational synthetic approach to (benz)imidazolium salts (Gravel and Schmitzer, 2017)

The final cyclization step does not necessarily have to be decreased to the use of aldehydes, high yields can be obtained using trialkylorthoformate (Huynh, 2017), but here it is necessary to reduce the imine to a secondary amine, accompanied by imidazolinium instead of imidazolium salt formation as shown in Picture 2. This can be used as an additional source of structural diversity in optimizing the relationship between structure and properties.

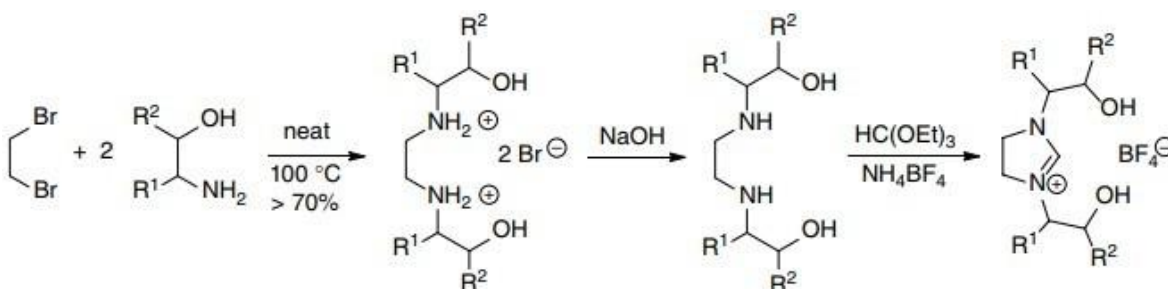


Figure 2: Condensation reaction with trialkyl orthoformate (Huynh, 2017)

In general, the maximum yields in terms of cyclization reactions are achieved by using trialkyl orthoformate (Huynh, 2017), but this reaction requires secondary amines and does not work on imines that are otherwise obtained in high yield, unlike secondary amines from alkylation reactions. To achieve maximum yields in both cases, reductive amination is used. Reductive amination involves the formation of an imine by the reaction of a suitable dicarbonyl and a primary amine, after which the imine is reduced to a secondary amine using reducing agents such as $NaBH_4$, followed by cyclization with trialkyl orthoformate (Picture 3). The only potential disadvantage of this preparative procedure is that the product are imidazolinium, not imidazolium salt.

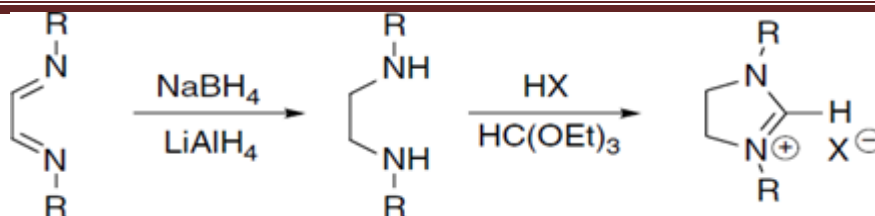


Figure 3: Reductive amination accompanied by cyclization (Huynh, 2017)

This approach is not explicitly limited to the use of a dicarbonyl and primary amines, namely any reaction resulting in a secondary diamine or diimine can serve as a basis for cyclization (Boydston et al., 2021). If the reaction is carried out with ethylenediamine and the corresponding carbonyl, the product is again a diimine which can be reduced and cyclized as shown in Picture 4.

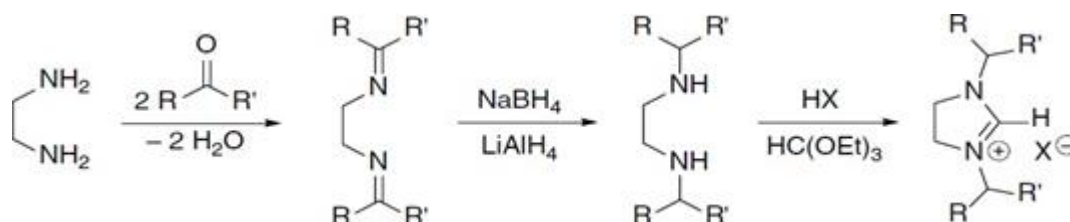


Figure 4: Reductive amination followed by cyclization with diamine precursor (Huynh, 2017)

Since the mentioned reducing agents easily reduce amides to amines, it is possible to include a variant with acid halides (Huynh, 2017) as shown in Picture 5.

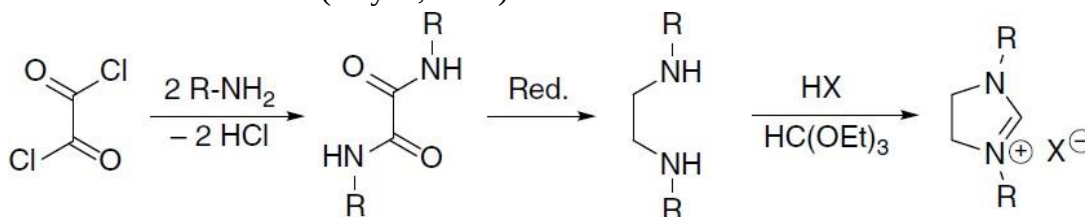


Figure 5: Reductive amination accompanied by cyclization (Huynh, 2017)

Therefore, condensation reactions are characterized by the best yields but not so much pronounced structural diversity, especially if the reaction is to be performed as a one-pot synthesis.

2.2 (Benz)imidazolium salts as *n*-alkylation reaction products

N-alkylation of imidazole or benzimidazole is the most commonly used synthetic route to (benz)imidazolium salts, since it is possible to obtain the same as asymmetric, i.e. substituents on *N* atoms do not have to be identical. This synthetic route can have many variations but the common feature of all is relatively low yield because *N*-alkylations are not easily feasible reactions and the usual yield depends on the effectiveness of the applied catalyst and the

characteristics of alkylating reagents. To prepare (benz)imidazolium salt means to quaternize one of the nitrogen atoms, so benzimidazolium salts are quaternary ammonium salts with *N* atom as part of the aromatic heterocycle (Gravel and Schmitzer, 2017). If the starting materials are unsubstituted imidazole or benzimidazole, the first alkylation is assisted by deprotonation of the hydrogen-bearing *N* atom with a base, resulting in the formation of an *N*-alkyl/aryl product which is subsequently quaternized using an additional alkylating agent summarized in Picture 6.

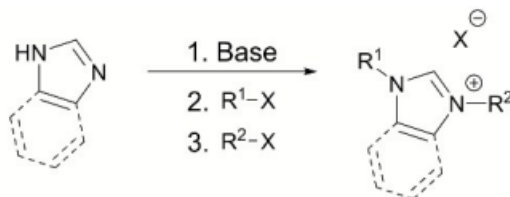


Figure 6: Double *N*-alkylation (quaternization) of (benz)imidazole (Gravel and Schmitzer, 2017)

The possibility of introducing different substituents through the *N*-alkylation/arylation reaction allows variations in the preparation of single-substituted (benz)imidazoles, so it is possible to combine aspects of condensation reactions with *N*-alkylation reactions.

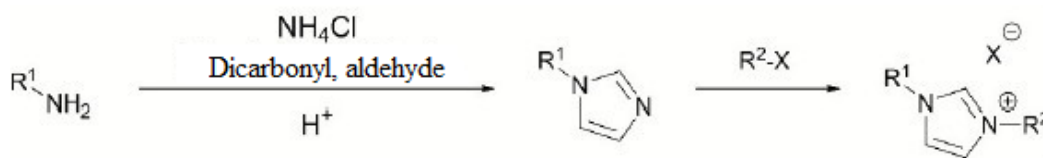


Figure 7: Imine cyclization accompanied by *N*-alkylation (Gravel and Schmitzer, 2017)

In this way, it is possible to prepare *N*-substituted imidazoles by manipulating stoichiometry in the first imine formation step (Picture 1. [A]). In some cases, the bioactivity of the (benz)imidazolium salt can be significantly affected by the anion of the same. If halides are undesirable, and they are the most common anions of this type of salt, it is possible to perform a double substitution based on differences in solubility (Ibrahim et al., 2021), which is usually done with virtually non-coordinating, indifferent anions such as tetrafluoroborate or hexafluorophosphate. Sometimes the tendency to synthesize this type of compounds with atypical anions opens up new synthetic approaches (Picture 8), so (benz)imidazolium salts can be obtained from alcohols using sulfonyl halides, wherein the first step an alkyl sulfonate is formed from the starting alcohol, and this sulfonate may serve as an alkylating agent for single *N*-substituted (benz)imidazole. The final product of this reaction are ionic (benz)imidazolium sulfonates where the sulfonate anion is subject to substitution with alkali metal salts which gives the possibility of introducing other anions (Gravel and Schmitzer, 2017).

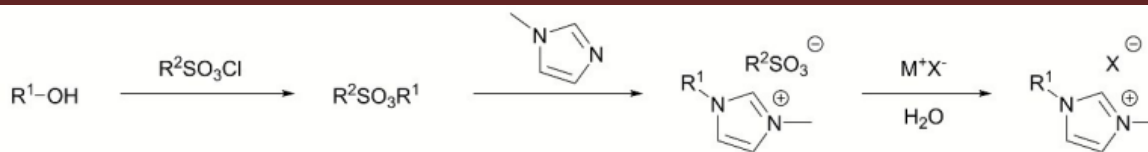


Figure 8: Imidazolium sulfonates derived from primary alcohols (Gravel and Schmitzer, 2017)

It turns out that *N*-alkylation/arylation reactions provide significant synthetic diversity to this class of compounds because they allow the synthesis of different structural analogs with subtle or drastic differences in one of the substituents, which is an extremely useful feature when evaluating biological activity and searching for new pharmacophores (Yetkinet al., 2021).

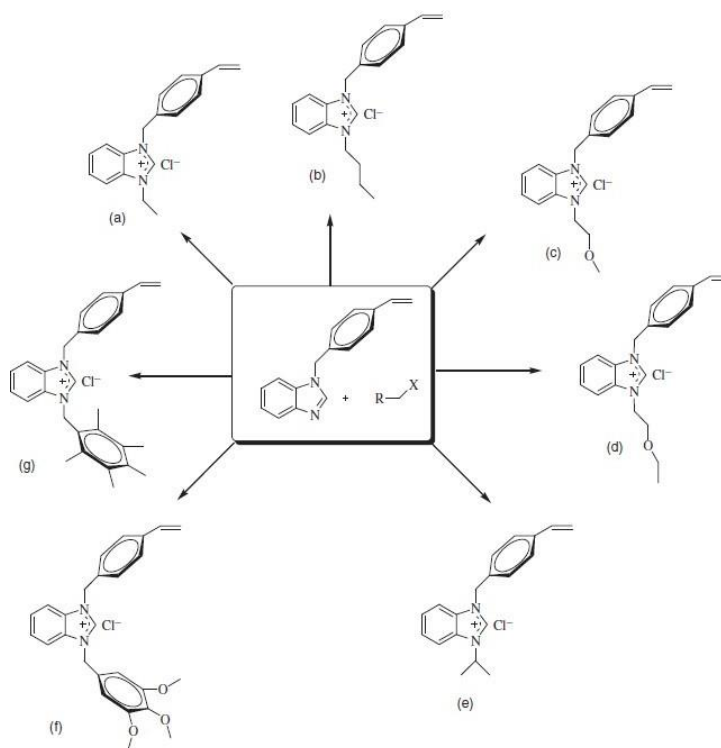


Figure 9: Quaternization of *N*-(4-vinylbenzyl)benzimidazole with alkyl chlorides (Yetkinet al., 2021)

Picture 9 shows a synthetic scheme illustrating the described, and the synthesized substances from (a) to (g) were prepared in yields of 78% to 90%, which represents very good results for this reaction.

2.3 (Benz)imidazolium salts as semi-synthetic reagents

The introduction of the (benz)imidazolium salt into a molecule as a final synthetic step means the use of these substances as semi-synthetic reagents. This may result in optimizing bioactivity, altering the physical properties of affected molecules, and the potential to build *N*-heterocyclic carbenes and their compounds with metals. Additionally, benzimidazolium salts exhibit versatility as synthetic reagents, facilitating the selective transfer of specific functional groups (Nawaz et al., 2023).

2.3.1 Affecting biological activity

It has been shown that the introduction of even structurally simple (benz)imidazolium salts, such as the reaction of a halogenated derivative of a given molecule with *N*-methylimidazole, in the structure of a molecule of a substance of known, characterized bioactivity, can significantly affect that bioactivity. This becomes obvious if we keep in mind that these compounds are salts, ie. they have a charge and thus the bioavailability, adsorption, transport, metabolism and excretion of the initial, bioactive molecules become significantly affected, in the optimal case, positively modified.

It is known that salicylic acid and its derivatives inhibit cyclooxygenase and have anti-inflammatory activity, but salicylic acid derivatives such as imidazolium salicylates (Picture 10) have antitumor effect, which is extremely surprising, but also encouraging for further research in this field (Gravel and Schmitzer, 2017).

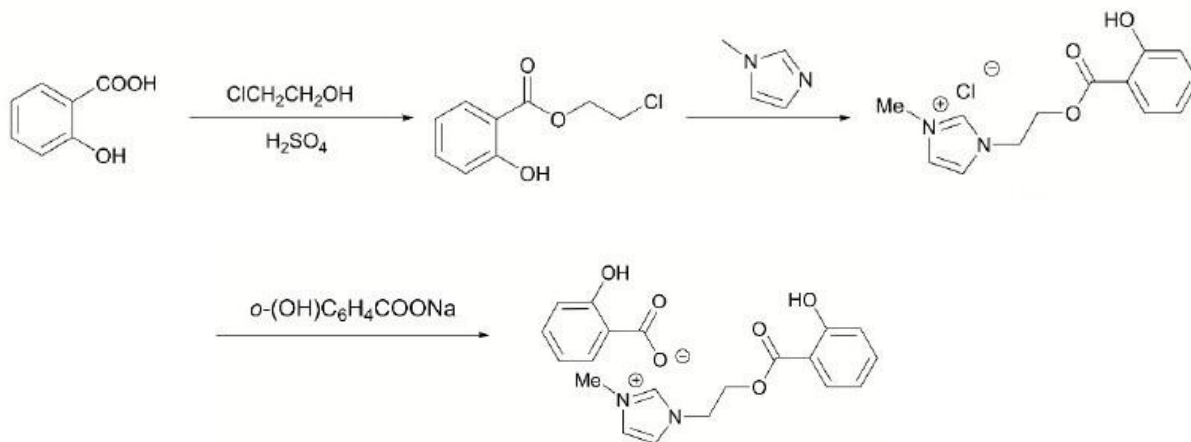


Figure 10: Synthesis of antitumor imidazolium salicylate derived from salicylic acid (Gravel and Schmitzer, 2017)

2.3.2 Compounds of metals and imidazolium salt

Some metal ions such as Ag^+ , Au^+ , Cu^+ , Pd^{2+} etc. react with (benz)imidazolium salts, substituting a hydrogen atom from the 2-position of said ring and forming a metal-carbon bond. Such compounds are called organometallic *N*-heterocyclic carbenes (NHC) and, in addition to their great importance in organometallic chemistry in general, they often have strong biological activity (Hussaini et al., 2019). Although most organometallics are not stable in air, nor are they resistant to hydrolysis, *N*-heterocyclic carbenes of these metals are characterized by significant thermodynamic stability and kinetic inertness (Huynh, 2017). *N*-heterocyclic carbenes of silver(I) and palladium(II) have been tested in vitro and in vivo and show strong antitumor effects.

From the point of view of this paper, the synthetic approach to these compounds is significant. It is easiest to prepare *N*-heterocyclic silver(I) carbenes because benz(imidazolium) salts dissolved in suitable, anhydrous solvents, and in the absence of light, dissolve i.e. react with silver (I) oxide to form Ag(I) organometallics (Picture 11) where the mentioned salt is the ligand (Gravel and Schmitzer, 2017).

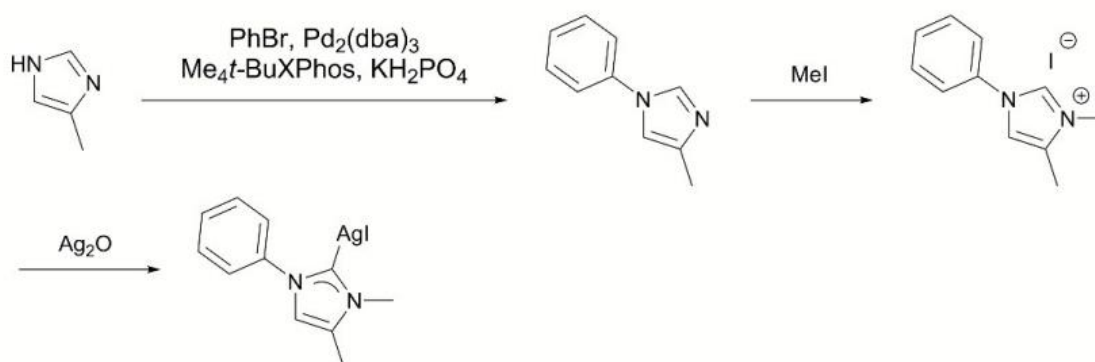


Figure 11: Synthesis of an antitumor *N*-heterocyclic carben-silver (I) complex (Gravel and Schmitzer, 2017)

N-heterocyclic carbenes of other metals are somewhat more difficult to obtain because the ligand needs to have better coordination properties (Picture 12) but can be more easily obtained by transmetallation reactions, where the halides of the desired metal react with *N*-heterocyclic silver(I) carbenes, isolating insoluble AgCl and formation of the desired organometallic compound.

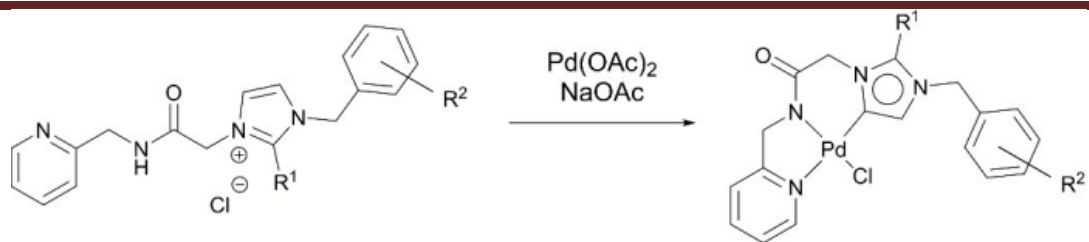


Figure 12: Synthesis of NHC-palladium(II) complex with coordination-suitable imidazolium salts (Gravel and Schmitzer, 2017)

3. CONCLUSION

(Benz) imidazolium salts represent an interesting class of compounds whose synthesis is achieved through relatively robust synthetic processes where fine optimization of the preparative pathway leads to better yield. Reactions resulting in structurally diverse compounds of this type, namely *N*-alkylation reactions, have lower yields than condensation reactions which, however, do not allow for significant structural diversity. Through this, it becomes clear that further research to address preparative issues must be aimed at finding better and more favorable catalysts (catalysts based on compounds of Cu(I) are favorable but have limited applicability, while catalysts based on Pd are expensive) to catalyze the synthesis of *N*-alkylation reactions, or to find better reactions to give selectively substituted imines (primary amines and dicarbonyls usually give a mixture of mono and disubstituted imine compounds or even other heterocycles as condensation products), and secondary amines. Fine control of the preparative pathway without loss of yield allows the optimization of the desired properties of imidazolium salts from their applicability as medicine to uses such as technical or laboratory solvents for specialized purposes.

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METAL CHELATING CAPACITY OF MELATONIN PRE-TREATED LEMON BALM AND VALERIAN PLANTS

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Key words: lemon balm, valerian, heavy metals, chelation

ABSTRACT:

Metal ions of iron (Fe^{2+}) and copper (Cu^{2+}) can catalyze the formation of free radicals. In living organisms, they are bound to proteins, which limits their participation in reactions that produce radicals and have an essential physiological function in living cells. Flavonoids have the ability to chelate metal ions, which contributes to their antioxidant activity in vitro, but it is not known whether they serve as effective metal chelators in vivo. They form a stable complex with transition group metals (Fe^{3+} , Al^{3+} , Cu^{2+} , Zn^{2+}) which depends on the nature of the molecule itself (catechol part) and the pH in which it is found. Chelation mainly takes place on the hydroxyl groups at positions 3' and 4' of the B ring, position 3 of the hydroxyl group of the A ring, and at positions 3 and 4 of the carbonyl group of the C ring. According to the results obtained in the control plants, valerian leaf extract reached the maximum metal chelation capacity of 65.76% at an extract concentration of 1 mg/mL, while the lowest capacity was shown by the lemon balm root at 41.93%. In the roots of both plant species grown on soil contaminated with Cd and Zn, there is a statistically significant increase in the ability to chelate iron (from 0.5 to 34.5%). Melatonin is simultaneously involved in the relocation of heavy metals and thus contributes to the reduction of oxidative stress in plants.

1. INTRODUCTION

During their lifetime, plants are exposed to various stressogenic factors (drought, extreme temperature conditions, increased light intensity, toxic metals, salinity and reduced nutrient content). In most aerobic organisms, factors such as pathogen invasions, UV and other forms of

radiation, air pollution, pesticides, ozone and others lead to increased production of free radical species (Malenčić et al., 2010). Certain heavy metals such as iron, cobalt, copper, zinc and manganese are necessary for numerous functions, normal growth and development of living organisms and are designated as essential. However, the accumulation of essential and non-essential heavy metals (cadmium, lead, arsenic, mercury) in living organisms reduces normal growth and development, causes acute poisoning, diseases, and death of organisms. Heavy metals have become one of the main abiotic factors with negative effects on living organisms. When consumed, plants play a significant role in the circulation of toxic metals because they enter the food chain and thus negatively affect human health. The accumulation and availability of toxic metals are particularly influenced by the type of soil, the pH value, as well as the content of organic matter in the soil (Zeremski, 2005). In addition, the accumulation of heavy metals in humans can cause serious damage to different organs, especially respiratory, nervous and reproductive and digestive systems. Biologically, metal chelation therapy is often used to treat metal toxicity. This process occurs through the interaction between the ligand and a central metal atom, forming a complex ring-like structure. After metals are chelated with appropriate chelating agents, their damage in metabolism can be prevented and efficiently removed from the body. On the other hand, heavy metals, including Zn, Fe and Cu, are necessary for the suitable functioning of different proteins including enzymes in metabolism. However, when the same metals accumulate at levels higher than the optimum level, they can easily become toxic and have harmful effects toward biomolecules. In this case, it induces the formation of ROS and nitrogen species (RNS) resulting in peroxidation of biological molecules such as lipids in the plasma membrane (Flora et al., 2008).

All living organisms need transition metals to maintain their vital functions. Metal ions have different functional roles in biological systems. In most cases, metals are tightly bound by forming coordination bonds with metalloproteins, and proteins acquire a three-dimensional structure (Gulcin i Dastan, 2007, Talaz et al., 2009). The chemical affinity of metal ions can be used by proteins to produce enzymes with strong catalytic activity. Metal chelating is usually considered as the most putative and common antioxidant method. Antioxidants have been reported to have an effective Fe-binding ability due to their functional groups that perform metal binding. The interaction of Fe ions with antioxidant compounds may also alter their biological effects including antioxidant properties (Han et al., 2018). It was found that curcumin, used as a food ingredient and is abundant in ginger and turmeric, chelates Fe^{2+} ions and prevents the formation of the Fe^{2+} -ferrozine complex. Curcumin can capture iron ions with a high binding affinity such as ferrozine. It has been reported that curcumin chelates iron ions with biological active -OH and -OCH₃ groups (Ak and Gulcin, 2008).

Metal chelators form complexes and reduce the reactivity of metals such as iron, making them inactive (Almhjell, and Mills, 2018). Flavonoids show bioavailability by chelating excess metal

ions in the human body. Such a metal chelating effect of flavonoids plays an important role in the detoxification of other heavy metals, such as Cr, Sn, Cd and Pb as well as binding excess Al. The chelating agents effectively chelate the toxic metal ions forming the complexes (Gosh et al., 2015).

The medicinal effect of lemon balm are spasmolytic, antibacterial and antifungal effects, and a relaxing effect on muscles. Water extract has an antiviral, i.e. virustatic effect, especially on the *Herpes simplex* virus, and it also acts as an antioxidant, where tannins, rosmarinic acid and caffeic acid derivatives have a special effect, which bind to the virus and prevent its penetration into new cells (Toplak - Galle, 2005).

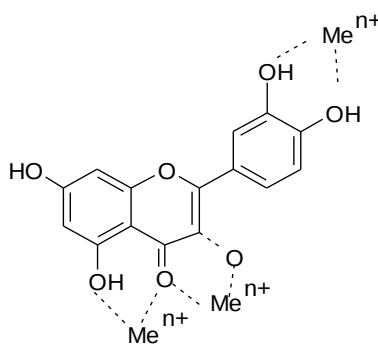


Figure 1: The positions in the flavonoid molecule where the chelation of the metal ion (Meⁿ⁺) takes place (Irina and Mohamed, 2012)

Valerian has antidepressant properties on the central nervous system and antispasmodic activity. Pharmacological studies of valerian and some other essential oil compounds of *Valeriana officinalis* indicate that the sedative effect can be attributed to the essential oil and valepotriate fractions. It is used as a sedative, mild sedative, hypnotic, antispasmodic, carminative, hypotensive (European Pharmacopoeia, 2005).

The aim of this study was to investigate the effects of heavy metal stress (cadmium and zinc) and exogenous melatonin application on the antioxidant capacity of ethanol extracts from the roots and leaves of lemon balm and valerian, by evaluating their ability to chelate iron ions and reduce copper ions. The goal was to assess whether melatonin could enhance the antioxidant potential of these medicinal plants under conditions of metal-induced oxidative stress.

2. MATERIAL AND METHODS

2.1 Metal chelating assay

Metal chelating assay by ferrozine reagent was used. The spectrophotometric method was developed taking into account the binding affinity between a reagent and a metal ion such as Fe²⁺. The Fe²⁺ chelation by ferrozine was evaluated by the Dinis method (Dinis et al., 1994).

Briefly, a different quantity of sample and standard compounds was transferred to a 0.05 mL of FeSO_4 solution (2 mM). The interaction between the sample and Fe^{2+} is supplied, that is, Fe^{2+} ions are chelated by the sample. The reaction was started by adding 0.2 mL of ferrozine reagent (5 mM) and the mixture was stirred and left standing at 25 °C for 10 min. Absorbance was measured spectrophotometrically at 562 nm. A mixture of ethanol, ferrozine and FeSO_4 without a sample was used as a control sample, and a mixture of ethanol and a sample without ferrozine and FeSO_4 as a blank. A lower absorbance value indicates a better activity, that is, the iron-ion chelating ability of the analyzed sample.

$$\% \text{ oxidation} = \left(\frac{A_0 - A_s}{A_0} \right) \times 100 \quad (1)$$

2.2 Antioxidant activity

The CUPRAC method by the Apak et al. (2007) was used. The method was originally developed as a spectrophotometric measurement of total antioxidant capacity, and later expanded with modifications. The selected chromogenic redox reagent is easily available, stable, selective and corresponds to all types of biologically important antioxidants (ascorbic acid, α -tocopherol, β -carotene, uric acid and bilirubin), as well as all types of food antioxidants (flavonoids, simple phenols and hydroxycinnamic acids), regardless of chemical type and hydrophilicity. In brief, aqueous CuCl_2 , ammonium-acetate buffer (pH=7) and an alcoholic solution of neocuproine are mixed together with a suitable aliquot of an unknown antioxidant solution, and the absorbance is read at 450 nm after 30 minutes in the dark. A reaction mixture of 1 mL of CuCl_2 solution (0.01 M), 1 mL of acetate buffer (1 mM), 1 mL of ethanolic neocuproine solution (7.5×10^{-3} M) and 0.5 mL of ethanol extract was prepared and left for 30 minutes at room temperature. The absorbance of the solution was measured spectrophotometrically at 450 nm in relation to the reference solution containing 1 mL of CuCl_2 solution (0.01 M), 1 mL of acetate buffer (1 mM), 1 mL of ethanolic neocuproine solution and 1.1 mL of deionized water. Trolox was used as a standard compound, and the reduction potential was expressed as $\text{TEA}_{\text{CCUPRAC}}$ in mmol equivalents of trolox g of dry matter (mmol/L). All experiments were repeated three times, and the result was expressed as the mean value of three measurements $\pm$ SD.

3. RESULTS AND DISCUSSION

The ability to chelate iron ions in the ethanol extracts of leaves and roots of valerian and lemon balm is shown in Figure 2. According to the results obtained in the control plants, valerian leaf extract reached the maximum metal chelation capacity of 65.76% at an extract concentration of 1 mg/mL, while the lowest capacity was shown by the lemon balm root at 41.93%. In the roots of both plant species grown on soil contaminated with Cd and Zn, there is a statistically significant increase in the ability to chelate iron (from 0.5 to as much as 34.5%).

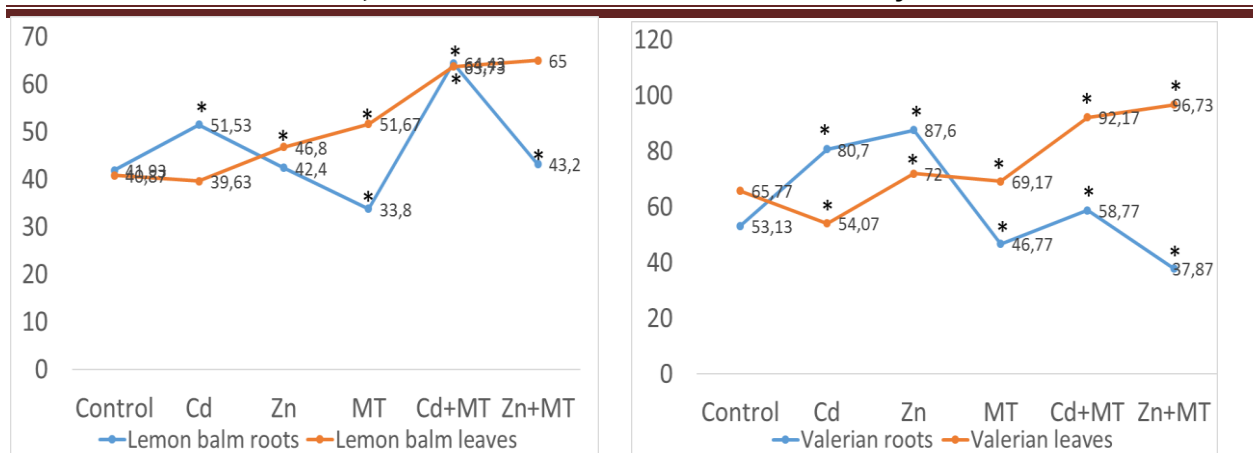


Figure 2. The influence of melatonin and heavy metals on the ability to reduce Cu²⁺/CUPRAC TEST/ in the root and leaf of lemon balm and valerian. Dots marked with an asterisk are statistically significantly different (Tukey test; $p < 0.05$)

In the leaf of both plant species treated with Cd, in contrast to the treatment with Zn, a reduced ability to chelate iron is observed, although the reduction in the leaf of the lemon balm (39.63%) is not statistically significant. Exogenous melatonin affected the increased ability to chelate iron ions in the leaves of lemon balm (51.66%) and valerian (69.16%) compared to the control, as well as reduced ability in the roots of both plant species. Interestingly, the maximum ability to chelate iron is observed in the roots and leaves of plants grown on soil contaminated with heavy metals, in combination with exogenous melatonin, which indicates the possible effect of exogenous melatonin in protection against oxidative stress. The maximum chelation is shown in valerian leaf treated with zinc ions and exogenous melatonin (96.73%)

Figure 2 shows that melatonin has a significant ability to bind iron, especially in the leaf of the examined plant species. Therefore, it can be assumed that its action in reducing lipid peroxidation is related to this ability.

Recent research points out that exogenously applied melatonin improves the adaptation of the Arabidopsis plant (*Arabidopsis thaliana*) to iron deficiency, by affecting iron remobilization. This process was found to be associated with cellular polyamine levels. Likewise, melatonin treatments could not alleviate iron deficiency-induced chlorosis in polyamine- or NO-deficient plants. Thus, the results obtained in this study indicate that exogenous melatonin increases the resistance of plants to iron deficiency through the induction of polyamine-mediated NO accumulation (Zhou et al., 2016).

In the results of the CUPRAC test, it is observed that the ability of lemon balm and valerian leaves to reduce Cu ions is significantly higher (three to four times) compared to the root of both plant species. Heavy metals had the opposite effect on the reduction of Cu ions in the roots of

lemon balm and valerian, in the sense that Cd caused a statistically significant increase and Zn a significantly decrease capacity for reduction (Figure 3).

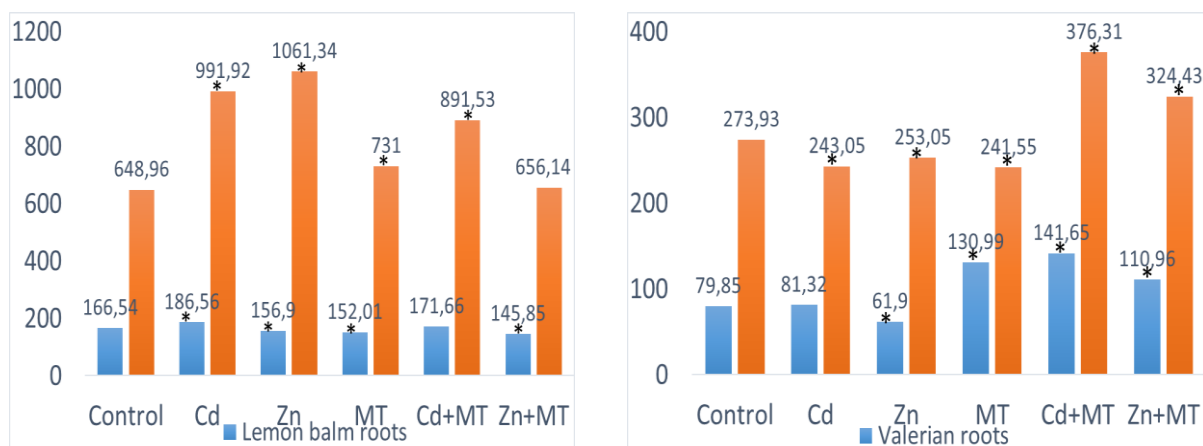


Figure 3. Effect of melatonin and heavy metals on the ability to chelate heavy metals for 1 mg/mL concentrations of root and leaf extracts of lemon balm and valerian. Columns marked with an asterisk are statistically significantly different (Tukey test; $p < 0.05$)

By exposing the lemon balm plants to cadmium ions, there is an increase in the ability to reduce Cu^{2+} in the roots (186.56 µg/g) compared to the control (166.54 µg/g), but also in the leaves (991.92 µg/g) compared to the control leaves (648.96 µg/g). In contrast, zinc ions in the roots of lemon balm caused a decrease in its reducing power (156.9 µg/g), which is again manifested in the roots of plants pretreated with melatonin with zinc treatment (145.82 µg /g).

Exogenous melatonin affected the reduced ability to reduce Cu ions in lemon balm root (152.01 µg/g) compared to control (166.54 µg/g) and valerian leaf (241.55 µg/g) compared to control leaves (273, 93 µg/g). In contrast, a statistically significant increase occurs in lemon balm leaf (731.00 µg/g) and valerian root (130.99 µg/g). Taking into account the fact that the root of the lemon balm, as well as the valerian leaf, are rarely used for medicinal purposes, the results obtained are encouraging. Exogenous melatonin in combination with heavy metal treatment also shows a significant positive effect on the reduction of Cu ions, increasing the ability of both roots and leaves of both plant species in this role. It is possible that the antioxidant activity of melatonin is enhanced in the roots of the analyzed plants. Since melatonin is very unstable, it is difficult to monitor its transport in plant organelles and organs (van Tassel and O'Neill, 2001). Similar results were obtained in other antioxidant activity test with same plant species (Hodžić et al., 2021). Namely, it was mentioned that mitochondria and chloroplasts can be the primary sites of melatonin synthesis in most cells. The root is probably the most frequently mentioned plant organ in earlier research as a potential site of melatonin biosynthesis. However, the evidence for this theory is still weak.

Based on the results obtained by different antioxidant capacity tests, the ethanol extracts of the leaves of both plant species show significant total antioxidant activity. Research results show increased antioxidant activity of plants, especially leaves in all applied biochemical tests. The assumption is that the increased antioxidant activity of the leaves of both plant species can be attributed to the increased content of total phenols, compared to the root (Hodžić et al., 2019).

Recent studies have shown that exogenous application of different concentrations of melatonin (0.1, 0.5 and 1 μM) significantly increases the antioxidant capacity of kiwifruit seedlings, exposed to increased salinity (100 mM NaCl). Also, damage to them was significantly reduced. It is interesting that melatonin concentration of 0.1 μM proved to be the most effective (Xia et al., 2017).

Treatment with exogenous melatonin (100 $\mu\text{mol/L}$) increases the endogenous melatonin content of ripe grape and wine berries. Also, in the same research, the content of total phenols (especially singer and coumarin acid), flavonoids and anthocyanins in wine increased, but also improved the antioxidant effect of wine analyzed through DPPH and FRAP biochemical tests (Xu et al., 2018). Exogenous melatonin application notably enhanced the iron-chelating ability in the leaves of both species, especially when plants were grown in contaminated soils. The maximum chelation capacity was recorded in valerian leaves treated with Zn and melatonin, suggesting that melatonin plays a key role in protecting plant tissues under metal-induced oxidative stress, possibly by modulating iron homeostasis.

4. CONCLUSION

It has been proven that soil pollution with cadmium and zinc ions leads to an increase in the reduction capacity of ethanolic root extracts, especially of lemon balm leaves (where a doubling of the reduction capacity is observed), and of valerian roots. Pretreatment with exogenous melatonin increases the reducing power in all examined plant organs, except in the valerian leaf, where there is no significant change. A very high degree of correlation with the content of total phenolic compounds and proteins was determined. The roots of both examined plant species pretreated with melatonin show a reduced ability to chelate heavy metals. The exact opposite effect of pretreatment is observed in plant leaves. A positive correlation was established between melatonin content and the ability of ethanolic extracts to chelate iron. Given the significant enhancement of antioxidant properties, particularly in plant parts traditionally used for medicinal purposes, the application of melatonin could be a promising strategy to increase the phytochemical value of medicinal plants grown in contaminated environments. Further research should aim to elucidate the underlying mechanisms of melatonin-mediated antioxidant responses and investigate its practical applications in sustainable agriculture and phytoremediation.

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Eighth International Scientific Conference
"June 5th - World Environment Day"

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AIR QUALITY COMPARISON IN UNA-SANA, ZENICA-DOBOJ AND TUZLA CANTON FROM 2020 TO 2023

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Key words: air quality, monitoring, pollution, SO₂, NO₂, CO, PM₁₀, PM_{2.5}

ABSTRACT:

Air quality impaired by the action of pollutants in concentrations that can significantly affect the quality of life, people's health or parts of the environment represents a public health problem of great importance and is associated with a series of harmful effects on human health. Air quality monitoring in the Federation of Bosnia and Herzegovina is the responsibility of the Federal Hydrometeorological Institute and the competent authorities of cantons and local self-government units. The operators that monitor ambient air quality at fixed locations in the Federation of Bosnia and Herzegovina are: Federal Hydrometeorological Institute, Institute of Public Health of Sarajevo Canton, Municipality of Kakanj, Metallurgical Institute „Kemal Kapetanović“ Zenica and Ministry of Physical Planning and Environmental Protection of Tuzla Canton.

In this paper, a comparison of data collected by the Federal Hydrometeorological Institute through automatic measuring stations was made. Three areas were observed: Una-Sana, Zenica-Doboj and Tuzla canton. The subject of comparison were concentrations of sulfur dioxide (SO₂), nitrogen dioxide (NO₂), ozone (O₃), particulate matter (PM₁₀ and PM_{2.5}) and carbon monoxide (CO) from 18 locations of fixed automatic stations. Mean annual concentrations were monitored over a period of four calendar years, namely 2020, 2021, 2022 and 2023. The results show extremely high and health-threatening concentrations of particulate matter in almost all locations. In some locations, health-threatening concentrations of sulfur dioxide and ozone have been recorded. Concentrations of nitrogen dioxide and carbon monoxide

are within the maximum allowed concentrations and exceedances are extremely rare. Also, the air quality in the Una-Sana canton compared to the Zenica-Doboј and Tuzla cantons is satisfying in average annual concentrations.

1. INTRODUCTION

The planet Earth is immersed in an invisible sea of gases and condensed water vapor. The Earth's atmosphere is characterized as a mixture of gases. This gaseous mixture becomes polluted when it's changed by the addition of particles, gases or energy forms (e.g., heat, radiation or noise) so that the altered atmosphere poses harm because of its impact on weather, climate, human health, animals, vegetation or materials. The concept of pollution entails a sense of degradation, a loss of quality and adverse environmental effects whether it's applied to the air, water or land (Godish et al., 2019). Studying the human health effects of air pollution has often been challenging, because it is difficult to isolate from other factors that also influence health such as smoking and exposure to poor indoor air quality (Baldasano & Valera et al., 2003).

Air quality in city and urban areas is the most important factor that directly influences the incidence of diseases and decreases the quality of life. Taking appropriate decisions in a timely period depends on the measurement and analysis of the parameters of the air, which creates the need for the development of real time air quality monitoring. The use of multiparameter air quality monitoring systems makes possible to do a detailed level analysis of major pollutants and their sources. These monitoring systems are important components in many smart city projects for monitoring air quality and for controlling the main pollutant concentrations in urban areas (Yang et al., 2009). Air pollution and quality monitoring is extremely important in today's world as it has a direct impact on human health. Air pollution is on the rise due to a number of anthropogenic activities and monitoring is of vital importance to mitigate certain measures to control it (Chaudhry, 2013). Air pollutants have major contributing factors to chronic diseases and mortality subsequently impacting public health. Air pollutants include ozone, sulfur oxides, carbon monoxide, nitrogen oxides, and particulate matter (Yang & Omaye, 2009). Suspended particles represent a complex mixture of organic and inorganic substances. They can roughly be divided into coarse and fine particles. The border between these two particle fractions is usually between 1 micron and 2.5 microns. The measurement of suspended particles smaller than 10 microns is significant because of their proven harmful effect on health. Polluting substances such as sulfur dioxide, suspended particles, nitrogen oxides, carbon monoxide and ground ozone are also considered air quality parameters. Hydrocarbons, fluorides, chlorine, heavy metals from the production and combustion processes are also present, but they aren't widely distributed in the ambient air, except in industrial areas (Čajić et al., 2016).

This paper presents the monitoring of air quality at 18 different locations in Bosnia and Herzegovina, taking into account their space prevalence and the presence of pollutants. The data

presented in this paper were collected in 2020, 2021, 2022 and 2023 years. The aim of this work is to show the harmful influence of pollutants in the atmosphere and their variations during the mentioned four years. The data were collected by the Federal Hydrometeorological Institute and competent bodies of cantons and local self-government units using the legally prescribed methodologies for air monitoring.

2. AIR POLLUTANTS AS PRODUCTS OF DIFFERENT PROCESSES

2.1 Sulfur dioxide

One of the most common pollutants is sulfur dioxide. The largest anthropogenic source of sulfur dioxide is the burning of fossil fuels. It is believed that a third of sulfur enters the atmosphere through the burning of fossil fuels (households, transport, thermal power plants and heating plants). In the atmosphere there is sulfur starting from the elemental form, through sulfur oxides, sulfur dioxide and sulfur trioxide and their acids (formed in reaction with water vapor from the air), as well as their salts, and is also encountered in the form of hydrogen sulfide. Determination of sulfur dioxide values are associated with soot values, because carbon particles, which are found in soot, serve as carriers of sulfur dioxide. Therefore, the standards for sulfur dioxide and soot are related. Inhaled (inhaled) sulfur dioxide is mostly dissolved in water (mucus) of the upper airways. Dissolving, it builds acid and acts on the mucous membrane of the respiratory tract irritatingly and inflammatory. With chronic exposure, most often irritant symptoms occur, but also the appearance of chronic diseases of the respiratory organs. Children are particularly sensitive to the effects of sulfur dioxide, as well as the elderly, asthmatics and chronic heart patients (Pirić et al., 2017).

2.2 Nitrogen oxides

Nitrogen monoxide and nitrogen dioxide are the most important oxides in air pollution. the most important oxides in the formation of photochemical smog, "summer smog". Nitrogen oxides and hydrocarbons accumulate in the atmosphere, and nitrogen monoxide is oxidized into nitrogen dioxide under the action of solar radiation with the release of nascent oxygen which, with molecular oxygen from the air, participates in the formation of ozone. With the presence of water vapor, aerosols are formed, which reduce visibility, and polluted air has a characteristic brown color. Nitric oxides act primarily on the exacerbation of asthma and other chronic respiratory diseases. In the lower layers of the atmosphere, as a result of complex photochemical reactions, ozone is formed as a secondary pollutant (tropospheric, ground or bad ozone) (Pirić et al., 2017).

2.3 Carbon dioxide

Carbon dioxide (CO₂) is a colorless gas, which is essential for life on Earth. It is a key component of photosynthesis, the process by which plants produce energy. CO₂ occurs naturally in the atmosphere, but its concentrations have increased significantly due to human activities

such as burning fossil fuels and deforestation. Carbon dioxide (CO₂) is a gas that plays a key role in the Earth's carbon cycle and climate system. It is colorless and odorless, and its presence in the atmosphere comes from natural processes such as volcanic eruptions, the respiration of living things, and the decomposition of organic matter, as well as from anthropogenic sources such as burning fossil fuels and industrial processes. Due to its greenhouse properties, CO₂ absorbs and radiates infrared radiation, which contributes to the warming of the atmosphere. The increase in CO₂ concentrations in the atmosphere over the last centuries, mainly as a result of human activity, has led to global warming and climate change. These changes affect ecosystems, weather patterns, sea level, and many other aspects of the global environment (Prentice et al., 2021).

2.4 Particulate matter

Particulate matter or aerosols are a two-phase system in which solid and/or liquid particles are dispersed in the air. They occur in different sizes, shapes, concentrations and compositions, mainly as a result of various natural and anthropogenic activities (Pöschl, 2005). These are particles of very small dimensions that can remain suspended in the air for several hours, weeks and even months before their final deposition to the ground, either dry (gravitational) or wet deposition (Harrison, 2004). Particulate matter is usually divided into three groups according to aerodynamic diameters: PM₁₀, PM_{2.5} and PM₁. PM₁₀ designates particles with an aerodynamic diameter of less than 10 µm, PM_{2.5} includes particles with an aerodynamic diameter of less than 2.5 µm, and PM₁ particles with an aerodynamic diameter of less than 1 µm. PM₁ fraction particulate matter is still insufficiently researched, although it has been proven that such particles linger longer in the atmosphere and are more dangerous to human health, because they penetrate deeper into the respiratory system. Throat and nose irritations, narrowing of the airways, pneumonia, tachycardia, increased blood pressure are just some of the symptoms associated with human exposure to particulate matter (Kampa & Castanas, 2008; Petrović & Antanasijević, 2010). On the other hand, they also have numerous harmful effects on the environment such as global warming, disruption of the hydrological cycle, nitrogen and sulfur cycles, contribution to the formation of acid rain, and also affect the reduction of visibility in many urban environments.

2.5 Carbon monoxide

Carbon monoxide (CO) is a highly toxic gas without smell and taste and is lighter than air. It belongs to the group of chemical suffocators, and causes general hypoxia due to irreversible binding with hemoglobin (Hb). The toxic effect of carbon monoxide occurs quickly and at low concentrations. Carbon monoxide is produced in the process of incomplete oxidation of organic matter. One of the most important sources of air pollution is the exhaust gas of an internal combustion engine where it can be from 1 - 14%, and the next major air pollutant is the metallurgical industry. In the USA, of the total amount of CO emitted into the atmosphere, 58% is accounted for by the exhaust fumes of cars and other internal combustion engines. Of all the

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fatal poisonings over 50% are caused by carbon monoxide. The intake and elimination of carbon monoxide is carried out through the lungs, and the ultimate toxic effect is hypoxia resulting from the formation of carbonyl compounds that take up space for oxygen binding (O₂) or for its transfer and utilization in tissues and cells (Čajić et al., 2016).

The rate of carbon monoxide binding to hemoglobin is slightly slower than oxygen binding, but hemoglobin's affinity for carbon monoxide is 210 to 220 times higher than for oxygen, so carbon monoxide has the advantage of binding with hemoglobin even when it is present in disproportionately smaller quantities over oxygen. The toxic effect of carbon monoxide on the body depends on the time of exposure and potential factors, that is, on the minute volume of breathing, muscle work, individual resistance of each individual concentration of carbon monoxide and intensity of hypoxia. The lethal dose for humans is 1000 -2000 ppm (0.1 to 0.2%) if the exposure lasts 30 minutes. At high concentrations of carbon monoxide in inhaled air, death can occur after 1 to 2 minutes. The maximum permitted concentration in industry is 50 ppm (0.005%) for 8 hours of exposure, and diving air shall not contain more than 10 ppm (0.001%).

3. MATERIAL AND METHODS

This paper is based on valid, annual basis reports for air quality in the Federation of Bosnia and Herzegovina. The data were collected by the Federal Hydrometeorological Institute and competent bodies of cantons and local self-government units using the legally prescribed methodologies for air monitoring. The methodology prescribed by law for monitoring the concentration of certain polluting substances in the ambient air is in accordance with the methodology prescribed in the countries of the European Union.

4. RESULTS AND DISCUSSION

Below are shown tables with the average annual concentrations of pollutants such as sulfur-dioxide (SO₂), carbon monoxide (CO), nitrogen oxides (NO₂), ozone (O₃) and particulate matter (PM₁₀ and PM_{2.5}) at 18 locations within the state of Bosnia and Herzegovina.

Table 1: Average annual concentrations for SO₂ and CO.

Location	Average annual concentrations							
	SO ₂ [µg/m ³]				CO [mg/cm ³]			
	2020	2021	2022	2023	2020	2021	2022	2023
Zenica Centar	86	91	71	47	0.6		0.6	0.9
Zenica Radakovo	92			58				0.4
Zenica Tetovo	78	73	58	62	0.8	1.0	0.8	0.7
Zenica Brist	97	87	92	56				
Zenica Vranduk		59	92	64				
Kakanj Doboј	87	67	68	51		0.4	0.4	0.3
Kakanj Centar				49				

Eighth International Scientific Conference
"June 5th - World Environment Day"

Bihać			5	4			0.5	0.7
Jajce Harmani	11	14						
Travnik				37				
Tuzla Skver	40	43	94	84			1.4	
Tuzla BKC	64	72	87	59			1.7	1.3
Tuzla Bukinje	64	51	76	49	1.2		0.7	0.7
Lukavac Centar	53	46	43	32	2.5			0.9
Živinice Centar	101	110	99	73	1.5		0.8	
Maglaj				48				
Tešanj		33	36					
Visoko	77	76	101	81				

*Limit values for SO₂ is >50 µg/m³ and the locations where the limit values were exceeded are marked in red. Limit values for CO wasn't exceeded at any location.

Annual concentrations of sulfur dioxide in 2023 were higher than the permitted value in Visoko, Zenica (Radakovo, Brist, Tetovo, Vranduk), Tuzla (Skver, BKC), Živinice and Kakanj (Doboj). These are mostly places where the limit values were exceeded in previous years as well. Values of average annual concentrations (multi-year presentation in table 1 above), in general, does not show significant changes compared to the earlier period. At all measuring points there are slight decreases or concentrations similar to those of previous years.

Table 2: Average annual concentrations for NO₂ and O₃.

Location	Average annual concentrations							
	NO ₂ [µg/m ³]				O ₃ [µg/m ³]			
	2020	2021	2022	2023	2020	2021	2022	2023
Zenica Centar			21	18			43	66
Zenica Radakovo			21	20		39	36	71
Zenica Tetovo	35			27			45	43
Zenica Brist		19	12	11			66	47
Zenica Vranduk						39	33	33
Kakanj Doboj	20	14	18	15		54	49	51
Kakanj Centar				22				
Bihać			10	10			71	
Jajce Harmani		12	10	11			52	62
Travnik				13				
Tuzla Skver	18	25	27	28	34		23	
Tuzla BKC	20	24	22	32	23			
Tuzla Bukinje	15	19	20	26	42			39
Lukavac Centar	23	26	25	27	38		39	40
Živinice Centar	20	25	22	26	25	37		32
Maglaj						66		
Tešanj		13	14	13		46	41	51
Visoko					40	41	37	44

Taking Nitrogen oxides into account, the average annual concentrations of nitrogen dioxide with slight oscillations show similar values as in previous years. There were no drastic reductions or increases in the emission of nitrogen oxides into the atmosphere. The limit value of the average

Eighth International Scientific Conference "June 5th - World Environment Day"

annual concentration of nitrogen dioxide is $40 \mu\text{g}/\text{m}^3$. Based on the presented data, the results collected during the previous 4 years show satisfactory results.

Furthermore, table 2 shows the statistical indicators of ozone measurement results. Concentrations of this pollutant can show significant variations from year to year. However, despite the mentioned variations, the measured values were within the permitted limits. Ozone concentrations depend on both natural-geographical factors and anthropogenic actions, and the possibilities of their reduction are significantly less than is the case with other pollutants in the air. In general, high values are expected in higher locations (such as mountainous areas, higher zones of cities with frequent traffic or specific industrial plants) and are directly related to sunlight and atmospheric turbidity, but also to the presence of ozone precursors (nitrogen oxides and volatile organic compounds) in the air.

Air pollution with particulate matter is the most characteristic type of pollution in our cities. Almost at all measuring points there are situations where the concentrations are above the legally prescribed. Annual concentrations of particulate matter indicate that it is the most prevalent pollutant that negatively affects air quality in our country. In earlier years, the limit values of the concentration of particulate matter PM_{10} were exceeded at almost all stations. In 2021, the lowest concentrations of floating particles were recorded compared to the previous period in which we have a slightly larger number of comparable data. In 2023, they were on average slightly higher than those in 2021, but they can still be considered lower compared to earlier years. Although very high and unhealthy concentrations of both hourly and daily values were recorded, and that their number and frequency is still worrying - the results from 2022 and 2023 show that this is the least polluted period in the last ten years since it has been available somewhat larger and comparable number of measured data. At almost all stations, a decrease in concentrations was recorded in terms of all statistical indicators related to particulate matter, which was most likely favored by slightly more favorable general meteorological conditions.

Table 3: Average annual concentrations for PM_{10} and $\text{PM}_{2.5}$.

Location	Average annual concentrations							
	$\text{PM}_{10} [\mu\text{g}/\text{m}^3]$				$\text{PM}_{2.5} [\mu\text{g}/\text{m}^3]$			
	2020	2021	2022	2023	2020	2021	2022	2023
Zenica Centar	53	47	44	37				
Zenica Radakovo	56	49	42	41				
Zenica Tetovo		63	55	47		41	43	37
Zenica Brist	49			37				
Zenica Vranduk	46	34	43		40	28	36	
Kakanj Doboј	29		33	36				
Kakanj Centar				47				
Bihać			30				25	
Jajce Harmani	35	30	32	21				
Travnik				20				17
Tuzla Skver					42	38	36	32

Eighth International Scientific Conference
"June 5th - World Environment Day"

Tuzla BKC					47	47	34	23
Tuzla Bukinje						27		
Lukavac Centar					44	40	35	
Živinice Centar					40	42		
Maglaj		42	42	36				
Tešanj		24	27	25				
Visoko	69	55	58	40				

*Locations where the limit values were exceeded ($>25 \mu\text{g}/\text{m}^3$ for $\text{PM}_{2.5}$ and $>40 \mu\text{g}/\text{m}^3$ for PM_{10}) are marked in red.

The average annual values of carbon monoxide are significantly below the prescribed limit values. We can consider that pollution with this pollutant is within the prescribed values and limited to smaller spaces and short periods. Based on the obtained results, we can conclude that this pollutant very rarely damages the air quality in our cities and the concentrations remain within the legal limits. Average annual concentrations are far below the prescribed limit values ($>3 \text{ mg}/\text{m}^3$)

In general, the measured concentrations of suspended particles PM_{10} and $\text{PM}_{2.5}$ in 2022 and 2023 show the lowest values in the last ten years. At the same time, the number of exceeding the limit values is still extremely high. The results of measuring nitrogen dioxide and carbon monoxide are satisfactory, limit values are rarely exceeded, and in most measuring places they do not even occur. As for the other recorded values, there are variations recorded by measurement, but they do not show any drastic increasing trend. [14]

All presented data are interrelated since the greatest impact of air pollution are mostly anthropogenic impact. Population migration leads to a reduction in economic activities and a reduction in economic activities leads to better air quality and vice versa. However, the reduction of population, industry, energy, agriculture and other activities is not the way of air protection from harmful pollution. It should strive to find a solution that will have a significant impact on reducing pollutant emissions into the atmosphere with long-term air protection goals. Air pollution represents one of the most dangerous threats to humans today. In addition to the impact on health, air pollution leads to climate changes that can be catastrophic for humanity in general.

5. CONCLUSION

At almost all measuring points in the Federation of Bosnia and Herzegovina, extremely high, health-threatening concentrations of floating particles and sulfur dioxide are evident. The results of measuring nitrogen dioxide and carbon monoxide are satisfactory, limit values are rarely exceeded and in most measuring places they do not even occur.

Air quality in the Zenica Canton is dangerously impaired by high concentrations of sulfur dioxide and floating particles to the extent that it can seriously harm people's health. Very high concentrations of the mentioned pollutants are evident at all measuring points. Very high concentrations of sulfur dioxide and floating particles were also recorded in the rural area (Vranduk). Air quality in the Tuzla Canton is seriously impaired to the extent that it can seriously

Eighth International Scientific Conference "June 5th - World Environment Day"

harm people's health due to very high annual concentrations of floating PM_{2.5} particles at all measurement points. The situation is similar with sulfur dioxide concentrations. The number of exceeding prescribed concentrations for other pollutants is very low. The annual concentration of sulfur dioxide is lower than the limit value. It is advisable to analyze the conditions affecting the air quality of existing industrial facilities. The air quality in the Una-Sana Canton is reflected in the occasionally very high and unhealthy concentrations of PM_{2.5} floating particles. Other monitored substances are satisfactory.

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Eighth International Scientific Conference
"June 5th - World Environment Day"

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DANGERS FROM CONSTRUCTION A RADIOACTIVE WASTE STORAGE CENTRE AT THE ČERKEZOVAC SITE, TRGOVSKA GORA, THE REPUBLIC OF CROATIA

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Key words: radioactive waste, storage centre, disposal site, Čerkezovac, Trgovska gora

ABSTRACT:

The planned construction of the Radioactive Waste Storage Centre (RWSC) at the Čerkezovac location, Trgovska gora in Croatia raises serious concerns due to its proximity to protected areas, populated places, and drinking water sources. This paper provides an overview of available relevant information regarding the planning of the RWSC construction, as well as potential risks and long-term consequences that such a facility could have on the environment and local community, including the risk to the water supply of the Novi Grad municipality. The RWSC at the Čerkezovac military storage would feature a central storage for the storage of radioactive waste (RW) and spent sources (SS) generated by the use of radioactive materials in the Republic of Croatia, a long-term storage for low and medium radioactive waste (LMRW) from the Krško Nuclear Plant generated during regular operation and its decommissioning, and a built disposal site for LMRW.

What adds further concern is that the construction of an LMRW disposal site within the Centre is also planned. The location of the disposal site would be within the Čerkezovac military storage or at another site within the preferred location on Trgovska gora.

Although the treatment of RW is necessary to reduce the risks that radioactive materials pose, the Čerkezovac location planned for the construction of the RWSC is not the most suitable due to its geographical and ecological characteristics, seismic instability, as well as proximity to protected areas and key drinking water resources. Negative impacts that the location of the Centre could have include: ecological contamination, health risks to humans, security risks,

Eighth International Scientific Conference "June 5th - World Environment Day"

social and economic impacts, issues with long-term monitoring, and ethical problems (burden to future generations without their consent). It can be concluded that the Čerkezovac location is not the most suitable for such a Centre. Therefore, the need for detailed consideration of alternative locations before making a final decision on the construction of the Centre is emphasized.

1. INTRODUCTION

The Krško Nuclear Power Plant (NEK) is located near the town of Krško in Slovenia, close to the border with Croatia. This power plant is a joint project between Slovenia and Croatia, and it started operating in 1983. The plant uses a Pressurized Water Reactor (PWR) with an installed capacity of 695 MW. The nuclear fuel consists of uranium dioxide (UO₂) in the form of sintered pellets (cylinders about 1 cm in size), which are placed in fuel rods. Each fuel assembly consists of 235 such rods. The reactor core is made up of 121 fuel assemblies, containing enriched uranium 235 (up to 5%, with the rest being uranium 238). The weight of a new fuel assembly is between 500 and 600 kg, and its height is about 4 meters.

In every production process, including at NEK, technological waste is generated – industrial and low- and medium-level radioactive waste (LMRW). LMRW consists of waste materials and outdated equipment in the radiologically controlled area, which cannot be decontaminated after use and thus have become radioactive. This includes used protective clothing, processed liquid waste, and materials generated during equipment maintenance in the radiologically controlled area.

In accordance with international standards, all LMRW generated since NEK began operations in 1981 has been stored in a temporary storage facility within the power plant. The total volume of LMRW amounts to 2488 m³ (approximately 20 m x 20 m x 6 m). The volume of waste is reduced through sorting, concentration, melting, and incineration processes. Incineration and melting services are performed abroad. Incineration can reduce the volume of waste up to 20 times. The amount of low- and medium-level radioactive waste produced depends on the stability of the plant's operation and the extent of maintenance. Annually, NEK produces about 30 m³ of low- and medium-level radioactive waste, equivalent to a cube with a side length of 3.1 meters.

There are two main types of facilities intended for the disposal of radioactive waste (RW): storage facilities and disposal sites. Storage facilities are places where RW is temporarily stored and isolated from the environment, waiting to be reused, processed, or permanently disposed of. The purpose of disposal sites is different. These are natural or artificially constructed facilities where radioactive waste is permanently deposited without plans for its future relocation. The main task of disposal sites is to isolate radioactive waste until the radioactive elements in it decay

to levels that are no longer dangerous to humans and the environment. It is assumed that LMRW needs to be isolated in a disposal site for a period of three hundred years.

The aim of this paper is to identify and analyze the dangers posed by the potential construction of a radioactive waste storage and disposal site at the Čerkezovac location, Trgovska gora, near the border with Bosnia and Herzegovina, based on available documentation from Croatia and Bosnia and Herzegovina. It specifically focuses on the risks of ecological contamination, health risks for people, social and economic impacts on the local community, as well as challenges related to long-term monitoring and ethical issues regarding the burden passed on to future generations.

2. THEORETICAL BACKGROUND

Plans for the construction of facilities for managing radioactive waste often face public resistance, which has sparked interest in researching the factors influencing the acceptance of these facilities. Particular attention is paid to the relationship between perceived risks and the acceptance of such facilities, as well as the impact of demographic data, perceived benefits, proximity to the facilities, and public trust (Hietala & Geysmans, 2020).

For the management of radioactive waste (RW) and spent nuclear fuel (SNF), the most important is Directive 2011/70/EURATOM, which establishes a formal framework in the EU for the responsible and safe management of spent nuclear fuel and radioactive waste (Directive, 2011). The basic requirements of Directive 2011/70 are: the establishment and maintenance of a National Policy for the management of RW and SNF, the establishment and maintenance of a national legislative, regulatory, and administrative framework for the management of RW and SNF, which defines responsibilities and coordination between relevant competent authorities.

According to the Intergovernmental Agreement between the Government of the Republic of Croatia and the Government of the Republic of Slovenia on the regulation of status and other legal relations regarding the investment, exploitation, and decommissioning of the Krško Nuclear Power Plant, Croatia is responsible for managing half of the radioactive waste generated by its operation (Narodne novine, 2002). The radioactive waste generated in nuclear power plants consists of used protective clothing, water filters, various tools, gloves, and other spent materials contaminated with radioactive substances. The Republic of Croatia is also responsible for half of the radioactive waste that will be generated during the dismantling and decommissioning of the Krško Nuclear Power Plant facilities after the planned end of operations in 2043. By volume, the majority of radioactive waste from the Krško Nuclear Power Plant is low and intermediate-level radioactive waste (LMRW). The operation of the Krško Nuclear Power Plant also produces spent nuclear fuel. The spent fuel will be stored at the Krško Nuclear Power Plant site until 2080.

Eighth International Scientific Conference "June 5th - World Environment Day"

On October 17, 2014, the Croatian Parliament adopted the Strategy for the Management of Radioactive Waste, Spent Sources, and Spent Nuclear Fuel (Croatian Parliament, 2014). The strategy defines the basic goals and guidelines for managing institutional radioactive waste (IRW) produced in Croatia, spent sources, LMRW, and spent nuclear fuel from the Krško Nuclear Power Plant, as well as for the remediation of sites with naturally occurring radioactive materials. The goals of the strategy include the establishment of long-term storage and disposal facilities for IRW, spent sources, and LMRW from the Krško Nuclear Power Plant. The strategy provides general guidelines on the legislative framework, responsibilities, financing, human resources, and public participation to achieve these goals. Additionally, the strategy offers an official interpretation of key articles of the Intergovernmental Agreement related to LMRW and SNF.

After adopting the Strategy, the Government of the Republic of Croatia adopted the National Program for the implementation of the Strategy (program for the period until 2025 with a perspective until 2060) (Republic of Croatia, 2018). The National Program sets deadlines for two goals listed in the Strategy by 2025: the establishment of a central national storage facility for institutional radioactive waste (IRW) and spent sources, and the establishment of a long-term storage facility for low and intermediate-level radioactive waste (LMRW) from the Krško Nuclear Power Plant. The long-term storage facility for LMRW from the Krško Nuclear Power Plant is planned for a period of 40 years. Since the establishment of the long-term storage facility for LMRW is planned for 2023, the construction of a disposal facility for LILW is not required before 2051. Therefore, activities related to the selection, characterization, and confirmation of the disposal site are not planned for the next 10 years, within the timeframe of this National Program.

The Strategy envisions the establishment of a special Center for Radioactive Waste Management. The preferred location for the Center is Čerkezovac, a military logistics complex that is expected not to be used by the military in the future. Čerkezovac is located in the municipality of Dvor, on the southern slopes of the Trgovska Gora massif. The status of this location is defined as a long-term non-perspective location for the Ministry of Defense and the Armed Forces of the Republic of Croatia.

The National Program cites the location of the Čerkezovac Military Storage Complex, located in the municipality of Dvor on the southern slopes of the Trgovska Gora massif, as the preferred location for establishing the Center for Radioactive Waste Management.

The Croatian Parliament adopted a document titled "Summary of the Third Revision of the Decommissioning Program for the Krško Nuclear Power Plant (NEK) and the Third Revision of the Radioactive Waste (RW) and Spent Nuclear Fuel (SNF) Disposal Program of NEK" (Croatian Parliament, 2020).

Eighth International Scientific Conference "June 5th - World Environment Day"

On March 31, 2020, the Fund for Financing the Decommissioning of NEK took over the location of the former Čerkezovac Military Storage Complex in the municipality of Dvor from the Ministry of Environment and Energy (now the Ministry of Economy and Sustainable Development).

The Strategy for the Management of Radioactive Waste, Spent Sources, and Spent Nuclear Fuel of the Republic of Croatia envisages that the Center for Radioactive Waste Management would include the following facilities: a central storage facility for RW and spent sources, a long-term storage facility for LMRW from the Krško Nuclear Power Plant, and a disposal facility for LMRW generated from the regular operation and decommissioning of the nuclear power plant. The storage facility would accept the Croatian portion of LMRW while the disposal site is being explored, and until the LMRW disposal facility is ready to accept LMRW for disposal.

The third facility of the Center would be the LMRW disposal site. The LMRW disposal site would be located at the Čerkezovac military storage site, if investigations confirm that the location is suitable, or it would be at another site within the preferred location on Trgovska Gora. After establishing the disposal site, the contents of the LMRW storage facility and the RW and spent sources from the central storage facility that meet the necessary criteria would be transferred to it.

The central storage facility for RW and spent sources would begin operations immediately after an agreement on the use of the Čerkezovac military storage site. The storage facility for LMRW from the Krško Nuclear Power Plant should be ready to accept LMRW by 2025. Currently, safety analyses and conceptual designs are being prepared to obtain the location permit, and a request has been submitted to the Ministry to define the content of the environmental impact study. In March, a public process was conducted to determine the content of this study, which included collecting opinions and comments from the public and relevant authorities from Croatia and Bosnia and Herzegovina. Investigative works have been completed at the site, establishing the baseline level of radioactivity, and an ambient dose rate measurement station has been set up. Additionally, seismological stations have been installed at the locations of the center, Rujevac, and Pobrđani, and have been integrated into the Croatian network of seismographs and accelerographs. At that time, investigative works aimed at selecting the best location for the LMRW disposal site should begin. According to current estimates, the LMRW disposal site should start operating in 2058.

Delić and colleagues (Delić, et al., 2016) conducted a study titled "Expert Opinion - Deficiencies of the Document 'Strategic Study for the National Program for the Implementation of the Radioactive Waste, Spent Sources, and Spent Nuclear Fuel Management Strategy (Program for the Period until 2025 with a Perspective until 2060)'. Their research findings indicated that the Trgovska Gora location does not meet ecological and safety standards. One of the biggest risks is

the potential contamination of soil, groundwater, and surface water with radionuclides that may leak from the disposal site. According to the documentation, the Čerkezovac area is located near protected zones such as NATURA 2000, the Una River, and its tributaries, which can have long-term harmful effects on flora and fauna. Groundwater in this region is extremely sensitive to contamination, which could threaten the quality of water used for drinking, irrigation, and other purposes. This area is rich in groundwater used for drinking water supply, and contamination could affect the water intake of the Novi Grad municipality, endangering water sources for the local population (Figure 1).

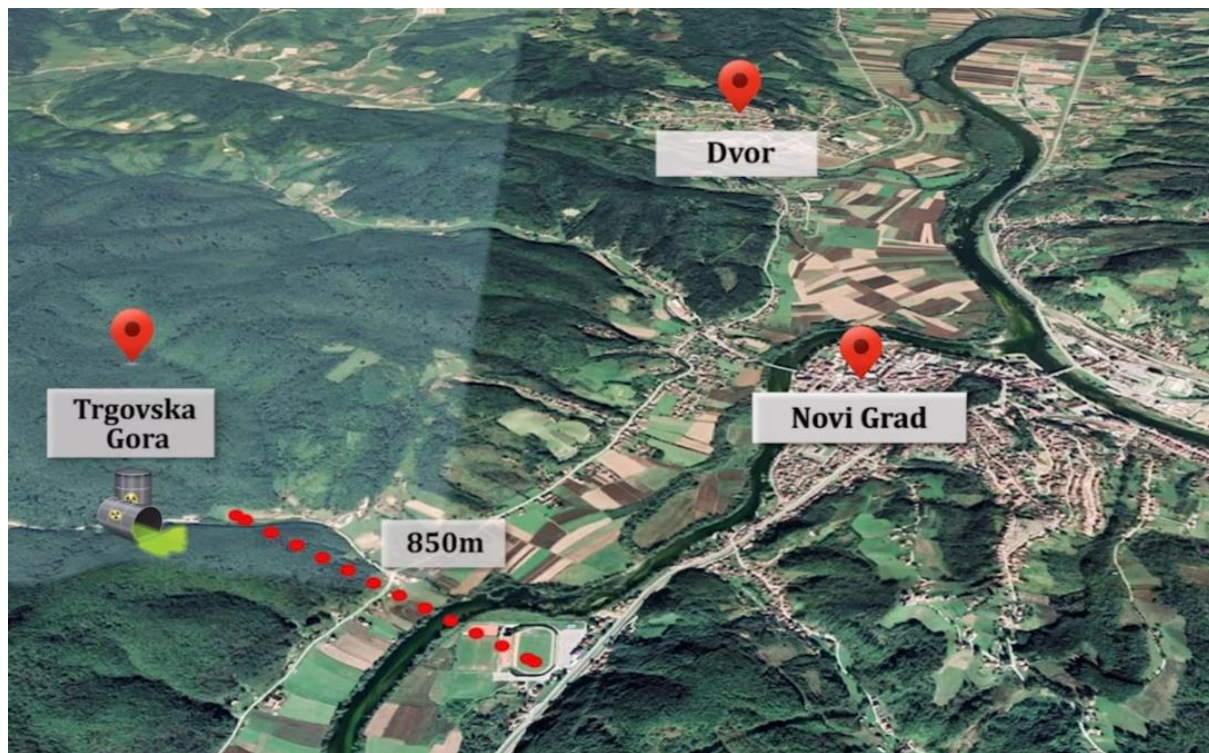


Figure 1: Čerkezovac location area (Source: RFE)

It has been emphasized that the Trgovska Gora area is not free from seismic activity and that potential earthquakes, which can have destructive effects, are seriously underestimated. For example, the 1969 earthquake in Banja Luka, which caused significant damage, was not adequately considered as a risk for the Čerkezovac location. Čerkezovac is located in a seismically active area, and according to the documentation, earthquakes with magnitudes up to VIII degrees on the Mercalli scale can cause significant damage to structures, including radioactive waste disposal facilities, increasing the risk of radioactive material leakage (Delić et al. 2016). Supporting this, the devastating earthquake and a series of hundreds of smaller earthquakes in Banija in 2020 further highlight these concerns. According to Professor Jahić, any earthquake with a magnitude of five on the Richter scale makes a location 70 or 80 kilometers from the epicenter unsuitable for waste disposal.

Eighth International Scientific Conference "June 5th - World Environment Day"

The area is prone to forest fires, and the presence of radioactive waste increases the risk of catastrophic consequences in the event of a fire. The lack of adequate firefighting infrastructure further exacerbates the situation. The authors stress the necessity of having a thorough analysis of potential fires and plans for their extinguishment (Delić et al. 2016).

Geological conditions on Trgovska Gora are unfavorable for the construction of a radioactive waste disposal site. Specifically, heterogeneous lithological and structural-tectonic conditions indicate potential risks, including the presence of faults and fracture porosity, which can affect the stability of RW storage (Delić et al., 2016). The same authors state that the hydrological conditions in the Trgovska Gora area were not adequately analyzed in the Study. The area contains high-quality underground aquifers and surface streams that could be threatened by RW storage. Additionally, the area is susceptible to flooding, which further increases the risks of water and soil contamination..

Trgovska Gora is composed of carbonate-clastic formations that are highly permeable, increasing the risk of groundwater contamination. The geological formations include shales, sandstones, siltstones, conglomerates, limestones, and dolomites, which can lead to soil instability and contaminant infiltration. Considering the hydrogeological characteristics, the complex of Paleozoic clastic deposits in Trgovska Gora as a whole is practically impermeable terrain predominantly without aquifers (EKONERG, 2016).

Competent institutions at all levels of government in Bosnia and Herzegovina unanimously oppose the disposal of radioactive waste near the border, as such a decision would endanger the health of residents in 13 municipalities in the Una River basin in Bosnia and Herzegovina, as well as the environment. The National Assembly of the Republic of Srpska and the Parliamentary Assembly of Bosnia and Herzegovina have adopted Resolutions rejecting the construction of storage and disposal facilities for radioactive waste, spent sources, and spent nuclear fuel, and opposing activities related to the construction of storage and disposal facilities on Trgovska Gora (National Assembly of the Republic of Srpska, 2015; Parliamentary Assembly of Bosnia and Herzegovina, 2016), while the House of Representatives of the Federation of Bosnia and Herzegovina and the Assembly of the Una-Sana Canton have adopted Declarations expressing opposition to the construction of radioactive waste storage facilities in the municipality of Dvor in the Republic of Croatia (Lalić et al., 2023).

Research conducted so far by the Republic of Srpska indicates that Trgovska Gora, which Croatia intends to use for radioactive waste disposal, is not a suitable location. Preliminary findings have revealed the presence of natural radioactivity, which will serve as a significant argument against Croatia's plans to dispose of waste in this area. A team of experts will soon conduct a detailed geological analysis. The construction of a disposal site on Trgovska Gora, in close proximity to the border between Croatia and the Republic of Srpska and the Federation of

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Bosnia and Herzegovina, could endanger more than 250,000 residents from several municipalities. The Public Health Institute confirmed that the area already has a low level of natural radioactivity. Geological investigations have refuted Croatia's claims that the carbonate deposits around Novi Grad and Trgovska Gora are impermeable to groundwater. The area is also prone to landslides, indicating the superficiality of Croatia's research. Preliminary investigations show some seepage, but additional drilling and measurements will be necessary to confirm these findings and use them as evidence that the terrain is not homogeneous but heterogeneous. Preliminary drilling was carried out at the Mlakve site, in the municipality of Novi Grad, which is closest to Trgovska Gora (RTRS, 2024).

The Federal Ministry of Environment and Tourism has presented a report on the activities conducted regarding the issue of radioactive waste disposal at Trgovska Gora, which is currently in the finalization phase of the first stage of research. Representatives from the Federal Hydrometeorological Institute informed those present about the Environmental Impact Study for the Radioactive Waste Management Center at the Trgovska Gora site, including the baseline conditions of hydrology, meteorology, and seismology. Representatives from the Federal Geological Institute provided data on the hydrogeological characteristics of the wider Trgovska Gora area, with particular emphasis on the issue of radioactive waste disposal at the preferred location of Čerkezovac in Dvor, Croatia, and the results of terrain reconnaissance.

Urgent upcoming activities include determining the baseline quality of water and radioactivity in the research area and developing a Hydrogeological Research Program. This program will encompass fieldwork such as monitoring springs, wells, piezometers, and surface watercourses, geophysical surveys, drilling, tracing, and sampling. The primary goal of the research is to assess the current water balance, identify aquifer environments that could be threatened by the construction of the disposal site, as well as identify springs and wells that may be at risk. It will also identify water infiltration zones along the Una River where sinkholes or groundwater recharge from the river occur. The results of these investigations will serve as the basis for establishing future monitoring of surface and groundwater in the area (Government of FBiH, 2024).

Politicians and journalists in Croatia agree that politics played a crucial role in selecting the Trgovska Gora location for radioactive waste disposal. Anka Mrak Taritaš, president of GLAS and a member of the Croatian Parliament, emphasizes that Trgovska Gora was chosen due to the least opposition from the local population and the area's sparse population, highlighting that lobbyists had a significant influence on the decision. Journalist Danijel Prerad adds that other potential locations were eliminated due to the political influence of powerful local politicians and interest groups. Marijana Petir, a long-time opponent of waste disposal at Trgovska Gora, notes that the selected locations were based on outdated information without field research and points out the disinterest of the political elites in thoroughly evaluating the options. According to Petir,

the decision was made based on the politicians' assessment that the least political damage would occur at Trgovska Gora (Portal "Misli o Prirodi").

The claims of experts, officials, and the public are justified, and the potential location of Čerkezovac poses a danger in the event of an accident, as evidenced by incidents at other radioactive waste storage facilities. In the case of the Asse storage facility in Germany, planners chose a poor location within a salt dome, a medium selected for its supposed ability to remain dry, ignoring warnings from local non-governmental organizations about flooding (Kallenbach-Herbert, 2011). Thus, there was a failure in both the design and the institution managing nuclear waste (Figure 2).



Figure 2: Leakage from the Asse waste storage facility (Source: National Geographic)

An example of an accident comes from the Waste Isolation Pilot Plant (WIPP) in the United States. For a long time, WIPP was seen as a model for how to handle radioactive waste until an explosion occurred in the storage facility in 2014. The accident is among the costliest in U.S. history, but the explosion resulted from a simple, seemingly minor decision: using an organic version of "cat litter" to absorb liquids in sealed drums instead of the mineral version (Vartabedian, 2016).

3. DISCUSSION

The discussion focuses on several key aspects related to the construction of a radioactive waste disposal site at the Čerkezovac location. Special attention is given to potential environmental contamination, health risks to humans, social and economic impacts on the local community, as well as challenges related to long-term monitoring and ethical issues concerning the transfer of burdens to future generations.

3.1 Environmental contamination

One of the biggest risks associated with nuclear waste disposal sites is the potential for contamination of soil, groundwater, and surface water by radionuclides that can leak from the disposal site. This can have long-term negative effects on flora and fauna, as well as on human health. Particularly concerning is the proximity of the disposal site to protected areas such as NATURA 2000, further endangering biodiversity and ecosystems in the wider area. Natura 2000 is a network of protected areas within the European Union aimed at preserving the most valuable and endangered species and habitats. The potential contamination of these areas poses a serious risk to the conservation of biological diversity and ecological stability.

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3.2 Health risks

Exposure to radioactive radiation can cause serious health problems, including cancer, genetic damage, and other diseases. The population near the disposal site may be particularly exposed to these risks, with potential long-term consequences for public health. The Čerkezovac area is populated, and the proximity to water catchment zones further increases the risk for the local population. According to the attached document, health risks include not only the immediate effects of radioactive radiation but also long-term consequences that can manifest across generations.

The document emphasizes that the Trgovska Gora area, where Čerkezovac is located, has significant hydrological resources, including drinking water sources that are vital for the local population. Contamination of these sources with radioactive materials could lead to permanent water pollution, which would have catastrophic consequences for human health and the ecosystem. Additionally, the lack of adequate studies on the transboundary health impact on the population of Bosnia and Herzegovina further complicates the situation, given the proximity to the border and the potential cross-border effects of radioactive pollution.

Exposure to radioactive radiation can lead to serious health issues, including cancer, genetic mutations, and other diseases. The population living near the disposal site may be particularly vulnerable to these risks, with possible long-term consequences for public health. Čerkezovac is a populated area, and the proximity to water catchment zones further increases the risk for the

local population. The Trgovska Gora area, where Čerkezovac is located, has significant hydrological resources, including drinking water sources that are crucial for the local population (Delić et al., 2016). Contamination of these sources with radioactive materials could lead to permanent water pollution, which would have catastrophic consequences for human health and the ecosystem. Moreover, the lack of adequate studies on the transboundary health impact on the population of Bosnia and Herzegovina further complicates the situation, given the proximity to the border and the potential cross-border effects of radioactive pollution.

Although LMRW from the Krško Nuclear Power Plant has low specific activity, minimizing the risk of external radiation, people can be exposed to this waste due to environmental contamination during transport. Contamination can occur due to an incident during the loading or unloading of LMRW, the release of material from packaging during transport, or as a result of a transportation accident. The risk during disposal depends on the geological stability of the location and the potential for site contamination due to leakage from packaging.

3.3 Social and economic impacts

The presence of storage and disposal sites for radioactive waste in the region can have profound and lasting negative consequences on the social and economic aspects of local communities. These impacts include, but are not limited to:

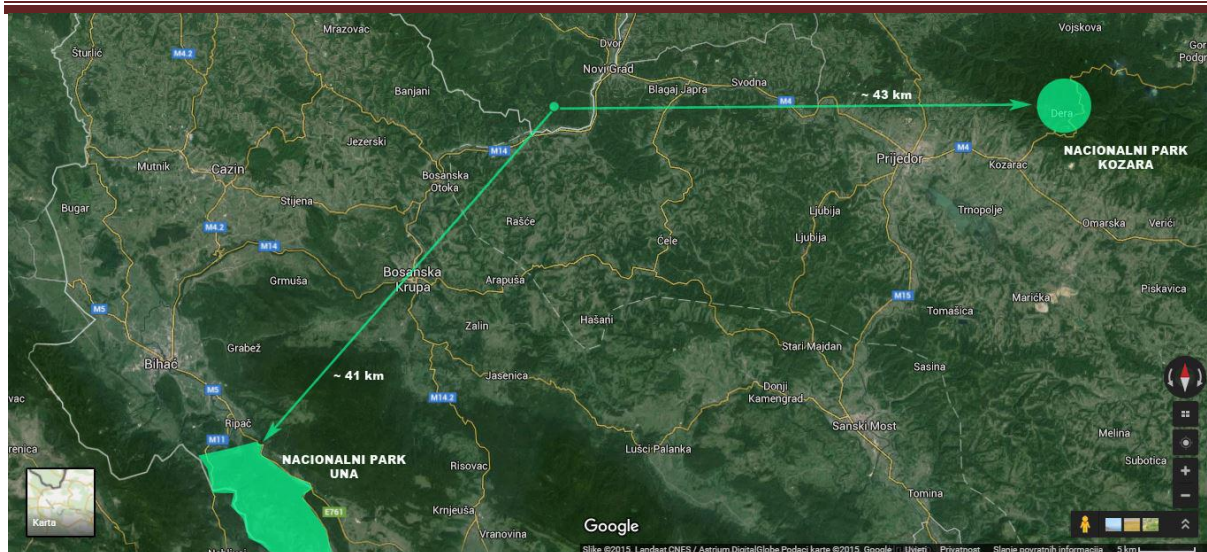
- ***Decrease in Property Values***

One of the first and most direct economic effects of the presence of a disposal site is the decrease in property values in the surrounding area. Buyers are less inclined to invest in areas associated with environmental risks, resulting in a decline in land and house prices. This decrease in value can have serious financial consequences for property owners who face difficulties in selling or retaining their assets.

- ***Impact on the Region's Tourism Potential***

Regions with radioactive waste disposal sites become less attractive to tourists. The region where Čerkezovac is located is known for its natural beauty, including the Una National Park, which attracts a large number of visitors each year. The Una River, with its crystal-clear waters, waterfalls, and canyons, is one of the most important tourist resources of Bosnia and Herzegovina. Tourism activities such as rafting, fishing, hiking, and ecotourism significantly contribute to the local economy. The presence of a radioactive waste disposal site can seriously threaten these tourism potentials, reducing the number of visitors and the revenue generated through tourism. Tourists seeking safe and environmentally clean places for vacation will also avoid such regions, which can reduce tourism income. In addition to the economic consequences, the degradation of the natural environment can have irreversible effects on the biodiversity and ecological balance of the region.

Eighth International Scientific Conference "June 5th - World Environment Day"



• *Loss of Jobs*

In the broader area around Trgovska Gora, downstream along the Una River towards Kostajnica, key resources for employment and economic development lie in the production of healthy food and the development of tourism. Industrial development has been lacking partly due to efforts to preserve natural values in the past (Delić, et al., 2016). Agriculture, fishing, and tourism are key economic activities in many rural areas. The presence of radioactive waste can negatively impact these sectors, leading to job losses and reduced income for the local population. This reduction in economic activity can further exacerbate the economic situation in the region.

• *Decreased Attractiveness for Investors*

Investors often avoid areas associated with environmental risks. This can result in a reduced influx of capital and hindered economic growth in the region. Existing businesses may consider relocating their operations due to perceived increased risks, further diminishing economic opportunities for the local population.

• *Increased Healthcare Costs*

Health problems caused by exposure to radioactive materials can lead to increased healthcare costs for local communities. This burden can further reduce available funds for other vital services and infrastructure projects, which can further affect the quality of life in the region.

• *Population Migration*

Fear of health risks and a decrease in quality of life can drive population migration from affected areas. This can lead to demographic changes and further economic decline as the working-age population moves in search of safer and more prosperous environments. This further exacerbates economic and social conditions in the region.

• ***Reduced Social Cohesion***

The presence of a disposal site often leads to polarized opinions within communities, which can weaken social cohesion. Conflicts between those who support the economic benefits and those who oppose due to health and environmental risks can cause long-term social tensions. These tensions can weaken the community and hinder collective efforts to improve living conditions.

• ***Negative Image of the Region***

The reputation of the region can be permanently damaged, making it more difficult to attract tourists and new residents. This stigmatization can have long-term economic consequences, reducing the potential for local economic development. A damaged image can permanently discourage economic initiatives and projects that could improve the local community.

3.4 Problems with long-term monitoring

Nuclear waste remains hazardous for many years, requiring long-term plans for safe management, monitoring, and maintenance of disposal sites. There is a risk that future generations may not be able or willing to continue adequate monitoring of the disposal sites.

According to Delić et al. (2016), one of the key issues is the inadequate assessment of long-term seismic risks, which can lead to damage to disposal structures and potential leakage of radioactive material. Additionally, the geological and hydrological conditions of the Trgovska Gora area have not been sufficiently studied, further complicating long-term safety planning. The area is prone to flooding, and there is a danger of groundwater contamination, which could have catastrophic consequences for the local population and the environment.

Long-term monitoring also requires continuous availability of technical and financial support. The document states that current plans do not include adequate financial mechanisms to ensure long-term monitoring and maintenance. This shortcoming could lead to a situation where future generations lack the necessary resources to properly manage the disposal site.

Furthermore, there is a risk of loss of institutional memory and expertise. In the event of political or social changes, continuity in the monitoring and maintenance of the disposal site may be lost. Without ongoing education and training of new generations of experts, the ability to adequately manage nuclear waste could be seriously compromised..

3.5 Ethical problems

The basic ethical principle that each generation must manage its own nuclear waste has become inoperative now that industrialized countries have generated waste for more than half a century and stored it in an increasing number of facilities for future generations (Andren, 2012). Ethical issues related to the disposal of nuclear waste include the burden placed on future generations without their consent. These generations will have to manage the potential risks and costs of

Eighth International Scientific Conference "June 5th - World Environment Day"

disposal sites they did not create. Nuclear waste remains hazardous for thousands of years, and long-term monitoring requires continuous technical and financial resources. Future generations will face the challenges of maintaining disposal site infrastructure, which includes regular inspections, repairs, and possibly updating safety systems. Any mistake or negligence could result in catastrophic consequences for the environment and human health.

Moreover, there are cultural and communication challenges. Methods need to be developed to convey information about the location and dangers of disposal sites across many generations. This involves using symbols and educational programs to ensure that each new generation understands the risks and important precautions. There is a real danger that knowledge about the location and nature of the waste could be lost over time, leading to unintentional disruption and exposure to radioactive materials.

The economic burden is also significant. Future generations will need to secure financial resources for the long-term management of the disposal site, including not only operational costs but also the costs of any remediation and repairs in the event of accidents or failures. The financial burden may limit resources available for other critical needs, such as education, healthcare, and infrastructure.

There is also a risk of a lack of political will and international cooperation in the future. Political changes, economic crises, or natural disasters can affect the ability of future generations to adequately manage the disposal site. Without strong international agreements and ongoing cooperation, there could be serious lapses in safety measures and oversight.

The ethical dilemma is further exacerbated by the fact that local communities often have no say in the decisions about the construction of disposal sites, and the burden and risks are shifted onto them and their descendants. This lack of fairness and participation in decision-making can lead to long-term social and environmental injustices, highlighting the need for a responsible and inclusive approach to nuclear waste management.

4. CONCLUSIONS

The establishment of the Radioactive Waste Storage Center at Čerkezovac site entails significant environmental, health, social, and ethical challenges. Potential contamination of soil, groundwater, and surface water, along with long-term health risks for the local population, underscores the need for thorough environmental impact assessments and robust safety measures. Additionally, the social and economic impacts, including the decrease in property values, reduction of tourism potential, and loss of jobs, highlight the broader consequences for the local community. Ethical considerations, such as the burden on future generations and the lack of involvement of the local community in decision-making, further complicate the situation.

Eighth International Scientific Conference "June 5th - World Environment Day"

Effective long-term monitoring and management of radioactive waste disposal sites require continuous technical and financial support, which current plans do not adequately cover. Ensuring the safety and well-being of future generations demands comprehensive strategies, robust international cooperation, and inclusive decision-making processes. The ethical, social, and environmental implications of managing radioactive waste in Čerkezovac call for a responsible and holistic approach to mitigate risks and ensure sustainable development.

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EFFECT OF FOSFOMYCIN ON BIOFILM FORMATION IN GRAM-NEGATIVE BACTERIA USING MICROBROTH DILUTION AND TISSUE CULTURE PLATE METHODS

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Key words: Biofilms, Antibiotics, Fosfomycin, *Escherichia coli*, ESBL

ABSTRACT:

The broad-spectrum antibiotic Fosfomycin has gained significant interest for its impressive efficacy in inhibiting the formation of biofilms by Gram-negative bacteria. This increased interest comes as a response to the urgent need for new therapeutic strategies against antibiotic-resistant strains, especially those which display extended-spectrum beta-lactamase (ESBL) resistance. This paper investigates the mechanisms by which Fosfomycin disrupts biofilm formation in Gram-negative bacteria, focusing on Escherichia coli and ESBL strains. We investigate in depth the intricate relationship between Fosfomycin and biofilm formation and how it prevents bacterial adherence in the early phases of biofilm formation. Fosfomycin's ability to interfere with adhesion molecules expressed on the bacterial surface acts as a critical barrier, preventing the establishment of robust biofilm structures in E. coli and ESBL-producing strains. Furthermore, our findings emphasize the ability of Fosfomycin to interfere with the synthesis of an extracellular matrix—the component that confers structural integrity to biofilms. Fosfomycin efficiently reduces the cohesiveness of biofilm matrices by targeting key enzymes involved in matrix formation, making the two types of bacteria highly susceptible to elimination. The complex mechanisms underlying Fosfomycin's influence on the biofilm formation of strains of E. coli and ESBL bacteria reveal the potential of the drug as an adjuvant treatment for bacterial infections that exhibit biofilm formation. Our discovery opens the door for the creation of new antibacterial strategies targeted at the successful treatment of resistant biofilm-associated diseases in a variety of bacterial populations, by unraveling these mechanisms.

1. INTRODUCTION

Biofilm represents a community of microorganisms that are irreversibly connected to the surface, producing extracellular polymeric substances (EPS), and at the same time show changed properties (phenotype) compared to the corresponding planktonic cells (Donlan, 2002). EPS is a highly dehydrated and chemically complex matrix that serves to store nutrients, and at the same time it can "trap" other microbes and non-cellular material such as minerals, crystals, and corrosion products. Within the biofilm, the cells function in a coordinated manner like a cooperative consortium, in a way mimicking a multicellular organism. At the same time, the process of development of this complex and highly differentiated association from individual cells requires complex genetic regulation (Flemming & Wingender, 2010). Resistance to antimicrobial agents is a highly complex phenomenon that may largely be caused by biofilms. In comparison to similar bacteria that are planktonic, bacteria living in biofilms can show a 10 to 1,000-fold increase in antibiotic resistance (Hoyle & Costerton, 1991). Bacteria's nutritional state, growth rate, temperature, pH, and previous exposure to low concentrations of antimicrobial drugs are thought to have an impact on their antibiotic resistance. The slower rate of bacterial species' growth in a biofilm, which reduces their susceptibility to bactericidal drugs, appears to be another significant reason. Antibiotic diffusion can be resisted by a biofilm matrix. Extremely charged or chemically strongly reactive substances, for instance, may not be able to penetrate the biofilm's deeper regions because the biofilm works as an ion exchanger, drawing such molecules out of solution (Socransky & Haffajee, 2002).

Fosfomycin is a broad-spectrum antibiotic originally discovered in the 1960s by Spanish researchers (Roussos et al., 2009). The outstanding feature of its chemical structure is that it has a phosphonic acid derivative, which forms another difference from various classes of antibiotics (Kahan et al., 1974). Fosfomycin acts by inhibiting an essential enzyme for peptidoglycan synthesis, enolpyruvyl transferase (MurA) (Kahan et al., 1974). This enzyme is essential in catalyzing the initial step of peptidoglycan synthesis, and thus, Fosfomycin inhibits the formation of the bacterial cell wall, leading to bacterial cell death (Kahan et al., 1974). One of the advantages of Fosfomycin is its action against a broad range of both Gram-negative and Gram-positive bacteria, including multi-drug-resistant strains (Falagas et al., 2010). This broad-spectrum activity makes it very useful for the treatment of various infections (Falagas et al., 2010). Fosfomycin is especially noted for its activity against *Enterococcus*, *Escherichia coli*, and *Staphylococcus* species, common pathogens responsible for UTIs (Falagas et al., 2010).

In current clinical practice, Fosfomycin is often used to treat uncomplicated urinary tract infections, mainly acute cystitis in women (Gupta et al., 2018). It is given as a single dose in oral formulation, enhancing patient compliance and ease of administration (Gupta et al., 2018). Furthermore, the safety profile of Fosfomycin is advantageous because it has a low incidence of

side effects, making it appropriate for a broad range of patients, including pregnant patients (Gupta et al., 2018).

In recent years, Fosfomycin has gained interest due to the growing problem of antibiotic resistance (Falagas et al., 2010). Traditional antibiotics are becoming less effective against resistant strains, and Fosfomycin's unique mechanism of action provides an alternative for some of these resistance mechanisms (Falagas et al., 2010). Studies have shown that Fosfomycin can be a useful addition to combination therapy by increasing the efficacy of other antibiotics and reducing the chance of resistance emergence (Falagas et al., 2010).

2. MATERIALS AND METHODS

2.1 Sample Preparation

Purified sterile water was used to dissolve Fosfomycin, and 190 mg/ml was the initial concentration used in the experiment.

2.2 Bacterial Strains

Escherichia coli ATCC 14169, *Escherichia coli* ATCC 25922 and *Extended-spectrum beta-lactamase (ESBL)* were plated on Mueller Hinton Agar for 24 hours at 37°C. For Minimum Inhibitory Concentration and Biofilm Forming Capacity testing, 0.5 Mc Farland inoculum (1.5×10^8 cfu/ml) was prepared in Tryptic Soy Broth (TSB).

2.3 Minimum Inhibitory Concentration (MIC)

By calculating the minimum inhibitory concentration, the lowest concentration of antimicrobial medicine that suppresses the observable growth of the target bacterium under certain, predefined conditions can be identified (MIC). The MIC test is used to assess the activity of antimicrobial drugs, such as antibiotics, as well as the sensitivity of a bacteria to medications. A standardized number of cells are added to 96-well plates, and the antimicrobial agent is serially diluted with varying dosages. After a 24-hour incubation period, the growth is assessed using an Elisa plate reader. If bacterial growth does not take place at the injected concentration, the MIC value is revealed.

2.4 Biofilm Forming Capacity

Using the tissue culture plate method, it will be determined whether the tested bacterial strains can create biofilms when exposed to varied concentrations of the tested antibiotic. The addition of subinhibitory antibiotic concentrations to TSB in 96-well plates should be followed by successive dilutions. The required bacterial suspension density should be 1.5×10^8 cfu/ml. After 24 hours of incubation at 37°C, the plates will be stained with 0.1% crystal violet and filled with 96% ethanol before being read with an ELISA plate reader. The results of the biofilm formation are classified into four categories: non adherent (NA), weakly adherent (W), moderately adherent

Eighth International Scientific Conference
"June 5th - World Environment Day"

(M), and strongly adherent (S). To classify each result, the optical density cut-off value (ODc) is calculated as three standard deviations of the negative control above the average OD of the negative control. The biofilm categories are determined as the following: $OD \leq ODc$: non adherent, $ODc < OD \leq 2 \times ODc$: weakly adherent, $2 \times ODc < OD \leq 4 \times ODc$: moderately adherent, and $4 \times ODc < OD$: strongly adherent.

3. RESULTS

3.1 Minimum Inhibitory Concentration (MIC) of Fosfomycin

Table 1: Represents minimum inhibitory concentrations (MIC) of 3 Gram-negative ATCC bacterial strains treated with 190mg/ml of Fosfomycin.

Bacterial strain	MIC (mg/ml) (TSB)
<i>Escherichia coli</i> ATCC 14169	0.0463
<i>Escherichia coli</i> ATCC 25922	0.0058
ESBL	0.0463

Table 2: Obtained TCP values after application of subinhibitory concentrations of Fosfomycin (mg/ml) on *Escherichia Coli* ATCC 14169 in TSB-first plate.

	NC	PC	190	95	47.5	23.75	1.19 E+01	5.94 E+00	2.97 E+00	1.48 E+00	7.42 E-01	3.71 E-01
<i>Escherichia Coli ATCC 14169</i>	0.061	0.883	0.052	0.049	0.048	0.052	0.052	0.051	0.049	0.05	0.05	0.055
	0.050	0.867	0.054	0.048	0.047	0.049	0.048	0.049	0.049	0.049	0.052	0.049
	0.050	0.848	0.056	0.049	0.048	0.049	0.048	0.049	0.049	0.05	0.05	0.051
	0.051	0.801	0.057	0.049	0.048	0.048	0.048	0.055	0.049	0.051	0.05	0.05
	0.051	0.793	0.056	0.049	0.049	0.049	0.049	0.05	0.049	0.049	0.049	0.05
	0.050	0.815	0.052	0.049	0.049	0.049	0.048	0.049	0.049	0.049	0.05	0.049
	0.050	0.815	0.052	0.049	0.049	0.049	0.048	0.049	0.049	0.049	0.05	0.049
Average	0.052	0.835	0.055	0.049	0.048	0.049	0.049	0.051	0.049	0.050	0.050	0.051
Stdev	0.004	0.036	0.0022	0.000	0.000	0.0014	0.0016	0.0023	0.0000	0.0008	0.0010	0.0023

NC- negative control; PC- positive control

Eighth International Scientific Conference "June 5th - World Environment Day"

Table 3: Obtained TCP values after application of subinhibitory concentrations of Fosfomycin (mg/ml) on *Escherichia Coli ATCC 14169* in TSB-second plate.

	1.86E-01	9.28E-02	4.64E-02	2.32E-02	1.16E-02	5.80E-03	2.90E-03	1.45E-03	7.25E-04	3.62E-04	1.81E-04	9.06E-05
<i>Escherichia Coli ATCC 14169</i>	0.058	0.055	0.052	0.288	0.207	0.664	0.621	0.858	0.913	0.939	0.838	0.893
	0.05	0.054	0.07	0.226	0.203	0.464	0.59	0.739	0.711	0.691	0.728	0.848
	0.05	0.051	0.05	0.319	0.322	0.565	0.691	0.721	0.643	0.584	0.56	0.816
	0.049	0.052	0.054	0.328	0.226	0.627	0.715	0.778	0.786	0.757	0.679	0.918
	0.051	0.051	0.065	0.349	0.345	0.625	0.735	0.806	0.83	0.709	0.751	0.965
	0.05	0.051	0.075	0.27	0.396	0.612	0.727	0.831	0.798	0.764	0.768	0.988
Average	0.051	0.052	0.061	0.297	0.283	0.593	0.680	0.789	0.780	0.741	0.721	0.905
Stdev	0.0033	0.0018	0.0104	0.0447	0.0819	0.0707	0.0603	0.0530	0.0939	0.1168	0.0944	0.0663

NC- negative control; PC- positive control

Table 4: Obtained TCP values after application of subinhibitory concentrations of Fosfomycin (mg/ml) on *Escherichia Coli ATCC 25922* in TSB-first plate.

	NC	PC	190	95	47.5	23.75	1.19E+01	5.94E+00	2.97E+00	1.48E+00	7.42E-01	3.71E-01
<i>Escherichia Coli ATCC 25922</i>	0.06	0.850	0.057	0.051	0.053	0.049	0.049	0.053	0.049	0.049	0.049	0.055
	0.051	0.816	0.056	0.058	0.049	0.048	0.049	0.049	0.049	0.049	0.05	0.049
	0.051	0.810	0.054	0.051	0.048	0.049	0.048	0.049	0.048	0.049	0.05	0.05
	0.052	0.798	0.055	0.049	0.048	0.049	0.05	0.049	0.049	0.05	0.049	0.049
	0.05	0.840	0.049	0.051	0.049	0.05	0.057	0.049	0.052	0.05	0.049	0.051
	0.051	0.848	0.052	0.05	0.049	0.049	0.048	0.049	0.05	0.05	0.05	0.05
Average	0.053	0.827	0.054	0.052	0.049	0.049	0.050	0.050	0.050	0.050	0.050	0.051
Stdev	0.0037	0.0219	0.0029	0.0032	0.0019	0.0006	0.0034	0.0016	0.0014	0.0005	0.0005	0.0023

NC- negative control; PC- positive control

Table 5: Obtained TCP values after application of subinhibitory concentrations of Fosfomycin (mg/ml) on *Escherichia Coli ATCC 25922* in TSB-second plate.

	1.86E-01	9.28E-02	4.64E-02	2.32E-02	1.16E-02	5.80E-03	2.90E-03	1.45E-03	7.25E-04	3.62E-04	1.81E-04	9.06E-05
<i>Escherichia Coli ATCC 25922</i>	0.056	0.05	0.049	0.051	0.058	0.113	0.302	0.524	0.679	0.803	0.801	0.899
	0.048	0.049	0.049	0.064	0.117	0.158	0.353	0.661	0.729	0.818	0.768	0.767
	0.049	0.049	0.048	0.049	0.068	0.067	0.28	0.524	0.693	0.779	0.799	0.794
	0.049	0.049	0.048	0.07	0.075	0.082	0.293	0.517	0.703	0.798	0.814	0.827

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	0.049	0.05	0.049	0.076	0.059	0.069	0.258	0.495	0.756	0.813	0.837	0.863
	0.049	0.049	0.048	0.049	0.066	0.082	0.293	0.514	0.765	0.775	0.811	0.84
Average	0.050	0.049	0.049	0.060	0.074	0.095	0.297	0.539	0.721	0.798	0.805	0.832
Stdev	0.0030	0.0005	0.0005	0.0118	0.0220	0.0349	0.0316	0.0606	0.0349	0.0175	0.0226	0.0473

NC- negative control; PC- positive control

Table 5: Obtained TCP values after application of subinhibitory concentrations of Fosfomycin (mg/ml) on *ESBL* in TSB-first plate.

NC- negative control; PC- positive control

	NC	PC	190	95	47.5	23.75	1.19 E+01	5.94 E+00	2.97 E+00	1.48 E+00	7.42 E-01	3.71 E-01
<i>ESBL</i>	0.057	0.872	0.052	0.05	0.05	0.05	0.048	0.049	0.05	0.053	0.049	0.055
	0.05	0.768	0.058	0.049	0.048	0.048	0.048	0.048	0.049	0.049	0.049	0.048
	0.052	0.739	0.055	0.05	0.049	0.048	0.049	0.047	0.049	0.049	0.051	0.049
	0.049	0.755	0.054	0.049	0.049	0.048	0.049	0.049	0.048	0.049	0.049	0.052
	0.05	0.758	0.055	0.046	0.048	0.048	0.049	0.048	0.049	0.049	0.05	0.049
	0.05	0.865	0.057	0.049	0.049	0.049	0.05	0.048	0.05	0.049	0.052	0.049
Average	0.051	0.793	0.055	0.049	0.049	0.049	0.049	0.048	0.049	0.050	0.050	0.050
Stdev	0.0029	0.0594	0.0021	0.0015	0.0008	0.0008	0.0008	0.0008	0.0008	0.0016	0.0013	0.0027

Table 6: Obtained TCP values after application of subinhibitory concentrations of Fosfomycin (mg/ml) on *ESBL* in TSB-second plate.

	1.86E-01	9.28E-02	4.64 E-02	2.32E-02	1.16E-02	5.80E-03	2.90E-03	1.45E-03	7.25E-04	3.62E-04	1.81E-04	9.06E-05
<i>ESBL</i>	0.052	0.055	0.067	0.199	0.328	1.009	0.976	1.222	1.104	1.243	1.176	1.146
	0.049	0.052	0.05	0.327	0.312	0.557	0.585	0.819	0.844	0.825	0.757	0.778
	0.049	0.051	0.05	0.283	0.313	0.529	0.632	0.718	0.75	0.737	0.731	0.75
	0.047	0.052	0.051	0.183	0.397	0.572	0.657	0.7	0.746	0.77	0.773	0.885
	0.049	0.051	0.132	0.163	0.401	0.65	0.698	0.795	0.817	0.87	0.895	1.154
	0.049	0.051	0.052	0.232	0.406	0.832	0.896	0.936	0.96	1.046	1.115	1.263
Average	0.049	0.052	0.067	0.231	0.360	0.692	0.741	0.865	0.870	0.915	0.908	0.996
Stdev	0.0016	0.0015	0.0325	0.0630	0.0463	0.1903	0.1577	0.1941	0.1386	0.1937	0.1935	0.2187

NC- negative control; PC- positive control

3.2 Biofilm Forming Capacity

3.2.1 Biofilm Forming Capacity for *Escherichia coli* ATCC 25922

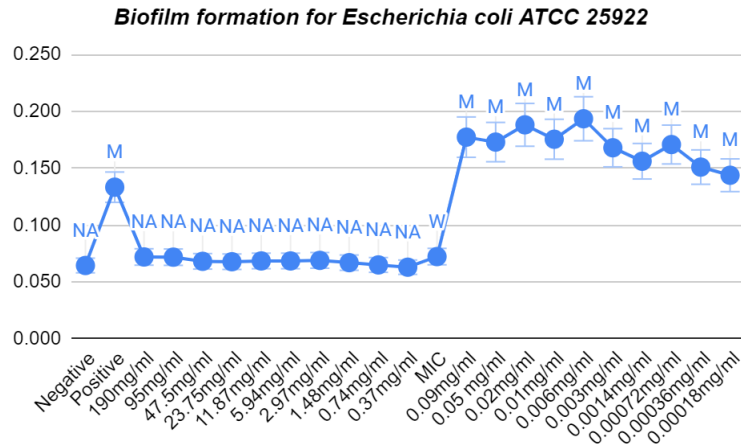


Figure 1: Effect of Fosfomycin on the biofilm formation of *Escherichia coli* ATCC 25922
NA – non-adherent, W – weakly adherent, M – moderately adherent, and S – strongly adherent.

Figure 1. shows the OD values for the effect of Fosfomycine on the biofilm-forming capacity of *Escherichia coli* ATCC 25922. The ODc value was 0.065. If the OD is smaller than 0.065 the biofilm is considered non-adherent; if the OD is between 0.067 and 0.130 it is considered weakly adherent; if the OD is between 0.130 and 0.260 it is considered moderately adherent; if the OD is larger than 0.260 it is considered strongly adherent.

3.2.2 Biofilm Forming Capacity for *Escherichia coli* ATCC 14169

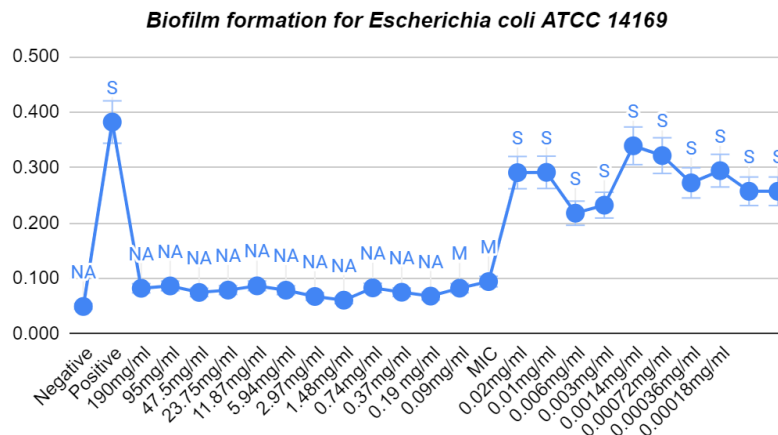


Figure 2: Effect of Fosfomycin on the biofilm formation of *Escherichia coli* ATCC 14169
NA – non-adherent, W – weakly adherent, M – moderately adherent, and S – strongly adherent.

Figure 2. shows the OD values for the effect of Fosfomycine on the biofilm-forming capacity of *Escherichia coli* ATCC 14169. The OD_c value was 0.065. If the OD is smaller than 0.050 the biofilm is considered non-adherent; if the OD is between 0.050 and 0.100 it is considered weakly adherent; if the OD is between 0.100 and 0.200 it is considered moderately adherent; if the OD is larger than 0.200 it is considered strongly adherent.

3.2.3 Biofilm Forming Capacity for *ESBL*

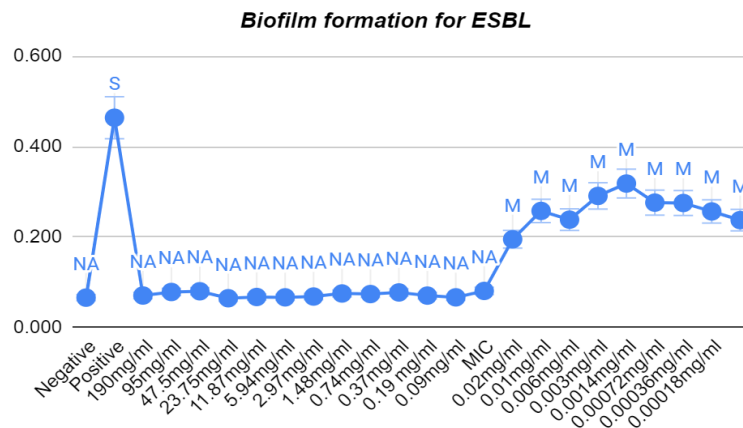


Figure 3: Effect of Fosfomycin on the biofilm formation of *ESBL*
NA – non-adherent, W – weakly adherent, M – moderately adherent, and S – strongly adherent.

Figure 2. shows the OD values for the effect of Fosfomycin on the biofilm-forming capacity of *ESBL*. The OD_c value was 0.066. If the OD is smaller than 0.066 the biofilm is considered non-adherent; if the OD is between 0.066 and 0.132 it is considered weakly adherent; if the OD is between 0.132 and 0.264 it is considered moderately adherent; if the OD is larger than 0.264 it is considered strongly adherent.

4. DISCUSSION

The Minimum Inhibitory Concentration of Fosfomycin in TSB was obtained for different bacterial strains. For *Escherichia coli* ATCC 14169, the MIC obtained was 0.0463 mg/ml. *Escherichia coli* ATCC 25922 had a very low MIC of 0.0058 mg/ml. The *ESBL*-producing strains had an MIC of 0.0463 mg/ml. This demonstrates that Fosfomycin exhibits effectiveness at low concentrations against these strains. However, the level of resistance varies slightly among different strains of *E. Coli*.

The non-treated *Escherichia coli* ATCC 25922 showed moderate biofilm-forming capacity (Figure 1). Treating the bacterium with different doses of Fosfomycin weakened the strain's ability for biofilm formation. The Fosfomycin concentrations starting with 0.190 mg/ml to 0.0927 mg/ml weakened the biofilm-forming capacity of *Escherichia coli* ATCC 25922 to non-

adherent. By treating it with sub-inhibitory concentrations, the biofilm-forming capacity rose to moderately adherent. The non-treated *Escherichia coli* ATCC 14169 showed strong biofilm-forming capacity (Figure 2). Treating the bacterium with different doses of Fosfomycin weakened the strain's ability for biofilm formation. The Fosfomycin concentrations starting with 0.190 mg/ml to 0.0231 mg/ml weakened the biofilm-forming capacity of *Escherichia coli* ATCC 14169 to non-adherent. By treating it with sub-inhibitory concentrations, the biofilm-forming capacity rose to be strongly adherent. The non-treated *ESBL* showed strong biofilm-forming capacity (Figure 3). Treating the bacterium with different doses of Fosfomycin weakened the strain's ability for biofilm formation. The Fosfomycin concentrations starting with 0.190 mg/ml to 0.0231 mg/ml weakened the biofilm-forming capacity of *ESBL* to non-adherent. By treating it with sub-inhibitory concentrations, the biofilm-forming capacity rose to moderately adherent.

5. CONCLUSION

The MIC results indicate that Fosfomycin is effective against *Escherichia coli* strains, including those implicated in biofilm formation. Regarding the challenge of biofilm-related infections, knowledge of antimicrobial susceptibility in such a context is, therefore, very important. The variations in the MIC values across the different *Escherichia coli* strains reflect a complex interaction between the mechanisms of antibiotic resistance and biofilm formation. The ability of Fosfomycin to inhibit gram-negative bacterial growth even in the presence of biofilms suggests its potential utility.

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Eighth International Scientific Conference
"June 5th - World Environment Day"

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EFFECT OF FOSFOMYCIN ON THE RESISTANCE PATTERNS OF GRAM-POSITIVE BACTERIA TO DIFFERENT CLASSES OF ANTIBIOTICS USING THE BAUER-KIRBY DISK DIFFUSION METHOD

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Key words: fosfomycin, gram-positive bacteria, antibiotic

ABSTRACT:

Modern antimicrobial research must comprehend how fosfomycin influences antibiotic resistance in Gram-positive bacteria. Because of its distinct mode of action, fosfomycin is becoming more and more useful in the treatment of illnesses brought on by Gram-positive bacteria that are resistant to drugs. This work provides crucial information for developing efficient antimicrobial treatments by examining how fosfomycin modifies the resistance patterns of Gram-positive bacteria to different antibiotic classes.

Various strains of Gram-positive bacteria, such as Methicillin-resistant Staphylococcus aureus ATCC 43300, Staphylococcus aureus ATCC 6538, and Staphylococcus aureus NCTC 12393, were cultivated and subjected to fosfomycin. After that, the Bauer-Kirby disk diffusion method was employed to assess the susceptibility of the bacteria to several antibiotic classes. We measured and compared the changes in inhibition zone diameters surrounding the antibiotic discs to control groups that did not get fosfomycin treatment.

Results showed that, in most cases, inhibition zones in fosfomycin-treated samples were smaller than those in the positive control (without fosfomycin). This suggests that fosfomycin reduces susceptibility of Gram-positive bacteria to tested antibiotics, potentially decreasing the effectiveness of these antibiotics in the presence of fosfomycin. However, exceptions were noted for Erythromycin (E15ug), Azithromycin (AZM15ug), and Streptomycin (S300ug), where S. aureus NCTC 12393 initially showed resistance but exhibited a small inhibition zone after

fosfomycin treatment. Results also revealed that the zone of inhibition for the same strain of bacteria (S. aureus NCTC 12393) with respect to the antibiotic ampicillin (AMP 2ug) increased after treatment with fosfomycin, suggesting a unique response to fosfomycin treatment for this antibiotic. This implies a possible synergistic effect between fosfomycin and these antibiotics, suggesting that fosfomycin may enhance bacterial susceptibility to them.

These findings highlight the complex interplay between fosfomycin and antibiotics in Gram-positive bacteria, emphasizing the need for further research to optimize antimicrobial treatment strategies.

1. INTRODUCTION

The development and spreading of diverse mechanisms behind antibiotic resistance in Gram-positive cocci has emerged as a significant public health concern (Rice, 2006). *Methicillin-resistant Staphylococcus aureus (MRSA)* in particular was first identified as a significant nosocomial infection. In the last ten years, community-acquired *MRSA* has received a lot of attention. It has distinct pathogenicity features, can exhibit an epidemic pattern, and usually affects young people (Grundmann et al., 2006).

Over the past several years, a number of novel antimicrobial drugs that target Gram-positive bacteria with advanced resistance to antibiotics have been brought into clinical practice (Ratnaraja and Hawkey, 2008), and other treatments are currently in the final phases of clinical development. However, the majority of the novel substances have particular medicinal uses, such as glycyclines, lipopeptides, streptogramins, and oxazolidinones.

Reassessing older antibiotics could be helpful in this sense as it increases our therapeutic options against modern infections that have sophisticated drug resistance patterns. Having been around for around 40 years, fosfomycin has demonstrated good antibacterial action against a wide range of infections, including Gram-positive bacteria. Its distinct mode of action involves blocking a preliminary stage of the formation of the cell wall. Although fosfomycin has only been used to treat simple UTIs in the majority of nations, it has also been used to treat systemic infections in some (Falagas et al., 2008). In this instance, our goal was to assess the efficacy of fosfomycin in treating infections brought on by Gram-positive bacteria that have developed sophisticated drug resistance to antibiotics.

An early broad-spectrum bactericidal antibiotic, fosfomycin prevents the bacterial cell from synthesizing. Among other reasons for its organ-penetration capacity is its tiny molecular size. The smallest molecular mass of any known antibacterial medication is found in fosfomycin. It works by preventing UDP-N-Acetylglucosamine Enolpyruvyl Transferase (MurA) from doing its job, which is what creates N-acetylmuramic acid, a precursor to peptidoglycan. The transporters of glucose-6-phosphate (G6P) and glycerol-3-phosphate (G3P) are necessary for fosfomycin to function. There have been no reports of cross-resistance with other antibiotics.

Fosfomycin exhibits a wide range of effects. According to Díez-Aguilar and Cantón (2019), it works well against *Staphylococcus aureus*, particularly *methicillin-resistant S. aureus (MRSA)*.

This study aims to investigate the effect of fosfomycin on the resistance patterns of Gram-positive bacteria to different classes of antibiotics using the Bauer-Kirby disk diffusion method. Specifically, we will examine the susceptibility of Gram-positive bacterial strains of *S. aureus* including *MRSA*, to a panel of antibiotics representing different classes, both before and after treatment with fosfomycin. By evaluating the changes in the bacteria's resistance patterns following fosfomycin treatment, we aim to gain insights into the potential use of fosfomycin as an adjunct therapy for combating multidrug-resistant Gram-positive bacteria.

2. MATERIALS AND METHODS

Three strains of Gram-positive bacteria (*S. aureus* NCTC 12393, *S. aureus* ATCC 6538 and *Methicillin resistant staphylococcus aureus (MRSA ATCC 43300)*) were tested for susceptibility to the following antibiotic discs: Ciprofloxacin (CIP 5µg), Doxycycline (DXT 30µg), Tobramycin (TOB 10µg), Gentamicin (CN 30µg), Levofloxacin (LEV 5µg), Tetracycline (TE 30µg), Vancomycin (VA 30µg), Cefoxitin (FOX 30µg), Erythromycin (E 15µg), Ampicillin (AMP 2µg), Augmentin (AUG 30µg), Rifampicin (RD 30µg), Azithromycin (AZM 15µg), Amikacin (AK 30µg), and Streptomycin (S 300µg).

These bacteria were cultured overnight on Mueller-Hinton (MH) agar at 37°C.

The Minimum Inhibitory Concentrations (MICs) of fosfomycin were determined for each bacterial strain using the microdilution broth method. The concentration after the MIC was used for subsequent antibiotic susceptibility testing. A 0.5 McFarland turbidity was prepared from these wells, containing 1.5×10^8 CFU/ml of bacteria. To achieve 5×10^5 CFU/ml, 100 µL of the 0.5 McFarland suspension was added to 9900 µL of Tryptic Soy Broth (TSB). The same procedure was followed for the untreated bacteria.

The Bauer-Kirby disk diffusion method was used for antibiotic susceptibility testing. A sterile swab was used to evenly spread treated and untreated bacterial suspension onto Mueller-Hinton agar plates. Antibiotic discs were then placed onto the agar surface using sterile forceps, ensuring adequate spacing. This process was repeated in triplicate to confirm the results. The plates were incubated at 37°C for 18-24 hours.

The results were interpreted based on the diameter of the zone of inhibition and the breakpoints established by CLSI or EUCAST for each antibiotic, classifying bacteria as susceptible, intermediate, or resistant. A larger zone of inhibition indicated greater susceptibility, while a smaller zone or absence of a zone indicated resistance.

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The diameter of the zones of inhibition around each antibiotic disk was measured using a ruler. The mean zone diameter for each antibiotic was calculated for both the fosfomycin-treated and control groups.

This methodology enables the assessment of fosfomycin's impact on the resistance patterns of Gram-positive bacteria to different classes of antibiotics using the Bauer-Kirby disk diffusion method.

3. RESULTS

Table 1: The zones of inhibition for all three Gram-positive bacteria positive control and treated with fosfomycin.

List of tested antibiotics	BACTERIA					
	<i>S. aureus</i> NCTC 12393		<i>S. aureus</i> ATCC 6538		<i>MRSA</i> ATCC 43300	
	Pos. Control	Fosfomycin 0.0014 mg/ml	Pos. Control	Fosfomycin 0.0014 mg/ml	Pos. Control	Fosfomycin 0.00072 mg/ml
Ciprofloxacin (CIP5)	28 mm	23 mm	31 mm	27 mm	32 mm	25 mm
Doxycycline (DXT30)	11 mm	13 mm	32 mm	26 mm	18 mm	13 mm
Tobramycin (TOB10)	23 mm	21 mm	26 mm	21 mm	26 mm	23 mm
Gentamicin (CN30)	27 mm	27 mm	31 mm	24 mm	31 mm	27 mm
Levofloxacin (LEV5)	31 mm	26 mm	31 mm	28 mm	30 mm	26 mm
Tetracycline (TE30)	14 mm	11 mm	29 mm	17 mm	15 mm	10 mm
Vancomycin (VA30)	21 mm	18 mm	19 mm	19 mm	20 mm	16 mm
Cefoxitin (FOX30)	16 mm	21 mm	30 mm	27 mm	15 mm	13 mm
Erythromycin (E15)	R	29 mm (DG)	30 mm	24 mm	11 mm	R
Ampicillin (AMP2)	8 mm	10 mm	34 mm	21 mm	11 mm	15 mm (DG)
Augmentin (AUG30)	25 mm	24 mm	42 mm	26 mm	20 mm	15 mm
Rifampicin (RD30)	40 mm	31 mm	40 mm	20 mm	42 mm	23 mm
Azithromycin (AZM15)	R	25 mm (DG)	27 mm	20 mm	14 mm	11 mm (DG)
Amikacin (AK30)	21 mm	20 mm	24 mm	20 mm	24 mm	21 mm
Streptomycin (S300)	R	24 mm (DG)	30 mm	22 mm	16 mm	16 mm

In this study three strains of Gram-positive bacteria (*S. aureus* NCTC 12393, *S. aureus* ATCC 6538 and *Methicillin resistant staphylococcus aureus* (MRSA ATCC 43300)) were tested on fifteen antibiotics from different classes (Ciprofloxacin (CIP 5µg), Doxycycline (DXT 30µg), Tobramycin (TOB 10µg), Gentamicin (CN 30µg), Levofloxacin (LEV 5µg), Tetracycline (TE 30µg), Vancomycin (VA 30µg), Cefoxitin (FOX 30µg), Erythromycin (E 15µg), Ampicillin (AMP 2µg), Augmentin (AUG 30µg), Rifampicin (RD 30µg), Azithromycin (AZM 15µg), Amikacin (AK 30µg), and Streptomycin (S 300µg)).

The initial concentration of fosfomycin for biofilm formation for all tested bacteria was 190 mg/ml. According to the results from biofilm formation, minimum inhibitory concentration

(MIC) of fosfomycin for *S. aureus* NCTC 12393 was 0.0028 mg/ml, for *S. aureus* ATCC 6538 was 0.0028 mg/ml and for *Methicillin resistant staphylococcus aureus* (MRSA ATCC 43300) was 0.0014 mg/ml.

Based on the data presented in Table 1, in most cases, the zones of inhibition in samples treated with fosfomycin were smaller compared to those in the positive control (without fosfomycin). This indicates that fosfomycin reduces the susceptibility of Gram-positive bacteria to the tested antibiotics, potentially diminishing the efficacy of these antibiotics in the presence of fosfomycin.

However, exceptions were observed for the antibiotics erythromycin (E 15µg), azithromycin (AZM 15 µg) and streptomycin (S 300µg), where *S. aureus* NCTC 12393 initially displayed resistance but showed a small inhibition zone after fosfomycin treatment. Furthermore, the results demonstrated that the zone of inhibition for the same strain of bacteria (*S. aureus* NCTC 12393) in relation to the antibiotics ampicillin (AMP 2µg) and doxycycline (DXT 30µg) increased following treatment with fosfomycin, indicating a distinct response to fosfomycin treatment for this antibiotics.

The most significant difference was observed for the same strain for the antibiotic cefoxitin (FOX 30ug) where the zone of inhibition increased from 16 mm to 21 mm. These findings suggest a potential synergistic effect between fosfomycin and these antibiotics, indicating that fosfomycin may enhance bacterial susceptibility to them.

Initially, with a zone of inhibition of 11 mm, the *MRSA* ATCC 43300 strain showed some susceptibility to the antibiotic, albeit at a low level. However, after treatment with fosfomycin, the zone of inhibition disappeared, indicating that the bacteria were no longer susceptible to the antibiotic and were now classified as resistant.

4. DISCUSSION

The findings show that Gram-positive bacteria are less susceptible to fosfomycin when combined with the tested antibiotics, which may lessen the antibiotics' effectiveness when fosfomycin is present.

It is interesting to note that *S. aureus* NCTC 12393 exhibits exceptions for erythromycin (E 15µg), azithromycin (AZM 15µg), and streptomycin (S 300µg), where the strain originally demonstrated resistance but thereafter displayed a modest inhibitory zone following fosfomycin treatment. This shows that the way this strain resists these antibiotics may be specifically affected by fosfomycin. This idea is further supported by the observation of an increase in the zone of inhibition for ampicillin (AMP 2µg) and doxycycline (DXT 30µg) in the same strain following fosfomycin therapy, suggesting that both drugs respond differently to fosfomycin treatment.

The observed increase in the zone of inhibition for ceftazidime in *S. aureus* NCTC 12393 from 16 mm to 21 mm following fosfomycin therapy is one of the most significant results reported. This significant rise implies that ceftazidime (FOX 30µg) and fosfomycin may work synergistically, suggesting that fosfomycin might increase *S. aureus* NCTC 12393's susceptibility to ceftazidime (FOX 30µg). Given that ceftazidime (FOX 30µg) is a frequently prescribed antibiotic for the management of Gram-positive infections, this finding is especially significant.

All things considered, these findings imply that fosfomycin might have a complicated and multidimensional effect on *S. aureus* NCTC 12393's antibiotic resistance patterns.

Following treatment with fosfomycin, the zone of inhibition for MRSA ATCC 43300 (*Methicillin-Resistant Staphylococcus aureus*) changed from 11 mm to resistant, indicating a change in the bacterial response to the antibiotic. This shift in susceptibility may be caused by a number of things. One possibility is that the treatment with fosfomycin caused the bacteria to evolve resistance mechanisms. A variety of methods, including the acquisition of resistance genes or mutations that change the antibiotic's target or lessen its capacity to penetrate the bacterial cell, can lead to the development of resistance in bacteria.

There exists an additional possibility that the susceptibility that was first observed was not indicative of the bacterial population's overall susceptibility. While the bulk of the population was already resistant to fosfomycin, there was a chance that a tiny subgroup of bacteria was the cause of the initial 11 mm zone of inhibition. It's possible that the most susceptible subpopulation was eradicated after therapy, leaving only the resistant pathogens.

Overall, after treating MRSA ATCC 43300 with fosfomycin, the zone of inhibition changed from 11 mm to resistant, indicating a loss of susceptibility to the antibiotic. This could be because of the emergence of resistance mechanisms or the eradication of a susceptible subpopulation. According to the study of Falagas et al, 2009, microbiological data show that fosfomycin has strong anti-MRSA action.

Information about the synergistic impact of fosfomycin with other antibiotics against *S. aureus* was obtained from a number of research. Notably, among the investigated strains of *S. aureus*, the synergistic effect of fosfomycin and ciprofloxacin (CIP 5µg) was shown to be the highest (Kastoris et al., 2010).

5. CONCLUSION

In conclusion, this research offers significant understanding of how fosfomycin affects Gram-positive bacteria's resistance to various antibiotic classes. The findings suggest that the effects of fosfomycin on bacterial susceptibility can vary, with certain antibiotics exhibiting enhanced susceptibility and others exhibiting decreased susceptibility or resistance following fosfomycin treatment.

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The findings suggest that fosfomycin may have a synergistic effect with certain antibiotics, such as cefoxitin (FOX 30µg), ampicillin (AMP 2µg), and doxycycline (DXT 30µg), leading to increased susceptibility of *S. aureus* NCTC 12393. This demonstrates how fosfomycin may improve the way these antibiotics work to treat infections caused by Gram-positive bacteria. These findings are in line with earlier research that showed how fosfomycin can increase the activity of other antibiotics by a variety of mechanisms, including the suppression of cell wall formation.

Nevertheless, the findings also demonstrate that, in *S. aureus* NCTC 12393, fosfomycin can lessen the sensitivity of Gram-positive bacteria to specific antibiotics, including erythromycin (E 15µg), azithromycin (AZM 15µg), and streptomycin (S 300µg). This implies that the bacterial resistance patterns to fosfomycin may alter, which could result in the emergence of antibiotic resistance. These results highlight how crucial it is to use fosfomycin cautiously in clinical settings in order to reduce the possibility of encouraging antibiotic resistance.

It is significant to remember that different bacterial strains and medications can react differently to fosfomycin treatment. The observed variations in susceptibility patterns demonstrate how intricate Gram-positive bacteria's antibiotic resistance systems are.

Further research is needed to elucidate the underlying mechanisms of these effects and to determine the clinical implications of these findings. Understanding the interactions between fosfomycin and other antibiotics is crucial for the development of effective treatment strategies against multidrug-resistant Gram-positive bacteria.

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Eighth International Scientific Conference
"June 5th - World Environment Day"

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EFFECT OF FOSFOMYCIN ON BIOFILM FORMATION IN GRAM-POSITIVE BACTERIA USING MICROBROTH DILUTION AND TISSUE CULTURE PLATE METHODS

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Key words: Biofilms, Antibiotics, Fosfomycin, Staphylococcus aureus, MRSA

ABSTRACT:

It is very challenging for antibiotics to eradicate biofilms, which are highly structured microbiome consortia adhering to surfaces, and antibiotics become ineffective. Composed of diverse bacterial colonies or single-cell groups, biofilms make diseases chronic, for which urgent and effective antimicrobial strategies are needed. However, in healthcare settings, the use of broad-spectrum antibiotics continues because of the difficulty in the prompt identification of pathogenic bacteria and the determination of their susceptibility to antimicrobial agents. This leads to an increase in the levels of resistance, and if proper infection control measures are not implemented, the spread of the resistant strains among the patients and the environment results in an increased rate of spread of the resistant strains. The bacteria use a variety of mechanisms of resistance, such as enzymatic inactivation and modification of antibiotic targets, and horizontal gene transfer; thus, this facilitates the ability of resistance traits to spread rapidly among bacterial populations. Antibiotic resistance generally develops when the minimum inhibitory concentration (MIC) increases due to sustained changes in bacterial physiology. In this case, fosfomycin is a drug of choice because of its ability to penetrate through the biofilms, which has been shown to be effective by many in vitro and biofilm infection model studies. These studies give evidence that fosfomycin, either as a sole antibiotic or in combination with other antibiotics, reduces or eradicates clinically significant bacteria in biofilms and causes structural changes to the biofilm matrix. This paper looks to evaluate the antibacterial effect of fosfomycin on Staphylococcus aureus and methicillin-resistant Staphylococcus aureus and its therapeutic

efficacy against the pathogens that cause biofilm-related infections. Therefore, this research contributes critical insights for the future development of successful strategies in the fight against biofilm-associated chronic diseases and against the emergence of antibiotic-resistant strains in a clinical setting.

1. INTRODUCTION

In biofilm-forming bacteria, several key features are different from those in planktonic bacteria, and this has significant consequences in both diagnosis and therapy. Since the bacteria in biofilm infections are clustered very closely together and are surrounded by the self-produced matrix, their morphology is very different. Biofilm is often visible in clinical samples (biopsies, pus, sputum) by light microscopy, but the correct identification of all the bacteria in a biofilm is only possible with DNA hybridization techniques, and specific staining techniques are required to identify the different constituents of the biofilm matrix (Hoiby et al., 2010). The biofilm is made up of extracellular polysaccharides, proteins, and DNA. EPS acts like a scaffold for cells, enzymes, and antibiotics attachment in addition to mediating surface adhesion and stabilizing the cells (Flemming & Wingender, 2010). *Staphylococcus aureus* is an example of persistent bacterium which is able to form biofilm and cause most of the wound infections (Archer et al., 2011). The contribution of the cells' diversity and metabolic status inside a biofilm is one of the key factors influencing antibiotic resistance. Besides the role played by persistent cells in largely being responsible for the reestablishment of the biofilm community, these cells are generally more resistant to antibiotics (Keren et al., 2004). As shown by studies on β -lactams, ciprofloxacin, tetracycline, and tobramycin, cells in the dormant regions of a biofilm usually remain unaffected by antibiotics (Pamp et al., 2008). Generally, antibiotic resistance is defined as an increase in the minimum inhibitory concentration value of an antibiotic due to a long-term change in the bacterium, such as mutation or resistance acquired by horizontal gene transfer (Stewart & Costerton, 2001). On the other hand, antibiotic tolerance is a phenotypic state that allows cells to tolerate the effects of an antibiotic. These two concepts fit when the cells are planktonic; the story is different for biofilms. Antimicrobial tolerance in biofilms is related to the biofilm's growth mode. In contrast, planktonic culture-grown bacteria are usually susceptible to antibiotics. That makes biofilms naturally resistant to antibiotics. Since bacteria have been developing ways of surviving the effects of natural antibacterial compounds for billions of years without human intervention, antibiotic resistance is also a natural phenomenon (Vega & Gore, 2014). The decline in effective medicines for treating infections caused by multidrug-resistant bacteria and the relative absence of new antimicrobial drugs is concerning. Reevaluation and repositioning of old antibiotics has become a pragmatic strategy to resolve this problem (Freire-Moran et al., 2011). Fosfomycin is one of those old antibiotics; it is a wide-spectrum antibiotic that was designed almost 45 years ago. Fosfomycin may be a promising antibiotic for treating various infections caused by MDR bacteria as well as by XDR and PDR pathogens, due to its

effectiveness both in vitro and in vivo (Dijkmans et al., 2017). However, these antibiotics never underwent the strict drug study procedures that are required nowadays in order for their sales to be approved. As such, pharmacological data is necessary to come up with the optimal dose schedules that induce the least toxicity with maximum effect (Bergen et al., 2012).

2. MATERIALS AND METHODS

2.1 Sample Preparation

Purified sterile water was used to dissolve fosfomycin, and 120 mg/ml was the initial concentration used in the tests.

2.2 Bacterial Strains

Methycillin-Resistant Staphylococcus aureus ATCC 43300, *Staphylococcus aureus* NCTC 12393, and *Staphylococcus aureus* ATCC 6538 were grown on Mueller Hinton Agar for 24 hours at 37°C. For Minimum Inhibitory Concentration and Biofilm Forming Capacity testings, 0.5 Mc Farland inoculum was prepared in TSB.

2.3 Minimum Inhibitory Concentration (MIC)

The microbroth dilution method in Tryptic Soy Broth (TSB) was used to measure the minimal inhibitory concentration (MIC). Fosfomycin serial dilutions were done in 96 well plates with a 100 µl final volume. A 20 µl bacterial inoculum of the tested bacteria was applied to each well, except for the negative control. The pure culture of each of the examined bacteria served as the positive control, while the negative control consisted only of TSB. The 96-well plates were incubated overnight. The absorbance values were read at a wavelength of 595 nm by the ELISA plate reader (Avdić et al., 2019).

2.4 Biofilm Forming Capacity

The content of each plate was decanted after incubation overnight. Then, plates were washed using distilled water and dyed for ten minutes with a 0.1% crystal violet solution. After washing and drying, the plates were treated for ten minutes with 96% ethanol. The absorbance readings were measured using a Biochrom EZ Read 400 ELISA plate reader at a wavelength of 595 nm. For each bacterial strain, mean values were evaluated based on the measurements of absorbance values that were obtained. The results in biofilm development were classified into four categories: non-adherent, NA, weakly adherent, W, moderately adherent, M, and highly adherent, S. The optical density cut-off value, OD_c, used in determining classification of each result, was calculated by multiplying the average, OD, of the negative control by three standard deviations of the negative control. Thus, the following biofilm categories were used: OD_c < OD ≤ 2 x OD_c: weakly adhering, OD_c < OD ≤ 4 x OD_c: moderately adherent and 4x OD_c < OD: highly adhering. OD ≤ OD_c: non-adhering (Avdić et al., 2019).

3. RESULTS

3.1 Minimum Inhibitory Concentration (MIC) of Fosfomycin

By treating 3 bacterial strains with 190mg/ml of Fosfomycin, minimum inhibitory concentrations (MIC) were discovered as shown in Table 1.

Table 1: Minimum Inhibitory Concentration of Fosfomycin in TSB

Bacterial strain	MIC (mg/ml) (TSB)
<i>MRSA</i> ATCC 43300	0.0014
<i>S. aureus</i> NCTC 12393	0.0028
<i>S. aureus</i> ATCC 6538	0.0028

The lowest concentration of Fosfomycin that inhibited the growth of tested bacteria in TSB was 0.0014 mg/ml for *MRSA* ATCC 43300, while the highest concentration of Fosfomycin was 0.0028 mg/ml in the case of *S. aureus* NCTC 12492 and *S. aureus* ATCC 6538.

Table 2: Absorbance at 595 nm for Minimum Inhibitory Concentration (MIC) determination of *S. aureus* ATCC 6538

Concentration (mg/ml)	OD values at 595nm (octuplet)						Average	St. Dev.
NC	0.400	0.049	0.050	0.051	0.050	0.044	0.050	0.001
PC	0.570	0.514	0.490	0.553	0.561	0.575	0.544	0.034
190	0.051	0.051	0.048	0.050	0.052	0.050	0.050	0.001
95	0.050	0.056	0.050	0.049	0.050	0.048	0.051	0.003
47.5	0.052	0.049	0.049	0.049	0.049	0.049	0.050	0.001
23.75	0.048	0.049	0.049	0.049	0.050	0.049	0.049	0.001
11.875	0.048	0.049	0.049	0.049	0.048	0.049	0.049	0.001
5.938	0.050	0.051	0.049	0.049	0.049	0.049	0.050	0.001
2.969	0.049	0.049	0.049	0.049	0.053	0.050	0.050	0.002
1.484	0.050	0.050	0.049	0.049	0.049	0.050	0.050	0.001
0.742	0.049	0.050	0.050	0.049	0.050	0.051	0.050	0.001
0.371	0.054	0.049	0.050	0.050	0.051	0.050	0.051	0.002
0.186	0.057	0.049	0.049	0.050	0.050	0.050	0.051	0.003
0.093	0.048	0.052	0.049	0.049	0.049	0.049	0.049	0.001
0.046	0.049	0.049	0.048	0.050	0.050	0.049	0.049	0.001
0.023	0.051	0.048	0.049	0.054	0.061	0.052	0.053	0.005
0.012	0.049	0.048	0.049	0.049	0.049	0.050	0.049	0.001
0.0058	0.052	0.049	0.050	0.048	0.063	0.048	0.052	0.006
0.0029	0.102	0.161	0.176	0.120	0.127	0.156	0.140	0.028
0.0014	0.211	0.153	0.156	0.183	0.112	0.127	0.157	0.036
0.0007	0.201	0.212	0.229	0.205	0.234	0.143	0.204	0.033
0.0004	0.237	0.273	0.329	0.289	0.366	0.352	0.308	0.050

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Table 3: Absorbance at 595 nm for Minimum Inhibitory Concentration (MIC) determination of *S. aureus* NCTC 12393

Concentration (mg/ml)	OD values at 595nm (octuplet)						Average	St. Dev.
NC	0.057	0.051	0.051	0.056	0.051	0.051	0.052	0.003
PC	0.413	0.413	0.456	0.422	0.499	0.484	0.448	0.038
190	0.050	0.049	0.052	0.053	0.048	0.048	0.050	0.002
95	0.050	0.055	0.051	0.051	0.049	0.050	0.051	0.002
47.5	0.050	0.050	0.049	0.050	0.050	0.050	0.050	0.000
23.75	0.050	0.050	0.049	0.049	0.050	0.050	0.050	0.001
11.875	0.049	0.049	0.049	0.049	0.050	0.049	0.049	0.000
5.938	0.050	0.050	0.049	0.049	0.048	0.050	0.049	0.001
2.969	0.054	0.050	0.050	0.050	0.050	0.051	0.051	0.002
1.484	0.053	0.050	0.050	0.051	0.050	0.050	0.051	0.001
0.742	0.051	0.050	0.051	0.050	0.050	0.053	0.051	0.001
0.371	0.052	0.051	0.050	0.052	0.068	0.050	0.054	0.007
0.186	0.059	0.049	0.050	0.051	0.049	0.051	0.052	0.004
0.093	0.054	0.052	0.056	0.141	0.052	0.051	0.068	0.036
0.046	0.052	0.051	0.068	0.051	0.051	0.050	0.054	0.007
0.023	0.053	0.050	0.054	0.049	0.048	0.048	0.050	0.003
0.012	0.053	0.061	0.050	0.050	0.055	0.097	0.061	0.018
0.0058	0.066	0.136	0.150	0.056	0.055	0.108	0.095	0.042
0.0029	0.139	0.137	0.147	0.122	0.117	0.141	0.134	0.012
0.0014	0.179	0.171	0.177	0.182	0.173	0.279	0.194	0.042
0.0007	0.575	0.480	0.470	0.588	0.464	0.553	0.522	0.056
0.0004	0.354	0.475	0.428	0.466	0.546	0.499	0.461	0.065

Table 4: Absorbance at 595 nm for Minimum Inhibitory Concentration (MIC) determination of *MRSA* ATCC 43300

Concentration (mg/ml)	OD values at 595nm (octuplet)						Average	St. Dev.
NC	0.059	0.051	0.050	0.051	0.050	0.052	0.051	0.001
PC	0.474	0.433	0.407	0.369	0.394	0.390	0.411	0.037
190	0.048	0.046	0.044	0.046	0.048	0.049	0.047	0.002
95	0.050	0.047	0.048	0.056	0.048	0.049	0.050	0.003
47.5	0.049	0.047	0.047	0.048	0.048	0.048	0.048	0.001
23.75	0.052	0.048	0.047	0.047	0.048	0.048	0.048	0.002
11.875	0.052	0.048	0.048	0.048	0.048	0.047	0.049	0.002
5.938	0.051	0.048	0.048	0.055	0.048	0.048	0.050	0.003
2.969	0.048	0.049	0.048	0.048	0.048	0.047	0.048	0.001
1.484	0.049	0.049	0.049	0.050	0.049	0.049	0.049	0.000
0.742	0.049	0.052	0.048	0.048	0.049	0.048	0.049	0.002
0.371	0.054	0.049	0.051	0.050	0.049	0.049	0.050	0.002
0.186	0.058	0.048	0.047	0.302	0.049	0.049	0.092	0.103
0.093	0.047	0.051	0.048	0.049	0.048	0.048	0.049	0.001
0.046	0.048	0.044	0.048	0.048	0.049	0.048	0.048	0.002

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0.023	0.053	0.048	0.048	0.048	0.048	0.049	0.049	0.002
0.012	0.048	0.048	0.048	0.049	0.048	0.048	0.048	0.000
0.0058	0.052	0.048	0.048	0.048	0.049	0.048	0.049	0.002
0.0029	0.062	0.075	0.056	0.073	0.093	0.053	0.069	0.015
0.0014	0.165	0.329	0.167	0.222	0.410	0.161	0.242	0.104
0.0007	0.316	0.308	0.318	0.302	0.311	0.278	0.306	0.015
0.0004	0.349	0.357	0.368	0.357	0.367	0.362	0.360	0.007

3.2 Biofilm Forming Capacity

3.2.1 Biofilm Forming Capacity for *S. aureus* ATCC 6538

Table 5: Absorbance at 595 nm for biofilm formation determination of *S. aureus* ATCC 6538

Concentration (mg/ml)	OD values at 595nm (octuplet)						Average	St. Dev.
NC	0.073	0.079	0.071	0.070	0.071	0.075	0.073	0.004
PC	0.475	0.522	0.428	0.459	0.425	0.464	0.462	0.036
190	0.078	0.079	0.081	0.081	0.078	0.087	0.081	0.003
95	0.081	0.082	0.087	0.083	0.074	0.095	0.084	0.007
47.5	0.081	0.073	0.115	0.081	0.076	0.096	0.087	0.016
23.75	0.074	0.075	0.077	0.078	0.074	0.080	0.076	0.002
11.875	0.073	0.081	0.086	0.073	0.086	0.073	0.079	0.006
5.938	0.084	0.084	0.081	0.077	0.087	0.084	0.083	0.003
2.969	0.081	0.080	0.080	0.098	0.083	0.079	0.084	0.007
1.484	0.080	0.080	0.083	0.090	0.092	0.087	0.085	0.005
0.742	0.078	0.077	0.076	0.083	0.080	0.084	0.080	0.003
0.371	0.090	0.073	0.078	0.074	0.072	0.072	0.077	0.007
0.186	0.088	0.161	0.150	0.072	0.076	0.072	0.103	0.041
0.093	0.107	0.162	0.075	0.074	0.071	0.074	0.094	0.036
0.046	0.135	0.173	0.107	0.110	0.144	0.078	0.125	0.033
0.023	0.167	0.184	0.077	0.179	0.187	0.119	0.152	0.045
0.012	0.079	0.065	0.109	0.081	0.079	0.075	0.081	0.015
0.0058	0.078	0.072	0.074	0.088	0.069	0.066	0.075	0.008
0.0029	0.077	0.069	0.091	0.066	0.072	0.106	0.080	0.015
0.0014	0.222	0.074	0.194	0.177	0.105	0.127	0.150	0.057
0.0007	0.198	0.126	0.231	0.198	0.221	0.182	0.193	0.037
0.0004	0.228	0.342	0.487	0.238	0.464	0.496	0.376	0.124

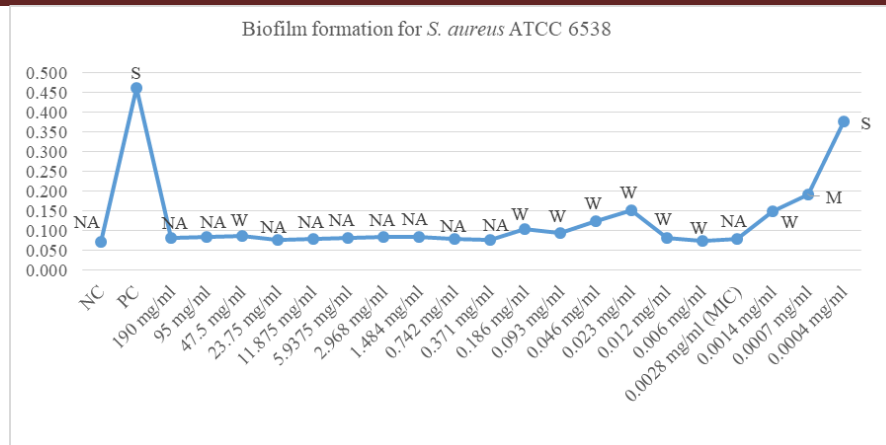


Figure 1: Biofilm formation for *S. aureus* ATCC 6538; PC - positive control; NC - negative control; NA - non-adherent; W - weak; M – moderate; S – strong

According to Figure 1, *S. aureus* ATCC 6538 when treated with 190 mg/mL to 0.0014 mg/ml of fosfomycin, decreased its biofilm formation from strong to non-adherent or weakly adherent. When treated with concentration lower than 0.0007 mg/ml, biofilm forming capacity turns to moderate or strong. After obtaining average and standard deviation values after absorbance measurement at 595nm (shown in Table 5), OD_c value was determined and was 0.085. If OD value is lower than 0.085, biofilm is considered as Non-Adherent (NA). If OD value is ranging between 0.0085 and 0.170, biofilm is considered as Weak (W). If OD value is ranging between 0.170 and 0.340, biofilm is considered as Moderate (M). If OD value is greater than 0.340, biofilm is Strong (S).

3.2.2 Biofilm Forming Capacity for *S. aureus* NCTC 12393

Table 6: Absorbance at 595 nm for biofilm formation determination of *S. aureus* ATCC 6538

Concentration (mg/ml)	OD values at 595nm (octuplet)						Average	St. Dev.
NC	0.085	0.075	0.078	0.077	0.074	0.073	0.076	0.002
PC	0.228	0.239	0.174	0.180	0.227	0.159	0.201	0.034
190	0.083	0.073	0.069	0.077	0.075	0.069	0.074	0.005
95	0.069	0.071	0.066	0.066	0.071	0.066	0.068	0.002
47.5	0.069	0.064	0.066	0.058	0.066	0.063	0.064	0.004
23.75	0.073	0.071	0.066	0.070	0.069	0.063	0.069	0.004
11.875	0.068	0.062	0.062	0.063	0.062	0.066	0.064	0.003
5.938	0.078	0.084	0.067	0.068	0.067	0.071	0.073	0.007
2.969	0.096	0.076	0.068	0.068	0.071	0.071	0.075	0.011
1.484	0.105	0.102	0.078	0.074	0.073	0.083	0.086	0.014
0.742	0.132	0.077	0.073	0.074	0.081	0.078	0.086	0.023
0.371	0.084	0.117	0.068	0.070	0.077	0.073	0.082	0.018
0.186	0.086	0.083	0.084	0.091	0.078	0.085	0.085	0.004
0.093	0.100	0.098	0.087	0.090	0.090	0.097	0.094	0.005

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0.046	0.085	0.097	0.125	0.077	0.085	0.079	0.091	0.018
0.023	0.100	0.144	0.081	0.080	0.079	0.080	0.094	0.026
0.012	0.097	0.114	0.083	0.093	0.072	0.075	0.089	0.016
0.0058	0.085	0.068	0.078	0.081	0.070	0.054	0.073	0.011
0.0029	0.067	0.064	0.115	0.052	0.048	0.052	0.066	0.025
0.0014	0.117	0.204	0.258	0.257	0.153	0.175	0.194	0.057
0.0007	0.128	0.135	0.190	0.148	0.119	0.168	0.148	0.027
0.0004	0.198	0.160	0.143	0.136	0.167	0.181	0.164	0.023

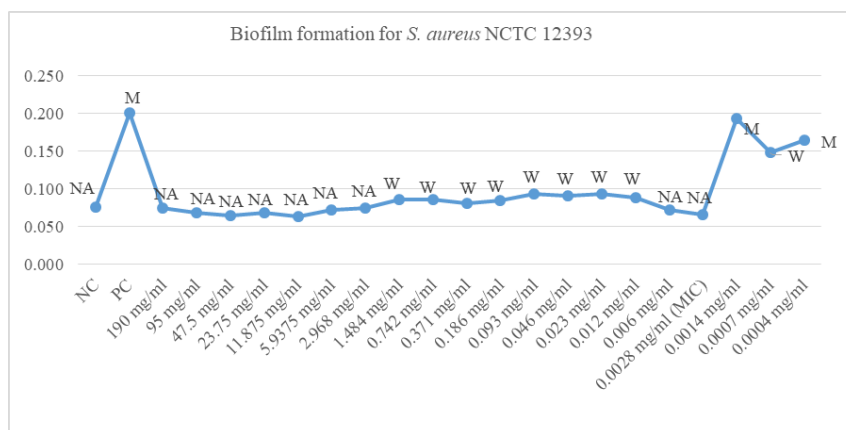


Figure 2: Biofilm formation for *S. aureus* NCTC 12393; PC - positive control; NC - negative control; NA - non-adherent; W - weak; M – moderate; S – strong

According to Figure 2, *S. aureus* NCTC 12393 when treated with 190 mg/mL to 0.0028 mg/ml of fosfomycin, decreased its biofilm formation from moderately adherent to non-adherent or weakly adherent. When treated with concentration lower than 0.0014 mg/ml, biofilm forming capacity turns to moderately adherent. After obtaining average and standard deviation values after absorbance measurement at 595nm (shown in Table 6), ODc value was determined and was 0.081. If OD value is lower than 0.081, biofilm is considered as Non-Adherent (NA). If OD value is ranging between 0.081 and 0.163, biofilm is considered as Weak (W). If OD value is ranging between 0.163 and 0.330, biofilm is considered as Moderate (M). If OD value is greater than 0.330, biofilm is Strong (S).

3.2.3 Biofilm Forming Capacity for *MRSA* ATCC 43300

Table 7: Absorbance at 595 nm for biofilm formation determination of *MRSA* ATCC 43300

Concentration (mg/ml)	OD values at 595nm (octuplet)						Average	St. Dev.
NC	0.087	0.077	0.090	0.093	0.074	0.077	0.082	0.009
PC	0.251	0.260	0.265	0.200	0.231	0.242	0.242	0.024
190	0.073	0.079	0.076	0.072	0.077	0.070	0.075	0.003
95	0.074	0.074	0.074	0.087	0.079	0.077	0.078	0.005
47.5	0.062	0.067	0.069	0.062	0.070	0.070	0.067	0.004
23.75	0.077	0.136	0.076	0.082	0.079	0.084	0.089	0.023
11.875	0.081	0.070	0.071	0.067	0.069	0.085	0.074	0.007
5.938	0.081	0.078	0.066	0.091	0.079	0.084	0.080	0.008
2.969	0.091	0.090	0.075	0.073	0.079	0.078	0.081	0.008
1.484	0.071	0.070	0.081	0.070	0.070	0.072	0.072	0.004
0.742	0.068	0.074	0.071	0.075	0.084	0.084	0.076	0.007
0.371	0.077	0.068	0.067	0.068	0.066	0.088	0.072	0.009
0.186	0.107	0.079	0.081	0.073	0.073	0.085	0.083	0.013
0.093	0.078	0.094	0.073	0.081	0.070	0.074	0.078	0.009
0.046	0.072	0.087	0.078	0.069	0.075	0.079	0.077	0.006
0.023	0.080	0.072	0.073	0.070	0.079	0.080	0.076	0.005
0.012	0.072	0.072	0.077	0.073	0.083	0.073	0.075	0.004
0.0058	0.077	0.072	0.082	0.076	0.073	0.077	0.076	0.004
0.0029	0.086	0.060	0.059	0.065	0.060	0.059	0.065	0.011
0.0014	0.210	0.171	0.166	0.199	0.116	0.170	0.172	0.033
0.0007	0.258	0.302	0.240	0.324	0.249	0.245	0.270	0.035
0.0004	0.157	0.233	0.157	0.097	0.074	0.084	0.134	0.061

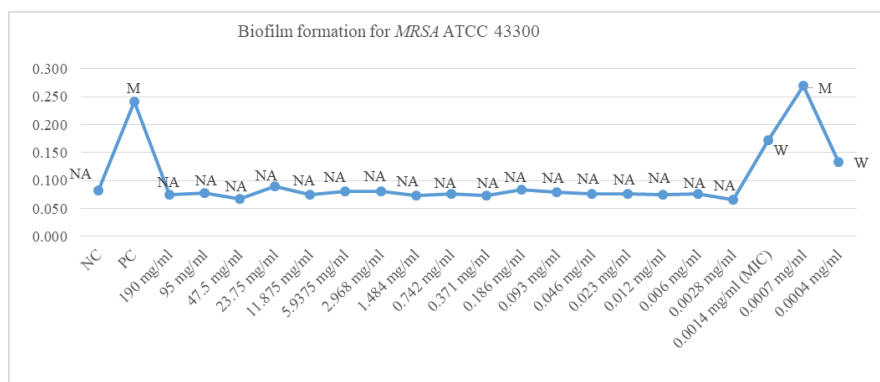


Figure 3: Biofilm formation for *MRSA* ATCC 43300; PC - positive control; NC - negative control; NA - non-adherent; W - weak; M – moderate; S – strong

According to Figure 3, *MRSA* ATCC 43300 when treated with 190 mg/mL to 0.0028 mg/ml of fosfomycin, decreased its biofilm formation from moderately adherent to non-adherent. When

treated with concentration lower than 0.0014 mg/ml, biofilm forming capacity turns to weakly and moderately adherent. After obtaining average and standard deviation values after absorbance measurement at 595nm (shown in Table 7), OD_c value was determined and was 0.110. If OD value is lower than 0.110, biofilm is considered as Non-Adherent (NA). If OD value is ranging between 0.110 and 0.220, biofilm is considered as Weak (W). If OD value is ranging between 0.220 and 0.440, biofilm is considered as Moderate (M). If OD value is greater than 0.440, biofilm is Strong (S).

4. DISCUSSION

The minimum inhibitory concentration of fosfomycin for *S. aureus* ATCC 6538 was 0.0028 mg/ml in Tryptic Soy Broth (TSB) (Table 2). Strain's ability to form biofilm after the application of different concentrations of fosfomycin was significantly reduced. This strain can form strongly adherent biofilm by itself, but after applying different concentrations of fosfomycin ranging between 190 mg/mL - 0.0014 mg/ml, biofilm formation was reduced to non-adherent or weakly adherent. After 0.0007 mg/ml, biofilm forming capacity turns to moderately or strongly adherent. In Tryptic Soy Broth (TSB), the lowest inhibitory concentration of fosfomycin for *S. aureus* NCTC 12393 was 0.0028 mg/ml (Table 3). After applying varying doses of fosfomycin, the strain's capacity for creating biofilm was greatly inhibited. This strain may produce moderately adherent biofilm on its own, however biofilm production was decreased to non-adherent or weakly adherent after applying several dosages of fosfomycin, which ranged from 190 mg/mL to 0.0028 mg/ml. The biofilm-forming capability changes to moderately to strongly adherent at 0.0014 mg/ml. For *MRSA* ATCC 43300, the lowest inhibitory concentration of fosfomycin in Tryptic Soy Broth (TSB) was 0.0014 mg/ml (Table 4). Following the administration of various doses of fosfomycin, the strain's capacity to build biofilm was considerably diminished. On its own, this strain can generate a moderately adherent biofilm, however when fosfomycin was added at varying dosages (190 mg/mL to 0.0028 mg/ml), the biofilm formed became non-adherent or weakly adherent. The ability to produce biofilms becomes moderately adherent at 0.0014 mg/ml.

5. CONCLUSION

From the analysis of the effect of fosfomycin on Gram-positive bacteria, it can be concluded that the Minimum Inhibitory Concentration (MIC) in Tryptic Soy Broth (TSB) for fosfomycin on Gram-positive bacteria (*S. aureus* NCTC 12393, *S. aureus* ATCC 6538, *Methicillin-resistant S. aureus* ATCC 43300) was detected as is shown in Table 1. The lowest amount of fosfomycin showing growth inhibition of bacteria was 0.0014 mg/ml for *Methicillin-resistant S. aureus* ATCC 43300. Moreover, an influence on biofilm formation was noted when the first concentration (190 mg/ml) of fosfomycin in Gram-positive bacterial strains, *S. aureus* NCTC

12393, *S. aureus* ATCC 6538, *Methicillin-resistant S. aureus* ATCC 43300, was added. The presented results suggest that fosfomycin is extremely effective against some Gram-positive bacteria at low concentrations, mainly *Methicillin-resistant S. aureus*. Besides, the noticed effect on biofilm formation suggests that fosfomycin acts not only through an inhibition of bacterial growth but probably may destroy the ways of protection developed by these bacteria, which is essential for chronic infection treatment. Therefore, fosfomycin may be considered a very important antibiotic for Gram-positive bacteria in combating resistant strains.

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MALACHITE GREEN ADSORPTION BY AMORPHA FRUTICOSA L. ACTIVATED CARBON

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Key words: adsorption, *Amorpha L. Fruticosa*, dye, Malachite green

ABSTRACT:

*The increasing presence of synthetic dyes in wastewater poses a significant environmental challenge, necessitating the development of efficient and sustainable removal methods. This study investigates the use of activated carbon derived from plant *Amorpha fruticosa* L. commonly known as false indigo bush, for the adsorption of synthetic dyes from aqueous solutions. Batch adsorption experiments were conducted to evaluate the removal efficiency of commonly used synthetic dyes malachite green (MG). Key parameters such as contact time, initial dye concentration, pH, and temperature were optimized to ascertain the maximum adsorption capacity. The adsorption kinetics followed a pseudo-second-order model, suggesting chemisorption as the primary mechanism. Equilibrium data were best fitted by the Langmuir isotherm model, indicating monolayer adsorption with maximum capacities of 27.07 mg/g MG.*

1. INTRODUCTION

Dyes are common industrial environmental pollutants because of the extensive application of the large-scale production. Due to their chemical and physical properties they represent significant harmful impacts on the ecosystem (Bhattacharyya et al. 2005). The removal of dyes in an economic way represents an important issue to the industry. Adsorption is most effective separation technique in terms of, simplicity of design, ease and flexibility of operation. In last decade, numerous approaches have been studied for the development ecological and effective adsorbents, so natural materials and waste materials from agriculture and industry are explored for production of non-conventional, low-cost adsorbents (Rafatullah et al, 2010). *Amorpha fruticosa* L., commonly known as false indigo bush, poses significant ecological problems in Croatia. This

invasive species aggressively colonizes riverbanks, wetlands, and other natural habitats, outcompeting native flora and altering ecosystems. Its dense thickets hinder native plant growth and reduce biodiversity, impacting local wildlife dependent on indigenous vegetation. Moreover, *Amorpha fruticosa* L. rapid spread complicates land management and conservation efforts. Efforts to control this species, such as mechanical removal and chemical treatments, are resource-intensive and often have limited success. The resilience and adaptability of *Amorpha fruticosa* necessitate ongoing monitoring and innovative management strategies to mitigate its ecological impact (Ožura et al. 2015)].

This study has been reported production of activated carbon from *Amorpha fruticosa* L. and its ability to remove synthetic dye malachite green. This method offers an eco-friendly solution for wastewater treatment by employing a renewable resource to remove harmful dyes from industrial effluents

2. MATERIAL AND METHODS

2.1 Preparation of Adsorbate

Malachite green (MG), a triarylmethane dye ($C_{23}H_{26}N_2O$, CI 42000) is a dark green crystalline solid obtained from E. Merck, India and was used in this study without further purification. The solution was prepared by dissolving the required amount of dye in distilled water.

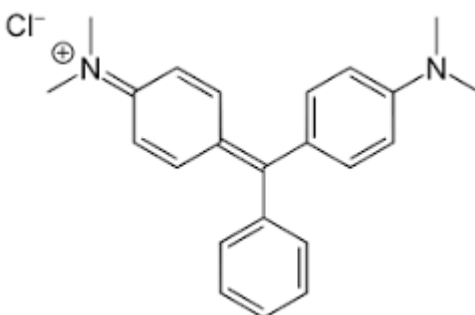


Figure 1: Structure of malachite green

2.2 Preparation of Adsorbent

Amorpha fruticosa L. seeds has been collected from Karlovac County, Croatia. The biomass was chopped and dried at 105°C for 24 h. The dried biomass was milled and sieved to 10 µm particle size. 10.0 g of prepared biomass was then added in small portion to 800 mL of 60% H_3PO_4 . After cooling to room temperature, the reaction mixture was poured onto cold water (3 L) and filtered. The resulting material was heated in an oven at 150 ° C for overnight, followed by washing with 3 L distilled water and then soaked in 1% $NaHCO_3$ solution overnight to remove

any acid. The obtained carbon was washed with distilled water until pH of activated carbon reached six and dried in oven at 150 ° C for 24 h (Cindrić et al. 2018).

2.3 Experimental protocol

The experiment was conducted in batch adsorption set up using 250 ml of laboratory glass. In 100 ml of malachite green solution with various initial concentrations was added adsorbate. The solute was mixed at magnetic stirrer at 150 rpm until the equilibrium is reached. After decantation and filtration, the equilibrium concentrations of dye in the solution were measured at $\lambda=614$ nm using UV-visible spectrophotometer (Shimadzu 2600). The pH of solution was adjusted with 0.1M HCl and 0.1 M NaOH solutions. The amount of dye adsorbed and percentage removal of MG were calculated using Eqs. (1) and (2), respectively.

$$q_e = (c_0 - c_t) \frac{V}{M} \quad (1)$$

$$\% \text{ Removal} = \frac{c_0 - c_t}{c_0} \times 100 \quad (2)$$

where:

q_e - amount of dye in mg per gram of adsorbent.

C_i and C_e - are respectively initial concentration and equilibrium time of MB (mg/l).

V - volume of solution.

M - mass of adsorbent

2.4 Adsorption isotherm study

The adsorption study was determined by Langmuir and Freundlich model. The Langmuir isotherm was based on assumption that adsorption of adsorbate onto adsorbents occurred in monolayer adsorption onto a surface [4]. The isotherm is represented by Eq. (3)

$$\frac{c_e}{q_e} = \frac{1}{b q_{max}} + \frac{1}{q_{max}} \quad (3)$$

The linear plot of specific adsorption (c_e/q_e) against the equilibrium concentration (c_e) was obtained. The constants b and q_e relate to the energy of adsorption and maximum adsorption capacity, and their values are obtained from the slope and interception of the plot.

Freundlich adsorption isotherm is obeyed by the adsorptions where the adsorbate forms a monomolecular layer on the surface of the adsorbent. The parameters, q_{max} represents the amount adsorbed per amount of adsorbent at the equilibrium (mg/g), c_e represents the equilibrium concentration (mg/L), and K_f and n are parameters that depend on the adsorbate and adsorbent.

$$\ln q_s = \ln K_f - \frac{1}{n} \ln c_s \quad (4)$$

where K_f and n are Freundlich constants which correspond to adsorption capacity and adsorption intensity, respectively.

3. RESULTS AND DISCUSSION

3.1 Effect of adsorbent dose and initial dye concentration

These experiments were carried out to determine the optimal dose of adsorbent. Experiments varying the dose of activated carbon derived from *Amorpha fruticosa* (from 0.1 to 1.0 g/100 ml) were conducted to determine the optimal dose for malachite green adsorption. As shown in the Fig 2, adsorption efficiency increased with higher adsorbent doses. Initial increases were significant, with adsorption efficiency rising from 20% at 0.1 g/100 ml to 75% at 0.6 g/100 ml. Beyond this point, the efficiency gains diminished, reaching a maximum of 82% at 1.0 g/100 ml. This suggests that the optimal adsorbent dose lies around 0.6 g/100 ml, where significant dye removal is achieved efficiently.

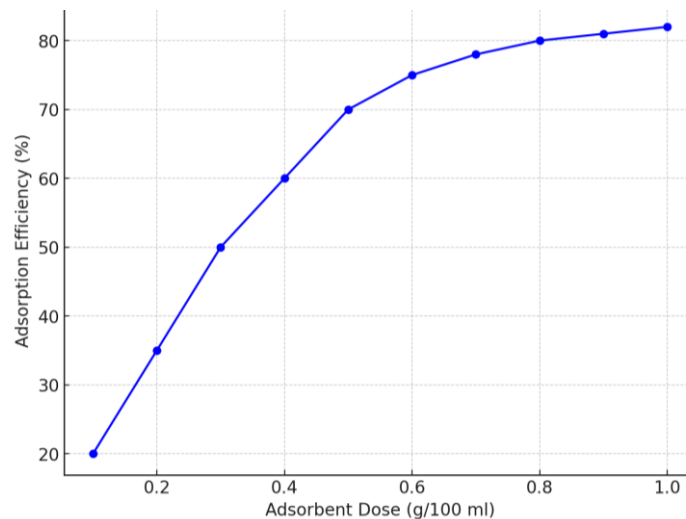


Figure. 2: Effect of AC adsorbent dose on malachite green adsorption

3.2 Effect of contact time

The amount of Malachite green adsorbed on AC increased for a short time and slowed gradually when equilibrium was approached. At beginning of the experiments, the active sites were completely free, so adsorbate was easily could bind to adsorbates. As the contact time increased, the active sites were covered by adsorbates until equilibrium was reached at 27 min.

3.3 Effect of pH

The efficiency of malachite green dye removal using activated carbon derived from *Amorpha fruticosa* was investigated across different pH levels (3, 5, 5.5, and 9). The adsorption efficiency was evaluated to determine the optimal pH for maximum dye removal. At pH 3, the adsorption efficiency was 40%, indicating moderate dye removal under acidic conditions. Increasing the pH to 5 significantly enhanced the adsorption efficiency to 60%. This trend continued, reaching a peak efficiency of 75% at pH 5.5, suggesting that slightly acidic to neutral conditions are optimal for malachite green adsorption. However, further increasing the pH to 9 resulted in a decrease in adsorption efficiency to 50%. This decline at higher pH levels can be attributed to the increased presence of hydroxide ions, which compete with the dye molecules for adsorption sites on the activated carbon surface. Additionally, the dye molecules might undergo structural changes at higher pH, reducing their affinity for the adsorbent. In conclusion, the optimal pH for malachite green removal using activated carbon derived from *Amorpha fruticosa* is around 5.5. These findings highlight the importance of pH control in optimizing the adsorption process for efficient dye removal from aqueous solutions.

Adsorption isotherm study

The adsorption isotherm study was conducted to determine the adsorption characteristics of Malachite Green (MG) onto Activated Carbon (AC) derivatives from *Amorpha fruticosa* L. The equilibrium data were fitted to the Langmuir and Freundlich isotherm models to derive the corresponding isotherm parameters.

Table 1: Results of Langmuir and Freundlich isotherm plots for the adsorption of MG onto AC.

Model	Isotherm parameters		
Langmuir	q_m (mg /g) 27.07	K_L (L/mg) 0.049	R^2 0.9987
Freundlich	n (mg/g) 2.12	(mg/g) 3.39	R^2 0.9313

The Langmuir isotherm model assumes monolayer adsorption onto a surface with a finite number of identical sites. The high R^2 value indicates a very good fit of the Langmuir model to the adsorption data, suggesting that the adsorption of MG onto AC predominantly occurs as a monolayer on a homogeneous surface. The R^2 value for the Freundlich model, while slightly lower than that of the Langmuir model, still suggests a reasonable fit, indicating the presence of heterogeneous adsorption sites on AC. The adsorption study indicates that the Langmuir isotherm model provides a better fit for the adsorption of MG onto AC, as evidenced by the higher R^2 value (0.9987) compared to the Freundlich model (0.9313). The maximum adsorption capacity (q_m) was found to be 27.07 mg/g, with a Langmuir constant (K_L) of 0.049 L/mg. The Freundlich constants, K_F and n , were determined to be 3.39 (mg/g) (L/mg) vs (1/n) and 2.12,

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respectively. The value of the term $(1/n)$ gives an indication of the favourability of the sorbent/adsorbate systems (Saira Bibi et al. 2020). These results suggest that the adsorption process is predominantly monolayer and occurs on a homogeneous surface with some degree of heterogeneity.

4. CONCLUSIONS

Utilizing *Amorpha fruticosa* L., an invasive species, for activated carbon production is economically advantageous and environmentally beneficial by turning a problematic plant into a valuable resource. The adsorption study showed that the maximum adsorption of MB onto AC was obtained at pH 5.5 and room temperature at the constant time of 27 minutes. Results of this study collectively indicate that activated carbon derivatives from *Amorpha fruticosa* L. has significant potential for efficient malachite green adsorption in water treatment applications.

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MONITORING OF CLIMATE ELEMENTS AND AIR QUALITY INDEX IN THE CITY OF BIHAĆ IN 2023

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Key words: air monitoring, air quality, climatic parameters, air quality indeks

ABSTRACT:

Clean air is the need of every living being on Earth. Along with the air, man inhales an increasing concentration of pollutants, which are the product of industrialization, traffic, accidents, but also everyday human activities. Air quality is the concentration of pollutants in the air over a period of time. Determination of air quality is preceded by air quality monitoring, which serves to preventively register exceeded limit values of air quality parameters affecting the health of the population and the environment. This paper presents the results of climatic parameters, namely: temperature, humidity, air flow rate, light intensity, ultraviolet (UV) and electromagnetic radiation, noise and pressure, which were measured to determine the physical state of the ground air layer. In addition to climatic elements, the results of measuring air quality parameters were presented, namely, concentrations as follows: particulate matter (PM_{2,5} and PM₁₀), carbon monoxide (CO), ozone (O₃), sulfur dioxide (SO₂) and nitrogen dioxide (NO₂), whose measurement is being performed by the Federal Hydrometeorological Institute of Bosnia and Herzegovina. The area of Una-Sana Canton and the city of Bihać in 2023 is not characterized by excessive pollution, except one day 17.10.2023. when concentration of CO (565) was indexed as dangerous but this does not diminish the need to establish an appropriate air quality management system. Air quality management in the city of Bihać is especially necessary in case of accidents, such as fires in factories and forest areas.

1. INTRODUCTION

The basis of human survival consists of three factors, namely air, water and food. We can survive without food even for several weeks, without water for several days, and without air we can survive only a few minutes. A person inhales about 20,000 liters of air every day, and at the same time an increasing concentration of harmful and toxic substances, created as a product of industrialization, transport, but also of everyday human activities. Air quality monitoring makes it possible to react in case of special situations in which air quality limit values, warning thresholds and alarm thresholds are exceeded, and which may endanger the life and health of residents and the environment. The aim of air quality monitoring is to determine the air quality in the stations of background pollution, regional and cross-border transmission, in the areas of national parks, protected landscapes, protected areas, natural monuments, sensitive environmental systems, and cultural and natural heritage and in settlements and industrial areas. At the Meteorological Station of the city of Bihać, there is an automatic air quality measuring station whose data is read by the Federal Hydrometeorological (FHMZ). The air quality index represents the state value of the observed pollutants measured at the measuring point. The index is expressed as a numerical value, marked with the corresponding color and indicates the state of air quality, which can be good, moderate, unhealthy for sensitive groups, unhealthy, very unhealthy and dangerous. The air quality index is determined based on the main sources of urban air pollution in traffic, trade and industry, and the parameters that are read are PM_{2.5}, PM₁₀, CO, O₃, SO₂ and NO₂. The results are available daily on the FHMZ website. The climate together with relief, fertile soil and the available amount of water are the basic natural factors for the gathering of the population in a certain territory and the monitoring of climatic elements is an integral part of air monitoring.

2. LOCATION DESCRIPTION

The town of Bihac, located in the Una-Sana Canton in the northwest of Bosnia and Herzegovina, exudes the beauty of emerald colors thanks to the enchanting river Una on whose banks it is located. The town lies in the spacious Bihac basin, which is closed from the west and southwest by the Pljesevica mountain, while it is bounded by the Grmec mountain to the east and southeast. Bihac is located at an altitude of 231 meters. Thanks to the favorable geographical and climatic conditions, this area has been inhabited since the prehistoric period. The relief in the area of the City of Bihac is diverse, and the majority of it is fields, hills and mid-mountain land. The area of the city of Bihać has a moderate mountain type of climate, which is diverse due to the influence of air masses from neighboring and distant areas. Summers are hot and dry, winters are cold with a lot of precipitation.

3. MATERIAL AND METHODS

In this work, devices for reading climate elements were used, namely: Multiparameter environment meter PCE EM 888, for measuring climate elements: temperature, relative humidity, wind speed, pressure and light intensity. To measure ultraviolet radiation, we used the device UV light meter PCE-UV34 [mw/cm] with accuracy $\pm (4\% \text{ FS} + 2\text{dgt})$. To measure noise, we used the device PCE-EM 882 - a multifunctional device that, in addition to noise, can also measure humidity, temperature and light intensity. To measure electromagnetic radiation, we used a PCE EMF 823 device with a precision of $\pm 4\%$.



Figure 1: Used measuring device (Multiparameter Environment Meter (Model: PCE EM 888))

4. RESULTS AND DISCUSSION

The following tables show the results of the measurement of climatic elements, as well as the concentration of pollutants and the air quality index:

Table 1: Climate elements

PARAMETER		TEMPERATUR E	RELATIVE HUMIDITY	CARBON DIOXIDE (CO) ₂	WIND SPEED	INTENSITY OF LIGHT	ELECTOMAGNETIC RADIATION	UV RADIATION	PRESSURE	NOISE
MEASURING UNIT		°C/F	%RH	ppm	m/s	Lux	mG	mW/cm ²	hPa	dB
DATE	10.10.2023	24°C	63,1	419	0	13,9	0,1	0,332	992,9	55,4
	17.10.2023	11°C	45,4	455	1,4	40,5	0,1	0,234	744,7	75,3
	24.10.2023	22°C	44,3	403	2,7	43,3	3,4	0,800	736,1	60,1
	31.10.2023	21.1°C	52,3	390	4,1	61,8	6,1	1,154	731,6	70,7
	07.11.2023	16°C	71,5	461	0,3	307,6	3,4	0,190	740,8	68,8
	14.11.2023	18.6°C	54,5	403	4,4	451,0	5,9	0,081	739,1	50,9
	28.11.2023	7°C	46,4	464	0,8	369,0	1,3	0,205	723,7	52,2
	05.12.2023	6°C	37,9	463	0,1	218,8	23,2	0,165	737,7	54,4
	12.12.2023	10°C	58,6	411	0,8	0	5,4	0,055	738,2	54,1
	26.12.2023	14°C	42,9	405	1,7	50,5	5,2	0,167	741,5	57

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Table 2: Air quality index

POLLUTANTS	PM _{2.5}	PM ₁₀	CO	O ₃	SO ₂	NO ₂	AIR QUALITY INDEX
DATE							
10.10.2023	41	30	4	13	2	8	
17.10.2023	19	21	565	66	6	10	
24.10.2023	14	13	2	88	2	1	
31.10.2023	14	10	2	79	2	2	
07.11.2023	20	10	4	33	3	9	
14.11.2023	18	9	3	28	2	7	
28.11.2023	16	6	27	28	1	5	
05.12.2023	59	29	8	23	1	10	
12.12.2023	18	7	6	23	1	*	
26.12.2023	58	28	9	8	1	*	

It doesn't measure up	Good (0-50)	Moderate (51-100)	Unhealthy for sensitive groups (101-150)	Unhealthy (151-200)	Very unhealthy (201-300)	Dangerous (301-500)

Air quality index of PM₁₀, SO₂ and NO₂ were measured as good (0-50). Concentration of PM_{2.5} were slightly increased in december (5th and 26th of december), as well as concentration of ozone in October. The concentration of CO were within allowed values except from 17.10.2023 when it was showed as dangerous.

5. CONCLUSION

Based on the research carried out in this paper, we can conclude that the concentrations of pollutants in the city of Bihać in 2023 are mostly within the permitted ratio.

The area of Una-Sana Canton and the city of Bihac in 2023 is not characterized by excessive pollution, except one day 17th October 2023. On this day, concentration of CO (565ppm) was indexed as dangerous but this doesn't request the need to establish an appropriate air quality management system. Air quality management in the city of Bihac is especially necessary in case of accidents, such as fires in factories and forest areas.

Monitoring of climate elements, pollutant concentration and air quality index provides information on the composition of the analyzed air. Monitoring enables action due to accidental situations and timely issuing of instructions and recommendations to residents to stay in closed rooms, because the increasing concentration of pollutants has a negative effect on the respiratory system. Climatic elements make up the climate of a certain area and they contribute to the movement and distribution of air pollutants.

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Eighth International Scientific Conference
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SUSTAINABLE ANIMAL AND PLANT PRODUCTION



INFLUENCE OF CULTIVATION SYSTEM ON STRAWBERRY QUALITY AND YIELD

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Key words: strawberry, Clery, cultivation system, high tunnels, open field

ABSTRACT:

The primary objective of this study was to investigate the differences in phenological and morphological-chemical characteristics of "Clery" strawberries cultivated under different cultivation systems (high tunnel cultivation compared to the standard open-field cultivation).

The obtained results indicate that the cultivation system factor had a statistically significant impact on all observed properties except for vitamin C content. Strawberries grown in high tunnels ripened earlier compared to those grown in the open field. Strawberries cultivated in high tunnels exhibited a higher number of plants per planting site and a greater yield per plant and planting site, compared to strawberries cultivated in the open field. Additionally, morphological fruit characteristics (weight, length, width, and fruit shape index) were higher in the high tunnel system compared to the open-field system. Higher values of chemical fruit properties such as dry matter content, vitamin C, and total acidity were obtained from fruits grown in the open field compared to those from high tunnels. The pH value of the fruit was higher in the high tunnel system compared to the open-field system.

The investigated "Clery" strawberry variety proved to be highly suitable for cultivation in the high tunnel system, enabling extended harvesting seasons, better yields, and improved morphological fruit traits. Moreover, the chemical characteristics of the fruit were not compromised in quality compared to fruits grown in the open field. In a general sense, strawberry production in high tunnels holds great economic potential for small-scale farmers.

1. INTRODUCTION

The strawberry (*Fragaria x ananassa* Duch) occupies second place in terms of economic importance within the group of pomological fruits in Bosnia and Herzegovina, right behind the raspberry (Behmen et al., 2020). It is one of the most popular fruit species in the world, containing numerous important nutritional components such as minerals and vitamins (Khammayom et al., 2022). Given that the strawberry is an important crop worldwide in terms of its commercial value (Nagamatsu et al., 2021), it is grown on all arable lands from the North Pole to tropical areas. Most of the production is in the northern hemisphere (98%), but there are no genetic or climatic barriers preventing its further spread to the southern hemisphere (Družić et al., 2006). The most significant impact on the quality of strawberry production, and hence the quality of the fruit, is the variety, as well as factors such as climate, irrigation, soil fertility, temperature, cultivation system, and harvest time (Himelrick and Galletta, 1990). Wysocki et al. (2012) emphasize that ensuring the quality parameters of strawberry yield requires the right choice of varieties, cultivation technology, as well as fruit harvesting and marketing. These factors have a greater or lesser impact on fruit quality depending on the cultivation system (Radunić et al., 2015). In Bosnia and Herzegovina, strawberries can be successfully grown in the southern parts of the country, where the Mediterranean climate prevails, as well as in areas with moderately continental and continental climates. In the south of the country, strawberries ripen up to a month earlier compared to northern areas, i.e., outside the market season, thereby achieving a high fruit price. Since out-of-season strawberry production commands high prices, farmers are interested in extending this period as much as possible. One way to extend the strawberry production period worldwide is the cultivation system in protected environments such as greenhouses, polytunnels, and high and low tunnels. The purpose of this study is to develop and evaluate the cultivation of strawberries in high tunnels to extend the production season and improve its quality. The high tunnel strawberry cultivation system has the potential to increase yields and fruit quality by reducing losses and damage mainly caused by precipitation, and through disease control.

2. MATERIALS AND METHODS

2.1 Materials of the Study

The research experiment was conducted on a family farm in the area of the City of Bihać during the 2023 growing season. The study monitored the phenological characteristics of flowering and fruit ripening, yield analyses, and morphological and chemical analyses of the fruit. The plantation where the experimental observations were carried out was established in August 2022. It covers an area of 1060 m², of which 910 m² is under a protected system-high tunnels-and 60 m² is open field. The type of seedlings planted was frigo, A+ quality, and the variety analyzed was "Clery". In both the protected environment and the open field, the strawberries were planted

on August 7, 2022, on machine-made beds 80 cm wide and 15 cm high, covered with black mulch film, using two drip irrigation lines. The planting density was set at 40 x 30 cm, with six plants per square meter. In early September 2022, PE film was installed over the tunnel arches and removed on December 7, 2022.

The average annual temperature based on analyses from the past ten years in the City of Bihać area is 12.46 °C, while the average precipitation is 118.82 l/m².

2.2 Phenological, Morphological, and Yield Testing Methods for Strawberries

The phenological observations conducted in this study were described by Tomić et al. (2018) and Milivojević et al. (2006). These observations included:

1. Flowering: Recording the start and end dates of flowering, and the duration in days.
2. Fruit Ripening: Recording the start and end dates of fruit ripening, and the duration in days.

The morphological analyses of the fruit and yield testing were performed on 60 plants and 60 fruits, with three repetitions of 20 plants/fruits each. The methodology for these analyses was a modified version of the one described by Milivojević et al. (2006) and Behmen et al. (2020), which includes the following measurements:

1. Number of Crowns per Planting Site: Counting the number of crowns at each planting site.
2. Yield per Crown (g): Measuring the yield per crown in grams.
3. Fruit Mass (g): Weighing individual fruits to determine their mass in grams.
4. Fruit Length (mm): Measuring the length of the fruit in millimeters.
5. Fruit Width (mm): Measuring the width of the fruit in millimeters.
6. Fruit Shape Index: Calculating the fruit shape index from the ratio of fruit length to fruit width.

2.3 Methods for Chemical Analysis of Strawberry Fruits

The chemical analyses of strawberry fruits was conducted on a homogenized sample of 3 kg of fruit, with three repetitions of 1 kg each. The selection of parameters for chemical analysis was based on the study by Družić et al. (2006). The following quality parameters were examined using standard analysis methods: Soluble Solids Content (SSC) Method: Refractometric analysis according to AOAC (1995). pH Value Method: Measured using a pH meter according to AOAC (1995). Vitamin C Content Method: Iodometric titration method according to Helmenstine (2007). Total Acidity Method: Titration method with NaOH according to AOAC (1984).

2.4 Statistical methods

The obtained results of yield, morphological, and chemical analyses of the fruit were analyzed using an appropriate statistical method, in this case, one-way analysis of variance (One-Way ANOVA) at a significance level of 5%. The entire data set was processed using the statistical analysis software Past (Hammer et al., 2001).

3. RESULTS AND DISCUSSION

3.1 Results of Phenological Research

The onset of vegetation for the "Clery" variety in the studied agroecological conditions was recorded on February 15, 2023. Subsequently, when optimal conditions for putting high tunnels into operation were achieved, i.e., when the risk of snowfall ceased, the PE film was installed on the tunnel structure on February 24, initiating the research experiment. Analysis of phenological data revealed that the "Clery" variety grown in high tunnels started flowering three weeks earlier compared to the open field. Specifically, flowering in the high tunnels began on March 20, while in the open field, it started on April 10. The duration of flowering in the high tunnels was 30 days, significantly shorter than in the open field (19 days). Tolić et al. (2010) also noted an earlier onset of flowering for the "Clery" variety in a greenhouse compared to the open field, with an average difference of nine days in their research. Similarly, fruit ripening and harvesting for the "Clery" strawberry variety began nine days earlier in the high tunnel system (April 27) compared to the open field (May 10). This can be attributed to the more favorable conditions for strawberry development in the protected environment compared to the open field. The end of fruit ripening in the high tunnels was registered on June 6, while in the open field, it was on June 15. The duration of the fruit ripening phase, i.e., harvesting for the examined variety in the high tunnels, was 41 days, compared to 37 days in the open field. These results indicate the justification for strawberry cultivation in high tunnels to extend the market supply of fresh fruit of this fruit species. Considering that the standard for strawberry cultivation in our conditions involves cultivation in the open field on raised beds covered with black mulch film and a drip irrigation system, in this cultivation system in this experimental trial, the market supply of fresh fruit lasted for 37 days, while in the high tunnels, it lasted for 41 days, and in a combination of these two cultivation systems, the period lasted for 50 days.

a. Strawberry Yield Results

The results of the research on yield parameters of the "Clery" strawberry variety, depending on the cultivation system, were analyzed using one-way analysis of variance and are presented in Table 1. Based on the obtained results of statistical data processing, it was found that the cultivation system factor has a significant impact ($p \leq 0.05$) on all investigated yield parameters of the "Clery" strawberry variety.

Eighth International Scientific Conference
"June 5th - World Environment Day"

The counting of strawberry crowns per planting site was conducted in spring 2023 during the flowering phase. The average number of strawberry crowns of the "Clery" variety planted in high tunnels was 2.87 per planting site, while in the open-field cultivation system, it was significantly lower at 1.57. Additionally, higher yields per crown and per planting site (338.19 g) of the "Clery" strawberry variety were observed in high tunnels compared to the open-field cultivation system (126.65 and 338.19 g), respectively. This indicates that the protected environment, i.e., higher temperatures in the protected environment, favor the development of a greater number of crowns and increased yield.

In the research by Tolić et al. (2010), the average number of crowns per planting site was almost the same in the greenhouse and open-field strawberry cultivation systems (2.73 and 2.72), which is close to the average in the high tunnel system in this study. Behmen et al. (2020) obtained higher average values for the number of crowns per planting site (high tunnel 3.70; open field 3.20).

Table 1: The average values of yield parameters for the "Clery" strawberry variety with the results of statistical data processing (One-Way ANOVA)

Observed properties	High Tunnel	Open Field	F - test
Average number of crowns per planting site	2.87 ± 0.83	1.57 ± 0.70	p≤0,05
Yield per crown (in grams)	126.65 ± 10.00	98.62 ± 41.71	p≤0,05
Yield per planting site (in grams)	338.19 ± 190.17	158.26 ± 66.36	p≤0,05

*One-Way ANOVA: p≤0,05 – statistically significant influence of the factor on the observed characteristic; p>0,05 – no statistically significant influence of the factor on the observed characteristic.

In the results of Behmen et al. (2020), the calculated average yield per crown was 199.64 g in the high tunnel system and 150.60 g in the open field. Regarding yield per planting site, Behmen et al. (2020) obtained double the yields of the "Clery" strawberry variety in both the high tunnel system (739.87 g) and the open field (481.93 g) compared to this study. Additionally, Tolić et al. (2010) also obtained higher yields compared to this study, with an average yield of 614.75 g in the protected environment and 602.67 g in the open field. Milivojević and Nikolić (2015) highlighted that the "Clery" variety in the raised bed cultivation system in the greenhouse had an average yield of 518.00 g.

b. Results of Morphological Analysis of Fruit

The obtained results of morphological analysis of "Clery" strawberry fruit, depending on the cultivation system, processed using one-way analysis of variance, are presented in Table 2. Similar to the analysis of yield parameters, the cultivation system factor has a significant influence ($p \leq 0.05$) on the weight, length, width, and shape index of "Clery" strawberry fruit.

Eighth International Scientific Conference "June 5th - World Environment Day"

Cultivation in high tunnels had a positive effect on the weight of "Clery" strawberry fruit, with obtained average values higher (17.00 g) compared to those determined in the open-field system (11.45 g). Given that the length, width, and weight of strawberry fruit are positively correlated (Grijalba et al., 2015), this means that the values of length, width, and shape index of the fruit accompanying its weight were higher in the high tunnel system (39.57 mm, 30.65 mm, and 1.29) than in the open-field system (30.76 mm, 25.50 mm, and 1.21). Regarding strawberry cultivation in the open field, all available studies record a higher average fruit weight for the "Clery" variety (ranging from 29.8 g to 12.27 g) Milivojević et al. (2010), Milivojević et al. (2009), Behmen et al. (2020), Tolić et al. (2010), Milivojević et al. (2007), and Kikas et al. (2016) compared to the average weight obtained in this study. Lower values of morphological fruit characteristics in this study can be explained by the high amount of precipitation and lower average temperatures in the months of April, May, and June of the experimental year.

Table 2: Average Values of Morphological Analysis of "Clery" Strawberry Fruit with the Results of Statistical Data Processing (One – Way ANOVA)

Examined properties	High tunnel	Open field	F - test
Fruit weight(g)	17.00 ± 5.19	11.45 ± 4.03	p≤0,05
Fruit length (mm)	39.57 ± 6.33	30.76 ± 5.07	p≤0,05
Fruit width(mm)	30.65 ± 4.02	25.50 ± 3.64	p≤0,05
Fruit Shape Index	1.29 ± 0.14	1.21 ± 0.11	p≤0,05

*One – Way ANOVA: p≤0,05 – statistically significant influence of the factor on the observed characteristic; p>0,05 – no statistically significant influence of the factor on the observed characteristic.

Radunić et al. (2015) record similar values for the average length and width of "Clery" strawberry fruit grown in high tunnels and in the open field compared to these studies, while Milivojević et al. (2009; 2010; and 2007) report higher average values for the length and width of strawberry fruit grown in the open field compared to these studies. The obtained results of the fruit shape index are consistent with the results published by Milivojević et al. (2006; 2007; 2009; and 2010) and Milivojević and Nikolić (2015). Considering these results, Milivojević et al. (2010) conclude that the "Clery" variety shows no tendency to vary in fruit shape depending on its size or under the influence of the cultivation system.

c. Results of the chemical analyses of the fruit

The results of the chemical analyses of the "Clery" strawberry fruit, depending on the cultivation system, processed using one-way ANOVA, are presented in Table 3.

Eighth International Scientific Conference "June 5th - World Environment Day"

Table 3: Average Values of Chemical Analyses of "Clery" Strawberry Fruit with the Results of Statistical Data Processing (One – Way ANOVA)

Examined properties	Visoki tunel	Otvoreno polje	F - test
Dry matter (°Brix)	6.56 ± 0.33	7.07 ± 0.13	p≤0,05
Fruit pH	3.00 ± 0.09	2.70 ± 0.04	p≤0,05
Vitamin C (mg/100g FW)	107.83 ± 5.97	107.92 ± 4.01	p>0,05
Total acidity (%)	0.60 ± 0.14	0.74 ± 0.05	p≤0,05

*One – Way ANOVA: p≤0,05 – statistically significant influence of the factor on the observed characteristic; p>0,05 – no statistically significant influence of the factor on the observed characteristic.

The results of statistical data processing using one-way analysis of variance (One-Way ANOVA) indicate that the cultivation system factor has a significant impact ($p \leq 0.05$) on the soluble solids content, pH reaction, and total acidity of the "Clery" strawberry fruit, while no significant impact ($p \geq 0.05$) was found on the vitamin C content in the fruits.

"Clery" strawberries grown in open fields had higher soluble solids content (7.07 °Brix) compared to those grown in high tunnels (6.56 °Brix). Additionally, the values of vitamin C content and total acidity were higher in fruits grown in open fields (107.92 mg/100g FW and 0.74%) compared to those grown in high tunnels (107.83 mg/100g FW and 0.60%). Only the pH reaction of the fruit was higher in fruits grown in high tunnels (3.00) compared to open fields (2.70). Several authors have recorded higher values of soluble solids content in both open field and high tunnel systems compared to this study (Radunić et al., 2015; Voća et al., 2008 and 2009; Kikas et al., 2016; Milivojević et al., 2007; 2006; 2009 and 2010; Đilas et al., 2011). Lower values of soluble solids content compared to this study were recorded in the study by Behmen et al. (2020). Regarding the pH reaction of the fruit, Radunić et al. (2015) and Voća et al. (2008 and 2009) recorded higher values compared to this study.

The vitamin C content in "Clery" strawberry fruits in this study was higher in both cultivation systems compared to the values published by Voća et al. (2008 and 2009) and Milivojević et al. (2009). Voća et al. (2006) highlight that the presence of vitamin C in strawberry fruit, as a powerful antioxidant, either acting alone or in combination with various phenolic compounds, significantly contributes to the exhibition of antioxidant activity. Its impact is also reflected in preventing browning and discoloration of the fruit, as well as increasing their shelf life (Milivojević et al., 2009). Regarding total acid content, Voća et al. (2009) obtained higher values in both open fields and high tunnels compared to this study. Additionally, Milivojević and Nikolić (2015) obtained higher values of total acid content in high tunnel systems compared to this study. The average total acid content of "Clery" strawberries grown in open fields is quite variable, ranging from 0.46 to 1.4% (Milivojević et al., 2010; 2006; 2009 and 2007; Đilas et al., 2011; Kikas et al., 2016). According to Đilas et al. (2011), the values of total acidity of the fruit

can indicate the degree of ripeness at the time of harvest, with a higher level of acidity corresponding to a lower degree of ripeness and vice versa. Additionally, Voća et al. (2008) emphasize that the ratio between total soluble solids and total acidity is very important in determining fruit quality. The ratio between sugars and acids in strawberries and other berries can be an important indicator of fruit flavor (Crespo et al., 2010).

4. CONCLUSIONS

The cultivation system factor had a statistically significant impact on all observed properties except for the vitamin C content. Strawberries grown in high tunnels ripen earlier compared to those grown in the open field, thus reaching the market first. Strawberries grown in high tunnels were characterized by a greater number of runners per planting site, higher yield per runner and per planting site compared to strawberries grown in the open field. The morphological characteristics of the fruit (weight, length, width, and shape index) were higher in the high tunnel system compared to the open field system. Higher values of fruit chemical properties such as dry matter content, vitamin C content, and total acidity were obtained from fruits grown in the open field system compared to those in high tunnels. A higher pH value of the fruit was recorded in the high tunnel system compared to the open field system. The studied "Clery" strawberry variety proved to be particularly suitable for cultivation in high tunnels, achieving an extended strawberry harvest season, better yields, and fruit morphological properties. Additionally, the chemical characteristics of the fruit were not inferior in quality compared to fruits grown in the open field. Overall, strawberry production in high tunnels has great economic potential for small farmers.

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Eighth International Scientific Conference
"June 5th - World Environment Day"

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MOLECULAR DETECTION AND IDENTIFICATION OF SELECTED ISOLATES ALTERNARIA SPP.

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Key words: *A. dauci* isolates, *A.alternata* isolates, PCR, primers, amplification, detection, identification

ABSTRACT:

Previous research has shown that the morphological and breeding characteristics are not sufficient for the proper determination and characterization of Alternaria spp. fungi of the carrot pathogen. Polymerase Chain Reaction (PCR) was used in order to detect the obtained Alternaria spp isolates originating from Bosnia and Herzegovina and Serbia through molecular detection, as well as to confirm the results obtained by conventional phytopathological methods. A total of 50 monosporous Alternaria spp. isolates were selected for molecular identification, of which 25 isolates belonged to group A and 25 isolates to group B, as well as two reference isolates from Serbia, namely: CLA-108 (A. dauci) and Aa- 82 (A. alternata). After the extraction of the total DNA of the tested Alternaria spp. isolates, PCR reactions were performed with a pair of universal primers ITS1/ITS4 in order to obtain a specific DNA fragment that includes the ITS genomic region (part of 18S rRNA, ITS1, 5.8 rRNA, ITS2 and part of 28S rRNA) and visualization of the obtained products by electrophoretic separation in agarose gel was performed. In addition to standard primers that amplify the ITS region of the genome, specific primers with target sequences within this region were also used for the detection of individual species within the Alternaria genus, namely: 1) ADF2/ADR1, which amplify the ITS region of the rDNA of A. dauci species, and 2) AAF2/AAR3, which amplify the ITS region of rDNA of A. alternata species. The analysis of amplified fragments of tested isolates originating from carrots

revealed the occurrence of fragments of the expected size of about 545 bp in all tested isolates. The positive reaction that was obtained in all samples confirms that a phytopathogenic fungus occurred in each of the analysed samples. No amplification occurred in the negative control (C).

1. INTRODUCTION

Previous research has shown that morphological and breeding characteristics are not sufficient for proper determination and characterization of fungi of the *Alternaria spp.* genus. For the aforementioned reason, molecular identification and characterization of the collected isolates was carried out in these researches. Polymerase Chain Reaction (PCR) was used in order to detect the obtained isolates of *Alternaria spp.* originating from Bosnia and Herzegovina and Serbia, as well as to confirm the results obtained by conventional phytopathological methods. Polymerase chain reaction (PCR) revolutionized molecular diagnostics and systematics of fungi and other microorganisms. This technique represents *in vitro* multiplication (amplification) of specific DNA sequences or loci of the examined organism, using specific or intermediate oligonucleotide sequences (primers) and thermostable DNA polymerase Živković (2007).

2. MATERIAL AND WORKING METHODS

After breeding monospored cultures of *Alternaria spp.* isolates according to the DNA extraction procedure, their DNA was extracted, after which PCR with one set of universal primers and seven PCR reactions with specific primers were applied and visualization of the obtained products by electrophoretic separation in agarose gel was performed. A total of 50 monosporous isolates of *Alternaria spp.* were selected for molecular identification, of which 25 isolates belonged to group A and 25 isolates to group B, as well as two reference isolates from Serbia, namely: CLA108 (*A. dauci*) and Aa82 (*A. alternata*).

2.1 DNA extraction

Colonies of tested *Alternaria spp.* isolates were grown on a potato-dextrose agar in the dark, at a temperature of 25 °C for 10 days. DNA extraction was performed according to the method described by Day et Shattock (1997). According to the recommended procedure, 10 mg of the mycelia of tested fungal isolates frozen at -80 °C in liquid nitrogen were homogenized in the presence of 800 µl of 2% CTAB buffer. The resulting suspension was transferred to a microtube with a volume of 2 ml and incubated for 1 h at a temperature of 65 °C. During the incubation, the contents of the tube were vigorously mixed every 15 minutes. After incubation, 800 µl of chloroform was poured into each microtube and mixed on a Vortex device for 10 s, then centrifuged for 10 minutes at 11,000 rpm (rpm = rotations per minute) in a centrifuge (Eppendorf 5417R, Germany) at a temperature of 4 °C.

After centrifugation, two phases were clearly separated. The resulting supernatant, approximately 500 µl in volume, was carefully pipetted into a new 1.5 ml microtube to which 300 µl of isopropanol was then added. The microtubes prepared in this way were then incubated for 10 minutes at room temperature, after which they were centrifuged again for 15 minutes at 11,000 rpm at 4 °C. After centrifugation, the supernatant was carefully poured off and the microtubes were washed with 600 µl of ice-cold 70% ethanol and centrifuged again for 10 minutes at 11,000 rpm. After that, the previously added ethanol was carefully discarded, after which the microtubes were left open for 10-15 minutes at room temperature to allow excess ethanol to evaporate. After drying, the DNA pellet was resuspended in 100 µl of TE buffer. The obtained DNA extract was kept in a freezer at a temperature of -20 °C until use.

2.2 Polymerase Chain Reaction, PCR

For the detection and identification of the tested isolates of *Alternaria spp.*, the PCR method was applied with eight different pairs of primers, namely: 1) ITS1/ITS4, which amplify the ITS region of rDNA of Eukaryotes; 2) ADF2/ADR1, which amplify the ITS region of *A. dauci* rDNA; 3) AAF2/AAR3, which amplify the ITS region of *A. alternata* rDNA; 4) ARF2/ARR3, which amplify the ITS region of *A. radicina* rDNA; 5) Pa2071/Pa2072, which amplify the ITS region of *A. radicina*/ *A. petrosellini* rDNA; 6) Alt-for/Alt-rev, which enable the amplification of the part for the $\alpha 1$ gene of *A. petrosellini*, 7) EF1-728F/EF1-986R, which enable the amplification of the part of the EF1- α gene of *A. petrosellini* and 8) Bt1a/Bt1b, which enable the amplification of the part for the β -tubulin gene of *A. carotiincultae*. Table 1 lists the sequences and expected amplicon sizes of the primers used.

The PCR reaction was performed in a working volume of 25 µl, using 12.5 µl of 2X PCR Master mix (Fermentas UAB, Lithuania), 9 µl of RNase-free water, 1.25 µl of each primer (100 pmol/µl) and 1 µl of extracted total DNA. As a negative control, RNase-free water was used, which was added to the prepared PCR mixture instead of the target DNA. The PCR reaction is 35 cycles consisting of denaturation for 2 min. at 94 °C, hybridization for 30 s at 57 °C, elongation for 1 minute at 72 °C, followed by final elongation at 72 °C for 10 min. A reference isolate (isolate from the collection of the Faculty of Agriculture, University of Belgrade) was used as a positive control, and RNase-free water, which was added to the prepared PCR mixture instead of the target DNA, was used as a negative control. One isolate from the collection of the Department of Phytopathology, Institute of Phytomedicine, Faculty of Agriculture, University of Belgrade was used as reference isolates for the species *A. dauci* and *A. alternata*.

Eighth International Scientific Conference "June 5th - World Environment Day"

Table 1: Overview of primers used for the detection of studied isolates of *Alternaria* spp.

Targeted sequence	Primer name	Sequence 5'– 3'	Fragment size (bp)	Source
ITS of Eukaryotes	ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	500–600	Konstantinova <i>et al.</i> , 2002
ITS of <i>A. dauci</i>	ADF2 ADR1	GCAATCAGCGTCAGTAACAACA CGCAAGGGGAGACAAAAA	~345	Konstantinova <i>et al.</i> , 2002
ITS of <i>A. alternata</i>	AAF2 AAR3	TGCAATCAGCGTCAGTAACAAAT ATGGATGCTAGACCTTTGCTGAT	~340	Konstantinova <i>et al.</i> , 2002
ITS of <i>A. radicina</i>	ARF2 ARR3	AATCAGCGTCAGTAAACAAACG AGAGGCTTTGTGGATGCTG	~251	Konstantinova <i>et al.</i> , 2002
ITS of <i>A. radicina</i> / <i>A. Petrosellini</i>	Pa2071 Pa2072	GGGCGTTATGCGAGATCAGG GTATTTGTAGGAATTTCCAG	~900	Pryor <i>et al.</i> Gilbertson, 2001; Infantino <i>et al.</i> (2009)
Alt a1 gene <i>A. petrosellini</i>	A. Alt-for Alt-rev	ATGCAGTTCACCACCATCGC ACGAGGGTGAYGTAGGCGTC	~510	Hong <i>et al.</i> , 2005 Stanković <i>et al.</i> , 2014
EF1- α gene <i>A. petrosellini</i>	A. EF1-728F EF1-986R	CATCGAGAAGTTCGAGAAGG TACTTGAAGGAACCCCTTACC	~280	Stanković <i>et al.</i> , 2014
β -tubulin gene <i>A. carotiincultae</i>	Bt1a Bt1b	TTCCCCCGTCTCCACTTCTTCATG GACGAGATCGTTCATGTTGAACTC	~480	Park <i>et al.</i> , 2008

2.3 Visualization and analysis of PCR reaction products

Visualization of amplified PCR reaction products was performed according to the method of Duduk *et al.* (2003), by electrophoretic separation of amplified nucleic acid fragments obtained from the PCR reaction in a 1% agarose gel in 1 × TBE buffer (90 mM Tris; 90 mM boric acid; 1 mM Na₂EDTA), at a voltage of 100 V. The gel was prepared by the required amount of agarose that was first added to 1 × TBE buffer and then heated to boiling in a microwave oven. An amount of 40 ml of the heated gel was poured into a mold in which the comb was previously immersed by placing it in the appropriate mold beds, after which the gel was left at room temperature until hardening. After hardening of the gel, the comb was removed and the gel was placed in a tub for horizontal electrophoresis (BlueMarine100, Serva Electrophoresis GmbH, Germany) into which 1 × TBE buffer was then poured until the gel was completely submerged in the buffer. Before applying the samples to the wells, 6 μ l of each sample was mixed with 1 μ l of loading dye (Loading Dye, Fermentas UAB, Lithuania). To determine the size of the amplified amplicon, a 1 kbp MassRuler™ DNA ladder marker (Fermentas UAB, Lithuania) with 10 bands ranging in size from 1,000 to 100 bp was used. Electrophoresis was performed at a constant voltage of 100 V. Staining was performed by immersing the agarose gel in a 0.1% ethidium bromide solution for 10 minutes. Amplified fragments in the gel were observed by using a UV transilluminator (Biometra,

UK). The appearance of product bands of the expected size for the particular type of primers used was considered a positive reaction (Table 1).

3. RESULTS

The analysis of amplified fragments of tested isolates originating from carrots revealed the presence of fragments of the expected size of about 545 bp in all tested isolates (Figure 1 and 2). Standard universal primers ITS1/ITS4 are found in the detection protocols of a large number of pathogenic fungi of different plant species. The positive reaction that was obtained in all samples confirms that a phytopathogenic fungus is present in each of the analyzed samples. Amplification did not occur with the negative control (K) (Figure 1 and 2).

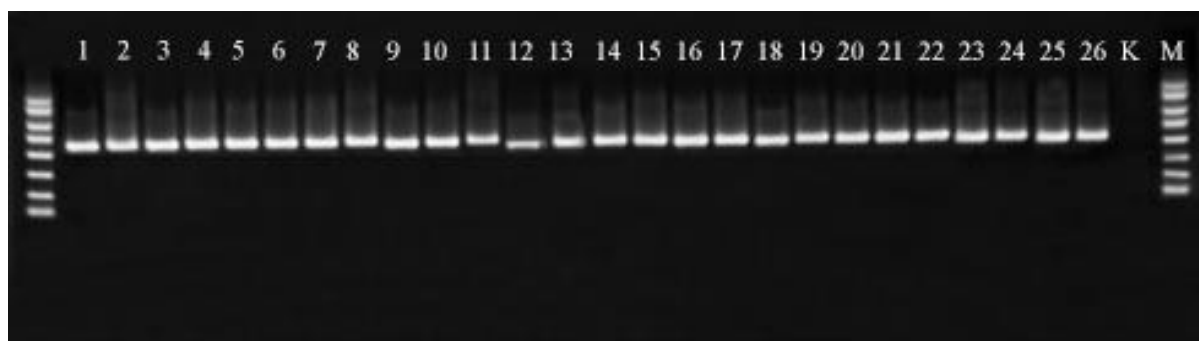


Figure 1: Visualization of the amplicon of the PCR product of the group A isolate, about 545 bp in size, obtained by using the pair of universal primers ITS1/ITS4 in a 1.5% agarose gel. Columns: 1) isolate FM-15; 2) isolate IM-4; 3) isolate BM-15; 4) isolate FM-17; 5) isolate Mr-90; 6) isolate Mr-10; 7) isolate BM-2; 8) isolate MM-100; 9) isolate Mr-15; 10) isolate Mr-30; 11) isolate Mr-31; 12) isolate Mr-91; 13) isolate Mr-125; 14) isolate MM-2; 15) isolate Mr-1; 16) isolate Mr-5; 17) isolate MM-11; 18) isolate Mr-3; 19) isolate MM-30; 20) isolate Mr-40; 21) isolate Mr-41; 22) isolate Mr-45; 23) isolate MM-37; 24) isolate Mr-46; 25) isolate MM-52; 26) reference isolate *A. dauci* (CLA-108); K) negative control (H₂O); M) MassRuler™ DNA Ladder

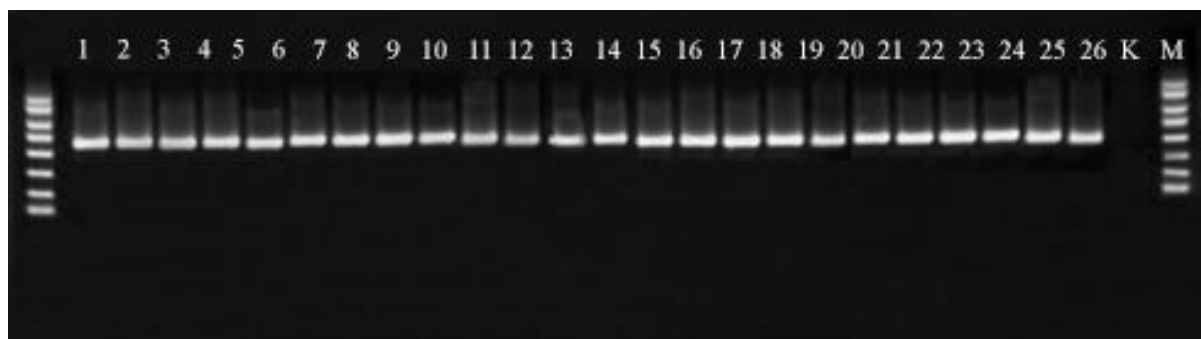


Figure 2: Visualization of the amplicon of the PCR product of the group B isolate of about 545 bp obtained by using the pair of universal primers ITS1/ITS4 in a 1.5% agarose gel. Columns: 1) isolate IM-3; 2) isolate IM-14; 3) isolate IM-15; 4) isolate MM-9; 5) isolate Mr-83; 6) isolate Mr-114; 7) isolate Mr-225; 8) isolate Mr-156; 9) isolate Mr-191; 10) isolate Mr-214; 11) isolate Mr-215; 12) isolate MM-1; 13) isolate Mr-80; 14) isolate Mr-

Eighth International Scientific Conference "June 5th - World Environment Day"

142; 15) isolate Mr-197; 16) isolate MM-4; 17) isolate Mr-305; 18) isolate MM-200; 19) isolate Mr-400; 20) isolate Mr-405; 21) isolate MM-205; 22) isolate Mr-60; 23) isolate Mr-71; 24) isolate MM-305; 25) isolate MM-400; 26) reference isolate *A. alternata* (Aa-82); K) negative control (H₂O); M) MassRuler™ DNA Ladder

Molecular detection and identification of studied isolates of *Alternaria spp.* by means of PCR reactions with the use of appropriate primers and sequencing of the ITS region of these isolates, made it possible to prove the presence of nucleic acids of two different species of the *Alternaria* genus and thus confirmed the distinction between morphological groups A and B. The obtained results are in agreement with the results reported by Ristić et al. (2014), who studied the morphological and molecular identification of isolates of *A. alternata*, a pathogen of caraway fruits in Serbia, using a pair of universal primers ITS1/ITS4 that enable the amplification of the ITS region of rDNA of Eukaryotes, and by comparing the amplified fragments of the selected isolates (CC1, CC2 and CC3) with the used marker (M), established the presence of fragments of the expected size of about 550 bp (base pairs). Amplification did not occur in the sample that represented the negative control (PCR mixture with RNase-free water).

The results of this research coincide with the previously identified species of the *Alternaria* genus pathogenic to carrots in numerous countries in Europe and beyond that were identified by other authors. So, for example, by applying molecular methods of identification using the PCR method and sequencing part of the genome of the isolated fungus in the Netherlands and Poland Konstantinova et al. (2002), Turkey Soylu et al. (2005) and Serbia Bulajić (2006); Bulajić et al. (2009); Bulajić et al. (2014) *A. dauci* was detected as a carrot pathogen, while using the same methods *A. alternata* was detected as a carrot pathogen in the Netherlands and Poland, Konstantinova et al. (2002), Serbia Bulajić et al. (2005a); Bulajić, (2006); Bulajić et al. (2009) and China Zhang et al. (2020).

4. CONCLUSION

For the purpose of molecular detection and identification of *Alternaria spp.* carrot pathogen isolates originating from Bosnia and Herzegovina and Serbia, as well as confirmation of the results obtained by conventional phytopathological methods, the Polymerase Chain Reaction (PCR) method and sequencing of amplified DNA fragments with the calculation of genetic sequence similarities of the isolates was applied. The tests included a PCR reaction with one pair of universal primers ITS1/ITS4 in order to obtain a specific DNA fragment that includes the ITS genomic region and seven PCR reactions with specific primers designed for different species of the genus *Alternaria* pathogenic for carrots and other plants from the Apiaceae family.

Molecular detection and identification of studied isolates of *Alternaria spp.* using PCR reactions with the use of appropriate primers and sequencing of the ITS region of these isolates made it possible to prove the presence of nucleic acids of two different species of the genus *Alternaria* and

Eighth International Scientific Conference "June 5th - World Environment Day"

thus confirmed the distinction between morphological groups A and B. Also, it was confirmed by used molecular methods of analysis that the isolates classified in group A on the basis of morphological characteristics belong to the species *A. dauci*, while the isolates of group B belong to the species *A. alternata*.

Molecular detection by PCR reactions, with the use of one pair of universal and two pairs of specific primers and sequencing of the ITS region, made it possible to prove the presence of nucleic acids of selected *Alternaria spp* isolates of carrot pathogens in Bosnia and Herzegovina and Serbia and thus confirmed the differentiation of the studied *Alternaria spp.* isolates members of morphological groups A and B. Also, using the mentioned primers, reference isolates of *A. dauci* and *A. alternata* originating from carrots from the collection of the Department of Phytopathology, Institute of Phytomedicine, Faculty of Agriculture, University of Belgrade were amplified, which indicates the specificity of the primers used and the possibility of their use in routine research.

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Eighth International Scientific Conference
"June 5th - World Environment Day"

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A CONTRIBUTION TO THE KNOWLEDGE OF THE REPRODUCTIVE BEHAVIOR OF CATTLE AND SHEEP AS A PARAMETER OF SUSTAINABILITY IN ANIMAL PRODUCTION

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Key words: behavior, reproduction, cattle, sheep, sustainability

ABSTRACT:

Production in domestic animals includes reproduction, which again depends on the health condition of the animals. However, the animal's health depends on its ability to cope with the living conditions of the environment in which it is placed, as well as on the way the farmer treats it. Proper nutrition, supply of clean and safe water, care, accommodation, health condition and removal of stressogenic factors from the environment will significantly ensure quality reproduction of domestic animals. Ethology is a science that studies the behavior of animals and humans. The focus of ethology is behavior in natural conditions. This paper describes the sexual behavior of cattle and sheep.

Breeds of domestic cattle are reproductively active throughout the year, but there are certain seasonal variations in the values of basic parameters, which measure reproductive efficiency in both male and female animals. The basic elements of a bull's sexual behavior are: courtship, erection and protusia, i.e. the exit of the penis from the foreskin, jump on the female, intromission, i.e. the introduction of the penis into the vagina, ejaculatory stroke and ejaculation and the end of the jump. One of the basic characteristics of the sexual behavior of cows and heifers during estrus is that they jump on other cows and allow themselves to be jumped on by other cows. As in most animals, sexual behavior in adult female cattle is manifested during a short estrous period, which repeats every three weeks, except for the period when the female is pregnant.

The basic phases of the ram's behavior during the act of mating are: smelling the female's ano-genital region, pushing the female with the head and shoulders, jumping up and ejaculation.

External signs of estrus are quite pronounced in sheep. This is why it is not possible to establish estrus without a ram. The characteristic signs of mating behavior are as follows: a sheep in estrus follows the male, pushes him with its neck and sides, makes quick movements with its tail and allows itself to be jumped, i.e. for the male to perform the entire act of mating.

1. INTRODUCTION

Reproduction of cows is a biological process and it includes the possibility of fertilization, pregnancy and giving birth to a healthy calf capable of life. This biological regularity takes place in certain time intervals. If the cow meets these requirements, it is fertile. When is the cow is fertile, the herdsman has no big worries, but the infertility of cows is quite common and hurts the herdsman many problems. Cattle reproduction is the basis of milk and meat production. Without after calving, milk production declines and finally stops. In the cow, the cyclic activity of the ovaries takes place throughout the year, which is why they are called polyester unipam animals (cattle are uniparous in 97 to 99% - only one calf) (Tomašković et al., 2007). Puberty in cattle occurs after sexual maturation, and is manifested by the appearance of the sexual cycle. A cow enters puberty at the age of seven to eighteen months (Tomašković et al., 2007). On appearance, puberty is affected by the breed (early-ripening breeds enter puberty at 8 months, late-ripening breeds after 10th month), diet, body weight and calving season (Peršak, 2018. Speranda, 2019.). As in other branches of animal husbandry, reproduction is the most important segment of breeding in sheep breeding, and without regular and physiological reproduction, there is no production. The sexual maturity of sheep varies from breed to breed, so in early-ripening it appears at 6 to 7 months of age, and in late-ripening at 10 to 12 months. It should be noted that sexual maturity in sheep depends much more on body weight than age, therefore sexual heat can appear in lambs when they reach a weight of 28 to 30 kg. The sexual cycle in sheep lasts an average of 17 days, while estrus lasts about 27 hours. Most sheep are seasonally polyestrous, which means that they mate at a specific time of year. Modern sheep farming uses artificial dosing of the amount of light to encourage moulting, because it is more natural and economically profitable than hormonal induction of estrus (Vince et al., 2017, Vučemilo and Kostelić, 2018). Temperature and nutrition are very important factors that play a role in observing behavior and detecting estrus (Ritter et al., 2019). Each estrus is accompanied by ovulation and external signs, which in sheep are manifested by restlessness, reduced appetite, jumping on other sheep, more frequent urination and other symptoms. Also due to the possible occurrence of "silent chasing", it is important to have "detector" rams in the herd that will detect ewes in estrus. Before the breeding season, it is very important to prepare animals for breeding, both sheep and rams. In rams, puberty begins at the age of six to nine months (Cergolj and Samardžija, 2006).

2. REPRODUCTIVE BEHAVIOR OF CATTLE

Breeds of domestic cattle are reproductively active throughout the year, but there are certain seasonal variations in the values of basic parameters, which measure reproductive efficiency in both male and female animals. Cows are in oestrus every 21 days, throughout the year. In each estrous cycle, they ovulate, most often, one egg cell, whereby ovulation is delayed 12 to 14 hours after the end of estrus. The establishment of the estrous cycle, followed by ovulation, is not inhibited by lactation, i.e. sucking and/or milking. Normally, in normal (healthy) cows, ovulatory estrus occurs within the first to three weeks after calving. Bulls produce fertile sperm throughout the year, although a certain intensity of spermatogenesis is usually observed during the warm summer months. Sexual maturity of bulls depends on feeding, housing conditions, environment and health (Dragin et al., 2016).

2.1 Reproductive behavior of the bull

A bull's libido depends on the level of androgenic steroid hormones that influence its aggressive behavior, mating and the function of all parts of the sexual organs. Sexual reflexes manifest themselves as reflexes of approach, erection, girdling, copulation and ejaculation (Cergolj and Samardžija., 2006). The basic elements of a bull's sexual behavior are: courtship, erection and protusia, i.e. exit of the penis from the foreskin, jump on the female, intromission, i.e. introduction of the penis into the vagina, ejaculatory stroke and ejaculation and completion of the jump. The desire to mate is called libido sexualis. The act of mating (copulation) in cattle lasts relatively short compared to horses and pigs. The first signs of a bull's sexual interest in a cow are noticed while she is in proestrus, i.e. about 4 days before the appearance of signs of estrus, i.e. the moment when the cow allows the bull to jump on her and, practically, is constantly near her. As it approaches estrus, the bull shows more intense interest in the cow, is more aggressive in trying to perform a jump and is more often in her immediate vicinity. More and more often he sniffs her external genitalia and urine, pushes her and tries and/or succeeds in performing a jump. In the work of the authors Sankar and Archunan (2014), the effect of saliva, feces and milk on the copulation of bulls and their communication with cows was investigated. It has been concluded that other body fluids also attract bulls during the stages of the sexual cycle (Sankar and Archunan., 2014). Sniffing ends with the characteristic stiff stance of the bull, in which he stretches his neck, turns his head a little to the left or right and raises his upper lip. In our language, there is no adequate term for this behavior of a bull, but in English terminology the term "flehmen" is used.



Figure 1: Flehmen bull

When a cow is in estrus, she allows the bull to perform the complete mating act. When making a jump, the bull quickly shifts his weight onto his hind legs, raises the front of his body, and makes a sudden forward movement to jump onto about half of the cow's back. Then the joints of the bull's front legs are firmly fixed to the loins of the cow, just behind the wings of the cow's pelvic bones. At that moment, the penis is directly in front of the entrance to the vagina. Penetration of the glans penis into the vagina very quickly. After the body of the penis enters the vagina, the sphincter muscles of the vulva contract around the body of the penis. This stimulus causes a fast and strong reflex contraction of the bull's abdominal muscles, which results in a sudden and strong approach of the bull's pelvis to the external genitalia of the cow, which causes further sudden penetration of the penis into the depth of the vagina, which leads to instant ejaculation. The contraction of the abdominal musculature during the ejaculatory impact is so strong that it instantly leads to the complete separation of the rear legs of the bull from the ground. Ejaculation is instantaneous, and sperm is ejected in one strong stream into the vicinity of the rear opening of the cervix of the uterus. After ejaculation, the contraction of the abdominal muscles relaxes and the bull slowly gets off the cow. Stimulants that cause and promote the bull's sexual behavior are numerous, but it has been shown that the most powerful are those that the bull receives through the sense of smell and hearing, although those received by the sense of touch, sight and taste are also important. Thus, it was established that a bull can be sexually aroused by the head of a cow, but the general opinion is that the strongest sexual arousal is achieved by the smell of the urine of a cow in estrus. It seems that tactile stimuli are not the most important for achieving an erection in the bull.

2.2 Reproductive behavior of cows

As in most animals, sexual behavior in adult female cattle is manifested during a short estrous period, which repeats every three weeks, except during the period when the female is pregnant. One of the basic characteristics of the behavior of cows and heifers during estrus is that they jump on other cows and allow themselves to be jumped by other cows. In this regard, while those that allow themselves to be jumped on are certainly in estrus, while those that jump on other cows cannot be said with certainty. Cows in estrus imitate other elements of male sexual behavior, such as sniffing the external genitalia of other females. Cows in estrus allow the male to perform the complete mating act. Cows that are in estrus at the same time stay together more

and, within the herd, form the so-called "sexually active group". In addition to cows in estrus, this group also includes cows in proestrus and metaestrus. These animals are very active in jumping each other, but they can sniff and push cows in diestrus, i.e. those outside the "sexually active group". Sometimes estrus cows manage to jump cows outside their group, i.e. those in diestrus. The stages of the cow's sexual cycle are divided into proestrus which lasts one to three days, then estrus which lasts 24 to 36 hours, metestrus which lasts three to four days and the last stage is diestrus which lasts 12 to 14 days. In the phase of metestrus, the formation of the corpus luteum occurs, while in the phase of diestrus, the activity of the corpus luteum itself occurs. An extended sexual period in which the sexual organs are at rest is called anestrus. A cow enters puberty at the age of seven to eighteen months (Tomašković et al., 2007).

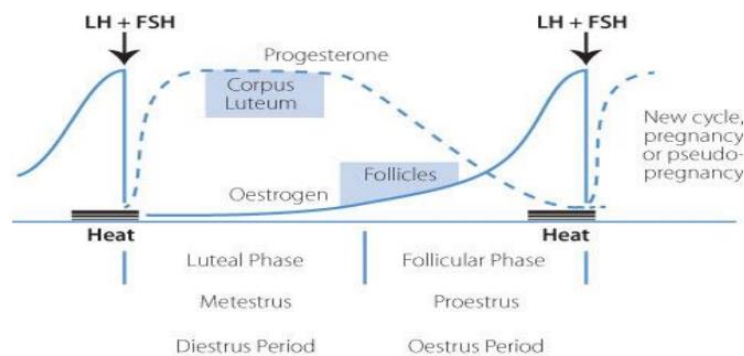


Figure 2: Phases of the estrous cycle of cows

Estrus lasts from 2 to 36 hours, but most often this period lasts 18 hours. Detection of estrus is very important in cattle breeding for the success of artificial insemination. The animal moves away from others, is nervous and has a reduced appetite (Stančić et al., 2014). The most noticeable change is in the pubis in the form of hyperemia and swelling. Oestrus mucus is clear and viscous and can extend from the pubis to the floor. Body temperature may be slightly elevated. The animal's tail can be raised and disheveled (Tomašković et al., 2007). Animals show a characteristic reflex of standing in the presence of males or by pressing on the back they jump on other animals and allow themselves to be jumped (Chenoweth, 1982). In cows, the standing reflex lasts very short, sometimes less than 24 hours, so it is important to detect estrus 4 times in 24 hours. Detection of estrus is carried out in the early morning and in the evening. If it shows anovulatory estrus, it is important to examine the female and find out the problem that could have led to this condition. The problems are most often related to cysts on the ovaries, pregnancy of cows and errors in keeping records (Stančić et al., 2014).



Figure 3: Basic characteristics of cows and heifers during estrus
(sniffing the external genitalia of other females and jumping on other cows, appearance of estrus mucus)

2.3 Cow behavior before, during and after calving

In cows, there is a progressive increase in irritability and separation from the group, into nature before calving. These and other characteristic changes in the cow's behavior occur in the interval from a few days to a few hours before calving. In this period, in addition to the aforementioned behavioral signs, others appear: the cow licks its sides and often wags and kicks its tail. Some authors have noted that the cow changes its behavior, especially in terms of increased anxiety, as early as 14 days before calving, with anxiety increasing significantly during the 36 to 24 hours before calving. During the calving process, the cow often changes position. During the first stage of calving, the cow lies down and gets up several times, during the first part of the second stage, it usually stands, while during the final part of the second stage, the cow lies on its side. After pushing out the fetus, the cow turns to the thoracic-abdominal position of the lying position, or stands up. Research numbers show that the largest number of cows calve between 18:00 and 06:00.



Figure 4: The first stage of calving (the cow lies down and gets up several times)



Figure 5: Other stages of calving and procedures with the calf after calving

In the event that the delivery is not too prolonged and difficult, the cow gets up very quickly after the end of pushing out the fetus. Soon after, he begins to lick the calf, usually starting with its head. Calf licking can last up to 1.5 hours. By licking, the cow removes the remains of the fruit sheaths and fruit liquid from the calf, while probably also marking the calf with its smell, for later recognition. In addition to licking, cow and calf advertise, which is also an important way of establishing mutual contact. The period immediately after childbirth is very critical, because the calf gets used to being suckled, but so does the cow to be suckled, especially young and inexperienced cows. The calf intensively starts looking for the teat, which helps it to stand on its feet. The desire to search for a breast decreases very quickly, if the first attempts were not successful. When contact is established naturally, the cow spends the whole day with her calf, leaving it only when she goes to drink water. Even in later weeks, the calves are constantly with their mothers, and they separate into special groups and usually lie down, while the mothers graze.

3. REPRODUCTIVE BEHAVIOR OF SHEEP

The basic feature of sheep reproduction is their seasonal sexual activity, which is conditioned by the seasonal difference in the duration of the daily photoperiod. Most breeds of sheep are seasonally polyestrous, which means that females manifest multiple estrous cycles within a single annual season. Breeds of sheep bred in northern latitudes exhibit sexual activity in the period between late summer and early winter. This period is called the breeding season or the estrus season. During the rest of the year, the animals are sexually inactive, so this period is called the season of sexual dormancy or the season of anestrus.

Modern sheep farming uses artificial dosing of the amount of light to encourage moulting, because it is more natural and economically profitable than hormonal induction of estrus (Vince et al., 2017, Vučemilo and Kostelić, 2018). The estrous cycle of a sheep lasts 16 to 17 days, while estrus lasts an average of 26 hours, ranging from 20 to 36 hours. Ovulation is spontaneous and occurs near the end of estrus. If fertile mating is absent, ewes exhibit 6 to 9 estrous cycles during the breeding season. The reproductive activity of rams is much less dependent on the annual season. Reproductive functions directly enable the process of reproduction, which begins with the appearance of puberty and lasts throughout the sexually active period of life and ends with old age. All reproductive functions are controlled by complex neurohormonal mechanisms, which can be modified to a greater or lesser extent by the influence of genetic and paragenetic factors.

The appearance of the first sexual drive in sheep depends on several factors, where the breed composition should be singled out. Early maturing sheep breeds reach sexual maturity at the age of 6 to 7 months, and late maturing at 10 to 12 months. However, with the first admission, you should wait until the sheep have acquired good physical development, which is approximately

when the female lambs reach about 65 percent of the final body mass. Young rams become sexually mature at the age of 6 months. However, in the case of male animals, one should wait until the complete completion of physical development, so breeding rams of precocious breeds are admitted after one year, and all others after 18 months.

3.1 Behavior of the ram during mating

The basic phases of the ram's behavior during the act of mating are: smelling the female's ano-genital region, pushing the female with the head and shoulders, jumping and ejaculation.



Figure 6: Sexual behavior of the ram (the ram smells the female and tries to jump on her)

Sniffing the smell of the urine of a sheep in estrus causes a characteristic behavior of the ram: it stretches its neck and raises its upper lip, intensively smells the female genitalia and pushes the female's sides. It is characterized by the extension of one front leg, with which it strongly hits the ground. If the female is in the estrus phase, when she allows mating, the ram jumps on her back, clings to her loins with his front legs and suddenly introduces the penis into the vagina.



Figure 7: Flehmen ram

The influence of breed and age on the sexual activity of rams was investigated, and it was proven that rams show more interest in females of the same breed compared to other breeds (Simitzis et al., 2004).

The methods of detecting bleating on the farm are mostly reduced to male testers wearing colored aprons and being introduced to the sections with the females. In sheep, along with a vaginal examination, the presence of a ram is the only method of detecting estrus (Grizelj et al., 2015). Today, "flushing" is most often used as a short-term method of increased feeding intensity

of sheep in poor condition, especially before mating, i.e. three to four days before the animals are admitted (Matejaš and Krumpović, 2004). It was observed that rams show signs of sexual behavior more intensively when they are more often in the presence of different ewes in estrus. Thus, some authors found a significantly higher frequency of sexual behavior of the ram when the same ram was replaced by an estrous ewe every 5 minutes, compared to the same value obtained when the ram was constantly with the same ewes, or when the same ewe was introduced every 5 minutes. .

An interesting question is how does the ram distribute its copulation evenly among a larger number of sheep? A partial answer to this question consists in the fact that rams prevent the mating of those ewes that are at the beginning of estrus and have not been mated until then. Not only that, rams seem to be able to recognize ewes that have been mated less times during estrus. Rams mate with ewes more often in the first half of estrus, and less often in the second half of estrus.

3.2 Behavior of sheep during mating

Temperature and nutrition are very important factors that play a role in observing behavior and detecting estrus (Ritter et al., 2019). Sexual maturation is under the control of the central nervous system hypothalamus-pituitary-ovaries. The hypothalamus stimulates GnRH, which is secreted from the pituitary gland, which secretes FSH, which stimulates the growth of follicles on the ovaries, and LH, which enables follicle maturation and ovulation (Kennedy, 2012). Fertilization is possible only if the animal is driven, i.e. when it is in estrus. Graafian follicles burst, i.e. ovulation, and external signs of urges and changes in behavior occur. If mating and fertilization occur during that period, pregnancy will occur (Samardžija et al., 2010). The sexual cycle is influenced by the breed, diet, temperature and amount of precipitation, the length of the light day, i.e. photoperiod and the presence of rams, while the seasonality of small ruminants is regulated by the level of melatonin in the circulation. It is secreted only during the night, from the pineal gland. By reducing light, i.e. by shortening the daylight hours, sexual activity is stimulated in sheep and goats, thus increasing the level of melatonin in the circulation, and by extending the daylight hours, anestrus is induced (Chemineau et al., 1992, Schoenian, 2019).

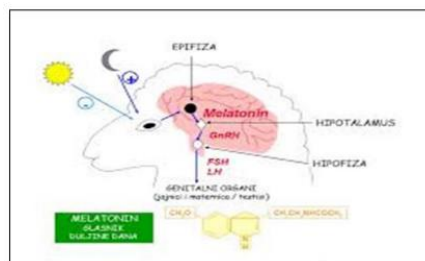


Figure 8: Action of melatonin

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Sheep have a more intense activity of the olfactory system in the sexually active period than goats, whose activity is conditioned by the presence of males (Cann et al., 2019).



Figure 9: Mating of sheep

The duration of estrus in sheep is usually 20-30 hours, but it can vary significantly, depending on the age of the ewe (it is shorter in young, pubescent females) as well as on the period of the mating season in which it occurs (at the beginning and end of the season, estrus is shorter). In farm conditions, it has been recorded that one ewe during estrus can be mated 0-18 times, and the average is 6.3 times. The frequency of mating depends on the age of the throat. Young females mate less often than adults. From the moment when the ram first discovers the female in estrus, it takes an average of 2 to 4 hours until the first copulation. Some researchers have found that mating shortens the duration of estrus. Perhaps because copulation provokes, to a certain extent, the beginning of ovulation, and after ovulation, external signs of estrus quickly cease to appear, due to the drop in the concentration of estrogen in the body's circulation.

3.3 Behavior before, during and after parturition

A progressive increase in agitation and separation from the group, as well as sometimes, kicking the ground, or sniffing the ground are characteristic signs of the imminent birth of the ewe. The described behavioral changes in sheep occur very shortly before lambing. In sheep, interest is often expressed in new-born lambs of other mothers, or in other sheep that are also about to lamb. It has been found that some sheep manifest this interest more than two weeks before lambing, but most often this interest manifests itself around 12 hours before lambing. During lambing, the ewe lies down, but can also stand during the final phase of pushing out the fetus. The duration of lambing is usually quite short, so that most lambs are born within an hour of the moment they first appear at the opening of the vulva. The second lamb is usually born in a shorter time. Most often in less than 45 minutes. In most breeds of sheep, it is observed that sheep most often lamb in the evening hours, i.e. between 5 and 9 p.m. Some authors find that there are significant differences in the distribution of lambs during the day, between certain breeds of sheep. Parturition in small ruminants is preceded by a drastic drop in progesterone, 12 to 24 hours before (Ghosh and Halдар, 2015).



Figure 10: Sheep lambing

After lambing, the ewe stands up within a few seconds, turns its head towards its newborn lamb and begins intensively licking it and eating the fetal membranes still on the lamb. Licking starts from the head, in order to remove the fetal membranes and mucus from the nostrils and thus prevent possible suffocation of the lamb. The intensity of licking decreases rapidly during the first hour after lambing, as the lamb dries. The intensive contact of the mother's mouth and nose with the lamb continues for several more hours. A well-behaved ewe will turn its head towards the lamb's head and stand still while the lamb tries to find the teat. Young sheep can often turn left-right from the lamb, so the lamb has a hard time finding the teat. Because of this, these attempts of the lamb suddenly lose their intensity. This can lead to the lamb not starting to suckle in time, or the ewe not receiving the lamb at all. Sight and voice are not particularly important in establishing contact between sheep and lambs. Smell is very important in this regard. The sheep intensively sniffs the body and anal region of the lamb, because that smell later serves as a basic criterion for the identification of the lamb. The described behavior of the mother around establishing contact with the lamb is not disturbed if her sense of smell is destroyed, but the ewe is then unable to recognize its own from other lambs. This points to the fact that other factors, probably heat and movement of the lamb, cause the described behavior of the mother. This also explains the fact that a sheep shows no interest in a stillborn or immobile lamb. A newborn lamb tries to stand up a few minutes after birth and normally does so within 30 minutes of birth. The lamb tries to suckle 30 to 45 minutes after standing up, ie within 90 minutes after birth. During the first day of the lamb's life, the ewe allows it to suck as many times and as long as it wants. After one to two weeks, the ewe begins to shorten in length and reduce the number of daily sucklings, usually by moving away from the lamb when trying to suckle. During the first to four weeks after lambing, the ewe allows the twins to suckle more often than if she had only one lamb. After one unsuccessful suckling attempt, the lamb manages to suckle in an interval of a few minutes to an hour. No difference was observed in the frequency of sucking during the dark and light part of the day. Ewes with two lambs usually allow one lamb to go without the other, but only during the first week after birth. Around the eighth week of life, some lambs from twin pairs learn this. In some cases, if one lamb reaches the ewe earlier, it returns to find its mate, and then they start sucking together.

4. CONCLUSIONS

- a) Knowledge of the ethology of cattle and sheep is an essential prerequisite that facilitates work and manipulation with them and helps in predicting their reactions. Knowledge of animal behavior prevents many injuries (both to humans and animals), eliminates radical animal restraint measures from zootechnical use, and generally speaking, enables a harmonious coexistence of animals and their owners.
- b) Reproduction of cattle is the basis of milk and meat production. Without calving, milk production declines and finally stops. As in other branches of animal husbandry, reproduction is the most important segment of breeding in sheep breeding, and without regular and physiological reproduction there is no production.
- c) The health and function of the reproductive system of farm animals depend significantly on the physical and mental health of the animals.
- d) Change in behavior in general or reproductive behavior is one of the first indicators of impaired health, i.e. impaired function of the reproductive system.

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SUSTAINABLE FOOD SYSTEMS AS PART OF THE GREEN DEAL

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ABSTRACT:

Emissions from the sectors of agriculture, forestry and land use are responsible for approximately a quarter of anthropogenic greenhouse gas emissions, and if they continue on the current trend they would prevent the achievement of the goals of the Paris Agreement. The European Green Deal defines the EU's future environmental policy to address the challenges of environmental protection in the face of climate change, and one of the essential elements of the Green Deal is a Farm-to-Fork strategy, with an emphasis on establishing an environmentally friendly food sector. Food production worldwide must be transformed in order to achieve the UN Sustainable Development Goals and to increase the level of resilience of production and processing activities to external factors.

The demand and need for food is growing as the world's population grows, but the amount of arable and pasture land supporting extensive agricultural production is decreasing due to increasingly noticeable climate changes. A part of the future demand for food and nutrition can be met by aquaculture, algae and insects, which, compared to livestock, represent an efficient way of producing protein with relatively low environmental costs. This paper presents some important advantages and disadvantages of the mentioned food systems, as well as additional efforts that must be made in order to achieve the best possible sustainability and acceptability of these systems.

1. INTRODUCTION

Climate change impacts all food system processes, reducing their capacity to ensure food safety and nutrition both now and in the future. In addition, emissions from the sectors of agriculture, forestry and land use are responsible for approximately a quarter of anthropogenic greenhouse gas emissions. If they continue on the current trend they would prevent the achievement of the goals of the Paris Agreement. Therefore, the challenge of feeding a growing population sustainably, goes beyond a simplified approach to producing more food, and the management of food systems should be closely linked to climate management. The transformations needed to support sustainable food systems, in current and predicted climatic conditions, require different governance mechanisms and a better understanding of how climate policies affect food security and nutrition security and the sustainability of food systems (Hidalgo et al. 2021). This paper will present some of the ways of sustainable food systems, which in the near future will gain more importance, and humanity will inevitably have to accept them as a mechanism of adaptation to the negative consequences of climate change.

2. METHODS

The literature was searched in academic databases: PubMed, Web of Science, ScienceDirect and Google Scholar. The literature used in the work covers the period from 2015 to 2024 and includes works in English, Croatian, Serbian, and Bosnian. Most sources refer to Europe and the Western Balkans, while international works are included for a broader context. Mainly, peer-reviewed scientific papers were used, ensuring the reliability of the data, with a smaller number of expert sources (expert book and official EU data). The literature was analyzed qualitatively using an inductive approach, through thematic categorization: traditional and sustainable aquaculture, algae as a protein source, and insects in human nutrition. Publications were reviewed and cross-referenced to identify similarities and differences in existing research. This enabled the synthesis of data with a view to defining trends and future research guidelines.

3. INTERNATIONAL STRATEGIC RESPONSE TO CLIMATE CHANGES

By signing the *Paris Climate Accords* on climate change in 2015 at the 21st United Nations Conference an agreement was reached to achieve the main goal; limiting the global temperature increase below 2°C, and ideally below 1.5°C, compared to the pre-industrial period. The accompanying goals are to increase the ability to adapt to the consequences of climate change while ensuring food supply and developing "green" technologies. The *European Green Deal* is a new strategy of the European Union (EU), adopted in 2019 by the European Commission, as a response to increasing global challenges and threats to the environment due to intensified climate

change. One of the essential elements of the *Green Deal* is the "*Farm-to-Fork*" strategy, with an emphasis on establishing an environmentally friendly food sector (Vasilkov et al. 2021).

The concept of food systems as complex socio-economic ecological systems has gained importance as an effective point for achieving these goals; reducing poverty and malnutrition and contributing to climate change adaptation and mitigation. Climate change is considered one of the most significant threats to modern food systems. The effects of climate change are expected to affect all processes and functions of food systems, hindering their potential to achieve food safety results in the future (Hidalgo et al. 2021). The world food production must be transformed in order to achieve the UN Sustainable Development Goals, as well as increase the level of resilience of production and processing activities to external factors. The current world population is projected to reach over 9.6 billion by 2050, which will require an increase in total food production by about 70 to 100% compared to current production, which is ultimately a tall order. The demand and need for food is growing as the world's population grows. The amount of arable and pasture land supporting extensive agricultural production is decreasing due to increasingly noticeable climate changes. Climate change is a key limiting factor contributing to the reduction of food production. The most widespread abiotic stresses in the world are: extreme temperatures, drought, salinity, floods, nutrient deficiencies and acidic conditions. Globally, the area of agricultural land is about 5 billion hectares, which is 38% of the Earth's land surface. Therefore, alternative and sustainable sources of protein and other nutrients must be researched and developed (Ijaola et al. 2024).

4. SUSTAINABLE FOOD SYSTEMS

4.1 Sustainable aquaculture

Aquaculture is the industry of growing marine and freshwater organisms under controlled environmental conditions; represents the biomanipulation of the life cycle of cultivated organisms and environmental conditions, including growth and reproduction control and elimination of causes of natural mortality. The aquaculture sector will grow in its production and, according to projections, by 2030 will account for 62% of the total share of global human nutrition by marine products (FAO, 1988 and FAO, 2016; Gulin, 2017). Part of the future demand for food and nutrition can be met by marine products. Compared to livestock production, aquaculture is an efficient way of producing protein with relatively low environmental costs. This set out international plans to increase aquaculture. Thus, in 2021, the European Commission published new strategic guidelines (COM(2021)0236) for a more sustainable and competitive EU aquaculture, in a way that will contribute to the *European Green Deal* and economic recovery after the COVID-19 pandemic. These guidelines also contribute to a "*Farm-to-Fork*" strategy, initiating a faster transition to a sustainable EU food system, while recognizing the potential of

sustainable aquaculture in providing food and feed with a low carbon footprint (Gerhard Brauer 2023).

Although there was a four-fold increase in global aquaculture production between 1990 and 2017, total EU production from around 1.2 million tonnes has not changed for a long time despite the Commission trying to improve the EU's production potential by publishing various strategic documents and guidelines (Gerhard Brauer 2023). The challenge for achieving strategic goals for the improvement of aquaculture production is compounded by the fact that ecological, socio-economic, financial and energy sustainable development needs to be integrated in parallel. Traditional breeding technologies are increasingly considered "polluting" and "unsustainable". The fish production process continuously generates solid and liquid waste, as well as carbon dioxide. Approximately 25% of the feed used for aquaculture farming is excreted as solid waste, while liquid waste includes ammonia, nitrites, nitrates, phosphates and other dissolved substances in the growing water. Limited amounts of water, negative impact on the environment and water quality, land price and other challenges, encourage growers and researchers to develop and apply new, more environmentally and economically acceptable breeding technologies that will meet the growing needs for food, but also the trend of healthy eating.

Aquaculture has the potential to increase the global food supply, as well a huge potential to address malnutrition and nutrition-related diseases. Therefore, a segment of the quality of cultivation itself is also important in order to obtain a high-value product, so the choice of the type of feed affects the nutrients available from aquaculture. Fish in the human diet is very beneficial from a health perspective. It provides high-value proteins and various necessary micronutrients: vitamins (A, B and D), minerals (calcium, iodine, zinc, selenium, iron) and polyunsaturated n-3 fatty acids, such as which are docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Starčević et al. 2022). With the growth of the population and increased consumer awareness of the health benefits of using fish in human nutrition, an increase in aquaculture is also expected. The contribution of aquaculture expansion in improving nutrition and increasing the global food supply is limited by the ecological sustainability of aquaculture (Fiorella et al. 2021).

4.1.1 Recirculating aquaculture

One of the alternatives to traditional breeding is recirculation aquaculture. It can be located in both urban and rural areas. The main characteristic of recirculation aquaculture systems is the purification and reuse of water from the the breeding areas. That significantly reduces the need for water changes, and an additional benefit is that they require significantly less space than traditional breeding methods. These systems integrate various individual processes for water purification, and processes for controlling and maintaining the desired water quality during an intensive production regimen. Controlled conditions enable the achievement of optimal breeding

conditions adapted to the specific species being cultivated, and thus it is possible to shorten the production cycle in relation to open uncontrolled systems. Better food conversion is also achieved, which means that less waste generated by food goes into the environment, thus creating small amounts of concentrated wastewater that can be processed relatively cheaply (Gavrilović and Jug-Dujaković, 2019).

4.1.2 Biofloc technology in aquaculture

Biofloc technology is considered an effective alternative system in aquaculture by which nutrients are recycled. Biofloc technology is based on the growth of microorganisms in an aqueous medium in order to improve water quality and treat waste products with minimal water exchange and an additional free source of protein for cultivated species.

For sustainable aquaculture, microorganisms rank high: they serve as a source of food for cultivated organisms and for the removal of waste generated in this activity. The sustainable approach of this system is based on the growth of microorganisms in culture medium, with minimal or zero water changes. Biofloc microorganisms have two main roles; maintaining water quality, through the uptake and conversion of nitrogen compounds into microbial protein and as an additional food source for cultivated species, increasing the profitability of the cultivated culture. Microbiological control leads to efficient decomposition of waste materials, efficient nitrification, and facilitates the control and recycling of nitrogen. Biofloc systems are most effective when applied to species of organisms that are able to directly consume flocculating particles. It is also important that the systems are suitable for species that can tolerate high concentrations of solids in the water and poorer water quality. Some species of prawns and tilapia have physiological adaptations to consume bioflocculating particles and digest microbiological protein. Almost all bioflok systems are used mainly for the breeding of prawns (*Penaeus monodon*, *Penaeus sp.*), Nile tilapia (*Nile tilapia*) or carp (*Ctenopharyngodon idell*) (Gulin, 2017).

4.1.3 Cyanobacteria and microalgae in human nutrition

Cyanobacteria and microalgae contain significant amounts of protein and lipids, which are highly digestible and nutritionally well balanced (Table1). Spirulina is a biomass of cyanobacteria (blue-green algae) used for the production of food supplements or as a whole food. Spirulina actually includes three species: *Arthrospira platensis*, *A. fusiformis*, and *A. maxima*. *Chlorella vulgaris* is a type of green microalgae from the phylum *Chlorophyta*, which is also used as a dietary supplement, and to improve the nutritional quality of cereal-based products, dairy products and others. Traditionally, *Spirulina* was collected from the wild and consumed as a protein-rich whole food in numerous cultures outside Europe and North America. Today, *Spirulina* and *Chlorella* species are commercially cultivated varieties and are integrated into a

Eighth International Scientific Conference
"June 5th - World Environment Day"

number of products: salad dressings, beverages and pastries, and are also sold as protein supplements. Proteins in algae contain all the essential amino acids essential for human consumption. In addition, biomass microalgae and cyanobacteria is extremely rich in numerous bioactive components, which further contribute to the preservation and improvement of human health (Ahmed et al. 2024).

Table 1: Nutritional composition of algae; *Spirulina* and *Chlorella vulgaris* (Ahmed et al. 2024)

Nutrient content in 100g	<i>Arthrospira</i>	<i>Chlorella vulgaris</i>
Fats	2,2 g	-
Proteins	63 g	60,1 g
Carbohydrates	22 g	40,1 g
Iron	58 mg	240,1 mg
Calcium	1 mg	33,1 mg
Zinc	3 mg	~1,5 mg
Potassium	1,4 mg	-
Magnesium	400 mg	~274 mg

It is important to note that the above two examples are only a small part of the total number of different types and forms of application of marine and freshwater algae in human nutrition. Algae are relatively new to the United States food market, but several types of algae have already received GRAS status from the Food and Drug Administration (FDA), among them: *A. platensis*, *A. protothecoides*, *C. vulgaris*, *Dunaliella bardawil* and others. This designation is assigned by the FDA to substances considered safe for human consumption.

Work should be done to increase the social acceptability of algae as a possible future source of nutrients, primarily because algae are characterized as a reliable and safe source of food. Agricultural activity needs approximately 75% of the total global fresh water, while animal protein requires 100 times more water than if the same amount of protein had been produced from plant sources. The "green alternative" is the commercial cultivation of algae, as most microalgae are photoautotrophs, relying on carbon dioxide as a carbon source and light as a source of energy (Ijaola et al. 2024). However, due to the high water content of the microalgae biomass, water must be removed during processing, which requires high costs and energy. The small cell size of microalgae (2-200 μm) and their growth in very diluted cultures (mass concentration $<1 \text{ g/L}$) are key reasons for the high process costs. Also, another challenge is the need to develop improved methods of extracting proteins from seaweed, and with the aim of increasing their bioavailability, because they are poorly digestible in their unprocessed form (Ijaola et al. 2024).

4.2 Insects in human nutrition

Insects are part of the common diet of many communities: they are consumed in Africa, South America, Oceania and Asia. In the underdeveloped and less developed countries of Central Africa, insects sometimes make up to 50% of the total protein in human consumption and have a significant market value. Currently, there are more than 2000 species of arthropods from the ranks *Coleoptera* (beetles) with 31 %, *Lepidoptera* (caterpillars) with 17 %, *Hymenoptera* (wasps, bees and ants), *Orthoptera* (crickets and grasshoppers) with 14 %, *Hemiptera* (hemiptera) with 11%, *Isoptera* (termites) with 3 %, *Odonata* (dragonflies), *Diptera* (flies) and others with 9 % (Rakić et al. 2022). The number of people who regularly consume insects is estimated at at least 2 billion (Čaklovica et al. 2021). Insects are a source of vitamins, minerals and proteins of animal origin. They require significantly less food to obtain mass and have a lower emission of greenhouse gases compared to the usual farm breeding of animals: pigs or cattle. For example, a house cricket (*Acheta domesticus*) needs 1.7 kg of dry food for 1 kg for obtain mass, compared to 2 kg for poultry, 3.8 kg for pigs or 7 kg for cattle (Rakić et al. 2022). Compared to other food sources, the advantage of breeding insects as a source of food and feed is that they require less space and water, have a short life cycle and better food conversion. They have significant benefits for the environment, economy and food availability (Starčević et al. 2022).



Figure 1: Realistic representation of insects as food: a) *Acheta domesticus* powder; b) *Alphitobius diaperinus*; c) *Tenebrio Molitor*; d) *Locusta migratoria*

Under the *Regulation (EU) 2015/2283* on novel foods, insects and products derived from insects are considered novel foods and are subject to an approval process, and whole edible insects and insect-derived ingredients can be lawfully placed on the market. Thus, by *Commission Regulation (EU) 2023/5* authorising the placing on the market of *Acheta domesticus* (house cricket) partially defatted powder as a novel food and amending *Implementing Regulation (EU)*

2017/2470, the placing of the said product on the market was approved for the company "Cricket One" for five years. This company, which is engaged in the production and distribution of insect-based food, has submitted a request that the partially defatted *Acheta domesticus* powder be approved for use in: bread and pastries from several types of cereals, dry premixes for bakery products, crackers, grain bars, biscuits, sauces, soups, processed potato products, dishes based on legumes and vegetables, pasta-based dishes, Whey powder, meat analogues and some other foods and products (Rakić et al. 2022).

One of the biggest obstacles to the use of insects in the diet of humans and animals is food safety. Eating insects carries the risk of chemical, physical and biological hazards and the occurrence of allergies. Cases of histamine poisoning have been recorded after eating fried insects that have a higher histidine content. These health risks when eating insects can be controlled by introducing a good manufacturing practice and hygiene practices (Starčević et al. 2022). Thus, Thailand was the first to describe Good Agricultural Practice (GAP) for breeding crickets. It is necessary to create standard operating procedures and Hazard analysis and critical control points systems (HACCP systems) for raw insects to ensure safe handling, processing and distribution to end consumers (Čaklovica et al. 2021). Countries where insect consumption is commonplace already have well-established breeding and production processes. In 2022, the International Platform of Insects for Food and Feed (IPIFF), as the EU's umbrella organization for the insect production sector, issued the document "Guide to best practices for hygienic and quality insect production". The document describes procedures in the primary production and processing of insects and recommendations for quality and hygiene control, including recommendations for the implementation of the HACCP system in this food sector (Čaklovica et al. 2021).

Before the placing on the market of the European Union of any insects is approved, the European Food Safety Authority (EFSA) provides a scientific opinion on its safety for consumption. Thus, on the EU market, in addition to the *Acheta domesticus* (house cricket) partially defatted powder as a novel food in 2023 (Image1-a), the European Commission approved the introduction of frozen, dried and powdery forms of *Alphitobius diaperinus* larvae (Image 1-b), better known as the small mealworm, which EFSA has determined are safe. In the list of insect foods approved by the EU, in addition, there is also the mealworm *Tenebrio Molitor* (Image 1-c) and the *Locusta migratoria* (Image 1-d) have been included since earlier (Commission Regulation (EU) 2022/188).

5. CONCLUSION

World food production must be transformed to achieve the sustainable development goals, and it is also necessary to increase the resilience of existing food production systems to the negative implications of climate change. Food needs increase as the world population grows, while the

Eighth International Scientific Conference "June 5th - World Environment Day"

amount of arable and pasture land for extensive agricultural production is decreasing. Therefore, alternative and sustainable food sources, such as sustainable aquaculture, microalgae cultivation, or insect use in human consumption must be researched and developed, and the public should be educated about the necessity of changes in traditional eating habits and increase the acceptability of these alternative sources of nutrients.

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Eighth International Scientific Conference
"June 5th - World Environment Day"

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EFFECT OF VERMICOMPOST ON ROOTING OF *NERIUM OLEANDER* L. CUTTINGS

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Key words: *Nerium oleander* L., cuttings, vermicompost, rooting parameters

ABSTRACT:

Nerium oleander L. as perennial ornamental plant is grown as a shrub or small tree worldwide in temperate and subtropical areas. Its propagation is usually done with its cutting and the rooting media is sand and peat. An investigation was carried out to study the effect of use of vermicompost based media on rooting of *Nerium oleander* L. cuttings. The rooting medias were combination of vermicompost and sand (pure sand, 50% sand 50% vermicompost, 70% sand 30% vermicompost, 30% sand 70% vermicompost, 20% sand 80% vermicompost). The cutting was taken from the central part of stem. The following root parameters were measured: percentage of rooting; no. of roots per cutting; the number of new leaves per rooted cutting; total root length and no. of secondary and tertiary roots. The results showed that pure sand and mixtures with lower percentage of vermicompost favored percentage of rooting, the number of new leaves and all the other followed parameters.

1. INTRODUCTION

Nerium oleander L. as perennial ornamental plant is grown as a shrub or small tree worldwide in temperate and subtropical areas. It is extensively used in landscape design mostly in parks and recently became very popular grown along roadsides. Due to its large use, its nursery propagation is very important. Since this plant is usually propagated using stem cuttings improvement of its rooting can considerably enhance nursery production and the choice of rooting media has very important role (Ochoa et al. 2003).

Vegetative propagation with cuttings in general is the most important way for clonal regeneration of many horticultural crops: ornamentals, fruits, nuts and vegetables, as well as for vegetative propagation of forestry plants (Hartmann et al., 2010). Substrates for rooting of ornamental cuttings can be classified as "light" substrates, consisting of natural components such as peat and sand, and artificial components which are the most often perlite (Davidescu et al., 2001). Rooting media directly influences the growth, development, ramification and functioning of rooting system. An ideal rooting medium should be loose, porous with high water holding capacity. Rooting medium holds cutting in place, holding moisture content for new emerged roots, congenial conditions for respiration and maintains optimum temperature for the root initiation (Kumar, et al. 2021).

Nerium oleander L. propagation is usually done with its stem cutting and recommended rooting media is sand and peat. Peat replacement became an issue recently and a few organic materials have been suggested either as substrates or as substrate constituents (Hernandez et al., 2014). Some of them is vermicompost.

An investigation was carried out to study the effect of use of vermicompost-sand based media on rooting of *Nerium oleander* L. cuttings.

2. MATERIAL AND METHODS

The research was conducted at the greenhouse of the Agromediterranean Faculty Dzemal Bijedic University of Mostar (longitude 43° 20' 35" N., latitude 17° 48' 29" E.) in the period from July to September 2019. It is an unheated greenhouse, with automatic ventilation, summer shading and automated watering and sprinkling. The usable area covers an area of 220 m². The position is east west.

Plants were of cutting origin and from a 8-year-old plant of *Nerium oleander* L. cultivated in the park of the faculty. Hardwood stem cuttings were collected on 03.07.2019. in the early morning hours from the previous year's growth cycle. The stems did not have traces of diseases and pests. With a sharp, clean knife, 10 cm long cuttings up to 2 nodes were taken from the central part of the stem. The upper cut was oblique, clean and smooth, and the lower cut was straight, clean, and smooth. On the upper part of the cutting, 2 to 3 leaves were left, which were cut in half, while the lower part of the cutting was defoliated.

The rooting medias (treatments T) were combination of vermicompost and sand as follows:

T1 - pure sand

T2 - 50% sand 50% vermicompost

T3 - 70% sand 30% vermicompost

T4 - 30% sand 70% vermicompost

T5 - 20% sand 80% vermicompost

There was no application of rooting hormone before planting into treatments and 200 cutting together (40 per treatment). Throughout the rooting period of three months, the appropriate environmental factors were ensured: temperature 22-25°C, humidity 95 – 98% with frequent watering of substrate. On the 03.09.2019, the cuttings were harvested after 3 months, relevant data were recorded (percentage of rooting; no. of roots per cutting; the number of new leaves per rooted cutting; total root length and no. of secondary and tertiary roots) and statistically analyzed.

3. RESULTS AND DISCUSSION

Investigated process of rhizogenesis was influenced differently depending on the rooting medias in which the cuttings were rooted.

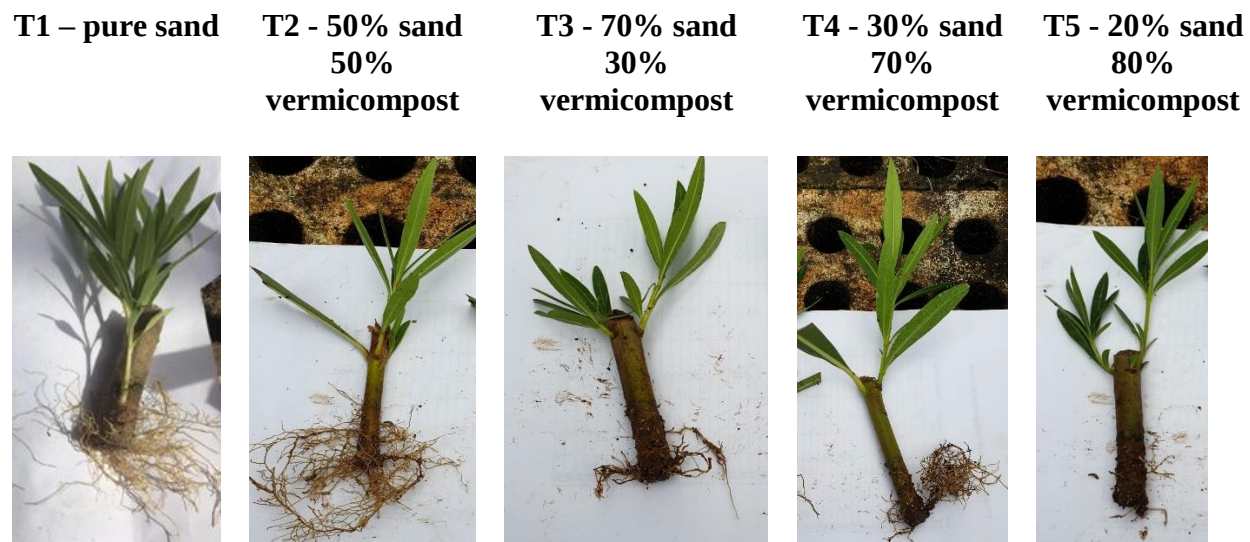
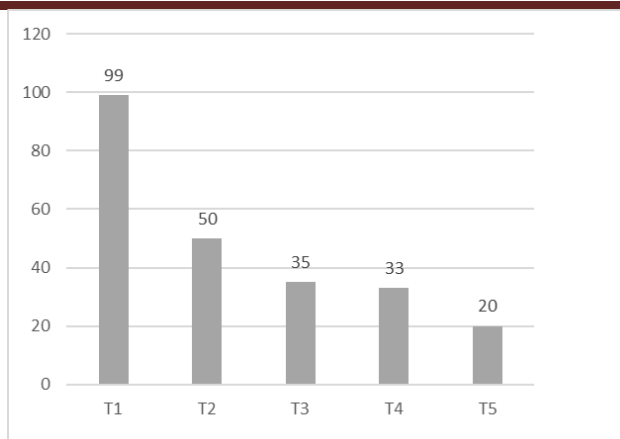


Figure 1: Rooted cuttings of *Nerium oleander* L. from all the treatments after three months

3.1 Percentage of rooting

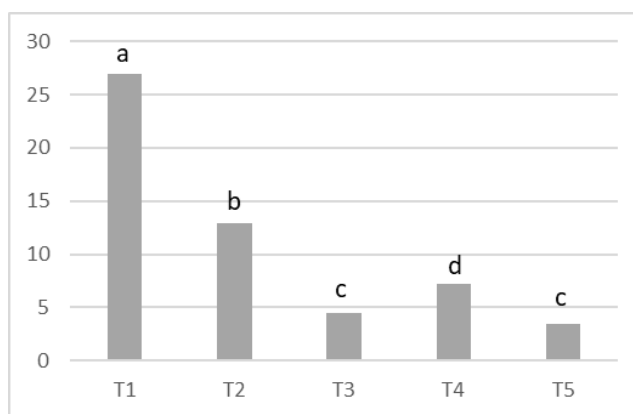
The percentage of rooting is shown in graph 1. The recorded values were different, and they valued from 99% to 20%.



Graph 1: The percentage of rooting (%)

As it can be seen in the graph 1, the highest percentage of rooting and number of roots per cutting was recorded for treatment T1 – pure sand and it gradually decreased following the decrease of sand percentage in the treatment. The rooting percentage in pure sand was investigated by Vasca - Zamfir et al, 2022, and recorded rooting percentage was 50%, while Ochoa et al. 2003 reported 66,8%. Obviously, in this experiment only 20% of rooting was recorded for treatment with the highest portion of vermicompost in media.

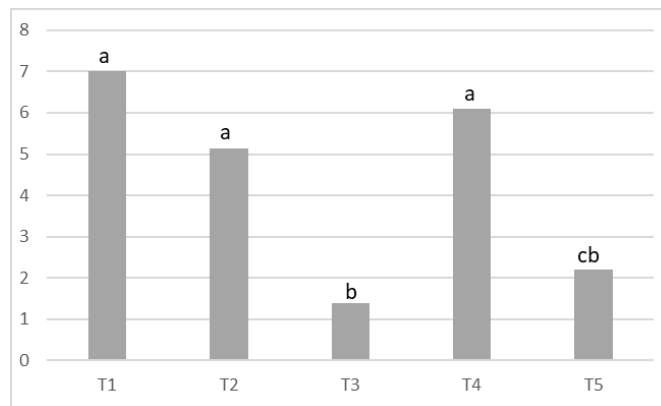
3.2 Number of roots per cutting



Graph 2: Number of roots per cutting

The highest number of roots per cutting was recorded for treatment T1 – pure sand and it also gradually decreased following the decrease of sand percentage in the treatment. Treatment T3 had lowest number of roots.

3.3 Number of new leaves per cutting

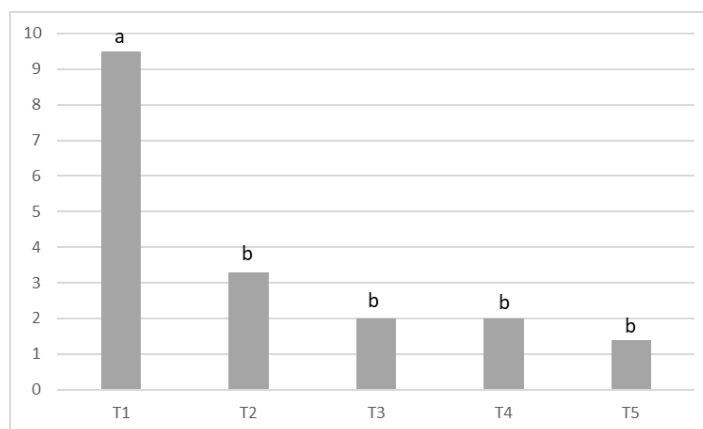


Graph 3: Number of new leaves per cutting

Graphs 3. presents the number of new leaves per rooted cutting. Treatments T1, T2 and T4 treatments effected creation of more new leaves, and the number of leaves itself for treatments 1, 2 and 4 had no significant difference.

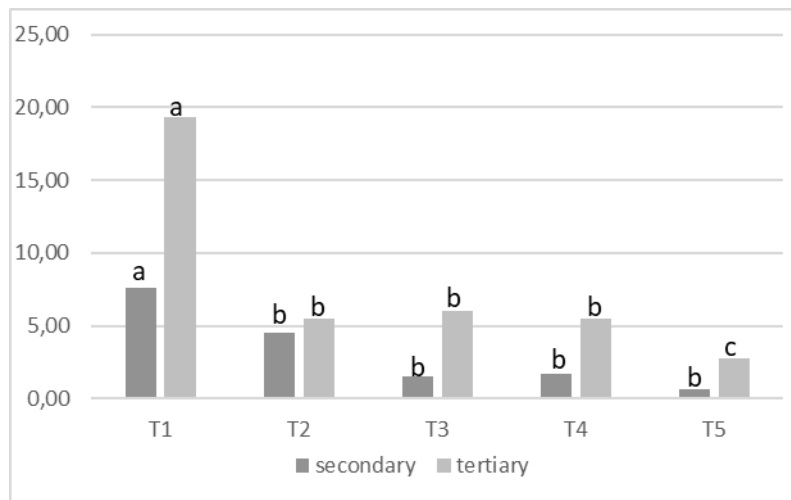
3.4 Total root length

The root lengths as it can be seen in the graph 4. was the same for all the treatments with added vermicompost. Results also indicate again that pure sand influences and the longest roots (9,47 cm), while the lowest roots were recorded for the cuttings with T5 (1,3 cm). Vasca-Zamfir et al. 2022. similarly reported 9.40 cm long roots in pure sand rooting media.



Graph 4: Total root length (cm)

3.5 Number of secondary and tertiary roots



Graph 5: Number of secondary tertiary roots in comparison

Graph 5. shows the number of secondary and tertiary roots after three months of rooting process. Pure sand treatment still has the highest influence on creation of secondary and tertiary roots, and it is obvious that development of tertiary roots enhances in the treatments with vermicompost addition. Similar recorded results of sand presenting the highest values for the number of secondary roots was obtained by Shaker et al. 2017.

Due to the optimum availability of water and nitrogen in the rhizosphere because added vermicompost in the rooting medium, it is obvious that secondary roots started with uptake of available nutrition, therefore tertiary roots started to develop in rooting medias with larger portion of vermicompost too.

4. CONCLUSION

The rooting medium based on pure sand had shown the most significant effect over other rooting media with vermicompost added. It is to be concluded that all parameters decreased gradually following the decrease of sand percentage in the treatment and increase of vermicompost percentage.

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INFLUENCE OF LAYER HEN FEEDING ON EGG QUALITY CHARACTERISTICS

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Key words: laying hens, garlic powder, egg quality characteristics

ABSTRACT:

A total number of 96 laying hens Isa Brown layers 28 weeks old divided into four equal groups were fed with designed meals for 4 weeks so that garlic powder was added to the standard feed mixture in concentrations of 2%, 4%, and 6%, and the fourth group was the control group without the addition of garlic powder. Diet and drinking water were provided ad libitum during the experimental period. It was examined the impact of adding garlic powder to the standard feed mixture on egg quality parameters such as egg mass and its components, egg shape index, yolk index, egg white height and value of Hogg units. The addition of the garlic powder in the standard feed mixture improves egg mass, as well as the mass of egg white and eggshell. The experimental group that took the addition of garlic powder of 6% in the standard feed mixture resulted in eggs of greater mass, greater mass of white and shell compared to the eggs of hens fed with the standard feed mixture ($p < 0.01$). The feeding significantly enhanced the albumen height value, as well as Hogg unit values. This applies only to the group with 2% of share of garlic powder, where the increase of albumen height value was 1.417 mm, and increase in Hogg units values was 5.909, and to the group with 6% garlic powder share, where the increase in the albumen height value was 1.250 mm and the increase in Hogg units value was 4.964, while the treatment had no statistically significant effect on shape index and yolk index. Egg shell thickness increased compared to the control group, with a statistically significant increase ($p < 0.05$) in the group receiving the 2% garlic powder supplement.

1. INTRODUCTION

(*Allium sativum*), according to the botanical classification, belongs to the lily family. It has been cultivated and consumed for more than 5000 years and is the oldest used spice. It originally came from Central and Central Asia, and after conquering Egypt, it arrived in Europe. The main active ingredient in garlic is allicin. The energy value of garlic is only 149 kcal per 100 g of fresh food. It contains more than 200 biologically active substances, including vitamins, minerals, amino acids, and enzymes. It is extremely rich in manganese, selenium, phosphorus, vitamin C, and vitamin B6. Among the medicinal substances, it contains essential oils that contain sulphur, have a specific smell, and have great volatility and sensitivity. Other substances include alliin, nitrogenous substances, vegetable fats, mineral substances, polyoses such as inulin, and compounds related to iodine. All the mentioned substances in garlic are in concentrated form, and the medicinal ones are already in very low concentrations (Navidshad et al., 2018). Garlic is used in a fresh and dried state as a paste, dehydrated powder or extract, macerates, and essential oils based on garlic (Amagase et al., 2001).

Dehydrated garlic powder. Today, dehydrated garlic powder is mainly used as an aromatic spice. Cloves of raw garlic are chopped or crushed, dried, and ground into powder. Since garlic powder is actually just dehydrated raw garlic cloves, the composition, and especially the allinase activity, of garlic powder is identical to that of raw garlic. However, the temperature during the dehydration process should not exceed 60 °C; above this temperature, allinase inactivation occurs (Banerjee and Maulik, 2002). Commercially available preparations based on garlic can be divided into two categories: rich allicin and not rich allicin. The former are produced from raw garlic, and the latter from processed garlic (Chowdhury et al., 2002). They can differ significantly in allicin content up to 10 times. As a result, different garlic preparations have up to 20 times different allicin content, which makes it difficult to compare results on the beneficial effects of garlic on human health (Reiner, 2000).

Egg quality characteristics. There are a large number of definitions that more closely explain and define the quality of the product, i.e., eggs. Pavlovski et al. (2007) state in their review the definition of quality taken from the Oxford English Dictionary, according to which quality is the degree of perfection of a product. Furthermore, that quality is a matter of personal perception, that is, the reception of stimuli through the five senses. Given that the last and most important link in the chain of production of table eggs is the consumer and that the success of the production largely depends on his attitude towards a certain product, it can be said that a quality egg is what consumers prefer. There is an increasing demand for eggs produced under acceptable conditions for the consumer, with a focus on organoleptic properties, the well-being of hens, and the preservation of the natural environment. In this sense, the term quality implies the sum of the mutual influences of people, chickens, environment, and equipment (Pavlovski et al., 2007; Akter et al., 2014; Augere-Granier, 2019).

a) Characteristics of the external quality of eggs

Egg mass. We can determine the mass of the egg by measuring it on a scale. According to the measured mass of eggs, they are classified into certain classes defined in each country by the respective regulations. In our country, considering the quality of eggs, they are classified into "A" class eggs and "B" class eggs. Class A eggs must be sorted by weight (Regulation on standards for putting eggs on the market in Official Gazette of BiH, No. 25/10).

Color, thickness, strength, and mass of the shell. The color of the shell can be measured with a refractometer or evaluated by a subjective assessment, scoring from 1 (light) to 5 (dark). The shell thickness is measured with a micrometer and ranges from 0.32 to 0.35 mm. Eggshell strength is determined in two ways: by determining its deformation or by determining the breaking force. Eggshell mass is measured by breaking the shell, separating the membrane, drying it, and then measuring its mass (Jurić et al., 2005).

Egg shape. Egg shape is a feature that is expressed through the shape index. A normal-shaped egg has a shape index of about 73%, and the values range from 61% to 86% (Duman et al., 2016).

b) Internal features and qualities of eggs

Yolk color. Yolk color is a very important quality characteristic that varies among consumers worldwide and ranges from light yellow to orange. It can be determined using the so-called Roche's fans by visually comparing the color of the yolk with the palette of colors on the fan. When the most similar color is determined, its value is read in points (the scale ranges from 1 to 15) (Perić and Birkhold, 2005).

Yolk index. The yolk index is the ratio of yolk height to yolk width. Normal values range from 32 to 58%. As the egg ages, the yolk index decreases (Perić and Birkhold, 2005). The quality of the egg white can be determined by measuring the height of the egg white, which is expressed in millimeters. The fresher the egg, the higher the height of the white. This parameter is used to calculate hog units; it is considered that the egg quality is better if the value of hog units is above 70 (Duman et al., 2016; Đukić Stojčić and Perić, 2018).

2. MATERIAL AND METHODS

2.1 Conditions for keeping laying hens

Dehydrated garlic powder used in the feed mixture for feeding laying hens consists of 12.80% protein, 3.75% fat, 5.05% ash, and 5.00% water.

Eighth International Scientific Conference
"June 5th - World Environment Day"

Table 1: Composition of the feed mixture for feeding laying hens (share in % of the total feed mixture) control and experiment

Composition of feed mixture%	Control group	Experimental groups			
	0 %	2% GP	4% GP	6% GP	
Corn	58.36	56.36	54.36	52.36	
Soybean meal, 44% SP	26.31	26.31	26.31	36.31	
Fodder chalk	8.19	8.19	8.19	8.19	
Dehydrated alfalfa	2.92	2.92	2.92	2.92	
Sunflower oil	2.92	2.92	2.92	2.92	
Sodium chloride	0.3	0.3	0.3	0.3	
Garlic powder	0	2	4	6	
Vitamin-mineral premix*	1	1	1	1	
TOTAL	100	100	100	100	
Calculated nutritional ingredients					
Metabolic energy (kcal ME/kg)	2311.86	2253.94	2196.02	2138.1	
Crude protein%	18.14	18.19	18.23	18.29	
Crude fiber%	5.46	5.36	5.26	5.16	
Ether extract%	4.95	4.79	4.63	4.47	
Ca %	3.1	3.1	3.1	3.09	
Total phosphorus%	0.44	0.47	0.46	0.45	
methionine %	0.24	0.24	0.24	0.24	
lysine %	0.96	0.96	0.95	0.94	

¹ 1 kg of vitamin-mineral premix contains 960,000 IU of vitamin A, 200,000 IU of vitamin D3, 2,000 IU of vitamin E, 160 mg of vitamin K3, 800 mg of Ca - D - pentathenate, 2,000 mg of nicotinic acid 360 mg of vitamin B6, 1,600 mg of vitamin B12, 4,000 mcg of biotin, 3,200 mg of zinc, 4,800 mg manganese, 1,200 mg of copper, 120 mg of iodine, 24 mg of cobalt, 30 mg of selenium, 420 mg of canthaxanthin, and 36,000 FTU of 6 phytase. 0% GP - control group, 2% GP - 2% share of garlic powder in the feed mixture, 4% GP – 4% share of garlic powder in the feed mixture, 6% GP – 6% share of powder in the feed mix.

Laying hens were fed on a private farm in the municipality of Bosanska Krupa, Una-Sana Canton, Bosnia and Herzegovina. Garlic powder was purchased from the company Kolmix d.o.o., Municipality of Velika Kladuša, Una-Sana Canton, Bosnia and Herzegovina. Laying hens of the "Isa-Brown" breed aged 28 weeks were divided into three experimental groups and a control group. They were fed designed meals over a period of 4 weeks. Hens were divided into three groups of 32 laying hens in each experimental group (cage breeding) plus a control group of 32 laying hens. Each group consists of 8 replicates, with 4 laying hens in each replicate, for a total of 96 laying hens. Garlic powder in concentrations of 2%, 4%, and 6% was added to the standard mixture for feeding laying hens. In the control group of laying hens, no garlic was added to the feed mixture. Water and food are provided *ad libitum*. Light period: 16 hours; dark period: 8 hours. At the end of the experimental feeding period, samples of fresh eggs were taken from each group using the random sampling method. For each of the three experimental groups mentioned, there were 12 fresh eggs and 12 eggs from the control group.

Eggs were stored at 2°C to 8°C before the analysis in the Laboratory of Biotechnical faculty Bihać. Egg mass was determined on an analytical balance (Mettler Toledo). The shape index was obtained according to the formula: (egg width/egg length) x 100 (Perić and Birkhold, 2005), and the yolk index was obtained according to the formula: (the average yolk height/the average yolk

width) x 100 (Perić and Birkhold, 2005). The height and width of the yolk were measured with the help of a calibrated compass. Thick egg white height: it was taken between the yolk and the outer border of the egg white. Egg shell thickness: shell thickness was measured by taking three pieces of shell from smaller, medium, and larger eggs. The membrane was separated from the eggshell, and the measurement was made using a calibrated caliper. The average thickness of the eggshell was taken. Hogg's units (HJ) were calculated according to the Haugh method from the height of the dense egg white (H) and the weight of the egg (W) in grams according to the following formula (Haugh, 1937):

$$HJ = 100 \log (H + 7,77 - 1,7W^{0,37})$$

Organoleptic analysis. The following were determined: smell, taste, and color (absence) of foreign impurities in the egg yolk. The evaluation was subjective, and the results were scored on a scale of 1–5 for each of the mentioned characteristics. A comparison was made with samples from the control group. The hedonic acceptability test was used (Watts et al., 1989). At the end of the experimental period, the respondents, a total of 30 respondents who consume eggs in their daily diet, received a questionnaire with questions to evaluate the degree of acceptability based on the following questions: 1 - I don't like it; 2 - I partly don't like it; 3 - I neither like it nor I don't like it; 4 - I partly like it; and 5 - I really like it, to rate each of the representative properties of eggs: smell, taste, and color. Statistical data processing and we used the method of descriptive analysis for statistical data processing. We calculated the arithmetic mean, variance, standard deviation, maximum and minimum variations, and coefficient of variation (Komić, 2000). Significance level, $\alpha=0.05$. * $p<0.05$ – statistically significant difference between groups, ** $p<0.01$ – highly statistically significant difference between groups, $p>0.05$, NS – no statistically significant difference between groups. We tested the results by applying the appropriate variance analysis model, analysis of variance with one factor of variability between the control group (the group that took the standard feed mixture) and the experimental groups (groups with the addition of garlic powder in the feed mixture in concentrations of 2%, 4%, 6%). We used the Microsoft Excel 2010 statistical package for data processing.

3. RESULTS AND DISCUSSION

Table 2 shows the mass of eggs used in the experiment (g); whole egg, whites, yolks and shell for the control group, the group without the addition of garlic in the feed mixture (0% garlic powder GP) and three experimental groups with 2%, 4% and 6% of garlic powder in the standard feed mixture.

Eighth International Scientific Conference
"June 5th - World Environment Day"

Table 2: Analysis of egg meat and its components – control and experiment (g)

Ingredient of feed mixture %	Control group	Experimental group			
	0 %	2% GP	4% GP	6% GP	
Whole egg mass	66.03	67.02	68.30	69.20	
Egg white mass	38.31	39.27	40.58	40.70	
Yolk mass	19.61	18.63	18.78	19.38	
Shell mass	8.20	8.34	8.90	9.12	
Ratio (W:Y) ¹	1.9:1	2.1:1	2.2:1	2.1:1	

¹ egg whites: egg yolks, ² 0% GP - control group, 2% GP - 2% share of garlic powder in the feed mixture, 4% GP - 4% share of garlic powder in the feed mixture, 6% GP - 6% share of powder in the feed mixture.,³ n = 12.

Table 3 shows the average proportions of egg components in the whole egg (% of the whole egg) for the control group, the group without the addition of garlic in the feed mixture (0% PA), and the three experimental groups with 2%, 4%, and 6% proportions of garlic powder in a standard feed mixture.

Table 3: Average proportion of egg components - control and experiment (% in whole egg)

Proportion in egg %	Control group		Experimental group	
	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$
	0% GP	2% GP	4% GP	6% GP
Egg whites	58.01±1.6177	58.79±4.0503	59.39±1.9816	58.53±2.7556
Egg yolks	29.69±1.9040	27.84±3.1239	27.52±2.4588	28.05±2.6643
Shell+membrane	12.43±0.8656	12.44±1.0245	12.95±2.7435	12.87±1.5044

¹ 0% GP - control group, 2% GP - 2% share of garlic powder in the feed mixture, 4% GP - 4% share of garlic powder in the feed mixture, 6% GP - 6% share of powder in the feed mixture.² $\bar{x}$ -mean, SD –standard deviation.³ n = 12.

From the Table 2 we can see that the mass of the whole egg increased with the addition of garlic powder in the feed mixture, as well as the mass of the egg white and the mass of the egg shell, while the mass of the yolk decreased compared to the control group. The proportion of egg white also increased compared to the control group (Table 3). The group with a 4% addition of garlic powder had the highest percentage of egg white (59.39%) compared to the control group (58.01%). Similarly, the share of yolk in the egg was the lowest in the group with 4% supplement (27.52%) compared to the control group (29.69%).

The proportion of shell in the egg increased with the increase in the concentration of garlic powder in the standard feed mixture, and in the control group it was the lowest (12.43%), while in the group with 4% addition it was 12.95%.

Tables 4, 5 and 6 present the statistical variation parameters for the mentioned parameters (whole egg mass (g), egg white mass (g), yolk mass (g), and shell mass (g)) and comparisons of each of the experimental groups (different concentrations of garlic powder in the feed mixture) with the control group (the group that did not have the addition of garlic powder in the standard feed mixture).

Eighth International Scientific Conference
"June 5th - World Environment Day"

Table 4: Statistical - variational parameters of the egg and its components (g) - control and experiment with 2% of garlic powder

Parameter	Control group	Experimental group	F-test value
Whole egg mass(g)	0% GP	2% GP	
$\bar{x}$	66.025 ⁿ	67.015 ⁿ	
SD	2.65231	4.85739	F=0.38528 ^{NS}
CV	0.04	0.072	
Egg white mass (g)	0% GP	2% GP	
$\bar{x}$	38.308 ⁿ	39.273 ⁿ	
SD	2.03714	2.04326	F=1.34002 ^{NS}
CV	0.053	0.052	
Egg yolk mass (g)	0% GP	2% GP	
$\bar{x}$	19.608 ⁿ	18.633 ⁿ	
SD	1.54653	2.26328	F=1.51823 ^{NS}
CV	0,079	0.121	
Egg shell mass (g)	0% GP	2% GP	
$\bar{x}$	8.200 ⁿ	8.342 ⁿ	
SD	0.587208	0.93853	F=0.19935 ^{NS}
CV	0.069	0.112	

¹ $\bar{x}$ - average value, SD - standard deviation, CV - coefficient of variation, F value - value of Fisher's test..² 0% GP - control group, 2% GP - 2% share of garlic powder in the feed mixture.³ NS –not determined statistically significant difference. ⁴ n = 12.

Table 5: Statistical - variational parameters of egg mass and its components (g) - control and experiment with 4% of garlic powder

Parameter	Control group	Experimental group	F-test value
Whole egg mass(g)	0% GP	4% GP	
$\bar{x}$	66.025 ⁿ	68.300 ⁿ	
SD	2.65231	4.862778	F=2.18295 ^{NS}
CV	0.04	0.0768	
Egg white mass (g)	0% GP	4% GP	
$\bar{x}$	38.308 ⁿ	40.575 ⁿ	
SD	2.03714	3.20939	F=4.26662 ^{NS}
CV	0.053	0.0579	
Egg yolk mass (g)	0% GP	4% GP	
$\bar{x}$	19.608 ⁿ	18.775 ⁿ	
SD	1.54653	1.85478	F=1.42892 ^{NS}
CV	0.079	0.099	
Egg shell (g)	0% GP	4% GP	
$\bar{x}$	8.200 ⁿ	8.900 ⁿ	
SD	0.587208	2.18299	F=1.15459 ^{NS}
CV	0.069	0.164	

¹ $\bar{x}$ - average value, SD - standard deviation, CV - coefficient of variation, F value - value of Fisher's test..² 0% GP – control group, 4% GP – 4% the proportion of garlic powder in the fodder mixture.³ NS – no statistically significant difference was found. ⁴ n = 12.

Eighth International Scientific Conference
"June 5th - World Environment Day"

Table 6: Statistical - variation parameters of egg mass and its components (g) - control and experiment with 6% garlic powder

Parameter	Control group	Experimental group	F-test value
Whole egg mass(g)	0% GP	6% GP	
$\bar{x}$	66.025 ⁿ	69.200 ^B	
SD	2.65231	2.60209	F=8.762155**
CV	0.04	0.038	
Egg white mass (g)	0% GP	6% GP	
$\bar{x}$	38.308 ⁿ	40.700 ^B	
SD	2.03714	1.86059	F=9.01776**
CV	0.053	0.046	
Egg yolk mass (g)	0% GP	6% GP	
$\bar{x}$	19.608 ⁿ	19.383 ⁿ	
SD	1.54653	2.25583	F=0.0812 ^{NS}
CV	0.079	0.116	
Egg shell mass (g)	0% GP	6% GP	
$\bar{x}$	8.200 ⁿ	9.116 ^B	
SD	0.587208	0.70946	F=12.13973**
CV	0.069	0.165	

¹ $\bar{x}$ -average value, SD –standard deviation, CV –coefficient of variation, F value –value of Fisher's test.² 0% GP – control group, 6% GP – 6% the proportion of garlic powder in the fodder mixture.³ A, B – average values in the same row marked with different letters are highly statistically significantly different from each other (p<0,01). ⁴ n = 12.

Table 7: Statistical - variation parameters for egg quality characteristics that were used in the work; shape index (%), yolk index (%), egg shell thickness (mm), egg white height (mm) and Hogg's units – control and experiment with 2% garlic powder

Average	Control group	Experimental group	F-test value
Shape index(%)	0% GP	2% GP	
$\bar{x}$	78.846 ^a	77.868 ^a	
SD	1.571	3.374	F=0.82751 ^{NS}
CV	0.019	0.043	
Yolk index (%)	0% GP	2% GP	
$\bar{x}$	44.493 ^a	45.014 ^a	
SD	2.424	2.451	F=0.27386 ^{NS}
CV	0.054	0.054	
Egg shell thickness (mm)	0% GP	2% GP	
$\bar{x}$	0.34 ^A	0.36 ^B	
SD	0.0072	0.0098	F=18.18367**
CV	0.021	0.027	
Egg white height (mm)	0% GP	2% GP	
$\bar{x}$	9.476 ^a	10.833 ^b	
SD	0.996	1.467	F=7.66024*
CV	0.105	0.135	
Hog's units	0% GP	2% GP	
$\bar{x}$	96.071 ^a	101.980 ^b	
SD	4.228	5.584	F=7.83035*
CV	0.044	0.055	

¹ 0% GP –control group, 2% GP – 2% the proportion of garlic powder in the fodder mixture.² $\bar{x}$ -average value, SD –standard deviation, CV – coefficient of variation, F value – value of Fisher's test.³ a, b – average values in the same row marked with different letters are statistically

Eighth International Scientific Conference "June 5th - World Environment Day"

significantly different from each other ($p < 0,05$).⁴ A, B –average values in the same row marked with different letters are highly statistically significantly different from each other ($p < 0,01$).⁵ NS – no statistically significant difference was found. ⁶n = 12

Table 8: Statistical-variational parameters of egg quality characteristics used in the work; shape index (%), yolk index (%), egg shell thickness (mm), egg white height (mm) and Hogg's units – control and experiment with 4% garlic powder

Average	Control group	Experimental group	F-test value
Shape index(%)	0% GP	4% GP	
$\bar{x}$	78.846 ^a	77.712 ^a	
SD	1.571	2.885	F=1.43022 ^{NS}
CV	0.019	0.037	
Yolk index (%)	0% GP	4% GP	
$\bar{x}$	44.493 ^a	44.843 ^a	
SD	2.424	3.159	F=0.09272 ^{NS}
CV	0.054	0.07	
Egg shell thickness (mm)	0% GP	4% GP	
$\bar{x}$	0.34 ^a	0.35 ^a	
SD	0.0072	0.0371	F=1.25317 ^{NS}
CV	0.021	0.106	
Egg white height (mm)	0% GP	4% GP	
$\bar{x}$	9.476 ^a	10.583 ^a	
SD	0.996	1.676	F=4.29482 ^{NS}
CV	0.105	0.158	
Hog's units	0% GP	4% GP	
$\bar{x}$	96.071 ^a	100.607 ^a	
SD	4.228	6.206	F=4.01269 ^{NS}
CV	0.044	0.062	

¹ 0% GP –control group, 4% GP – 4% share of garlic powder in the feed mixture. ² $\bar{x}$ -average value, SD –standard deviation, CV- coefficient of variation, F value– value of Fisher's test.³ NS – no statistically significant difference was found.⁴ n = 12.

Table 9: Statistical-variational parameters of egg quality characteristics used in the work; shape index (%), yolk index (%), egg shell thickness (mm), egg white height (mm) and Hogg's units – control and experiment with 6% garlic powder

Average	Control group	Experimental group	F-test value
Shape index(%)	0% GP	6% GP	
$\bar{x}$	78.846 ^a	78.833 ^a	
SD	1.571	2.656	F=0.00023 ^{NS}
CV	0.019	0.032	
Yolk index (%)	0% GP	6% GP	
$\bar{x}$	44.493 ^a	44.509 ^a	
SD	2.424	2.569	F=0.00024 ^{NS}
CV	0.054	0.057	
Egg shell thickness (mm)	0% GP	6% GP	
$\bar{x}$	0.34 ^a	0.35 ^a	
SD	0.0072	0.0144	F=0,51163 ^{NS}
CV	0.021	0.041	
Egg white height (mm)	0% GP	6% GP	
$\bar{x}$	9.416 ^A	10.666 ^B	

Eighth International Scientific Conference
"June 5th - World Environment Day"

SD	0.996	1.073	F=9.32450**
CV	0.105	0.101	
Hog's units	0% GP	6% GP	
$\bar{x}$	96.071 ^a	101.035 ^b	
SD	4.228	4.242	F=7,88453**
CV	0.044	0.042	

¹ 0% GP – control group, 6% GP – 6% share of garlic powder in the feed mixture. ² $\bar{x}$ – average value, SD – standard deviation, CV – coefficient of variation, F value – value of Fisher's test. ³ a, b- average values in the same row marked with different letters are statistically significantly different from each other (p<0,05). ⁴ A, B – average values in the same row marked with different letters are highly statistically significantly different from each other (p<0,01). ⁵ NS – no statistically significant difference was found. ⁶ n = 12.

The experimental group that took the addition of garlic powder of 6% in the standard feed mixture produced eggs of greater mass and greater mass of white and shell compared to the eggs of hens fed with the standard feed mixture. The values are highly statistically different (p<0.01), the other two experimental groups of laying hens (2% and 4% addition of garlic powder) had no statistically significant difference (p>0.05) in the mentioned parameters compared to the control.

The shell thickness increased highly statistically significantly (p<0.01) in the group with 2% garlic powder (0.36 mm) compared to the control group (0.34 mm) (Table 7); in the group with 4% and 6% addition, no statistically significant difference (p>0.05) was recorded in the eggshell thickness compared to the control group (Tables 8 and 9). The values for the height of the egg white were the highest in the group with 2% garlic powder in the feed mixture and the group with 6% of the supplement. The height of the egg white was statistically significantly different in the experimental group with 6% addition of garlic powder (10.666 mm) (p<0.01) compared to the control group (9.416 mm) (table 11). In the group that took 2% addition of garlic powder, a statistically significant difference (p<0.05) was observed in the height of the egg white compared to the control (Table 7). The value of Hog's units was the highest in the group with 2% addition of garlic powder (101.98) in relation to the control group (96.071). The values of Hog's units for the experimental groups with 2% and 6% addition were statistically significantly different in relation to the value in the control group (p<0.05) (Tables 8, 9).

Table 10 shows the influence of feeding with garlic powder on the organoleptic properties of smell, taste, and color of the laid eggs that were used in the test for all four groups: the control group (0% garlic powder GP) and the experimental groups with 2%, 4%, and 6% of garlic powder in the feed mixture. The point scale is expressed as an interval from 1 to 5.

Eighth International Scientific Conference
"June 5th - World Environment Day"

Table 10: Statistical - variation parameters organoleptic analysis of eggs; smell, color, taste of eggs (scoring scale from 1-5) – control and experiment

Organoleptic properties of eggs	Control group	Experimental group				F-test value
Smell	0% GP	2% GP	4% GP	6% GP		
$\bar{x}$	5	5	5	5		
SD	-	-	-	-		-
CV	-	-	-	-		
Taste						
$\bar{x}$	5	5	5	5		
SD	-	-	-	-		-
CV	-	-	-	-		
Colour						
$\bar{x}$	4.97 ^a	5 ^a	5 ^a	5 ^a		
SD	0.0887	-	-	-		F = 1.96231 ^{NS}
CV	0.017	-	-	-		

¹ 0% GP – control group, 2% GP – 2% % share of garlic powder in the feed mixture, 4% GP – 4% % share of garlic powder in the feed mixture, 6% GP – 6% % share of garlic powder in the feed mixture.² $\bar{x}$ – average value, SD – standar deviation, CV – coefficient of variation, F value value of Fisher's test.³ NS – no statistically significant difference was found.⁴ n = 1 (four eggs per subject for all listed organoleptic properties).

From Table 10, it can be seen that there was no statistically significant difference ($p > 0.05$) in the smell, taste, and color of eggs between the group without garlic control and all three experimental groups. The eggs had no garlic smell.

The mass of eggs increased with the increase in the proportion of garlic powder in the standard feed mixture, despite the fact that a statistically significant difference ($p < 0.01$) was observed only in the group with a 6% addition of garlic powder compared to the control group. Egg mass in the experimental group with 6% garlic powder was 69.2 g compared to the control group (66.025 g), which is an increase of 3.18 g. An increase in egg white mass, as well as egg shell mass, was also observed with an increase in the concentration of garlic powder in the standard feed mixture. The weight of egg whites in the group of laying hens that received a 6% addition of garlic powder (40.7 g) was statistically significantly different ($p < 0.01$) from the weight of egg whites in the control group of laying hens (38.308 g). The egg white mass of laying hens that received 6% garlic powder in the feed mixture increased by 2.39 g compared to the control group. The same trend was observed in the eggshell mass, where the shell mass in the group with the addition of 6% garlic powder (8.916 g) was highly statistically significantly different ($p < 0.01$) compared to the eggshell mass of the laying hens from the control group (8.2). The eggshell mass increased by 0.716 g in total. The addition of garlic powder had no significant effect on egg yolk mass (Table 6). In the other two experimental groups (2%, 4% share of garlic), no statistically significant difference was observed in the weight of the whole egg, the weight of the egg white, the yolk, or the weight of the egg shell (Tables 5, 6). The addition of garlic powder had a favourable effect on egg quality parameters, egg white height, and Hog's

units. Statistical significance ($p < 0.05$) was observed in the group that took garlic powder supplement 2% (10.833 mm) and in the group with 6% garlic supplement (10.666 mm), highly statistical significance ($p < 0.01$), compared to the control group (9.416 mm). Which is an increase in egg white height by 1.417 mm for the group with 2% addition and 1.25 mm for the group with 6% addition of garlic powder (Tables 7, 8, 9). The values of Hog units were for 2% addition: 101.98, 4%, 100.607, 6%, and 101.035, while the control group had a value of 96.071. Which is an increase of 5.909 for the group with the addition of 2%, 4.599 for the 4%, and 4.964 for the 6% addition of garlic powder in the feed mixture (Tables 8, 9). A statistically significant increase ($p < 0.05$) of Hog's units was observed in the experimental groups with 2% and 6% addition of garlic, while in the group with 4% addition, the increase was not statistically significant ($p > 0.05$). The addition of garlic did not have a statistically significant ($p > 0.05$) effect on the egg shape index or on the egg yolk index. These results are in accordance with the results reported by Olobatoke and Mulugeta (2011), and Kralik and Lovreković (2018). The diet with the addition of garlic powder had no adverse effect on the organoleptic properties of eggs in terms of color, taste, and smell ($p > 0.05$), and the respondents also did not smell the garlic in the eggs (Table 10). Ademola et al. (2011) state that the addition of garlic oil to the standard feed mixture improved the overall organoleptic properties of the egg yolk, while Olobatoke and Mulugeta (2011) state that the addition of 5% garlic powder caused a strong garlic smell in the eggs.

4. CONCLUSION

Based on the above, we can conclude that the addition of garlic had a favourable effect on the mass of the whole egg and the mass of the egg white. The concentration of garlic powder between 2% and 6% in the standard feed mixture had a favourable effect on the parameters of egg quality, egg white height, and Hog's units. The addition of garlic powder had no negative effect on the organoleptic properties of the egg yolk, taste, smell, or color.

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Eighth International Scientific Conference
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Eighth International Scientific Conference
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CONCENTRATION OF VITAMIN C IN LEAVES OF LIMEIRA LETTUCE DEPENDING ON DEVELOPMENT PHASE

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Key words: Lactuca, Limeria, beby leaf, vitamin C

ABSTRACT:

The research determines the content of vitamin C from the "baby leaf" to the formed plant, as well as the content of other components: K, Ca, P, Mg and Fe. The Limeria lettuce hybrid was grown in a greenhouse according to the principles of classic conventional production. The content of vitamin C increases from the 30th, 60th to the 90th day of physiological maturity, the recorded value is 20.28 mg/100 g, and after 60 days of vegetation it decreases to a value of 18.75 mg/100 g. Other investigated parameters (K, Ca, P, Mg, and Fe), sugar and nitrite content show lower values in the young leaf in the interior of the salad compared to the outer, more developed leaves.

1. INTRODUCTION

Lettuce is the most profitable fresh vegetable crop on the market. Chopped and bagged ready-to-eat products first appeared on the market in the 1980s (Claracson et al. 2003). Today, salad mixes include a mix of six to eight young leaves that is tasty, nutritious and visually interesting. As a result, baby-leaf lettuce is increasingly popular among consumers who want to eat healthy, convenient foods, while lettuce consumption has decreased (Cook 2011; Glaser et al. 2001). In the cultivation of lettuce, hybrids are mainly used, which form more uniform plants, with greater vigor and a higher yield (Matotan, 2004). Research by the author Pandjaitan et al., 2005 reveals that medium ripeness has a higher content of bioactive compounds, while other research (Zhao et al., 2007) showed a higher content of antioxidants. In the case of lettuce, some authors reported

that stage of maturity negatively affected vitamin C content (Leon et al. 2009). For the conducted research, the Limeria lettuce hybrid was grown in a protected agricultural area according to the principles of conventional production, and all measurements were made after 30, 60 and 90 days from the day of sowing, and the values of the mentioned parameters were monitored.

2. MATERIALS AND METHODS

The research was conducted in a protected environment (greenhouse) over a period of three months. The aim was to evaluate the effects of lettuce hybrid and harvest time on various plant parameters.

Biometric measurements included the determination of biological and marketable plant weight. Additionally, mineral content in the lettuce leaves was analyzed, including iron (Fe), phosphorus (P), potassium (K), calcium (Ca), and magnesium (Mg). Furthermore, the content of sugars and vitamin C was measured, along with nitrate and nitrite concentrations, during the three-month growing period. These analyses were conducted at three developmental stages of the plant: baby lettuce, medium-developed lettuce, and fully mature lettuce.

The experiment was designed as a two-factorial study conducted over one year using a randomized complete block design with four replications. Factor A represented the lettuce hybrid, while factor B corresponded to the technological maturity stage.

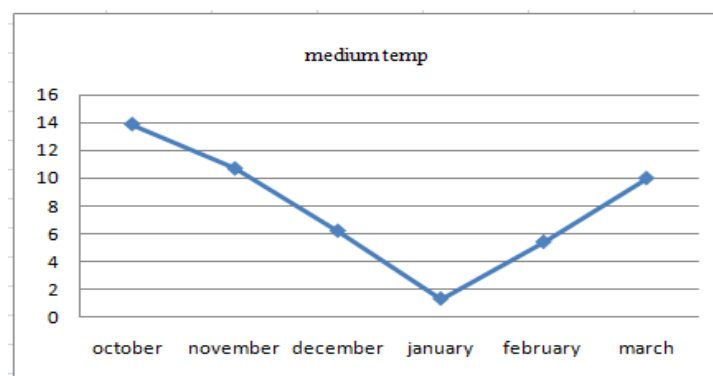
2.1. Applied laboratory methods

Soil sampling was conducted prior to establishing the experiment to determine the basic characteristics and available nutrient content in the soil. Sampling was performed from a 25 cm soil depth layer. Total nitrogen content was measured using the Kjeldahl method, while soil pH was determined in both water and KCl solution. Humus content was assessed via the bichromate oxidation method. Plant-available phosphorus and potassium were analyzed using the Egner-Riehm-Domingo method (Paige, 1982). Nitrate concentration in the soil was quantified by the N-min method, which is based on the adsorption of ultraviolet rays by nitrate ions at a wavelength of 210 nm.

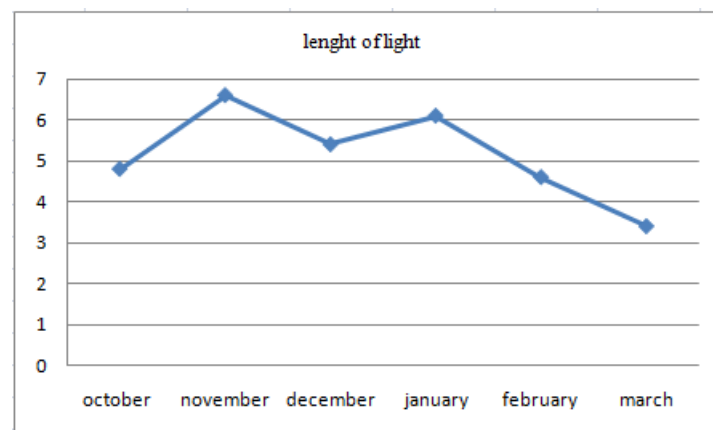
Vitamin C content was determined using the iodometric titration method following Helmenstine (2007). Phosphorus concentration was measured photometrically using ammonium molybdate with spectrophotometric readings according to ISO 6878 (1998). Potassium, calcium, and magnesium contents were determined by flame photometry according to ISO 9964-3 (1996). Total sugars were analyzed by the Luff-Schoorl method, and nitrite concentration was measured using ion chromatography.

3. RESULTS AND DISCUSSION

In order to investigate the aforementioned problem, the experiments were set up in a protected area (greenhouse) without an installed heating system, in the northwestern part of Bosnia and Herzegovina, in the vicinity of the city of Bihac. Lettuce, like spinach, and most other leafy vegetables are nitrophilous plants with a high ability to accumulate nitrates, so special attention should be paid to all the factors that contribute to this. Climatic conditions, especially insolation, is one of those factors, because in gloomy, cloudy weather, a larger amount of nitrate accumulates (Gent, 2017). Meteorological and climatic factors, necessary for the growth and development of the lettuce plant in the winter growing period (temperature and amount of light), are available on the website of the Federal Hydrometeorological Institute, and a graphical display of the measured values was made with statistical processing (graphs 1 and 2).



Graph 1: Average monthly temperatures for the growing period



Graph 2: Length of lighting for the growing period

Average monthly temperatures for the vegetation period for the Bakšaiš locality were: 13,9° C, 10,7 °C, 6,2 °C, 1,3 °C, 5,4 °C and 10,0 °C (min. -1,8° C, max. 20,9° C) (graph 1), and the temperature in the greenhouse is 7 °C higher on average. The number of hours of sunshine in the

Eighth International Scientific Conference
"June 5th - World Environment Day"

research months (October, November and December 2019, as well as January 2020) was higher at all analyzed stations compared to the thirty-year average (1961-1990). When it comes to the amount of light, lettuce requires about 12 hours of sunlight. In conditions of a reduced amount of light by 30 to 40%, there is an increase in the concentration of nitrates in the leaves. Cometti et al. (2013) by researching the influence of unfavorable conditions and concentration of the solution on the quality of lettuce, they proved that light plays a very important role in the life of the plant, because photosynthetic activity is conditioned by the quality and quantity of solar radiation they receive. Anusiya and Sivachandiran (2019) research on the influence of shade and shading on the quality and yield of lettuce conducted experiments with three different levels of shade (0%, 50% and 75%), in three repetitions. The results indicate a significant increase in the growth and yield of leafy lettuce was observed at the level of 50%, and the use of LED lighting in the agricultural sector has become popular in recent years. Soils with good physical and biological characteristics are suitable for growing different crops, and it is necessary to maintain them at the same level with balanced soil fertilization. Soil analysis shown in table 1.

Table 1: Chemical composition of the soil

sample	depht (cm)	pH in		Humus (%)	CaCO ₃	N (%)	AL – method mg/100 g soil	
		H ₂ O	1M KCL				P ₂ O ₅	K ₂ O
Bakšaiš	0-30	7,85	7,79	6,25	38,93	0,41	72,43	37,18
Metod		BAS ISO 10390:2009	BAS ISO 10390:2009	Tjurin	BAS ISO 10693:2015	Kjeldah	Al metod	
sample		I		II		III	IV	
		mg NO ₃ ⁻ /kg		mg NO ₃ ⁻ /kg		mg NO ₃ ⁻ /kg	mg NO ₃ ⁻ /kg	
N-min metod		24,49		25,64		25,09	26,43	

Examining the total weight of the head per plant, and finally the yield per unit area, significant statistical differences were also determined depending on the sampling time (table 2).

Table 2: Yield per plant based on technological maturity

yield	Min.	Max.	Mean	Var.	St. dev.	K.var.	St.error
	98	190	132,75	1090,214	33,01839	24,87261	11,67376
	163	381	285,375	6673,125	81,6892	27,37803	28,88149
	299	633	465,25	13513,36	116,247	24,98591	41,09951
	30 day		60 day			90 day	
	131±1,86 ^b		190±1,17 ^a			132±1,86 ^b	
	361±5,81 ^a		381±5,71 ^a			248±4,96 ^c	
	496±7,12 ^b		633±6,49 ^a			396±8,67 ^{bc}	
	p≤ 0,05		p≤ 0,05			p≤ 0,05	
	ANOVA	Sum	df	Mean	F	p≤0,01	

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Technological maturity	148937	3	7092,23	0,588	0,02961**
Kruskal - Walis test					
H (ch ²):17,8		H _c (tie corrected): 17,8		p (≤0,01): 0,0001367	
There is a statistically significant difference between the samples.					

Research by Kosma et al. (2013) studying the yield and yield components depending on the amount of light and cultivation method conducted experimental research in two seasons (spring and winter) in a greenhouse with an installed heating system using a hydroponic system. The results were based on the determination of the content of chlorophyll, vitamin C, the amount of nitrates, as well as the yield per plant. Depending on the hybrid, the yield per plant ranged from 167.67 to 426.67 g, depending on the time of harvest, which is in accordance with our research. The specifics of the vitamin C content are reflected in the fact that the time period of the plant's development had an impact on the vitamin C content, whereby a statistically significant difference in values was determined at a time period of 60 days of development, after which a decrease in the amount of vitamin C was recorded (table 3).

In addition to the investigated parameters, analyzes were also carried out on lettuce samples and the content of: vitamin C mg/100, sugar % and K, Ca, P, Mg, and Fe was determined in three repetitions (30, 60 and 90 days of development from day of sowing), results shown in table 3.

Table 3: Vitamin C content % tehnological maturity

ANOVA	Sum	df	Mean	F	p≤0,01
Technological maturity	2036,54	3	96,9569	0,758	0,02874*
Kruskal - Walis test					
H(ch²): 2,555		Hc (tie corrected): 2,555		p (≤0,01): 0,2787	
There is a statistically significant difference between the samples.					

Aćamović-Djoković et al. (2011) investigating the content of vitamin C in different cultivars conducted experimental research in an open field with 5 different varieties of lettuce (buterica): Plenti, Murai, Kibou, Batavija and Levistro. Lettuce was grown on vertisol on beds covered with PE foil with an irrigation system, and the spring of 2010 was used as the growing season. The results showed values from 10.41% (Batavia) to 31.08% (Kiboa), depending on the variety, which is in line with our research, but also with the research of other researchers who conducted similar experimental research such as Himesh et al. (2012), who, in addition to the vitamin C content, also determined the quantification of ascorbic acids in salad components.

Research by Bajwa and Kwatra (2013) states that dietary potassium is usually limited to 2000-3000 mg per day -1 for patients suffering from chronic kidney disease. Thanks to the rapid increase in kidney disease patients, Zang et al. (2017); Zhang and Rothenbacher (2008) increased the demand for fresh lettuce with a low potassium content. The accumulation of potassium during our research did not significantly depend on the time of harvest, and the K value ranged from 2.517 mg/100 g to 4.165 mg/kg.

Eighth International Scientific Conference
"June 5th - World Environment Day"

The closing of the leaves and the formation of the head leads to a reduced concentration of Ca, which is often manifested by visually visible injuries of the tips of the leaves that look like a burn injury. Barta and Tibitts (2000) studied the concentration of Ca in leaves depending on different feeding systems, cultivars, as well as the rate of plant development, that is, the formation of the head. The first injuries on the tips of the leaves were noticed on the 22nd day of plant development from the time of emergence, when the leaves were about 3 cm long, however, in the phase of head formation, 83% of the leaves had visible injuries due to Ca deficiency. The measured amounts of Ca from 261 mg/100 g to 449 mg/100 g showed a decrease in Ca concentration depending on the time of harvest.

Since the soil on which the experiment was built was evaluated as extremely well supplied with phosphorus in the amount of 72.43 mg/100 g of soil, no deficiencies of P were observed in the plants. The recorded values were uniform based on the examined parameter and ranged from 31 mg/100 g to 48 mg/100 g. Recorded Mg values ranged from 122 mg/100 g to 146 mg/100 g, and no significant statistical differences were found, as well as visible symptoms on the plant. Based on the recommended values of the daily intake of lettuce, the most important bioactive components are: iron, folates, vitamin C, β -carotene, lutein, total phenol content, which play a prominent role in the prevention of many chronic diseases (Kim et al., 2016).

No statistically significant differences were found regarding Fe content, and the recorded values ranged from Fe 1.3 mg/100 g to 3.2 mg/100 g (table 4).

Table 4: Nutrient content mg/100 g based on technological maturity

	K	Ca	P	Mg	Fe
30 day	3,062	312	309	289	36
60 day	4,165	475	467	412	36
90 day	3,888	298	287	261	48

Similar experimental research was carried out by Shwerif et al. (2018) where they monitored the influence of different temperature regimes on the sugar content of two types of lettuce grown in a controlled environment. The results of the research proved that the temperature regimes had a very significant influence on the sugar content of both examined varieties of lettuce (Dixter and Exbury). The highest sugar content was recorded at low temperature (12/8 °C), and it decreased with increasing temperature, while Dixter lettuce had a higher concentration of sugar than Exbury lettuce. The sugar content depending on the time of harvest is shown in table 5, depending on the technological maturity at the level of significance ($p \leq 0.05$), where a smaller statistical difference was determined, and the highest sugar content was 26 mg/100 g after 90 days, and the lowest of 4.14 mg/100 g after 30 days of cultivation.

Eighth International Scientific Conference "June 5th - World Environment Day"

Table 5: Sugar content depending on technological maturity

ANOVA	Sum	df	Mean	F	p≤0,05
Technological maturity	53627,8	3	2553,71	0,002401	0,9963

Kruskal - Walis test

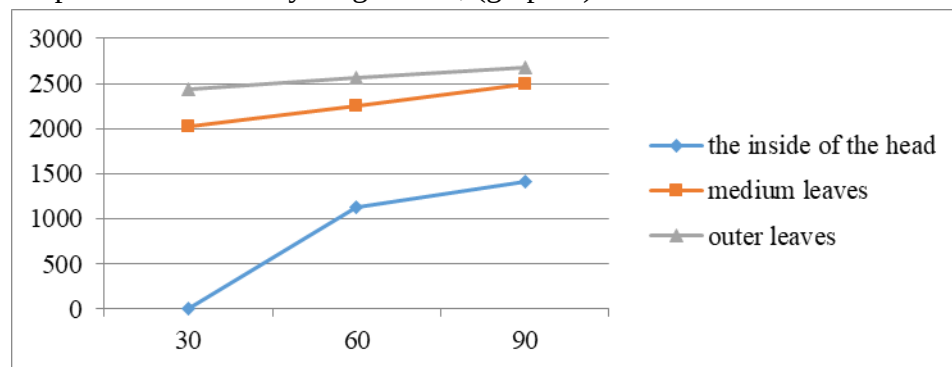
H (ch²): 0,285

H_c (tie corrected): 17,8

p (≤0,01): 0,000867

There is a statistically significant difference between the samples.

The obtained results of this research proved that after 90 days of development from the time of sprouting, the content of harmful substances increases, because the amount of nitrates found in the leaves is three times higher, but it was also proven that the outer leaves have a higher content of nitrates compared to the inner younger ones, (graph 3).



Graph 3: Nitrite content depending on the position in the head and technological maturity

The amounts of nitrates are prescribed by the Ordinance on the Maximum Permitted Amounts of Contaminants in Food (OG 154/2008. OG 78/2019), for lettuce (*Lactuca sativa* L.) (grown in a greenhouse) and harvested by March 1, the allowed amount of nitrates is up to 4000 mg/kg, while in the open field that amount is 2500 mg/kg, and the measured values are within the permitted amounts.

4. CONCLUSION

The final goal of a one-year experimental study of Limeria lettuce hybrids grown according to the principles of conventional agriculture in a protected agrospace (a greenhouse without a heating system) is to determine the most optimal harvest date when the quantitative and qualitative characteristics of the lettuce are in question.

Based on this study, it is evident that a greater amount of natural light resulted in positive effects on the growth and development of the plant in all measured parameters, but also the lack of it resulted in an increased amount of harmful substances in the leaves. The positive and negative influence of the harvest time was observed for most of the examined parameters when the quantity and quality of the product was in question. It is recommended to harvest lettuce 60 days after sowing.

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POWDERY MILDEW OF BULBOUS WILD BARLEY (*Hordeum bulbosum*) IN BİNGÖL UNIVERSITY CAMPUS AREA, TÜRKİYE

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Keywords: *Hordeum bulbosum*, powdery mildew, Bingöl University, Türkiye

ABSTRACT:

Bulbous wild barley (Hordeum bulbosum) is a species commonly found in Türkiye. This species can be used to improve the ability of barley to withstand both biotic and abiotic stress factors. In May 2023, a survey was conducted in the campus area of Bingöl University, located in Bingöl, Türkiye, to investigate the presence of powdery mildew disease caused by Blumeria graminis f. sp. hordei in H. bulbosum populations. Twenty-five locations with dense H. bulbosum populations were examined in the Bingöl University campus area, and it was found that powdery mildew was present in 24 of those locations. The percentages of diseased plants ranged from 0.25 to 46.25 for the disease in the affected areas. The severity values of the powdery mildew ranged from 1 to 9. Hordeum bulbosum populations in the Bingöl University campus area demonstrated a wide range of powdery mildew resistance.

1. INTRODUCTION

Barley (*Hordeum vulgare*) is a type of cereal that grows in cool climates and is used as animal feed, malt material, and human food in many countries around the world, including Türkiye. It is a popular crop due to its easy cultivation and ability to grow in different soil and climate conditions (Geçit, 2016).

Wild barley species, *Hordeum spontaneum* and *Hordeum bulbosum*, are resistance sources to various biotic and abiotic factors. While *H. spontaneum* has been studied extensively, *H.*

bulbosum is also used as a genetic source of disease-resistance genes (Ruge-Wehling and Wehling, 2014; Çelik and Karakaya, 2017). *H. bulbosum* is considered in the secondary gene pool of barley because it is difficult to cross with barley. However, after crossing, chromosome pairing and recombination may occur. There are diploid and tetraploid forms of *H. bulbosum*, and doubled haploids can be formed using this plant in cereal breeding studies. The bulbous wild barley contains disease-resistance genes, which can be transferred to barley through introgression (Ruge-Wehling and Wehling, 2014).

Powdery mildew is a disease caused by *Blumeria graminis* f. sp. *hordei* and it is a significant economic concern in most barley-producing regions. It is a destructive foliar disease that affects barley crops worldwide (Mathre, 1997). Researchers are constantly searching for gene pools that can provide new resistance genes to combat the disease (Jorgensen and Wolfe, 1994; Czembor and Johnston, 1999; Czembor and Czembor 2021; Çelik Oğuz and Karakaya, 2021). *H. bulbosum* populations may provide a valuable source of new resistance genes for powdery mildew. Therefore, the goal of this study, conducted under natural field conditions, was to determine the powdery mildew disease situation of field-grown *H. bulbosum* populations.

2. MATERIALS AND METHODS

In May 2023, a survey was conducted at the campus area of Bingöl University, which covers an area of 3,115,616.85 square meters. The campus is known for its dense population of *Hordeum bulbosum* plants. The survey involved examining 25 locations within the campus, each containing at least 10 to 50 *H. bulbosum* plants. Samples of diseased plants with typical symptoms of powdery mildew were collected from naturally grown *H. bulbosum* plants on the campus. The powdery mildew fungus was identified using Mathre (1997). The severity of the disease was determined using the 1-9 scale developed by Saari and Prescott (1975). To calculate the average incidence of powdery mildew, *H. bulbosum* plants with and without disease were evaluated together.

3. RESULTS AND DISCUSSION

This study conducted at Bingöl University campus in Turkey revealed the presence of powdery mildew disease caused by *B. graminis* f. sp. *hordei* in 24 *H. bulbosum* populations (Table 1). The highest incidence of the disease was found in population 7 (46.25%), while the lowest incidence was observed in population 2 (0.25%). Interestingly, one population (population no: 13) did not show any signs of powdery mildew. The differences in disease incidence among different locations showed that the resistance of *H. bulbosum* to *B. graminis* f. sp. *hordei* varied in this area. Further research under controlled conditions is necessary to confirm this variation, and the pathotypes/races of the pathogen should be identified.

Eighth International Scientific Conference
"June 5th - World Environment Day"

A survey was conducted in 2018 to determine the diseases affecting *H. bulbosum* plants in the Bingöl province of Türkiye. The survey examined 27 naturally occurring *H. bulbosum* populations from the central district of Bingöl, as well as the districts of Solhan, Karlıova, Genç, Kiğı, and Adaklı. Out of these populations, 5 were found to be disease-free. Powdery mildew was identified as the most prevalent pathogen, followed by leaf rust (Karakaya et al., 2020). Our current study also revealed the variation in resistance among the *H. bulbosum* populations at Bingöl University campus, Türkiye. This campus area was relatively smaller, however, *H. bulbosum* populations with no disease or with low disease were also found. Saraç Sivrikaya et al. (2023) examined two *H. bulbosum* populations in the Harran district of the Şanlıurfa province of Türkiye and found no powdery mildew in these 2 populations.

Table 1: Powdery mildew observed on *Hordeum bulbosum* populations examined in the Bingöl University campus area, Bingöl, Türkiye.

Population no	Powdery mildew	Incidence (%)	Severity (Mean)
1	present	2,25	3
2	present	0,25	1
3	present	2,75	1-5 (1,5)
4	present	3,75	5
5	present	25,75	3-7 (3,75)
6	present	2,5	3
7	present	46,25	5-9 (5,25)
8	present	37,5	5-9 (3,5)
9	present	15	5
10	present	8,75	3
11	present	8,75	3
12	present	22,75	1-7(2)
13	No disease	0	0
14	present	7,5	5-7(3)
15	present	13,75	3-5(3,25)
16	present	7,5	3

Eighth International Scientific Conference
"June 5th - World Environment Day"

17	present	6,25	3
18	present	3,75	1
19	present	0,5	1-3(1)
20	present	1,25	5
21	present	7,5	1-3(1,75)
22	present	5	5
23	present	10	3-5(2)
24	present	3,25	3
25	present	1	1-3(1)

In 2019, Czembor et al. conducted a study to test the resistance of recombinant lines to powdery mildew. These lines were derived from crosses and backcrosses between two common barley cultivars, Emir and Golden Promise, and *H. bulbosum*. The researchers used a differential set of 14 *Blumeria graminis* isolates to test the resistance of 24 lines. The results showed that 22 of the tested lines exhibited resistance to powdery mildew.

4. CONCLUSION

In various sampling locations at the Bingöl University campus area, *H. bulbosum* plants were found with different levels of *B. graminis* f. sp. *hordei*, regardless of direction, precipitation, and altitude. This study suggests that the *H. bulbosum* populations found in these locations could potentially be used as sources of resistance to barley powdery mildew caused by *B. graminis* f. sp. *hordei*. In order to identify specific genes that are responsible for resistance to powdery mildew and to understand how they work, further extensive studies will be needed.

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Eighth International Scientific Conference
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FOREST MANAGEMENT



COMPARATIVE ANALYSIS OF SOIL QUALITY FOR BEECH GROWTH IN THE AREAS OF NATIONAL PARKS KOZARA AND SUTJESKA, BASED ON TAKSATION DATA

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Key words: National Park, Kozara, Sutjeska, soil, beech

ABSTRACT:

Forests represent the most complex terrestrial community of organisms and are considered a key component of landscape stability. Therefore, sustainable management has been regarded as one of the most important challenges for researchers in recent decades. The subject of research in this paper is two National Parks, namely NP Sutjeska and NP Kozara, both located in the II protection zone. The National Park "Sutjeska" is the oldest and largest national park in Bosnia and Herzegovina. The Park's area is 16,052.34 hectares, of which over 80% is covered by forests, while the rest consists of meadows, pastures, and clearings above the upper forest limit. Kozara Mountain was declared a National Park "Kozara" in April 1967. The current area of the park is 3,907.54 hectares. Beech in Southeastern European countries is one of the most valuable species of forest trees, but it has only received due attention in the last few decades. Research on the pedological properties of soils in the mentioned Parks was conducted on the same soil class (brown or cambic soils), on different parent substrates. Alongside the analysis of soil samples, a comparative assessment of beech condition was performed based on taksation data for the researched areas. All results were processed and presented through tables, graphs, and presented

using GIS application. Traditional editing methods are sophisticated, facilitating, and significantly speeding up with great accuracy. After the completion of soil analyses and processing of taksation data, it is concluded that the habitat quality in NP "Sutjeska" is better compared to the habitat quality in NP "Kozara".

1. INTRODUCTION

Forests are considered key components of landscape stability, and this research was further motivated by this fact, particularly because it pertains to the areas of the Sutjeska and Kozara National Parks. The National Park "Sutjeska" is the oldest and largest national park in Bosnia and Herzegovina (BiH), covering an area of 16,052.34 hectares, with over 80% of it covered by forests, while the rest comprises meadows, pastures, and barren lands above the upper forest limit. Mount Kozara was declared a National Park in April 1967 with the aim of preserving nature and protecting numerous cultural-historical monuments. The current park area is 3,907.54 hectares. This study focused on the beech stands, which represent one of the most valuable species of forest trees in Southeastern Europe. The application of Geographic Information Systems (GIS) enables more productive forest management through access to a large amount of data, facilitating their collection, monitoring, analysis, and modeling. The primary aim of this research was to determine if there are differences in beech stands under similar stand conditions in different habitats. Differences were identified through the assessment data of beech stands and pedological profiles opened on the dominant soil type.

2. MATERIAL AND WORK METHODS

The research was conducted within the boundaries of the "Kozara" and "Sutjeska" National Parks, specifically:

- a) section 239, subsection a – National Park "Kozara" (2nd degree of protection)
- b) section 152, subsection b – National Park "Sutjeska" (2nd degree of protection).

The research sites were located in two municipalities. The first location is in the northeastern part of the Prijedor municipality, and the second location is in the southwestern part of the Foča municipality.

Based on the research objectives, the following tasks were defined:

The data collection methodology was based on original data collected through fieldwork at the research sites, focusing on gathering primary data for the area. The survey of assessment data was conducted for the sections under study. The collection of assessment data was performed using a sample within the sections under study, comprising individual sample elements organized into a system of concentric sample areas (circles) systematically distributed in a square grid.

Eighth International Scientific Conference
"June 5th - World Environment Day"

Pedological profiles were opened, one in each of the two sections under study, and samples were taken from the horizons in a disturbed state. The profiles were opened to a depth of 100 cm, up to the appearance of the parent substrate. One profile was opened on each dominant soil type in a forest where beech stands predominate. The obtained results were statistically processed and interpreted in accordance with the defined objectives (using GIS tools). The initial task was to cartographically display the arrangement of samples (in the form of ordinary and detailed circles) and the positions of pedological profiles on all surfaces under study. For each pedological profile, x, y, and z coordinates were determined (Gauss-Krüger MGI Balkans zone 6 coordinate system).

3. RESEARCH RESULTS AND DISCUSSIONS

The forests of the National Parks "Sutjeska" and "Kozara" fall under the category of "forests for special purposes." Forests for special purposes are considered "protected forests," and they were once "protected forests" safeguarded by specific laws aimed at maintaining and enhancing all forest functions, preserving natural values, and the rarity of other forest and woodland functions. The study was conducted at two sites.

The Sutjeska site in section 152 has Eutric Cambisol soil type.

The soil profile at the Sutjeska site was opened on a forest glade of approximately 0.5 hectares, located on a more leveled part of an alluvial terrace. Eutric brown soil is maintained permanently and has the widest distribution in semi-humid areas with an average annual precipitation of 600–700 mm and a pronounced dry summer period (Manuševa, 1969). The properties of the parent substrate are crucial for the occurrence of Eutric Cambisols, as well as for the physical and chemical properties of this soil type.

Table 1: Physical properties of soil at the Sutjeska location

Profile 1	Horizon	Depth in cm	Texture			Texture classification
			Sand %	Silt %	Clay %	
Sutjeska	A	0 – 20	27.40	24.06	45.40	g. clayey soil
	Bv	20 – 40	23.01	24.92	52.06	i. clay
	BC	40 – 70	25.80	17.46	56.73	p. clay
	B/C	70 – 90	3.46	22.93	73.60	Clay
	C	90 - 100	10.40	19.86	69.73	Clay

Based on the textural composition, the soil can be characterized as silty clay loam in the A horizon, with a change in texture composition as you move deeper into the profile. At a depth of 20-40 cm, it transitions to silty clay, then sandy clay, and finally ends with a clayey horizon that transitions into a clayey parent material. The slightly higher clay content in the B/C horizon compared to the other horizons, where the values are lower, suggests a gradual change in soil texture with depth. Additionally, the gentle slope of the terrain and slightly lower clay content in

Eighth International Scientific Conference
"June 5th - World Environment Day"

the surface A horizon compared to deeper horizons indicate potential erosion of fine particles from the soil surface.

Table 2: Description of soil chemical properties in the open profile at the Sutjeska location

Profile 1	Horizon	Depth in cm	pH		Organic matter	Humus type	Physiologically Active Soil Content (mg/100 g)	
			H ₂ O	KCl			P ₂ O ₅	K ₂ O
					%			
Sutjeska	A	0–20	6.48	5.46	4.03	Base saturation	5.33	20.49
	Bv	20–40	6.76	5.74	2.96	Base saturation	1.37	13.22
	BC	40–70	6.90	6.14	2.20	Base saturation	1.00	13.90
	B/C	70–90	7.18	6.42	1.17	Base saturation	0.32	7.06
	C	90–100	6.84	6.05	1.10	Base saturation	0.03	5.17

The soil reaction throughout the entire profile is neutral to slightly acidic, with the lowest pH observed in the A horizon, which can be attributed to the presence of undecomposed organic matter or soil leaching processes. Similar views are shared by the authors (Košanin and Knežević, 2006). The humus content decreases with depth. The significantly higher humus content in the A horizon is a result of residual leaf litter, which was in the Ol or Of form and is present across the entire surface. The P₂O₅ content is highest in the A horizon at 5.33 mg/100 g of soil, which indicates a moderate phosphorus content on the scale. The K₂O content in the B/C horizon is much lower than in the other horizons. The reason for this potassium distribution in the soil can be explained by the humidity of the climate in this area and the terrain slope.

Kozara Site, Section 239, Soil Type: Brown Forest Soils on Dolomites or Limestones – Dystrophic Cambisol

This soil is named after its red color, which comes from the mineral hematite. It is also referred to as "red Mediterranean soil." Ičanović and Marković (2022) state that these terms are used specifically for soils formed on limestone. Dystrophic brown soils, like brown soils on limestone, are formed from the insoluble residue of pure limestone, and the influx of silicate material by aeolian means cannot be ruled out, especially considering the prolonged genesis process of these soils.

Table 3: Physical Properties of Soil at the Kozara Location

Profile 2	Horizon	Depth in cm	Texture			Texture classification
			Sand %	Silt %	Clay %	
Kozara	A	0–20	6.91	31.58	61.50	Clay
	Bv	20–45	51.20	20.40	29.06	s. clay loam
	BC	45–60	68.48	13.98	17.53	l. s.sand
	B/C	60–80	75.66	9.73	14.60	s.l. sand
	C	80–100	53.33	17	29.66	l. s. sand

Eighth International Scientific Conference
"June 5th - World Environment Day"

Table 4: Profile 2 Horizon Depth in cm Texture Texture Class

Profile 2	Horizon	Depth in cm	Texture			Texture classification
			Sand %	Silt %	Clay %	
Kozara	A	0–20	6.91	31.58	61.50	Clay
	Bv	20–45	51.20	20.40	29.06	s. clay loam
	BC	45–60	68.48	13.98	17.53	l. s.sand
	B/C	60–80	75.66	9.73	14.60	s.l. sand
	C	80–100	53.33	17	29.66	l. s. sand

The texture designation for this type progresses from clay in the surface horizon, then transitions through silty clay loam, silty sandy loam, and ends with sandy clay.

Table 5: Description of Soil Chemical Properties in the Open Profile at the Kozara Location

Profile 2	Horizon	Depth in cm	pH		Organic matter	Humus type	Physiologically Active soil content (mg/100 g)	
			H ₂ O	KCl			P ₂ O ₅	K ₂ O
					%			
Kozara	A	0–20	6.90	6.16	7.33	Base saturation	4.53	22.52
	Bv	20–45	7.41	6.88	0.96	Base saturation	0.60	6.26
	BC	45–60	7.34	6.41	0.08	Base saturation	0.13	3.66
	B/C	60–80	7.77	6.82	0	-	0	2.76
		80–100	7.02	6	0	-	0	3.2

Based on the pH value in 1M KCl, it can be observed that the soil is neutral to moderately alkaline throughout the entire profile depth. The highest organic matter content (7.33%) is in the A horizon, indicating highly humic soil, while in the other horizons, these values do not exceed 1%. The physiologically active phosphorus content in this profile's surface horizon is 4.53 mg/100 g of soil, classified as low phosphorus content on the scale. The accessible K₂O content in the Ap horizon is high at 22.52 mg/100 g of soil, then decreases in subsequent horizons, corresponding to the phosphorus values.

Taxation data for sections

Taxation data for the sections under investigation were obtained from samples evenly distributed across the section's surface. Samples are arranged in concentric circles. In section (152/02) in the "Sutjeska" National Park, 52 circles were sampled, while in section (239/01) in the "Kozara" National Park, 51 circles were sampled.

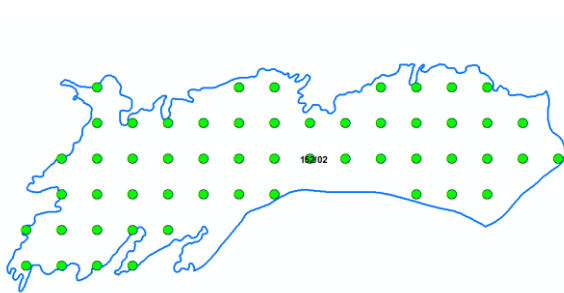


Figure 1: Layout of the NP 'Sutjeska' Circles

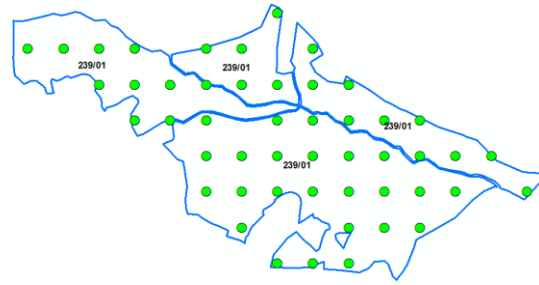


Figure 2: Layout of the NP 'Kozara' Circles

Table 6: Taxation Data for NP "Kozara" and "Sutjeska"

National Park Name	Section/Department	Area	Total Volume of Spruce	Total Volume of Beech	Total Volume of Other Tree Species	TOTAL
		ha	m ³	m ³	m ³	m ³
"Kozara"	239/01	47.53	301.1	11,012.0	3,220.0	14,533.0
"Sutjeska"	152/02	53.86	806.5	15,635.6	413.4	16,855.5

The table shows the total volumes obtained for the entire section. The areas of the analyzed sections are different. To better understand the differences between these two sections, the taxation data will be averaged per hectare and presented in the following table.

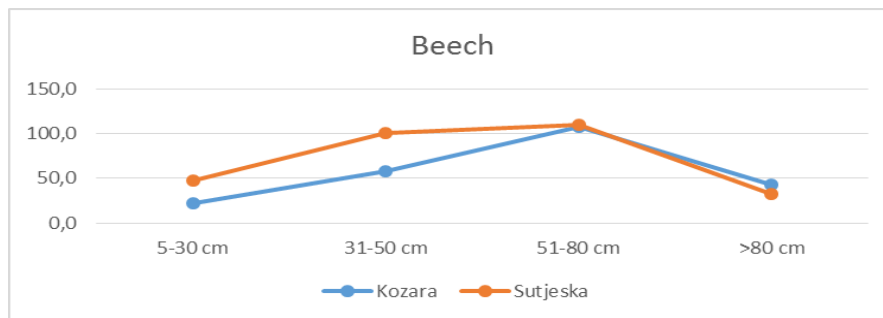
Table 7: Taxation Data for NP "Kozara" and "Sutjeska" (Per Hectare)

National Park Name	Section/Department	Area	Total Volume of Spruce	Total Volume of Beech	Total Volume of Other Tree Species	TOTAL
		ha	m ³ /ha	m ³ /ha	m ³ /ha	m ³ /ha
"Kozara"	239/01	47.53	6.3	231.7	67.7	305.8
"Sutjeska"	152/02	53.86	15.0	290.3	7.7	313.0
Difference Between NP-a "Sutjeska" i NP-a "Kozara"		6.33	8.6	58.6	-60.1	7.2

Looking at the previous table, it can be observed that the average volume of all represented species per hectare is higher in NP "Sutjeska" compared to NP "Kozara." When considering only the volume of beech, this species is 25% more represented per hectare in NP "Sutjeska" than in NP "Kozara." The contribution of other tree species (22% in volume) is higher in NP "Kozara," which increases the diversity in that section, whereas in NP "Sutjeska," it can be described as a pure beech stand with a small proportion of spruce and other deciduous trees (below 8% in volume). The distribution of beech volume, as the main species in both sections, across diameter

Eighth International Scientific Conference "June 5th - World Environment Day"

classes over 50 cm does not differ between stands in NP "Kozara" and NP "Sutjeska," but there are differences in lower diameter classes (below 50 cm). This indicates that besides the presence of larger diameter trees in NP "Sutjeska," there is also a higher number of smaller diameter trees, unlike the stand in NP "Kozara."



Graph 1: Distribution of Beech Stand

The spatial distribution of volume across the section area in NP "Kozara" shows uneven spatial distribution of volume. Not only are their volumes unevenly distributed across the section area, but spruce and other tree species are not represented in all parts of the section. Beech extends throughout the section, but with varying volume distribution across the section.

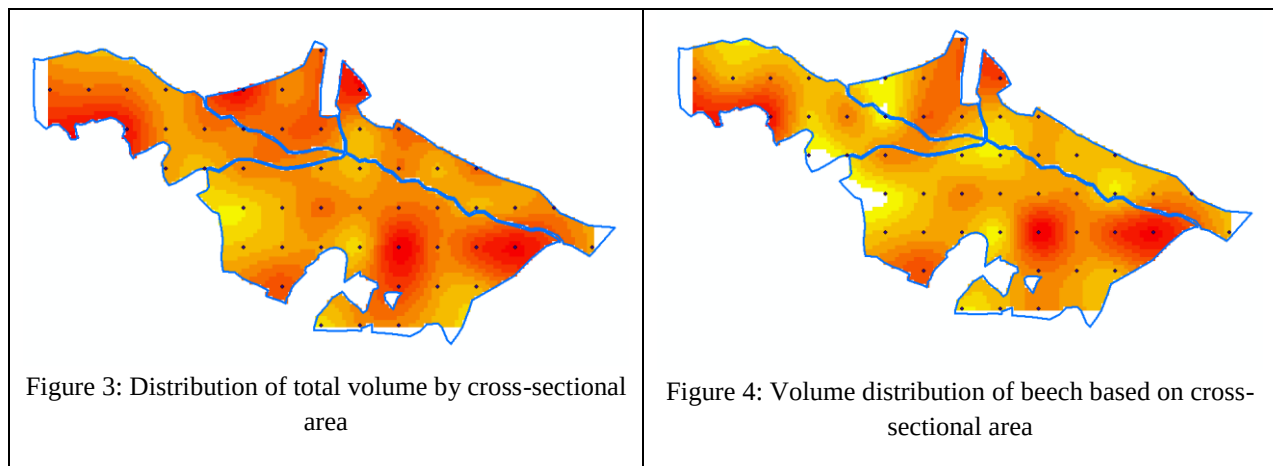
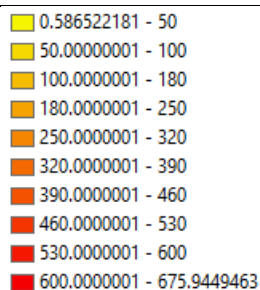


Figure 3: Distribution of total volume by cross-sectional area

Figure 4: Volume distribution of beech based on cross-sectional area



Legend - Distribution of Volume in NP "Kozara" and NP "Sutjeska" expressed in m³/ha

Eighth International Scientific Conference
"June 5th - World Environment Day"

Similarly to NP "Kozara," the volume is unevenly distributed across the area in NP "Sutjeska." In the section located in NP "Sutjeska," beech is present throughout the section with varying volume distribution. Spruce is less present across the area but is more concentrated in certain locations where it appears. Other tree species are less represented in the section and are unevenly distributed across the area. The different presence of tree species across the section area, as well as the varied volume distribution, is influenced by microclimatic conditions in smaller localities.

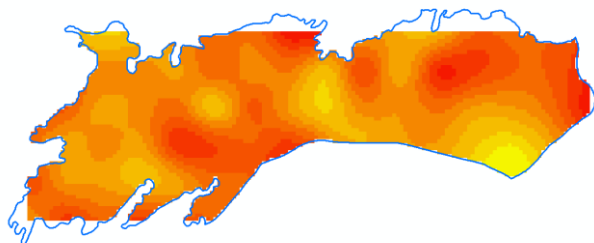


Figure 5: Distribution of total volume by cross-sectional area

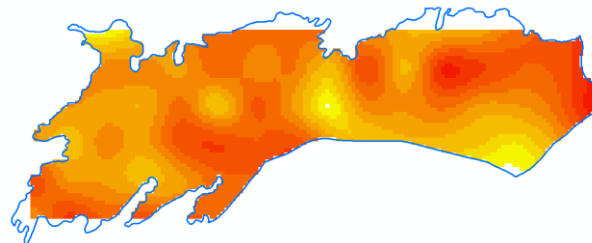


Figure 6: Distribution of beech volume by cross-sectional area

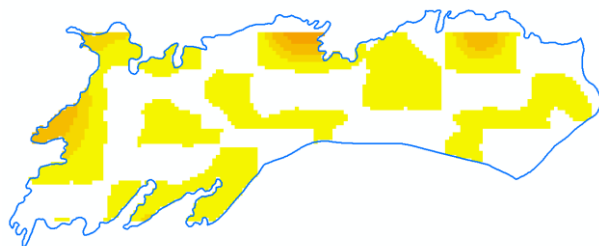


Figure 7: Distribution of spruce volume by cross-sectional area

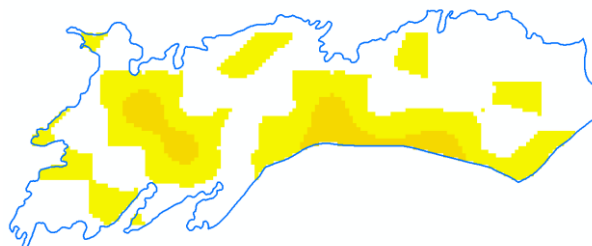


Figure 8: Distribution of volume of other species by cross-sectional area

The timber stock of beech is higher in the "Sutjeska" National Park compared to "Kozara" National Park. The average volume in "Sutjeska" is 348.04 m³/ha, with the majority of the volume accounted for by trees with diameters over 31 cm (82%). In "Kozara," the average beech volume is 223.45 m³/ha, with most of the volume concentrated in trees with diameters over 31 cm (89%).

Eighth International Scientific Conference
"June 5th - World Environment Day"

Table 8: Beech wood stock

National park	Department/section	Timber stock of beech (m3/ha)
"Kozara"	239/01	223,45
"Sutjeska"	152/02	348,04

During the assessment of silvicultural and technical classes, it was found that "Sutjeska" National Park is expected to have better quality (around 22% in I silvicultural and technical class and 25% in I technical class) compared to "Kozara" National Park (around 6% in I silvicultural and technical class and 12% in I technical class).

4. CONCLUSION

After completing soil analyses and processing of tachometric data, it is concluded that the habitat quality in "Sutjeska" National Park is superior to that in "Kozara" National Park. This is confirmed by the soil analysis in two sections and tachometric data derived from forestry tables, i.e., breast diameter and height of trees included in the measurement based on concentric circles. In general, it can be concluded that the soil in the "Sutjeska" location has better conditions for cultivation and better yield of beech and fir stands compared to the soil profile at the "Kozara" location. This can be confidently asserted based on parameters obtained from analyses, as well as on the depth of the solum and textural composition, which are more conducive from the perspective of better plant rooting, i.e., beech and fir stands. The average volume of all represented species per hectare is higher in "Sutjeska" National Park compared to "Kozara" National Park. When considering only the volume of beech, it is observed that this species is 25% more represented per hectare in "Sutjeska" compared to "Kozara". The proportion of other tree species (22% share in volume) is higher in the "Kozara" section, and this greater share contributes to increased diversity in the mentioned section, while it can be said that "Sutjeska" National Park consists of a pure stand of beech with a small share of fir and other deciduous species (below 8% share in volume). The timber stock of beech is higher in "Sutjeska" National Park compared to "Kozara" National Park. The average volume in "Sutjeska" is 348.04 m3/ha, with the majority of the volume accounted for by trees with diameters over 31 cm (82%). In "Kozara," the average beech volume is 223.45 m3/ha, with most of the volume concentrated in trees with diameters over 31 cm (89%).

During the assessment of silvicultural and technical classes, it was found that "Sutjeska" National Park is expected to have better quality (around 22% in I silvicultural and technical class and 25% in I technical class) compared to "Kozara" National Park (around 6% in I silvicultural and technical class and 12% in I technical class).

Based on the silvicultural and technical classification, tree quality in "Sutjeska" National Park is more present, and the assessment of cultivation quality is also influenced by soil quality.

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ENVIRONMENTAL IMPLICATIONS OF WOOD MODIFICATION PROCESSES

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Key words: Wood modification, thermal and chemical processes, LCA methodology, environmental impact

ABSTRACT:

Wood modification processes are crucial for enhancing the properties of wooden materials, but they can also have a significant impact on the environment. This paper explores various wood modification techniques, including thermal, chemical, and biological modification, and analyzes their environmental impact. We examine greenhouse gas emissions, chemical usage, and energy consumption in the process, as well as potential environmental side effects such as wastewater and waste materials. We also investigate possible environmentally sustainable alternatives and strategies to reduce the negative impacts of wood modification processes. Through a systematic analysis of environmental impacts, this paper provides a foundation for understanding the ecological aspects of wood modification processes and encourages the development of more sustainable approaches in the wood processing industry.

1. INTRODUCTION

Nowadays, wood modification is referred to as a process used to improve the physical, mechanical, or aesthetic properties of sawn timber, veneer or wood particles used in the production of wood composites. This process produces a material that can be disposed at the end of a product's life cycle without presenting any environmental hazards greater than those that are associated with the disposal of unmodified wood (Chu et al., 2017; Demiano et al., 2023). Modification is applied to overcome weak points of the wood material that are mainly related to moisture sensitiveness, low dimensional stability, hardness and wear resistance, low resistance to

bio-deterioration against fungi, termites, marine borers, and low resistance to UV irradiation (Johnes, 2020).

Choosing the right material can be a difficult decision, as there are numerous options available. The selection process typically involves considering the expected lifespan and cost of the materials. However, accurately predicting the service life of wood is difficult, as it is affected by various factors such as the type of wood used, the protection measures employed, and local climate conditions (Isaksson, 2013). Moisture content during exposure is of great interest because in addition to the material-inherent durability, the moisture and temperature conditions inside the wood, i.e., the material climate, are the most critical factors influencing the ability of fungi to decompose wood. These factors are affected by the design of the construction, the exposure conditions, and local climatic conditions (microclimate) (Van Acker et al., 2015; Brischke et al., 2007). The main challenges associated with wood, such as poor dimensional stability and limited biological durability, are primarily due to the high content of hydroxyl groups (OH) in the main polymers of the wood cell wall, as indicated by previous studies (Rowell et al., 1985; Gerardin, 2016).

In the pursuit of wood protection without toxic chemicals, modification emerges as a viable solution. This method can enhance species with inferior properties, particularly softwoods, transforming them into new products with superior characteristics (Papadopoulos, 2010; Teng et al., 2018). Modification of wood is a promising technique that offers an environmentally friendly and effective way to improve wood durability and hydrophobicity, as supported by several studies (Ugovšek et al., 2015; Hasanagić et al., 2021; Hasanagić, 2022; Hasanagić, 2019; Liang et al., 2023).

In recent years, there have been significant advancements in wood processing technologies, especially within the commercial sector, as highlighted by Hill (2011). According to Boonstra (Boonstra, 2008), this resurgence of interest is driven by the declining production of durable wood, increased demand for sustainable building materials due to deforestation, and stricter regulations limiting the use of toxic chemicals.

The aim of this study was to examine the environmental consequences of using wood as a material, with a specific focus on determining the additional environmental benefits provided by the use of modified wood compared to its unmodified equivalents. This paper analyzes relevant published data in this field and considers the necessary information for a proper evaluation of the research question.

2. ENVIRONMENTAL IPACT OD WOOD MODIFICATION PROCESSES

The burgeoning demand for modified wood stems from regulatory constraints on hazardous preservatives and the imperative for sustainable material alternatives. Augmenting wood properties is achievable through ecologically benign methodologies, notably the Thermo-Hydro-Mechanical (THM) treatment, leveraging temperature, moisture, and mechanical perturbations. Conversely, chemical adaptations, exemplified by acetylation, confer dimensional stability and decay resistance but engender prospective environmental and health hazards.

2.1 Thermal Modification: Advancements in Eco-Friendly Wood Enhancement

Thermal modification techniques, prominently represented by the Thermo-Hydro-Mechanical (THM) treatment, represent a pivotal advancement in sustainable wood enhancement strategies. THM operates at the nexus of temperature, moisture, and mechanical forces to imbue wood with superior properties while preserving its environmental integrity. By subjecting wood to controlled thermal conditions, inherent characteristics such as dimensional stability, moisture resistance, and decay resilience are significantly bolstered. Notably, the evolution of these methodologies from rudimentary experiments to sophisticated industrial applications underscores their efficacy in meeting contemporary environmental imperatives. (Sandberg et al., 2012; Bao et al., 2017)



Figure 1: Thermal Modification- Thermo ASH (20)

2.2. Chemical Modification: Balancing Performance with Environmental Considerations

Chemical modification emerges as a potent avenue for augmenting wood's structural integrity and longevity through the introduction of reactive substances. This process, often exemplified by acetylation, imparts remarkable dimensional stability and resistance to decay. However, the pursuit of enhanced performance must be judiciously weighed against the potential ecological ramifications. (Mantani et al., 2017) Chemical treatments, by their nature, may engender environmental and health risks stemming from emissions and residual chemical residues. Consequently, while chemical modification offers notable benefits, its implementation necessitates stringent adherence to eco-centric principles to mitigate adverse environmental impacts.

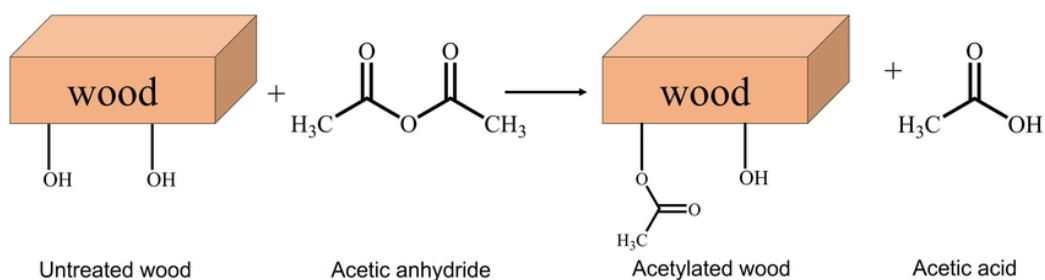


Figure 2: Schematic view of the acetylation of wood (Johne et al., 2020)

3. MODIFIED WOOD IN A CIRCULAR ECONOMY

The utilization of various wood modification processes, characterized by potential parameter variations, yields a spectrum of modified wood products endowed with distinct properties, facilitating diversified product lines. Concurrently, these processes entail differential environmental footprints, thereby transposing environmental considerations into material attributes, constituent elements, and ultimate product functionalities (Van der Lugt et al., 2016). A systematic evaluation encompassing process parameters, resultant product properties, and environmental ramifications is imperative to steer the development of innovative modification techniques and manufacturing methodologies, aligning with the 'cradle to cradle' paradigm (Araujo et al., 2019). Integral to this paradigm shift is the incorporation of recycling, up-cycling, and end-of-life disposal modalities within a fully realized industrial ecology framework. Strategic adoption of intelligent material reuse and up-cycling frameworks holds promise in curbing landfill-bound waste and mitigating downstream down-cycling implications (Piccardo et al., 2022).

The quest for optimal wood modification processes necessitates a comprehensive understanding of pertinent process factors, necessitating the formulation of robust chain of custody protocols spanning the entire lifecycle. In this context, initiatives have galvanized interdisciplinary

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research endeavors aimed at curtailing CO₂ emissions, alongside advocating for the proliferation of Life Cycle Assessment (LCA) studies appraising wood modification processes and their resultant products, an area currently underexplored (John et al., 2020).

Critical to the sustainability agenda is the assurance of modified wood's non-toxicity during service tenure, coupled with the prevention of toxic substance release upon cessation of service (Araujo et al., 2019). Modification processes may induce physical and/or chemical alterations in wood constituents, engendering heightened durability or diminished hygroscopicity, thereby enhancing material resilience.

Life Cycle Assessment (LCA) emerges as a pivotal tool for quantifying the environmental implications of products, processes, or activities across their entire lifecycle. Anchored within the ISO Standards (Hill, 2006; ISO-14044, 2006), LCA delineates a structured framework encompassing 'Goal and Scope Definition', 'Inventory Analysis', 'Impact Assessment', and 'Interpretation'. By scrutinizing inputs and outputs across the product lifecycle continuum, from extraction to disposal, LCA elucidates global and localized environmental impacts, thereby facilitating informed decision-making and fostering sustainable production paradigms.

While LCA studies have proliferated within the wood products domain, scant attention has been directed towards modified wood, particularly concerning the influence of timber provenance on overall environmental performance. Addressing this lacuna mandates a holistic evaluation encompassing social performance and risk dimensions, ensuring alignment with evolving sustainability imperatives.

3.1 Circular Economy in the Wood Industry

Existing traditional linear production methods are unsustainable as sectors of the wood industry, including construction, furniture manufacturing, and pulp and paper industry, significantly contribute to environmental pollution and waste generation. Sustainable production and consumption increasingly aim for circularity and the use of sustainable natural resources. There is a need to increase resource efficiency, i.e., produce more with less, and decouple economic growth from long-term environmental degradation.

Circular economy (CE) is a model aimed at maintaining materials and products at their maximum value in terms of utility and time, with the intention of waste elimination. It is a regenerative approach in which raw materials, waste generation, emissions, and energy are minimized by promoting material circularity achieved through repair, reuse, production, and recycling of wood resources.

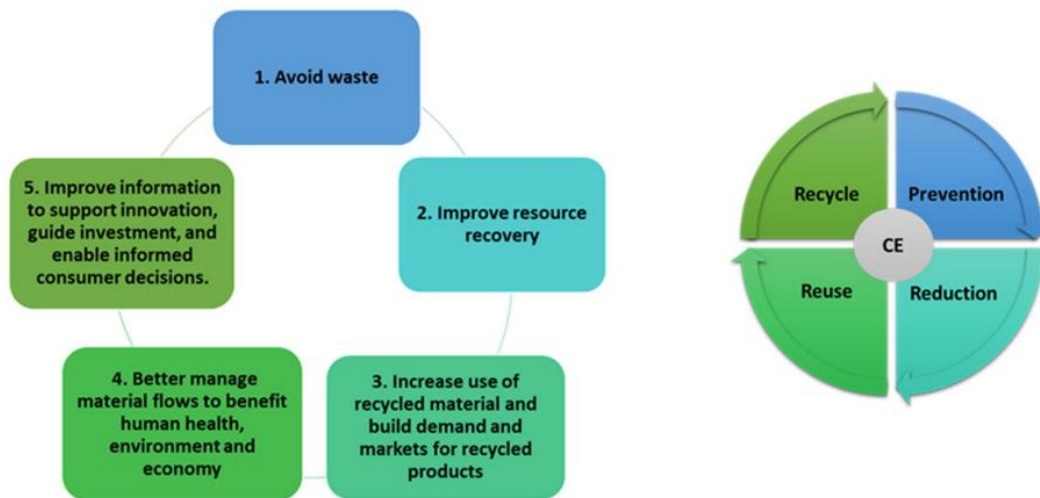


Figure 3: Presentation of the circular economy (ISO-14044, 2007)

In addition to increasing the efficiency of wood resource utilization, circular economy aims to achieve a balance between the environment and society, encompassing the principles of:

- a) preserving and enhancing natural capital by managing finite reserves and balancing renewable energy sources.
- b) optimizing production, product manufacturing, components, and materials.
- c) encouraging system efficiency by identifying and eliminating negative traits.

Essentially, circular economy represents a relatively new concept, which helps explain the lack of studies in this area. Due to its novelty, this model is still in its nascent stage of development, and its proliferation will undoubtedly yield positive outcomes for both the economy and ecology (Jaham et al., 2022).

3.2 Sustainability and Reuse of Modified Wood

The sustainability of modified wood pertains to the ability to produce and use modified wood in a manner that minimizes environmental footprint, supports ecological balance, and encompasses the following aspects (Araujo et al., 2019):

- a) Environmental sustainability: Wood modification processes can reduce the need for chemical treatments like pesticides, thereby mitigating negative environmental impacts. Additionally, certain modification processes can enhance wood's resistance to decay and degradation, thereby extending the lifespan of products made from modified wood.
- b) Reuse: Materials made from modified wood can be reused or recycled after use, contributing to waste reduction and better resource utilization.

- c) Greenhouse gas emission reduction: The use of modified wood can reduce greenhouse gas emissions compared to other materials like steel or concrete, especially when considering the entire lifecycle of modified wood products.
- d) Energy efficiency: Wood modification processes can be energetically efficient compared to traditional wood processing and preservation methods, contributing to reduced energy consumption and negative environmental impacts.

The sustainability of modified wood is becoming increasingly important in the context of global environmental challenges such as climate change, deforestation, and the growing demand for sustainable materials in construction and industrial applications. Integrating sustainable practices in the production and use of modified wood is crucial for creating environmentally viable solutions in modern society.

4. THE USE OF MODIFIED WOOD IN A HEALTHY LIVING ENVIRONMENT

Decking for terrace, balcony, platforms, and similar structures represents one of the most prominent applications for modified wood, including thermally modified timber, acetylated, and furfurylated timber. Following closely behind are façade cladding applications, which may include shading elements. These applications constitute the primary market for modified wood products and are well-established within the industry. While some suppliers have introduced the use of modified timber for window scantlings, comprehensive data on actual sales in this domain remain unavailable. Notably, a significant portion of thermally modified timber finds application in sash cores of polyurethane-coated windows. Additionally, modified wood is increasingly utilized in garden furniture, playground equipment, as well as various gardening and landscaping purposes such as fences, poles, and screens. Thermally modified timber, owing to its aesthetically pleasing appearance, is also employed in interior applications, predominantly in flooring and paneling, a trend not observed in acetylated and furfurylated timber. In interior settings, adherence to existing emission requirements is paramount, particularly for thermally modified timber.



Figure 4: Thermally modified wood for outdoor use (31)

This widespread recognition has fueled the increasing adoption of modified wood across Europe. In addition to the three primary wood modification processes (acetylation, furfurylation, thermal modification), there has been a notable surge in the commercialization of alternative processes. Furthermore, there has been a proliferation of companies engaged in the production of thermally modified wood, especially for localized usage within specific countries. This trend suggests a growing preference for modified wood among architects, specifiers, and end-users, indicating a sustained success trajectory for modified wood products across Europe.

The resistance of modified wood to moisture and mold not only mitigates the development of allergies and respiratory issues but also fosters an environment conducive to the health and comfort of occupants (Hasanagić et al., 2024). In spaces such as bedrooms, living rooms, or office areas, where individuals spend a significant portion of their time, ensuring high indoor air quality is imperative. Modified wood's resistance to moisture inhibits the buildup of conditions conducive to mold and bacteria growth, thereby reducing the risk of allergic reactions and respiratory ailments among residents or employees. Moreover, the low maintenance requirements of modified wood facilitate the maintenance of space hygiene. By incorporating modified wood into construction projects, not only is the aesthetic appeal of the space enhanced, but the quality of life of its occupants is also improved, promoting their health and well-being.

Furthermore, modified wood can contribute to health by attenuating noise levels in indoor environments. Certain varieties of modified wood exhibit acoustic properties that aid in sound absorption, thus minimizing reverberation and echo. This attenuation of noise pollution proves particularly advantageous in residential or workspaces where maintaining a tranquil environment is essential for concentration, relaxation, and overall well-being.

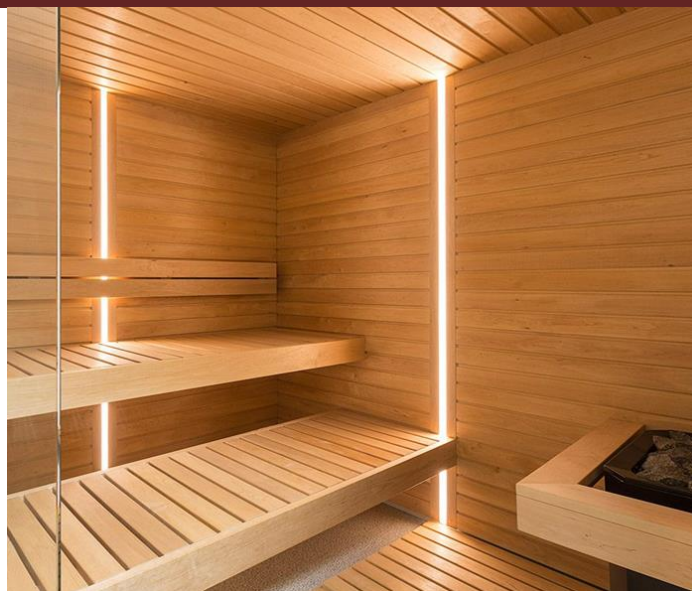


Figure 5: Finnish sauna made of thermally modified wood (33)

5. CONCLUSION

The research on modified wood's environmental impact highlights its significant influence on key factors like air and water quality, conforming to ISO standards. Certain modification processes enhance water resistance and durability, reducing the need for resource replacement and repair. Life Cycle Assessment (LCA) is crucial for evaluating modified wood's ecological performance, aiding informed decision-making. Modified wood finds wide use in construction, furniture, and outdoor elements, serving as a sustainable alternative. Continuous research and LCA integration are vital for ensuring its sustainable future use.

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COMPARATIVE PHYSICAL CHARACTERISTICS OF WOOD SPECIES FROM ZAGROS FORESTS

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Key words: Zagros Forests, wood species, physical characteristics, density, shrinkage

ABSTRACT:

*This paper presents a comparative analysis of the physical characteristics of three wood species, namely Persian oak (*Quercus brantii* Lindl.), hawthorn (*Crataegus azarolus* L.) and wild almond (*Amygdalus eburna*), grown in the Zagros Forests region. The study aims to provide insights into the physical properties (e.g. oven-dry density, basic density, volumetric shrinkage, and swelling). Through comprehensive research and analysis, the paper outlines the specific physical attributes such oven-dry density, basic density, volumetric shrinkage, and swelling of selected wood species. Samples from three adult trees per species (Persian oak, hawthorn, wild almond) were analyzed using ISO 13061-14 (2016) standards. Wild almond exhibited the highest oven-dry density ($0.91 \text{ g}\cdot\text{cm}^{-3}$), while Persian oak showed the greatest volumetric swelling (17.70%). The findings of this study contribute to a better understanding of the diverse range of wood resources available in the Zagros Forests, forest management practices and promote the conservation of biodiversity in the region.*

1. INTRODUCTION

The Zagros Forests in Western Asia are renowned for their vast biodiversity and the wide variety of tree species they harbor. Covering approximately 5 million hectares, these forests make up around 40% of Iran's forested areas (Sagheb- Taleb et al. 2004). The climate in the Zagros region is influenced by westerly disturbances and the Azores high, leading to distinct wet winters and dry summers (Azizi et al. 2013). Main hardwood species found in the Zagros forests include various oak species, wild pistachio, hawthorn, and pear trees (Jazirehi, Rostaghi 2003). The Zagros Forests are not only biodiversity hotspots but also vital for local economies, providing timber, fuelwood, and ecosystem services like soil conservation. Over 1.5 million people depend on these forests for livelihoods (Sagheb-Talebi et al., 2004), underscoring the need for sustainable utilization.

Quercus spp., which include over 500 oak species worldwide, are crucial components of the Zagros forests, with eight species naturally occurring in Iran (Willis, 1998). The region boasts the highest oak species diversity in the country, with Persian oak (*Quercus brantii* Lindl.) being the dominant species, covering more than half of the Zagros forests. These oak trees often grow alongside other species like *Crataegus azarolus*, *Acer monspessulanum*, and *Pistacia atlantica*. *Crataegus* species, belonging to the Rosaceae family, are small trees or shrubs with around 300 species distributed across Europe, North Africa, West Asia, and North America (Phipps 1997).

Almonds, with their origins believed to lie in Iran, exhibit a broad geographical distribution extending from southwest to central Asia (Gradziel, 2011). Iran is considered significant for almond biodiversity, hosting numerous wild almond species, some of which are endemic (Zohary & Hopf, 2000). Zagros forests, which make up 40% of Iran's forested areas, are crucial for the growth and conservation of almond species. Almonds, adapted to arid environments, can be found growing between 600 to 2200 meters above sea level in the region. Understanding the physical properties of wood species in the Zagros Forests is important for their optimal use and the promotion of sustainable forestry practices. This study aims to compare the physical characteristics of selected wood species in the area to underscore their unique qualities and potential applications.

2. MATERIALS AND METHODS

To conduct this study, a selection of prominent wood species from the Zagros Forests was identified and collected for analysis. Disks were obtained from the logs for the determination of physical properties. In detail, specimens with dimensions of $3 \times 2 \times 2$ cm³ were prepared in accordance with ISO 13061-14 (2016) for the investigation of oven-dry density and volumetric shrinkage. In total 270 specimens from different parts of disks (10 specimens per each disk) were prepared. Sample dimensions were measured in green (saturated) and oven-dry condition with a slide calliper; oven-dry mass was determined with an electric balance to the nearest 0.01 g. The

volumetric shrinkage was calculated using the dimensional change from the green to oven-dry condition. The results obtained from these tests were then analyzed to identify trends and variations among the different wood species.

3. RESULTS AND DISCUSSION

The comparative analysis of the physical characteristics of wood species from the Zagros Forests revealed interesting insights into their unique properties. Table 1 shows the average values of physical properties in the three studied wood species.

Table 1: The average values of physical properties in the three studied species.

Wood species	Oven-dry density (g·cm ⁻³)	Volumetric swelling (%)
Persian oak (<i>Q. brantii</i> L.)	0.78	17.34
Hawthorn (<i>C. azarolus</i> L.)	0.71	17.70
Wild almond (<i>A. eburna</i>)	0.91	16.11

In the study area, Wild almond's high density (0.91 g·cm⁻³) suggests suitability for flooring or furniture, while Persian oak's pronounced swelling (17.70%) may require stabilization treatments for outdoor use. These insights guide species selection for industry-specific needs. It is reported that there are several factors affecting the density of the wood, such as anatomical properties, e.g. vessel and fibre morphology, provenance, moisture content, and chemical composition (Bahmani et al. 2018; Nazari et al. 2020; Nazari et al. 2021; Fathi et al. 2022). Persian oak, on the other hand, displayed higher volumetric shrinkage as compared to the other wood species.

The findings of this study underscore the diversity and versatility of wood species in the Zagros Forests in terms of their physical characteristics. Understanding these differences is crucial for selecting the right wood species for specific applications based on their inherent properties. Moreover, awareness of the distinct features of each wood species can guide sustainable forest management practices and promote the conservation of biodiversity in the region. By comprehensively comparing the physical characteristics of wood species from the Zagros Forests, this study contributes valuable insights to the forestry and woodworking industries, aiding in informed decision-making for resource utilization.

4. CONCLUSION

This study has provided a comparative analysis of the physical characteristics of wood species grown in the Zagros Forests, highlighting their diverse properties and potential applications. By examining factors such as physical properties, the study offers valuable insights into the unique

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attributes of each wood species. Moving forward, further research and exploration of wood species in the Zagros Forests can enhance our understanding of their properties and support sustainable forest management practices. Overall, the findings of this study contribute to the body of knowledge on wood resources in the Zagros Forests and offer practical implications for the woodworking industry.

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**THE INFLUENCE OF NATURAL DISTURBANCES
ON THE HARVEST PLAN AND TIMBER PRODUCTION
FROM MOUNTAINOUS FORESTS**

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Key words: forest management, natural disturbances, weather extremes, sanitary felling, assortment structure, sudden yield

ABSTRACT:

The success of achieving goals in forestry operations depends on quality of multicriterial planning. Production in forestry takes place in large areas with the presence of various climatic factors. This complicates the planning and assumes higher production costs. The determination of annual cutting yield is a complex and comprehensive process, the realization of which needs to meet the requirements of sustainable forest management. There are regular fellings prescribed by the plan (regular yield) or sanitary fellings after the natural disturbances (sudden yield). The occurrence of sudden yield decreases the stability of the ecosystem and makes it difficult to manage due to the disturbed stand structure and health. Such stands are additionally exposed to various biotic and abiotic factors. The financial effect of timber production after sanitary felling is extremely negative. The reason for this is low timber volumes over a large area, with a lot of "idle" human and mechanized work. Sanitary fellings result in low quality timber logs, whether as the result of pests or weather extremes. The aim of investigation was to determine the production structure and performance of the production in the compartments where sanitary fellings were carried out. Results were obtained and then compared to the timber production from regular fellings. The analyses were carried out according to the timber volume and quality. In case of sanitary fellings direct production costs were higher and revenues were lower. The planned financial income per 1 m³ was higher than achieved, despite the higher production in total wood volume. In regular fellings timber production was higher than planned

but financial income per 1 m³ was lower than planned. The research points to dependence of the planning on the quality of input data and proposes improvements of planning process in case of fellings after natural disturbances and extreme weather events.

1. INTRODUCTION

Successful achievement of goals in forestry creates the need for multicriterial planning, which includes the principles of other scientific and professional disciplines. Modern forest management are becoming increasingly important taking into account ecology, water regulation, environmental and nature protection, climate change mitigation, adaptation to climate change, etc. The main indicator of performance is represented by acquiring total revenue, however, if we talk about performance in forestry management, then it depends not only on the total income gained, but also on the structure of production costs. In forestry, timber production takes place in large areas exposed to natural and climatic influences, which makes it difficult to use machinery and human resources, due to the constant need for adaptation. In addition, it requires knowledge of a large number of qualitative and quantitative information, interactively related with each other, which is also an aggravating circumstance for the planning process, as well as the process of forestry production itself. In the context of the sustainable development as a fundamental principle of modern forest management, the determination of timber yield is a complex and comprehensive approach that should meet all the requirements of multipurpose and integrated forest management.

Sabadi et al. (2001) point out that the principle of sustainable management with all the consequences should be inviolable, i.e., felling can be up to an incremental quantity, assuming it includes only and exclusively the wood that should be harvested from the stands for regeneration reasons. Annual yield is a instrument of a forestry policy that guides the development of forests with regard to the structure of the existing requirements towards forests and the forest purpose that results from it. Today, the annual yield is expressed by the volume of felling trees and the area on which felling is planned. The yield expressed by volume and its distribution by tree species, diameter, and qualitative log classes, is a good starting point for determining the value of trees for felling and planning expected income. When the yield is in relation to the wood stock and the volume increment of the stand, it refers to the type of felling (thinning, regeneration, preparatory or sanitary felling) and the intensity of management. The yield expressed as an area primarily shows the obligation arising from forest management (regeneration, restoration). It is an element of establishing and maintaining spatial layout, i.e., a normally landscaped forest according to the area with regard to a certain way of management (even or uneven aged forests). Yield can be planned and realized, i.e., achieved. The planned yield is the volume or area prescribed by the management plan for logging during the management period at the stand level, management class, management unit or forest management area. Since it refers to a period,

usually ten years, it is a periodic yield. The realized yield is the volume of trees that have been cut down, i.e. the area on which felling was carried out at the end of the planning period as a result of the records of the forest operations carried out. When it comes to uneven aged stands, on whose examples the results of this paper are based, the yield within such stands is classified into main (regular) yield, irregular yield, and sudden yield (Klepac 1965).

The emphasis of this paper is precisely on the main and sudden yield of uneven aged forests. Main yield means the volume cut down and prescribed by the management plan. Sudden yield consists of the harvested volume, which not prescribed by the management plan, and realized by illegal and sanitary felling. When we talk about sanitary logging that are not planned, and how they are occurred, it is clear that there is a violation of several factors of stability and sustainable management within a stand, starting from the stand structure itself, to its state of health, where the stand is further, as such exposed to various influences, with urgent for its recovery. As a rule, the financial effect of such felling is extremely negative, primarily due to a low volume dispersed randomly on a large area, whereby a lot of time is spent both for workforce and mechanization, and the recovery of the stand through regeneration activities is a very expensive and time-consuming process. Sanitary logging usually results the low-quality assortments, which influences not only by pests and diseases, but with snowthrows, icebreakers, windbreaks and winthrows.

What is the difference in the assortment structure, the number of assortments, as well as the price of all the works necessary to obtain these logs, will be shown and explained in the rest of the paper.

2. PROBLEM

The timber production consists of three main phases (Kulušić, 1977), namely felling of trees and production of wood assortments, collection and extraction (transport of round and split assortments to the landing) and long-distance transport of logs. Logging is the first phase in the process of production and represents a tool in forest management which regenerates and exploits the forest. As stated by Krpan and Pičman (2001), the final forestry product are timber assortments, so management aims to achieve their best quality. When extracting roundwood in a market-oriented economy, it is important to understand the classification of products by quality (Lipoglavšek, 1996). In modern forestry, knowledge of the share of wood assortments in certain forest communities is an important forest-economic, market and organizational information. The forest engineering consists of the analysis, the establishment of technological systems and their on-going improvement (Heinimann, 2007). By producing wood assortments in stands of different health status categories, a timber assortment structure achieved often significantly deviates from the planned ones, and thus from the planned revenues (Paladinić et al., 2011). A more precise assessment of the assortment or qualitative structure of the stand and the enables

more efficient management planning.

Knowledge of the quantity and quality of wood is necessary for more economical utilization in felling and production (Bojanin, 1960). In general, the determination of the percentage share of logs by quality classes is negatively affected by those trees that do not contain all the assortments that they potentially could contain by their dimensions. In addition, the absence of a certain assortment of roundwood does not mean a worse assortment structure of a particular tree. (Vuletić, 1999). The primary interest of forestry operations is the knowledge of the assortment structure that can be achieved by implementing a certain type of cutting according to forest breeding principles. Prka and Krpan (2007) points out that the assortment structure of the stand varies in a wide range. Frequent significant deviation of the planned production of assortments from the realized one indicates an open problem and the need to develop a model for the assessment of the assortment and/or qualitative structure of stands (Vondra, 1995).

Tables of the share of timber assortments in yield (assortment tables) are important tool, but their production, precision and practical application is associated with large and numerous difficulties, which are the result of biotic and abiotic factors (Prka, 2008). Foresters in the field need assortment tables with as few inputs as possible. Teslak et al. (2013) in their paper on the qualitative structure of pedunculate oak trees, note that the quality of tree assumes the types, i.e. the quality of assortments that can be made from it. Tables of timber assortments that consider the type of felling as one of the inputs allow a more reliable assessment of the assortment structure of the felling and more precise planning of the yield. In doing so, the criterion of (positive) selection during the marking trees for felling, which is applied until preparatory felling, was taken as a influencing factor in even-aged forests (Prka and Poršinsky, 2009). Precise models of assessment of the development of assortment tables can reliably define for the level of location (stands/groups of trees), which could be feasible in operation (Paladinić and Vuletić, 2006). In the preparation of assortment tables, the permanent laws of biological (natural) growth and development of stands (trees) conflict with the provisions of standards for the classification of timber products and the timber trade policy (Prka and Poršinsky, 2009). The authors emphasize that the norms for roundwood were prescribed by humans but also changeable over time. Paladinić et al. (2011) stated there not all information taken into account when choosing appropriate assortment tables in income planning, e.g. phytocenosis type, ecological-economic type, quality grades of trees and the like are not considered. Therefore, the application of assortment tables in the planning of the production is a possible main cause in cases of significant differences between the plan and the realization in the forest.

Changes in the natural, biological, and ecological balance, result in disturbance of stability, and degradation of forest ecosystems (Seletković and Tikvić, 2004; Anić et al., 2002). Pothier and Mailly (2006) point out that the health status of the forests influencing productivity of forest stands. It can be biotic and/or abiotic factors, individual competition with each other, insects, negative climatic phenomena, human influence, etc. The quality and technical usability of wood

depends on the state of health, wood defects and dimensions, which are the main factors that affect financial income. The value of timber and timber assortments changes in terms of changes in society, changes in market and economic relations, the application of innovative technologies and the action of other factors (Rebula, 1996). The health of stands due to the consequences of logging is indirectly affected by the structure of production percentage of sawlogs and energy wood (Danilović, 2015), and timely interventions in the form of sanitary felling can prevent losses of wood volume and its value, thus reducing the share of damaged trees in the stand (Zečić et al., 2009).

For each economic component of forest management, it is necessary to know the amount of income and costs that occurred in the production cycle (Beljan, 2015). The question arises whether the same regulations are applicable in forestry production when achieving regular and sudden yield, especially in high forests where we expect high-value logs, and whether the estimation of the assortment structure can or should be done according to the same rules?

3. RESEARCH AREA

The research was conducted in the northwestern part of Bosnia and Herzegovina, which administratively belongs to the entity Federation BiH and Una-Sana County. The data was collected within the forest stands of the forest management unit FMU "Plješevica". This unit extends to the western and southwestern part of the City of Bihać, along the border with the Republic of Croatia. It is located within the forest management area FMA "Una", and occupies an area of 7,553.2 ha. On its entire surface it is managed by the forest company ŠPD "Unsko-sanske šume" LLC, Bosanska Krupa, and is located within the forest office FO Bihać. Of the forest stands, within this FMU we find high forests (4,286.6 ha), coppice forests (2,215.6 ha), forest cultures and plantations (435.7 ha) and unproductive area (615.3 ha). Data on logging and records of timber production of the FO Bihać were collected. In the FMU "Plješevica" the most damaged are high mixed forests, namely beech and fir forests, with spruce, and the greatest damage, sprouts and fractures, are visible on coniferous trees (fir and spruce). A total of 39 departments in this FMU were treated with sanitary felling, and a total of 15,710 m³ of gross conifers and 2,643 m³ of deciduous were in 2018, mostly broken trees. Also, within this FMU, regular, planned felling determined by the forest management plan (FMP) were carried out. For the analysis of sudden yield, data from compartments 68, 69, 70, 74 and 75 was used, and for regular yield from compartments 41, 42, 65, 74 and 76.

4. METHODS OF WORK AND MATERIALS

Methods of analysis, synthesis and data comparison were applied to obtain the results. The steps that needed to be carried out included: determination of assortment structure, selection of hit

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departments for analysis of regular and random income, comparison of quantitative and qualitative structure of timber assortments, economic analysis, assessment of the impact on the stands, comparison of results obtained by analysis of regular and sudden yield, assessment of the criteria for determining assortment structure, defining proposals for possible corrections in determining the assortment structure regular and random income of high forests.

The planned assortment structure in the investigated departments was determined by a computer application used in the ŠPD "Unsko-sanske šume". It is based on "Tables of wood masses, journals and others" (Eić, 1965) and the silvicultural-technical classification of trees (Matić et al., 1971). This application is used to determine the assortment structure and regular and sudden yield. In this application, based on the input of the species, the diameter of the tree, the breeding and technical class and the habitat, the assortment structure of each individual tree was obtained, and by further data generation procedures, the total assortment structure of the compartment or subcompartment is obtained.

Classifications of trees are based on descriptive characteristics and have great importance in the execution of forestry operations. In Bosnia and Herzegovina, when it comes to the classification of wood, it is still applied standards for forest wood assortments from Yugoslavia - JUS (no. D.B4.029-1979, no. JUS D.B4.028-1979 and no. JUS D.B1.023-1980).

5. RESULTS

The marked wood volume of the sanitary felling departments showed that the total allocated volume of the departments where sudden yield has been observed amounted to 2,852 m³ gross, or 2,334 m³ net. From the above it can be seen that conifers predominate in sudden yield, and the reasons are of a different– shallow rooting of conifers, dominance of conifers in the stand due to greater harvest of deciduous during the regular harvest, crown density, etc.

Table 1: Marked trees and volumes per yield type

Compartment	Area (ha)	Marked trees			Marked volume		
		Coniferus	Deciduous	(N·ha ⁻¹)	Gross volume (m ³)	Net volume (m ³)	Net volume (m ³ ha ⁻¹)
Sanitary felling (Sudden yield)							
68	77.0	218	81	4	427	355	4.61
69	137.7	81	29	1	118	91	0.66
70	47.7	247	153	8	476	397	8.37
74	124.7	120	78	2	215	169	1.36
75	142.1	660	278	7	1617	1322	9.30
Σ / MEAN	528.9	1326	619	4	2852	2334	4.41
Selection felling (Regular yield)							
41	152.1	1792	2167	26	5019	4271	28.08
42	128.5	398	1461	14	5175	4291	33.39
65	72.3	1052	628	23	2000	1666	22.12
74	124.7	864	1480	19	1417	1201	9.63
76	110.8	746	1330	19	5003	4212	38.01
Σ / MEAN	588.4	4852	7066	20	18614	15641	26.58

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Out of a total of 1,945 trees allocated, 68% refers to conifer trees, and the total net volume allocated is 2,333.68 m³ which is 4.41 m³ha⁻¹. Also, a small number of marked trees per ha is noticeable, and average it is 4 treesha⁻¹.

Due to the low wood volume per unit area, and in accordance with the normative acts for the execution of works in forestry in the ŠPD "Unsko-Sanske šume", all works of harvesting, production and extraction of wood assortments automatically fall into the V category of work, because the volume per ha is less than 25 m³. This is the most expensive category of heavy and low productive working areas, and therefore the costs have drastically increased.

Table 2: Comparison of the harvest plan and timber production in sanitary fellings

Compartment	Coniferus (m ³ ha ⁻¹)								Deciduous (m ³ ha ⁻¹)								Total (m ³ ha ⁻¹)
	Sawlogs (class)				Tunnel wood	Long pulpwood	Short pulpwood	Σ	Sawlogs (class)				Long firwood	Stacked firwood	Σ		
	I	II	III	Σ					I	II	III	Σ					
	Harvest plan																
68	0.87	1.40	0.95	3.21	0.26	0.40	0.27	4.14	0.05	0.05	0.06	0.15	0.16	0.15	0.47	4.61	
69	0.06	0.20	0.10	0.37	0.08	0.08	0.08	0.57	0.01	0.01	0.01	0.03	0.03	0.04	0.09	0.66	
70	0.78	2.19	0.76	3.73	0.00	0.96	0.64	5.33	0.21	0.40	0.52	1.13	1.15	0.76	3.04	8.37	
74	0.05	0.49	0.29	0.83	0.00	0.32	0.08	1.23	0.01	0.01	0.02	0.03	0.06	0.04	0.13	1.36	
75	1.66	3.14	1.13	5.93	0.00	1.47	0.58	7.98	0.10	0.13	0.18	0.41	0.47	0.44	1.32	9.30	
MEAN	0.67	1.41	0.61	2.69	0.06	0.63	0.29	3.66	0.06	0.08	0.11	0.25	0.27	0.23	0.75	4.41	
	Timber production																
68	0.27	1.90	1.37	3.54	0.00	1.17	0.00	4.71	0.08	0.10	0.05	0.23	0.41	0.00	0.64	5.35	
69	0.03	0.25	0.09	0.38	0.00	0.45	0.00	0.83	0.01	0.02	0.01	0.04	0.05	0.00	0.08	0.91	
70	0.05	1.39	1.68	3.12	0.00	1.52	0.23	4.86	0.22	0.55	0.60	1.37	1.27	0.04	2.68	7.54	
74	0.05	0.37	0.35	0.77	0.00	0.30	0.00	1.08	0.00	0.00	0.00	0.00	0.06	0.01	0.07	1.15	
75	0.43	3.20	3.40	7.03	0.00	2.25	0.79	10.07	0.08	0.23	0.18	0.50	0.85	0.52	1.87	11.94	
MEAN	0.18	1.41	1.37	2.96	0.00	1.10	0.23	4.30	0.06	0.13	0.11	0.30	0.43	0.15	0.87	5.17	
	Difference (%)																
%	-73.23	0.27	126.35	10.36	-100.00	73.27	-18.42	17.27	3.40	60.58	0.47	20.76	56.11	-35.61	16.63	17.16	

Table 3: Comparison of the harvest plan and timber production in regular fellings

Compartment	Coniferus (m ³ ha ⁻¹)								Deciduous (m ³ ha ⁻¹)							Total (m ³ ha ⁻¹)
	Sawlogs (class)				Tunnel wood	Long pulpwood	Short pulpwood	Σ	Sawlogs (class)				Long firwood	Stacked firwood	Σ	
	I	II	III	Σ					I	II	III	Σ				
	Harvest plan															
41	2.08	6.39	2.07	10.54	1.43	0.00	2.34	14.30	1.19	1.67	2.39	5.25	0.00	8.53	13.78	28.08
42	1.11	3.43	1.55	6.09	0.77	0.80	1.20	8.85	1.39	2.27	4.19	7.85	6.20	10.48	24.54	33.39
65	2.03	6.54	2.32	10.90	1.15	2.63	0.00	14.67	0.50	1.05	1.73	3.28	0.00	5.09	8.37	23.04
74	0.36	1.00	0.38	1.74	0.42	0.00	0.65	2.82	0.51	0.70	1.01	2.22	0.00	4.60	6.81	9.63
76	3.91	8.22	3.07	15.21	0.84	1.67	1.12	18.83	1.56	2.37	3.52	7.45	6.44	5.30	19.19	38.02
MEAN	1.84	4.96	1.82	8.63	0.92	0.81	1.21	11.58	1.07	1.65	2.63	5.35	2.57	7.09	15.01	26.58
	Timber production															
41	1.70	4.37	3.38	9.44	0.01	2.98	2.44	14.87	1.72	2.02	1.75	5.49	6.08	5.43	16.99	31.86
42	0.16	1.74	2.12	4.02	0.00	2.32	0.00	6.34	1.05	1.61	1.47	4.13	5.66	0.24	10.03	16.37
65	1.60	3.29	4.81	9.70	0.00	1.52	2.54	13.76	2.01	1.54	1.12	4.67	3.18	2.18	10.03	23.79
74	0.10	0.86	0.87	1.83	0.00	0.52	0.63	2.97	0.84	0.91	0.77	2.53	2.66	2.34	7.53	10.50
76	1.89	7.96	8.59	18.43	0.00	5.16	1.18	24.78	4.17	3.69	2.84	10.71	7.41	4.69	22.81	47.58
MEAN	1.05	3.59	3.73	8.37	0.00	2.54	1.30	12.21	1.88	1.95	1.61	5.45	5.15	3.10	13.71	25.92
	Difference (%)															
%	-43.23	-27.64	105.05	-2.99	-99.65	213.26	7.07	5.51	75.52	18.35	-38.61	1.85	100.73	-56.22	-8.66	-2.49

Although the produced wood volume is higher than planned by 18%, a lower financial income of 1 m³ can be expected. The reasons for such a decrease in revenue per unit volume can be found in the lower quality assortment structure than planned, especially in more valuable assortments

(sawlogs). With regular yield, it is clearly visible a larger number of marked hardwood trees, contrary to sudden yield, where conifers dominate.

6. DISCUSSION

For appropriate economic evaluation and comparison of different management types and intensities, the analysis needs to be done simultaneously and in the same or similar conditions (Hanewinkel 2002). Accordingly, the assessment of the impact and consequences of natural disturbances on the production of timber assortments was carried out by comparative analysis, in different departments of the same forest management unit. One group consisted of departments where sanitary felling was carried out, the other departments where there was a regular yield.

Analyzing the results of the marking trees in Table 1, a large difference in the allocated wood volume of regular and sudden yield can be observed, namely $26.58 \text{ m}^3\text{ha}^{-1}$, or $4.41 \text{ m}^3\text{ha}^{-1}$. Therefore, all other costs, i.e. direct production costs, from felling to transporting wood, have increased, and revenues are significantly lower, which is in accordance with the laws of mechanization of forestry works (Grammel, 1988). In sanitary felling, the planned revenue is higher than achieved despite the higher achieved total wood volume than planned. The reason for this is the lower quality of produced wood assortments than planned. On the other hand, with regular yield we there is situation where are no significant differences, which favors planning method as appropriate.

When we compare the planned values of wood of sudden and regular income, although it is a far different amount of wood volume, it is evident that the percentage values of the assortment structure are approximate, even in terms of the participation of the highest quality logs, and the reason for this lies in the planning, i.e. the same criteria in the evaluation and evaluation of trees. As a rule, in sanitary felling on large areas, a small amount of less valuable assortments are cut down, which requires high costs in the felling itself because there is no pattern, but the workers and machinery randomly moves on the site looking for trees, wasting a lot of time, which affects the effective working hours and reduces the effect. Similar situation has been confirmed by Klobučar et al. (2012) during sanitary thinning of even-aged oak forests in lowlands. The cost of operation of forest machines is often doubled, because of broken trees. Since trees in such fellings are rare and random throughout the felling site, and the extraction of logs has much more so-called "idle work", which again increases the cost of production.

After the realization of salvage logging and sanitary felling (Table 2), it is evident that it assortment structure deviates from the planned, both in quantity and in quality. In the total volume, more conifers were produced (334.47 m^3 or 17.26%), of which sawmill logs 147.12 m^3 or 10.35%, and pulpwood 217.78 m^3 (or 44.79%). In hardwood, variations are also quite high. A total difference of 65.94 m^3 in deciduous was realized, or more by 16.63%, of which sawmill logs 27.27 m^3 , or 20.76%, while firewood (in long and stacked) was realized by 38.67 m^3 or

14.58% more than planned. As far as the assortment structure is concerned for the regular yield, the deviation of the planned and realized yield is also visible, both in total quantities, but also in quality (Table 3). The total produced net volume is lower by 389.61 m³, or 2.50 %. In conifers, it is slightly larger and is 375.42 m³ more, or 5.50% more. Sawmill logs were realized 151.79 m³ more, or 3% more, while pulpwood is significantly different, as 1,069.31 m³ more was realized, or 190% more than planned. With deciduous, less 765.03 m³ was achieved, or 8.70% less than planned. Sawmill logs were achieved more than planned for 58.36 m³ or 1.85%, while the largest abortion compared to planned was achieved in firewood, i.e., 823.37 m³ less than planned, or 14.49% less. Such a deviation in hardwood can be justified by the uncut part of department 42, where less than 1,864.14 m³ was cut down compared to planned, which is less by 59.12 % of the total planned mass of hardwood in the department. If the uncut volume were added to the realized volume, then it would be 1,099.11 m³ or 12.45 %, while in the total net wood mass it would be 1,474.48 m³ and 9.40%, respectively. The difference has emerged in pulpwood, where almost twice as much as planned was achieved, and the biggest differences are in compartments 41 and 76.

As for deviations by species and timber assortments, there is question of the application of the standards of silvicultural and technical classification of trees, as well as the standards for the wood assortments. Namely, both are based on an individual approach of the forest engineers and operators. Knowledge of these standards, as well as an individual approach to each individual tree is crucial in the planning and implementation of assortment production, because it is evident that deviations of planned and realized are significantly lower in regular income than with sudden ones.

7. CONCLUSIONS

For establishing the optimal state of the forest stand, and to create preconditions for the recovery, it is necessary to conduct the rehabilitation of the damaged forest in the shortest period of time after the disturbance had occurred. This implies forest management activities, such as marking of broken trees, planning of forest operations, felling, bucking and extraction of timber to the landing for further transport. Sanitary felling in some departments were conducted within a period of two years after the storm, which combined with the insects (*Ips* sp.) can lead to deterioration of wood volume, so we produce logs of lower quality. Although the works were conducted within the legally stipulated deadline, if they were carried out earlier, the financial profit, as well as the quality of wood assortments obtained would increase and mitigate the negative impacts of disturbances on the forest ecosystem.

Variability in the total marked wood volume and the quality ratio of assortments occurs in regular and sudden yield. The reasons could be conservative planning methods, methodology of assessing the technical quality of a tree, subjective impact in the application of wood standards or

multiple measurement of the same log (both after bucking and before transport). Deviations of plan and production in assortment structure and quality assortment ratio in regular yield are significantly less than in sudden yield. The reason may be in the assortment tables used for planning. In case of sanitary felling, conifer trees predominate as the result of more intensive harvest of hardwoods in the past, so conifer trees have prevailed and are therefore more susceptible to the negative influences of the atmospheric conditions. The fir is deeply rooted only in the early stages of life and the root system is not dispersed as in hardwoods (beech), because prevailed shallow soils on which deep rooting cannot occur. Spruce has a shallow, thinner root, so it can be concluded coniferous trees suffered the most damage during the windstorm.

The costs of salvage logging are higher; the reason is a large area with a low wood volume per hectare and less effective work time for employees and mechanization. Engagement of mechanization regarding time consumption is almost twofold because the trees felled over each other, and it was necessary to separate them with winch and rope. Also, it was not always possible to collect logs in one place, which further increased production costs. It is necessary to create norms for timber logging for sudden yield because the cost of works is incomparably higher than the cost in regular felling and planning is difficult, also. There are significant costs of the company from the aspect of silvicultural activities after the sudden yield occurs; letter involves disruption of the structure of the stand, preparation of the soil for regeneration and afforestation, the possibility of weeding, the occurrence of diseases and calamities of insects.

Considering the economic feasibility of preparing the area for afforestation and procurement of seedlings, it can be concluded the cost of stand recovery is extremely high. All of the above is necessary in order to restore the stand as successfully as possible. In regular felling the costs are not as high, since the stand is simultaneously logged, nurtured, and rejuvenated (regeneration felling). Sanitary logging is not profitable from an economic point of view, but they is necessary; further deterioration of stands and habitats can be prevented with such measures.

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Eighth International Scientific Conference
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WASTE MANAGEMENT



ANALYSIS OF SOIL COMPOSITION NEAR THE CITY WASTE LANDFILL

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Key words: soil pollution, heavy metals, waste, city landfill

ABSTRACT:

Municipal waste is waste generated in households, public institutions, public areas and production plants. In our country, waste is currently disposed of in landfills. In Bosnia and Herzegovina, an increasing problem for the environment and human health is represented by wild landfills located near populated areas, as well as the fact that very little waste is reused. In this paper, the composition of the soil surrounding the city landfill was analyzed. There is a large list of pollutants that easily accumulate in the surface layers of the soil and degrade its quality.

The experimental part of the paper referred to the analysis of physical and chemical properties of soil from two samples. Parameters such as: organic and mineral part of the soil, moisture, soil pH, carbonates, total lime, humus, nitrates, nitrites, phosphorus and concentration of heavy metals were determined. The analysis of the soil composition determined the amount of humus of 2.29% in the 0-30 cm layer in sample number 1 and 2.45% in the 0-30 cm layer in sample number 2. In the sample number 1, the concentration of nitrite was 1.4 mg/100g, and in the sample number 2 1.2 mg/100g, while the concentration of nitrates in the sample number 1 was 43 mg/100g and 51.1 mg/100g in sample number 2. The concentration of all heavy metals (Co, Cd, Pb, Fe, Zn) in both soil samples was in accordance with the prescribed values, except for the cadmium. The concentration of cadmium in sample 1 was 47mg/kg and in sample 2 2.8 mg/kg. Prescribed MAC value for cadmium is 1.5 mg/kg.

Research has established that dumping waste at the city landfill changes the composition of the land in terms of certain parameters where the quality of the land is impaired, but many parameter values were still within the limits of the prescribed values.

1. INTRODUCTION

The soil as a place for waste disposal is under the direct harmful influence of waste in the environment. In landfills, a series of uncontrolled reactions occur that have unpredictable endings. In addition to being dumped on the ground, the waste goes to the underground water via leachate, evaporates through various substances and smells, and the inhabitants of the landfill such as birds, rodents, insects and pathogenic microorganisms also appear. Landfills represent one of the main sources of pollutants, especially heavy metals, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) that accumulate in the soil and change its composition [1]. Heavy metals can cause changes in the chemical, physical and biological properties of the soil, a decrease in chemical and microbial activity, and in the last case, make it impossible to use it [2]. They are the result of accumulation from parent material and anthropogenic influence [3]-[7]. Through the analysis of soil composition, the concentration of a total of five heavy metals (Co, Cd, Pb, Fe and Zn) was monitored in this paper. Also, due to the increased use of artificial fertilizers in agriculture and the disposal of waste, a large amount of nitrates, nitrites and phosphorus reach the environment every year, which easily reach the groundwater [8]. The content of nitrates, nitrites and phosphorus in the examined soil samples was determined by the spectrophotometric method and presented in detail by graphs. While the physical properties of the soil determine its adaptability when growing various crops, the chemical properties speak of its chemical environment and nutrient status [9]. Determining the physico-chemical properties of the soil, using appropriate analytical methods, was the first step in assessing the impact of the city's waste disposal site on the composition of the soil in this research.

2. MATERIAL AND METHODS

Two composite soil samples from two locations were used for this work. Sample number 1 represents a soil sample from the city landfill, and sample number 2 represents a soil sample from the landfill's surroundings (400 m). Individual samples were taken at depths of 0-30 cm, the procedure was repeated evenly over the surface of the plot 20-25 times. After the excavation of individual samples, composite samples were made by mixing. Plant parts and stones were removed from the sample, and larger lumps were converted into smaller forms. Before starting the determination of soil properties, samples were prepared by drying on paper in a layer of about 2 cm, at room temperature for several days. After drying, the samples were crushed and

Eighth International Scientific Conference
"June 5th - World Environment Day"

passed through a sieve of the appropriate diameter. Soil composition was determined by measuring organic and mineral content, humidity, soil pH, carbonate, total lime, humus, nitrate, nitrite, phosphorus and heavy metal concentration. Determination of physical and chemical soil parameters was done according to standard methods [10].

3. RESULTS AND DISCUSSION

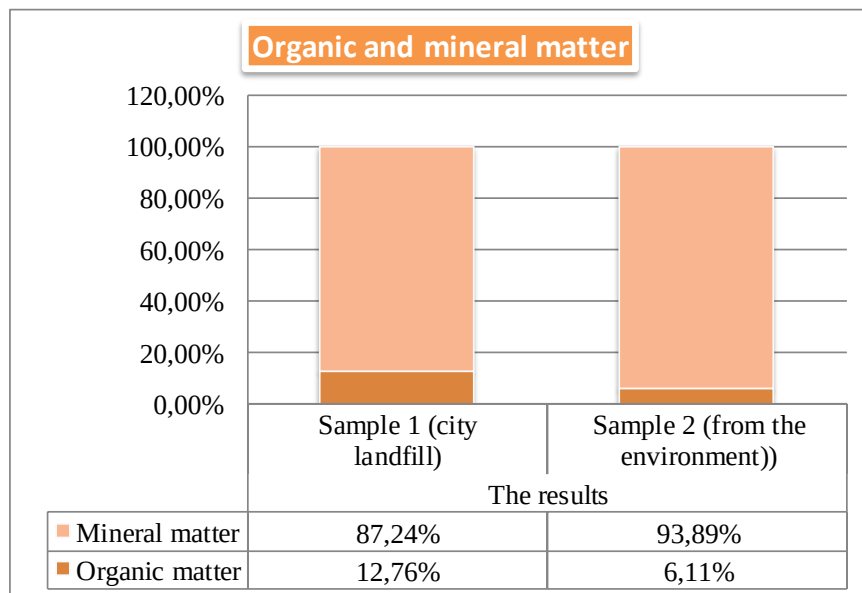
This paper presents the results of the soil composition analysis. The analysis process took place in laboratory conditions and on that occasion the physico-chemical parameters of the soil were examined. As already mentioned, two composite soil samples from two locations were used.

Table 1 shows the results of basic soil parameters.

Table 1: Physical-chemical analysis of soil from and around the city landfill.

Parameters	Measuring unit	Analysis results	
		SAMPLE 1 (city landfill)	SAMPLE 2 (from the surroundings, at a distance of 400m)
Hygroscopic moisture (Hy)	%	8,536	17,776
Humus	%	2,29	2,45
Character of humus		slightly acidic	slightly acidic
Active acidity		6,91	6,82
Total acidity		6,42	6,26
pH in calcium acetate		6,22	6,01
Hydrolytic acidity (y1)	%	0,5	1

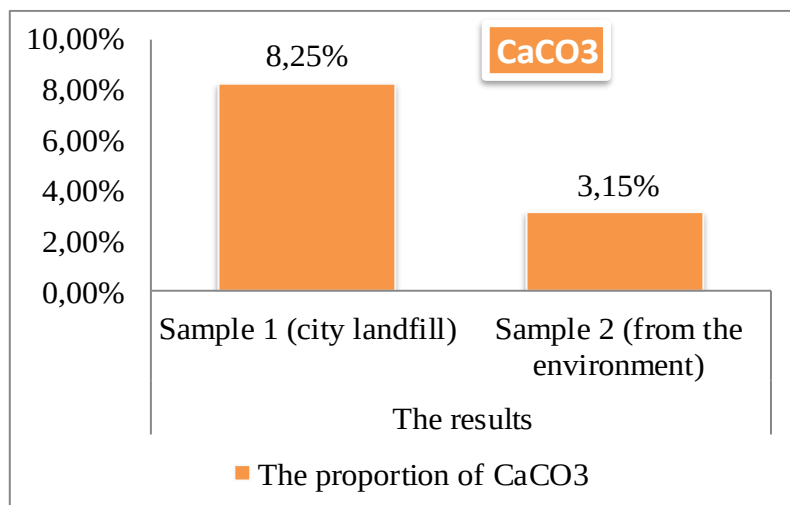
Table 1 shows the results of the analysis of physical and chemical soil parameters. Looking at the table, we can conclude that the obtained results were approximately the same for both tested samples. The exception is the value of hygroscopic moisture, which was slightly higher in the sample from the environment. According to the amount of moisture contained in both soils, we classified them in the textural class of loam, which does not allow water to pass through due to high humidity, but when it dries, water and other water-soluble substances easily pass through it. Also, according to the obtained pH values from three different procedures, the soils had a slightly acidic to neutral pH reaction. Based on the analysis of the humus in the soil samples, we assessed the humus content as slightly humus, and according to the character of the humus, it was slightly acidic humus in both samples. The results of the analysis showed that both soil samples contain small amounts of physiologically active lime, 0.5% for the sample from the city landfill, and 1% for the soil sample from the surrounding area (400 m).



Graph 1: Organic and mineral matter in soils

On graph 1, we can see that the soil from the city landfill has a higher percentage of the organic matter (12.76%), while the soil from the environment has a higher percentage of the mineral matter (93.89%).

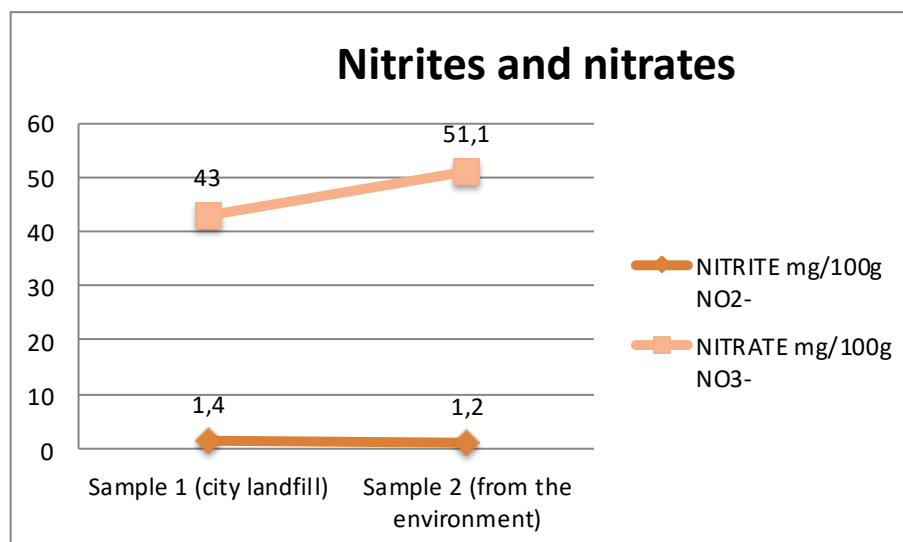
Graph 2 shows the proportion of carbonates, where we see that both soil samples contained less than 10% CaCO_3 , which makes them low carbonate soils.



Graph 2: The proportion of CaCO_3

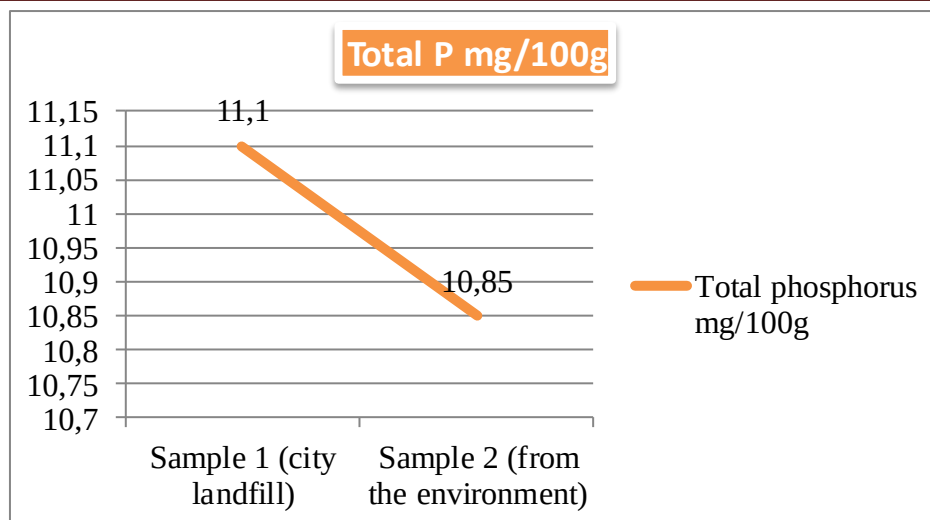
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In the soil samples from the city landfill, the presence of nitrite was recorded at 1.4 mg/100g, and in the sample from the area around the landfill, a slightly lower 1.2 mg/100g. Nitrates in the soil, which are formed by nitrite oxidation, were recorded in concentrations of 43 mg/100g in sample number 1, and 51.1 mg/100g in sample number 2 from the environment (Graph 3). The graph shows that there has been an increase in the concentration of nitrates, because ammonia and nitrites turn into nitrates through oxidation. Because loam predominates in the examined area (less permeable soil), the movement of nitrates is slowed down.



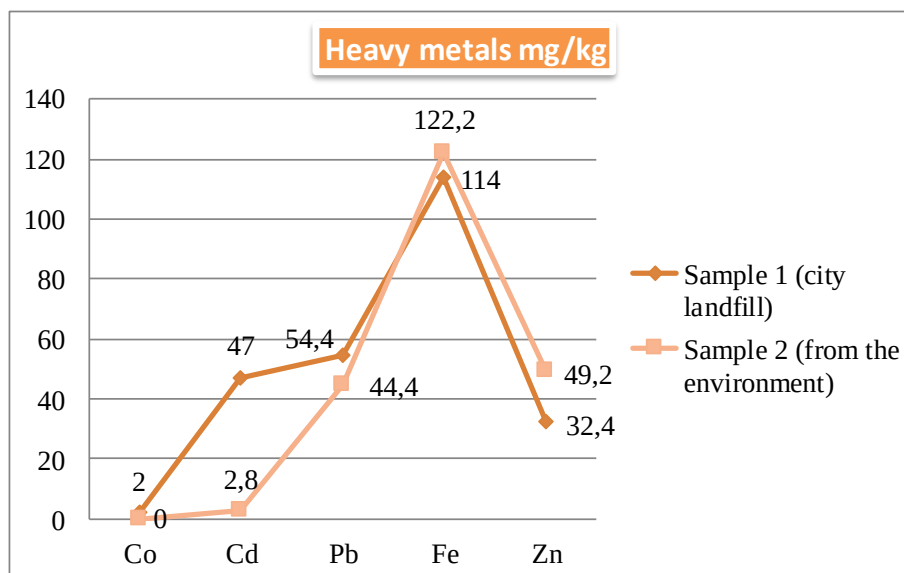
Graph 3: Concentration of nitrite and nitrate in soil samples

The value of phosphorus in both soil samples was also determined and we can notice that the concentration of phosphorus is reduced in the soil that is considered polluted and that is not cultivated, in contrast to the concentration that is characteristic of agricultural lands.



Graph 4: Concentration of total phosphorus in soil samples

The presence of heavy metals in soil samples was monitored through the analysis of a total of five heavy metals, which are presented in graph 5. The concentrations of analyzed heavy metals (Co, Cd, Pb, Fe and Zn) were mostly in accordance with the legal regulations as prescribed. The exception was cadmium, whose concentration exceeded the MAC many times over. The concentration of cadmium was 47 mg/kg in the soil from the city landfill, which exceeds the prescribed MAC of 1.5 mg/kg. The concentration of cadmium in the soil sample from the surrounding area (distance 400 m) also exceeded the MAC and amounted to 2.8 mg/kg.



Graph 5: Concentration of heavy metals in soil samples

4. CONCLUSION

Based on the obtained test results, it was determined that waste disposal at the city landfill definitely changes the composition of the soil in terms of certain parameters where the quality of the soil is impaired, but many parameter values are still within the limits of the maximum allowed concentrations. We can also confirm that the landfill has an impact on the surrounding land, where an increased concentration of cadmium, which can sample various diseases, has been recorded. The landfill is far from the local population and the inhabited area, but regardless of that, it can have a harmful effect on the quality of the land and people's health through groundwater. Most of the waste ends up in landfills, which should be the last stage in the waste management system. Unfortunately, this problem is not the only one, but only one in a series. This research did not analyze the leachate, but based on the results of the soil analysis (primarily pH value, concentration of ammonia and heavy metals), it can be concluded that it is a medium-old landfill.

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THE ROLE OF DIGESTATE IN THE CIRCULAR ECONOMY

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Key words: digestate, circular economy, bio-waste management, renewable energy

ABSTRACT:

Circular economy and renewable resource usage are some of the major goals in the EU strategies. Up to 90% of the raw materials (substrate) for biogas plants are returned as a by-product of anaerobic digestion in the form of organic fertilizer - digestate, while the energy from organic matter of raw materials has been used to produce biogas (biomethane) or electricity and heat generation thereafter.

There are many advantages of digestate use – sustainable waste management, soil enrichment, lower GHG emissions, and less odour. Even though Europe needs significant quantities of digestate, there are many controversies regarding digestate final use and its compliance with some EU directives and national laws that regulate the use of fertilisers in agriculture. Because of them and other reasons, composting, incineration and landfilling are used as alternatives to municipal waste management.

This work gives an overview of digestate's benefits and existing regulations applicable to digestate usage in the EU. It explores the ways of proper waste management of municipal bio-waste via the final by-product – digestate, which is used as a raw material for land enrichment and circular economy goals enhancement.

1. INTRODUCTION

A circular economy concept spreads from production over consumption to recycling of products. It was launched in 2015 in EC's communication Closing the loop – An EU action plan for the Circular Economy (European Commission, 2015). According to this concept, the waste hierarchy

establishes a priority order from prevention, preparation for reuse, recycling and energy recovery through to disposal, with the aim to deliver the best overall environmental outcome. The way the waste is collected and managed can lead either to high rates of recycling and to valuable materials finding their way back into the economy, or to an inefficient system where most recyclable waste ends in landfills or is incinerated, with potentially harmful environmental impacts and significant economic losses. When waste cannot be prevented or recycled, recovering its energy content is in most cases preferable to landfilling it, in both environmental and economic terms.

Even though biogas upgraded to biomethane takes a special place in renewable energy policies as it can be used in electricity, heat and fuel production, the focus of the paper is digestate, a form of organic fertilizer that is produced as the by-product of anaerobic digestion process in biogas plants. Digestate's treatment as either waste or fertilizer is crucial in its commercial exploitation. It is estimated that 60-70% of European soils are unhealthy due to climate change, unsustainable soil management, extreme weather events, intensification of agricultural practices, etc. The costs of soil degradation exceed EUR 50 billion per year (European Commission, 2023). This paper presents waste treatment options for organic fraction of municipal bio-waste, benefits of using the digestate, and highlights the crucial factors for digestate market development.

2. MUNICIPAL AND BIO-WASTE MANAGEMENT IN THE EU

Annual waste generation from all economic activities in the EU amounts to 2.5 billion tonnes, or 5 tonnes per capita a year, and each citizen produces on average about half a tonne of municipal waste (European Commission, 2020). Bio-waste from municipal waste comprehend food and kitchen waste, and green waste (biodegradable garden and park waste).

In 2018, industrial and household bio-waste generated in the EU-27 amounted to 147 million tonnes of dry matter, of which 90% was recovered and either recycled or incinerated. Of household bio-waste, 60% was food waste, representing 173 kg per EU citizen per year (European Environment Agency, 2020). Across the EU-27, 38 million tonnes of municipal bio-waste per year (tpa) are composted and digested and it is expected that it will rise to 78 million tpa by 2035 to fulfil the EU goal to recycle/re-use 65% of collected municipal waste (European Compost Network, 2022).

Composting dominates over anaerobic digestion for separately collected bio-waste, but a high proportion of bio-waste still ends up in the mixed waste that is landfilled or incinerated, even in the countries with well-established separate municipal waste collection systems. Anaerobic digestion and composting share many positive features such as nutrient recycling, contribution to soil health, odour reduction and sanitation, but composting does not recover electric energy in combined heat and power plants after anaerobic digestion. Incineration, landfilling and untreated waste do not contribute to soil health at all, but, depending on the substrate, there is some nutrient recycling in ash after incineration, and landfilling. Anaerobic digestion seems to be the

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preferred process of waste management compared to others as it recovers heat and electricity from biogas or enables production of the biomethane that can be injected into gas grid or in transport vehicles running on gas. In addition, composting requires a large area and longer time to generate quality product, landfilling has significant GHG emissions, while incineration requires management of toxic emissions and ash residues (M. Logan and C. Visvanathan, 2019).

The differences between rates of recycling (both anaerobic digestion and composting treatment), incineration and landfilling are very big between the EU member states, as presented by the data in Table 4. Recycling rate is the highest in Germany, Austria, Slovenia and the Netherlands, while incineration rates are the highest in Sweden and Finland. Landfilling is the highest in Cyprus, Malta, Greece, Romania, Bulgaria and Croatia, as well as Portugal, Latvia and Spain. The countries with high rate of landfilling typically have a waste management problem and should invest into the waste treatment facilities. Interestingly, some of these countries have seasonal problems as their economy is tourism dependent. All EU countries are obliged to introduce separate bio-waste collection systems by the end of 2023. Yet, even if such systems are in place, there is no guarantee that people will separately collect and throw away different types of waste. In addition, digestate produced, especially if in the liquid form, cannot be transported far, which means that there must be the demand from agriculture to use digestate nearby biogas plants.

Table 4: Recycling, landfill and incineration rates for municipal waste in EU-27, data for 2020.

Country	Recycling rate	Landfill rate	Incineration rate
Germany	70%	1%	30%
Austria	62%	2%	36%
Slovenia	59%	7%	13%
Netherlands	57%	1%	42%
Luxembourg	53%	4%	43%
Italy	51%	20%	19%
Belgium	51%	1%	49%
Denmark	46%	1%	53%
Slovakia	45%	46%	7%
Lithuania	45%	16%	26%
Finland	42%	1%	57%
France	42%	26%	32%
Ireland	41%	16%	42%
Czechia	40%	48%	13%
Latvia	40%	53%	3%
Poland	39%	40%	22%

Eighth International Scientific Conference
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Sweden	38%	0%	60%
Spain	38%	51%	11%
Bulgaria	35%	62%	3%
Hungary	32%	54%	12%
Croatia	29%	60%	0%
Estonia	29%	15%	43%
Portugal	27%	57%	21%
Greece	21%	78%	1%
Cyprus	17%	67%	1%
Romania	12%	74%	5%
Malta	11%	83%	0%

Source: (European Environment Agency, 2020)

Anaerobic digestion used in biogas plants, and composting are two methods used for waste recycling. They both create fertilizer-like by-products: digestate, and compost. Composting decomposes organic matter in the presence of air (oxygen) and produces solid fertilizer and heat (water in gaseous vapor) as well as water as liquid leachate and volatilized gases (ammonia, CO₂, occasionally gases perceived as odours). Anaerobic digestion decomposes organic matter without air (oxygen) and creates biogas and liquid fertilizer. Both processes require moisture and specific bacteria. Composting is most efficient with a moisture content of around 50 percent while anaerobic digestion systems operate best at 100 percent moisture content (Coker, 2014). In composting, glucose is converted to carbon dioxide and water. In anaerobic digestion, glucose is converted to carbon dioxide and methane. Both compost and digestate affect the GHG balance of arable land as 58% of soil organic matter is carbon. Both composting and anaerobic digestion last about 90 days.

Biogas facilities are typically larger, and they require smaller space to install compared to composting plants. Biogas plants require at least 13,000 tonnes of biowaste per year, while composting plants need at least 8,000 tonnes of biowaste yearly (M. Logan and C. Visvanathan, 2019; Cucina, 2023). Biogas plants are very suitable for the treatment of municipal bio-waste, but they are more complex to operate compared to composting facilities, which is mainly the reason why composting is the prevailing method of recycling bio-waste. In addition, the potential for GHG savings is higher with anaerobic digestion which makes it a preferred waste treatment method despite higher CAPEX. According to (Murphy and Power, 2007), composting can potentially save 1,190 kg CO₂/t waste treated, while anaerobic digestion can save up to 1,451 kg CO₂/t waste treated.

Anaerobic digestion is praised for its circular economy effect – the feedstock used are transformed into electricity and heat, or gas, and organic fertilizer – digestate that is used as an organic fertilizer in nutrition of soil and plants. These plants are edible, and a part of them

eventually ends up as bio-waste / feedstock for biogas plants. The feedstock for anaerobic digestion must have organic matter, such as bio-waste, agricultural plants' residues, cattle manure, wastewater sludge, energy crops, restaurant and catering waste.

The choice for anaerobic digestion as a method of bio-waste treatment often depends on renewable energy policies. For example, the target for biogas / biomethane share in transport fuel led to the choice of anaerobic digestion as a preferred treatment for bio-waste in Sweden (European Compost Network, 2019). However, no country ever introduced any type of subsidy for organic fertilizer / digestate production in biogas plants.

3. BENEFITS OF DIGESTATE USAGE

Digestate is a valuable by-product of biogas plants that can be used as an organic fertilizer in agriculture instead of artificial (mineral or inorganic) fertilizers. Biogas is produced by anaerobic digestion process which is a perfect example of circular economy. Apart from energy production, up to 90% of the substrates are transformed into digestate (Kisielevska et al 2022).

Three primary nutrients in digestate are: nitrogen (N), phosphorus (P) and potassium (K), followed by three secondary nutrients: calcium (Ca), magnesium (Mg) and sulphur (S), and eleven micronutrients. Plants need a permanent availability of all the nutrients in proportion to their daily needs. These nutrients need to be replenished in the soil after the plant harvest and this is done using organic and/or mineral fertilizers.

Recycling digestate back to soil preserves nitrogen, potassium, phosphorus and organic carbon for plants. There are various research that investigate the role of digestate's application for various plants. It primarily improves potassium content, followed by phosphorus, as it is shown in Table 5.

Table 5: Difference in the content of soil before and after digestate application

Feature	Before digestate application	After digestate application
Reaction (pH)	7.56	7.63
Phosphorus (mg/100g soil)	31.40	37.30
Potassium (mg/100g soil)	7.20	16.40
Magnesium (mg/100 g soil)	13.40	13.80

Source: (Koszel and Lorencowicz, 2015)

One tonne of food waste entering the digestion process results in about 1.4 t of liquid digestate and 0.2 t of solid digestate due to tap water added in the process (Chiew et al, 2015). Biogas plants typically produce liquid digestate, but digestate can be separated into liquid and solid digestate. Hence, the research on digestate's impact differs based on the fact whether liquid or solid digestate is applied (Table 6). The greatest value of digestate as an organic fertilizer is mostly not valorised, especially if digestate is sold in the liquid form.

Eighth International Scientific Conference "June 5th - World Environment Day"

The data in Table 6 suggests that solid digestate is rich in organic nitrogen, and phosphorus and that it possesses up to 100% of fibrous material. In addition, it is easier for transport as it has only 10-20% of volume of original feedstock. Liquid digestate is rich in ammonia, potassium and phosphorus, but it needs to be applied on the area close to the biogas plant as the volume of the feedstock is up to 90% preserved. From 50-80% of carbon from digestate can be stored in soil (Reuland at all, 2022.)

Table 6: Difference between the content of solid and liquid digestate.

Feature	Liquid digestate (fertilizer)	Solid digestate
Distinguishing characteristic	Directly nourishes plants in the short-term	Nourishes the soil, rich in stable organic matter, P and K
NH ₄	95%	5%
Organic N	5%	95%
Potassium (K)	80%	20%
Phosphorus (P)	Up to 50%	51-99%
Fibrous material	Close to 0%	100%
Humus forming fraction	Little present	Mostly present
Volume	80-90%	10-20%
Application	Directly, or upgraded	It can be used as soil fertilizer or further treated (composted, dried and pressed into fertilizer pellets)

Source: (European Biogas Association, 2024)

A comparison between organic and mineral fertilizers is given in Table 7. Organic fertilizers are praised for being beneficial for the plants in the long term. They are also more favourable for human health and the environment as replacing 1 tonne of synthetic fertilizer with digestate preserves approximately 108 tons of water and 4 tons of CO₂eq GHG emissions (Akbar at all, 2021). Despite organic fertilizers should be preferred to mineral fertilizers, the latter are more used in Europe as a source of nitrates.

Table 7 demonstrates the application of mineral vs organic fertilizers in Europe, with striking differences in application of organic fertilizers in the CEE region compared to Western Europe. There is plenty of room for application of organic fertilizers in the CEE region, especially because this region is not abundant with mineral fertilizer plants.

Eighth International Scientific Conference "June 5th - World Environment Day"

Table 7: Mineral and organic fertilizers usage in Europe, in million tonnes, data for 2022.

Feeding element	Mineral fertilizers	Organic fertilizers	Organic fertilizers – Western Europe	Organic fertilizers – CEE
Nitrogen (N) / digestate fertilizers	9.3	8.4 / 1.7	6.7	1.7
Phosphate (P) / digestate fertilizers	2.3	4.5 / 0.3	3.5	0.9
Potash (K) / digestate fertilizers	2.5	9.9 / 0.2	8.0	1.9

Source: (Fertilizers Europe, 2024)

Table 8 compares the characteristics of both organic and mineral fertilizers. The price between organic and mineral fertilizers seems comparable when both short-term and long-term effects are considered.

Table 8: A comparison between organic and mineral fertilizers.

Characteristics	Organic fertilizers	Mineral fertilizers
Inputs for production	Bio-waste, manure, plant matter = organic matter	Potentially hazardous chemicals: they are synthesized artificially or mined from non-living materials
Nutrient supply	Predictable amounts of nutrients, not immediate but steady supply of nutrients to the soil throughout the plant growth cycle	Nutrients are precisely calculable and concentrated, and they can be immediately taken up by the plant
Soil organic matter	Increased, as the carbon capture capacity of soils is improved and less CO ₂ is released to the atmosphere	Deficiency of secondary and micronutrients occur in soil and crop, if the common NPK type is consistently used; reduce soil organic matter; increase soil acidity; degradation of soil physical properties and structure leads to increased soil erosion
Support to micro-organisms growth	Yes, improve soil health and promote healthy root growth	No
Nutrient loss	Need to be applied continuously	Minimized, less likely to be washed away by rain or irrigation, unless the rain falls soon after the application
Impact on environment	Environmentally friendly, do not contain any chemicals or pollutants, help reduce the number of synthetic fertilizers, reduce water pollution and soil erosion; safe for the environment, the body and free of pesticides	Over-application leads to nutrient runoff and potential environment damage as they leach in the waterways, pollute them and harm aquatic life - 50% of N and 90% of P applied through chemical fertilizers are lost to water and the atmosphere; increase soil salinity;

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"June 5th - World Environment Day"**

		human carcinogens;
Impact on taste and texture of food	Beneficial	May cause nutrient imbalance and plant damage
Price	More expensive upfront, but they last longer than mineral fertilizers	Cheaper upfront, but need to be purchased often; in general, more expensive
Quantity	Greater quantity is needed compared to mineral fertilizers	Concentrated, so less quantity is needed
Ease of application	Difficult, bulky and heavy	
Storage	Difficult, they are sensitive to moisture and temperature, must be stored in cool and dry place	Suitable for changing climate conditions
Overall benefits to the plants	Long-term	Short-term

Source: Authors' compilation.

4. STIMULI AND CONSTRAINTS FOR DIGESTATE USAGE IN THE CEE AND SEE REGION

The stimuli for more frequent digestate application in Eastern and Southern part of Europe are:

- Arable land and developed and growing agriculture
- Availability of biogas plants and feedstock such as bio-waste
- More favourable market price compared to mineral fertilizers
- Care for human health and environment
- Sustainable waste management and intention to produce renewable energy, and
- Favourable regulatory treatment.

More arable land requires more fertilizers. The average share of arable land in total land area in EU-27 countries fell from 31.8% in 1961 to 24.7% in 2021. The decline is present in all countries, and the differences between the countries range from 9% (Slovenia) to 37.3% (Romania). However, agriculture is on decline as it contributed only 1.3% to EU GDP in 2023.

The availability of biogas plants is not necessarily related to arable land but to subsidy policy for renewable energy production. However, the countries with the highest number of biogas plants per capita, such as Germany, Austria, Switzerland, have the highest waste recycling rate in Europe.

Three types of digestate markets are distinguished (Fourcory et al., 2021):

- Direct application of digestate on agricultural soils following solid-liquid separation, which is affordable and allows better nutrient management, cheaper transport costs, and better storage; the waste producers bring their wastes, residues and by-products to the anaerobic digestion plant that integrates them as substrates. Later, the anaerobic digestion plant gives digestate to farmers; selling prices for such digestate are often very low and

Eighth International Scientific Conference "June 5th - World Environment Day"

can even be null or negative and land application costs are often shared between the farmers and biogas plant; this concept is favoured by EU renewable energy policies

- Anaerobic digestion plant sells or gives digestate to farmers without receiving their waste; waste is collected from industries, municipalities or farmers; specific categories of digestate are eliminated through incineration (rarely used)
- Digestate is upgraded to the products with higher market value, such as processed digestate, manufactured fertilizer, soil improver; solid fraction of post-digestion sludge can be further processed through second drying or palletization; this concept is favoured by EU bioeconomy and circularity ambitions.

Average liquid digestate's price per tonne was EUR 4.42, while average solid digestate's price per tonne was EUR 7.62 in Lithuania (Jurgutis at all, 2021). While liquid digestate price was mostly determined by nitrogen (EUR 2.14 per tonne) and potassium value (EUR 0.98 per tonne), solid digestate value was overwhelmingly a sum of four elements – organic carbon (EUR 3.27 per tonne), nitrogen (EUR 3.02 per tonne), phosphorus (EUR 2.86 per tonne), and potassium (EUR 1.04 per tonne). Both liquid and solid digestate contained the same value of trace elements such as copper, zinc, iron, and magnesium, estimated to combined value of EUR 0.51 per tonne. Although the typical feedstock biomass for biogas production can be applied to soil directly as natural biofertilizer, strict environmental requirements encourage the integration of anaerobic digestion process prior to biomass application on arable lands (Jurgutis at all, 2021). In addition, unclear regulation causes problems with selling digestate at its real price. Instead, the biogas producers often give it for free to get rid of it.

Waste / product status and land application limits play a critical role in digestate market dynamics. The utilization of digestate is complicated when it is classified as waste. A survey conducted by (European Compost Network, 2022) reveals that digestate achieves either zero or negative price (EUR 0 to minus 10 per tonne), while compost has a weighted average price of EUR 10.1 per tonne of fresh mass. As a comparison, one tonne of ECN certified compost (and only 25% of all produced compost in EU-27, the United Kingdom and Norway is ECN certified) contains nutrients valued at EUR 63 per tonne. 10 tonnes of compost per hectare is applied to improve soil by adding organic matter and to help restore productivity (European Compost Network, 2022). Overall, 1 tonne of mineral fertilizer is used per 8.78 hectares, and they are applied to the arable surface of 123.8 million hectares (Fertilizers Europe, 2024).

The production of digestate from all feedstocks is estimated to 31 million tonnes of dry matter. However, only a part of that is applied as organic fertilizer. The rest of nutrients comes from further digestate upgrading or composting.

Table 9 shows the applicable EU regulatory framework for digestate application. There are some rules common to the EU member states, but EU member states can determine their own policies up to a certain extent.

Eighth International Scientific Conference
"June 5th - World Environment Day"

Table 9: Governing regulatory framework for digestate in the EU.

Regulatory framework	Waste / product status	Land application limits
EU Circular Economy Package	The target is to extract the maximum value and use from all raw materials, products and waste, fostering energy savings and reducing greenhouse gas emissions.	The Nitrates Directive is derived from the Circular Economy Package.
Nitrates Directive	Sets the limit of 170 kg N/ha/year from livestock manure in nitrate-vulnerable zones.	Restrains the use of digestates as fertilizers in livestock-intensive regions, encouraging producers to upgrade and export digestate.
Fertilising Products Regulation	Digestate is defined as product under certain conditions, with the possibility to be traded on the EU internal market and used in fertilizers manufacturing.	Up to 2.5 g plastics per kilogram of compost (dry matter) is allowed; however, several countries apply more stringent limits
Waste Framework Directive	Sets the basic concepts and definitions related to waste management including provisions for end-of-waste status, recycling and recovery.	Foresee separate collection of organic waste streams and its treatment according to the waste hierarchy.
Landfill Directive	Defines different categories of waste and applies to all landfills, defined as waste disposal sites for the landfilling of waste, preventing or reducing negative effects of landfilling of waste on the environment, particularly on surface water, groundwater, soil, air and on human health.	Landfilling of untreated waste and liquid waste is not allowed. Biodegradable waste must not exceed 35% of the total amount (by weight) of biodegradable municipal waste produced in 1995. Landfilling must be reduced to a maximum of 10% of municipal waste generated, by 2035
Water Framework Directive	Defines environmental objectives for surface water, groundwater and protected areas.	Sets the targets for achieving good environmental status of water bodies, with combined approach of quality standards and emission limits

Source: Authors' compilation.

As per Waste Framework Directive provisions, biowaste shall not be burned or landfilled and EU member states are obliged to collect bio-waste separately (or recycle it at source) by 31 December 2023. Following the fertilising product regulation, starting in July 2022, compost and digestate can be placed as CE marked fertilising products on the European market. Product certification is a key to obtaining sustainable and acceptable price and to deepen trust of farmers into the quality of product. Hence, the member states that do not have such a system of certifying

Eighth International Scientific Conference
"June 5th - World Environment Day"

digestate and digestate-based products as well as compost, should consider giving a quality label to these valuable organic products.

The digestate's potential is high, yet it is expected that both composting and anaerobic digestion will grow at the same rate, i.e.100%, in the next decade to reach the EU target of recycling 60% of municipal waste by 2035. It would raise overall production of digestate and compost up to 65 million tonnes per year by 2035 as shown in Table 10. While composting is suitable for communities of 120 thousand of people on average, anaerobic digestion is applicable to almost twice larger population of 225 thousand on average. In addition, composting requires less skilful work compared to anaerobic digestion.

Table 10: Statistical data on municipal and bio-waste treated by composting and anaerobic digestion in EU-27, data for 2022.

	Final product produced in M tpa	Municipal bio-waste treated			No. of treatment plants	Average number of		Potential	
		to recycling in M tpa	in kg/per capita / p.a.	in kg/per capita / p.a.		people served by the plant (in 000)	employees in a plant	production by 2035 in M tpa	number of treatment plants by 2035
Bio-waste to compost production	17.6	36	72	61	3,800	120	4.7	39.2	7,600
Bio-waste to digestate production	11.4	24	48	26	2,000	225	4.9	25.4	4,000

Source: (European Compost Network, 2022)

5. CONCLUSION

EU countries should divert the organic fraction of municipal bio-waste from landfilling and incineration to recycling in the form of either composting or anaerobic digestion. The latter is a favoured bio-waste treatment option as apart from digestate, which can be a valuable organic fertilizer, it also converts feedstock energy to either electricity and heat or heat and renewable gas, injectable into the public gas network or gas-running vehicles. Apart from that, environmental and human health benefits from digestate / organic fertilizers application exceed the benefits of mineral fertilizers.

The potential of organic fertilizers' application seems to be very high in the CEE region as four times fewer organic fertilizers are used there compared to the Western Europe. In addition, there are less mineral fertilizer plants and less biogas plants in the Central and Eastern Europe despite unresolved problems for bio-waste recycling.

Production of biogas is a sustainable solution to manage bio-waste, especially if integrated with a market system that introduces digestate and digestate-like products, to close the nutrients cycle and reduce greenhouse gas emissions.

Eighth International Scientific Conference "June 5th - World Environment Day"

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Eighth International Scientific Conference "June 5th - World Environment Day"

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QUANTIFICATION OF THE ENVIRONMENTAL BENEFITS OF THE INTRODUCTION AND IMPLEMENTATION OF THE ISO 14001 STANDARD IN THE MEAT INDUSTRY SHOWN BY MONITORING THE CONCENTRATIONS OF PHOSPHORUS AND SULFUR COMPOUNDS IN WASTEWATER

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Key words: ISO 14001, internal benefits, external benefits, total phosphorus, sulfates, equivalent number of inhabitants, daily wastewater load

ABSTRACT:

ISO 14001 is the most well-known standard of the ISO 14000 series and sets out the requirements for all organizations to implement an EMS (Environmental Management System). The implementation of ISO 14001 brings to companies external and internal benefits. This paper quantifies the benefits of the implementation based on the assessment of the ecological harm of environmental pollution indicators, before and after the introduction and implementation of ISO 14001 in the company MS Alem d.o.o. Bosanska Krupa through phosphate and sulfur loading. The concentrations of total phosphorus (mg/l) and sulfate (mg/l) were measured and statistical processing was performed. The t-test of paired samples compared the values of the harmful equivalent (ENI), and it was shown that there is a statistically significant difference before and after the introduction of ISO 14001, for total phosphorus, Sig. = 0.006, at the level of statistical significance $p = 0.05$. For sulfates, it was proven that there is no statistically significant difference, Sig. = 0.185, at the level of statistical significance $p=0.05$ (measured by the daily load of wastewater). It has been proven that there is a statistically significant difference in the

distribution of the daily load of total phosphorus expressed through ENI (equivalent number of inhabitants), before and after the introduction of ISO 14001, $p = \text{Sig.} = 0.006$. The Man-Whitney test confirmed that the average values of the median daily load with sulfate compounds before and after the application of ISO 14001 do not statistically significantly differ, $\text{Sig.} = 0.240$, at the level of statistical significance $p = 0.05$. The Mann-Whitney U test confirmed the acceptance of the null hypothesis that the distributions of the daily load of wastewater with sulfate compounds are the same before and after the introduction of ISO 14001, $\text{Sig.} = 0.310$.

1. INTRODUCTION

The international standard ISO 14001 is the most well-known standard of the ISO 14000 series and specifies the requirements for all organizations, of any type or size, to implement an environmental management system. The Environmental Management System is a practical tool for environmental initiatives that can ultimately generate financial benefits. The implementation of the ISO 14001 standard brings to companies internal benefits, such as: cost reduction, improvement of environmental protection, increased productivity, increased profit margin, improvement of internal procedures, improvement of employee morale, as well as external benefits, which include: improvement of corporate image, increased market share, increased customer satisfaction, improved relations with communities and improved relations with authorities. ISO 14001 is based on the following elements: setting goals and making plans, implementing plans within a structured management framework, measuring achieved goals against planned goals and correcting plans and improving results. The key factors of successful ISO 14001 implementation are: management support and commitment; awareness, involvement and competence of employees; government initiatives and commitment; sufficient organizational resources; adoption of a continuous, integrative and collaborative approach and the use of external consultants (Zilahy, 2017). In this research, wastewater was sampled at the location of the company, meat industry, MS-ALEM d.o.o. Bosanska Krupa. Sampling of waste water was carried out before its discharge into public sewage systems, i.e. into another receiver, in accordance with the Regulation on conditions for discharge of waste water into natural recipients and public sewage systems (Official Gazette of the Federation of Bosnia and Herzegovina; number 26/20). Analytical tests were performed using accredited methods. Concentrations of total phosphorus (mg/l) and sulfate (mg/l) were measured and statistical processing was performed. The benefits of the implementation were quantified based on the assessment of the ecological harm of environmental pollution indicators, before and after the introduction and implementation of ISO 14001 in the company MS-ALEM d.o.o. Bosanska Krupa based on the daily load of wastewater with concentrations of total phosphorus and sulfate compounds (Muminović, 2023).

2. ENVIRONMENTAL BENEFITS OF THE INTRODUCTION AND IMPLEMENTATION OF THE ISO 14001 STANDARD IN THE MEAT INDUSTRY

The methodological approach involved monitoring the concentrations of total phosphorus and sulfate compounds before and after the introduction and implementation of the ISO 14001 standard, which was accompanied by statistical analysis. Sampling, as well as wastewater testing, was carried out in the authorized laboratory of Herkon d.o.o. Mostar. Wastewater was sampled at the location of the company, meat industry, MS-ALEM d.o.o. Bosnian Krupa. The potentially polluted area was represented by a stream. The place of sampling was the revision shaft. The main catchment area is the Una river basin. The water area is the water area of the Sava River. Sampling of waste water was carried out before its discharge into public sewage systems, i.e. into another receiver, in accordance with the Regulation on conditions for discharge of waste water into natural recipients and public sewage systems (Official Gazette of the Federation of Bosnia and Herzegovina; number 26/20). The accredited test methods for total phosphorus and sulfate compounds were as follows: total phosphorus - BAS EN ISO 6878:2006 and sulphate compounds - EPA 375:3. All obtained values were related to mean daily averages based on a 24-hour composite sample proportional to the wastewater flow (Muminović, 2023).

2.1 Significance of the ISO 14001 standard

The International Organisation for Standardization 14001 Environmental Management System standard provides a guideline for an organization to perform continuous improvement to their environmental performance. In light of continued concerns over global environmental impacts and climate change, the ISO 14001 standard serves to demonstrate organizational commitment to sustainable production processes (Hengky et al; 2018). The international standard ISO 14001 is the most well-known standard of the ISO 14000 series and specifies the requirements for all organizations, of any type or size, to implement an environmental management system. An environmental management system is a practical tool for environmental initiatives that can ultimately generate financial benefits through market competition or organizational improvement: cost reduction and revenue growth. These benefits have encouraged companies to implement the ISO 14001 standard, which is today the most popular environmental management standard. The implementation of the ISO 14001 standard brings benefits to companies that adopt it, which are visible through measures to improve environmental protection, profitability, efficiency, image improvement, customer satisfaction and improvements employee satisfaction. Benefits and advantages can be manifested through various effects: internal effects such as cost reduction, improvement of environmental protection, increased productivity, increased profit margin, improvement of internal procedures, improvement of employee morale, then external marketing effects such as improvement of corporate image, increased market share, increased customer satisfaction, increased time and delivery to customers. In addition, there are advantages in relation to the environment such as improved relations with communities, improved relations

with the authorities. The six key factors of the successful implementation of ISO 14001 in order of importance are as follows: management support and commitment; awareness, involvement and competence of employees; government initiatives and commitment; sufficient organizational resources; adoption of a continuous, integrative and collaborative approach and the use of external consultants. During the introduction and implementation of the standard, the following steps are defined: environmental protection policy and analysis of problems in environmental protection, identification of key factors that have an impact on the environment, introduction and implementation of the ISO 14001 standard, measurement and evaluation of implemented activities, and certification and work on continuous improvement (Zilahy, 2017).

2.2 Average values of total phosphorus before and after the introduction and implementation of ISO 14001

Phosphorus is a non-renewable element, naturally present in the environment, important for microorganisms, plants, animals and people (Zhang et al; 2020). The increased concentration of phosphorus causes the growth of plant life in the waters (eutrophication), which reduces the penetration of sunlight and plankton begins to die. That plant mass is decomposed by aerobic bacteria, which consume oxygen for this, and this leads to a decrease in oxygen in the water. Total phosphorus includes all types of phosphate: ortho-phosphate, organically bound phosphate and condensed phosphate or polyphosphate (Jurenić, 2015). Average values of total phosphorus in the period before the introduction of the ISO 14001 standard ranged from 0.38 mg/l measured in March to 0.87 mg/l average value measured in April, while the range of average values of total phosphorus in the period of implementation of the ISO 14001 standard it was a minimum of 0.23 mg/l measured in October to a maximum of 0.71 mg/l measured in November. In the period before the introduction of the standard, the average value of the concentration of total phosphorus was $\text{Me} = 0.6767 \text{ mg/l}$ with $\text{SD} = 0.17108 \text{ mg/l}$ and the error of the average estimate $\text{SE} = 0.06984 \text{ mg/l}$, with a range of values of minimum 0.38 mg/l to a maximum of 0.87 mg/l, while in the period of application of the ISO 14001 standard, the range of average concentration values was $\text{Me} = 0.388 \text{ mg/l}$, with $\text{SD} = 0.16916 \text{ mg/l}$, and $\text{SE} = 0.06906 \text{ mg/l}$ and with a range of values from a minimum of 0.23 mg/l to a maximum of 0.71 mg/l (Muminović, 2023).

2.3 Environmental damage from total phosphorus

Based on the estimated average value of the concentration of total phosphorus (cp), daily flow (q) and the time factor of waste water observation (24 hours), the daily load of waste water with total phosphorus was calculated. The equivalent of harm from total phosphorus measured by the equivalent number of inhabitants based on the daily load of wastewater was calculated. In the period before the introduction of the standard, the lowest total equivalent of harm from phosphorus compounds was achieved in the month of March and was equivalent to the pollution of 129,96 inhabitants, and the maximum equivalent was achieved in the month of April and was equivalent to the pollution of 290,232 inhabitants. The minimum equivalent of harm from total

phosphorus compounds in the period after the introduction of the standard was in the month of October and was equal to the equivalent of harm of 61,824 inhabitants, and the maximum equivalent of harm was in the month of November and was equal to the harm that would be achieved by 153,36 inhabitants. The t-test of paired samples showed that there is a statistically significant difference in the equivalence of harm from total phosphorus compounds in the period before and after the introduction of the ISO 14001 standard $t(10) = 3.497$, Sig. = 0.006, at the level of statistical significance $p = 0.05$, and the average value of the difference between the equivalent of harm $M(R) = 105.17800$, and the error of estimation of the average difference of $SE = 30.07364$ and 95% CI: from 38.16975 to 172.18625 (Muminović, 2023).

2.4 Average values of sulphate compounds before and after the introduction and implementation of ISO 14001

Sulfate in wastewater can originate from various sources like industrial processes, mining, agriculture, and urban runoff. High sulfate levels in wastewater can contribute to environmental issues such as increased salinity, changes in aquatic ecosystems, and potentially harmful effects on human health. The toxicity associated with sulfate in wastewater primarily arises from its conversion to hydrogen sulfide gas (H_2S) by sulfate-reducing bacteria under anaerobic conditions (Haritha et Kannayiram, 2023). The average value of sulfate concentration in the period before the introduction of the standard ISO 14001 was from a minimum of 42.90 mg/l measured in May to a maximum of 61.30 mg/l measured in February, while the range of average values of sulfate concentration measured during the period of implementation of the standard ISO 14001 were from the minimum of 39.90 mg/l in September to the maximum concentration of 82.10 mg/l measured in July. The average value of sulfate concentration in the period before the introduction of the standard was $Me = 51.9667$ mg/l, with $SD = 7.92052$ mg/l, and $SE = 3.23354$ mg/l with a range of values that was in the interval from a minimum of 42.9 mg/l to a maximum of 61.3 mg/l, while the average value of sulfate concentration during the period of implementation of environmental protection standards was $Me = 50.1333$ mg/l with $SD = 16.42908$ mg/l, $SE = 6.70714$ mg/l and a range of values from Min. = 39.9 mg/l to Max. = 82.1 mg/l (Muminović, 2023).

2.5 Environmental damage from sulfate compounds

Based on the calculated average value of the concentration of sulfate compounds (cSF) and wastewater flow (q) at the daily monitoring level (24h), the environmental damage from the total sulfate compounds that are excreted into the wastewater during the production cycle was evaluated based on the daily load (g/day) of waste water. Before assessing the daily load of wastewater, the deviation of the average values of the concentration of sulfate compounds from the limit value $LV = 2000$ mg/l of the presence of sulfate compounds in wastewater was calculated. The analysis showed that in the period before the introduction of the standard, the difference between the average concentration and the permissible limit value of $LV = 2000$ mg/l,

ranged from 1938.70 mg/l, which was in the month of February, to 1957.10 mg/l, which was the difference measured in May month. In this period, no exceeding of the maximum permissible limit value of sulfate compounds in wastewater was recorded. In the period of application of the ISO 14001 standard, the difference between the calculated concentration of the presence of sulfate compounds ranged from the minimum difference in the amount of 1917.90 mg/l to the maximum difference of 1960.10 mg/l measured in September. T-test of paired samples showed that the differences in harmfulness of the concentration of sulfate compounds measured by the daily load of wastewater in the period before and after the introduction of the ISO 14001 standard on environmental protection are not statistically significant, $t(10) = 1.422$, Sig. = 0.185 at the level of statistical significance $p = 0.05$, with the average value of the difference between the daily load $M(R) = 6925.72$, g/day with an error of estimation of the average difference of $SE = 4869.9737$ and 95% CI: from 3925.25761 to 17776.69761 g/day. The Mann-Whitney U test confirmed that the average value of the median daily load of sulfate compounds during the period before the introduction of the standard ($N = 6$; $Md = 29765.400$, $SD = 9609.66640$) is statistically not significantly different from the average value of the median daily load of wastewater measured in during the period of application of the ISO 14001 standard on environmental protection ($N = 6$; $Md = 22299.12$, $SD = 7067.8267$), $U = 10.000$, $z = -1.281$, Sig. = 0.240, at the level of statistical significance $p = 0.05$. The Mann-Whitney U test confirmed that the null hypothesis should be accepted that the distributions of daily wastewater load with sulfate compounds are the same before and after the introduction of standard 14001, Sig.=0.310 (Muminović, 2023).

3. CONCLUSION

The introduction and implementation of ISO 14001 brings to the company many benefits that result in the company's progress on the market. In this research, wastewater sampling was performed prior to discharge of wastewater into public sewer systems. Analytical tests were performed using accredited methods in an authorized laboratory. Benefits based on environmental damage that can cause concentrations of total phosphorus and sulfate compounds before and after the implementation of ISO 14001 were quantified. Average concentrations of total phosphorus were lower after implementation. Namely, the median concentration of total phosphorus before implementation was $Me = 0.6767$ mg/l, while at the time of implementation it was $Me = 0.388$ mg/l. Before the implementation of ISO 14001, the range of the equivalent number of inhabitants (harm equivalent) was from 129.96 to 290,232 inhabitants, while after the implementation it was 61,824 - 153.36. The t-test showed that there is a statistically significant difference in the harm equivalent of total phosphorus in the period before and after the implementation of ISO 14001 ($t(10) = 3.497$, Sig. = 0.006, $p = 0.05$). The average value of the sulfate concentration in the period before the introduction of the ISO 14001 standard was $Me = 51.9667$ mg/l, while the average value of the sulfate concentration in the period of application of

the standard was $Me = 50.1333 \text{ mg/l}$, so the concentrations of sulfate compounds were lower after the implementation. Given that in the periods before and after the implementation of the ISO 14001 standard, the average concentrations of sulfate compounds did not exceed the limit value of $LV = 2000 \text{ mg/l}$, and were somewhat lower after the implementation than before, the t-test showed that the differences in harmfulness from concentrations of sulfate compounds measured by the daily load of wastewater before and after the implementation of ISO 14001 are not statistically significant ($t(10) = 1.422$, $\text{Sig.} = 0.185$, $p = 0.05$). The Mann-Whitney U test confirmed that the average value of the median daily load of sulfate compounds during the period before the introduction of the standard ($N = 6$; $Md = 29765.400$, $SD = 9609.66640$) is statistically not significantly different from the average value of the median daily load of wastewater measured in during the period of application of the ISO 14001 standard on environmental protection ($N = 6$; $Md = 22299.12$, $SD = 7067.8267$), $U = 10.000$, $z = -1.281$, $\text{Sig.} = 0.240$, at the level of statistical significance $p = 0.05$. The Mann-Whitney U test confirmed that the null hypothesis should be accepted that the distributions of daily wastewater load with sulfate compounds are the same before and after the introduction of standard 14001, $\text{Sig.} = 0.310$.

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SPATIAL-PLANNING ASPECT OF WASTE MANAGEMENT IN VARAŽDIN COUNTY

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ABSTRACT:

The processes of globalization and industrialization, which shaped world history, had a significant impact on the dynamics of waste generation, disposal, collection and processing. Through time development, these processes have resulted in a greater amount of different types of waste, exposing the environment to serious challenges. The negative impact of this rapid growth of waste is manifested through ubiquitous pollution, which has serious consequences for the ecological environment, both for the human community and for all living organisms. In the light of global and European initiatives for sustainable development, Varaždin County as a regional unit acts as a unique key actor in achieving the goals of the circular economy and sustainable waste management. The aim of this work is based on statistical analysis, which will investigate, analyze and present the complexity of the challenges facing Varaždin County in the context of waste management in the midst of globalization and industrialization. The result of the work is based on the obligations of the County in the implementation of legal regulations, and at the same time the creation and implementation of Waste Management Plans as key instruments for achieving a sustainable balance between economic activity, environmental protection and social needs. The work highlights the importance of raising awareness among the population as a key factor in achieving a reduction in waste generation, while the promotion of circular economy and the creation of green jobs will be recognized as a key element of the waste

management strategy and plan. The interdisciplinary research approach combines economic, ecological and social aspects in order to achieve long-term sustainability of waste management in Varaždin County from a spatial-planning aspect through efficient and sustainable waste management.

1. INTRODUCTION

The Republic of Croatia has a long tradition in spatial planning and development. Regional spatial planning became part of planning practice relatively early, already during the 1960s, with the creation of the first republic spatial plan and regional spatial plans. This was due to a developed methodology and defined components from which the final plan document emerged. Since the second half of the 20th century, the spatial planning system in the Republic of Croatia has been characterized by frequent changes in legislative regulations that prescribe in detail the content, preparation, and adoption of spatial planning documents. As a consequence of these changes, the problem of outdated plans arises, i.e., inconsistency of previously created plans with the current law. The Spatial Development Strategy of the Republic of Croatia (Spatial Development Strategy of the Republic of Croatia, Official Gazette No. 106/17.) is the fundamental document of the state for directing spatial development, including waste management. Environmental protection policy lies in the necessity of establishing an effective waste management system that encompasses a broader area of sustainability, including rational use of food, improvement of energy efficiency, promotion of sustainable development, and rational and prudent exploitation of natural resources, among which drinking water, forests, and soil are particularly highlighted (Tišma et al., 2017). In line with the evolution of green policies, it is noteworthy that international organizations, with a focus on the European Union, as well as civil society organizations, play a crucial role in promoting environmentally responsible approaches. Collaboration and initiatives of these organizations are crucial for achieving global sustainability and effective environmental protection policies (Rios and Picazo-Tadeo, 2021). Every year, we witness an alarming increase in waste production and intensive exploitation of natural resources, a phenomenon that directly threatens not only human health but also the entire ecosystem. Sustainable waste management becomes a crucial element in the system to mitigate negative impacts on the environment and ensure resource conservation. This escalation poses a serious challenge, driven not only by technological and industrial advancements but also by ubiquitous consumerist lifestyles characteristic of modern society (Vergara and Tchobanoglous, 2012). The integration of these ecological principles within the context of spatial planning can ensure strategies that promote sustainability and efficient spatial management, taking into account the needs of both current and future generations. As space is constantly influenced by human activities, adjustments, and organization, it is necessary to control these activities to prevent uncontrolled and uneven development. This is precisely the focus of spatial planning, which integrates ecological principles to ensure sustainability and efficient spatial management,

Eighth International Scientific Conference "June 5th - World Environment Day"

considering the needs of both current and future generations. Waste management is a crucial issue for the sustainability of every community, especially for Varaždin County, which is recognized for its commitment to environmental protection. In Varaždin County, waste management encompasses a variety of activities conducted in accordance with the latest legislation and best global practices. These activities include waste collection, transportation, recycling, and disposal, along with supervision of these processes. Waste management in the Republic of Croatia is governed by the Waste Management Act (Official Gazette" No. 84/21). In the context of Varaždin County, which includes 6 cities and 22 municipalities (Figure 1.), sustainable waste management holds particular significance.

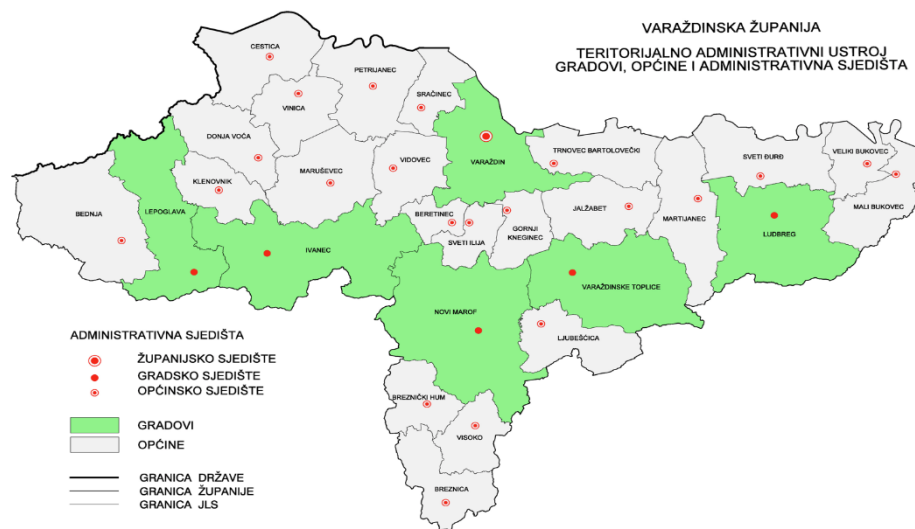


Figure 1. Administrative-territorial division of Varaždin County (Srpak, 2022)

The city of Varaždin, as a regional center, together with sub-regional centers such as Ivanec, Ludbreg, Novi Marof, Lepoglava, and Varaždinske Toplice, plays a crucial role in implementing the Law. Through their polycentric settlement networks, cities provide the infrastructure and resources necessary for efficient waste management. Therefore, further encouragement of the development of a properly structured network of cities within the County can further enhance this process.

2. ANALYSIS OF WASTE MANAGEMENT LEGAL FRAMEWORK IN THE REPUBLIC OF CROATIA

The framework for European waste management policy has been evolving over decades, with its origins contained in the Council Resolution on Waste Management Strategy (97/C76/01), based on the then-applicable Framework Directive on Waste (75/442/EEC) and other European regulations in the field of waste management. The European Union's waste policy represents a

Eighth International Scientific Conference "June 5th - World Environment Day"

complex system encompassed in numerous documents of various types and levels of authority. Through analysis and consultations, this research has selected key European documents (the Seventh Environment Action Programme of the European Union (European Commission, 2013), the European Union Action Plan for the Circular Economy (European Commission, 2015, COM(2015) 614 final) as well as the key consolidated directives currently shaping the regulatory framework for waste in the European Union: Directive 94/62/EC of the European Parliament and of the Council (Official Journal of the European Union, L365/10), Council Directive 1999/31/EC (Official Journal of the European Union, L182/1), Directive 2008/98/EC of the European Parliament and of the Council (Official Journal of the European Union, L312/3). In presenting and analyzing, the latest versions of documents, including amendments and supplements, are taken into account. In accordance with the Waste Management Act, waste management is of interest to the Republic of Croatia, and therefore waste management and its efficiency are ensured by the Government and the relevant Ministry by prescribing waste management measures, while local and regional self-government units are obliged to ensure the conditions and implementation of prescribed waste management measures within their respective areas. Moreover, multiple local and regional self-government units can agree to jointly implement waste management measures. At the national level, the Waste Management Strategy ("Official Gazette", No. 130/05) has been adopted, which Perković (Perković, 2021) describes as idealistic, almost utopian. His thoughts are supported by a quote: "The concept of waste management in the Republic of Croatia is the so-called landfill-free concept that is aimed at as an ideal. To achieve this, it would be necessary to close the loop from waste prevention, reduction in quantity and harmfulness, recycling and recovery (mechanical, biological, energy) to the utilization of inert residue. The prerequisites for achieving this goal include continuous education and training of all target groups and citizen involvement from the initial concept to implementation and management. In 2023, the Decision on the Adoption of the Waste Management Plan of the Republic of Croatia for the period 2023-2028 was made ("Official Gazette", No. 84/23). In this segment, new regulations were adopted, including the Waste Management Act and corresponding sublegal acts: the Waste Management Regulation ("Official Gazette", No. 106/22) the Waste Disposal Sites Regulation ("Official Gazette", No. 4/23) and the Regulation on Construction and Asbestos-containing Waste ("Official Gazette", No. 69/16). According to the obligations and responsibilities in waste management arising from the Waste Management Act, the State is responsible for managing hazardous waste and waste incineration. The County is responsible for managing all types of waste (except hazardous waste and waste for thermal treatment) and is obliged to ensure the conditions and implementation of prescribed waste management measures within its territory, collaborating with local self-government units in their implementation. Cities and municipalities are responsible for ensuring the provision of public waste collection services within their territories in a quality, consistent, and economically efficient manner, avoiding unjustifiably high costs, in line with the principles of sustainable development and environmental protection. The quantity of collected municipal waste largely depends on the population (with

Eighth International Scientific Conference
"June 5th - World Environment Day"

emphasis on urban areas) and can also be influenced by the population's lifestyle, economic power, average wages, and the number of employed individuals. Tourism activity also has a significant impact on the generation of municipal waste (Grbeš et al., 2016), (Grbeš, 2017). Local self-government units (hereinafter: LGUs) are also required to ensure separate collection of waste paper and cardboard, glass, metal, plastic, biowaste, wood, textiles, packaging, electrical and electronic equipment, batteries and accumulators, and bulky waste by providing the functioning of one or more recycling yards or mobile units. To ensure the availability of separate collection services for hazardous municipal waste and other municipal waste, the Executive Body of the local self-government unit is obliged to: in areas with 3000 inhabitants or less, where no recycling yard is located, ensure the availability of a mobile recycling yard. Based on the current regulation, the executive body of the local self-government unit is obliged to ensure the implementation of prescribed measures in the system of collecting special categories of waste, prevent littering in a manner contrary to the Law, and remove such littered waste, implement the Waste Management Plan of the Republic of Croatia for the period 2023-2028, implement the waste management plan of the local self-government unit, conduct educational and informational activities within its area, and organize waste collection actions. Since the regional self-government unit is obliged to adopt a waste management plan by January 1, 2024, until the adoption of the plan, the Waste Management Plans of the local self-government units adopted based on the Sustainable Waste Management Act ("Official Gazette", No. 94/13, 73/17, 14/19, 98/19) remain in force. However, according to the current Waste Management Act, it is necessary to ensure a location for the construction and operation of a recycling yard for separate waste collection in municipal waste management, or the operation of a mobile unit, as well as the placement of appropriate containers for separate waste collection, and a location for managing construction waste and asbestos-containing waste. The Regulation on construction waste and asbestos-containing waste introduces certain novelties to local self-government units, as well as to manufacturers of construction products, construction contractors, and owners and transporters of construction and asbestos waste. Among other obligations, the municipality is required, according to the Regulation, to invite owners or users of buildings containing asbestos to provide data on the locations of those buildings, an assessment of the quantities, types, and status of materials likely to become asbestos waste. Once the data is collected, the municipality submits it to the Environmental Protection and Energy Efficiency Fund. The disposal of asbestos waste is carried out by disposal in a cassette for asbestos waste within the waste landfill, in accordance with special regulations governing waste disposal (Srpak and Zeman, 2017). All producers of waste other than municipal waste must be registered in the Environmental Pollution Register, and industrial waste and special categories of waste must be collected separately from municipal waste and disposed of in accordance with the Law. Biodegradable waste from gardens and parks, food and kitchen waste from households, restaurants, catering establishments, retail outlets, and similar waste from food production, known as biowaste, must be separately collected with the aim of composting, digestion, or energy recovery of biowaste. Waste recycling is on the rise, but

food waste still ends up in landfills where its decomposition produces methane (Kalambura et al., 2014). Locations for buildings in the waste management system must be planned in spatial plans according to categories and placed outside settlements in separate economic-production construction areas, within settlements on areas designated for economic-use, predominantly industrial, and on areas designated for business purposes, communal service, or in areas designated for waste management.

3. METHODS AND MATERIALS FOR WASTE MANAGEMENT ANALYSIS IN VARAŽDIN COUNTY

Sustainable waste management in the Varaždin County area is defined based on prescribed obligations and guidelines from national legislative regulations. Therefore, the analysis of regulatory acts addressing waste and regulating its management is conducted, using qualitative content analysis as the methodological approach. Key regulatory documents at three different levels of jurisdiction are examined: the European Union as the highest authority, the Republic of Croatia as the national level, and specifically, Varaždin County as the regional unit. At the regional level (Varaždin County), the Waste Management Strategy of Varaždin County (2006) and the Waste Management Plan of Varaždin County for the period 2008-2015 ("Official Gazette of Varaždin County", No. 9/08) have been adopted. According to this Plan, the County conceptually focused on establishing a comprehensive waste management system through the County Waste Management Center. The implementation of the Waste Management Plan was regularly monitored through the periodic preparation of reports on its implementation. The Sustainable Waste Management Act did not envisage further adoption of county waste management plans; instead, this remained an obligation for the state and local self-government units. According to the then-existing Waste Management Plan in Varaždin County, commitments were defined regarding a comprehensive county waste management system, which includes the county waste management center "Motičnjak" in the city of Varaždin, along with three transfer stations (in Ludbreg, Novi Marof, and Ivanec), as well as an appropriate number of recycling yards. In further actions of the County, aiming to find the optimal and most acceptable solution, the commitment of Varaždin County in waste management is a regional approach. This approach would involve three neighboring counties in addition to Varaždin County, and within this framework, the formation of a public regional waste management center in the area of Koprivnica-Križevci County is planned (at the "Piškornica" location in Koprivnički Ivanec). This commitment was indicated through the Report on the Implementation of the Waste Management Plan of Varaždin County for the period 2008-2015 ("Official Gazette of Varaždin County", No. 22/11), established for the period from April 2008 to September 2010, and began to be realized by signing the Agreement on the Establishment of the limited liability company "Piškornica" d.o.o. Regional Waste Management Center of Northwestern Croatia (2009). This was further confirmed at the session of the County Assembly by adopting a Conclusion within the

Eighth International Scientific Conference "June 5th - World Environment Day"

acceptance of the Report on the Implementation of the Waste Management Plan of Varaždin County for the period 2008-2015 ("Official Gazette of Varaždin County", No. 22/11). As a result, there was no longer a need for planning a County Waste Management Center or other potential locations for the construction of waste treatment and disposal centers in Varaždin County. The regional concept includes, in addition to the center itself located in Koprivnica-Križevci County, several transfer stations, and envisages that existing local landfills (used until the establishment of the regional waste management center) become potential locations for other waste management facilities (transfer stations, sorting facilities, composting plants, recycling yards). The location for the regional waste management center for the northwestern Croatia region at the Piškornica site was also planned through Amendments and Supplements to the Spatial Plan of Koprivnica-Križevci County ("Official Gazette of Koprivnica-Križevci County", No. 8/07), along with several transfer stations. Consequently, it can be considered that a comprehensive waste management system has been conceived and that activities are being implemented to operationally establish the Regional Center, in which Varaždin County appropriately participates. On June 14, 2014, the Government of the Republic of Croatia adopted a Decision declaring the project titled Regional Waste Management Center (RCGO) Northwestern Croatia Piškornica as a strategic project of the Republic of Croatia. Since the Regional Waste Management Center is not yet operational, waste management in Varaždin County is carried out by collecting mixed municipal waste and biodegradable municipal waste through authorized companies entrusted with the public service of collecting these types of waste, along with sorting and separate collection in all local self-government units (LSGs). The collected waste is deposited in controlled landfills. It is planned that all landfills in Varaždin County should be closed with the establishment and start of operation of the Regional Waste Management Center. In the County, there were operational waste disposal sites (for collected municipal and non-hazardous industrial waste) at the following locations: "Gornje Vratno" in the Municipality of Cestica, "Čret" in the City of Novi Marof, and "Meka" in the City of Ludbreg, while the waste disposal site at the "Jerovec" location in the City of Ivanec is still in operation. At the "Brezje" location in the City of Varaždin, baled waste is stored, and although the location was designated for temporary storage, baled waste is still located there, and activities are underway to remediate the site. Municipal and non-hazardous waste collected in the cities of Ivanec and Lepoglava and the municipalities of Bednja, Donja Voća, Klenovnik, and Maruševac is deposited at the Jerovec landfill in the City of Ivanec. Waste collected in the other 22 local self-government units is deposited at the "Johovača" landfill in the Municipality of Velika Trnovitica (near Garešnica in Bjelovar-Bilogora County), the "Baćanska" landfill in the Municipality of Davor (Brod-Posavina County), the "Doroslov" landfill in the City of Donji Miholjac (Osijek-Baranja County), the Kutina landfill in the City of Kutina (Sisak-Moslavina County) and the "Piškornica" landfill in the Municipality of Koprivnički Ivanec (Koprivnica-Križevci County).

4. RESULTS AND DISCUSSION ON WASTE MANAGEMENT IN SPATIAL PLANNING DOCUMENTS OF VARAŽDIN COUNTY

From the analysis of document content, it is evident that waste management is heavily regulated at various levels – European, national, and local. Each level has different types of documents, including strategic, planning, and legislative ones. At the European level, the emphasis is on the circular economy and adhering to the waste hierarchy, which prioritizes waste prevention, preparation for reuse, recycling, and energy recovery before disposal. Through the III. amendments and supplements to the Spatial Plan of Varaždin County, a location outside the County has been designated for the disposal of asbestos-containing waste - specifically, the "Košer" location in the Municipality of Pučišće, Split-Dalmatia County. Additionally, within the Regional Waste Management Center of Northwest Croatia "Piškornica" Ltd. (located in the Koprivnica-Križevci County, Municipality of Koprivnički Ivanec), a transfer station is planned in Varaždin (Poljana Biškupečka), which will be situated within a designated construction area outside settlements, intended for economic purposes - waste management. Through comparative analysis of these documents, several relevant conclusions have been drawn. Within the current spatial plans of local self-government units (LSUs), precise locations or possibilities for waste management have been defined, as detailed in Table 1 in the subsequent research.

Table 1: Waste Management in Spatial Planning Documents of the Municipalities of Varaždin County.

No.	Town/Municipality	Location Name
1.	Varaždin	Motičnjak - designated for a transfer station, recycling yard, and recycling yard for construction waste (location of the Colas company), buildings for sorting, mechanical processing, and packaging of municipal and non-hazardous industrial waste, as well as the remediation of sites where waste management occurred. Brezje - in accordance with the Waste Management Plan of the City of Varaždin, remediation of the Motičnjak illegal landfill sites and the Brezje location, where baled municipal waste was temporarily stored, has been designated. Poljana Biškupečka - designated for a recycling yard and a recycling yard for construction waste, for sorting, mechanical processing, and packaging of municipal and non-hazardous industrial waste with short-term storage, as well as an alternative location for a transfer station. Bio-waste disposal is envisaged within the biopurification plant in the neighboring Municipality of Trnovec Bartolovečki.
2.	Ivanec	Jerovec - the location of the existing Jerovec waste landfill is planned for conversion into a transfer station and recycling yard for separate collection of waste in municipal waste management/recycling yard for construction waste.
3.	Ludbreg	Meka - in accordance with specific regulations, the remediation and closure of the landfill are planned, followed by conversion into a transfer station and recycling yard for separate collection of waste in municipal waste management/recycling yard for construction waste, as well as space for biodegradable waste (composting facility).
4.	Novi Marof	The location of the recycling yard for separate collection of municipal waste

**Eighth International Scientific Conference
"June 5th - World Environment Day"**

		is planned at the site of small and medium-sized enterprises near the bus station in Novi Marof. The location of the recycling yard for construction waste is planned in the area west of the "Čret" landfill site undergoing remediation.
5.	Lepoglava	Očura - a recycling yard for separate collection of municipal waste and for construction waste at a single location - within the planned economic zone in the quarry area, providing the opportunity for construction waste processing. For temporary waste disposal from the city area, the Jerovec landfill site is used.
6.	Varaždinske Toplice	The placement of recycling yards for separate collection of municipal waste and construction waste is planned, alternatively, at four locations: within a separate zone reserved for waste processing and storage (location near Bednja) and/or within the economic zones in Petkovec, Škarnik, and Lovrentovec. The Waste Management Plan of the City of Varaždinske Toplice ("Official Gazette of Varaždin County" no. 33/12 and 29/14) specifies possible locations for recycling yards in accordance with the Spatial Plan of the City. The selection of the location for the recycling yard in Škarnik (within the economic zone) was determined by the Decision of the City Council of Varaždinske Toplice on October 30, 2013 ("Official Gazette of Varaždin County" no. 61/13). The selected location for the recycling yard is situated in the western part of the zone on cadastral plot no. 3365/1, cadastral municipality Svibovec (according to the latest available data obtained from the Cadastral Office of Varaždin, and in the current Spatial Plan of the City of Varaždinske Toplice, it is cadastral plot no. 2566/1, cadastral municipality Svibovec). The remaining locations for the recycling yard are retained as alternative options.
7.	Bednja	The unique location of the recycling yard is planned within the economic production zone in Bednja (in its southern part), and it consists of a recycling yard for separate collection of municipal waste, as well as a recycling yard for construction waste and waste containing asbestos.
8.	Beretinec	The location of the recycling yard for municipal waste and construction waste is planned in the settlement of Beretinec as a separate construction area outside the settlement in the zone reserved for economic purposes.
9.	Breznica	Nije definirana lokacija za gospodarenje otpadom.
10.	Breznički Hum	Radešić - location for the construction of a recycling yard for construction waste next to the stone quarry; within the Breznički Hum economic zone - location of the recycling yard for separate collection of municipal waste.
11.	Cestica	Gornje Vratno - in the area east of the existing waste disposal site (which has since been remediated), a recycling yard is planned as a unified location for municipal and construction waste.
12.	Donja Voća	The construction area of the settlement, intended for industrial-commercial purposes in the village of Gornja Voća, is planned for a recycling yard.
13.	Gornji Knežinec	Within the separate construction area designated for communal-service purposes (central recycling yard for the municipality / labeled RD / municipal recycling yard for construction waste / labeled RDG / landfill for depositing excess excavation material representing mineral resources during construction works / labeled VI).
14.	Jalžabet	A potential conditional location for processing non-hazardous technological waste and for landfilling municipal and inert waste in the settlement of Jalžabet, as one of the possible locations according to the County Spatial

Eighth International Scientific Conference
"June 5th - World Environment Day"

		Plan (PPŽ), includes an agreement with Gornji Kneginec for the use of the recycling yard.
15.	Klenovnik	The plan does not specify a location for a new landfill within the municipality's area; instead, waste will be transported to the shared landfill in Jerovec.
16.	Ljubešćica	The municipality of Ljubešćica has reserved space for the establishment of a recycling center for separate collection of municipal waste and a recycling center for construction waste within the spatial plan and the Detailed Urban Plan (UPU) of the Ljubešćica Entrepreneurial Zone. In accordance with the legally prescribed possibility for multiple local and regional self-government units to jointly ensure the implementation of waste management measures, the municipality of Ljubešćica has reached an agreement with the city of Novi Marof on the shared use of the recycling center once it is built within the territory of Novi Marof.
17.	Mali Bukovec	The waste management location has not been defined..
18.	Martijanec	The economic zone in the village of Martijanec includes two variant locations: in one variant, it's in the northwest part of the zone (north of existing construction), while in the other variant, it's in the northeast part of the expanded economic zone. The specific area of the location in the second variant will be defined by the Urban Development Plan (UPU) to be prepared for the expanded part of the economic zone.
19.	Maruševac	In Donje Ladanje (on the border with the Vinica Municipality) and in the area of Čalinec village, there are two locations planned for recycling yards.
20.	Petrijanec	The provisions envisage the placement of a recycling yard in one of the larger municipal settlements.
21.	Sračinec	Turnišće - a recycling yard as a single location for municipal and construction waste.
22.	Sveti Đurđ	Sveti Đurđ and Hrženica - locations of recycling yards as single sites for separate collection of municipal waste and for construction waste.
23.	Sveti Ilija	Within the Tomaševac Biškupečki - Križanec economic zone, a location is designated for a recycling yard for separate collection of municipal waste and a recycling yard for construction waste and waste containing asbestos within the business - communal-service designated area for waste management, in the western part of the economic zone. During the transitional period, an agreement with the Municipality of Gornji Kneginec for the use of the established recycling yard in Gornji Kneginec.
24.	Trnovec Bartolovec	The placement of the recycling yard is planned as a single location (for separate collection of municipal waste and construction waste) in the northern part of the Trnovec settlement within the economic zone. The southern part of the Zamlaka settlement is designated for composting.
25.	Veliki Bukovec	The location for waste management has not been defined.
26.	Vidovec	Recycling centers are planned to be located in the Cargovec settlement.
27.	Vinica	In Vinica, a unified location for recycling centers is defined in the eastern part of the settlement, south of the local road L25034, within an area of agricultural land surrounded by forested areas. It is recommended to utilize the western part of the location for managing construction waste.
28.	Visoko	Within the economic zones in the settlements of Visoko and Vinično, variant locations for recycling centers for separate collection of municipal waste and construction waste are planned.

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During the process of drafting amendments and supplements to spatial plans of local self-government units, locations for recycling centers, recycling yards for construction waste, transfer stations, and potentially other types of waste management facilities are being determined in accordance with the legal obligations in force at the time of drafting or amending the spatial plans of local self-government units (Third amendment and supplement to the Spatial Plan of Varaždin County, "Official Gazette of Varaždin County", No. 96/21).

5. CONCLUSION

Waste management and the implementation of green policies are indeed crucial aspects of contemporary social and environmental contexts. The complexity of waste management requires a serious and scientifically grounded approach. Waste management, especially municipal waste, poses a significant challenge for modern societies. To effectively manage waste, it is necessary to develop and implement comprehensive strategies that involve various aspects such as waste reduction, recycling, composting, and energy recovery. Spatial planning documentation plays a crucial role in this process, enabling the planning and organization of waste management infrastructure in a way that minimizes negative impacts on the environment and human health. Varaždin County, like many other counties, faces challenges in waste management. This multidisciplinary approach includes economic, environmental, social, and spatial factors to create a comprehensive picture of the challenges and opportunities related to waste management. Landfills, as the final destinations for municipal waste, play a crucial role in researching the long-term environmental impacts. Therefore, green policies provide a framework and promote sustainable practices that reduce waste production and encourage recycling (using clean technologies and renewable energy sources, which can contribute to reducing greenhouse gas emissions). Waste management and green policies are key topics that require further research and innovation. Through a multidisciplinary approach and collaboration, sustainable solutions can be found.

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OPTIMIZATION OF THE PROBLEM OF THE LOCATION OF TRANSFER STATIONS IN THE SYSTEM OF INTEGRAL WASTE MANAGEMENT

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Key words: Communal Waste, Landfill, Optimization, Location Problems, Transferring Stations

ABSTRACT:

The waste directives of the European Union and the Resolution on waste policy require member states and countries in transition to first of all reduce waste production, then to recycle, and purpose-built facilities with the most modern sophisticated technologies with the application of the "polluter pays" principle. According to the definition, municipal waste is household waste, as well as other waste that is similar in nature or composition to household waste. The increase in the amount of generated waste represents a growing and significant problem in the context of environmental protection and the population's health. The impact of municipal waste on the environment in USK is multiple negative. Waste is disposed of in unorganized landfills, while there is also an evident problem of inadequate organization of official locations of municipal and city landfills and low awareness of citizens about the need to preserve the environment and the negative consequences of waste on civilization. Optimization of the waste collection system in the world has been the subject of research for many years, but only in some segments of that system. Optimization in terms of the choice of transfer station locations with the alternative inclusion of mechanical-biological treatment (MBO) of waste at several locations will be the subject of interest in this work. The establishment of MBO could be considered one of the priorities, because the construction is much faster than the construction of the landfill itself, and they could be built one by one.

1. INTRODUCTION

According to the definition, municipal waste is household waste, as well as other waste that is similar in nature or composition to household waste. According to the Law on Waste Management, the collection and treatment of municipal waste is carried out according to a special regulation on municipal activities. The increase in the amount of generated waste represents a growing and significant problem in the context of environmental protection and the health of the population. The impact of municipal waste on the environment in USK is multiple negative. Waste is disposed of in unorganized landfills, while there is also an evident problem of inadequate organization of official locations of municipal and city landfills, and low awareness of citizens about the need to preserve the environment and the negative consequences that waste has on civilization. Environmental management at the local level in BiH is very complex. Each canton in FBiH has its own government and passes its own laws.

There is no single form of organization or policy for ministries dealing with environmental protection issues at the cantonal level. One of the important tasks is the establishment of the Regional Sanitary Landfill for USK, to which the area of Drvar was added, and in the following text, the area of USK and Drvar will be called the Bihać region. The experiences of neighboring countries and EU countries show that the best model for dealing with waste disposal is a system of integrated municipal waste management at the regional level. The integrated waste management system follows the regional concept, i.e. it is created in the area of several municipalities that are united into regions, for which Cantonal waste management plans will be drawn up (Figure 1).

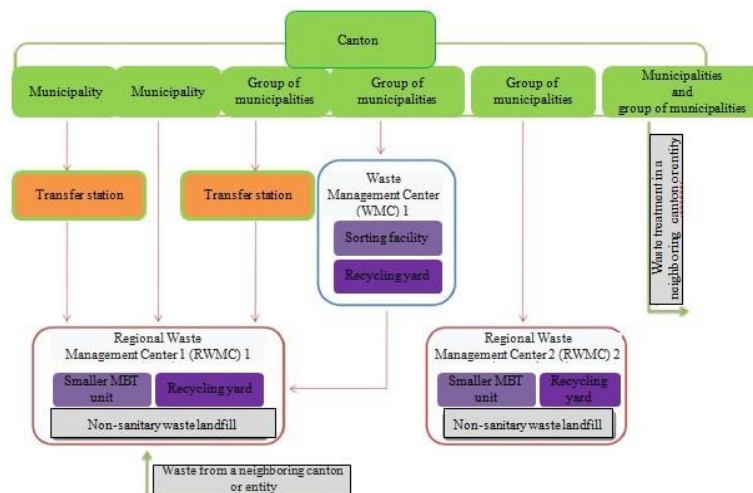


Figure 1: Scheme of the regional concept of the integrated waste management system in the territory of one canton

2. PRINCIPLES OF WASTE MANAGEMENT

In recent decades, the legal regulations of developed countries have followed the intensive development of the concept of integral waste management, with a focus on reducing the amount of disposed waste. The waste directives of the European Union and the Resolution on waste policy require member states and countries in transition to first of all reduce waste production, then to recycle, and purpose-built facilities with the most modern sophisticated technologies with the application of the "polluter pays" principle, that is, covering cost management costs. In an effort to reduce the amount of waste to be disposed of in landfills, there is a growing need to increase the share of recycled and biologically processed waste in the total amount of waste. Prevention of waste generation, as one of the measures to reduce the generation of waste, is certainly the most desirable method for solving the problem of waste management. In an effort to reduce the amount of waste for disposal in landfills, there is a growing need to increase the share of recycled and biologically processed waste in the total amount of generated waste. The basic principles of waste management are:

- Prevention of waste generation (education and work with the public, promotion, stimulation, motivation and sustainable consumption).
- Reducing the amount of waste - reuse (environmental education develops new knowledge in the individual and builds changes in behavior).
- Recycling (separate collection of usable waste at the point of origin, because in this way separate streams of different types of usable waste materials are formed - green islands and recycling yard).
- Treatment (through a mechanical, thermal, chemical or biological process, including sorting, the properties of the waste are changed in order to reduce the amount, and it facilitates the handling and improves the utilization of the waste. MBO (eng. Mechanical Biological Treatment - MBT) is a concept of waste disposal that was developed in Germany as a result of the desire to reduce the amount of biodegradable waste that was disposed of in landfills and to enable the recovery of useful raw materials from waste through an automatic separation system).
- Final disposal (a sanitary landfill is a facility for the final disposal of waste that can no longer be used (recycled, composted, used as fuel, etc.).

A sanitary landfill is a closed and controlled system that includes the following: proper selection of the location - the base on which it is built must be impermeable and stable, construction of a special base in order to protect the soil from leachate, collection and treatment of leachate and stormwater, construction of a leachate treatment plant water, a special way of disposing of waste in cells where the waste is spread out, compacted and covered with a layer of soil every day after disposal, collection and disposal of landfill gas through a degassing system in compliance with

regulations on the amount of polluting substances and constant monitoring of waste disposal, as well as regular work and maintenance.

2.1. General characteristics of the Bihać region

The region of Bihać is located in the northwestern part of Bosnia and Herzegovina and covers an area of 4,714. Most of the region belongs to the Una-Sana Canton (USK), whose administrative center is located in Bihać. According to the administrative division, USK consists of the following municipalities: Bihać, Bosanska Krupa, Cazin, Bosanski Petrovac, Ključ, Sanski Most, Velika Kladuša and Bužim. The area defined by the region of Bihać includes all the municipalities of USK, as well as the municipality of Drvar, which belongs to the Livno Canton. According to the estimate of the Federal Bureau of Statistics as of December 31, 2013. in the Bihać Region, there are 299,042 inhabitants. Based on these data and data for several previous years, the number of inhabitants in the Bihać region was estimated for the period 2012 to 2040, and it follows that there is a negative trend of population growth in the analyzed area - a decrease at an average annual rate of about 0.02%. The assessment of the annual amount of waste is carried out for the last complete calendar year, i.e. 2012 year. According to the known data obtained in the Bihać Region in 2012, approximately 299,065 inhabitants lived, of which approximately 223,555 inhabitants were included in the collection and removal of waste to landfills (approximately 75% of the population). The amount of municipal waste disposed of in landfills in 2012 was estimated based on data on the type and number of vehicles for waste collection, and the number of their deliveries to landfills. It is estimated that approximately 61,452 tons of municipal waste were generated in the analyzed area in 2012, of which approximately 45,330 tons came from the permanent population. Based on these data, the specific amount of municipal waste generated in the analyzed area per inhabitant covered by organized collection for 2012 was calculated. The calculation also took into account the amount of bulky waste created, which amounted to 782 tons. The estimated specific amount of waste is within the framework of average values of areas with similar characteristics and is calculated according to the following expression:

$$\text{specific quantity} = 46.112 \text{ tons} \div 1.000 / (223,555 \text{ inhabitants} \div 365 \text{ days}) = 0.565 \text{ kg/st./day.}$$

Based on this expression, the total amount of waste in the USK Bihać Region was calculated, which is shown in table 1, as well as the estimate of the number of inhabitants.

Eighth International Scientific Conference
"June 5th - World Environment Day"

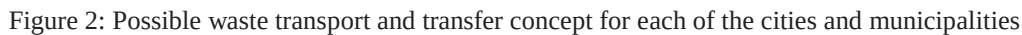
Table 1: Annual amounts of waste and number of inhabitants for the region of Bihać

Year	Total number citizen	Forecast commune. waste t/year	Not dangerous commun./ind. waste t/year	Biodegradation. waste with public area t/year	Quantity bulky waste t/year	Forecast total communal waste t/yea
2012	299.041	61.634	4.534	1.342	795	68.305
2015	298.620	65.533	5.063	2.839	847	74.282
2020	298.180	70.412	5.736	4.078	911	81.137
2025	297.808	75.293	6.463	4.366	976	87.098
2030	297.486	80.175	7.253	4.675	1.040	93.143
2035	297.201	85.057	8.107	5.006	1.104	99.274
2040	296.945	89.939	9.024	5.362	1.169	105.494

For the region of Bihać, several studies were conducted in order to choose the most suitable place for the regional sanitary landfill. According to those surveys, the best ranked location was Grabež-Drenovo Tjjesno (Bosna-S Co, 2007). There are two possible concepts of transportation/transfer of waste streams from production areas to the final destination, i.e. to the regional sanitary landfill:

- Direct transport - waste is taken away by vehicles that are involved in the collection and transport of waste
- Transportation via transshipment stations (PS) with MBO technology - waste is transported by vehicles that collect waste first to the location of transshipment stations where it is unloaded and reloaded into larger containers, which are usually larger than the capacity of local collection vehicles, and then unloaded into a reception pit MBO plant.

Figure 2 shows one of the possible waste transports and the transport concept for each of the cities and municipalities.



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graph LR; A[MIXED MUNICIPAL WASTE] --> B[SORTING FACILITY FOR MIXED MUNICIPAL WASTE]; B --> C[RECYCLING YARD]; B --> D[BIODEGRADABLE WASTE]; B --> E[RESIDUAL WASTE]; D --> F[COMPOSTING ZONE]; F --> G[RESIDUAL WASTE]; E --> H[LANDFILL]; G --> H;
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417

With an appropriate waste preparation method, conditions can be ensured to separate the useful part of the useful raw materials contained in municipal waste from the coarse fraction on the sorting line. Useful parts, individual parts of plastic, paper and cans are separated on the sorting line and baled in the same way as the part of useful waste that is separated at the place of origin. The rest of the waste that can be reused is disposed of. Depending on the composition of mixed municipal waste, the amount of waste that needs to be finally disposed of with the use of MBO is reduced by 50-60% by weight, and up to 80-90% by volume. After MBO processing, a part of the waste that cannot be used must be taken to a landfill, in the same place it can also be transferred to appropriate trucks with a larger capacity, so the establishment of MBO in some municipalities would represent an organized system of transfer stations. One of the most important parameters for organizing a transfer station system is the cost of transportation from the collection point to the transfer stations, i.e. to the landfill. The effect of the transfer station, according to data in studies, shows that the number of necessary transports is reduced by half, regardless of the way in which the transport is carried out, because the waste is loaded into trucks with a large capacity, and thus the cost of transport is reduced by 50%. Transfer stations can also be centralized, i.e. waste from several areas is brought to the local central transfer station, the least distant from the landfill. The system is optimized in such a way that sorting and mechanical-biological processing of waste is assumed instead of centralized transshipment stations. For the implementation of MBO, costs depend on the degree of automation and work technology and can be divided into the following:

- Machinery and equipment
- Landscaping
- Operating costs (labor and other)

Processing mixed waste before final disposal reduces the volume and weight of waste that must be disposed of. By organizing a system of centralized transshipment stations along with sorting and mechanical-biological processing of waste, the volume is additionally reduced by at least 50%, so that transport costs are reduced to a quarter, i.e. 25% of the original cost of direct transport to the landfill.

The costs of collection and transportation to the MBO plant, as well as the costs of transportation to the landfill, with an assumed reduction of quantities to 25% of the initial weight, i.e. quantities driven to the landfill and costs of MBO processing investment and operating costs are the data used for the mathematical model.

3. DEFINING THE MATHEMATICAL MODEL

The integral municipal waste management system is influenced by a large number of parameters that were arrived at based on the results of previous research on this topic and based on the operation of landfills in Bosnia and Herzegovina and in the surrounding area. The influencing parameters are:

- Number of municipalities covered by the transfer station
- Amount of waste generated by one municipality during one year
- Distance between municipalities and transshipment stations
- Transport costs (taken as a coefficient of 1 because it is assumed that they are the same in the entire Bihać Region per kilometer of distance) □
Distance between transfer stations and the regional landfill
- Costs of opening a transfer station

To define a model for waste management in the Bihać Region, it is necessary to define the objective function and input data. Locating new transshipment stations creates fixed costs, the number of transshipment stations is not determined, the task is to determine the number of transshipment stations and their location with the aim of keeping the total costs as a sum of fixed costs and transport costs as low as possible. For the transportation of waste from the place of its origin (households, industry, ...) the road network for the traffic of freight vehicles is used, while avoiding unpaved roads, toll roads and otherwise inappropriate roads. In the usual setting of the location-allocation problem, the costs of "opening" transfer stations are also respected, with the assumption of sorting and mechanical-biological treatment of waste at the location. Also, the restrictions define whether the user (transfer station with MBO) receives garbage exclusively from one city or municipality or from several cities and municipalities.

Using mixed integer programming, the problem can be defined as follows (Kulenović F., 2014):

Minimize

$$F = \sum_{j=1}^m f_j y_j + \sum_{i=1}^n \sum_{j=1}^m c_{ij} x_{ij} \quad (1)$$

with restrictions:

$$\sum_{i=1}^m x_{ij} = 1, j = 1, 2, \dots, n, \quad (2)$$

$$x_{ij} \leq y_j, i = 1, 2, \dots, m, j = 1, 2, \dots, n, \quad (3)$$

$$x_{ij} \geq 0, y_j = 0 \text{ ili } 1, i = 1, 2, \dots, m, j = 1, 2, \dots, n. \quad (4)$$

where is:

m – number of potential transfer stations with MBO

n – number of municipalities

c_{ij} – transportation costs of one unit from the municipality i to the transfer station with MBO j

f_j – fixed costs of opening and operating a transfer station with MBO j

x_{ij} – the number of units supplied by the customer i to the transfer station with MBO j , $y_j = 1$ if the transfer station is with MBO j open, 0 otherwise.

The objective function (1) to be minimized denotes the total fixed costs of opening all transfer stations and the total costs of transportation in EUR to all municipalities. Constraints (2) ensure that the demands of all municipalities will be satisfied, i.e. each municipality must have its own transfer station.

If it is $x_{ij} > 0$ for some j , because of (3) it must also be $y_j > 0$, and because of (4) then it must be $y_j = 1$. Therefore, the demand from a certain municipality is met only in the place where the transshipment station is opened. The total costs of garbage transportation include different types of costs. However, when it comes to choosing the location of transshipment stations, transportation costs and fixed costs of opening transshipment stations play the most significant role.

For the transportation of garbage through transfer stations, transportation costs decrease and fixed costs increase with the increase in the number of transfer stations. At the same time, the minimum total costs are achieved with the best mutual relationship between these two types of costs, which is shown in Figure 4. The problem of locating transfer stations is based on the optimization of the ratio of transport costs and the opening of transfer stations. Increasing the number of transshipment stations increases the fixed cost, but reduces the transportation cost as a result of reducing the transportation distance.

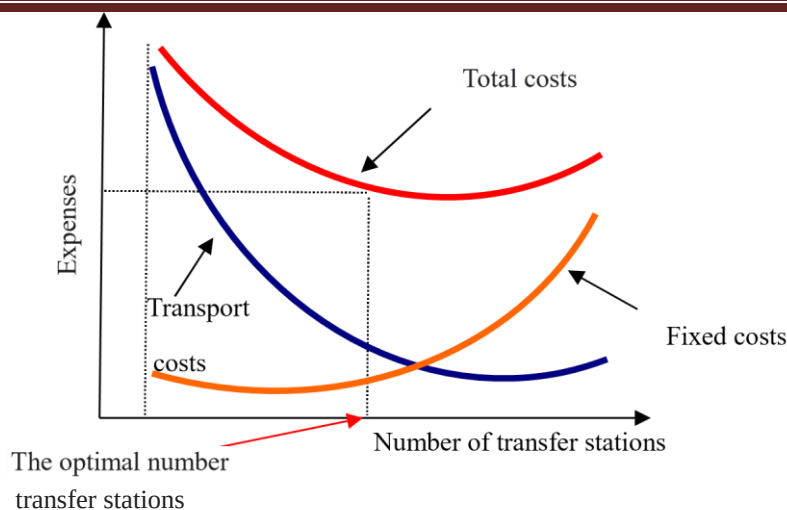


Figure 4: Total costs in relation to the number of transfer stations

The formulated problem represents the so-called mixed-integer linear programming problem and often its form is modified and adapted to apply some of the methods for solving the LP problem. Since the landfill is planned in the area of the municipality of Bihać, taking into account the fact that we explained previously that the organization of the system of centralized transshipment stations will reduce transportation costs to a quarter, i.e. 25% of the original cost of direct transport to the landfill. Therefore, corrected distances between all cities and municipalities were calculated, taking into account the existence of transfer stations. Therefore, all combinations of municipality (city)-municipality (city) transportation were taken into account, then the use of a transfer station (reducing transportation costs to 25%) and then transportation from the municipality (city) to the landfill.

In solving the problem of optimizing the transportation of waste to the landfill and the number of transfer stations, several possible versions of the problem solution were examined using Lpsolve. Lpsolve is a publicly available free tool for solving mixed integer programming problems. Lpsolve successfully solves common linear programs, allows the use of integer and binary variables (via the branch-and-bound algorithm), allows jumps in variable values, and the use of special ordered sets. There is no built-in limit on model size, however the larger the model the longer it will take to solve.

First, the case where transshipment stations are not used was examined, i.e. when there is no reduction in the amount of garbage, i.e. this means that the waste is driven directly to the landfill located in the area of the city of Bihać. In the second solution, transfer stations were used, and after the arrangement of all 36 combinations, it was concluded that the combination with the lowest costs would have transfer stations established in Bihać (the landfill itself), in Cazin and San. Most. The transfer station at the landfill would be used by Bihać, Bos. Krupa, Bos. Petrovac and Drvar, the transfer station in Cazin would be used by the municipalities of Bužim and Vel. Kladuša and the town of Cazin, while the transfer station in San. Most was used by Ključ and

San. Most. If we compare the costs that would be from 2014.-2040. without transshipment stations with the costs in 2014.-2040. with transshipment stations, we see that the costs are much lower (Figure 5).

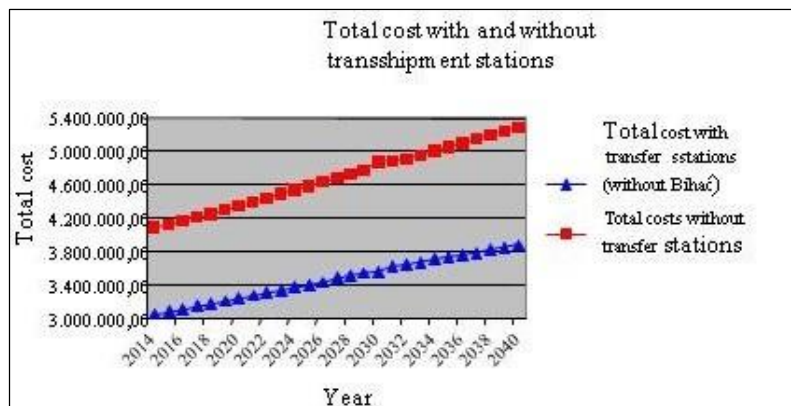


Figure 5: Total costs without and with transfer stations (without Bihać)

The resulting cost in 2040. without transshipment stations compared to the cost with transshipment stations would be higher by EUR 1,410,310.25 or 26.6%. From the presented results, it can be concluded that the use of transfer stations over the years is more profitable, regardless of the fixed cost, because the total cost is lower, from 25.4 to 26.6% from 2014-2040. From everything presented, the recommendation is to open transshipment stations at the landfill itself, in Cazin and San. Most.

Once the model has been analytically applied for cost optimization, the experimental application of the model in practice can be planned and implemented through specific phases of pilot projects. Based on the analytical results of the model, one or more areas can be selected for the experimental implementation of a transfer station and MBO plant. According to the model, a smaller or modular transfer station would be built that allows for temporary storage and reloading of waste. In the first phase, the goal could be to test operational efficiency, capacity and waste flows.

3. CONCLUSION

The increase in the amount of generated waste represents a growing and significant problem in the context of environmental protection and the health of the population. The waste directives of the European Union and the Resolution on waste policy require member states and countries in transition to first of all reduce waste production, then to recycle, and purposebuilt facilities with the most modern sophisticated technologies with the application of the "polluter pays" principle. The introduction of MBO with centralized transfer stations is the best solution for the Bihać Region because it reduces transport costs, reduces the required landfill volume and disposal

Eighth International Scientific Conference "June 5th - World Environment Day"

costs, and in the long term fulfills the legal obligations arising from EU approximation. The establishment of MBO could be considered one of the priorities, because the construction is much faster than the construction of the landfill itself, and they could be built one by one. In addition, the obtained compost could be used as a mulch for the rehabilitation of existing landfills. In the work, in addition to the mentioned parameters for building the mathematical model, other parameters could be taken, such as the utilization of recycled waste-compost. Of all the types and methods of waste disposal known today, one of the most economical and environmentally friendly is composting as a way of managing organic waste. The need to protect the environment stimulated the awareness of citizens to start thinking about converting the so-called of useless waste natural organic material into a form that the plant can use as food. This is one of the parameters that will be considered in some future work or research.

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ENERGY EFFICIENCY AND RENEWABLE ENERGY SOURCES



A CONSTRUCTED WETLAND FOR WASTEWATER TREATMENT AS A PRODUCT

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Key words: constructed wetland, product, wastewater treatment, life cycle

ABSTRACT:

Constructed wetlands are engineered man-made systems that aim to simulate the selfpurification of wastewater observed in nature when polluted water is released into natural ecosystems. The constructed wetland as an innovative and competitive product meets today's requirements, especially in light of the problem of waste management, i.e. untreated wastewater that causes damage to the environment. A product is a material result of production, everything that we can offer someone to satisfy their need or desire, i.e. a product is the final result of a production activity that exists even after the production process has been completed. The basic goal of production systems is to produce an adequate product, as requested and on time, with the least costs and maximum quality. The product in question aims at systemic control and the greatest possible improvement of the wastewater treatment system and, at the same time, reducing the harmful impact on the environment. The product life cycle goes through five characteristic stages: product development, introduction, growth, maturity and decline. The aim of this paper is to prove that the constructed wetland as a product, that is, as an innovative product, remains in its mature stage for a very long time, and as proof of this is the fact that this phase of the life cycle has been occurring in nature since the dawn of time. From all of the above, one can expect that the application of constructed wetlands will grow even more, and especially their application will be primarily oriented towards smaller capacities (up to approximately 2000 PE). In such conditions, constructed wetlands feature

simple operation, high purification effect and relatively low costs of construction, operation and maintenance.

1. INTRODUCTION

A product is the result of human work that was created to directly or indirectly satisfy a human need. Production systems, depending on their specificity, determine the basic goal that can be defined by the following sentence: "To produce an adequate product, as requested and on time, with the lowest possible costs and maximum quality" (Klarić, 2012). Development of settlements and the increase in population standards cause pollution of the human environment, and among the most critical forms of pollution we certainly include water pollution. The consumption of water for various needs is increasing, which also causes an increase in the amount of waste water, which significantly endangers the human environment. Today, in some parts of developed and undeveloped settlements of municipalities and cities, there are no constructed sewers, but drainage is solved individually by discharges into road ditches, and part of the constructed sewage systems flows directly into watercourses. Such a solution does not meet today's needs and standards when it comes to housing and water protection. Collection of stormwater and waste water is considered one of the most important communal functions of municipalities and cities. It is evident that for the high-quality performance of wastewater collection and treatment in municipalities and cities, as well as the achievement of higher standards regarding protection, it is necessary to construct a complete system and a constructed wetland. Wastewater treatment can be divided into two groups: conventional waste water treatment technologies and non-conventional waste water treatment technologies. As one of the non-conventional wastewater treatment technologies, it is necessary to present the constructed wetland as an innovative and competitive product that meets today's requirements. Like any product, the constructed wetland goes through a production process, i.e. a life cycle, all for the reason of meeting a predetermined set of goals and standards.

2. CONSTRUCTED WETLAND

The constructed wetland consists of several pools through which waste water passes. Along the way, the water is purified using biological (aerobic and anaerobic microbial decomposition, incorporation into the body of plants), physical and chemical processes (evaporation, sedimentation and suction). The bottom of the pools is lined with impermeable foil or loam. On this base, a substrate is placed in which swamp and wetland plants are planted. The natural property of plant aquatic organisms to effectively remove various compounds of nitrogen, phosphorus and other toxic substances from waste water, thereby protecting groundwater, sources of drinking water, streams and lakes, began to be used in a targeted way about thirty

years ago, although this principle has been happening in nature since time immemorial (Mlaus and Vouk, 2011). Constructed wetlands can function as stand-alone units, but can also include other units, including reedbeds, wetlands, lakes, grasslands and even forests. They can work as a surface or subsurface filtration system suitable for the functioning of the most favourable physical and chemical mechanisms for the removal of pollutants to balance the aerobic and anaerobic processes of biological decomposition (Šeparac et al., 2013).

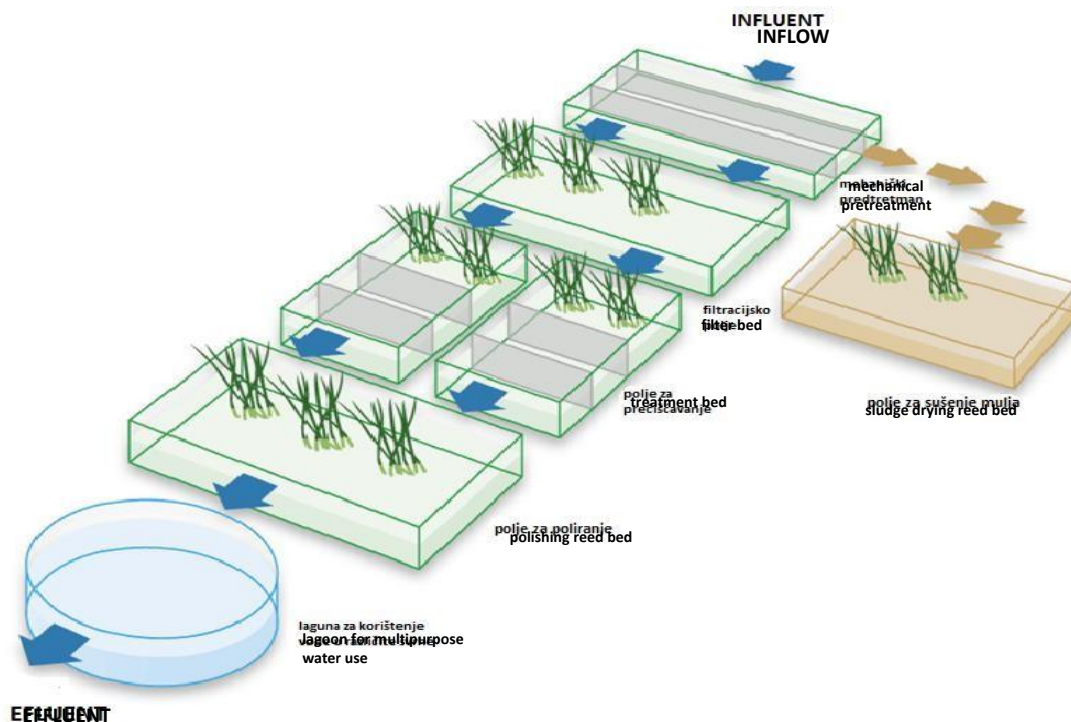


Figure 1: Constructed wetland

Constructed wetlands are engineered man-made systems that aim to simulate the selfpurification of wastewater observed in nature when polluted water is released into natural ecosystems. Those with subsurface flow can have horizontal flow, vertical flow or a combination of the two flows (Zrnčević, 2022). In horizontal systems, water that has previously been mechanically purified flows through filtration basins near the surface and leaves the filtration basin at the bottom, in a slow longitudinal flow. The location of the inlet is adapted so that the flow of wastewater is always uniform. This can be ensured by a distribution structure or by using a filtration basin filled with particles of different granulometric composition at the inlet.

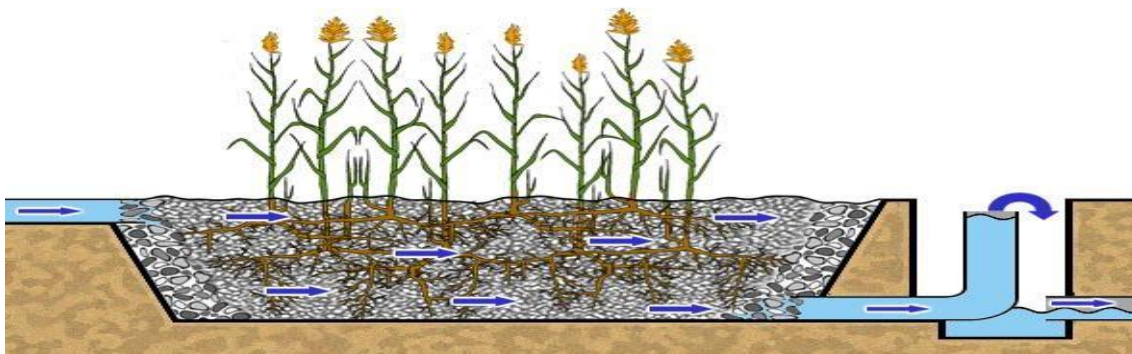


Figure 2: Horizontal flow constructed wetland

In the case of a vertical system, the wastewater inlet is located close to the surface, and the balanced distribution of the wastewater is ensured by parallel pipes. Purified water is collected at the bottom and flows out through drainage pipes.

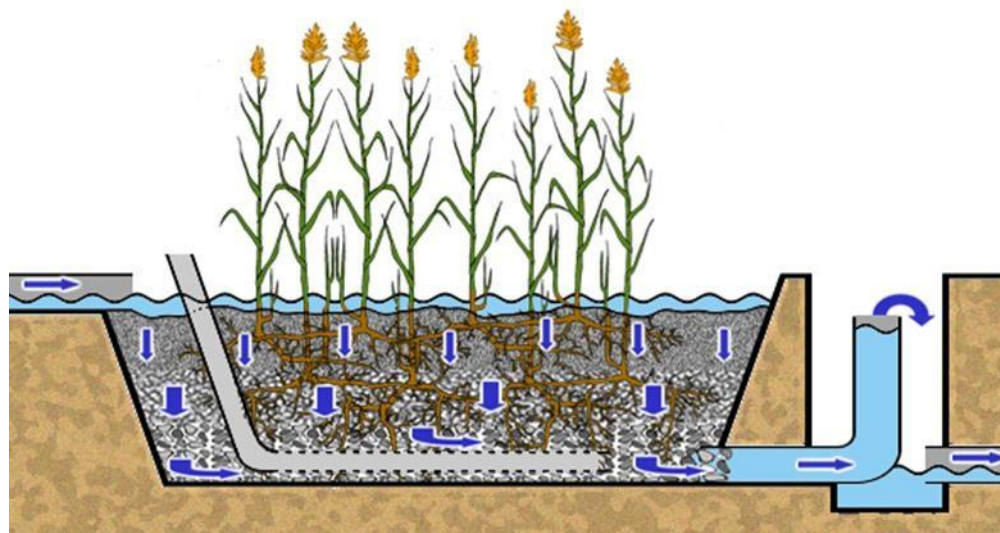


Figure 3: Vertical flow constructed wetland

In both cases, it is necessary to ensure the possibility of regulating the water level at the outlet, so that the retention period can also be regulated and the water supply during the vegetation period can be continuous.

2.1. JUSTIFICATION OF BUILDING A CONSTRUCTED WETLAND

The fact that a larger percentage of the population lives in small settlements (less than 2,000 people) where they use permeable septic tanks with overflow or directly discharge impure water that goes into our environment is one of the essential elements for justifying the use of

this product. The constructed wetland is a good solution for our conditions in Bosnia and Herzegovina, considering the low construction costs and the local workforce for construction and maintenance. In addition to these properties, it is important to state that this product generally does not require energy or mechanical equipment for operation. Operation and maintenance are simple and there are no big costs. Constructed wetlands function continuously even with large changes in flow and pollution load which is present in smaller settlements. Additional treatment beds can also be added in case of increased load. Disadvantages that occur when using constructed wetlands in the wastewater treatment process are: greater need for land, in the absence of a favourable slope mechanical equipment and pumps for lifting wastewater are necessary, greater sensitivity to anaerobic conditions and weed control which is a problem during the growing season initially until beneficial plant communities develop.

Not all products follow the life cycle. Some products are introduced to the market and die quickly. Others remain in the mature stage for a very long time until they enter the decline stage and are then recycled back to the growth stage with the help of strong advertising or repositioning (Klarić, 2012).

The process of making a product is called the life cycle of the product and this process represents a set of procedures organized so that the resulting product, and in this case a constructed wetland, meets a predetermined set of goals and standards. The manufacturing process itself is a more complex concept than the procedure itself (Baraković, 1998). The life cycle of a constructed wetland is a series of the following steps, or phases:

1. Idea as the first phase of the project. In this phase, through preliminary research or studies, by analysing the situation and defining the problem, and by thinking critically about the problem, the problem is discovered and defined, and in this case it is the problem of the generation of wastewater.
2. Planning of the constructed wetland as the second phase has the task of defining the strategy and ways of solving the problem defined in the previous phase of the wastewater problem, but also to elaborate and describe the product in detail.
3. Realization, that is, production of the product, in this case a constructed wetland.
4. Checking and applying the constructed wetland is the final stage in which the results obtained by the product are monitored, but also by its introduction into the system in order to eliminate possible defects caused by carelessness through the previous three stages. In this phase, the product "lives", so its effects are constantly monitored and errors are corrected if necessary.

2.2. LIFE CYCLE OF THE CONSTRUCTED WETLAND AS A PRODUCT

The process of making a product is called the product life cycle (Ivović, 2012).

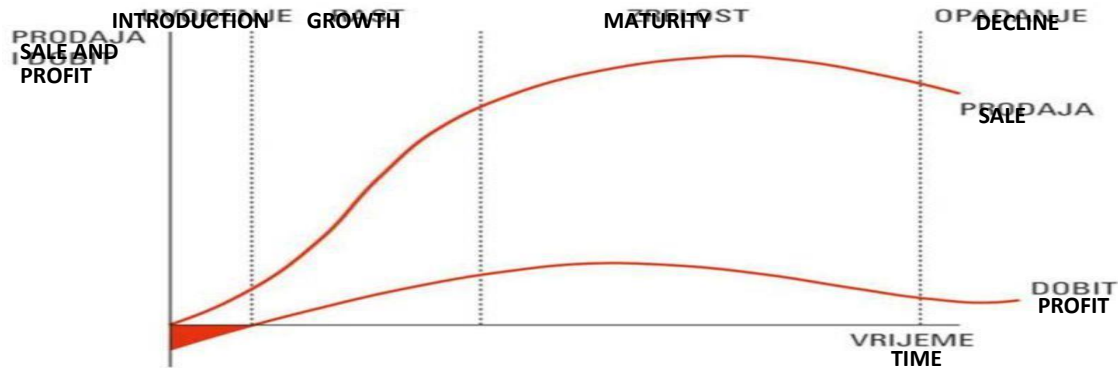


Figure 4: Product life cycle

The product life cycle goes through five characteristic stages:

1. Product development where sales are at zero and investment costs are increasing. It is a time of spending resources with absolutely no income.
2. Product introduction. In this period, profits are negative or low due to high costs for distribution and advertising.
3. Product growth is the period in which the new product, if it meets the needs of the market, starts a rapid rise in sales, which is the result of a larger number of customers who decide to try the new product.
4. The maturity stage in the product life cycle is the stage when sales stabilize or slow down. In this phase, a turning point is reached when the costs of defining the product are fully covered and the profit made, in addition to covering current costs and advertising activities, can already be directed to planning the development of new products or extending the life of the existing product.
5. The decline stage begins at the moment when sales and profits begin to decline. Often, products enter the phase not because of the wrong strategy, but simply because of changes that occur in the environment (Klarić, 2012).

3. CONCLUSION

The constructed wetlands as a product are a simple, acceptable and economically justified way of protecting water and the environment in general. In addition, the construction of such systems that function without a large consumption of energy, but with the maximum utilization of processes that normally take place in nature, perfectly fits into the idea of sustainable development and, in general, into the ecological approach to water protection, which in the world today takes on a major meaning. In the above stated, we have described what a product is and the life cycle of a product, that is, in this case, a constructed wetland as a product. As we mentioned, the product life cycle goes through five characteristic phases: product development, product introduction, product maturity and decline. Taking into account the above, we can say that the constructed wetland as a product does not follow the life cycle of the product completely, i.e. it remains in the mature stage for a very long time and as such will function continuously. The proof of this is that this stage of the life cycle cycles in nature has been occurring since time immemorial. With regard to today's requirements, the use of renewable energy sources, the reduction of CO₂ emissions, the use of unconventional technologies, the use of natural purification methods and, among other things, a coned wetland that requires lower initial investment costs, but also lower operating and maintenance costs while achieving satisfactory results, it is evident that this is a product that meets all requirements on a global level.

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ASSESSMENT OF *ARUNDO DONAX* IN A CONTINUOUS BIOGAS PRODUCTION SYSTEM

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ABSTRACT:

The increase in the production and consumption of biofuels has, for a long time, been mainly encouraged by policies that initially promote the use of biofuels regardless of origin of the raw material. Current EU regulation is focused on the sustainability of the energy production. Although corn silage has historically served as a primary raw material for biogas production, there is an obvious shift towards more sustainable sources, such as energy crop Arundo donax. To determine its potential as a possible transforming factor the production of biogas in the EU, the potential of the crop as a substrate for AD was assessed in this study. Biogas/biomethane production of A. donax and a mixture of corn silage and A. donax in two ratios (1:2 and 2:1) through the process of anaerobic digestion, along with COD, DM, VS, pH value, and FOS/TAC of digestate were continuously monitored. During the process, the comparative analysis accentuated the viability of both, A. donax and corn silage, as well as their mixtures as potent feedstocks for biogas production. The biogas yields ranged from 356.81 to 548.81 NL kg⁻¹ VS. It was observed that a 100 % A. donax substrate exhibited a superior biogas yield initially; however, an intriguing boost in methane proportion was noted with a mixture at a 1:2 ratio, underlining the essence of the synergistic interaction between the feedstocks in methane production. Notably, the levels of H₂S were within permissible limits, eliminating the need for further desulphurization procedures, which is important. Further studies are necessary to

*identify the optimal mixture ratios of corn silage and *A. donax* that maximize biogas and methane production and should also explore the feasibility of storing the crop, considering the continuous process of AD and the seasonality of feedstock, to ensure more consistent and efficient biogas production.*

1. INTRODUCTION

Due to the approaching energy crisis, the EU has published the RePowerEU in May 2022 with a focus on encouraging a 20 % increase in biogas and biomethane production in EU member states. until 2030. This increase in production aims to strengthen the energy independence of the EU. Biogas production and consumption in the EU27 has grown significantly over the past 15 years, with production exceeding 14,000 thousand tonnes of biogas oil equivalent in 2020. The process of anaerobic digestion (AD) involves the activity of anaerobic microorganisms, which break down organic matter and produce methane, carbon dioxide, and trace gases as a product. Biogas production and its efficient use play a key role in sustainable development and reduction of GHG emissions. Today, many biogas plants in the EU27 use corn silage as a primary feedstock. Relevant data indicate an increase in the market price of corn silage in the last 18 months by more than 60 %, which consequently disrupted the livestock production sector. Given that corn silage is primarily a highly nutritious food intended for livestock, the challenge is to find sustainable alternative, sustainable raw materials for biogas production by 2030.

One promising avenue in biogas production is the use of perennial energy crops, such as *Arundo donax*, as a substitute for traditional feedstocks like corn silage. *A. donax*, also known as giant reed, is a fast-growing perennial grass that has shown potential as a feedstock for biogas production due to its high biomass yield and low input requirements. Advantages of using *A. donax* in biogas production are its high availability, high biomass yield compared to annual crops, reduced competition with food crops, and carbon sequestration.

From the chemistry point of view, *A. donax* has a very similar composition to corn silage. This lignocellulosic biomass consists of cellulose, hemicellulose, lignin and small amounts of other extractives, in different proportions. The relative proportions of cellulose and lignin are one of the factors that influence the determination of the plant's suitability for further processing and energy production. Therefore, the harvest period is a factor that affects the biomass structure; depending on the harvest date, the biomass has different cellulose/lignin ratios and can be used in different conversion pathways. For the purpose of using it in the AD system and biogas production, a favourable cellulose/lignin ratio of *A. donax* is achieved during the late summer, when the raw material has low lignin and high water content.

The objective of this study was to assess the feasibility of using a perennial *A. donax*, as a substitute for corn silage, in a continuous biogas production system. For that purpose, the productivity of biogas, CH₄ and H₂S was monitored. In line with the main principles of the

circular bioeconomy, utilization of digestate was also considered by continuous monitoring of its quality parameters.

2. METHODS OF WORK AND MATERIALS

Samples of *Arundo donax* biomass were collected in September 2021 from its natural habitat, on the island of Pag (Croatia, 44°29'48" N 14°57'33" E). After their collection, the samples were stored in a freezer at -18 °C. In this way, the original quality of the samples was preserved, primarily the moisture content, in order to investigate the potential of the *A. donax* fresh biomass for direct use in the anaerobic digestion (AD) process. Prior to AD process, the samples were milled to a maximum particle size of $\phi=10$ mm. For the AD setup, the inoculum from the nearest biogas plant was taken, as well as the corn silage sample. Continuous AD was performed in a 30 L bioreactor (CROTEH d.o.o., Croatia), at the temperature of approx. 39 °C and with the retention time of 136 days. The bioreactor was fed every 24 ± 2 h to maintain a consistent ORL. The process started with a 100 % corn silage as raw material for several days, then continued with the addition of *A. donax* to achieve the (*A. donax* : corn silage) ratio 1:2; the process proceeded to the (*A. donax* : corn silage) ratio 2:1 until a 100 % *A. donax* was reached. The production of biogas and biomethane (NL kg⁻¹ DM) was monitored and the quality of biogas from each setup was compared. During the AD process, dry matter (DM, %), volatile solids (%), chemical oxygen demand (COD, mg kg⁻¹ DM), pH and FOS/TAC of digestate were monitored to ensure proper process execution.

Statistical analysis was performed by using TIBCO STATISTICA 13.3.0 software (StatSoft TIBCO Software Inc., Palo Alto, CA, USA).

3. RESULTS

Results shown in Table 1 reveal the specific biogas production (SBP), specific methane production (SMP), and hydrogen sulphide (H₂S) concentrations for used feedstock over a five-month period.

Table 1: Specific biogas, specific methane, and hydrogen sulphide production

Sample	Month	SBP	SMP	H ₂ S (ppm)
Arundo 1-2	1	547.7±108.86 ^c	320.76±72.48 ^e	265.27±115.77 ^{defg}
	2	530.23±84.5 ^{bc}	317.8±60.68 ^{cde}	279.17±134.62 ^{efg}
	3	479.01±71.8 ^{abc}	283.46±52.68 ^{bcd}	202.07±144.76 ^{bcd}
	4	548.81±93.95 ^c	338.9±60.48 ^e	218.90±108.27 ^{cdef}
	5	507.48±86.04 ^{bc}	315.56±50.81 ^{de}	151.15±83.94 ^{abcd}
Arundo 2-1	1	543.15±158.67 ^{bc}	282.89±84.49 ^{bcd}	331.40±130.96 ^g
	2	356.82±150.94 ^a	186.5±82.11 ^a	258.53±135.76 ^{defg}

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	3	497.52±90.26 ^{bc}	256.98±71.94 ^{abcd}	168.67±113.94 ^{abcd}
	4	401.16±120.7 ^{abc}	218.26±65.8 ^{abc}	116.47±97.05 ^{abc}
	5	443.89±73.7 ^{abc}	244.34±43.05 ^{abcde}	283.25±99.15 ^{defg}
Arundo 100 %	1	430.71±53.67 ^{abc}	221.6±44.72 ^{abcd}	225.80±148.13 ^{cdefg}
	2	479.78±128.78 ^c	253.62±68.2 ^{bcde}	292.90±162.92 ^{efg}
	3	359.07±57.47 ^{ab}	193.13±26.55 ^{ab}	321.87±142.21 ^{fg}
	4	426.24±51.17 ^{abc}	226.58±28.58 ^{abcd}	101.37±116.12 ^{ab}
	5	416.62±79.24 ^{abc}	221.91±42.71 ^{abcde}	75.90±70.49 ^a
Minimum		356.81	186.49	75.90
Maximum		548.81	338.89	331.40
Statistical significance		*	*	219.51
				*

Abbreviations: "Arundo 1-2" - mixture of *A. donax* and corn silage at ratio 1:2; "Arundo 2-1" - mixture of *A. donax* and corn silage at ratio 2:1; "Arundo 100 %" - 100 % *A. donax*; SBP – specific biogas production; SMP – specific methane production. Note: Different letters in the same column represent the difference between the observed values according to the Tukey HSD post hoc test ($p < 0.05$).

Table 1 shows the SBP values that ranged from a minimum of 356.81 NL kg⁻¹ VS to a maximum of 548.81 NL kg⁻¹ VS. The highest SBP was observed in the sample Arundo 1-2 during the fourth month (548.81±93.95c), indicating a strong biogas production capability during this period. Conversely, the lowest SBP was recorded for the sample Arundo 2-1 in the second month (356.82±150.94a). This variation can be attributed to the differences in feedstock composition and operational conditions. SMP values varied between 186.49 NL kg⁻¹ VS and 338.89 NL kg⁻¹ VS. The highest SMP was noted for the sample Arundo 1-2 in the fourth month (338.9±60.48e), suggesting efficient methane production from this feedstock mixture. The lowest SMP was observed for the sample Arundo 2-1 in the second month (186.5±82.11a). These findings highlight the significant impact of feedstock ratios and digestion time on methane yield. H₂S concentrations ranged from as lowest as 75.90 ppm to 331.40 ppm. The maximum concentration was detected in the sample Arundo 2-1 during the first month (331.40±130.96g), while the minimum concentration was found in the sample Arundo 100 % during the fifth month (75.90±70.49a).

The data provided in Table 2 present detailed measurements of dry matter (DM), volatile solids (VS), pH values, chemical oxygen demand (COD), and the ratio of volatile fatty acids to total alkalinity (FOS/TAC) for the used feedstocks during anaerobic digestion process.

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Table 2: Digestate analysis during anaerobic digestion process

Sample	Month	DM (%)	VS (%)	pH	COD (mgO kg ⁻¹ DM)	FOS/TAC
Arundo 1-2	1	0.05±0 ^{gh}	0.71±0.01 ^{ab}	7.4±0.17 ^{bcd}	46302.5±2747.08 ^{ef}	0.27±0.02 ^{ab}
	2	0.05±0 ^{defg}	0.75±0.04 ^{ab}	7.46±0.07 ^{cde}	42666.25±3021.18 ^{def}	0.28±0.07 ^{ab}
	3	0.04±0 ^{ab}	0.8±0.04 ^b	7.28±0.08 ^{abc}	39707.78±3069.6 ^{cde}	0.29±0.06 ^{ab}
	4	0.03±0.01 ^a	0.76±0.09 ^{ab}	7.2±0.06 ^a	28440±6073.2 ^a	0.28±0.05 ^{ab}
	5	0.04±0.01 ^{abc}	0.7±0.1 ^{ab}	7.32±0.13 ^{abc}	27890±3653.53 ^a	0.26±0.04 ^a
Arundo 2-1	1	0.05±0 ^h	0.7±0.02 ^{ab}	7.65±0.27 ^{ef}	49761.25±3506.09 ^f	0.31±0.02 ^{ab}
	2	0.05±0 ^{defgh}	0.75±0.01 ^{ab}	7.56±0.11 ^{def}	44697.14±3843.88 ^{def}	0.28±0.08 ^{ab}
	3	0.04±0 ^{bcd}	0.78±0.03 ^{ab}	7.31±0.1 ^{abc}	49801.11±2916.46 ^f	0.35±0.06 ^b
	4	0.04±0.01 ^{cdef}	0.77±0.03 ^{ab}	7.2±0.05 ^a	39713.75±11694.43 ^{cde}	0.32±0.06 ^{ab}
	5	0.04±0 ^{cde}	0.72±0.05 ^{ab}	7.2±0.09 ^{ab}	30591.25±7098.11 ^{ab}	0.35±0.06 ^{ab}
Arundo 100%	1	0.05±0 ^{efgh}	0.75±0.03 ^{ab}	7.71±0.17 ^f	48382.22±3011.05 ^f	0.33±0.09 ^{ab}
	2	0.05±0 ^{cdefg}	0.76±0.03 ^{ab}	7.31±0.15 ^{abc}	48586.67±5517.18 ^f	0.3±0.07 ^{ab}
	3	0.05±0.01 ^{fgh}	0.69±0.18 ^a	7.25±0.06 ^{ab}	37040±2271.62 ^{bcd}	0.33±0.02 ^{ab}
	4	0.05±0.01 ^{defgh}	0.78±0.06 ^{ab}	7.19±0.08 ^a	33185±1669.83 ^{abc}	0.36±0.04 ^{ab}
	5	0.06±0 ^h	0.78±0.02 ^{ab}	7.18±0.06 ^a	33108.75±1239.73 ^{abc}	0.34±0.03 ^{ab}
Minimum		0.03	0.69	7.18	27890.00	0.26
Maximum		0.06	0.80	7.71	49801.11	0.36
Statistical significance		*	*	*	*	*

Abbreviations: "Arundo 1-2" - mixture of *A. donax* and corn silage at ratio 1:2; "Arundo 2-1" - mixture of *A. donax* and corn silage at ratio 2:1; "Arundo 100 %" - 100 % *A. donax*; DM – Dry matter; VS – volatile solids; COD – Chemical Oxygen Demand; FOS/TAC - volatile organic acids/total inorganic carbon. Note: Different letters in the same column represent the difference between the observed values according to the Tukey HSD post hoc test ($p < 0.05$).

Table 2 shows that the DM content across the samples ranged from a minimum of 0.03 % to a maximum of 0.06 %, with the lowest value observed for Arundo 1-2 during the fourth month, whilst the highest value was recorded for Arundo 100 % during the fifth month. Volatile Solids (VS) varied between 0.69 % and 0.80 %. The highest VS content was noted in Arundo 1-2 during the third month (0.80±0.04b), suggesting a high organic matter content available for biogas production. Conversely, the lowest VS content was found in Arundo 100 % during the third month (0.69±0.18a). The pH values ranged from 7.18 to 7.71, with the most alkaline pH recorded in Arundo 100 % during the first month (7.71±0.17f) and the most acidic pH in Arundo 100 % during the fifth month (7.18±0.06a). COD values fluctuated between 27,890 mgO kg⁻¹ DM and 49,801.11 mgO kg⁻¹ DM. The highest COD was observed in Arundo 2-1 during the third month (49801.11±2916.46f), indicating a high concentration of organic pollutants in the feedstock mixture. The lowest COD was noted in Arundo 1-2 during the fifth month (27890±3653.53a). The FOS/TAC ratio, an indicator of the stability of the anaerobic digestion process, ranged from 0.26 to 0.36. The highest FOS/TAC ratio was observed in Arundo 100 % during the fourth month (0.36±0.04ab), suggesting a balanced production and consumption of

volatile fatty acids. The lowest FOS/TAC ratio was found in Arundo 1-2 during the fifth month ($0.26 \pm 0.04a$).

4. DISCUSSION

The analysis of the presented data shows considerable variability in the chemical and physical properties of the starting material mixtures. This variability underlines the complexity and dynamics of the anaerobic digestion process. One of the key observations from the data is the range of SBP values, which fluctuated between a minimum of $356.81 \text{ NL kg}^{-1} \text{ VS}$ and a maximum of $548.81 \text{ NL kg}^{-1} \text{ VS}$. Notably, the highest SBP was achieved for Arundo 1-2 mixture in the fourth month, indicating its substantial potential for biogas production during this period. In contrast, the Arundo 2-1 mixture recorded the lowest SBP in the second month. Giertl et al. (2022) reported that corn silage managed to produce more biogas during continuous anaerobic digestion process than *A. donax* which confirms that higher ratio of *A. donax* and corn silage resulted with a decreased biogas production. The SMP values also exhibited considerable variability, ranging from $186.49 \text{ NL kg}^{-1} \text{ VS}$ to $338.89 \text{ NL kg}^{-1} \text{ VS}$. The peak SMP value, observed for the Arundo 1-2 mixture during the fourth month, underscores the efficiency of this feedstock combination in producing methane. On the other hand, the lowest SMP value recorded for the Arundo 2-1 mixture during the second month highlights potential inefficiencies in methane production for this particular mixture. These results are in line with the findings from Baldini et al. (2017) and Shilpi et al. (2019). With the addition of corn silage to the *A. donax* mixture, methane production increased, confirming that the addition of corn silage increases biogas quality. H_2S concentrations varied across different samples and months, but according to Moreno-Andrade et al. (2020) concentrations remained at the permitted level of 500 ppm.

The digestate analyses during the anaerobic digestion process revealed several noteworthy trends and comparisons with existing literature. Our study found that the dry matter content ranged from 0.03 % to 0.06 %, which is significantly lower than the findings of Möller and Müller (2012), who reported DM values ranging from 1.5 % to 13.2 %. This discrepancy could be attributed to differences in feedstock composition, reactor conditions, and operational parameters. In terms of pH values, our results ranged from 7.18 to 7.71, which aligns closely with the findings of Trisakti and Sitompul (2021), who reported pH values between 6.8 and 7.3 during anaerobic digestion. Maintaining a stable pH within this range is crucial for optimum microbial activity and biogas production. Chemical oxygen demand varied between $27,890 \text{ mgO kg}^{-1} \text{ DM}$ and $49,801.11 \text{ mgO kg}^{-1} \text{ DM}$. These values are consistent with those reported by Parawira et al. (2004), who found COD values ranging between 27 and 37 $\text{mgO kg}^{-1} \text{ DM}$. Similar COD values indicate comparable levels of organic pollutants in the feedstock mixtures and the efficiency of the anaerobic digestion process in reducing these pollutants. The FOS/TAC ratio, an important indicator of the stability of the anaerobic digestion process, ranged from 0.26

to 0.36 in our study. Mézes et al. (2011) reported FOS/TAC ratios ranging from 0.14 to 0.29, which is slightly lower than our findings.

5. CONCLUSION

The aim of this study was to evaluate the potential of biogas production from *A. donax* and its mixtures with corn silage by continuous anaerobic digestion. The results showed a significant variability in specific biogas production (SBP) and specific methane production (SMP) in different feedstock mixtures and retention periods (months). The Arundo 1-2 mixture, in general, produced higher SBP and SMP values, especially in the fourth month, indicating its significant potential for biogas and methane production. In contrast, the Arundo 2-1 mixture showed significant fluctuations, with the lowest production values recorded in the second month, indicating a need for further optimization. Analysis of the digestate showed variations in dry matter, volatile solids, pH, chemical oxygen demand, and FOS/TAC ratio, highlighting the importance of optimizing feedstock blends and monitoring process parameters. Overall, the study underlines the potential of *A. donax* as a sustainable, alternative feedstock for biogas production, especially in combination with corn silage. Further research is required to determine the optimum mixing ratios.

6. ACKNOWLEDGMENT

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Eighth International Scientific Conference
"June 5th - World Environment Day"

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THE MECHANISM OF CROSS-BORDER SETTLEMENT OF CARBON EMISSIONS AND ITS IMPORTANCE IN THE EUROPEAN GREEN DEAL

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Key words: European Green Deal, Environment, Climate Change, CO₂, CBAM

ABSTRACT:

The European Green Deal is the sustainable development strategy of the EU for the 21st century. The central problem addressed by this strategy is the environment and climate change - how to develop the EU economy without accelerating climate change. The goal of this strategy is for Europe to become the first neutral continent with zero net greenhouse gas emissions by 2050. The European Green Deal covers all economic sectors, especially transport, energy, agriculture, building maintenance and construction, and industries such as steel, cement, and textile production, and chemicals. Greenhouse gas emissions do not stop at the EU borders. Both other countries and the countries of the Western Balkans are important partners of the EU in the context of trade and geopolitical value. By signing the Sofia Declaration 2020, the countries of the Western Balkans committed themselves to the implementation of the Green Agenda for the Western Balkans as a "map" for a series of concrete actions and measures aimed at low-carbon development of the region and economic growth in accordance with the principles of sustainable development. The existing European Emissions Trading System (ETS) covers EU countries, while the Carbon Border Adjustment Mechanism (CBAM) will apply to products manufactured outside the EU. The implementation of CBAM, a new carbon regulation that helps combat climate change, addresses the issue of carbon emissions. This paper will outline the CBAM implementation procedures and their importance in the European Green Deal.

1. INTRODUCTION

Climate change is a global problem that requires global solutions. As a leader in climate protection and a driver of the global environmental agenda, Europe aims to become the world's first climate-neutral continent by 2050. The European Green Plan (EGP), adopted in 2020, is a roadmap of tax and non-tax policy initiatives designed to achieve this ambitious goal.

The EU has announced its ambition to reduce emissions by 55% compared to 1990 levels by 2030. To meet this target, the European Commission has adopted a series of legislative proposals designed to facilitate the necessary acceleration of greenhouse gas emission reductions by 2030 and aligned EU policies on climate, transport, land use, energy and taxation with this key goal (Prokopowicz, 2020).

The current regulation is Commission implementing Regulation (EU) 2023/1773 Official Journal of the European Union, L 228/94 (EU 2023/1773, 2023).

Although the EU is raising its own climate change ambitions, there is still a risk of so-called carbon leakage in many non-EU countries with less stringent climate policies.

Picture 1 shows the European Green Deal from July 2021 under the caption "Suitable for package 55". According to the United Nations, as of June 2023, there are 195 signatories to the Paris Agreement to limit their greenhouse gas (GHG) emissions. However, the Paris Agreement allows countries to set their own ambitions within certain parameters (<https://kpmg.com>).

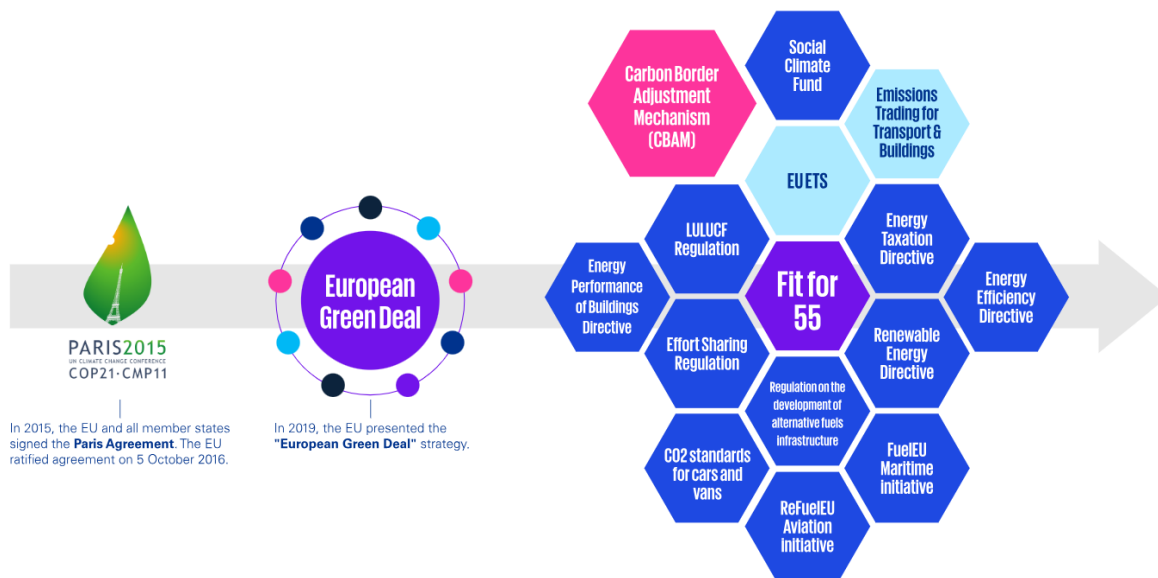


Figure 1: The European Green Deal through Package 55 (<https://kpmg.com>)

The European Green Deal is the EU's sustainable development strategy for the 21st century. The central issue addressed by this strategy is the environment and climate change - how to develop the EU economy without accelerating climate change (EU 2023/956, 2023).

Eighth International Scientific Conference "June 5th - World Environment Day"

The goals of the European Green Deal are to reduce gas emissions (zero net greenhouse gas emissions by 2050), improve the state of the natural environment, protect wild animals, create new economic opportunities, and improve the quality of life of all citizens (EU 2023/956, 2023).

The benefits of the European Green Deal are (EU 2023/956, 2023):

- fresh air, clean water, healthy soil, and biological diversity,
- cleaner energy and modern technological innovations,
- renovated, energy-efficient buildings,
- longer-lasting products that can be repaired, recycled, and reused,
- healthy and affordable food,
- globally competitive and resilient industry,
- greater prevalence of public transport, and
- future-proof jobs and skills training.

The fact is that greenhouse gas emissions do not stop at the borders of the EU. The countries of the Western Balkans are an important partner of the EU in the context of trade and geopolitical value.

With the signing of the Sofia Declaration in 2020, the countries of the Western Balkans committed to implementing the Green Agenda for the Western Balkans as a "map" for a series of concrete actions and measures aimed at low-carbon development of the region and economic growth in accordance with the principles of sustainable development. The Green Agenda for the Western Balkans is an instrument for implementing the European Green Deal in the Western Balkans.

Bosnia and Herzegovina, within the negotiation chapters for EU accession and economic cooperation programs, is obliged to adopt and implement the measures proposed by the Green Agenda and its Action Plan for the implementation of the ZAZB.

Bosnia and Herzegovina's production sector needs to fulfill all requirements, directives, and obligations, which refer to:

- Requirements of EU customers who must harmonize their business with ESD (reduction of waste, preferably use of renewable energy sources, measurement of CO₂ emissions, use of recycled materials,...).
- EU directives that are in the revision phase in accordance with the objectives of ESD.
- Obligations assumed by Bosnia and Herzegovina by signing the Sofia Declaration of ZAZB.

The European Emissions Trading System (EU ETS) is one of the key tools with which the European Union wants to combat global warming and effectively reduce greenhouse gas (GHG) emissions at economic costs. The EU ETS aims to apply "polluter pays", meaning that the costs of pollution should be borne by those who cause it (Olasehinde-Williams and Akadiri, 2024).

2. CBAM - PART OF THE EUROPEAN GREEN AGREEMENT AND THE "FIT FOR 55" PACKAGE

Although the EU is implementing measures to achieve climate neutrality and is already successfully reducing greenhouse gas emissions, it recognizes that many other countries have not yet reduced or even increased emissions. The EU therefore hopes to make a global impact on the fight against climate change and solve the problems of potential carbon leakage through the implementation of the Carbon Border Adjustment Mechanism (CBAM), which is proposed under the EU Green Deal and is an essential element of the Fit for 55 package (<https://kpmg.com>).

The Carbon Cap Adjustment Mechanism Regulation, which was adopted on May 17, 2023, and entered into force on October 1, 2023, is designed to counter the risk of carbon leakage and works by imposing a charge on the embedded carbon content of certain goods imports equal to fees imposed on domestic goods under the European Emissions Trading System (ETS). To ensure that there is no double benefit for EU producers, the CBAM will replace the free ETS emissions currently allocated to EU producers assessed to be at high risk of carbon emissions. Therefore, by imposing an equivalent carbon price on the import of covered goods, the conditions are leveled for producers from the EU and for importers of such goods from the EU, as partner countries are encouraged to decarbonize their production processes (<https://kpmg.com>).

2.1 Affected products

The new rules will initially affect goods imported from countries outside the EU that are particularly carbon-intensive, certain goods within the production sectors of fertilizers, electricity, hydrogen, cement, iron and steel, and aluminum (Picture 2). CBAM covers imports of covered goods from non-EU countries, except those participating in or linked to the EU ETS.

Products initially covered by CBAM

The regulation applies to goods listed in Annex I to the Regulation.

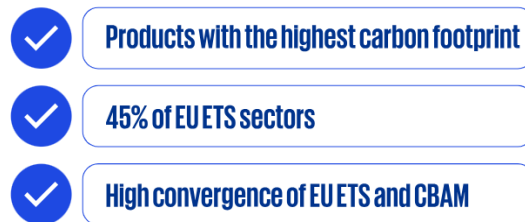
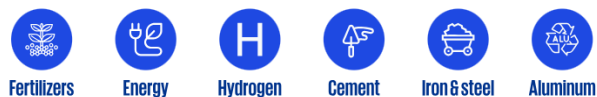


Figure 2: Products subject to CBAM reporting (<https://kpmg.com>)

After implementing CBAM, the European Commission plans to expand the scope of application to all sectors subject to EU emissions trading until 2030.

2.2 Phases of Introduction of CBAM

The current transition phase of CBAM lasts from October 1, 2023. until 31.12.2025. year, and in its full context will be applied from 01.01.2026. years. Figure 3 shows the gradual introduction of CBAM.

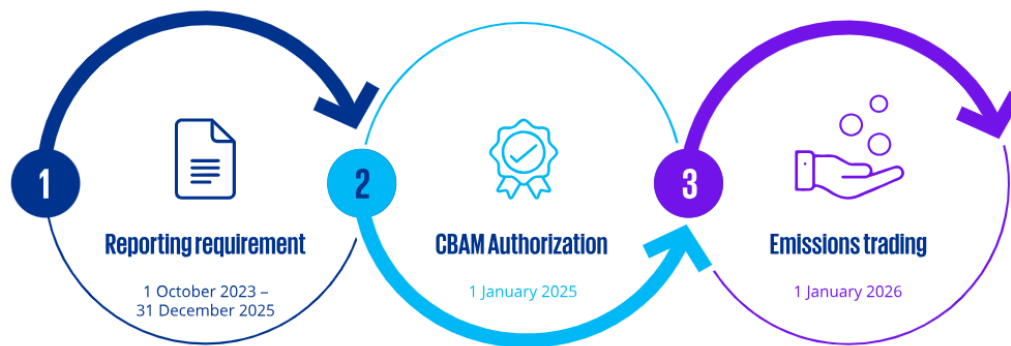


Figure 3: Gradual introduction of CBAM (<https://kpmg.com>)

The goal of the transition phase is to serve as a pilot phase and a learning phase for all participants (importers, producers, and competent authorities) and to collect useful information on incorporated emissions to improve the methodology for the full phase, and to harmonize the price of carbon produced in the EU with the price of carbon-intensive goods imported into the EU. During the transition period, importers of goods within the scope of the new rules will have to report only the emissions of greenhouse gases (GHG) incorporated in their imports (direct and indirect emissions), without the need to purchase and submit certificates. Indirect emissions will be included in the scope after the transition period for some sectors (cement and fertilizers), based on the defined methodology specified in the Implementing Regulation published on August 17, 2023, and its accompanying guidelines.

By the end of 2024, companies will have a choice of reporting in three ways:

1. full reporting according to the new methodology (EU method);
2. reporting based on the equivalent method (three options); and
3. reporting based on default benchmarks (until July 2024 only).

From 01.01.2025., only the EU method will be accepted, and estimates (including default values) can only be used for complex goods if these estimates represent less than 20% of total embedded emissions.

This gradual introduction of CBAM is aligned with the gradual abolition of the allocation of free emission units under the EU Emissions Trading System (ETS) (<https://kpmg.com>).

3. THE INFLUENCE OF CBAM ON THE BUSINESS OF COMPANIES IN BOSNIA AND HERZEGOVINA

3.1 Obligations of the company during the transition period

During the transitional period, the obligations arising from the import of goods subject to CBAM are:

- ✓ Submit CBAM reports on a quarterly basis: Importers of goods (or their indirect customs representative) must report greenhouse gas (GHG) emissions embedded in their imports (direct and indirect emissions) during a given quarter of the calendar year, without submitting any financial payments or adjustments. Reports are submitted no later than one month after the end of that quarter.
- ✓ Registration in the transitional CBAM register: Developed by the European Commission to assist traders in creating and submitting CBAM reports, this register will also enable communication between the Commission, competent authorities, national customs authorities, and traders.

According to Regulation (EU) 2023/956 of the European Parliament of the Council and Commission implementing Regulation (EU) 2023/1773, the rules for reporting obligations on the amount of imported goods, direct and indirect emissions contained in them, and all carbon prices for these emissions.

If Bosnia and Herzegovina does not introduce its CO₂ emissions trading system comparable to the EU system by the beginning of 2026, BH exporters will still be required to submit CBAM reports.

In that case, BH companies, and thus the BH economy, would find themselves in a new unenviable situation, because their products could become more expensive by up to 20%. This would greatly affect their competitiveness in the European market.

Companies from the cement, iron and steel, aluminum, artificial fertilizers, electricity, and hydrogen industries already have to accept the job, because they are obliged to submit their reports on CO₂ emissions. The amount of the penalty for incorrect or incomplete submission of the CBAM report will be determined by the individual member. It is important to note that the price of the CBAM certificate will be linked to the EU ETS (the average price of the EU ETS in 2023 – 100 Eur/ton of CO₂), and that fines will come into force for EU companies that illegally import goods from countries outside the EU without CBAM certificate.

3.2 Models and instruments for the application of the green economy

For the application of the green economy in the BiH economic sector, general models and instruments can be listed, in Figure 4.:

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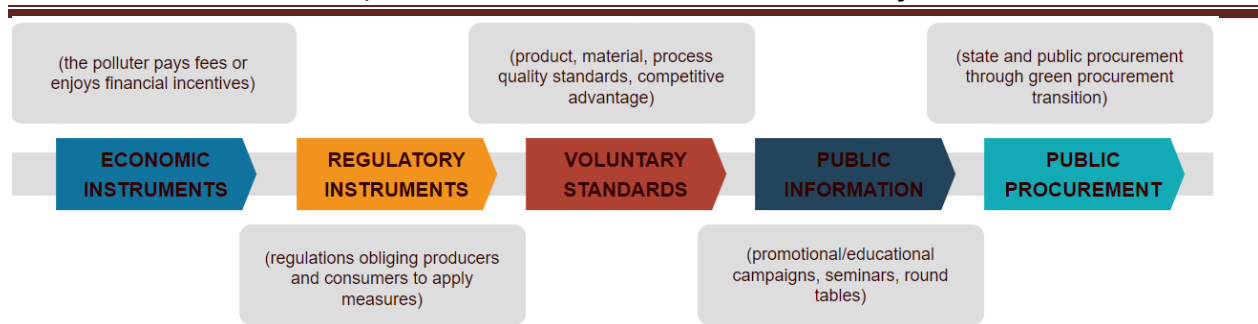


Figure 4: Models and instruments for the application of the green economy

Innovative business models, tools, and measures that can be applied in the green economy (Bajramović, et al., 2024), (Paleari, 2024):

- Reduce the consumption of natural resources through sustainable procurement (less hazardous materials), optimal delivery (optimization of transport routes), review of raw material procurement and reliability of suppliers, primary measure for energy and raw material efficiency, application of international standards, e.g. standard for determining costs and benefits per environment ISO 14007:2019.
- Reduce the amount of generated waste and emitted pollution through the recovery of materials, semi-products, and by-products, the application of some of the techniques (reuse, maintenance/repair, renewal/refurbishment, re-production, recycling), the application of a zero-emissions or zero-waste approach, extending the life span products/materials.
- Reduce the need to buy from consumers (change of habits, information) through instructions for proper use, storage, maintenance and disposal, without product ownership/leasing, sharing, renting and use services.
- Production/transportation with greater use of renewable energy sources: replacement of the use of fossil fuels with renewable energy sources, process optimization, application of cleaner production measures, introduction of modern vehicle fleet and logistics.
- Design a product/service that does not harm the environment with less harmful materials in the product, more recyclable materials, eco-labeling, design a product suitable for the environment and easy to disassemble at the end of its life, a standard for the inclusion of ecodesign, analyze the entire life cycle of the product, application of international standards, e.g. guidelines for the application of material circulation in product design and development.
- Application of standards in the field of Material handling and eco-design (ISO 14006, ISO 14007, ISO 14008 and ISO 14009), Environmental reporting (ISO 14016, ISO 140063, and ISO 14097), Climate change and GHG (ISO 14064-1, ISO 14064 -2, ISO 14064-4, ISO 14080, ISO 14090, ISO 14091 and ISO 19694) and Carbon neutrality (ISO/FDIS 14068).

4. CONCLUSION

Most of the CBAM will concern more industrialized countries, such as Russia, China, the UK, and Norway, the countries expected to be most affected in relative terms are Mozambique, Bosnia and Herzegovina, Ukraine, Serbia, North Macedonia, Montenegro, Zimbabwe, Moldova and Albania.

In May 2022, the European Parliament called on the EU to "provide financial support, which is at least equal to the financial value of the income generated from the sale of CBAM certificates, to support the efforts of the least developed countries towards the decarbonization of manufacturing industries", but no concrete commitments have been made to date.

And if there are still many open questions, for BH companies that export their products to the EU, in terms of CBAM readiness, the most important is:

- Stay informed - monitor the development of regulations in the EU.
- Implement the processes of data collection and processing required for CBAM reporting in the transition period.
- Invest in cleaner production:
 - improving the efficiency of production processes, digitization and automation,
 - energy efficiency of production processes and facilities, i
 - production of own energy from renewable sources (solar and wind power plants).
- Evaluate own supply chain to improve competitiveness or reduce indirect exposure to CBAM.
- Introduce and implement standards related to the environment and climate change, as the best tool for all of the above.

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COUNCIL OF EUROPE AND ENVIRONMENTAL PROTECTION

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Key words: Council of Europe, right to a healthy environment, normative framework, conventions, criminal liability

ABSTRACT:

Although the European Convention for the Protection of Human Rights and Fundamental Freedoms (ECHR) does not guarantee the right to environmental protection as an independent right, the practice of the European Court of Human Rights (ECtHR) found cases that were in the function of environmental protection rights. Thanks to the extensive interpretation of the ECHR's provisions, ECtHR considered the right to environmental protection indirectly: through the right to life, the right to respect for private and family life and other rights guaranteed by the ECtHR. In addition to the above in terms of re-actualizing the relevant judicial practice, this paper provides an overview of the documents of the Council of Europe on environmental protection, especially the Convention on Civil Liability for Damage resulting from Activities Dangerous to the Environment, the Convention on the Protection of the Environment through Criminal Law, the Convention on the Conservation of European Wildlife and Natural Habitats and Council of Europe Landscape Convention.

Bosnia and Herzegovina is a member of the Convention on the Conservation of European Wildlife and Natural Habitats (ETS No. 104) and the Council of Europe Landscape Convention (ETS No. 176). The Convention on the Protection of the Environment through Criminal Law (ETS No. 172) has not entered into force, as only Estonia has ratified it to date. The Convention on Civil Liability for Damage resulting from Activities Dangerous to the Environment (ETS No. 150) also did not enter into force, as it was not ratified by any member state of the Council of Europe.

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1. INTRODUCTION

The Council of Europe was established by the London Treaty of May 5, 1949. It is the first regional international organization that was created on European soil after the Second World War with the aim of contributing to the unification of the countries of Europe.

The main goals of the Council of Europe are:

- protection of human rights, democracy and the rule of law,
- improving awareness and encouraging the development of cultural identity and diversity of European countries,
- searching for solutions to the problems facing European society,
- providing assistance in consolidating democracy through the implementation of political, legislative and constitutional reforms.

Council of Europe bodies make various decisions: conventions, declarations, resolutions and recommendations. The most important legal documents are conventions that have binding legal force for the countries that have signed and ratified them. Among the conventions, the most significant is the European Convention for the Protection of Human Rights and Fundamental Freedoms from 1950 with protocols, and in the area of environmental protection, the Convention on Civil Liability for Damage resulting from Activities Dangerous to the Environment (ETS No. 150), the Convention on the Protection of the Environment through Criminal Law (ETS No. 172), the Convention on the Conservation of European Wildlife and Natural Habitats (ETS No. 104) and the Council of Europe Landscape Convention (ETS No. 176).

2. EUROPEAN CONVENTION FOR THE PROTECTION OF HUMAN RIGHTS AND FUNDAMENTAL FREEDOMS AND THE RIGHT TO A HEALTHY ENVIRONMENT

The European Convention for the Protection of Human Rights and Fundamental Freedoms does not guarantee the right to environmental protection as an independent right. This means that the violation of the right to environmental protection is not taken independently, but only in connection with some other protected right. Nevertheless, some cases that were in the function of environmental protection rights, through the right to life, the right to respect for private and family life and other convention rights, were brought before the European Court for the Protection of Human Rights.

The European Court was invited to develop its case law in environmental protection issues due to the fact that the realization of some rights from the Convention could be threatened by the existence of environmental damage and exposure to environmental risks.

Eighth International Scientific Conference "June 5th - World Environment Day"

In its practice so far, the Court has considered several cases related to the right to a healthy environment in relation to the right to life. Thus, in the case of *Öneryıldız v. Turkey*, the Court found a violation of Art. 2. Convention (right to life) due to dangerous industrial activities. The applicant's place of residence was built without approval on the land next to the waste disposal site that was jointly used by four municipal councils. In April 1993, a methane explosion occurred at the landfill, and debris erupting from the waste pile covered more than ten houses located below the landfill, including a house belonging to the applicant who lost nine close relatives. In particular, the applicant complained that no measures were taken to prevent the explosion despite the expert report that drew the attention of the authorities to the need for preventive action. The court found a violation of Art. 2 of the Convention in its substantive part due to the lack of appropriate steps to prevent the accidental death of nine of the applicant's close relatives, and the violation of Art. 2. in its procedural aspect due to the lack of adequate protection of the law that preserves the right to life. In particular, the Court noted that the Government of Turkey had not provided slum dwellers with information about the risks they faced by living there. Even if she had, she would still be liable for failing to take the necessary practical measures to avoid risk to human life. The regulatory framework proved to be defective because the landfill was open and operated without a unified monitoring system. Urban policy was also inadequate and undoubtedly played a role in the chain of events that led to the accident.

Based on texts adopted by the Parliamentary Assembly of the Council of Europe and the case-law of the European Court of Human Rights, the Committee of Ministers has entrusted the Steering Committee for Human rights (CDDH) with elaborating a manual on human rights and the environment. The Committee of Ministers approved the publication of Manual on Human Rights and the environment (2006)⁷ and a revised manual has been republished in 2012.⁸

In the meantime, Recommendation 1885 (2009)⁹ on 'Drafting an additional protocol to the European Convention on Human Rights concerning the right to a healthy environment' was prepared by the Parliamentary Assembly.

The Parliamentary Assembly of the Council of Europe, referring to Resolution 2396 (2021)¹⁰ on Anchoring the right to a healthy environment: need for enhanced action by the Council of Europe, reiterated the need to admit a new generation of human rights. In Recommendation 2211

⁷ Council of Europe, Manual on human rights and the environment - Principles emerging from the case-law of the European Court of Human Rights, 2006.

⁸ Council of Europe, Manual on human rights and the environment (2nd edn.), 2012.

⁹ Parliamentary Assembly, PACE - Recommendation 1885 (2009) - Drafting an additional protocol to the European Convention on Human Rights concerning the right to a healthy environment.

¹⁰ Resolution 2396 (2021): Anchoring the right to a healthy environment: need for enhanced action by the Council of Europe.

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(2021)¹¹ the Assembly expressed anxieties because of the rate and extent of environmental degradation, the loss of biodiversity and the climate crisis, which has a direct impact on human health, dignity and life.

The Assembly asked the Committee of Ministers develop an additional protocol to the European Convention on Human Rights (ECHR) on the right to a safe, clean, healthy and sustainable environment, based on terminology used by the United Nations. At the same time, they proposed the draft text for the protocol, which is an integral part of Recommendation 2211 (2021).¹²

According to the Recommendation, the right to a safe, clean, healthy and sustainable environment requires going beyond an approach based on individual rights alone and defines the right to a healthy environment as an autonomous right of humanity.¹³

For the purpose of the additional protocol 'the right to a safe, clean, healthy and sustainable environment' means the right of present and future generations to live in a non-degraded, viable and decent environment that is conducive to their health, development and well-being.¹⁴

The draft text of the additional protocol to the ECHR enshrines an enforceable right to 'a safe, clean, healthy and sustainable environment'. Members of the Assembly have agreed on the following general principles, in interest of conducting: (a) principle of transgenerational responsibility, equity and solidarity (Article 2); (b) principle of environmental non-discrimination (Article 3); (c) principle of prevention, precaution, non-regression and in dubio natura (Article 4).

Finally, the substantive and procedural rights are also included. The adoption of the proposed additional protocol would give the European Court of Human Rights a non-disputable base for decisions in connection with human rights violations arising from environment-related adverse acts.

¹¹ Parliamentary Assembly Recommendation 2211(2021), Council of Europe. Text adopted by the Assembly on 29 September 2021 (27th sitting).

¹² Appendix - The proposed text for an additional protocol to the European convention on Human Rights, concerning the right to a safe, clean, healthy and sustainable environment.

¹³ Recommendation 2211 (2021), Preamble.

¹⁴ Recommendation 2211 (2021), Article 1.

3. CONVENTION ON CIVIL LIABILITY FOR DAMAGE RESULTING FROM ACTIVITIES DANGEROUS TO THE ENVIRONMENT

The Convention aims at ensuring adequate compensation for damage resulting from activities dangerous to the environment and also provides for means of prevention and reinstatement. It considers that the problems of adequate compensation for emissions released in one country causing damage in another country are also of an international nature.

The Convention first explains some technical terms as "dangerous activity", "dangerous substance", "genetically modified organism" and so on. The system of the Convention is based on objective liability taking into account the "polluter pays" principle. However, specific rules are provided concerning the fault of the victim, causation, joint liability of the operators of installations or sites for damage, and a compulsory financial security scheme to cover liability under the Convention.

The Convention provides that persons interested have the right to access to information held by bodies with public responsibility for the environment.

The Convention sets up a Standing Committee which is responsible for the interpretation and implementation of the Convention. The Committee may make recommendations as regards the implementation of the Convention and propose any necessary amendments to the Convention.

4. CONVENTION ON THE PROTECTION OF THE ENVIRONMENT THROUGH CRIMINAL LAW

In this Convention, the various signatory states agreed to adopt specific provisions to protect the environment in their criminal law. It therefore contains minimum provisions on environmental criminal law. Interestingly, many of the ideas presented above can be found in this convention, more particularly the three types of provisions mentioned above.

The core of the Convention is-not surprisingly-a concrete endangerment crime, which can be found in Article 2. This Article includes a long list of behaviors that the signatory states will criminalize on the basis of their respective national laws. Article 2(lb) refers to: „The unlawful discharge, emission or introduction of a quantity of substances or ionizing radiation into air, soil or water, which causes or is likely to cause their lasting deterioration or death or serious injury to any person or substantial damage to protected monuments, other protected objects, property, animals or plants.“¹⁵

Here, one recognizes clearly the criminalization of concrete endangerment. It is the unlawful emission that is penalized. This provision goes much further than provisions that would merely aim at penalizing the failure to abide by administrative obligations.

¹⁵ Convention on the Protection of the Environment through Criminal Law, Article 2(lb).

Eighth International Scientific Conference
"June 5th - World Environment Day"

The Convention goes further and stakes out an independent crime aimed at serious pollution in Article 2. 1(a): "The discharge, emission or introduction of a quantity of substances or ionizing radiation into air, soil or water, which: (i) causes death or serious injury to any person, or (ii) creates a significant risk of causing death or serious injury to any person[.]" Note that in this particular case, when an emission has the serious consequences of causing death, serious injury, or creating a significant risk of death or serious injury, the Convention abandons the "unlawful discharge" requirement. This is truly independent in the sense that this provision applies irrespective of the violation of administrative obligations. The idea is that emissions that cause death or serious injury, or create a significant risk of such damage, can never be justified under administrative law.

Finally, the Council of Europe Convention also has the traditional abstract endangerment provisions, but they occupy a less important place in the Convention. More particularly Article 4 refers to the unlawful operation of a plant. Note, however, that in this particular case it holds: "Each party shall adopt such appropriate measures as may be necessary to establish as criminal offences or administrative offences, liable to sanctions or other measures under its domestic law."

The drafters of the Convention clearly recognized that in the case of abstract endangerment, ecologic values are not endangered in the same way as with a concrete endangerment. For those, Member States can rely on administrative offences.

The Council of Europe Convention demonstrates a new attitude towards environmental criminal law. If environmental criminal law is indeed supposed to play an important role in supporting sustainable development, it should not limit itself to sanctioning administrative obligations. Rather, criminal law should grant more direct protection to ecological values. This is clear where the Convention refers to unlawful emissions and recognizes pollution with serious consequences as an independent crime. The Convention preserves the criminal law. In addition, the Convention explicitly refers to administrative offences for crimes of abstract endangerment. This facilitates a toolbox approach, whereby the criminal law may be reserved as an enforcement method of last resort.

5. CONVENTION ON THE CONSERVATION OF EUROPEAN WILDLIFE AND NATURAL HABITATS

The Convention aims to ensure conservation of wild flora and fauna species and their habitats. Special attention is given to endangered and vulnerable species, including endangered and vulnerable migratory species specified in appendices.

The Parties undertake to take all appropriate measures to ensure the conservation of the habitats of the wild flora and fauna species. Such measures should be included in the Parties planning and

Eighth International Scientific Conference "June 5th - World Environment Day"

development policies and pollution control, with particular attention to the conservation of wild flora and fauna. The Parties undertake to promote education and disseminate general information concerning the need to conserve species of wild flora and fauna and their habitats.

The Convention establishes a Standing Committee on which the Parties are represented by their delegates. The Committee's principal task is to monitor the provisions of this Convention in the light of development of the wild flora and the assessment of its needs. For this purpose, the Standing Committee is especially competent to make recommendations to the Parties and amendments to the appendices where these protected species are specified.

6. COUNCIL OF EUROPE LANDSCAPE CONVENTION

The Convention aims to encourage public authorities to adopt policies and measures at local, regional, national and international level for protecting, managing and planning landscapes throughout Europe. It covers all landscapes, both outstanding and ordinary, that determine the quality of people's living environment. The text provides for a flexible approach to landscapes whose specific features call for various types of action, ranging from strict conservation through protection, management and improvement to actual creation.

The Convention proposes legal and financial measures at the national and international levels, aimed at shaping "landscape policies" and promoting interaction between local and central authorities as well as transfrontier cooperation in protecting landscapes. It sets out a range of different solutions which States can apply, according to their specific needs.

The Council of Europe intergovernmental committees will be supervising the convention's implementation. The text also provides for a Council of Europe Landscape award, to be given to local or regional authorities or an NGO which introduced exemplary and long-lasting policies or measures to protect, manage and plan landscapes.

7. CONCLUSION

As environmental protection is directly related to the survival of humans and other living beings on the planet, it is of great practical importance how the legal framework will be designed, which will enable the prevention of such phenomena and the remediation of harmful consequences. The legal framework, which should enable effective environmental protection, is based on the reaffirmation of the preventive approach. In order for the values treated by the norms that regulate the ecological relationship to come to life, they must be protected. The threat of sanctions is necessary for unwanted threats and violations of these values.

Bosnia and Herzegovina is a member of the Council of Europe, and is bound by the norms of the conventions it has ratified. Of the four "environmental" conventions, Bosnia and Herzegovina is bound by two. In the coming period, the necessary activities related to the signing and

Eighth International Scientific Conference "June 5th - World Environment Day"

ratification of the remaining two conventions should be undertaken, as well as the harmonization of national legislation with the conventions, especially in the direction of preventive action.

Bosnia and Herzegovina is a member of the Convention on the Conservation of European Wildlife and Natural Habitats (ETS No. 104) and the Council of Europe Landscape Convention (ETS No. 176). The Convention on the Protection of the Environment through Criminal Law (ETS No. 172) has not entered into force, as only Estonia has ratified it to date. The Convention on Civil Liability for Damage resulting from Activities Dangerous to the Environment (ETS No. 150) also did not enter into force, as it was not ratified by any member state of the Council of Europe.

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Eighth International Scientific Conference
"June 5th - World Environment Day"

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CRIMINAL OFFENSES AGAINST THE ENVIRONMENT IN THE REPUBLIC OF SERBIA

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ABSTRACT:

Our environment is our planet Earth. During the development of the human population, first, as much as was necessary for survival and life was taken from the environment, and then people, in their desire to make a profit, began to take much more, thus disrupting the natural balance. Ecocentric behavior has brought man into a world that recognizes criminal acts against the environment, which are regulated by criminal law.

Through a review and comparative analysis of statistically available data on environmental crimes in recent years, which define eighteen crimes according to the Criminal Code of the Republic of Serbia, the authors of the paper provide an overview and analysis of the most common crimes, the material damage caused by these crimes, and the damage to life the environment and the health of all people. By comparing with the data of the countries of the region, behavioral trends were observed.

1. INTRODUCTION

When we talk about the environment, we often come across the following terms: environment, natural environment, working environment, human environment, etc. Each of these expressions refers to a specific part of the space of the planet Earth and does not depend on its size. Human evolution has developed procedures and technologies for cleaning the so-called own

Eighth International Scientific Conference "June 5th - World Environment Day"

environment, while on the other hand, pollution is spontaneously or intentionally moved from one environment to another, contributing to further pollution. This further indicates that no "environment" is and cannot be isolated from the others, thus indicating that our environment is actually made up of our planet Earth as a unique system. By disturbing only one of the parameters, we affect a series of changes in these complex interdependent processes.

The main cause of environmental damage is the desire to make a profit. In order to achieve environmental protection, it is necessary to reduce profits. That is why environmental protection is more or less neglected in favor of profit (Đarmati Š et al. 2008).

Environmental law represents one of the branches of legal regulation that began to develop more intensively at the beginning of this century, even if many laws in this area were passed much earlier. The process of accession to the European Union further accelerated the development of this legal discipline, especially the part related to the establishment of the criminal legal framework for environmental protection, so the turning point was the year 2004, when four systemic laws were adopted: the Law on Environmental Protection, the Law on strategic environmental impact assessment, Law on environmental impact assessment and Law on integrated prevention and control of environmental pollution (Čavoški and Trajković 2021) and then the Criminal Code, which for the first time systematizes criminal acts against the environment into a single entity. In the criminal legislation of the Republic of Serbia, with the beginning of the implementation of the new Criminal Code, for the first time, all criminal offenses aimed at protecting the environment are listed in one place. Therefore, now all criminal offenses are classified under Chapter XXIV and bear the title: "Criminal offenses against the environment". Also, a certain number of criminal acts against the environment are regulated by special secondary legislation and are provided for in other laws. Crimes against the environment do not protect the classic human rights, which are life and health, as they did before, but an independent good, which is the environment, or more precisely, the human right to a preserved environment. In earlier years, the environment was protected by some criminal acts such as criminal acts against people's health, criminal acts against the economy, criminal acts against general security, etc. In our country, legal entities could only be held liable for misdemeanors and economic offenses, and criminally only since 2008, when the Law on Liability of Legal Entities for Criminal Offenses was adopted (Joldžić 2007).

Until the second half of the 20th century, there was no global approach to this problem on the international level, but mostly interest was focused on certain segments of the environment, such as endangered plant and animal species or sea pollution with oil, etc. Only in the second half of the 20th century, the constant activities of international organizations in the field of the environment resulted in the creation of institutional frameworks and the constitution of legal norms embodied in the Declaration of the United Nations Conference on the Environment adopted in Stockholm in 1972. In the Declaration, the main goal of ecological development is the

Eighth International Scientific Conference "June 5th - World Environment Day"

reduction of risks in terms of living conditions and the improvement of the quality of life, while special attention is paid to the protection of the environment. Two decades later, in 1992, the UN Conference on Environment and Development was held in Rio de Janeiro, with concerns expressed over the issues of global warming and loss of biological diversity. Very important principles were adopted that determined further activities in this area. In this regard, the Constitution of the Federal Republic of Germany guarantees animals protection by prescribing the responsibility of the state to protect natural sources of life and animals by law (Article 20a), while Switzerland in its Constitution emphasizes the dignity of creatures as a value that is inherent to every living being (Article 120, paragraph 2), while the Constitution of the Republic of Serbia does not include an explicit provision guaranteeing the dignity and protection of animals (Bajović 2023).

Among the many international directives, the European Union Directive on responsibility in the field of environmental protection from 2004 should be mentioned, which established the "polluter pays" principle as a legal means of environmental protection. Measures and procedures are foreseen that prevent or mitigate the consequences of the resulting damage. Although in practice there were dilemmas and vagueness in the application and implementation of this Directive into national legislation, it still focused attention on responsibility for damage to the environment, which was also adopted by our country.

It should also be mentioned the Law on Liability of Legal Entities for Criminal Acts (Official Gazette of the RS, No. 97/08), which provides for the criminal liability of legal entities. When the Republic of Serbia ratified the Stabilization and Association Agreement with the EU in 2008, the legislative initiative in this matter was further accelerated.

In science, the prevailing view is that criminal law is an autonomous branch of law, although it does not itself create the values it protects, but mostly borrows them from other legal or extralegal areas of social relations (Stojanović 2011). According to the Law on Environmental Protection of the Republic of Serbia (Official Gazette of the RS, No.135/2004, 36/2009, 36/2009 – another law, 72/2009 – another law, 43/2011 decision of the Constitutional Court, 14/2016, 76/2018, 95/2018 – another law and 95/2018 – another law) the terms pollution, endangerment, burden and degradation of the environment can be defined in particular, which unites the most common actions of the human factor that disrupts the natural balance.

Criminal law is a set of legal regulations which, in order to suppress crime, determine which human behaviors will be qualified as criminal acts and which criminal sanctions, as well as under what conditions, can be imposed on the perpetrators of those acts.

2. METHODOLOGY OF WORK

The methodology of this work included a review of available literature, collection and analysis of statistical data officially published as part of the Annual Reports of the Public Prosecutor's Office of the Republic of Serbia, from 2023, under the title of the Supreme Public Prosecutor's Office, available on their official website, with the aim of drawing attention to criminal offenses against the environment, which is the basis for the life and health of all living beings.

3. RESULTS AND DISCUSSION

In the Criminal Code of the Republic of Serbia (Official Gazette of the RS, no. 85/2005, 88/2005-corrected, 107/2005 - corrected, 72/2009, 111/2009, 121/2012, 104/2013, 108/2014 , 97/2016 and 35/2019) eighteen criminal offenses against the environment are singled out in Chapter XXIV:

1. Pollution of the environment (Art. 260)
2. Failure to take environmental protection measures (Art. 261)
3. Illegal construction and commissioning of facilities and equipment that pollute the environment (Art. 262)
4. Damage to facilities and devices for environmental protection (Art. 263)
5. Damage to the environment (Art. 264)
6. Destruction, damage and export abroad and import into Serbia of protected natural property (Art. 265)
7. Bringing dangerous substances into Serbia and illegal processing, disposal and storage of dangerous substances (Art. 266)
8. Unauthorized construction of nuclear facilities (Art. 267)
9. Violation of the right to information about the state of the environment (Art. 268)
10. Killing and abuse of animals (Art. 269)
11. Transmission of infectious diseases in animals and plants (Art. 270)
12. Negligent provision of veterinary assistance (Art. 271)
13. Production of harmful means for the treatment of animals (Art. 272)
14. Contamination of food and water for consumption, i.e. animal feeding (Art. 273)
15. Devastation of forests (Art. 274)
16. Forest theft (Art. 275)
17. Illegal hunting (Art. 276)
18. Illegal fishing (Art. 277)

According to the official statistical data of the Supreme Public Prosecutor's Office of the Republic of Serbia, shown in the past six years (from 2018 to 2023), on the territory of the Republic of Serbia, the largest number of reported and prosecuted crimes against the

Eighth International Scientific Conference "June 5th - World Environment Day"

environment are forest thefts, for which the perpetrators of the crime have a motive had in achieving economic profit. According to the data summarized by the review of statistical data in the last six years, one can see a trend of decreasing the number of unsolved cases and thus the number of new reports, where it can be concluded that the trend of committing this criminal offense is decreasing. Regardless, the number of prison sentences did not change significantly, but a proportional decrease can be seen in the total number of convictions (Table 1).

Table 1: Overview of cases of the criminal offense of forest theft in the Republic of Serbia from 2018 to 2023

Year	The number of pending cases from an earlier period	Number of new applications	Sum	Rejected	Indictment proposal	Prison sentence	A monetary penalty	Work in the public interest	Conditional sentence	Judicial warning	Total number of guilty verdicts
2023.	870	982	1852	750	319	43	78	7	157	2	287
2022.	826	1198	2024	692	355	52	72	66	153	5	348
2021.	1051	938	1989	776	375	40	69	9	193	7	318
2020.	1078	1094	2172	737	384	24	60	7	198	/	289
2019.	1410	1299	2709	1070	559	43	83	13	271	2	412
2018.	1752	1543	3295	1179	649	49	55	9	348	9	471

Comparing the data on the gender representation of the perpetrators of criminal acts, the dominant gender is male, while women were prosecuted significantly less. In a small number of cases, minors were also recorded as perpetrators of criminal acts against the environment in the Republic of Serbia.

Apart from forest theft as the most prevalent criminal offense against the environment in the Republic of Serbia, table 2 provides an overview of the number of convictions for other criminal offenses against the environment in Serbia, for the period from 2018 to 2023. After forest thefts, in the last year of 2023, the most reported crimes were due to crimes: executions for polluting the environment, bringing dangerous substances into Serbia and illegal processing, disposal and storage of dangerous substances and killing and abusing animals.

Table 2: Ratio of the number of total reports and legally convicted perpetrators of crimes against the environment in Serbia, in the period from 2018 to 2023.

Criminal act	2023.		2022.		2021.		2020.		2019.		2018.	
	Total number criminal reports *	Condemning judgments	Total number criminal reports *	Condemning judgments	Total number criminal reports *	Condemning judgments	Total number criminal reports *	Condemning judgments	Total number criminal reports *	Condemning judgments	Total number criminal reports *	Condemning judgments
Environmental pollution	302	137	46		43	2	41	1	30		35	
Failure to take environmental protection measures	139	43	30		26		11		10		15	
Illegal construction	8		3		3		2		2			

Eighth International Scientific Conference
"June 5th - World Environment Day"

and commissioning of facilities and plants that pollute the environment												
Damage to facilities and devices for environmental protection	5											
Damage to the environment	20		24	1	12	1	7		10		9	1
Destruction, damage and export of protected natural assets abroad and import into Serbia	29	6	19	2	11	2	11	4	11	3	17	2
Bringing dangerous substances into Serbia and illegal processing, disposal and storage of dangerous substances	231	54	75		31	2	37		40	2	26	6
Illegal construction of nuclear facilities	1		1		1		1		1			
Violation of the right to information about the state of the environment	18		48		99	2	3		3		3	
Killing and abusing animals	278	34	269	30	303	29	263	19	266	24	251	30
Transmission of infectious diseases in animals and plants	7	1	7		20	2	1		5			
Negligent provision of veterinary care	2				3		10		6			
Production of harmful means for the treatment of animals	1	1										
Contamination of food and water for feeding, i.e. feeding animals	10	1	5	1	3		2		2		3	1
Deforestation	107	18	131	18	108	13	139	16	168	12	213	37
Forest theft	1849	287	2013	348	1989	318	2172	289	2709	412	3295	471
Illegal hunting	116	7	148	15	151	17	119	6	154	10	212	23
Illegal fishing	68	16	47	13	49	1	44	5	49	6	50	6
SUM	3191	605	2866	428	2852	389	2863	340	3466	469	4129	577

* The total number of applications represents the total number of pending cases from the previous period and new applications in the reporting period

Eighth International Scientific Conference "June 5th - World Environment Day"

Comparing the total number of processed reports in all years, with the total number of convictions in previous years, it can be concluded that there was a total reduction in the number of criminal offenses against the environment until 2023, where a significant increase in the number of reports for crimes involving the introduction of dangerous material into Serbia and the illegal processing, disposal and storage of hazardous materials, but also the act of environmental pollution. These data are worrisome considering the negative impact such acts have on human health and the entire environment.

The devastating data are the numbers that indicate that the crime of "killing and abusing animals" is still significantly represented, which shows a growing trend in the total number of cases in recent years. According to the Criminal Code of the Republic of Serbia, whoever kills, injures, tortures or otherwise abuses an animal in violation of the regulations, will be punished with a fine or imprisonment for up to two years. Amendments to the Criminal Code (Official Gazette RS 35/2019) which entered into force on December 1, 2019. the threatened prison sentence for the basic form of this criminal offense was increased from one year in prison to two years in prison. This shows that the importance of punishing this act is recognized. The prison sentence can be higher (up to three years) with a mandatory fine and a fine if a person for profit organizes, finances or hosts a fight between animals of the same or different species, as well as for those who organize or participate in betting on such fights. Considering the possibility of punishment, such "events" are organized in secret, and for this reason they often go unpunished. According to the Ministry of Internal Affairs of Croatia, "Killing or torturing animals" as a crime in the neighboring Republic of Croatia is the most common crime against the environment in that country, which according to their official statistical report from 2021, recorded a trend of reducing material damage by 80% in 2020 compared to 2019.

One of the positive aspects in the Republic of Serbia shown by the aforementioned statistics is that no cases of the criminal offense of "production of harmful means for the treatment of animals" (Article 272) have been reported or they have not been recorded and publicly reported to the competent authorities.

A special challenge in practice is the collection, securing and further use of evidence in the prosecution of criminal offenses in the field of environmental protection, according to the nature of the criminal offences, for which public prosecutors, as managers of the pre-investigation procedure, do not have adequate professional knowledge (Samuilov 2023). In practice, it is not often the case that the public prosecutor invites the environmental protection inspector to provide professional assistance to the public prosecutor during the investigation, which would contribute to better recognition and fixation of relevant evidence. Due to these shortcomings, as well as situations in which the public prosecutor rejects the criminal complaint due to the lack of relevant evidence about the criminal act and the perpetrator, the real perpetrators often go unpunished. This is the most common case when citizens or non-governmental organizations file

Eighth International Scientific Conference "June 5th - World Environment Day"

criminal charges, as persons who found the situation, not knowing what preceded the events that resulted in such an act, making subjective claims that have no basis in the actual factual situation. The ratio of the number of total applications and the number of rejected applications in previous years on the territory of the Republic of Serbia (Table 1) shows that almost a third of applications are rejected, which confirms the previously mentioned problem.

It should be emphasized that the provisions of the Criminal Code do not regulate trade in protected plant and animal species, but this type of offense is legally sanctioned based on the Rulebook on Cross-Border Traffic and Trade in Protected Species (Official Gazette of the RS, No. 99 from 1.12.2009. and 6 from 24.1.2014.) which resulted from the articles of the Law on Nature Protection (Official Gazette of the RS No.36/09) and Article 28, paragraph 5 of the Law on Environmental Protection (Official Gazette of the RS, No. 135/04,36/09 i 72/09 – another law). Special attention should be paid to that topic, especially if we look at the facts that indicate that the pandemic situation with the COVID-19 virus, according to the announcement of the World Health Organization, is a zoonotic disease that has passed from animals to humans.

Protecting endangered species has many benefits for the entire community, and contributes to human well-being and satisfaction, among other things. If a species were to become extinct, in addition to the reduction in gene stocks, the benefits that humans had from it, especially agricultural and medical benefits, would also be significantly reduced. Plants and animals in themselves have a right to life, which should be respected even if they are of little use to humans because humans did not create the diversity of life on Earth, so they should not destroy that creation either.

Competent authorities can find out about criminal acts against the environment primarily based on reports from inspection bodies and institutes. In order for the public prosecutor's office to act on the basis of the submitted reports, it is necessary that they contain evidence of a certain quality, which is often a problem, and for this reason a large number of criminal offenses do not "enter" the procedure or later criminal reports are rejected due to lack of evidence. For this reason, it is important to adequately train employees in the competent inspection bodies and institutes so that they can recognize the grounds of suspicion that indicate the existence of criminal acts against the environment, as well as evidence that is important for initiating criminal proceedings and indicting the perpetrators of those acts. In addition to significant collection of evidence of the crime, when it comes to these crimes, often a big problem is the impossibility of determining the identity of the perpetrator of the crime, but also the failure to recognize the crime itself, given that the perpetrators can be sanctioned based on a large number of misdemeanors and economic offenses that are prescribed in the Law on Environmental Protection. For this reason, competent inspection bodies must be well trained and familiar with all the details of criminal acts against the environment so that the perpetrators of those acts are adequately sanctioned. What does not support this is that the Law on Environmental Protection does not explicitly prescribe the obligation of inspection authorities to submit a criminal

Eighth International Scientific Conference "June 5th - World Environment Day"

complaint to the competent prosecutor's office if, during the supervision, they determine that there are grounds for suspicion that indicate the existence of a criminal offense. However, the Law foresees the obligation of the inspector to, in the course of the inspection, assess that, in addition to the violation of the law, other laws and regulations regulating matters of importance for the protection of the environment or a particular part of it have been violated, to notify another competent authority in addition to taking the measures for which he is authorized .

An important role in environmental protection, reporting and reducing the number of crimes against the environment should be played by the media, which would draw the attention of the public with their reports and raise the awareness of the population, who would be encouraged to report the observed criminal acts in their surroundings to the competent authorities.

In March 2022, for the first time in the Republic of Serbia, the Unit for Suppression of Crime and Environmental Protection was established, whose task is to ensure a unique and professional procedure in detecting and proving this group of criminal acts. Through direct cooperation with all police administrations and judicial authorities in order to enable the timely and efficient prosecution of all natural and legal persons responsible for destroying or damaging the environment in any way, a greater number of prosecuted acts were recorded during the previous year. As of August 2023, this Unit was reorganized into the Environmental Crime Suppression Department of the Crime Suppression Service of the Criminal Police Directorate of the Ministry of Internal Affairs of the Republic of Serbia.

4. CONCLUSION

In the last few years, we have witnessed the changes that are taking place in the Republic of Serbia in relation to raising the awareness of citizens and the state's reaction to the increasingly pronounced need for environmental protection. However, this topic is still insufficiently present in the public eye, as evidenced by the data on anthropogenic negative impacts on the environment. The Constitution of the Republic of Serbia recognizes the right to a healthy environment as one of the basic human rights and represents a good legal framework for the detailed legal regulation of this very important topic, not only for the citizens of Serbia, but also for the entire society. Many laws regulating this area have been adopted, international treaties have been ratified, numerous by-laws have been adopted, and many are being prepared. For almost two decades in our Criminal Code, environmental crimes have been given due importance, because these crimes have been systematized in a separate chapter of the Code. The existence of the Department for Suppression of Environmental Crime within the criminal police of the Ministry of Internal Affairs of the Republic of Serbia is a good basis for improving the situation in this area with a tendency to reduce crimes. Our laws are largely harmonized with international legal frameworks, but there is a need for public prosecutors to be additionally

Eighth International Scientific Conference "June 5th - World Environment Day"

trained for these crimes and to use more of the specialized professional knowledge of environmental protection inspectors during the investigation. Also, it is necessary to give additional guidelines and provide a technique for adequate collection of evidence, as well as their preservation during the duration of the criminal procedure.

The overview of offenses and the analysis given in this paper for the period 2018-2023. year, serve only as an illustration to obtain an appropriate picture for further directing attention and improving the System of protection, preservation and improvement of the environment, all its media and all living beings.

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THE IMPACT OF TRAFFIC NOISE IN THE UNA-SANA CANTON

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Key words: noise, protection, laws, traffic, Una-Sana Canton

ABSTRACT:

Noise is defined as any unwanted sound, which at higher volume can have harmful and even pathological effects. In the urban environment, where most of the human population is located, whose health may be at risk, the main source of noise is traffic. Although people differ in their sensitivity to noise, prolonged exposure is sure to have consequences for everyone. Any noise above 85 decibels is considered to damage hearing. In addition to hearing damage, prolonged exposure can also lead to health problems such as high blood pressure, nervous breakdown, accelerated heart rate and even heart attack. The Law of Bosnia and Herzegovina prescribes noise protection measures that also apply to the Una-Sana Canton. The aim of this work is to find out whether and to what extent these protection measures are respected and implemented in the Una-Sana Canton, because traffic is an indispensable sphere of life, and then to propose measures to reduce it.

1. INTRODUCTION

Today's everyday life is a constant noise that surrounds man from all sides. Is it possible to know true silence today and what kind of fear would prevail among people? However, why can't a person feel real silence? With its arrival, the modern era brought a large number of innovative devices and machines, without which a person's life would be incomplete. Unfortunately, the side effects of most of them are the sounds they make, no matter how small those sounds seem. In the urban environment, where most of the human population is located, whose health may be

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at risk, the main source of noise is traffic. Although people differ in their sensitivity to noise, prolonged exposure is sure to have consequences for everyone. Although the Constitution of Bosnia and Herzegovina prescribes laws that prescribe the permitted noise level, noise protection measures, the method of noise measurement and recording, noise limit levels according to the environment, the purpose of the space and the time of day (day or night), for the purpose of protecting people's health, working and life space and surroundings and the guidelines of the FBiH and RS Road Directorate for planning and maintenance of structures for noise protection, the non-implementation of these is visible. In addition, a strategy and action plan called BiH ESAP 2030+ is being developed, which for the first time includes the issue of noise management in the strategic planning of environmental protection at all levels of government in Bosnia and Herzegovina. This strategy will enable the creation of noise maps, strategic noise maps, noise action plans and noise monitoring. Mirković researched the legal regulations and the impact of air traffic on noise, highlighting the "Nikola Tesla" Airport, which since 2014 is under a legal obligation to measure noise, create strategic noise maps and create an action plan for noise protection (Mirković, 2015). However, due to the possible construction of an airport in the area of the Unsko-Sana Canton, an environmental impact assessment study was also conducted for JP Aerodrom Bihać, which was the only one to examine the impact of air traffic noise on all surrounding populated areas. Xiodong Lu and colleagues talk about the reasons for increased noise and make suggestions for improving the quality of life in urban areas (Lu, Kang, Zhu, Ca, Guo, et al., 2019), while Weijun Yang and colleagues do an assessment of noise pollution based on noise maps (Yang, He, He and Cai, 2020). Zambon et al emphasize the importance of proper mapping of noise maps and give suggestions for reducing traffic noise at night (Zambon, Roman, Smiraglia, and Benocci, 2018). Đorđić et al. deal with the influence of combinations of noise from substations and traffic (Đorđić, Đurić, and Milotić, 2021). Turčinović carried out noise control and analysis with permissible values defined by legal provisions and offered a choice of possible solutions for reducing the impact of noise (Turčinović, 2020), while Mošić et al analyzed noise reduction due to the use of protective barriers (Mošić, Vidović and Đorić, 2019). Lai Fern Ow and S. Ghosh talk about the reduction of road traffic noise through vegetation at different planting intensities (Ow and Ghosh, 2017). Traffic noise has an enormous impact on the mental health of people, which is being investigated by O. Klompmaker and colleagues (Klompmaker, Hoek, Bloemsma, Wijga, Brink, et al., 2019). Through this paper, an overview of the places in the Una-Sana Canton, the number one canton of the Federation of Bosnia and Herzegovina, with the most influential traffic noise will be given, and compliance with legal regulations for the protection of people and the environment from noise will be determined, and then measures will be proposed to combat this type of noise. noise in order to improve the quality of life and protect people's health.

2. APPLICATION OF THE NOISE PROTECTION LAW IN THE UNA-SANA CANTON

The Una-Sana canton, with its area of 4125 km², is the canton that connects the rest of Bosnia and Herzegovina with the countries of the European Union. It can be said that precisely because of this, transit traffic dominates, and therefore transit tourism. Noise protection using walls and protective panels is mostly applied on highways. However, not a single highway passes through the Unsko-Sana Canton. This does not mean that traffic noise protection should not be applied, because in this canton traffic is carried out via the main roads, of which there are many (M4.2, M5, M11, M14, M14.2, M15). Although the canton is not densely populated (273,261 inhabitants), a large number of main roads pass through city hubs. This is exactly why noise is inevitable and affects the population around it to a great extent. Although the laws are clear, they are not put into practice. During data collection, no noise measurement was found in road traffic in the Una-Sana Canton, although its presence is visible. The only study that has been done is a study on the impact of air traffic noise on the environment for Bihać airport in Golubić. In the territory of the Federation of Bosnia and Herzegovina, the Law on Noise Protection ("Official Gazette of the Federation of Bosnia and Herzegovina", number: 110/13) is applied. This law was promulgated on December 7, 2012 and adopted by the Parliament of the Federation of Bosnia and Herzegovina at the session of the House of Representatives and the session of the House of Peoples. It was published in the Official Gazette of the Federation of Bosnia and Herzegovina, number 110/13. The assessment of the impact of noise on the environment and people is also carried out on the basis of the provisions of the Law on Noise Protection ("Official Gazette USK", number: 1/04 and 11/04). The deadline for equating noise protection with the permitted noise level prescribed by this law is 10 years from the date of entry into force (until December 2022). However, although it is mandated by law, it is not implemented in reality. There are no noise measurements, nor measures to protect people and the environment from noise sources. The close connection between residential buildings and schools with main roads primarily affects people's health.

In the following chapters, places on the main roads in the Unsko-Sana canton will be presented, where the implementation of noise protection measures would significantly improve the living standards of the population living near these roads and the proposal for these measures.

3. BEDEM STREET – BIHAĆ, M5

Bedem Street is located in the city center of Bihać and one of the busiest roads passes through it, which is part of the M5 highway. The road consists of one carriageway, i.e. two traffic lanes in the same direction. On the right side there is a village and an elementary school, and on the left side there is a row of residential buildings. The noise produced by vehicles is unavoidable. The belt around the road is relatively narrow.

A proposal to reduce noise, in addition to replacing it with a higher-quality road curtain, is also transparent panels that do not create a feeling of anxiety in drivers and do not separate them from the landscape. Also, due to their transparency, they do not reduce the light needed by residential buildings. In order to reduce noise as effectively as possible, it is necessary to reduce and limit the speed on a given route. Figure 1 shows a map of the considered street Bedem, part of the M5 highway.



Transparent panels are shown in Figure 2, from which it can be seen that, due to their transparency, they provide safety to road users. At the same time, the surrounding area (in this case, the school and residential buildings) will be protected from noise to a greater extent.

4. VRTOČE VILLAGE - BOSANSKI PETROVAC, M5

The main road M5, E761 passes through the village of Vrtoče. It is the section that connects Bihać and Bosanski Petrovac. On both sides of the road there are residential buildings and an eco village. The noise produced by vehicles is unavoidable. The belt around the road is wider. A proposal to reduce noise, in addition to replacing it with a higher-quality road curtain, is also transparent panels that do not create a feeling of anxiety in drivers and do not separate them from the landscape.

Figure3 shows a map of the Vrtoče village through which part of the M5 highway passes.

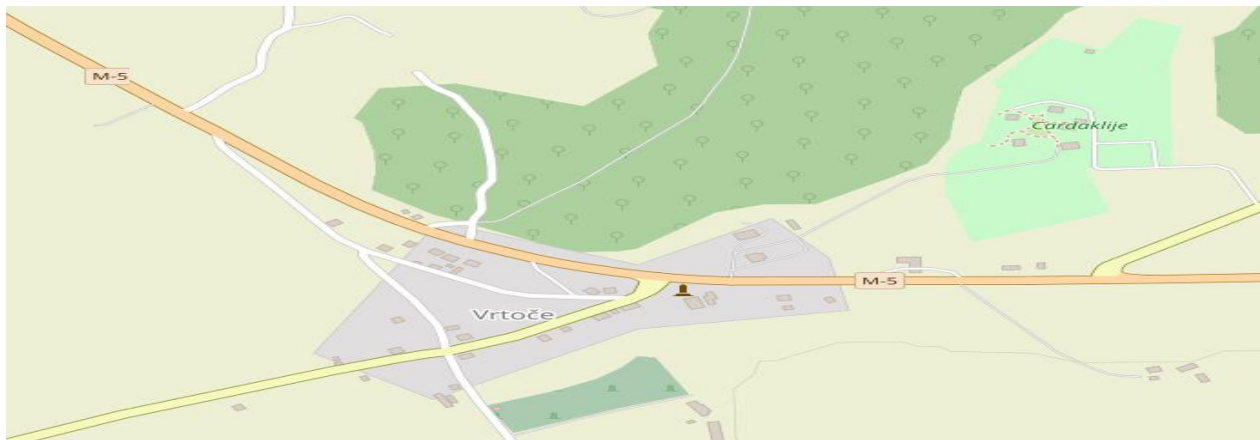


Figure: View of the main road through the village of Vrtoče

Due to their transparency, they do not reduce the light needed by residential buildings. Since the belt around the road is much wider, other sound barriers can be applied, such as aluminum panels and wooden fences.

5. BIHAĆ AIRPORT, GOLUBIĆ VILLAGE

During data collection, no noise measurement was found in road traffic in the Una-Sana Canton, although its presence is visible. The only study that has been done is a study on the impact of air traffic noise on the environment for Bihać airport in Golubić. The study showed that the impact of noise will be a permanently present problem and that it will affect the following settlements of the city of Bihać: Sokolac, Golubić, Pritoka, Orljani and Novo Naselje.



Figure 4: View of the existing runway of the airport in Bihać

However, it should be emphasized that this impact will not be significant in the initial phase of use due to the sporadic frequency of take-offs and landings. This does not mean that the influence of noise as such will not pose a problem for the surrounding population. First of all, it is necessary to implement all the laws into reality and thus stop the displacement of the population in the vicinity of the airport. The law of the FBiH stipulates that the owner of the noise source is obliged to ensure sound insulation of the windows in all endangered objects, so that the noise level is in accordance with the table of permitted noise levels from external sources in the premises according to their purpose, within 6 months, regardless of the quality window was previously. However, this law has not found its way into reality. Since the project of the airport in Bihać is a current topic, when allocating and ensuring the costs, it is necessary to take into account the aforementioned law, in order to better protect the health of people in the immediate vicinity.

A view of the existing runway of the current sports airport in the Golubić settlement is shown in Figure 4.

It is important to emphasize that the first presence of large-scale noise will occur at the beginning of construction. Then the actual noise level should be measured and measures to reduce it should be planned based on that. The construction of the airport in Bihać is a current topic and all funds for its construction have already been secured. It is assumed that the first planes will take off from the airport in approximately 3 to 4 years. However, it was not stated whether the investor provided the funds for the insurance of nearby facilities for noise protection, which, in addition to being legally prescribed, is also an important investment for people's health, but also for preventing the displacement of people from these settlements of the city of Bihać.



Figure 5: The project of the future airport in Bihać

Figure 5 shows a 3D model of the airport planned to be built in Bihać.

6. CONCLUSION

Based on the research conducted during the preparation of this work, it can be concluded that, in the modernized world we live in, noise is inevitable. Although it has extremely harmful effects on people and their lives, both in the whole of Bosnia and Herzegovina and in the Una-Sana Canton, almost nothing has been done to reduce it, and the laws for implementation do exist.

Throughout this paper, legal legislation on noise protection is mentioned, in which traffic noise is specifically singled out. The laws were adopted both in the entire state of Bosnia and Herzegovina, as well as in the Federation of Bosnia and Herzegovina and in the Una-Sana Canton. However, the extent to which these laws are respected and implemented in reality is a big question. Also, protection investments in existing facilities are questionable. Noise is all around us, it comes from various sources, but people are not aware of the danger it carries with it. As a small contribution to raising awareness of this topic, proposals were made to reduce traffic noise in populated areas on the M5 highway. In addition to the main roads, the increasingly realistic construction of an airport in the vicinity of the city of Bihać, i.e. in the settlement of Golubić, was also mentioned.

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UNDERSTANDING SOUVENIRS IN TOURIST CONSUMPTION OF EU TOURISTS IN BOSNIA AND HERZEGOVINA

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Key words: Role of souvenirs, Factors Influencing Tourists to Purchase Souvenirs, Souvenir Shopping, Tourist Experience, Bosnia and Herzegovina souvenirs

ABSTRACT:

Souvenirs are an integral and intangible part of the tourist experience, acting as a guiding element for tourists. Purchasing souvenirs is considered the most universal of all tourist activities, and souvenir sales are often seen as an effective way for communities to leverage the economic advantages of tourism. Besides economic factors, the role of souvenirs in tourism is considered a significant component of the tourist experience. The importance of souvenirs can be viewed as an efficient promotional tool for tourist attractions. Therefore, it is essential to explore the role of souvenirs in consumer behavior, triggers, motivations, and the meaning of souvenirs for consumers to contribute to better tourism offerings.

This study is based on a focus group of EU tourists who visited Bosnia and Herzegovina during summer of 2002. The research focused on the role of souvenirs in consumer behavior, exploring the role of souvenirs in tourism, souvenirs as memory triggers, souvenirs as holiday experiences, souvenirs as gifts, and collectible items. Additionally, factors influencing souvenir purchases by tourists such as motives, motivation, perception, perceived quality, and value were investigated. Thus, the impact of emotional factors and souvenirs in Bosnia and Herzegovina was explored.

The results of the research on the target group indicate that EU tourists recognized the souvenirs as something valuable, something that should be unique, handmade, or art craft, i.e., the object that has value. The main function of souvenirs is to be reminded of the visited place, as well as a gift to the close people. Understanding favorable souvenirs and comprehending the factors

influencing souvenir purchases are crucial in tourism research. This study will contribute to souvenir traders and tourism as a whole to tailor their offerings to tourists, thereby contributing to the strengthening of tourism in Bosnia and Herzegovina.

1. INTRODUCTION

Souvenirs are an integral and intangible part of the tourism experience, acting as an integral part of guiding tourists (Singh, 2018). The tourism sector has noticeable effects on the growth of the economy, especially in developing countries (Ibadat, 2016). Shopping is said to be the most universal of all tourist activities, and selling souvenirs has been repeatedly considered an effective way for communities to take economic advantage of tourism. The development of the tourism industry contributed to the rapid development of the tourist souvenir market (Wu et al, 2022). A souvenir is a representative reminder of some special moments, places, events, and people (Siregar et al, 2017). Besides that, souvenir purchase motivation provides an important understanding of consumer behavior (Wilkins, 2009). Also, some authors like (Siregar et al, 2017) determined the four factors that affect the tourists' behavior such as personal factors, social factors, and psychological and situational factors. The importance of souvenirs can be seen as an effective promotional tool for tourist attractions (Singh, 2018).

2. MULTIPLE ROLES OF SOUVENIRS IN TOURISM

Souvenirs are highly significant material objects that can keep and remind tourists of travel memories. Souvenirs are memorable reminders of special moments while traveling (Trinh et al, 2014). According to the research of these authors, a souvenir is both a memory keeper and a memory trigger. The study reveals that souvenir serves as a symbol of travel that evokes memories after the journey. Furthermore, Wilkins (2013) explained that there is an important connection between memories and souvenirs in the post-purchase behavior of tourists. Purchased souvenirs serve as a memory of the visited place. Also, the importance of memory is seen in hospitality consumer behavior, because it enables consumers to recall or recognize previous experiences.

Based on the studies of Wilkins (2013), souvenirs reflect the importance of travel experiences in the construction of the self and as status consumption products. Also, Lunayai et al (2008) researched the tourist vacation experience and the age difference among tourists. The older tourists appreciate more the value of souvenirs and the experience that they had while traveling. They would buy souvenirs that they tend to use at home, so the experience while buying souvenirs is really important to them. Tourists who had a better experience while buying souvenirs tend to buy more local products. Hugh (2011) argues that souvenirs are one of the most important components of the tourist experience because tourists like to be reminded of special moments in their travels by purchasing souvenirs.

The importance of gift-giving is especially important in some cultures like collectivist cultures. In East Asian cultures, gift-giving is one of the ways to build up a relationship with family or friends, and that is one of the main reasons why travelers purchase souvenirs (Kim, 1997). Almost every tourist activity involves purchasing a gift while visiting some place (Heroux and Church, 2014). Another essential point in tourism and souvenirs that Berberović and Kurtović (2021) pointed out is the importance of diaspora tourists and product stocking when they return to their country of origin. Diaspora tourists tend to purchase through two phases:

1. Symbolic representation filling phase
2. Mainstream trend purchase phase (Berberović and Kurtović, 2021).

The diaspora tourists tend to purchase and consume homemade, hand-made, traditional, and local products for the immediate phase (mainstream trend purchase phase), or the products with the visible representation of their country of origin (symbolic representation filling phase. This type of tourist finds local products important, even if they can consume or purchase the same products in the countries of current living, and they like to share their experiences and products with friends abroad.

3. RESEARCH METHODOLOGY

3.1 Research Problem

Tourism has a tremendous influence on to environment, culture, and society (Ibadat, 2016). For developing countries where economic activity is often limited, tourism helps and has noticeable effects on the growth of the economy. The tourism sector provides jobs to increase people's income which is one of the most important sources of economic and social progress (Ibadat, 2016). Different authors and studies focused on different topics and research, but there is a lack of studies on the intentions of consumers to buy souvenirs. Due to the high number of tourist arrivals in Bosnia and Herzegovina in past years, and the significant influence of souvenirs on tourism and the economy overall, there is a need to investigate the intentions of souvenir purchases, the decision process, and the value of souvenirs to tourists. To gain a further understanding of souvenir purchase behavior, this study is based on a qualitative research design, and data was collected through focus groups.

3.2 Research Questions

Based on the research problem, the following research questions were defined:

1. What are the main factors that influence tourist behavior in terms of buying souvenirs?
2. What product categories of souvenirs do tourists buy?

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"June 5th - World Environment Day"

3. What motivates tourists to buy souvenirs?

4. How do emotional factors affect the purchase of souvenirs?

3.3 Sample and Framework

COVID-19 affected the number of tourist arrivals in Bosnia and Herzegovina, and through 2020 and 2021, the number of tourist arrivals decreased compared to 2019. According to the Agency for Statistics of Bosnia and Herzegovina (2022), this country had 960.383 total tourist arrivals in 2021. The focus group data collection method provided a greater understanding of EU tourists and their behavior in terms of tourism and souvenir purchases. The focus group contained six participants.

4. RESEARCH RESULTS

4.1 Coding the EU focus group

Table 1: Coding the EU focus group

Interviewee	Purchased souvenirs and favorable souvenirs	Purpose and description of souvenirs
Participant 1, female (25) Spain	- Handmade products - Unique products - Handmade magnets	- Symbols that represent country, city, or village - Do not purchase mass-produced souvenirs
Participant 2, male (22) Germany	- Purchasing and collecting postcards	- Souvenirs an object that reminds of a visited place
Participant 3, female (22) Germany	- Casual stuff, shoes - Collecting natural souvenirs (flowers) - Do not buy magnets or postcards - Likes extraordinary things	- Not spending money on magnets or postcards to save money for more useful stuff - Buying local cuisine - Purchasing souvenirs for close people
Participant 4, male (22) Germany	- Do not purchase souvenirs	- Memorizing the place without purchasing souvenirs - Using photos or videos as a reminder to trip
Participant 5, male (23) Germany	- Purchasing souvenirs for himself, family, and friends - Purchased coffee pot, baklava, and sweets in B&H	- Souvenirs are attraction - The remainder of the trip - Memories

Eighth International Scientific Conference
"June 5th - World Environment Day"

Participant 6, female (20) Cyprus	- Handmade products for family and friends	- Using souvenirs as a memory trigger

4.2 Souvenir purchases (which items tourists purchase and the frequency of souvenir purchases)

The first section represented the frequency of souvenir purchases generally. In response to the question "Could you please explain to me the process of buying souvenirs?" participants explained the process of buying souvenirs while traveling before. The process of buying souvenirs was similar to all respondents, where they explained how they bought souvenirs in souvenir retail shops in the countries they visited and souvenirs that they liked, except one participant who said:

"So, actually I do not buy souvenirs, I memorize the place I have been to, without their help". (Participant 4, male (22), Germany).

In response to the question: "What does a souvenir mean to you?" all respondents affirmed that for them it means a memory of a visited place, and explained how souvenirs represent the remainder of the trip, or they use it as a gift for friends and family, except one participant who explained the meaning of souvenir in another way:

"I think a souvenir is a kind of symbol that represents country or city or village. Also, for me, it's a tourist trap" (Participant 1, female (25), Spain).

In addition, respondents were asked, "Which items do you usually buy as souvenirs?" The majority of respondents bought similar souvenirs such as magnets and postcards, while one participant had a different answer:

"For me, in the last three years, I decided to buy some casual stuff and for example, I bought shoes from this city and it is also a souvenir for me. (Participant 3, female (22), Germany).

All respondents agreed that they purchase souvenirs mostly in the tourist village or in the city center, in response to the question, "Where do you buy souvenirs?". For the question regarding frequency of souvenir purchase, i.e. if they visit more than one city in the country, do they buy souvenir/s in every city or just in particular one, the majority claimed that they only purchase souvenirs in the one city that they spent the most days in because, in that way, one city represents the whole country to them.

"I do not buy souvenirs in every place when I travel, because I travel a lot, and I would not have money to travel if I spend all the money sooner, but only in the places that were special to me" (Participant 1, female (25), Spain).

While responding to the questions, participants often mentioned the uniqueness of the souvenirs. The next question was related to the artisan work and creativity of souvenirs. All respondents

agreed that they prefer unique and creative souvenirs, and they also mentioned unique and artisan souvenirs in previous questions. The majority of participants are not interested in mass-produced souvenirs and souvenirs that do not have authenticity from a particular country.

"I always look for handmade magnets. I do not like the massive production of magnets and postcards. Usually, I cannot find them, but then I find something that you cannot find in every shop. And I also want to support small business, and all these channels". (Participant 1, female (25), Spain).

4.3 Meaning of souvenirs

When describing the motives and motivations for buying souvenirs, the majority of participants agreed that their main motive is to have a reminder of the visited place and to feel good about the purchased souvenir, but the motivation is family and friends, as well as themselves.

"For me, my motivation is to buy souvenirs for my family and friends, especially if I find something handmade for my family and friends. (Participant 6, female (20), Cyprus).

These findings confirm the previous findings from Siregar et al (2017) that claim that purchase decisions, especially on souvenirs the most influenced by the opinions of family, friends, and the closest people of travelers. Sharing experiences with the family and the closest social group is the reason why tourists purchase souvenirs while traveling.

Regarding the question "For what purpose do you buy souvenirs?" the majority of respondents mentioned that they buy it as a gift for close people such as family and friends. The highlighted answers were from two participants:

"My family decided not to buy magnets and postcards, to save money for more useful stuff. When I see something that for example my siblings or my parents need and also some parts of local cuisine, I could buy it as well. But, if it is not so extraordinary or something new for us, I prefer not to buy it. (Participant 3, female (22), Germany), and

"So, I do it both for myself and my family as well. Because when you are walking on the street and there are a lot of shops selling these souvenirs, they are just like attraction to see it and buy it and I like just buying for myself and friends". (Participant 5, male (23), Germany).

Most participants agreed that they collect souvenirs and mostly they are magnets and postcards. One participant stated:

"I collect postcards". (Participant 2, female (29), Poland.)

And also one participant explained the collection of natural souvenirs:

"I do not collect souvenirs, but flowers. Flowers from different countries". (Participant 3, female (22), Germany).

The answer from Participant 3 confirms the studies from Pabian and Brzezinski (2019) that pointed out that young people collect natural souvenirs because of their uniqueness, and because they can bring back memories that cannot be purchased in stores.

In response to the question „Which factors influence your souvenir purchasing“? i.e., cultural factors, and family factors, all respondents agreed that culture is not the factor influencing souvenir purchase, while as previously mentioned in the responses, family factors play the important role in purchasing souvenirs. These findings contradict some studies from (Gordon, 1986) that explained that souvenirs as gifts are traditionally and generally accepted by cultures as obligatory.

4.4 Souvenirs in Bosnia and Herzegovina

The first question about souvenirs in Bosnia and Herzegovina was „Have you bought any souvenirs in Bosnia and Herzegovina (B&H)“? All participants except one agreed that they have not bought it yet. The participant who bought souvenirs visited Bosnia and Herzegovina before stated:

„I bought a coffee pot for a friend of mine because I know they like how it looks, but they do not use it for coffee. I always buy sweets when I go back, for some baklava, hurmašica and such stuff. Yeah, I bought them and I already brought them to them“. (Participant 5, male (23), Germany).

The next question was related to an opinion about souvenirs in B&H, where only one participant who already visited Bosnia and Herzegovina answered this question „Do you think that souvenirs are related to the history of B&H“? answered:

„They are all related. They are all historically related and for example, if you are on Bašćaršija you will see the pen in the form of this shooting staff because of the reminder of the war or such staff“. Also, ashtrays, like putting cigarettes and everything in there in form of the gun staff as a reminder of war and everything. On another side, you have magnets with the pictures of Sarajevo, like Sebilj, mosques, and such stuff. They all have different meanings, but they are all pointing to Bosnia and Herzegovina and everything that is nice. (Participant 5, male (23), Germany).

Regarding the question „Have you had any problems finding typical B&H souvenirs“? and what type of souvenirs would you buy in Bosnia and Herzegovina, one participant answered:

„I tried to look for handmade magnets and I found one but I did not like it. But in my opinion, I did not find many handmade magnets.“ (Participant 1, female (25), Spain).

Also, this answer can be related to the importance of uniqueness and handmade souvenirs that previously have been discussed. The studies from Wang et al (2018) confirm these findings that

for creating value for customers, souvenirs should also be authentic and they need to represent the local heritage and unique culture of the community.

The last question from the section about souvenirs in B&H was: „Are they any types of souvenirs that you avoid, and that you wouldn't buy“? majority of participants agreed that they would not buy big souvenirs that they cannot transfer from B&H to their country because they do not have space in their luggage and they look for souvenirs and food that are not in the liquid form because of airport regulations. The participant who previously explained that does not buy souvenirs in the form of fridge magnets stated:

„I avoid magnets. We had so many magnets in our family that our fridge was absolutely full and they were just falling down, they were broken, and that's one of the reasons we decided not to buy it“. (Participant 3, female (22), Germany).

4.5 Post-purchase behavior

Participants of the EU focus group were asked about post-purchase behavior. The first question was „What do the souvenirs mean to you after buying them and getting them home“? participants agreed that they sometimes spent some time checking them and trying to use them as a memory trigger. One participant stated:

„Whenever I need a reminder of my good days abroad, I usually look at my photos or videos on my phone.“ (Participant 4, male (22), Germany).

Another stated:

„Sometimes when I organize my stuff I look back at the memories and I remember the places I've been because sometimes I forgot about them, but when I see them I look to think about memories“. (Participant 2, female (29), Poland).

Also, others stated:

„I remember them when I put this stuff on my desk, on my table, and when I work and when I take a rest time, I look at this stuff. It's making me good.“ (Participant 6, female (20), Cyprus).

These findings confirm previous studies and research from (Lai and Deng 2006; Smith and Reid 1994; Paraskevaidis and Andriotis 2015) that claim that souvenirs have a role as a memory trigger, and that tourists use them as a reminder of past travel, and that it can recall previous experiences.

In response to the question „What do you consider to be a quality souvenir“? and all of the participants agreed that value is connected with the people to whom they are buying souvenirs and their usage of souvenirs. One participant replied

„I do not know how to respond to that question, because I always buy postcards and magnets“. (Participant 2, female (29), Poland).

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The last question was related to the money that they were willing to spend on souvenirs. The majority of participants agreed that they do not like to spend too much money on souvenirs, except two participants who stated:

„If I see something that I know someone will like, I do not care that much about the money. Last time, one figure was handmade and I really like it for my mom, and it was quite expensive, it was like 17€, but in that case, if it is worth it, I do not care that much“. (Participant 1, female (25), Spain).

„Depends on the person I think. Mostly I do not buy so expensive stuff. But if I see that, for example, watches and they are really good and my father is into it, why not buy him these watches from Switzerland or something like that“. (Participant 3, female (22), Germany).

These findings are the opposite of the findings of Gordon (1986) that claims that tourists are not careful too much on vacations like in ordinary times while spending money.

5. FINDINGS AND ANSWERS TO RESEARCH QUESTIONS

Tourists' behavior is mainly influenced by close people (family and friends) for whom they usually buy souvenirs. Complementary to this, the majority of respondents buy souvenirs for themselves and they claim that for those reasons souvenirs need to be creative and representative. The participants of the focus group also identified the vital factors of souvenirs such as emotional factors and memories that they find in the purchased souvenirs, and they explained that those factors also influence their buying behavior. Responses from the participants are correlated with previous studies and research (Siregar et al, 2017; Smith and Reid, 1994; Verdugo et al, 2016) that assert the importance of emotional connections and unique memories that souvenirs represent, and how souvenirs evoke travel memories and how they are used to keep the memories tangible. These findings also confirm the research from Singh (2018) that states that factors that influence the purchase are related to moments, feelings, locations, people, and situations and that the main reason for purchasing souvenirs is to “make people go back in time” when they were traveling.

The souvenirs that the majority of tourists usually purchase are fridge magnets, postcards, and small souvenirs that are not expensive and that can be carried out easily to their country of living. The main motives for purchasing souvenirs for most participants is to giveaways to their family and friends as well as other close people, but also to have the memory of the visited place. Most of the respondents explained the emotional factors related to their souvenir purchase when they described post-purchase behavior. Wilkins (2011) explained that there is an important connection between memories and souvenirs in the post-purchase behavior of tourists. Purchased souvenirs serve as a memory of visited places, which is correlated with these findings. The emotional factors are recognized among the majority of participants, and they are mostly seen in post-

purchase behavior. Most of the respondents prefer to use souvenirs after a trip to evoke travel memories, and in that way, they recall feelings, moments, experiences, and visited locations. These findings support the research from (Singh, 2018; Wu et al, 2022; Yuksel, 2007), which reports that souvenirs have an emotional value that allows tourists to relate to the moments, enhance the memorable tourist experience, and have a positive impact on post-purchase behavior.

6. CONCLUSION

This research provides an understanding of the role of souvenirs in tourist consumption, and purchase behavior about souvenirs. The research used EU focus groups who visited Bosnia and Herzegovina in August of 2022 to gain a deeper understanding of the purchasing behavior of tourists generally, as well as the purchasing behavior in Bosnia and Herzegovina. There are several findings obtained from the research. Even if there have been numerous research from different authors regarding understanding the role of souvenirs from different perceptions, such as perceived quality of souvenirs, perceived value, motivations, and emotional factors from souvenirs, previous research focused on the individual factors, more than overall factors that have been investigated into this research. Also, there is a lack of studies about the studies of souvenirs in Bosnia and Herzegovina and the meaning and understanding of the issues and cons of how tourists perceive souvenirs.

Firstly, the research investigated the factors of “souvenirs as a memory trigger”. The research supports the findings from most authors and researchers about this topic. Previous research found that souvenirs evoke travel memories and are used as a memorable reminder of special moments and that there is an important connection between memories and souvenirs in the post-purchase behavior of tourists (Wilkins, 2011). The findings from this research are also in correlation with the studies of Paraskevaidis and Andriotis (2015) which states that souvenir serves as a memory trigger and that the main reason for purchasing souvenirs is to remember traveled places.

Secondly, findings show the importance of souvenirs as a gift, which confirms the research done by Lunayi et al (2008), which states the importance of souvenirs as gifts, and how many travelers express love by purchasing the souvenirs for close people. The findings from this research support the studies of these authors, and they are also supporting the findings of Pabian and Brzezinski (2019) who pointed out how young people collect natural souvenirs, whereas one participant from the EU focus group is collecting natural souvenirs.

Furthermore, the factors influencing tourists to purchase souvenirs were researched. It has been shown that the main motives for purchasing souvenirs are emotional connections with them, as well as the satisfaction of giving away them as a gift. Another essential point presents the motivation of tourists to purchase souvenirs, which are social factors, psychological factors, and social factors (mostly purchasing for gifts for family and friends), by also including the unique

products and affordable prices as mentioned by (Siregar et al, 2017). By studying the factors which influence tourists to purchase souvenirs, the perceived quality, value, and satisfaction of souvenirs were investigated.

Lastly, the souvenirs in Bosnia and Herzegovina were researched, where tourists explained the authenticity of the souvenirs from B&H, as well as the process of purchasing souvenirs, and the issues that they could have by purchasing them. The results showed that the participants did not have problems finding souvenirs that are related to the history of Bosnia and Herzegovina, but that they had problems finding unique souvenirs (participants who were looking for unique souvenirs). Also, the majority of participants agreed that they would not buy souvenirs that they are not easily able to carry in their luggage.

Knowledge about favorable souvenirs and understanding the factors that influence tourists to purchase souvenirs is essential in tourism research. This research could be useful for researchers, souvenir retailers, and tourism overall. Moreover, this study revealed how different cultures have different views of souvenirs and factors that influence them to purchase souvenirs.

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