Current trends of ecology

Edited by

Stephka Chankova, Vlada Peneva, Roumiana Metcheva,
Michaela Beltcheva, Kiril Vassilev, Galina Radeva,
Kalina Danova

BioRisk (Special Issue)

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Greetings

Dear participants, dear colleagues,

On behalf of the Organizing Committee and I, it is my honor and pleasure to welcome you to the 13th Annual International Seminar on Ecology 2021.

In the last 12 years, this seminar has become a traditional and expected scientific event. These annual meetings gave us the opportunity to present our results, to gain new knowledge, and to exchange thoughts and ideas. New contacts and future creative scientific teams were established.

Unfortunately, our long-term tradition to meet each other in the hall of Gagarin str 2 has been broken due to reasons out of our control. However, the global pandemic of COVID-19 did not stop the willingness of students, Ph.D students, young researchers and well-known scientists to share their research results. Your participation is an illustration of how important and useful this Seminar of Ecology is.

Today we are not personally together, but by participating in the e-version of the 13th International Seminar of Ecology, we show how strong and brave we are together.

Let me wish you successful presentation of your scientific results, fruitful discussions, and stimulating comments!

I am sure that with your support the Seminar will be successful.

Thank you for your participation!

Many thanks to Organizing Committee for their selfless work.

Many thanks to our sponsors: PensoftPublishing and BulGap.

Good luck and success!

Prof. Stephka Chankova
Head of Section "Biology", Union of Scientists in Bulgaria
Head of the Organizing Committee

Dear colleagues,

It is an honor and pleasure for me on behalf of the government of the Union of Scientists in Bulgaria and personally on myself to congratulate the participants in the traditional annual International Seminar on Ecology – 2021.

Ecology is of a fundamental importance among the complex of biological scientific fields with a separate subject, specific methods and field of application. It is one of the fastest growing scientific disciplines in the world.

The success achieved in raising the ecological culture of the society and creating a neutral public opinion will significantly facilitate the solution of the problems related to the protection of the environment, and thus the conditions for normal and sustainable development will be improved.

The participation of young scientists contributes to the exchange of fresh opinions and ideas, as well as to greater empathy.

I wish all participants in the seminar successful and fruitful work!

Believing in your future successes, I wish you health, more optimism, personal happiness, new ambitious tasks and success in science!

Prof. Diana Petkova, D.Sc.
President of Union of Scientists in Bulgaria

Dear colleagues and friends,

I am very glad this year again I have the opportunity to congratulate you on the opening of the Seminar of ecology. This is a scientific forum that over the years has become a necessary event and not only for young scientists. We cannot meet live, but I hope you all will have the opportunity to present the results of your research and have the opportunity to work productively.

Allow me to express my gratitude to the colleagues from the Organizing committee and especially to Professor Chankova, who made it possible to hold the seminar.

I wish success to all!

Anna Ganeva
Director of IBER-BAS

Dear Organizers of the International Seminar of Ecology,
Dear Colleagues and Friends,

I have the pleasure to greet you on behalf of the Governing Body of the Bulgarian Academy of Sciences.

Although, due to the pandemics, the seminar will take place on-line for the second consecutive year, I am sure that it will be held on the traditional high scientific level, and there will be many interesting and important scientific reports.

The ecology, as an interdisciplinary science, covers almost all aspects of the everyday life – from the conservation of nature and biodiversity to the environmental quality and environmentally-friendly technologies. Therefore, the participants will have many opportunities to report the results of their research, and to discuss the most important and new issues in the respective domains of the science of ecology. I believe the seminar will be useful to all participants and will inspire new ideas among the researchers and research organizations participating.

I am also very glad to outline the leading role of the Institute of Biodiversity and Ecosystem Research (IBER) in the organization and important contribution to the high scientific level of the event.

I wish all participants creative inspiration for a very successful and fruitful Seminar, and for further development of the science of ecology.

Assoc. Prof. Ina Aneva, PhD,
Scientific Secretary of the Bulgarian Academy of Science.

EDITORIAL

International seminar of ecology – 2021 "Current trends of ecology"

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Introduction

The term "ecology" was used for the first time in 1866 by Ernst Haeckel, who saw both the descriptive natural history and observation field studies, as the main objectives of ecology. During the last 155 years, ecology has evolved to the present multidisciplinary field by cooperating with biogeochemistry, climatology, evolution, genetics, hydrology, statistics, economics, social science, and others, in order to clarify and solve environmental problems provoked by climate changes, invasive species, environmental pollution, etc. (Murrel et al. 2001; Carpenterand and Folke 2006; Corlett 2015). Humans, as rational beings, aim to reduce and prevent environmental problems some of which were discussed at the "International Seminar of Ecology - 2021", titled "Current trends of Ecology".

About the seminar

The annual Seminar of Ecology has a long-lived history. The first scientific event was organized in 2007 by Section Biology, Union of Scientists in Bulgaria, and Central laboratory of General Ecology, BAS as a platform for students, and scientists to share their results and to discuss major ecological problems. The annual seminar became a very popular forum for well-known researchers, young scientists, BSc, MSc, and PhD students from Bulgaria and abroad. Each year, guest lecturers present their latest

reports at the main thematic areas of the Seminar, covering current and interesting topics. Over the years, the seminar has been broadened. In 2014 it became a Seminar of Ecology with international participation, and in 2019 it became known as the "International Seminar of Ecology". Due to the pandemic situation, the last two meetings of the International Seminar of Ecology were carried out online. Through the years, the Organizing Committee has kept the tradition to award the best oral presentations and posters, of students and young scientists, with certificates and books. After a review by independent reviewers, proceeding books were published with a corresponding ISBN. In the last two years, selected papers were published in the journals Ecologia Balkanica, BioRisk, and Phytologia Balcanica.

About this issue

This thematic special issue of BioRisk compiles materials presented at the International Seminar of Ecology - 2021. The articles published here illustrate, approximately to the full extent, the problems presented and discussed at the Seminar. During the first session, devoted to the biotic and abiotic impact on the living nature, ecological risk, and bioremediation, two complementary plenary reports were presented to the audience. "Ecocide" introduced the auditora to the global effect of pesticides, radionuclides, and petroleum products on target and non target organisms. The "Green and nano- pesticides" report discussed their use, as alternatives to chemical pesticides.

A set of reports focused on the anthropogenic and environmental impact of heavy metals, fungicides, polycyclic aromatic hydrocarbons (PAHs), and natural radionuclides provoked the interest of the participants and sparked a serious discussion. Based on the correlation found between bacterial abundance, soil properties, and heavy metal pollution, the discussion was focused on soil properties as a factor that can modulate the effect of heavy metals, present in chronically contaminated soils. Different approaches to overcome environmental pollution were presented and discussed: zeolites as detoxifying tools, microalgae for the treatment of contaminated water bodies, and a newly developed bio-fertilizer, based on activated sludge combined with a bacterial strain with detoxifying and plant growth-promoting properties. There is a clear need to extend the existing monitoring programs, by including more bioindicators and markers, in order to achieve a more detailed assessment of the environmental impact. For example, sexually-manifested variations in the pigmentation of *Boeckella poppei* (Copepoda: Calanoida) from the Livingston Island (Maritime Antarctica), were described as a suitable indicator, reflecting the ongoing global environmental changes in Antarctica. Changes in the antioxidant system of commercially important fish species and mussels from the Bulgarian Black Sea coastal area, were recommended as markers for the ongoing global environmental changes.

It was shown that by using various markers for the evaluation of environmentally induced stress response at different levels (microbiological, molecular, biochemical), it was possible to gain insights of the organisms' protection and the mechanisms involved

in the formation of resistance to environmental impact. The contribution of increased DNA repair capacity and AOS to the development of environmental tolerance or adaptation was also shown. Important results for understanding the processes of photoprotection, in either cyanobacteria or algae, and higher plants were obtained by *in vitro* reconstitution of complexes of stress HliA protein with pigments. The crucial role of the cellular physiological state, as a critical factor in determining the resistance to environmental stress with Q cells was demonstrated. Several papers were focused on the action of bioactive substances with plants origin. The bioactivity was shown to depend strongly on chemical composition. *Origanum vulgare hirtum* essential oil was promoted as a promising candidate for the purposes of "green" technologies. Analyzing secondary metabolites of plants, it was shown that their productivity *in vitro* is a dynamic process closely related to the plant's growth and development, and is in close relation with the interactions of the plant with its environmental conditions.

In the plenary report of the ecological agriculture session, the impact of agriculture on the environment, human health, energy crises, and climate changes was shown to enforce policymakers and farmers to rethink the recent model of agricultural production. It was pointed that, designing and implementing such an agricultural model requires significant changes in the management of the farming systems, natural resources, food-chain, and scientific approaches, in order to meet environmental and societal demands. In this aspect, the link and interrelationship between traditional, organic, and new plant breeding techniques including GMO and precision farming were also considered.

In this context, the influence of the agricultural system type on essential oil production and antioxidant activity of industrially-cultivated *Rosa damascena* Mill. in the Rose valley (Bulgaria) was reported, comparing organic vs. conventional farming. The rose extracts from organic farming were shown to accumulate more phenolic compounds, corresponding to the higher antioxidant potential of organic roses.

A comparative study, based on official data from the statistics office of the European Union and the Member countries, concerning viral infection levels in intensive and organic poultry farming, demonstrated that free-range production had a higher incidence of viral diseases with a high zoonotical potential.

Pollinators of *Lavandula angustifolia* Mill., as an important factor for optimal production of lavender essential oil were analyzed. It was concluded that, although lavender growers tend to place beehives in the fields for optimal essential oil production, it was also crucial to preserve wild pollinators.

New data reported that essential oils and alkaloid-rich plant extracts had the strongest acetylcholinesterase inhibitory activity and could be recommended for further testing for insect control.

The thematic session "Biodiversity. Conservation Biology" started with 3 plenary lectures concerning the vegetation diversity on the territory of Bulgaria, genetic diversity in cork oak plantations in Portugal, and nematodes in two cold deserts - the Antarctic and the Arctic. It was reported that the vegetation diversity of Bulgaria was still not fully investigated. Grasslands, broad-leaved forests, and wetlands are the best-

investigated habitats. Data concerning ruderal, shrubland, fringe and chasmophytic vegetation in Bulgaria are scarce. The second report "Cork oak landscape: a sustainable multi-use resource" presented the main results from a very long-lasting investigation, spanning the last 2 decades, in order to understand cork oak genetics and to identify "genes of interest for high-quality cork production".

In „Nematodes of Extremes: Polar deserts", both nematodes' fauna and ecology in two polar deserts - the Arctic and the Antarctic, were presented along a short historical review of exploring these regions. Special attention was focused on the specific survival adaptation strategies of nematodes and the good opportunity for the polar deserts to be regarded as an „excellent model habitat - a natural laboratory to study and monitor the impact of the global changes in the biotic communities".

Other important problems were reported and discussed in this session: the opportunity of pest control using pteromalids as natural enemies of pests in various crops; the main reasons responsible for the population decrease of bumblebees - habitat destruction, loss of floral resources, emerging diseases, and increased use of pesticides (particularly neonicotinoids); the strong impact of temperature and wind on the distribution of zooplankton complexes in Mandra Reservoir, in Southeastern Bulgaria; an alternative approach for the *ex-situ* conservation of *Stachys thracica* based on *in vitro* shoot culture and its subsequent adaptation under *ex vitro* conditions.

The plenary report in the session "Ecosystem research and services. Landscape ecology" asks the question: is it possible for us to become ecosystem-friendly people again? In this report, ecosystems, as the fundamental functional units of the living planet were discussed. The main problems, responsibilities, and goals of ecosystem management were presented, as well as, the necessity to integrate ecological, economic, and social goals into a unified management approach that requires urgent reorganization of science and education.

In the next reports, new information was presented concerning pre-monitoring geochemical research of river sediments in the area of Ada Tepe gold mining site (Eastern Rhodopes). The obtained results illustrate that the explored landscapes have been influenced by natural geochemical anomalies, as well as, impacted as a result of human activity. The forests habitat diversity of Breznik Municipality was revealed, following the EUNIS Classification and initial data from the Ministry of Environment and Water and the Forestry Management Plans. It was shown that in addition to the dominant species *Quercus dalechampii*, *Quercus frainetto*, *Fagus sylvatica*, *Carpinus betulus*, some artificial plantations with *Pinus nigra* and *Pinus sylvestris* were also present, as well as non-native species, such as *Robinia pseudoacacia* and *Quercus rubra*. Models for Predicting Solution Properties and Solid-Liquid Equilibrium in Cesium Binary and Mixed Systems were created. The results are of great importance for the development of strategies and programs for nuclear waste geochemical storage.

In conclusion, many results in different areas of ecology were presented in the Seminar, followed by interesting discussions. A lot of questions were answered, however many others remained open. A good platform for further discussion will be the next International Seminar of Ecology - 2022, entitled "Actual problems of Ecology".

Acknowledgements

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RESEARCH ARTICLE

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Ecocide – global consequences
(pesticides, radionuclides, petroleum products)

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Abstract

The problem of environmental pollution is becoming increasingly important on a global scale. Man has oversaturated the environment of his habitat with harmful and most often toxic waste. It is difficult to describe all the toxic substances, as a separate book can be written for each group. The term "ecocide" has been introduced, which reflects large-scale destruction of the natural environment. We will focus only on three classes of pollutants that are of particular concern, creating environmental conflicts. These are:

• Pesticides are extremely toxic and create large amounts of non-degradable waste. It accumulates in tissues and organs of target organisms, becoming toxic and causing serious pathological changes in the body, mainly at the cellular and subcellular levels, causing various diseases and as a result, serious changes in the structure and functions of the populations and the whole ecosystem are increasingly observed.
• Waste from the nuclear industry and radioactive fallout from nuclear explosions. It is especially dangerous that radioactive elements can be concentrated in certain organs.
• Petroleum products - often large quantities end up in the seas and oceans, along with industrial waste of various kinds, impossible to compensate for by nature and they pose a serious threat to ecosystems, many of which have already been destroyed.

At the submolecular level, chemical and physical effects can lead to genetic rearrangements (mutations); destructive ionization in the tissues of every living being, sometimes with completely unexpected consequences for humans.

Keywords

Pesticides, petroleum products, radionuclides

Introduction

The word "ecocide" combines 'eco', which comes from ancient Greek word 'οικος', meaning house which nowadays means 'habitat' or 'environment' and -cide' comes from the Latin verb 'caedere', meaning 'to cut down' or 'to kill'. Ecocide literally means "to kill the environment", or destruction of large areas of the natural environment as a consequence of human activity.

The problem of environmental pollution is becoming increasingly important on a global scale. Man has oversaturated the environment of his habitat with harmful and often toxic waste. It is difficult to describe all the toxic substances, so the focus will be stressed only on three classes of pollutants that are of particular concern, creating environmental conflicts. These are:

1. Pesticides. Their use is sometimes unavoidable, yet they pose serious hazards to ecosystems due to runoff in freshwater ecosystems and biomagnification along food chains.

2. Radionuclides. Localized anthropogenic contamination can be dangerous to ecosystems due to a tendency of fission products such as ^{131}I, ^{137}Cs, and ^{90}Sr to bioaccumulate in the terrestrial and aquatic biota.

3. Petroleum and petroleum waste products. Large quantities of petroleum and its derivatives have ended up contaminating ocean ecosystems and shorelines, producing damage that is hard to overcome.

Ecocide can be irreversible when an ecosystem is damaged beyond its capacity for self-repair. It is generally associated with damage caused by an organism, which might cause ecocide directly by destroying enough species in an ecosystem to disrupt its structure and function. Ecocide can also result from pollution such as high concentrations of pesticides which decimate the local biodiversity.

Pesticides

Pests are the most serious problem in agricultural production. Since the discovery of DDT, farmers use pesticides as the most effective means against destruction of crop production. Pesticides significantly damage the environment as well as humans, they damage water and soil quality, which has a dangerous effect on animals, birds, plants and humans.

The degree of pesticide toxicity strongly depends on its environmental behaviour. They enter in the ecosystems by two different pathways depending on their solubility. Water-soluble pesticides enter groundwater, streams, rivers and lakes and in this way harm non-target species. Fat-soluble pesticides enter organisms along food chains and have a strong tendency towards biomagnification. They are absorbed in the fatty tissues and result in persistence of pesticides in food chains for very long periods. These persistent pollutants are transferred up the food chain faster than they are broken down or are excreted. Therefore, the higher trophic levels of the food chains will contain higher

pesticide concentration. This disrupts the normal functioning of the whole ecosystem as the species in higher trophic levels will die due to greater toxicity.

The threats associated with the use of these toxins cannot be ignored. It is of paramount importance to study the pesticide impact on populations of aquatic and terrestrial ecosystems. Accumulation of pesticides along food chains is of greatest concern as it directly affects terrestrial predators and raptors. Indirectly, pesticides can also reduce the quantity of plants and primary consumers, on which higher orders feed. Spraying with insecticides, herbicides and fungicides has also been associated with reduction in the population of rare species of animals and birds.

Pesticides enter the water via rain, by runoff, leaching through the soil or they may be applied directly to water surfaces, for instance, for the purpose of controlling mosquitoes. Water contaminated with pesticides is a serious threat to aquatic life forms. It can affect aquatic plants, decrease dissolved oxygen in the water and can cause physiological and behavioural changes in fish populations. These pesticides are not only toxic themselves but also interact with stressors which include harmful blooms of algae. Aquatic animals are exposed to pesticides in three ways: direct absorption via skin; uptake via gills during breathing and via drinking contaminated water.

Pesticides in terrestrial ecosystems are able to cause sublethal and lethal effects on plants. As early as 1977 Kelley and South (1977) note that herbicides cause considerable damage to fungal species in soil by inhibiting the growth of symbiotic mycorrhizal fungi that help plant nutrient uptake. Glyphosate, a broad-spectrum herbicide, reduces the growth and activity of nitrogen-fixing bacteria in soil (Kanissery et al. 2019). Even low doses of herbicides have a great impact on the productivity and diversity of the natural plant communities and wildlife. Beneficial insects like bees and beetles can experience significant population decline due to the use of broad-spectrum insecticides (Pisa et al. 2015). Synergistic effects of fungicides and neonicotinoid insecticides are very harmful to bees. Even a low dose of them reflects negatively on their feeding behaviour. Since 2006, each year, honeybee populations have dropped by 29–36% (Pisa et al. 2015).

Some reports have confirmed that only about 10% of pesticides reach the target groups of organisms in crops. (Pisa et al. 2015; WHO 2017) The majority of pesticides react with non-target organisms (WHO 2017). If the toxicity expression of a pesticide is measurable, the non-target organisms can be used as bioindicators. There are various options on the choice of species for pesticide monitoring. They depend mostly on the feeding habitat of the species. The non-targeted part of an applied pesticide moves through the ecosystem and a significant portion of it accumulates in the lower trophic levels. By the mechanisms of bioaccumulation, it reaches higher trophic level organisms and affects normal physiological processes of the organisms, thereby putting the whole ecosystem at risk (EEA 2013).

Gutierrez et al. (2012) reported the response of the copepods and the cladocerans as early bioindicators of endosulfan toxicity. Ecotoxicological risk to the copepods *Acartia margalefi*, *A. latisetosa* and the mysid *Siriella clausi* can be used as early indicators to assess the risk to marine ecosystems.

Due to specific morphological features, bees can carry pesticides which may be brought to the hive. Thus beehives may also be polluted. The spraying of beehives

during honey collection may be the reason for pesticide adulteration of honey and bees-wax (Kujawski and Namieśnik 2011). This indicates the use of honey bees as a potential bioindicator to determine the amount of pesticide levels in pollinator communities.

Earthworms are common organisms in the soil ecosystem and play an important role in soil health (Spurgeon et al. 2003). They play a significant role as bioindicators of soil contamination and as models for soil toxicity. Their reduction may alter the nutrient cycling and nutrient availability to plants (Rizhiya et al. 2007). Pesticides produce neuro-toxic effects in earthworms and after exposure they are strongly physiologically damaged, with DNA damage, changes on feeding activity and loss of vitality (Zhang et al. 2020).

Bird feathers are one of the best indicators for the presence of pesticides in the body. Several studies showed a significant correlation between the contamination level in sea-birds' food and their feathers. Feather collection is easy and minimally invasive and is very important from the viewpoint of conservation biology. Moreover, feathers indicate toxicant exposure during an annual cycle. There is a wide concentration range of pesti-cides that can be traced using feathers from birds in Patagonia (6.49 ± 5.95 µg/g) (Mar-tínez-López et al. 2015) to relatively high concentration in birds from Spain (870.48 ± 614.48 ng/g) (Espín et al. 2012). Feathers can be used as bioindicators throughout the wide range of different geographic regions of the world. For biomonitoring of OCPs in Antarctica, penguin feathers are a very good tool (Metcheva et al. 2017).

A lot of studies show that herbivorous mammals, and especially rodents, are one of the best species that fulfil the requirements for a good bioindicator for pesticide contamination due to their large population number, good representation of spatial and ecological niches, sufficient knowledge about their physiology, great reproductive potential, as well as their dietary composition (Tataruch and Kierdorf 2003).

Since use of pesticides is unavoidable, early monitoring is essential to prevent or control the damage caused by pesticides to humans and ecosystems. It is a timely need to integrate the studies of different disciplines including toxicology, environmental chemistry, population biology, community ecology, conservation biology and land-scape ecology to understand the direct and indirect effects of pesticides on the envi-ronment. In the future, chemical pesticides can be used in combination with natural treatments and remedies, resulting in more sustainable elimination of pests. This com-bination not only promises environmental health, but also has diverse applications in controlling urban pests and invasive species.

Nowadays, is very important to control the use of pesticides and to find ways to apply appropriate substances; to encourage farmers to reduce pesticide overuse. It is necessary to develop and apply various techniques for remediation of pesticides from the environment. Adsorption and bioremediation have been found to be most suitable as environmentally friendly, cost-effective and less toxic by-products. Environmental protection organisations, farmers, health professionals, producers, and governments have to commit to and adopt joint initiatives to reduce the negative effects of pesti-cides. Immediate action is needed to effectively control pesticides and to adopt strict laws and regulations in this area. Integrated pest management is very useful for the management and further application of pesticides, as well as for their best control.

Radioactive contamination

Radionuclides are nuclides that have excess nuclear energy, making them unstable. This instability is due to excess energy in the atomic nucleus, leading to the release of particles with different energies in a process called radioactive decay. Natural radionuclides emit alpha (α), beta (β-) and gamma (γ). Of these types, α-particles have the strongest biological effects, causing 20 times more biological damage than an equivalent dose of β- or γ radiation (ICRP 2007). While α and β- particles do not penetrate deeply into matter, γ-radiation, especially at the higher end of the energy spectrum, has high penetration. Biologically, α and β- emitters are only relevant if incorporated into living organisms, while γ-emitters are relevant both as internal and external radiation sources. Some technogenic radionuclides emit other types of radiation. Medical PET isotopes such as ^{18}F, ^{11}C, ^{13}N, ^{15}O, are positron (β+) emitters. Other radionuclides such as ^{252}Cf are capable of emitting neutrons. Both positron and neutron emitters require special equipment for handling and detection of radiation sources (Hall and Giaccia 2006). Some radionuclides emit multiple radiation types. The technogenic radionuclide ^{137}Cs emits β- particles at two energies: 511 and 1173 kiloelectronvolts (keV), and γ-rays at 661.6 keV.

The biological effects of radionuclides are mainly due to the emitted ionizing radiation (IR). Researchers have elucidated the biological effects of high and medium doses of radiation. However, low-dose effects are still insufficiently understood (Hall and Giaccia 2006; Kosti 2019). Currently, IR risk is extrapolated linearly to the low doses by using the Linear Non-Threshold (LNT) mathematical model (Trott and Rosemann 2000; Hall and Giaccia 2006). Other hypotheses include radiation hormesis, which is the idea that small doses of radiation are beneficial due to the induced protective stress responses (Schirrmacher 2021), and low-dose hypersensitivity, which is the assumption that low doses of radiation are more mutagenic (Joiner 2001). While radiation hormesis has been well researched recently (Shankar et al. 2006; Schirrmacher 2021) it has still not been taken into account in radiation protection calculations, where every minimal dose of radiation is assumed to carry a small but non-negligible risk (ICRP 2007). On the other hand, the low-dose hypersensitivity hypothesis is supported by recent studies, raising questions about the validity of current assumptions in radioprotection (Heuskin et al. 2013). Organisms have different radiosensitivities. The champion of radioresistance is the bacterium *Deinococcus radiodurans*, which can withstand an acute dose of 5000 Gy with almost no loss of viability. Similarly, tardigrades can withstand 5000 Gy with 50% loss in viability (LD_{50}=5000 Gy). For comparison, the LD_{50} for humans is around 6 Gy, for mice around 6.4 Gy, and for goats only around 2.4 Gy (Bond 1963).

A significant concern in radionuclide-contaminated areas arises from the process of bioaccumulation. Similarly to other elements from their respective groups, radioisotopes are incorporated preferentially into different target organs and tissues. Thus, ^{90}Sr, an analogue of calcium, has a strong affinity for bone and hematopoietic tissue. Some of the properties of the three most significant anthropogenic radionuclides are presented below (Table 1):

Table 1. The most significant anthropogenic radionuclides and their biological effects (data adapted from Besson et al. 2009 and Holm 2006).

Radionuclide	Symbol	Half-life (λ)	Emitted radiation	Target tissue	Biological effects
Cesium-137	[137]Cs	30.17 years	β- (511, 1173 keV), γ (661.6 keV)	nerve, muscle	different cancers
Strontium-90	[90]Sr	28.8 years	pure β- (546 keV)	Bone	bone cancer, leukemia
Iodine-131	[131]I	8.02 days	β- (333.8, 606.3 keV), γ (364.5, 636.9 keV)	thyroid gland	thyroid cancer

As evident from the table, the most significant environmental contaminants of the above are ^{137}Cs and ^{90}Sr due to their long half-lives and persistence in nature. [131]I was only a very significant risk in the first year following the Chernobyl accident, causing ~4000 excess thyroid cancers in the most significantly affected populations of the former USSR (Williams 2006).

Natural radioactivity, including external terrestrial γ-radiation, internal α, β- and γ-radiation from terrestrial radionuclides, cosmic radiation, and exposure to radon ([222]Rn) and thoron ([220]Rn) and their radioactive progeny molecules accounts for ~95% of the annual radiation dose for the terrestrial biota (Hall and Giaccia 2006; ICRP 2007). Globally, there are areas with high natural radiation, mostly due to thorium ([232]Th) deposits. Two such areas are Guarapari, Brazil, and Kerala in southern India. The area of Ramsar, Iran, has increased natural radioactivity due to radioactive hot springs containing [222]Rn and its progeny. Although annual doses in these areas reach an average of 35–40 mSv/a, compared to 3.6 mSv/a average in Europe, epidemiological studies report no excess cancer risk (Dobrzyński et al. 2015; Kosti 2019).

In contrast, environmental contamination by man-made radionuclides poses serious risks. The Chernobyl accident is the most prominent example of technogenic environmental damage, although it is not the only one; Chernobyl caused significant chronic morbidity and mortality in people and enormous damage to the environment and economies in Europe. This is mostly due to ^{131}I, ^{137}Cs, and ^{90}Sr, and their tendencies for bioaccumulation and biomagnification in terrestrial ecosystems (Chesser et al. 2001, UNSCEAR 2020). Although the Chernobyl accident is the best-known example, there are other significant events in the period 1945–2011. The Fukushima accident in 2011 presents a new precedent – the reactors in the plant were nearing the end of their design life (UNSCEAR 2020). Since this is true for many of the currently operating reactors, crumbling nuclear infrastructure may present a significant radiation hazard in the future.

Some of the risks to ecosystems posed by radionuclide contamination are well understood. They include, at high doses >1 Gy acute dose, teratogenesis in developing embryos, stunted plant growth, visible damage to the flora and fauna. These are known as deterministic effects, because they occur definitely after exposure to strong doses of ionizing radiation and are dose-dependent. More worrying are the so-called stochastic effects, which occur with a small probability even at low radiation doses. These include radiation mutagenesis and, as a consequence of it, radiation carcinogenesis (Hall and Giaccia 2006; ICRP 2007). Based on data from mouse experiments and results from the monitoring of survivors of Hiroshima and Nagasaki, it is estimated that the

doubling dose of radiation-induced mutagenesis is 1 Gy; an acute exposure to 1 Gy of γ-rays doubles the spontaneously occurring rate of mutation (Russell et al. 1958). However, this perspective is being challenged. For example, Goncharova and Riabokon (1998) observed transmission of chromosomal damage in the progeny of wild rodents from the vicinity of Chernobyl, indicating genomic instability. Another, more recent venue of research with significant progress, is the radiation-induced bystander effect (RIBE) phenomenon, in which non-irradiated cells show similar cytotoxicity and genetic damage to their irradiated neighbours (Osterreicher et al. 2003; Wang et al. 2018). The results from bystander effect studies generally support the theory of low-dose hypersensitivity and highlight possible molecular mechanisms for increased radiation risks in the low-dose range (Osterreicher et al. 2003; Wang et al. 2018). Dubrova et al. (1996) report a higher mini- and microsatellite mutation rate in the children of Chernobyl liquidators. The same concern was raised in a more recent study (Bazyka et al. 2020). Both of these findings support the theory that even low doses of radiation can be harmful to the biota. Radiation risk is still to be taken very seriously, and every effort should be made to keep radioactive contamination of ecosystems to a minimum.

Petroleum products

All types of oil differ by their chemical composition, weight, prior refinement, concentration of heavy metals, sulphur, and other impurities. Oil spills involve accidental contamination by oil ranging from various grades of crude oil to different refined products, from heavy fuel oil to light, less persistent, but very toxic fuels. The chemical composition of the spilled oil, and the associated weathering reactions, determine their fate, behaviour, and impact in the marine environments. Oil spills are of great concern due to the long period of oil and gas exploitation and the adverse impacts of the marine environment and these various undesirable repercussions have been documented. (Murawski et al. 2021).

On February 15, 1996 the oil tanker "Sea Empress" lost 72,000 t of crude light oil and 370 t of heavy fuel oil of her cargo in the North Sea. Over 100 km of coastline were affected. Estimates suggest that overall, 200 km of coastline has been affected. A further 25,000 tons of waste were created by the clean-up operation. The "Sea Empress" ranks as one of the world's top 10 oil spills (Johnson and Butt 2006).

The 2010 "Deepwater Horizon" oil spill is considered the largest marine environmental disaster in North America. Over 200 million gallons of oil poured into the Gulf and contaminated the coast. It is estimated that up to 170,000 people worked to clean up the Gulf oil spill. This event is now considered to be the worst environmental disaster in US history, with massive ecotoxic effects on sea life and human habitats. The ecological effects were drastic and longstanding, affecting all biota of all trophic levels ranging from microorganisms and algae to pelagic fish, marine invertebrates, mammals, and seabird populations, marine mammals from whales to otters, and plankton populations (Lee et al. 2015).

Crude oil releases the most harmful toxins into the water and air within a short time. The rest of the toxins are broken down by microorganisms in the sea water, but before this, crabs, shellfish and fish concentrate toxins in their bodies. The toxins are then bioaccumulated in higher trophic levels. It could take decades to understand how oil affects the next generation of whales, coral, sea turtles, birds, fish, and other marine life.

The toxic effects of oil spills to wildlife can be categorized as lethal and sublethal. Basically, assessments of environmental impacts of oil spills are based on evaluating concentrations of pollutants required to kill 50% of individuals in test animals' toxicological experiments to estimate lethal concentrations or other effective concentrations (Bejarano et al. 2014). In this way considerable research was conducted to assess traditional biomarkers of biological endpoints (Mitchelmore et al. 2020) and to develop and apply suites of sublethal indicators of aquatic biota health in order to understand the induction of health effects involving immune system function, genomic changes, reproductive success, growth effects, and impairment of various organ systems in affected species (Sherwood et al. 2017; Grosell and Pasparakis 2021; Rodgers et al. 2021). Most often, research on pollutant effects on gene expression is conducted with model organisms. At the sub-molecular level, chemical and physical effects can lead to genetic rearrangements (mutations); destructive ionization in the tissues of every living being, sometimes with completely unexpected consequences for humans.

Ten years after it happened, the "Deepwater Horizon" oil spill continues to harm wildlife. The spill affected 320 miles of shoreline and affected the rich and complex ecosystem of the Gulf. The future duration and magnitude of that impact is uncertain, principally because scientists do not know how the pollutants will affect the Gulf ecosystem in the long term. Observations of damaged corals indicate impact at a depth of 1,370 m, 11 km from the site of the blowout.

Deep-water colonial corals together with ophiuroid symbionts may provide a more sensitive indicator of the impact from petroleum hydrocarbons. They are important habitats for shrimp, crabs and other marine life. Coral colonies presented widespread signs of stress, including varying degrees of tissue loss, sclerite enlargement, excess mucus production, bleached commensal ophiuroids, and are covered by brown flocculent material (floc).

Shellfish can digest oil, which could cause changes in reproduction, growth rates or even death. Fish in oil spill areas show reduced reproduction even years after the spill, because oil remaining in the environment is still toxic to fish larvae. Oil exposure in fish can lead to cancer and eventually to death, but it can also result in reproductive changes. Particularly the nesting habitats of sea turtles are affected. At least 402,000 were exposed to oil during the spill. Sea turtles are extremely sensitive to the effects of contact with oil. Young and juvenile turtles have been found to starve to death when their beak and oesophagus have become blocked with petroleum residue. Birds were among the hardest-hit animals immediately after the spill. The oil coating their feathers had reduced their ability to regulate their body temperatures due to feather damage. Marine mammals face a more expansive threat than most other coastal biota due to their large geographical range. Physical contact with oil has shown to have substantial negative and lethal effects on many varieties of marine mammals, although

the cumulative long-term effects of consumption of petroleum-laden food sources are ongoing (Geraci 2012). Thousands of dolphins died in the months following the spill, after they ingested toxins. They are important indicators of the overall health of the ocean. Humans suffer from oil-related cancers. For many other species, the damage is not clear. Many species have been difficult to study. That's because scientists knew little about the habits of many deepwater marine mammals before the spill, so have trouble detecting changes from current data (Lee et al. 2015).

China, the United States, India and Russia are four of the world's top polluters. At least 10 countries have national ecocide laws, including Vietnam, which enacted the law in 1990. Oil spills in remote high-energy locations will quickly disperse, and are difficult to reach or remediate through dispersal methods. The removal methods are expensive, labour-intensive, cause further environmental degradation, and are overall ineffective (Lee et al. 2015).

Conclusions

The complete destruction of an ecosystem due to human activities may result from exploitation of resources, nuclear warfare or the dumping of harmful chemicals. Ecocide includes all major environmental disasters which would have severe consequences on the Earth's ecological system. Even years after the accidents it is still much too early to assess the full impact. Decontamination will continue for a long period, probably more than 40 years.

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RESEARCH ARTICLE

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Correlation between bacterial abundance, soil properties and heavy metal contamination in the area of non-ferrous metal processing plant, Southern Bulgaria

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Abstract

In the present study, the correlation between bacterial abundance and soil physicochemical properties along the heavy metal contamination gradient in the area of non-ferrous metal processing plant was assessed. Our results showed that bacterial abundance (number of heterotrophic bacteria and number of 16S rRNA gene copies) decreased with 45–56% (CFU) and 54–87% (16S rRNA gene) along the Zn, Pb and Cd contamination gradient. The total bacterial abundance (16S rRNA gene) increased exponentially in contrast to the abundance of heterotrophic bacteria. The reduction of bacterial abundance in heavily contaminated soil indicated that the soil properties (soil pH, total organic carbon, inorganic ions, soil texture) could modify the effects of heavy metals and the response of microorganisms to that stress in long-term contaminated soils.

Keywords

Bacterial abundance, 16S rRNA gene, heavy metals, soil contamination, soil properties

Introduction

Soil sustains a great abundance and diversity of microorganisms, which modify its physical and chemical environment and play an essential role in the mineralization of organic matter and nutrient recycling (Martinez-Toledo et al. 2021). Microbial communities are strongly susceptible to soil physicochemical properties and to the effect of various soil pollutants, such as heavy metals (HMs). HMs are one of the most common pollutants, which accumulate in soils of industrial and mining areas. The most common HMs found in contaminated sites are Zn, Cd and particularly Pb (Wuana and Okieimen 2011; Fajardo et al. 2019). Long-term contamination with HMs is a threat to human health and ecosystems due to their non-biodegradability, bioaccumulation, environmental stability, persistence and biotoxicity characteristics (Ali et al. 2021).

Previous studies showed that HMs severely affect soil microbial communities by reducing their diversity (Chodak et al. 2013; Zampieri et al. 2016), richness (Cui et al. 2018), microbial biomass, metabolic activity (Chen et al. 2014; Hong et al. 2015; Zampieri et al. 2016; Fajardo et al. 2019) and by altering their structure (Cui et al. 2018; Feng et al. 2018; Zhang et al. 2018). Recently, many studies were focused on the ecological effects of HMs on microbial community structure and diversity using next-generation DNA sequencing technologies (NGS) (Gołębiewski et al. 2014; Fajardo et al. 2019; Jiang et al. 2019; Xiao et al. 2019; Zhao et al. 2019; Huang et al. 2021). Gołębiewski et al. (2014) found that the diversity and abundance of soil microorganisms near a Pb-Zn mining area have been reduced and that Zn was the largest selective factor. Zhao et al. (2019) reported that HMs (Cu, Zn, Pb) affected the abundance and structural diversity of microbial communities in the mining area. Fajardo et al. (2019) showed significant phylogenetic and functional shifts in the bacterial community during the soil exposure to Pb, Cd, and Zn. Xiao et al. (2019) revealed that the bacterial community structure was mainly altered by soil organic matter, HMs (Cr) and pH. According to Huang et al. (2021), pH and HMs (Cr, Cu, Ni, and Zn) were among the most powerful factors, which change the community structure in HM contaminated soil under remediation.

Many studies reported that soil physicochemical properties (soil pH, soil texture, organic matter, etc.) moderate HMs' toxicity and therefore, HMs play a key role in shaping the community diversity and structure (Wang et al. 2022).

Taking into consideration that soil is highly heterogeneous, it is necessary to investigate the microbial communities at different sites and scales (Wang et al. 2022). In this term, the aim of this study was to assess the correlation between soil bacterial abundance and soil physicochemical properties, including HM content along the Zn, Pb and Cd contamination gradient in the area of non-ferrous processing plant KCM-2000. KCM-2000 is the largest lead-zinc smelter in the country, located in the vicinity of Plovdiv city, Southern Bulgaria. We hypothesized that long-term HM contamination reduced the abundance and changed the composition of indigenous soil bacterial communities, and these effects might be modulated by the local soil properties.

Materials and methods

Study area and soil sampling.

The study area is located in the region of a non-ferrous metal plant KCM 2000- Plovdiv, Southern Bulgaria (42°03'40.8"N, 24°48'52.0"E) (Fig. 1). Topsoil samples (0–20 cm) were collected in June 2020 along a gradient of contamination with Zn, Pb and Cd. Five points have been selected from a monitoring map, considering the direction of spread of the diffuse pollution as follows: KCM_1, named "Green belt of decorative trees", (42°03'31.68"N, 24°49'19.2"E), located at a distance of 0.5 km – south of the smelter; KCM_2 (42°03'5.76"N, 24°49'19.6"E) – 2 km south of the smelter; KCM_3 (42°02'6"N, 24°49'19.2"E) located close to the village of Dolni Voden – 3 km south of the smelter; KCM_4 (42°03'31.68"N, 24°49'45.12"E) – 1 km south-east of the smelter; and KCM_5 (42°04'23.52"N, 24°49'45.12"E) – 1 km east of the smelter. Five subsamples per site were pooled and used for further analyses. At all sites, the soil was classified as alluvial, being crop managed, except at KCM_1, where it was classified as technogenic.

Soil physicochemical properties and heavy metal content.

Soil pH was measured in 0.1M $CaCl_2$ according to ISO 10390:2005. Soil texture was determined by the Kachinsky method (1958). The total organic carbon (TOC) was determined according to Chen et al. (2014). Soil nitrate (NO_3-N) and ammonium (NH_4-N) nitrogen and inorganic phosphates (P_2O_5) were deter-
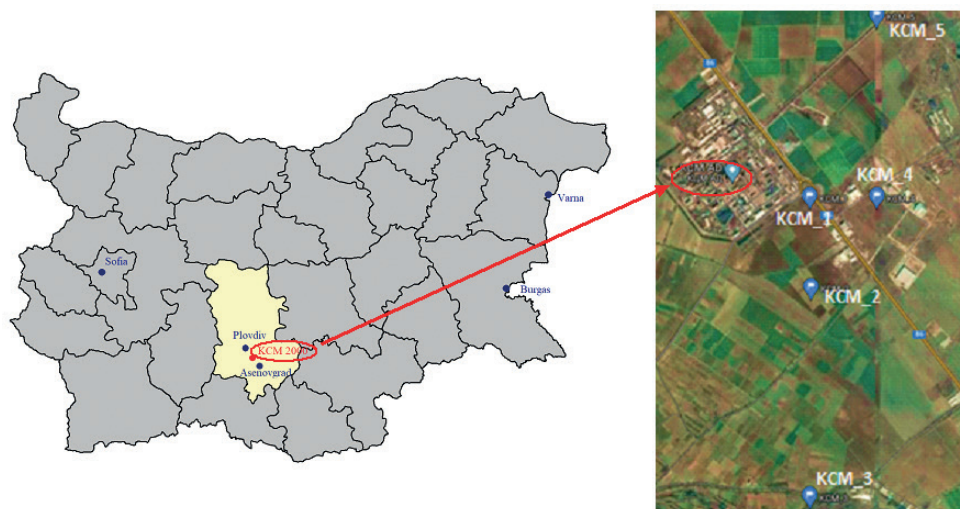
Figure 1. Map of the study area in Southern Bulgaria and sampling sites.

mined according to the methods of Keeney and Nelson (1982), and Olsen (1982), respectively. Soil moisture (SM) was calculated after oven drying (105 °C). The concentration of heavy metals was measured by ELAN 5000 Inductively Coupled Plasma Mass Spectrometer (Perkin-Elmer, Shelton, CT, USA) according to ISO 11047:1998 after soil decomposition by *aqua regia* (total HMs) and soil extraction with 0.01M $CaCl_2$ (bioavailable forms of HMs). Nemerow's pollution index (NPI) was calculated to evaluate the overall pollution of heavy metals in the soil samples (Zhao et al. 2019).

Enumeration of heterotrophic bacteria.

The bacterial abundance was estimated by the use of colony-forming units (CFUs) in serial dilution in R2A medium at 25 °C for 2 days. The selected dilution from each test sample was 10^{-4} and it was plated in triplicate. For this analysis, we used 10–100 colonies per plate.

DNA extraction.

The genomic DNA was extracted from 0.5 g soil using the E.Z.N.A DNA soil kit (Omega Bio-tek, USA) using the manufacturer's recommended protocol. The soil DNA quality was controlled by a spectrophotometer (NanoDrop 1000, ThermoScientific, USA) and agarose gel electrophoresis.

Quantitative PCR (qPCR) of the 16S rRNA gene

Bacterial abundance was quantified by real-time quantitative PCR (qPCR) with bacterial universal primer pairs Eub338f (5'-ATTACCGCGGCTGCTGG-3')/Eub518r (5'-ATTACCGCGGCTGCTGG-3') for 16S rRNA gene (Fierer et al. 2005). The qPCR reactions were set up using iTaqTM Universal SYBRGreen Supermix (BioRad) as described in Aleksova et al. (2020) and PCR efficiency was 90% (R^2 =0.9879).

Statistical analyses

Soil properties and HM concentrations were compared using principal component analysis (PCA). Prior to the analysis the data was normalized and checked for outliers. Linear correlations between the resulting indicator of pollution and the copies of 16S rRNA gene found in each sample were plotted and evaluated (r2 metric of plotted trendline). Additionally, the significance of the correlation between the two variables was evaluated with the Student T-test.

The PCA statistical analyses were carried out using Primer 7.0. Univariable statistical correlations were tested using STATGRAPHICS Centrion XVII software package. (Karamfilov et al. 2019).

Results

Environmental variables

The values of studied soil properties are shown in Table 1. The soils were determined as sandy loam textured. The soil pH was neutral (pH 6.7 to 7.2) and the total organic matter content (TOC) ranged from 6.45 g kg^{-1} to 14.07 g kg^{-1}. Soils were abundant with inorganic nitrate (especially KCM_1 and KCM_2) and inorganic phosphates. Probably, the high NO$_3$-N concentration in KCM_1 and KCM_2 was due to soil fertilization.

The HM concentrations at KCM_3 were under (Zn) and slightly higher (Pb and Cd) than the maximum permissible concentration (MPC) allowed under Bulgarian Regulation 3/2008 (http://eea.governement.bg/bg/legislation/soil), and this sample was considered as a control in our study. Pb was the most serious soil pollutant, and its concentration was over 100 (KCM_1), 57 (KCM_4), 13.7 (KCM_2) and 3 (KCM_5) times higher than the guideline limit. Cd was the other most serious soil pollutant and its concentrations exceeded the MPC in the following order: KCM_1 (92.45 times) >KCM_4 (43 times) >KCM_2 (7.85 times) >KCM_5 (4.0 times). The order of Zn soil contamination comparing to MPC was: KCM_1 (29.5 times) >KCM_4 (21.0 times) >KCM_2 (4.8 times) >KCM_5 (2.0 times). Nemerow's Pollution index (NPI) assessed the overall level of soil contamination (Zhao et al. 2019), and the soils were classified as heavily contaminated (NPI>3), except KCM_3, which had precautionary values of contamination (Table 1).

Table 1. Soil physicochemical properties and concentrations of heavy metals (total and bioavailable forms) in the area of KMC-2000.

Soil parameter	Soils				
	KCM_1	KCM_2	KCM_3	KCM_4	KCM_5
pH	7	7.1	7.2	6.7	6.8
Sand (%)	17.6	53.6	47.1	49.0	51.7
Silt (%)	39.2	30.9	31	30.8	36.2
Clay (%)	43.3	15.5	21.9	20.2	12.1
Soil moisture (SM) (%)	16.7	12.3	9.3	14.7	22.7
TOC (g kg^{-1})	9.65	14.07	6.45	12.33	7.035
NO$_3$-N (mg g^{-1})	43.38	16.32	3.01	5.13	[†]ND
NH$_4$-N (mg g^{-1})	6.62	5.13	3.26	2.25	2.22
P$_2$O$_5$ (mg kg^{-1})	5.69	24.02	7.42	6.89	[†]ND
Zn (mg kg^{-1})	9452	1558.2	216.2	6872	740
Pb (mg kg^{-1})	11569	1370.1	135.6	5723	335
Cd (mg kg^{-1})	184.9	15.7	3.9	86.2	9.3
Zn$_{b‡}$ (mg kg^{-1})	8.2	0.1	0.1	3.3	0.3
Pb$_{b‡}$ (mg kg^{-1})	2.6	0.2	0.9	0.2	0.8
Cd$_{b‡}$ (mg kg^{-1})	9	0.2	0.5	1.1	0.4
NPI[§]	73.27	8.74	1.00	37.46	3.11

[†]ND – No data; ‡$_b$ – Bioavailable forms of the heavy metals; [§]NPI – Nemerow Pollution Index.

Soil bacterial abundance

The results from the bacterial abundance of heavily contaminated soils were compared as a percent to KCM_3, which was a control soil in the experiment (Fig. 2). The highest number of cultivable heterotrophic bacteria (CFU) and 16S rRNA gene copies were reported for KCM_2. This was the only site, where bacterial abundance was around 27% (CFU) and 55% (16S rRNA) higher than that of the control (KCM_3). The lowest bacterial abundance was detected in the most contaminated site KCM_1, where it decreased by 56% (CFU) and 87% (16S rRNA gene copies) compared to the control soil. The soil bacterial abundance in KCM_4 and KCM_5 decreased by 47% (CFU) and 64% (16S rRNA) for KCM_4, and by 45% (CFU) and 17% (16S rRNA) for KCM_5 compared to KCM_3.

Correlation between soil properties and heavy metals.

Soil properties (total organic content, inorganic ions, soil particles of silt, clay and sand) and HM concentrations in soils were compared using principal components analysis (PCA) and the results are presented in Fig. 3. PC1 explained 65.7% of the total soil variation and showed a significant negative correlation with HM concentrations (total and bioavailable forms), concentrations of nitrate, nitrogen, soil pH and silt particles (Table 2). PC2 explained 17.7% of the total soil variation and positively correlated with sand particles and negatively with TOC and phosphates. PCA ordination showed a clear gradient of contamination level and differences in soil char-
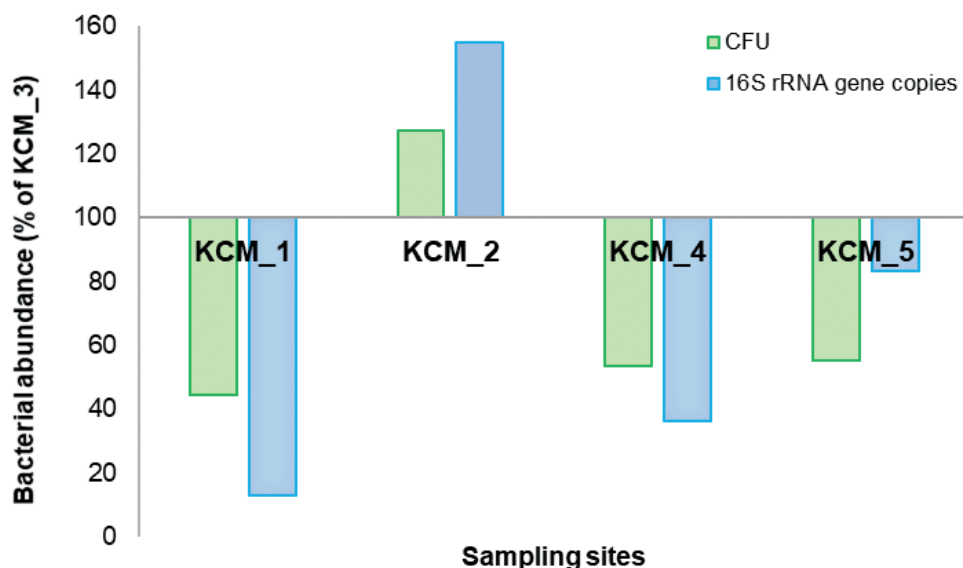
Figure 2. Soil bacterial abundance (% of KCM_3) of cultivable heterotrophic bacteria (CFU) and 16S rRNA gene copies.

Table 2. PCA axes scores of measured soil variables.

Variable	PC1	PC2	PC3	PC4
Variation explained (%)	**65.7**	**17.7**	**13.4**	**3.2**
pH	-0.001	**-0.308**	**-0.618**	**-0.327**
TOC	-0.011	**-0.501**	**0.437**	0.205
Sand	**0.324**	-0.047	0.119	0.110
Silt	-0.248	**0.266**	-0.137	**0.708**
Clay	**-0.316**	-0.027	-0.102	**-0.358**
Zn	**-0.284**	-0.019	**0.357**	-0.211
Pb	**-0.313**	-0.028	0.219	0.127
Cd	**-0.316**	0.000	0.197	-0.117
Zn_b	**-0.321**	0.032	0.156	-0.119
Pb_b	**-0.296**	0.142	-0.276	0.067
Cd_b	**-0.328**	0.019	-0.057	0.037
NO_3-N	**-0.305**	-0.216	-0.080	0.199
NH_4-N	-0.244	**-0.357**	-0.240	0.251
P_2O_5	0.068	**-0.620**	-0.022	0.116
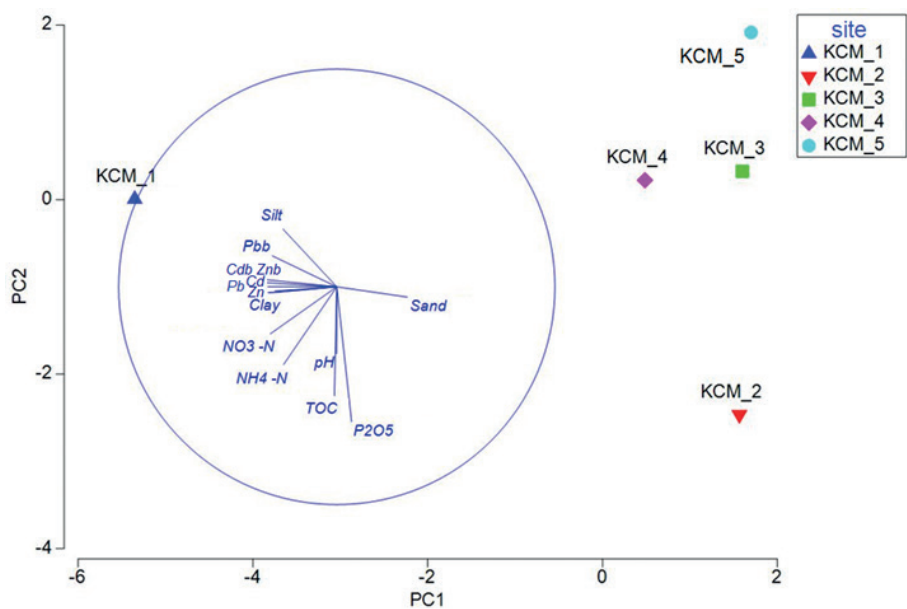
Figure 3. Principal component analysis of soil properties and heavy metals in the area of KCM-2000. Principal components axis 1 (PC1) explains 65.7% of the total soil variation and PC2 – 17.5%.

acteristics between different sampling sites (Fig. 3, Tables 2, 3). Thus, the resulting PC1 score for each sampling site can be regarded as an integral index of heavy metals pollution throughout the study area. Based on this evaluation, the soils of KCM_3 (PC1=1.6) and KCM_2 (PC1=1.57) were qualified as the least impacted by HMs, followed by KCM_5 (PC1=1.7), and the highly impacted soils of KCM_4 (PC1=0.486) and KCM_1 (PC1=-5.35) (Table 3).

Table 3. PC1 score and 16s rRNA gene copies.

Sample	PC1 score	16S rRNA gene copies ×10¹⁰
KCM_1	-5.35	1.49
KCM_2	1.57	17.80
KCM_3	1.6	11.50
KCM_4	0.486	4.17
KCM_5	1.7	9.57
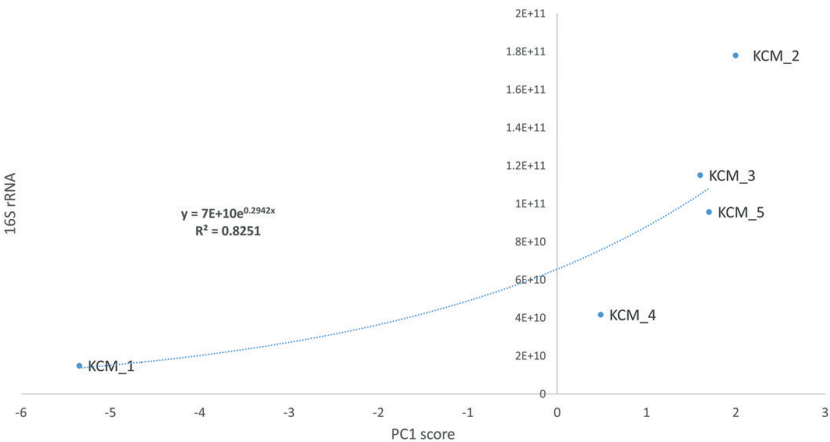
Figure 4. Exponential correlation between integrated HM contamination status (PC1 score) and bacterial abundance (16S rRNA gene copies) in the studied soils (R^2=0.8251) from the area of KCM – 2000.

Correlation between bacterial abundance and soil properties.

The soils had a high abundance of bacteria, whose number varied from 17.80×10^{10} (KCM_2) to 1.49×10^{10} (KCM_1) 16S rRNA gene copies (Table 3). To evaluate the impact of HM contamination on soil bacterial abundance, estimated as 16S rRNA gene copies, an exponential correlation was performed. The good exponential correlation between the values of PC1 pollution score, obtained by the PCA (Table 3), and 16S rRNA gene copies (R^2=0.8251) was demonstrated in Fig. 4. The results indicated both a dramatic decrease of soil bacterial abundance at KCM_1 and its exponential increase in soils along the gradient of contamination with Zn, Pb and Cd. The same analysis was conducted with the abundance of soil heterotrophic bacteria. The influence of increasing heavy metal contamination on microbial abundance was also confirmed by the significant correlation between the 16S rRNA gene copies and the PC1 pollution score (Student T-test, p=0.017).

Discussion

The present study focused on the correlation between bacterial abundance and soil properties in long-term contaminated soils in the area of non-ferrous metal processing

plant KCM-2000. The gradient of Zn, Pb, and Cd concentrations in the soil from KCM_3 to KCM_1 provided a good soil pattern for estimating the changes that occurred in soil bacterial abundance under the power of long-term HM contamination. The soil of KCM_3 was determined in this study as a control (NPI=1.00). In KCM_3, the concentration of Pb was slightly higher than the MPC, and Pb bioavailable forms were equal to that of KCM_5, exceeded by 2.5 times that of KCM_2 and KCM_4, and was by 3.0 times lower than Pb_b of KCM_1.

The distribution of bacterial abundance of unculturable and cultivable bacteria along a gradient of contamination was estimated through quantitative PCR of 16S rRNA gene and numbers of colony-forming units (CFU). In general, the soils of the site of interest showed a high bacterial abundance – 1.49×10^{10}-17.80×10^{10} 16S rRNA gene copies (total bacterial abundance) and 1.30×10^6 -3.70×10^6 CFU (abundance of heterotrophic bacteria).

Although, the HM gradient of soil contamination determined a gradient of soil bacterial distribution, only in the case of KCM_2 bacterial abundance was higher (around 55% for 16S rRNA and 27% for CFU) compared to that of the control. We suggested that this inconsistency with the model of general bacterial distribution could be due to the toxicity of the much higher Pb_b concentrations in KCM_3, or attributed to the modulating effects of higher concentrations of TOC, NO_3-N and P_2O_5 in KCM_2 compared to KCM_3 soil. Bacterial reduction in HM contaminated soils varied between 45–56% (CFU) and 54–87% (16S rRNA), except for the relatively low decrease in 16S rRNA gene copies in KCM_5 (17% compared to KCM_3). This fact could be explained by the relatively low level of soil contamination compared to the other studied sites (NPI=3.11). The obtained results were consistent with our previous study, where bacterial abundance (CFU and 16S rRNA gene copies) decreased in long-term contaminated with Cu, Zn and Pb soils in the area of copper mine and smelter (Aleksova et al. 2020; Palov et al. 2020). Similar findings for the decrease of CFU (Pacwa-Płociniczak et al. 2018) and 16S rRNA gene copies (Yin et al. 2015) under a long-term HM pollution in soils were observed by other authors. Fajardo et al. (2019) explained the decrease in bacterial abundance under HMs by a decrease in the metabolic activity of *Bacteria* in microbial soil communities. Other authors showed the opposite trend, which manifested that HMs (Cu, Zn, Pb and Cd) affected slightly the abundance, but strongly the diversity of bacterial communities (Tipayno et al. 2018; Huang et al. 2021).

To elucidate the effects of HMs and soil properties on the distribution of bacteria, an exponential correlation between the values of PC1 scores, and both 16S rRNA gene copies and CFU was conducted. There was found to be a good correlation between soil variables and 16S rRNA gene copies, but a low relationship between soil characteristics and CFU. We assumed that the lack of significant correlation between soil variables and heterotrophic bacteria (CFU) might have resulted from the limitation of cultivation technique or the high ecological tolerance of cultivable representatives of bacterial communities. Some authors (De Leij et al. 1994) suggested that cultivable bacteria could be classified as r-strategic opportunists, which grow fast, tolerate environmental fluctuations and are highly resistant to outside impacts.

Conclusions

The total bacterial abundance (estimated by the quantified 16S rRNA genes) increased exponentially in contrast to the abundance of heterotrophic bacteria, which could be explained by the limitations of the cultivable method. Regarding the statistical analysis, the bacterial abundance, expressed by the 16S rRNA gene copies, was considered as a more valuable indicator of the HM contamination effects on the soil inhabitants in comparison to the heterotrophic bacterial abundance. The reduction of bacterial abundance in heavily contaminated soil indicated that the soil properties (soil pH, total organic carbon, inorganic ions, soil texture) could modify the effects of heavy metals and the response of microorganisms to that stress in long-term contaminated soils. Further studies are needed for investigating the shifts in bacterial community structure in this area in response to the HM contamination gradient.

Acknowledgements

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RESEARCH ARTICLE

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Differences in bacterial functional profiles from loamy sand and clay loam textured soils under fungicide QuadrisR impact

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Abstract

The non-target effect of the fungicide QuadrisR on the bacterial community from grassland loamy sand (LS) and cropland clay loam (CL) soils with unknown history of fungicide usage was investigated. QuadrisR was applied to soil mesocosms at 0.0 mg kg^{-1} (Az0), 2.90 mg kg^{-1} (Az1), 14.65 mg kg^{-1} (Az2) and 35.0 mg kg^{-1} (Az3) calculated towards the active ingredient azoxystrobin (Az). Response of bacterial communities to QuadrisR was investigated during a 120-day incubation experiment, evaluating the shifts in bacterial catabolic profiles by the community-level physiological profiling (CLPP) technique and Biolog EcoPlates™ method. QuadrisR decreased the overall catabolic activity (AWCD) of soil bacterial communities and the rate of decrease was independent of soil type and fungicide concentration. Fungicide affected negatively the utilisation of amines and positively that of amino acids in both soil types, whereas the effects on other carbon guilds (carbohydrates, carboxylic acids and polymers) corresponded closely to the respective soil type and fungicide concentration. Results indicated the presence of non-target effects of QuadrisR on bacterial functioning; hence, it is important to address the fungicide side-effects on soil health.

Keywords

Average well colour development, community-level physiological profiling, fungicide azoxystrobin, QuadrisR, soil bacterial communities

Introduction

Plant diseases are a common occurrence, often having a significant economic impact on yield and quality; thus, managing diseases is an essential component of production for most crops. For this reason, fungicides are used to kill fungi by damaging their cell membranes, inactivating critical enzymes or proteins or by interfering with key processes, such as energy production or respiration (McGrath 2004). One of the widely used classes of fungicides is strobilusrins (Howell et al. 2014). The first patent for a strobilurin fungicide (azoxystrobin) was introduced in 1996 (Bartlett et al. 2002) and, subsequently, a series of strobilurin fungicides (pyraclostrobin, fluoxastrobin, kresoxim-methyl, trifloxystrobin, picoxystrobin, mandestrobin and metominostrobin) were developed and marketed (Rodrigues et al. 2013). Strobilurin fungicides specifically bind to the quinol oxidation (Q_o) site of cytochrome b and inhibit mitochondrial respiration (Bartlett et al. 2002). Strobilurin fungicides control an unusually wide array of fungal diseases, including diseases caused by water moulds, downy mildews, powdery mildews, leaf spotting and blighting fungi, fruit rotters and rusts (Vincelli 2002). They are used on a wide variety of crops, including cereals, field crops, fruits, tree nuts, vegetables, turf-grasses and ornamentals.

These pesticides are designed to manage fungal pathogens, although their broad-spectrum mode of action also produces non-target impacts. Due to the unique mechanism of action, strobilurins may directly affect soil fungi by inhibiting mitochondrial respiration, inducing a shift from fungal to bacterial dominance in soil activities (Baćmaga et al. 2015). Strobilurins may affect not only soil fungi, but also soil bacteria (Baćmaga et al. 2015), archaea (Howell et al. 2014) and invertebrates (Han et al. 2014). For instance, Baćmaga et al. (2015) reported negative effects of azoxystrobin not only on soil fungi, but also on soil organotrophic bacteria and actinomycetes. Most of the earlier studies reported low to negligible effects of Az alone or Az containing fungicides on bacterial diversity, but the knowledge of fungicide effects on bacterial metabolic activity is still insufficient.

The aim of this study was to elucidate the effects of Quadris[R], Az containing fungicide, on soil bacterial metabolism. The study suggested that Quadris[R] can potentially cause long-term adverse effects on soil nutrient turnover, affecting bacterial metabolism, although bacteria are considered as fungicide non-target organisms.

Material and methods

Sampling site and preliminary soil preparation

In this study, two soils with different histories of management practice were used. Five subsamples were pooled randomly from the surface layers (0–20 cm) of grassland and cropland located near Gabra Village (Sofia Region, Bulgaria): 42°31'48.36"N, 23°37'28.20"E (Fig. 1). The subsamples per soil type were sieved through a 2 mm mesh and mixed in aliquots after determining the dry weights of 1 g sample at 105 °C in an oven for 24 hr.

Figure 1. Map of Gabra Village territory (red line and red mark) and the sampling site (blue mark).

Mesocosm experimental design

Four sets of three replicated mesocosms (2 kg) were prepared for each soil type. The following treatments were studied: control (Az0) and Quadris[R] amendments of 2.90 mg kg^{-1} (Az1), 14.65 mg kg^{-1} (Az2) and 35.00 mg kg^{-1} (Az3), calculated towards the active ingredient – Az. Soil water content was adjusted to 60% of the maximum water holding capacity and it was maintained with sterile distilled water during the experiment. The mesocosms were incubated at 22 ± 1 °C in dark to prevent physical degradation of Az by light. Soil samples were collected randomly in triplicates from each mesocosm on the 1st (D1), 30th (D30), 60th (D60), 90th (D90) and 120th (D120) day after fungicide application.

Soil physico-chemical properties

Soil texture was defined and classified according to ISO 11277 (2009) and SSDS (1993), respectively. Soil pH was measured potentiometrically (HANNA Instruments) after mixing soil in 0.01 mol l^{-1} CaCl$_2$ solution and shaking for 30 min (1:5; weight: volume). Soil nitrate (NO$_3$-N) and ammonium (NH$_4$-N) nitrogen and phosphates (P$_2$O$_5$) were determined spectrophotometrically, according to the methods of Keeney and Nelson (1982) and Olsen (1982), respectively.

Az residues in soil

The method of Az soil residues extraction and determination was explained in detail in Aleksova (2020). Recovery rates of Az in each sample were satisfactory at 80.0%–85.0%. Modelling of Az dissipation in soils was conducted according to the recommendations of the FOCUS group (2006), using an Excel file (FOCUS_DEGKIN V2) provided online by the group. The same file was used to calculate the time of 50% reduction in Az soil concentrations (DT50).

Community level catabolic activity and physiological profiling

EcoPlates (Biolog Inc., Hayward, CA, USA) were used to establish the changes in CLPPs over time. The procedure of plates' inoculation, cultivation and monitoring (every 12 hr for 5 days) was described in detail in Kenarova et al. (2014). During the initial data processing, the control OD was subtracted from the OD of each carbon source (CS) well and the CSs with corrected OD < 0.25 were considered as non-oxidised and their values were set to zero (Garland 1996). Biolog CSs were grouped according to Weber and Legge (2009) into five carbon guilds (CGs) depending on their chemical moieties: carbohydrates (CH; 10 CSs), polymers (Polym; 4 CSs), carboxylic acids (CA; 9 CSs), amino acids (AA; 6 CSs) and amines/amides (Amin; 2 CSs). The Biolog-derived data were used to evaluate the bacterial metabolic activity (AWCD) (Garland and Mills 1991) and the pattern of CLPP (Kenarova et al. 2014).

Data analysis

Each data point in the paper represented the mean value of the respective Az soil amendment $\pm$ standard deviation. One-way ANOVA, followed by Tukey's test, was performed to examine the differences in the means of soil (pH, NO_3-N, NH_4-N, P_2O_5, Az) and bacterial (AWCD and CLPP) parameters. Principal component analysis (PCA) was performed with soil abiotic data to assess the differences in soil physical environments after Quadris[R] application. The differences in CLPPs between soil types and amongst fungicide concentrations were assessed with the graph 'one-to-one' technique. The above statistics were performed with the package PAST (Hammer et al. 2001) at a level of significance $p < 0.05$.

Results

Soil environments

The soil textures of grassland and cropland were classified as loamy sand (LS; 2% clay, 15% silt and 83% sand) and clay loam (CL; 27% clay, 37% silt and 36% sand), respectively. Soils were well abundant in organic carbon (LS: 21.92 $\pm$ 1.41 g kg^{-1} and CL: 23.4 $\pm$ 3.11 g kg^{-1}) and Kjeldahl nitrogen (LS: 2.20 $\pm$ 0.21 g kg^{-1} and CL: 2.64 $\pm$ 0.34 g kg^{-1}), both of them fluctuating insignificantly during the incubation time. Soil pH was moderately acidic (5.63) at LS and neutral (6.99) at CL and, during the incubation, it decreased significantly (LS: by Az1 – 8%, Az2 – 12% and Az3 – 14%) and insignificantly (CL: by Az1 – 0.8%, Az2 – 1.1% and Az3 – 1.3%) in fungicide amended soil mesocosms. Quadris[R] application increased the overall soil NO_3-N – in LS by 10% (Az1), 13% (Az2) and 20% (Az3) and, in CL, by 70% (Az1), 34% (Az2) and 19% (Az3). On the other hand, the overall soil NH_4-N concentrations decreased by 15% (LS) and 39% (CL). Soil concentrations of P_2O_5 were much more

stable than those of the inorganic nitrogen, decreasing during the incubation by 8.6% (LS) and 14.3% (CL).

Az soil residues decreased over time and the rate of decrease was higher for LS than those for CL – DT50 ranged for LS from 36.5 ± 7.1 (Az1) to 86.6 ± 4.1 (Az3) days, whereas those for CL ranged from 130.8 ± 7.8 (Az1) to 212.1 ± 3.2 (Az3) days.

PCA, based on soil physico-chemical properties and Az soil residues, was conducted in order to elucidate the similarity amongst soil physical environments (Fig. 2) and the analysis indicated: 1) the respective LS and CL mesocosms differed significantly from each other, except Az1 where fungicide input approximated to the physical environments of LS and CL on D90; 2) significant differences within-soil physical environments were detected, except those of Az1 and Az2 at LS on D60; 3) temporal fluctuations of CL physical environments were smaller than those of LS.

Bacterial metabolic activity

The AWCD of Az0 (CL) -1.69 OD was calculated to be around 35% higher than that of Az0 (LS) -1.25 OD. Quadris[R] application decreased the overall mean value of AWCD (except Az1 at LS and Az2 at CL) and the changes were significant (Az3 at
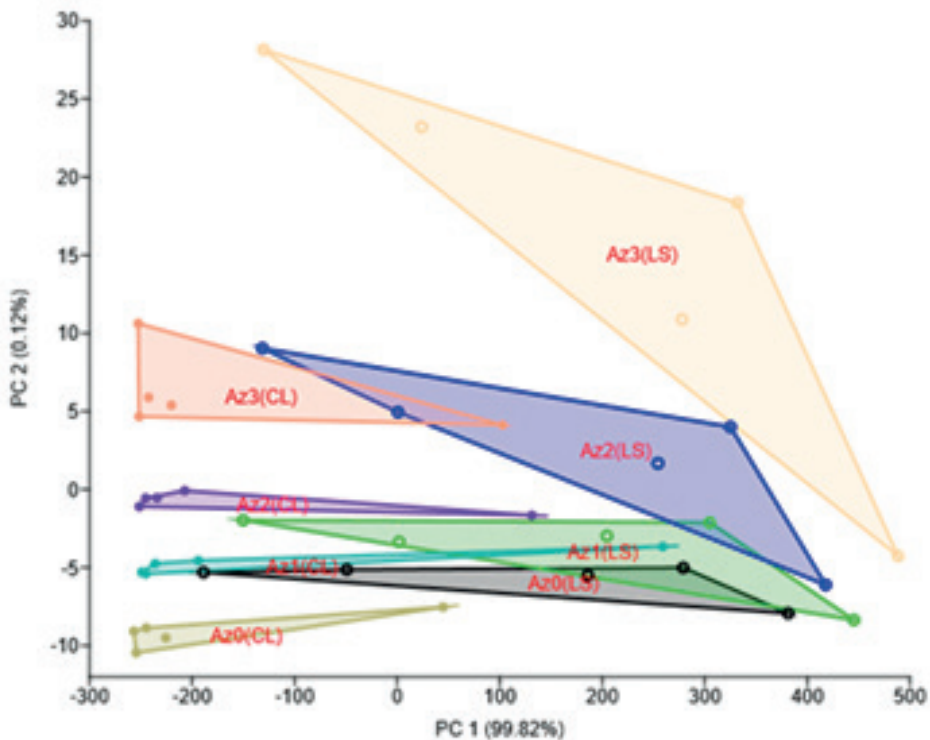
Figure 2. Spatial projection of the first two principal components (PC 1 and PC 2) with an ordination plot related to soil physical environments of Quadris[R] amended (Az1 – Az3) and un-amended (Az0) loamy sand (LS) and clay loam (CL) soil mesocosms.

LS and Az1 and Az3 at CL) and insignificant (Az2 at LS). A stimulation effect was recorded for Az1 (LS) and Az2 (CL), being significant only for the second one. Temporal Quadris[R] effects on bacterial metabolism were very similar, independent both on fungicide concentration and soil type – AWCD profiles manifested a decrease in bacterial metabolism for at least two months (D1 – D60), followed by recovery (D60 – D90) and stimulation (D120). Different metabolic profiles over time were formed for Az2 and Az3 at CL – permanent stimulation (Az2, except on D1) and dramatic decrease (Az3 after D60) after fungicide application. The values of Quadris[R] that influenced AWCDs were much higher than that of Az0 on D120 (except Az3 at CL) and the rates of stimulation were in reverse- (LS) and non- (CL) relationships with the applied fungicide concentrations.

One-way ANOVA showed that the respective AWCD means of Az1 and Az3 did not differ significantly between LS and CL ($F < 2.05$, $p > 0.16$), opposite to that of Az2 ($F = 18.5$, $p = 0.000$).

Community level physiological profiling

It was obvious that bacterial metabolism was changed under Quadris[R] impact, but AWCD was not sufficiently powerful to demonstrate the differences of these changes as a dependence of the applied fungicide concentration and soil properties. Therefore, after grouping the EcoPlate carbon sources into carbon guilds (CG), the CLPP approach and 'one-to-one' analysis were used to elucidate the intrinsic nature of AWCD changes (Fig. 3). Between-soil analysis indicated that, after Quadris[R], LS differed from CL by the utilisation of: 1) CH and CA – Az1, 2) all CGs, except CH – Az2, and 3) all CGs – Az3. Within-soil 'one-to-one' analysis demonstrated the effects of increasing fungicide concentrations on the utilisation of CGs in the respective soil type and they were significant at: 1) LS – all fungicide concentrations influenced the utilisation of CH (positively at Az1 and Az2 and negatively at Az3) and Amin (negatively at Az1 – Az3); Az1 and Az2 stimulated the utilisation of AA; Az3 decreased the utilisation of Polym and 2) CL - all fungicide concentrations influenced the utilization of-Amin (positively at Az2 and negatively at Az1 and Az3); Az1 and Az2 stimulated the utilisation of AA and CA (except Az1); Az3 decreased the utilisation of CH and Polym.

Metabolic diversity

In order to understand the insights of changes in CG utilisation rates under Quadris[R], the overall number of utilisable CSs were counted – metabolic richness, as well as the index of carbon sources' utilisation evenness per CG.

Most of the changes occurred in CGs related to shifts into the "evenness" rather than the "richness" of utilisable CSs. For example, the richness changes under Quadris[R] were detected only for the utilisation of CHs and CAs at CL - utilisation of carbohydrate D-Xylose (Az1 – Az3) was inhibited, whereas that of carboxylic acids, γ-Hydroxybutyric acid (Az1) and 2-Hydroxy benzoic acid and α-Ketobutyric acid (Az2 and Az3), was
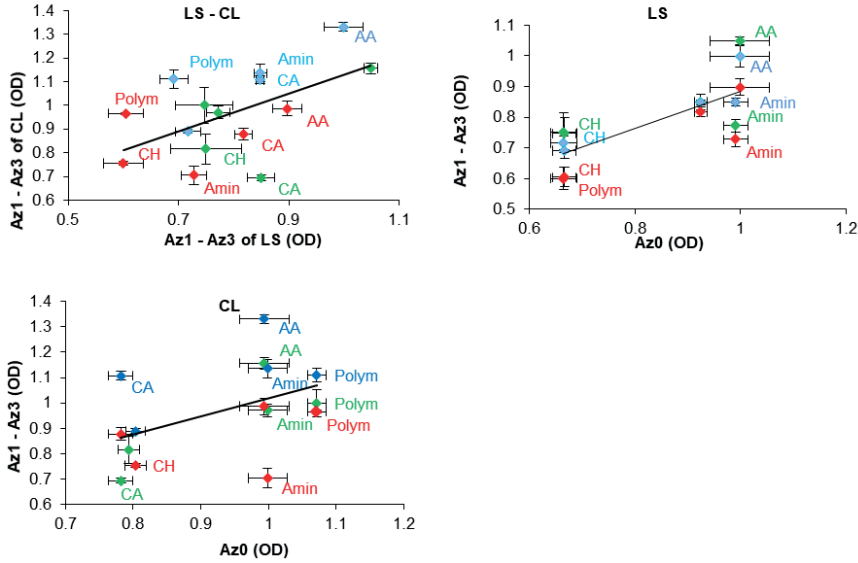
Figure 3. 'One-to-one' comparison of CLPPs between loamy sand and clay loam (LS – CL) soil mesocosms and between Quadris[R] un-amended (Az0) and fungicide-amended soil mesocosms (Az1 – Az3) per soil type. Diamond symbols illustrate the mean (n = 3) utilisation rate of the respective carbon guild, bars illustrate the standard deviations and colour denotes the fungicide concentration - Az1: green, Az2: blue and Az3: red.

stimulated. Much more changeable amongst soil mesocosms was the index of metabolic evenness. The greatest between-soil type differences were detected at: 1) Az1, where Quadris[R] increased the metabolic evenness of Polym, CA and Amin at LS and decreased that at CL and 2) Az3, where Quadris[R] increased the metabolic evenness of CH, Polym and CA at CL and decreased that at LS. The between-soil type similarity was found at Az2, where Quadris[R] increased the metabolic evenness of CA, AA and Amin.

Discussion

Soil physical environment

Soil amendments with Quadris[R], even by the lowest fungicide concentration, created new physical environments referring mainly to changes in soil pH, nitrogen pool and presence of allochthones substrates (Az and Quadris[R]'s adjuvants). Similar soil acidification after Az application was also reported by earlier studies (Ghosh and Singh 2009; Singh et al. 2010), explaining this fact by the formation of azoxystrobin acid as the major product of fungicide degradation. In this study, the decrease in pH was recorded immediately after fungicide application (D1), assuming that azoxystrobin acid was not the only determinant of soil acidification. Probably, some of

the Quadris[R] ingredients contributed also to soil acidification. We supposed that soil acidification might influence directly and/or indirectly bacterial metabolism, shifting community composition into growth of acidophiles and influencing nutrient solubility (bioavailability).

Soil amendments with Quadris[R] influenced the soil nitrogen pool, changing bioavailable concentrations and forms of inorganic nitrogen which could be related to the adjuvants presented in fungicide commercial formulations (Devare et al. 2007; Mijangos et al. 2009), fungicide metabolisation (Cycoń et al. 2011; Baćmaga et al. 2017) and accumulation and degradation of proteins released from killed soil inhabitants (Wu et al. 2014; Zhang et al. 2014). Since nitrification and mineralisation play important roles in nutrient turnover (Edwards et al. 1995), it seems that shifts in soil inorganic nitrogen could disrupt these processes and impact overall soil quality and productivity. Additionally, proportions of soil NO_3-N and NH_4-N could also influence the utilisation rates of nitrogen containing CSs. We supposed that Az was more bioavailable in LS compared to CL and it influenced its persistence, half-life and toxicity to soil organisms. According to DT50, Az could be considered as a low to medium persistent fungicide in LS and highly persistent in CL.

The ordination of soil physical environments demonstrated that Quadris[R] application in increasing concentrations influenced soil properties, creating new physical environments. We supposed that newly-created environments might influence soil bacteria to adapt their metabolism.

Fungicide effects on soil bacteria

In this study, we evaluated the Quadris[R] effects on soil heterotrophic bacteria, which display a substantial role in plant growth rates, mineralising dead organic matter and detoxifying a range of exogenous substances. Bacteria are considered as Az non-target organisms, due to the fungicide mode of action on mitochondrial respiration (Bartlett et al. 2002). In fact, earlier reported data, referring to bacterial community composition (Howell et al. 2014) and functioning (Sułowicz et al. 2016; Wang et al. 2020) under Az, were very contradictory. We hypothesised that fungicides influenced soil bacterial metabolism indirectly by changes in soil biotic and abiotic properties. Two main metabolic criteria were followed during the soil mesocosms' incubation: 1) community metabolic activity expressed by AWCD and 2) community metabolic profiles (CLPP) expressed by carbon guilds' utilisation rates and metabolic diversity. In the case of detected fungicide impacts, it was important to mention if there were any relationships to soil type and, in particular, with some of the studied soil properties.

Community metabolic activity (AWCD)

Quadris[R] application influenced bacterial metabolism for at least four months, decreasing it in most of the soil mesocosms, except at Az1 (LS) and Az2 (CL). The most serious negative effects were detected during the first two months after fungicide application,

followed by recovery and stimulation of bacterial metabolic activity (except Az3 at CL). Some researchers reported inhibitory effects of fungicides on bacterial metabolic activity (Zhang et al. 2014), although others advocated none or stimulation effects on AWCD (Muñoz-Leoz et al. 2011). We suppose that these contradictions arise from the fungicide chemical origin, applied concentrations and soil properties. In our study, soil properties were of significant importance for the differentiation of Quadris[R] effects on overall AWCD at Az2, but not at the other fungicide concentrations. Probably, the delayed stimulation effects at Az1 decreased the differences in AWCD between LS and CL, whereas the very high value of Az3 minimised the modulating effects of soil peculiarities on fungicide mode of action.

Community level physiological profiles (CLPP)

Carbon guilds' utilisation rates

Earlier studies (Bending et al. 2007) and our investigations (Aleksova et al. 2021) reported that azoxystrobin did not affect bacterial community structure, suggesting that the fungicide shifted bacterial metabolism via chemical toxicity and/or phenotypic bacterial adaptation to environmental changes. The dissimilarity between LS and CL under Quadris[R], applied at a field recommended concentration, was referred towards CH and CA utilisation. These results confirmed the findings of some authors (Kenarova et al. 2014; Yu et al. 2020) that the utilisation of carbohydrates and carboxylic acids was sensitive to environmental disturbance and it could be used to indicate the alterations that occurred in bacterial functional profiles under stress. Additionally, in this study, we related that fungicide-affected soil physical environments. Probably, the differences in pH, clay content and organic matter concentration between the two soil types reflected differently on CH and CA bioavailability; hence, on bacterial adaptations to changed soil nutrient pools. Great differences in temporal profiles of CH and CA utilisation (not shown here) were detected in the late fungicide exposure stage (D90–D120), when the utilisation of the two CGs increased dramatically at LS (by 45%, on average) and stabilised (CH) or decreased by 15% (CA) at CL.

Higher fungicide concentrations (Az2 and Az3) widened the spectrum of impacted CGs, but these effects could be related to soil chemical pollution, rather than to the controlled use of Quadris[R] for plant protection.

Metabolic diversity

In both soil types, Quadris[R] application influenced metabolic evenness rather than on metabolic richness, which might be explained by the intrinsic bacterial community capacity to be metabolic resistant, resilient and redundant (Fenchel and Finlay 2004; Meyer et al. 2004). Some bacteria show a high degree of metabolic tolerance (resistance) to changing environmental conditions (Meyer et al. 2004), whereas others

are capable to adapt quickly to the new nutrient inputs for rapid growth (resilience) (Fenchel and Finlay 2004). Further, the extremely high abundance and diversity of bacteria are arguments for their metabolic redundancy, ensuring ecosystem functioning (nutrient turnover), even in extreme conditions.

We assumed that Quadris[R] significantly changed soil nutrient pools and the changes might occur due to soil accumulation of dead fungal biomass, induction of detoxification agents (mainly proteins) - molecules that can later be metabolised by the same microbiota (Degens et al. 2000), changes in soil pH and/or fungicide adjuvants' inputs (Syngenta 2021). Changes in soil mesocosms affected in different ways bacterial capacity to use CSs – ranging from inhibition to stimulation. Interesting was the stimulation effects of Quadris[R] on the utilisation of γ-Hydroxybutyric acid, 2-Hydroxy benzoic acid and α-Ketobutyric acid at CL. Stimulated utilisation of 2-Hydroxy benzoic acid and α-Ketobutyric acid under fungicide tetraconazole was observed earlier by Sułowicz et al. (2016).

Conclusions

The study showed that soil properties (soil texture, pH and organic and inorganic substances) were of significant importance for the fate of applied fungicide Quadris[R], as well as its effects on bacterial metabolism. The fungicide decreased for at least four months the overall bacterial activity (AWCD), shifted the metabolite profiles (CLPPs) of bacterial communities and changed the preferred carbon sources and metabolic diversity. Fungicide also affected the mode of environmental control on bacteria, in accordance with soil peculiarities.

Acknowledgements

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RESEARCH ARTICLE

Radiation status of soils from the region of the Eastern Rhodopes (Southern Bulgaria)

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Abstract

Local values of natural radiation background in soils from unexplored regions in the Eastern Rhodopes were established. The impact of anthropogenic activity as a potential risk for increase in radiation background was assessed. Soil samples from areas near the liquidated lead-zinc mines – Madzharovo, gold mine – Ada Tepe, Krumovgrad, lead-zinc complex – Kardzhali, Neochim – Dimitrovgrad, deposits for extraction of gneiss, marble quarries, etc. were analyzed to study possible contamination. Specific activity of natural radionuclides ^{210}Pb, ^{238}U, ^{226}Ra, ^{235}U, ^{232}Th, ^{40}K and technogenic ^{137}Cs in the studied samples was determined by gamma spectrometric analysis with Multichannel analyzer DSA 1000, production of CANBERRA and HPGe-detector.

Keywords

Anthropogenic activity, natural and technogenic radionuclides, radiation monitoring of soils, radiation pollution, radioecology

Introduction

The Rhodopes are the largest mountain range in our country. The relief of the Eastern Rhodopes is mainly lowland and hilly with an average altitude of about 300 m. The main rocks are sedimentary and volcanic (andesites, rhyolites, tuffs, etc.), as the Eastern Rhodopes were occupied by a water basin with active under-

water volcanism in the past. Deluvial and cinnamon soils are the most widespread. The soil-forming rocks are mainly granites, marble, gneiss and shale characterized by relatively high content of uranium and other natural radionuclides. The extraction of heavy and rare metals in the area, as well the production of some mineral fertilizers, carries the potential risk of further pollution of the environment with natural radionuclides.

The aim of the research was to study undisturbed soils, i.e. soils unaffected by industrial activity from areas in close proximity to industrial sites to assess the impact of anthropogenic activity on potential increase in radiation background. A large region was covered to collect initial data on the radiation status of the soils and for planning further studies in areas where high content of natural radionuclides was found.

The aim of the study was also to register the soil status in the region in terms of technogenic pollutant cesium-137.

A comparison was made with the radiation status of soils from other regions of Bulgaria.

Methods

Soil samples from representative points close to anthropogenically affected areas were collected and analyzed. Four expeditions were carried out to collect soil samples from areas near the liquidated Madzharovo lead-zinc mines, Ada Tepe gold mine, Krumovgrad, Kardzhali mining complex, Neochim, Dimitrovgrad, gneiss mining sites and marble quarries (Figures 1 and 2). Five samples were collected for each point according to BSS 17.4.5.01:1985.

Collection and preparation of samples were performed following ISO 18589–2,3 (2007) Sampling from 0 to 5 cm depth was carried out to monitor surface contamination and up to 20 cm to characterize the process in depth. Soil samples were air-dried, homogenized and ground and sieved through a 2 mm sieve. The Marinelli samples containers of 0,5 l volume and geometry 4π were used for performing gamma spectrometric analyses. The statistical reliability of the gamma analysis result is achieved through the duration of the measurements. The samples were measured from 19 to 24 hours.

The specific activity of natural radionuclides ^{238}U, ^{226}Ra, ^{232}Th and ^{40}K and technogenic ^{137}Cs in soil samples was determined by gamma spectrometric analysis following ISO 18589-3. A DSA 1000 Multi-Channel Analyzer, CANBERRA, with ultra-pure germanium detector, with 35% efficiency and 1.8 keV resolution was used allowing simultaneous and direct measurement of a large number of gamma emitters with energies from 50 to 2000 keV. ^{238}U was measured by the daughter product ^{234}Th (63.3 keV and 92.3 keV). ^{226}Ra was determined by the maximum energy peak at 186.3 keV, with correction for ^{235}U (185.6 keV), ^{210}Pb – by the gamma line at 46.6 keV. ^{232}Th was determined by the daughter product ^{228}Ac (911.0 keV)

Figure 1. Eastern Rhodopes – map of the studied area.
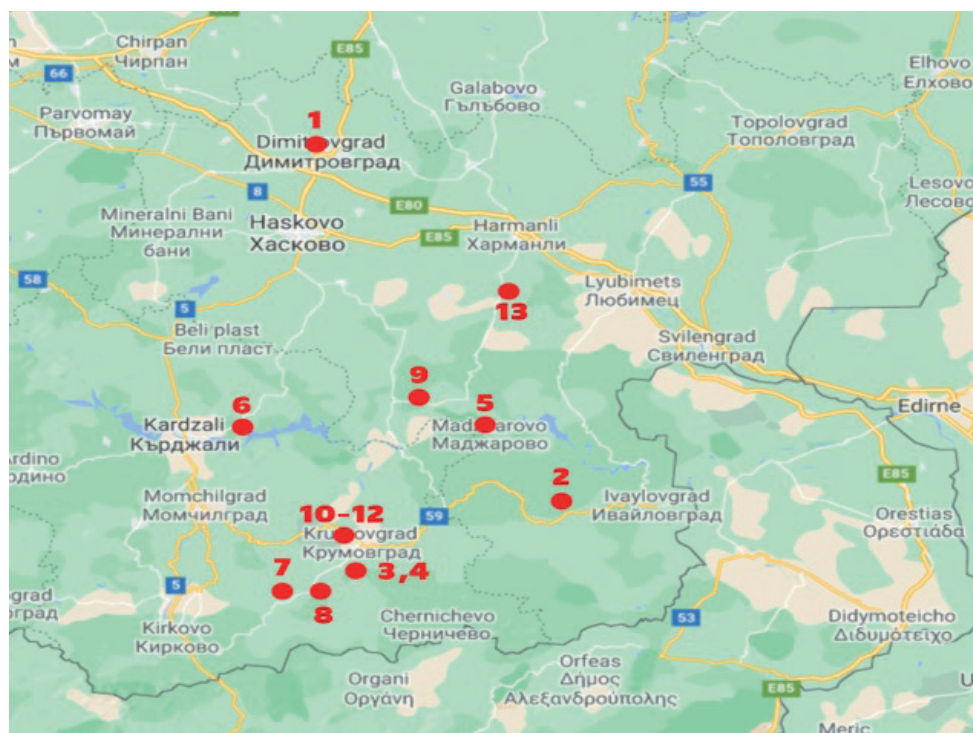
Figure 2. Ada Tepe gold mine.

and ^{40}K – by the 1461 keV full energy peak. Technogenic ^{137}Cs was measured by the gamma line at 661.6 keV.

To assess the radiation risk to the population in the studied areas radium equivalent activity (Ra$_{eq}$) and external hazard index (H$_{ex}$) were calculated by the formulas given below.

$$Ra_{eq} = A_{Ra} + 1.43A_{Th} + 0.077A_K, \text{ (Beretka and Mathew 1985)},$$

where A_{Ra}, A_{Th} and A_K are the activities in Bq/kg of ^{226}Ra, ^{232}Th and ^{40}K respectively. The limit value is 370 Bq/kg.

$$H_{ex} = A_U / 370 + A_{Th} / 259 + A_K / \ 4810 \le 1, \text{ (Krieger 1981)},$$

where A_U, A_{Th} and A_K are activities in Bq/kg of ^{238}U, ^{232}Th and ^{40}K, respectively.

Results

The data of the content of ^{40}K, ^{210}Pb, ^{235}U, ^{238}U, ^{232}Th and ^{226}Ra, as well as technogenic ^{137}Cs in the soil samples from the studied region of the Eastern Rhodopes are presented in Table 1 in Bq/kg dry weight and combined standard uncertainty. The table indicates the anthropogenically affected areas near where the soil samples have been taken.

The results are discussed in the context of possible increase in background radiation exposure due to continuing human activity in the areas.

The radiation hazard was assessed by calculating the radium equivalent activity (maximum permissible level – 370 Bg/kg) and the external hazard index presented (maximum permissible level – 1) in Figures 3 and 4.

Table 1. Content of radionuclides in soils from the region of the Eastern Rhodopes in Bq/kg dry weight.

No	Sampling	Depth cm	^{210}Pb	^{137}Cs	^{40}K	^{238}U	^{226}Ra	^{235}U	^{232}Th
1	Neochim, Dimitrovgrad	0–5	34±5	28±1	670±10	41±4	30±3	1.7±0.5	38±3
2	Quarry, village of Cherni rid	0–5	55±6	< 1	850±20	50±5	73±8	2.5±0.5	47±4
3	Ada Tepe mine, Krumovgrad, bypass road	0–5	60±5	26±1	360±10	18±3	21±4	1±0.5	25±2
4	Ada Tepe mine, Krumovgrad	0–5	-	-	1000±10	9±4	-	-	-
5	Madzharovo mine,	0–5	52±6	5±1	1432±20	55±6	92±10	2.5±1.0	40±3
6	LZC, Kardzhali	0–5	64±7	1±0.5	750±20	56±7	65±8	2.6±0.5	70±8
7	Marble quarry, Golyama Chinka Village	0–5	26±4	12±1	260±10	30±5	52±7	1.5±0.5	22±2
8	Fossils after the village of Kandilka	0–5	45±5	7±2	300±10	42±5	53±6	2±0.5	16±2
9	Arable soil the village of Razhenovo	0–5	60±5	6±1	850±20	62±5	70±8	3±0.5	82±7
10	Arable soil, Krumovgrad	0–5	50±8	26±4	26±4	40±5	36±5	2±0.5	30±4
11 - 1	Arable soil, Krumovgrad (yard)	0–5	36±6	87±4	540±10	28±4	42±5	1.5±0.5	35±4
11 - 2	Arable soil, Krumovgrad (yard)	5–10	52±8	76±3	570±10	35±6	33±5	1.8±0.5	33±3
11 - 3	Arable soil, Krumovgrad (yard)	10–20	57±10	76±3	570±10	38±5	30±4	1.7±0.5	34±3
12	Undisturbed soil, Krumovgrad	0–5	28±5	7±1	540±20	35±5	25±4	1.6±0.5	35±5
13	Arable soil, Leshnikovo village	0–20	45±6	35±3	670±20	35±6	31±6	2±0.5	40±3
14	min and max value	0–20	26 - 64	< 1 - 87	26 - 1432	9 - 62	21 - 92	1 - 3	16 - 82
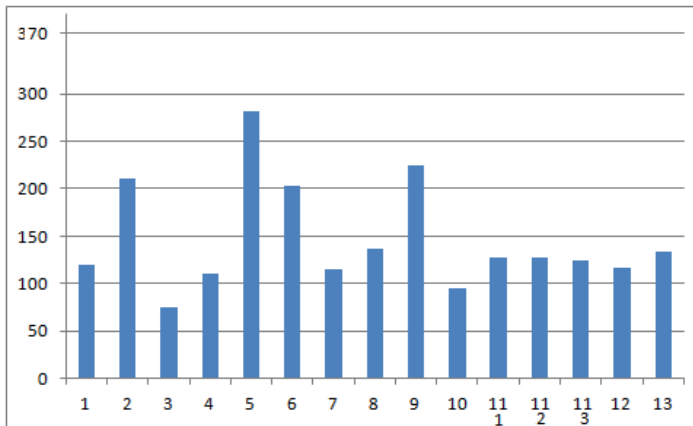
Figure 3. Radium equivalent activity (Ra$_{eq}$).
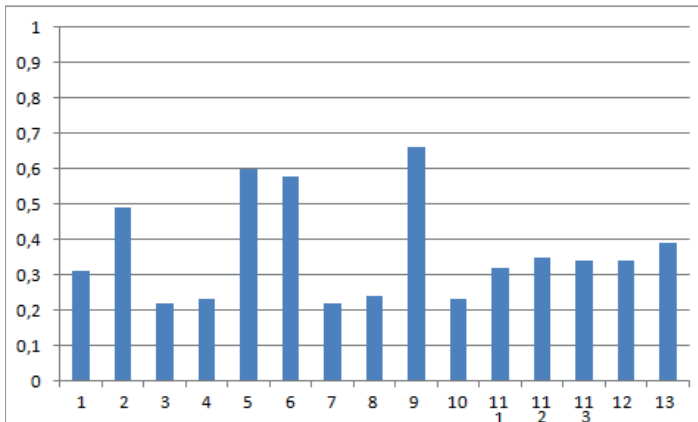
Figure 4. External Hazard Index (H$_{ex}$).

Discussion

The study covers unexplored areas of the Eastern Rhodopes affected by a variety of human activities, the result of which may cause additional radiation pollution to the soil. Sampling was carried out in close proximity to such areas.

Natural radioactivity

Radioactivity levels in the environment depend on geological aspects, mainly on the composition of rocks and soil, where natural radionuclides are found in varying concentrations (Raykov 1978; Négrel et al. 2018). The Rhodopes are rich in uranium and other ore deposits. Exposure to various human activities can increase the proportion

of natural radioisotopes in the effective dose. This can pose a potential risk to humans and living organisms, as 75% of the radiation received by mankind is due to natural sources of radiation. (Ghiassi-Nejad et al. 2001).

A comparison is made between the radioisotopes content in the soils from the studied areas and the average values of natural radionuclides in undisturbed soils from different regions of the world, Bulgaria, the Western Rhodopes, and the Sofia field. (Yordanova et al. 2005, 2015; Lazarova et al. 2019).

The publications of the United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR 2000) report average values of the content of natural radionuclides in Bq/kg in undisturbed soils from different regions of the world. The data is presented in Table 2.

For the study region, the specific activity of the natural radionuclides ^{238}U, ^{226}Ra, ^{232}Th, ^{40}K, ^{210}Pb in Bq/kg was in the following range: ^{238}U – 9÷62; ^{226}Ra – 11÷92; ^{232}Th –16÷82; ^{40}K – 260÷1432 and ^{210}Pb – 26÷64 (Figs 5–7).

Content of natural radionuclides in the studied soils was within the background amounts and was comparable to global averages (Figs 5–7). The differences between the Eastern and Western Rhodopes could be explained by the predominance of sedimentary rocks in the Eastern Rhodopes. Sedimentary rocks, especially of biogenic origin, have a very low content of radioactive elements.

Table 2. Content of ^{238}U, ^{226}Ra , ^{232}Th and ^{40}K in Bq/kg in various regions of the world.

	^{40}K	^{238}U	^{226}Ra	^{232}Th
World average	400	35	35	30
Europe	40÷1650	2÷330		2÷190
Bulgaria	40÷800	8÷190	12÷210	7÷160
average	(400)	(40)	(45)	(30)
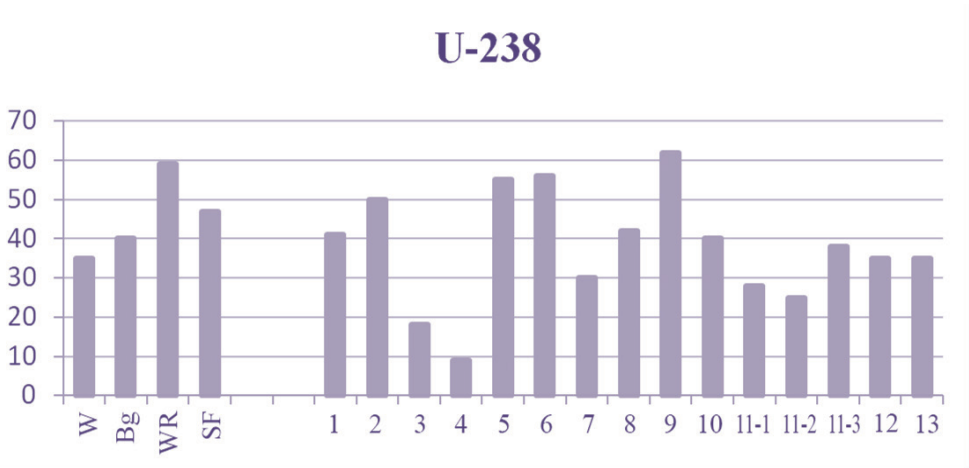
Figure 5. Content of ^{238}U in soil samples – world average (W), average for Bulgaria (Bg), average for the Western Rhodopes (WR), average for the Sofia field (SF) and in the studied samples.
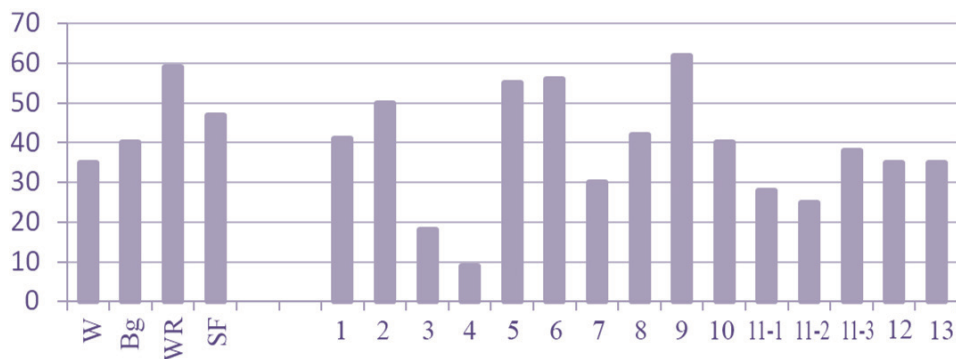
U-238

Figure 6. Content of ^{226}Ra in soil samples – world average (W), average for Bulgaria (Bg), average for the Western Rhodopes (WR), average for the Sofia field (SF) and in the studied samples.

U-238
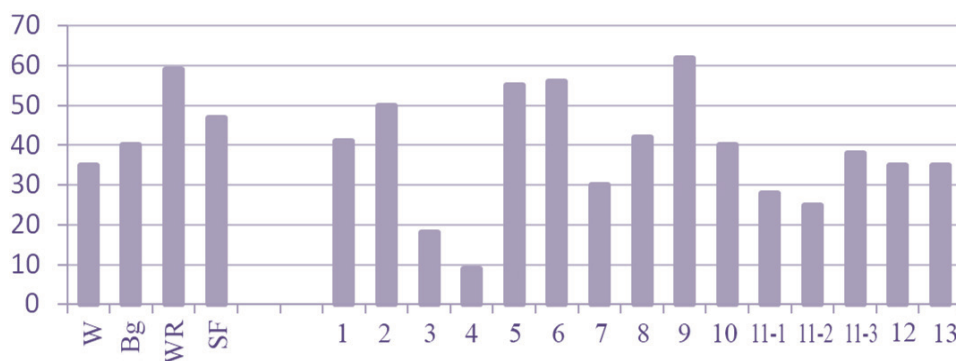
Figure 7. Content of ^{232}Th in soil samples – world average (W), average for Bulgaria (Bg), average for the Western Rhodopes (WR), average for the Sofia field (SF) and in the studied samples.

Potassium is an important element. It is found in all living organisms. The importance of the radioactive isotope ^{40}K is mainly due to its long half-life (1.28 × 109 years) and its ubiquity.

World average specific activity of ^{40}K (activity per unit mass of soil) is 370 Bq/kg, ranging from 100 to 700 Bq kg (Mcaulay and Moran 1988).

Content of ^{40}K in Bulgarian soils varies significantly – from 40 to 800 Bq/kg. For the studied area it ranges from 30 to over 1400 Bq/kg (Fig. 8). This wide range is characteristic of cinnamon forest soils, predominant in the studied region, due to the great variety of soil-forming rocks in these soils. In the soil samples from the Ada Tepe

U-238
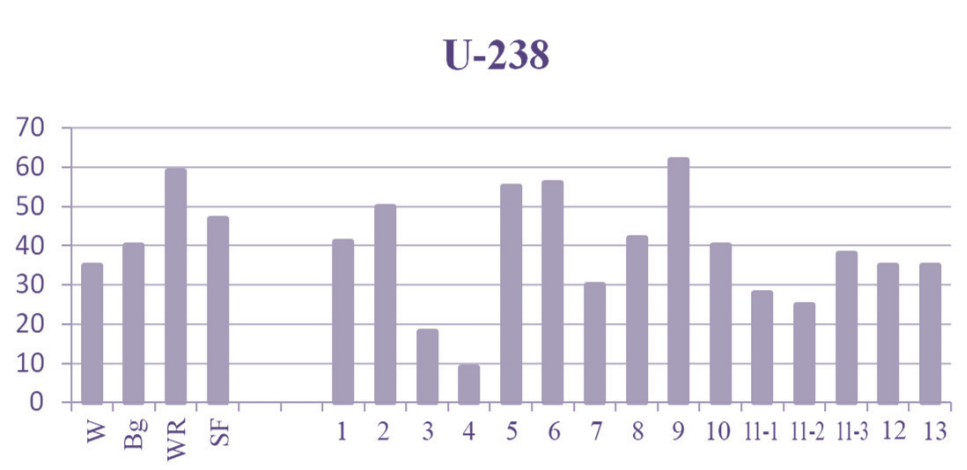
Figure 8. Content of ^{40}K in soil samples – world average (W), average for Bulgaria (Bg), average for Western Rhodopes (WR), average for the Sofia field (SF) and in the studied samples.

gold mine and the Madjarovo mine, the measured values were two times higher than the world average. This can be explained by the presence of sedimentary rocks rich in organic remains.

A shift in the radioactive equilibrium between ^{238}U and ^{226}Ra and higher content of radium was detected in some of the soil samples. It may be due to the lower mobility of radium in soils, the low humus content and the greater solubility of uranium salts allowing U migration along the soil profile.

No significant differences were found in the radiation status of arable and non-arable soils. The results of the studied radionuclides correspond to the average or slightly above average values, typical for the geographical latitude of Bulgaria and were within the values cited in the literature as normal for the respective regions.

Technogenic radioactivity

Radioactive contamination introduces new elements into the ecosystem. As a result of nuclear tests in the 1960s and the Chernobyl accident, ^{137}Cs entered the environment. For the study area, the specific activity of the technogenic radionuclide is in the range (1–87) Bq/kg.

After the Chernobyl nuclear accident, the southern part of Bulgaria was most affected. Four to five times higher activity concentrations were measured in the soils of Southern Bulgaria than in those of the Northern part (Yordanova et al. 2007). The Rhodopes have received significant amounts of radioactive ^{137}Cs. Through the rains, it entered the soil, bound to the surface soil layer and was redistributed into the ecosystem, where it remained for a long time due its long half-life.

From the data on the specific activity and dynamics of ^{137}Cs it can be seen that the soil pollution was non homogeneous as a result of air transport. It varied between

3 and 1700 Bq/kg, even within small areas (tens of square meters) (Yordanova et al. 2007). This was also confirmed by the present studies where its content was ranging from <1 to 87 Bq/kg (Fig. 9). The total technogenic γ-activity in the soils of Bulgaria has increased between 10 and 300 times after the Chernobyl accident. For ^{137}Cs this excess was 3–10 times, in some cases reaching up to 50 times above the characteristic background values. BFSA (2012).

Until 1986, the average value of ^{137}Cs specific activity in Northern Bulgaria was 10 Bq/kg and in Southern Bulgaria – 26 Bq/kg. (Naydenov and Staneva 1987a, b). After 1990, cesium-137 could be detected in all soil samples. (Yordanova et al. 2014). In 1996, due to the high inhomogeneity, the average values of the isotope varied between 160 and 280 Bq/kg for Southern Bulgaria and between 40 and 60 Bq/kg – for Northern Bulgaria. (Yordanova et al. 2007). According to the data of the Executive Agency for Environment (2016), the highest values were registered in the Western Rhodopes (Chetroka region – 261/kg and the town of Laki – 189 Bq/kg;). 35 years after the Chernobyl accident, the present study recorded specific activity of cesium-137 from <1 to 87 Bq/kg. This decrease of ^{137}Cs activity in the surface soil layer was mainly due to its radioactive decay.

The Eastern Rhodopes are rich in water. Sites such as the Ada Tepe gold mine, the Madzharovo mines, the Kardzhali Lead-Zinc Complex, are located in close proximity to settlements or to large water sources. A study on the radiological impact of uranium mining on surface waters and sediments closure shows that the migration of U_{nat}, ^{226}Ra, ^{210}Pb and ^{232}Th through surface water is one of the major pathways for contamination spread. (Ivanova et al. 2015). Most of the sites are near surface water bodies and are potentially dangerous for groundwater bodies. This poses an environmental risk in case of possible pollution, not only for humans but also for all living organisms, as the Eastern Rhodopes have the greatest species variety in Bulgaria and is one of the richest terri-
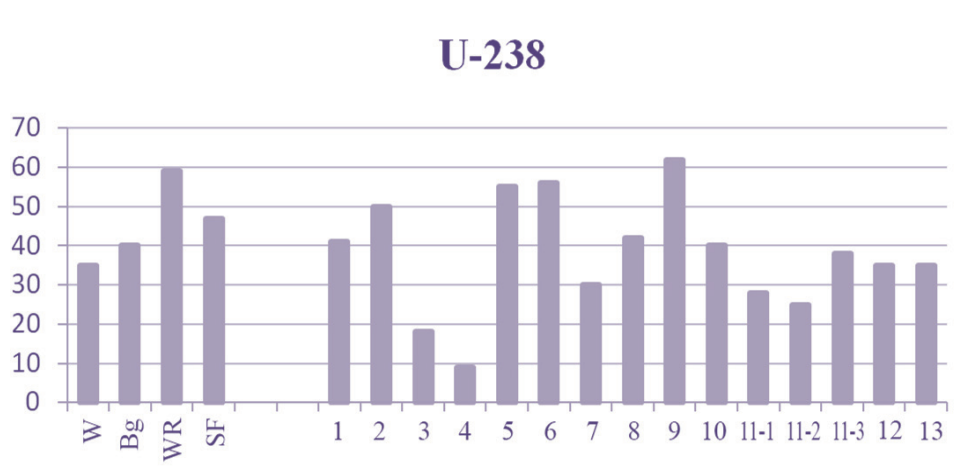
Figure 9. Content of ^{137}Cs in soil samples – average for Western Rhodopes (WR), average for the Sofia field and in the studied samples.

tories in aspect of biodiversity in Europe (Ministry of Environment and Water 2013). Many Balkan endemics and highly endangered species inhabit the region. The studied areas are close to regions of the European ecological network Nature 2000 and are of growing interest for tourism. In this regard a further research and assessment of the radiological status of ground and surface water in the region could be recommended.

The radium equivalent index (Ra_{eq}) and the external hazard index (H_{ex}) were used to assess the results obtained with respect to potential radiation hazard to the population. The data for H_{ex} do not exceed the permissible upper limit 1. Thus, with an external hazard index below 1 and low Radium equivalent activity, in relation to natural radionuclides in the soil, the study area of the Eastern Rhodopes is within normal background amounts and does not pose a radiation hazard for the population and biota in the area.

Conclusion

The analysis of data obtained showed the natural radionuclides content in studied soils does not differ considerably from the average values for our latitudes cited in the literature.

The measured ^{137}Cs content in the samples was as a result of the global fallout and the Chernobyl accident.

No additional pollution and impact of industrial activities on the content of radionuclides was found.

The External Hazard Index (Hex) showed the content of the studied radionuclides was not dangerous for the biota in the region from radiological point of view.

Due to the systematic use of unregulated drinking water sources in the region, a recommendation is given for the radiological assessment of ground and surface water in the studied areas.

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Supplementary material 1

Figure S1
Authors: Milena Hristozova, Radoslava Lazarova
Data type: jpg file
Explanation note: Content of 238U, 232Th and 226Ra in soil samples – world average (W), average for Bulgaria (Bg), average for the Western Rhodopes (WR), average for the Sofia field (SF) and in the studied samples.
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Link: https://doi.org/10.3897/biorisk.17.77432.suppl1

Supplementary material 2

Figure S2
Authors: Milena Hristozova, Radoslava Lazarova
Data type: jpg file
Explanation note: Content of 238U, 232Th and 226Ra in soil samples – world average (W), average for Bulgaria (Bg), average for the Western Rhodopes (WR), average for the Sofia field (SF) and in the studied samples.
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Link: https://doi.org/10.3897/biorisk.17.77432.suppl2

Supplementary material 3

Figure S3
Authors: Milena Hristozova, Radoslava Lazarova
Data type: jpg file
Explanation note: Content of 238U, 232Th and 226Ra in soil samples – world average (W), average for Bulgaria (Bg), average for the Western Rhodopes (WR), average for the Sofia field (SF) and in the studied samples.
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Supplementary material 4

Figure S4
Authors: Milena Hristozova, Radoslava Lazarova
Data type: jpg file
Explanation note: Content of 40K in soil samples – world average (W), average for Bulgaria (Bg), average for Western Rhodopes (WR), average for the Sofia field (SF) and in the studied samples.
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Supplementary material 5

Figure S5
Authors: Milena Hristozova, Radoslava Lazarova
Data type: jpg file
Explanation note: Content of 137Cs in soil samples – average for Western Rhodopes (WR), average for the Sofia field and in the studied samples.
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RESEARCH ARTICLE

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Environmental impact assessment of discharge of treated wastewater effluent in Upper Iskar sub-catchment

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Abstract

The upper Iskar sub-catchment is one of Bulgaria's most important economic and socially significant water sources because of its role in supplying Sofia with drinking water. Among the critical factors that carry potential high-risk levels for water quality in this hydrosystem are the discharge from the Samokov Wastewater Treatment Plant (WWTP), diffuse pollution from agriculture, and the percolation of untreated sewage from the small villages. In this study, we assessed the effect of treated wastewater effluent on water quality, and on the ecological state and microbial communities in the river sector of Samokov's WWTP discharge area. The assessment was based on the complex use of chemical and microbiological indicators and biological quality elements. The concentrations of organics, nutrients and microcomponents were determined with results confirming the expected increase for parameters associated with the discharge of urban wastewater. The ecological state, according to macrozoobenthos indicators, was "good" throughout the river sector but local deterioration was registered in a proximal location downstream of the WWTP outfall. The analysis of stream water and bed sediment microbial communities by a fluorescent technique showed the high metabolic activity and intensive transformation processes in addition to high abundance registered with standard cultivation methods. The importance of the studied sub-catchment for the functioning of the urban water cycle, and for the quality of Sofia's drinking water, underlines the need to extend an existing monitoring program with a more detailed assessment of the environmental impact of wastewater discharge.

Introduction

The increasing release of different organic and inorganic pollutants, associated with rapid urbanization and industrialization, is one of the global environmental threats for the quality and ecological status of aquatic ecosystems. The prevention of pollution, and protection of the quality of natural and drinking water, is a primary concern for society in order to ensure healthy living conditions, as well as a high standard of public health (Fernandez-Luqueno et al. 2013; Giri 2021; Saravanan et al. 2021). Inland waters, especially rivers, are specific cross points in urban water cycles, considering their role as both freshwater sources and main recipients of wastewater effluents. The extensive use of wastewater treatment plants (WWTPs), implementation of different treatment facilities and advanced technologies for removal of pollutants in waste flows is currently an environmental standard in water management practice and significantly reduces the release of pollutants in the aquatic environment (Wang et al. 2022). Despite the innovations that are being applied to wastewater treatment processes, the effluent characteristics always remain worse than those in the receiving natural waters. That is why the continuous discharge from the WWTP is considered as a factor with a high-risk level for the functioning and ecological status of aquatic ecosystems (Drury et al. 2013). Effluents from WWTPs are significant sources of organics, nutrients, hazardous micropollutants and pathogenic microorganisms (Kiedrzyńska et al. 2014). The river ecosystems respond to this impact by a realization of different abiotic and biotic transformation processes known as self-purification. The specific transitional areas of WWTP discharges are important nodes in system functionality and keys for achieving effective utilization of pollutants. In the biotic part of self-purification, the contribution of microbial communities and their enzyme systems is fundamental for the fate of pollutants, carbon fluxes and nutrient cycles (Findlay and Sobczak 2000; Panigrahi et al. 2019). Microbial activity is a driving force in the processing of organic matter and the whole ecosystem metabolism in aquatic habitats (Zeglin 2015). In this context, the standard use of biological indicators, based on macroorganisms such as macrozoobenthos, macrophytes or fishes, can be successfully extended to include the assessment of microbial communities (Lau et al. 2015; Chen et al. 2021). The changes in microbial diversity, community structure and functionality have the potential to be sensitive indicators for the assessment of pollution impact and associated environmental risks from WWTP effluent discharge (Lear et al. 2012; Zhang et al. 2021; Sun et al. 2022). Another important factor must also be taken into account when evaluating the complex processes of pollution/self-purification in disposal areas of WWTP – the structural complexity of rivers. The sediment component with its stability and

heterogeneity has a dominant role in the functioning of these aquatic ecosystems as a trap for some pollutants but also as a habitat for metabolically active organisms, forming the specific sediment microbiome.

In Bulgaria, the upper Iskar sub-catchment (Danube River Basin) is one of Bulgaria's most important economic and socially significant water sources in Bulgaria because of its role in supplying the capital Sofia with drinking water and upholding the city's urban water cycle. The biggest reservoir in Bulgaria (Iskar Reservoir) is situated in this sub-catchment and provides more than 70% of Sofia's drinking water. According to official data, the waters of the Iskar River basin are in good/moderate ecological status to Sofia, after which the status is moderate except for the section after inflow of Vladayska River, where the status is categorized as bad (http://www.bd-dunav.org/uploads/content/files/upravlenie-na-vodite/ocenka-na-sustoianieto/povurhnostni-vodi/BDDR_analiz_SWB_2019-2020.pdf). The critical factors with potential high-risk levels for water quality in the upper Iskar aquatic ecosystems are the discharge from the WWTP in the town of Samokov, diffuse pollution from agriculture, and the penetration of untreated sewage from the small villages. Failing to make maximum use of the capacity of the treatment plants, exacerbated by an inflow of untreated water, as well as several diffuse sources, constitute some of the background explanations for pollution in the upper valley of Iskar (Ministry of Environment and Water 2021).

This study aims to assess the environmental impact of treated effluent discharge from the WWTP in Samokov municipality in the upper part of Iskar River. We use a complex approach with a combination of indicators (chemical, microbiological, biological quality elements) to assess local changes in water quality, ecological status and microbial communities. The paper is structured as follows: (1) Firstly, we discuss the effect of WWTP discharge on physicochemical parameters and nutrient concentrations on the water of the disposal area; (2) An analysis of the concentrations of selected hazardous and specific pollutants (microelements) in water samples is also presented; (3) The abundance and activity of microbial communities are assessed in water and sediments by standard cultivation and fluorescence techniques; (4) Finally, an assessment of ecological status in river sector by quality element "macrozoobenthos" is conducted and then discussed.

Materials and methods

Study area

The study area is located in the upper part of the Iskar River before the Iskar Dam in Northern Rila, Bulgaria. Iskar is the longest (wholly) Bulgarian river (368 km) with a river basin of 8 650 km². The study river sector is 10 km in length, and 25–35 m in width; its depth ranges from 50 to 200 cm and the bottom substratum consists of pebbles, coarse and medium sands. The seasonal character of flow is determined with summer and winter low flow (1–3 m³/s), a little increase in flow during the autumn

(6–10 m³/s) and very expressive spring high water level (15–25 m³/s). According to a map of the land use, agricultural land, pastures and forests dominate the area (Topalova et al. 2013; Todorova et al. 2017). In this river sector, one significant source of point pollution is registered – the discharge from WWTP Samokov (44 100 m³/day). The treatment plant treats the mixed wastewater from the town of Samokov (municipal, industrial and storm) and works on the denitrification/nitrification scheme.

Sampling and field analyses

The sampling design included sites upstream and downstream of the WWTP. We carried out two sampling campaigns in November 2020 and March 2021 when the average water flow is 5–8 m³/s. Paired water and sediment samples were collected from four sampling sites (Fig. 1).
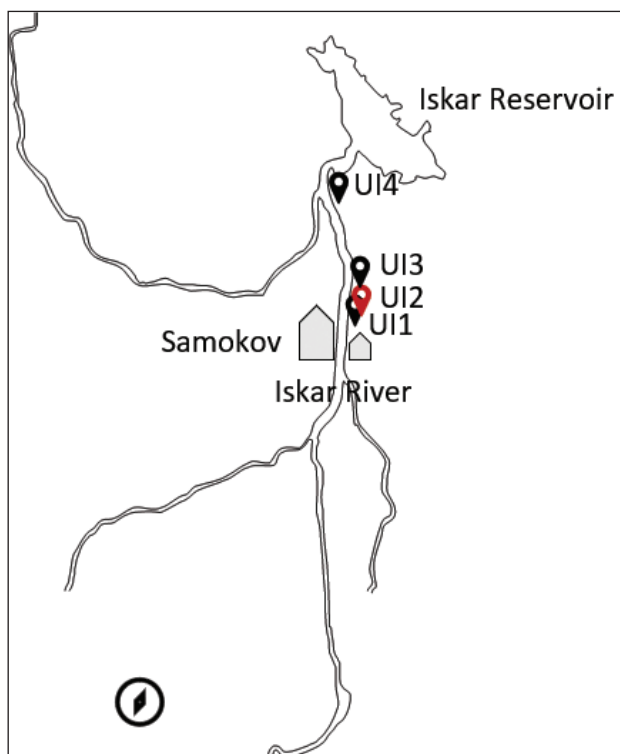
Figure 1. Location of the sampling sites in the study area of the Upper Iskar subcatchment

Sampling site UI1 – Iskar above WWTP Samokov
Sampling site UI2 – Discharge of WWTP Samokov
Sampling site UI3 – Dragushinovo village, under WWTP Samokov
Sampling site UI4 – Iskar River, near the Villa zone Mechkata (site from monitoring system for surface water bodies in Bulgaria)

We analyzed the physicochemical parameters (temperature, oxygen concentration, conductivity and pH) of the water *in situ* immediately after sampling with a portable oxygen and pH meters. The hand net was used for collecting the macroinvertebrates – up to 10 sub-samples were collected on one site according to multihabitat approach (EN ISO 10870:2012, EN ISO 16150: 2012). The assessment of the ecological status was conducted by metrics "biotic index" in ranges for river types R4 (semi-mountain rivers in 12 Ecoregion "Pontic Province"). The sediment samples for chemical, microbiological and fluorescent analyses were collected by manual dragging. The all water and sediment samples were transferred in sterile containers at 4 °C storage and processed within 24 h.

Chemical analyses

The determination of the organic loading in the water was performed by measuring the chemical oxygen demand (COD) by colorimetric dichromatic method. Nitrogen was measured as dissolved inorganic forms – ammonium and nitrate ions (colorimetric methods). The concentrations of phosphorus were defined as phosphates also by use of colorimetric method. Procedural details for measuring nutrients and organics were in line with standard methods recommended in EN ISO standards. The concentrations of selected microelements in the water samples were determined with inductively-coupled plasma mass spectrometry (ICP-MS, PerkinElmer SCIEX Elan DRC-e). The number of the decimal places is related to the precision of the measurements. The estimation of accuracy was conducted by the analysis of two water standard reference materials: SPS-SW2 (Reference Material for Measurement of Elements in Surface Waters, Spectrapure Standards, Norway) and NWTM-23.5 (Environmental matrix reference material, a trace element-fortified sample, Environment and Climate Change, Canada). The experimental results were in very good agreement with the certified values.

Data from chemical analyses of water and assessment of ecological status was compared with the requirements of Bulgarian legislation (Regulation No. H-4 of 14.09.2012 on characterization of surface water and Regulation No. 12 of 18.06.2002 on the quality of surface water intended for drinking and household purpose). Selecting microelements and ranking hazardous and specific pollutants was conducted on the basis of the EU-list of priority substances (Water Framework Directive 60/2000/EC (WFD) and Regulation on environmental quality standards for priority substances and some other pollutants, 2015) and the above-mentioned regulations.

Analyses of microbial communities

The total microbial count and number of coliforms was determined by the use of standard count-plate technique on Nutrient agar and Lactose TTC Agar with Tergitol 7 (Merck Millipore). The sediment samples were preliminarily treated with ultrasonic disintegrator VCX 750, Sonics & Materials Inc. (3 times × 10 sec). The data for microbial counts were normalized and presented as ln CFU/mL or ln CFU/g dry weight.

For analysis of changes in microbial activity of sediment communities, we used a modification of CTC/DAPI staining method with fluorescence imaging. CTC (5-cyano-2,3-ditolyl tetrazolium chloride) enters in cells and is reduced to CTC-formazan (fluorescent red signal). The content of reduced compound depends on the electron transport activity (activity of the dehydrogenase enzymes in viable cells) and is considered as an indicator of their metabolic activity. DAPI (4′,6-diamidino-2-phenylindole) is fluorescent dye that specifically binds to nucleic acids but enters both in live and fixed cells. It is widely used for the enumeration of bacterial abundance. The combined staining with CTC and DAPI is applied to distinguish the active fraction in different microbial communities (Topalova et al. in press). The epifluorescent microscope Leica DM6 B was used to make fluorescence images, and then a digital image analysis (using the software *daime*) was applied to assess the total area, the area of fluorescent objects, and mean fluorescence intensity. From these parameters the percent of live, metabolically active cells in images was calculated (Daims et al. 2006; Topalova et al. in press).

Results

Effect of WWTP discharge on physicochemical parameters and nutrient concentrations in water

In the study area, the water temperature showed typical seasonal dynamics and varied from 4.2 °C at sampling site UI3 to 8.9 °C at sampling site UI2 in November (Table 1). The highest temperature in both seasons was reported in the water from the discharge area of WWTP. The values of pH varied between 7.63 and 8.80 – the highest value was measured at UI2. The content of dissolved oxygen is usually high for this part of the Iskar River because of its rapid flow rate. The lower concentrations in all sampling sites for this parameter were measured in March, when the water level and turbidity were higher as snow melted, and with the onset of the wet season. At the point of WWTP discharge, the oxygen concentration was significantly lower during both samplings and the conductivity was higher than the site located above. In March, the measured values for oxygen and pH in UI2 exceeded the recommended norms for high status but the fluctuations were assimilated downstream.

In Fig. 2 we present the impact of WWTP effluent on the dynamics of nutrients (nitrogen and phosphorus) in surface water for both sampling campaigns (Fig. 2A

Table 1. Physico-chemical parameters of the waters in the upper valley of the river Iskar.

Sites	Temperature °C		Oxygen, mg/L		Conductivity µS/cm		pH	
	Nov.	March	Nov.	March	Nov.	March	Nov.	March
UI1	4.7	5.0	12.14	7.71	114	295	8.21	7.63
UI2	8.9	8.8	9.39	6.04	291	341	8.00	8.80
UI3	4.2	5.3	12.80	8.25	90	209	8.19	7.68
UI4	4.9	6.2	12.33	8.73	177	422	7.75	7.73
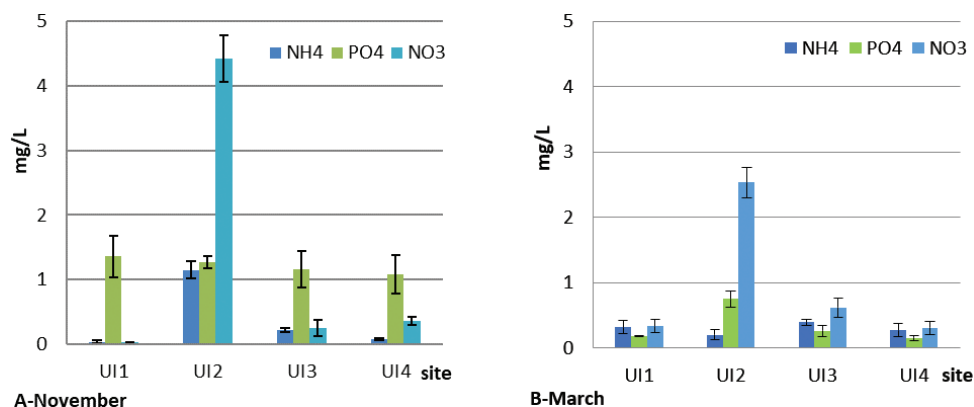
Figure 2. Dynamics of ammonium ions, phospahates and nitrates in surface water of study area in upper Iskar River (A – November, B – March).

– November and Fig. 2B – March). With regard to nitrogen, there was a significant impact on concentration of nitrates and ammonium ions from the discharge from the WWTP effluent. The highest values of nitrates were registered in water of sampling site UI2 during the both samplings. The concentration of nitrates increased almost 20-fold in comparison with the previous site in November and 8-fold in March. The situation was similar for ammonium during the autumn – the increase was more than 15-fold. Phosphate concentrations in surface waters were higher in November (between 1.087÷1.358 mg/L) compared to the values observed in March (0.188÷0.750 mg/L). During the autumn, the phosphate concentration retained similar very high levels in the whole river sector. In March, the discharge of WWTP effluent affected the phosphate concentration in the main river channel and the measured value at UI2 site was 0.750 mg/L. The nitrates were in the range acceptable for surface waters, although the WWTP effluent affected their concentration, but the ammonium ions and phosphates exceeded the values admissible according to the stipulations of Bulgarian legislation.

The values for chemical oxygen demand (COD) during the two samplings at three of the four sampling sites were below 10 mgO_2/L. The highest concentration was measured in site UI2 in March – 11.98 mgO_2/L.

Analyses of selected microelements (metals and metalloids) in water

The concentrations of the selected microelements in the water samples are presented in Table 2. The data showed an increased level of risk, associated with seasonal dynamics of Hg, being a priority pollutant (Regulation on environmental quality standards for priority substances and some other pollutants, 2015) and Cu – the measured values in November were higher than those measured in March for all sampling sites. The highest mercury concentration was determined in the water of site UI3 (after discharge of WWTP) – 1.42 μg/L; for copper – 44 μg/L was measured at site UI2. The concentrations of Cu increased more than 10–30 times in the autumn. The concentrations of the

Table 2. Concentrations of selected microelements in the water of the study area (the values exceeding the maximum admissible concentrations are marked in gray).

		Pb	Cd	Hg	Ni	Mn	Cr	As	Cu	Fe	Zn
		µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	mg/L	mg/L
UI1	Nov.	0.150	0.031	0.12	1.04	2.20	1.830	1.15	21.60	0.120	0.062
UI1	March	0.113	0.028	<LOD	0.480	1.10	0.166	0.163	1.56	0.098	0.004
UI2	Nov.	0.032	0.073	0.13	0.90	0.18	0.820	0.85	44.00	0.051	0.045
UI2	March	0.011	0.352	0.27	2.470	0.43	0.526	0.408	1.48	0.006	0.019
UI3	Nov.	0.084	0.030	1.42	0.30	1.13	1.100	0.59	21.30	0.112	0.058
UI3	March	0.022	0.035	<LOD	0.548	0.37	<LOD	0.245	1.29	0.027	0.003
UI4	Nov.	0.008	0.071	0.24	0.46	0.90	1.039	0.50	24.20	0.094	0.076
UI4	March	0.167	0.072	<LOD	1.640	1.46	0.498	0.310	1.81	0.184	0.003

rest of the microelements were below the admissible values in both seasons. As a result of increased anthropogenic activity in the post-summer period, significant seasonal changes are worth noting in all sampling sites for Zn (till 25 times in UI4), Cr (till 11 times in UI1) and As (till 7 times in UI1). To a certain extent, the same tendency is also established for Fe with the exception of sampling place UI4. The contents of the toxic elements Cd, Ni, Pb, being also priority pollutants, and Mn showed relatively close values in the studied sampling sites and significant stability between the seasons.

Analyses of water and sediment microbial communities

The data from determination of total microbial count and coliforms in water and sediments are presented in the figures below (Fig. 3, 4).

In water samples from the studied part of the river, we enumerated stable values for total culturable microflora – in the range of $10^3 \div 10^4$ CFU/mL. The coliforms were constantly presented in water samples with abundance of $10 \div 10^2$ CFU/mL. According to site location, the numbers of two indicator groups showed a slight increase in the sampling site of WWTP discharge during the each season studied. In the sediment samples, the total microflora was more abundant and variable. The numbers fluctuated between $10^5 \div 10^7$ CFU/g. In the sediments of discharge area of WWTP, the increase in numbers of two indicator groups was significant, especially for coliforms (3 000 ÷ 11 000 CFU/g).

The mean fluorescence intensity and percent live cells were calculated from the total area and the area of fluorescent objects on images from CTC/DAPI analysis (Table 3). The fluorescence intensity as an indicator of viability and activity of cells was assessed on CTC staining images. It can be seen that the parameter was up to 2.3 times higher in U13 when compared to the other two sites. The metabolic activity of the bacteria in that site remains high also in March of the following year (with up to 35%). The share of live cells was calculated as ratio of bacteria with metabolic activity (CTC) to total bacteria (DAPI). At sampling site UI3, a high share of live cells (2.20%) was also detected in November, but their share decreased by 10.5 times in March. The data for UI2 revealed clear differences in the share of live cells in sediment samples compared to
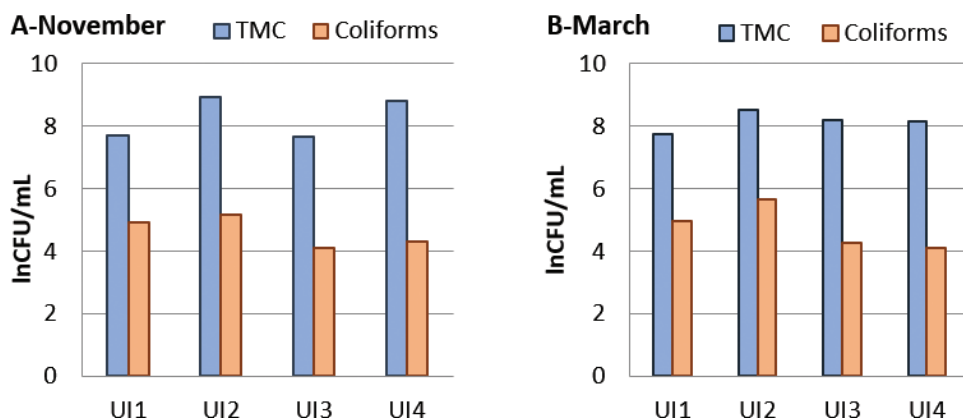
Figure 3. Total microbial count and coliforms in surface water **A** November **B** March.
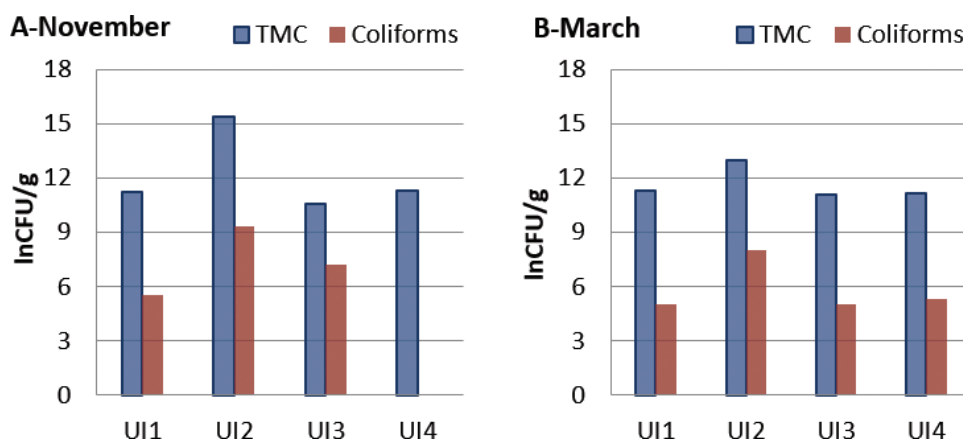
Figure 4. Total microbial count and coliforms in sediments **A** November **B** March.

UI1 – the live cells were about 3% in both seasons. The fluorescent images of sediment communities in sampling site UI2 were presented in Fig. 5.

Assessment of ecological status

The ecological status of the studied river sector was assessed as "good" according to WFD and Regulation N-4/2012 using macroinvertebrate bioindicators. The metrics Biotic index had a score of 4 in both sites for macrozoobenthic analyses (UI1, UI3). Despite similar ecological status, in sampling site UI1 species tolerant to deterioration in environmental conditions were found – *Hydropsyche* sp. (Trichoptera), Chironomidae (Diptera), *Erpobdella octoculata/monostriata* (Hirudinea). After discharge of WWTP Samokov, in sampling site UI3, the total taxa number was higher and *Baetis* sp. (Ephemeroptera) was also found.

Table 3. Mean intensity and percent live cells in sediment samples.

Sites	mean fluorescence intensity, CTC		percent live cells, CTC/DAPI	
	November	March	November	March
UI1	80.25	99.67	0.40	0.83
UI2	79.67	96.67	3.07	2.81
UI3	182.33	130.00	2.20	0.21
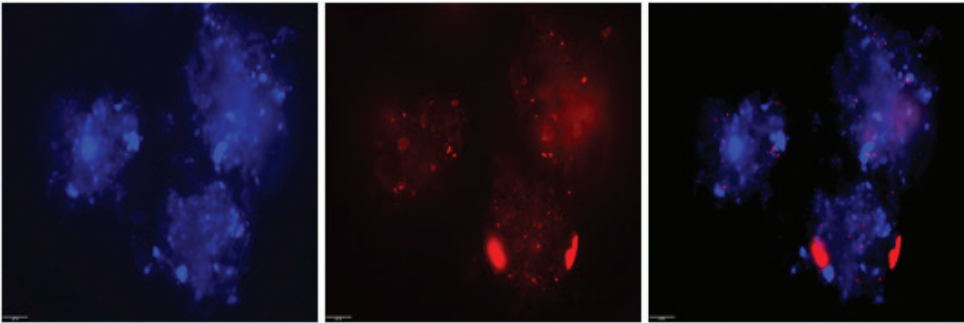
Figure 5. Fluorescent images of sediment communities in sampling site UI2 (left – DAPI staining, middle – CTC staining, right – CTC/DAPI staining).

Discussion

Many previous studies have discussed the response of aquatic ecosystems to WWTP effluents by assessing various indicators, but in most cases, the analyses were performed on specific communities or groups of indicators (Todorova and Topalova 2009; Drury et al. 2013; Zeglin 2015; Todorova et al. 2017; Kenderov and Trichkova 2020; Wang et al. 2022). In our study, we applied a comprehensive approach to assess the impact of WWTP effluent on water quality characteristics, the changes to the number and functioning of aquatic and sediment microbial communities, as well as an overall assessment of the ecological status. The synthesis of obtained results demonstrates the expected negative impact of WWTP discharge on water quality – the waters in the area of discharge had higher values of temperature, conductivity, nitrates, ammonium ions, phosphates and lower concentration of dissolved oxygen. This impact of WWTP effluent is partially assimilated along the river, but the analyses performed in other sampling sites reveal the salient problems affecting the whole study area – high concentrations with seasonal fluctuations of ammonium ions, phosphates and some hazardous/specific pollutants (Hg, Cu). At subcatchment scale, considering the location and strategic role of Iskar Reservoir, it is necessary to pay special attention to these results and implement better control measures for these parameters. The risks, associated with the potential eutrophication of stagnant water bodies and toxic effects of Hg and Cu, are serious threats for water quality and ecological status.

The data about nitrates, ammonium ions and phosphates also are of interest when we refer them to the measured low organic content in the waters of the studied area. The discharge of the WWTP does not lead to additional organic loading and in the

aqueous phase there is a imbalance in the ratio C:N:P, which further complicates the utilisation of nitrogen and phosphorus by heterotrophs. However, if we look at the river ecosystem in its heterogeneity, namely the sediment component, the high abundance of microorganisms in the sediment microbiome is impressive. The sediments are stable, active habitat where the predominant part of the transformation processes is probably quickly realized. The more diverse redox and oxygen regimes, the different ecological niches, and the longer retention of organic matter suggest that the sediments are the habitat where the full variety of self-purification processes unfolds. This is confirmed by the high metabolic activity and the share of live cells in sediments – these indicators have the higher values in the discharge area of WWTP and at the sampling site located downstream. The registered high fluorescence intensity at the site under the treatment plant shows that the activity of sediment microbiome remains high downstream, despite the fact that the number and share of live cells decreased. The assessment of the ecological status confirms the role of the sediments for the retention of the organics and the fast realization of the self-purification processes after the discharge. At the same time, the fact that the sediment habitat also serves as a potential refuge for opportunistic and pathogenic microorganisms must be taken into account. The high abundance of coliforms shows the potential role of sediments as a "natural depot" and bring to the fore the recommendation for including the sediment component into the system of water quality monitoring in the upper subcatchment of Iskar River.

Compared to our earlier studies in this part of river subcatchment (Todorova and Topalova 2009; Todorova et al. 2017; Kenderov and Trichkova 2020), the current ecological status and water quality have deteriorated. The discharge from WWTP is combined with negative effects of intensive agriculture and livestock breeding in area – the cumulative environmental impact is significant and can have consequences in the future, although self-purification is currently working effectively enough.

Conclusion

Along with the positive aspects of the increased number of WWTPs worldwide, the associated environmental risks of their operation must be taken into account, especially in terms of the functioning of urban water cycles. The importance of the studied sub-catchment of Upper Iskar for the quality of drinking water of Sofia enforces the extension of an existing monitoring program with a more detailed assessment of the environmental impact of wastewater discharge.

Acknowledgments

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RESEARCH ARTICLE

BioRisk

Trace metal accumulation in tissues of wedge clams from sandy habitats of the Bulgarian Black Sea coast

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Abstract

The aim of the present study was to carry out an initial screening of trace metals bioaccumulation in soft tissues of the wedge clam (*Donax trunculus* Linnaeus, 1758) from different localities of the Bulgarian Black Sea coastal area and to evaluate the bioindicator potential of this species. Wedge clams were collected in June and September 2020 from sublittoral sandy habitats at different localities of the Bulgarian Black Sea coast. Soft tissues of individual clams were digested with nitric acid followed by ICP-OES analytical determination. The content of trace metals in the wedge clams differed significantly amongst localities. Higher metal content was present in wedge clams from Sveti Vlas, Shkorpilovtsi, Slanchev Bryag, Ahtopol and Kranevo. The highest values of lead (Pb) (2.51 mg/kg) and cadmium (Cd) (0.32 mg/kg) were found in samples from Sveti Vlas and the highest concentration of copper (Cu) (34.12 mg/kg), iron (Fe) (269.52 mg/kg) and nickel (Ni) (0.32 mg/kg) were detected in wedge clams from Shkorpilovtzi. Maximum content of chromium (Cr) (0.58 mg/kg) was present in samples from Slanchev Bryag, together with high values of Fe. The highest concentration of zinc (Zn) (18.04 mg/kg) together with high values of Cr and Fe were measured in wedge clams from Irakli. In conclusion, the wedge clams from the localities known to have higher coastal inflows and touristic pressures, i.e. Varna, Shkorpilovtsi, Sveti Vlas, Slanchev Bryag and Ahtopol accumulated significantly higher metal elements in their tissues. Only few significant seasonal differences in the concentration of metal elements in wedge clams were present and the observed

seasonal variations were probably connected to the hydrological parameters of the ecosystems. The wedge clam *D. trunculus* is a suitable bioindicator for assessment and monitoring of metal pollution in the Bulgarian Black Sea environment.

Introduction

Trace metals constitute a significant proportion of the pollutants in the Black Sea (Mirinchev et al. 1999; Jitar et al. 2013; Bat et al. 2018a; Doncheva et al. 2020). The highest concentrations of metals are present in marine sediments from where they can be released back to water. Due to the migratory nature and bioaccumulation of metal elements, their discharge into the Black Sea can have profound effects on the entire marine environment (Islam and Tanaka 2004; Zaitsev 2008; Jitar et al. 2013). Metal pollution poses serious problems as it has a specific role in the impacts on marine ecosystems (Tchounwou et al. 2012). Unlike other pollutants, trace metals are characterised by their long time persistence in the environment (soils and waters) with periods ranging from hundreds to thousands of years (Bowen 1979). Being filter feeders and primary consumers in the food chain of the Black Sea, bivalves can accumulate metals and other pollutants in their tissues and shells (Romeo et al. 2005; Bat and Öztekin 2016). The capability of marine bivalves to accumulate metal elements makes them suitable pollution bioindicators (Bat and Öztekin 2016). Clam species are being reported as reliable bioindicators to monitor pollution in marine coastal areas (Adjei-Boateng et al. 2010; Bat et al. 2018b).

Although the spatial variation of metal concentrations in sediments of the Bulgarian Black Sea coastal areas is well documented (Simeonov and Andreev 1989; Andreev and Simeonov 1992; Jordanova et al. 1999; Doncheva et al. 2020), no comprehensive studies on the bioaccumulation and concentration of metal elements in clams from the Bulgarian Black Sea coastal habitats were carried out. In addition to their key role in coastal ecosystems, clams recently obtained significant economic importance for Bulgaria and this is quickly increasing.

The aim of the present study was to carry out an initial screening of trace metals bioaccumulation in soft tissues of the wedge clam (*Donax trunculus* Linnaeus, 1758) from different localities of the Bulgarian Black Sea coastal area and to evaluate the bioindicator potential of this species to assess the level and distribution of metal contamination in sandy habitats.

Methods

Wedge clams (length 23–35 mm) were collected manually or were obtained from commercial providers from sublittoral sandy habitats at representative localities along the

Point	Locality
S1	Kranevo
S2	Varna Bay
S3	Shkorpolovtzi
S4	Sveti Vlas
S5	Slanchev Bryag
S6	Sozopol
S7	Primorsko
S8	Arkutino
S9	Ahtopol
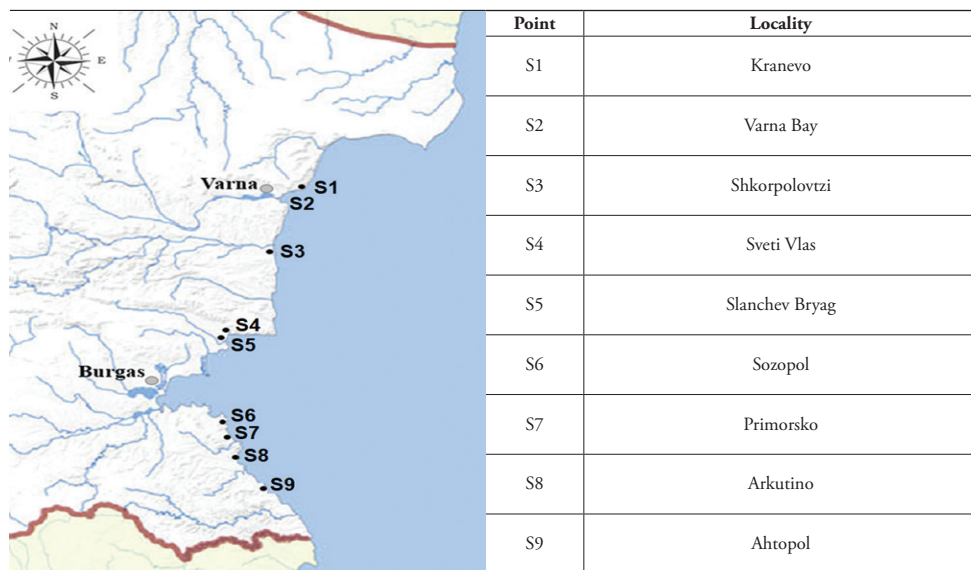
Figure 1. Localities of wedge clam sampling in 2020 along the Bulgarian Black Sea coast.

Bulgarian Black Sea coast (Figure 1) in 2020, before and after the intensive touristic season (June and September). The soft tissues of individual clams were digested with nitric acid followed by Inductively Coupled Plasma – Optical Emission Spectrometry (ICP-OES) determination of elements (Cd, Cr, Cu, Fe, Ni, Pb and Zn).

The measurements were carried out in the certified chemical laboratory at the Department of Chemistry of the Medical University-Varna. Significance of difference in means was estimated by the t-statistic and patterns of similarities between sites in metal bioaccumulation in clam tissues were studied by cluster analysis using the STA-TISTICA 10 package.

Results

The concentration of trace metals in the wedge clams collected in June 2020 differed significantly amongst the studied localities (Table 1). Higher metal content was present in tissues of *D. trunculus* from Sveti Vlas, Shkorpilovtzi, Slanchev Bryag, Ahtopol and Kranevo.

It can be seen from the data in Table 1 that the highest values of Pb (2.51 mg/kg) and Cd (0.32 mg/kg), together with high concentration of Cr (0.424 mg/kg) and Cu (29.7 mg/kg) were found in wedge clams sampled from Sveti Vlas. High metal bioaccumulation levels were established in wedge clams from Shkorpilovtzi – Cu (26.13 mg/kg), Fe (192.1 mg/kg) and Ni (0.187 mg/kg). Maximum concentration of Cr (0.504 mg/kg) was present in wedge clams from Slanchev Bryag, together with the highest content of Fe (244.4 mg/kg). The highest concentration of Zn was measured in wedge clams from Varna Bay (15.98 mg/kg). Significantly higher concentrations of

Table 1. Metal content (ME ± SD mg/kg wet weight) in tissues of wedge clams gathered in June 2020, from different localities of the Bulgarian Black Sea coastal area in (* - significance of difference at p < 0.05 from locality s[n]; nd – not detectable).

Site	Locality	Pb	Zn	Cd	Cu	Cr	Fe	Ni
S1	Kranevo	0.237	8.475	0.054	19.231	0.378	137.3	0.104
		± 0.14	± 0.13	± 0.008	± 3.83	± 0.16	± 62.41	± 0.04
S2	Varna Bay	0.281	15.98	0.062	24.201	0.24	68.6	0.062
		± 0.01	± 1.04 *s[1,8]	± 0.01	± 2.4	± 0.09	± 14.6	± 0.055
S3	Skorpilovtzi	0.629*	13.884	0.158	26.131	0.313	192.1	0.187
		± 0.59 s[1,6,8]	± 3.53	± 0.12 *s[1,2,6,7,8]	± 10.1 *s[1,6,7,8]	± 0.11	± 155.76	± 0.14 *s[2,6,8]
S4	Sveti Vlas	2.514	14.455	0.322	29.754	0.424	186.6	0.109
		± 2.36 *s[1,2,3,5,6,7,8]	± 0.45 *s[1,8]	± 0.203 *s[1,2,6,7,8]	± 0.48 *s[1,6,7,8]	± 0.13 *s[2,6,7,8]	± 19.63 *s[2,6,7,8]	± 0.012
S5	Slanchev Bryag	0.267	14.375	0.144	22.391	0.504	244.4	0.147
		± 0.12	± 1.59 *s[1,8]	± 0.02	± 2.91 *s[6,7,8]	± 0.09 *s[2,6,7,8]	± 49.92 *s[2,6,7,8,9]	± 0.02 *s[2,6,8]
S6	Sozopol	0.068	12.613	0.066	5.774	0.066	57.01	0.015
		± 0.002	± 0.06	± 0.001	± 0.06	± 0.001	± 0.91	± 0.004
S7	Primorsko	nd	12.224	0.057	5.150	0.121	36.4	nd
			± 0.24	± 0.001	± 0.35	± 0.05	± 8.89	
S8	Arkutino	0.065	10.222	0.054	5.982	0.053	46.01	0.016
		± 0.008	± 0.08	± 0.003	± 0.03	± 0.003	± 0.46	± 0.005
S9	Ahtopol	0.249	12.988	0.151	25.427	0.204	74.03	0.168
		± 0.12	± 2.987	± 0.04 *s[1,2,6,7,8]	± 7.2 *s[6,7,8]	± 0.14	± 37.26	± 0.04 *s[2,6,8]

Zn (14.573 mg/kg), Cr (0.504 mg/kg) and Fe (244.4 mg/kg) were measured in wedge clams from Slanchev Bryag.

The pattern of the similarities between the studied localities, based on the metal bioaccumulation in wedge clams sampled in June 2020, was studied by cluster analysis using Euclidean distance as a measure (Figure 2).

The analysis revealed the presence of two main clusters of localities, based on the similarity of the metal concentrations in wedge clams (Figure 2). The first cluster (upper part of the diagram) comprised localities situated in the northern coastal area - Shkorpilovtzi, Sveti Vlas, Kranevo and Slanchev Bryag. This cluster clearly indicated that the localities from the northern coastal part (first cluster) had similar metal bioaccumulation in the wedge clams amongst them, which however differed significantly from the metal concentrations in the wedge clams of the localities along the southern coastal part, grouped in the second cluster (lower part of the diagram). Amongst the northern localities (first cluster), Shkorpilovtzi and Sveti Vlas showed the highest similarity in the metal concentrations in the wedge clams sampled there.

The second cluster included the localities Primorsko, Arkutino, Sozopol and Ahtopol from the southern coastal area. In this cluster, Varna Bay was grouped together with Ahtopol as a result of the similarity of the metal concentrations in the wedge clams between them.
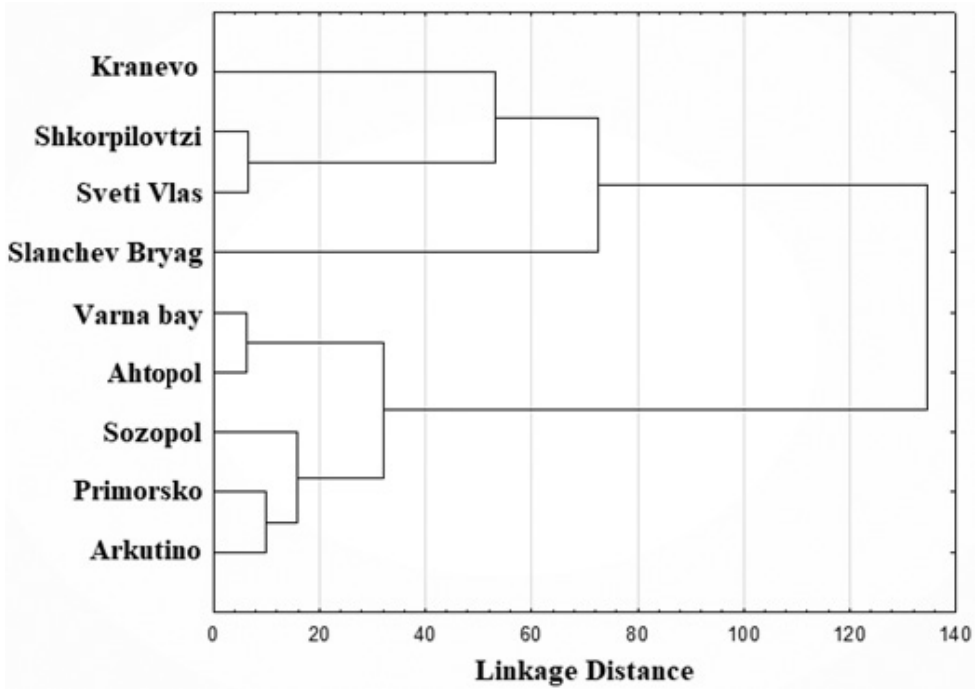
Figure 2. Cluster analysis of the similarity of localities based on metal microelements bioaccumulation in wedge clams sampled in June 2020.

Table 2. Seasonal variations (June and September 2020) in metal microelement concentration (ME ± SD mg/kg wet weight) in wedge clams from four localities (* - significant difference from June at p < 0.05; nd – not detectable).

Locality	Month	Pb	Zn	Cd	Cu	Cr	Fe	Ni
Kranevo	June	0.237	8.47	0.054	19.20	0.37	137.20	0.104
		± 0.14	± 0.13	± 0.01	± 3.83	± 0.16	± 62.4	± 0.037
	September	nd	13.02*	0.053	4.46*	0.02*	26.20*	nd
			± 0.08	± 0.01	± 0.1	± 0.01	± 0.32	
Varna Bay	June	0.281	15.98	0.062	24.20	0.24	68.60	0.062
		± 0.01	± 1.04	± 0.01	± 2.4	± 0.09	± 14.6	± 0.055
	September	0.232	14.91	0.050	27.50	0.14*	51.90	0.070
		± 0.036	± 0.04	± 0.01	± 0.1	± 0.01	± 0.71	± 0.009
Primorsko	June	nd	12.22	0.057	5.20	0.12	36.40	nd
			± 0.239	± 0.001	± 0.3	± 0.05	± 8.89	
	September	nd	10.09	0.077	6.40	0.06*	55.80	0.202
			± 0.017	± 0.001	± 0.1	± 0.01	± 0.99	± 0.008
Arkutino	June	0.068	10.22	0.054	5.90	0.05	46.01	0.016
		± 0.01	± 0.083	± 0.003	± 0.1	± 0.01	± 0.46	± 0.005
	September	nd	13.10	0.058	5.60	0.04	38.10	nd
			± 0.04	± 0.002	± 0.1	± 0.01	± 0.79	

Specifically, the levels of metal bioaccumulation in wedge clams from the northern localities was significantly higher, as a whole, than in the wedge clams from the southern localities, thus indicating different metal pollution levels of the northern and southern Bulgarian Black Sea coastal areas (see also Table 1).

Amongst the cluster of southern localities, Primorsko and Arkutino were most similar in the metal content in the wedge clams sampled there (Figure 2). The similarities between the southern localities seemed to be most probably due to the lower level of metal contamination of the marine environment at the southern localities compared to the northern ones. The Ahtopol locality was an exception showing the lowest similarity with the other southern localities, due to the relatively higher values of Cu, Cd and Ni in the wedge clams sampled there (Table 1) and, hence, was grouped together with Varna Bay.

Available data from four localities sampled in June and also September 2020 (before and after the intensive touristic season), were analyses for seasonal changes in the metal accumulation in the wedge clams (Table 2). As a whole, little seasonal differences were present in the concentration of metal elements in the wedge clams from the studied localities. Some exceptions, however, were present. In particular, in wedge clams from Kranevo, the concentration of Cu, Cr and Fe was significantly lower in September samples compared to June samples. The content of Cr in wedge clams from Varna Bay and Primorsko was also significantly lower in September (Table 2).

Discussion

The pollution of the Black Sea with metals is a major environmental challenge (Strezov 2008). Metal elements are introduced into the marine environment by natural geological processes, rivers or direct discharge of industrial waste (Mitryasova et al. 2020). Recent studies have revealed variable average concentrations of metals in seawater and surface sediments from the Bulgarian Black Sea coastal zone and pollution hotspots were designated (Peycheva et al. 2017; Doncheva et al. 2020). Bivalves, in particular the mussel *Mytilus galloprovincialis* (Lamarck, 1819), have been traditionally used as biomonitors of Black Sea metal pollution (Bat 2012; Bat and Öztekin 2016). However, available data on the spatial and temporal distribution of metal accumulation in benthic clam species from the Bulgarian Black Sea coast is quite scarce.

In this paper, we present the first comprehensive study on the distribution and bioaccumulation of metal elements in the wedge clam *D. trunculus* from different localities of the Bulgarian Black Sea coastal area. The content of trace metals in the wedge clams differed significantly amongst the studied localities. Higher metal content was present in the tissues of wedge clams gathered from several resort beaches at Sveti Vlas, Shkorpilovtzi, Slanchev Bryag, Ahtopol and Kranevo. Highest values of Pb (2.51 mg/kg) and Cd (0.32 mg/kg) were found in wedge clams from Sveti Vlas and of Cu (34.12 mg/kg), Fe (269.52 mg/kg) and Ni (0.32 mg/kg) in the wedge clams from Shkorpilovtzi.

Patterns in the similarity amongst localities, based on metal bioaccumulation in the wedge clams, were studied by cluster analysis using Euclidean distance. The analysis showed the presence of two main clusters of localities according to the metal content in the wedge clams. The first one included the localities Shkorpilovtzi, Sveti Vlas, Kranevo and Slanchev Bryag which are situated in the northern coastal area and appeared more polluted since the wedge clams had higher concentrations of metal elements. Amongst these

localities, Shkorpilovtzi and Sveti Vlas showed the highest similarity, obviously due to the high accumulated levels Pb, Cd and Cu in the wedge clams sampled there. The second cluster was formed by the localities Primorsko, Arkutino and Sozopol which are situated along the southern coastal area and appeared to have less accumulated metal elements in the wedge clams living there. Our findings correspond, at least in part, with the data on the concentration of metal elements in sediments from these regions (Peycheva et al. 2017).

We did not find many significant seasonal differences in the concentration of the studied in June and September 2020 trace metal elements in wedge clams with the exception of Kranevo where the content of Zn, Cu, Cr and Fe was significantly lower in September compared to June. The observed seasonal variations could have been mainly connected with local changes in the hydrological parameters of the ecosystem.

Recent studies on trace elements (Cd, Cr, Cu, Fe, Ni, Pb and Zn) concentration in commercial wedge clams from the Bulgarian Black Sea coast were carried out with respect to consumption risks for humans and indicated that this species is, as a whole, safe for human consumption (Peycheva et al. 2021). In general, our data also showed that the concentrations of the accumulated metal elements in wedge clams from the studied in 2020 localities were below the maximum residual levels prescribed by different local and international regulations for seafood. There was only one exception connected with the established high concentration of Pb (2.51 mg/kg) in wedge clams from Sveti Vlas in June which significantly exceeded the national and European regulations (1.5 mg/kg, Commission of the European Communities 2008) for seafood.

Conclusion

The wedge clam *D. trunculus* plays a significant role in maintaining functionality of marine ecosystems and, as a filter feeder, can accumulate considerable amounts of trace metal elements. Wedge clams from localities with high coastal inflows and touristic pressures, i.e. Varna Bay, Shkorpilovtzi, Sveti Vlas, Slanchev Bryag and Ahtopol accumulated significantly higher metal elements in their tissues. No significant seasonal variations in the concentration of accumulated metal elements in wedge clams were present. The few observed significant seasonal differences were probably connected with changes in the local hydrological parameters of the ecosystems. Our data showed that concentrations of accumulated metal elements in wedge clams from the studied localities were below the maximum residual levels prescribed by local and international regulations for seafood. The wedge clam *D. trunculus* proved to be a suitable bioindicator for assessment and monitoring of the metal pollution levels of the Bulgarian Black Sea environment.

Acknowledgements

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RESEARCH ARTICLE

BioRisk

Heavy metal stress response of microalgal strains *Arthronema africanum* and *Coelastrella* sp. BGV

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Abstract

The present study compared the stress response of two microalgal strains – *Arthronema africanum* (Cyanoprokaryota) and *Coelastrella* sp. BGV (Chlorophyta), after heavy metals' treatment. Changes of algal growth, pigment and protein content were analyzed after adding Cu, Cd and Pb (50 µM and 100 µM) to the nutrition medium. It was found that Cd and Pb significantly inhibited growth and protein biosynthesis of microalgae, but the effect of Cu remained less pronounced. In both strains, a decrease of chlorophyll content was observed, while carotenoid content markedly increased, especially in *Coelastrella* sp. BGV biomass. The addition of 100 µM Cd and 100 µM Pb to the medium caused a strong enhancement of malondialdehyde in both microalgal strains, which corresponded to the significant increase of superoxide dismutase and catalase activity. The antioxidant enzymes appeared to be differently altered by heavy metals' exposure. The activity of SOD in the *Arthronema africanum* cells was most strongly affected by Cd, in contrast to *Coelastrella* sp. BGV that was highly increased by 100 µM Pb. The application of 100 µM Cd and 100 µM Pb increased in a similar manner catalase activity in both microalgae. The strains that were studied showed a high absorption capacity for metal ions, especially for Pb, which was absorbed largely than Cd and Cu. For that reason, we assumed that both microalga and, in particular, *Coelastrella* sp. BGV, could be successfully used for treatment of contaminated water bodies.

Keywords

Arthronema africanum, catalase, *Coelastrella* sp., Cu, Cd, Pb, pigments, superoxide dismutase

Introduction

Heavy metals are among the most common environmental pollutants nowadays. In addition to natural sources, heavy metal pollution often comes from anthropogenic activities – mining, refining, chemical and metallurgical industries, etc. (Sibi 2019). A large number of microorganisms, including bacteria, algae and fungi, have the ability to absorb and uptake metals and metalloids (Al-Amin et al. 2021; Spain et al. 2021). Microalgae (prokaryotic and eukaryotic species) may be used as an efficient and eco-friendly alternative to the existing physicochemical methods for heavy metal removal (Bestawi 2019; Cui et al. 2021). Cyanoprokaryotes and green microalgae have been recently studied, due to their high potential for disposal of various pollutants and their use in phytoremediation (Kalambate et al. 2019; Pham et al. 2020).

Heavy metals that have been usually tested for uptake were Cu, C, Ni, Pb, Zn, Hg, Cr (Kumar et al. 2015). Cu, Cd and Pb are known to be among the most common pollutants in natural ecosystems. Cd and Pb, being highly toxic metals, are nonessential for the growth and development of plant organisms, but Cu, although a necessary trace element, in elevated concentrations could be also damaging. High Cd content in the medium leads to significant inhibition of growth and photosynthesis, expressing its toxic effect via complexing to SH-groups of proteins and inhibiting cellular respiration. Excess Pb causes reduced growth and chlorosis, suppresses photosynthesis, mineral nutrition and water balance (Dao and Beardall 2016). Despite their micronutrient copper requirement most algae may be damaged by Cu, which decreases rates of photosynthesis and chlorophyll biosynthesis, imbalances cell division, etc. (Yruela 2005).

Arthronema africanum (Cyanoprokaryota) is a filamentous, nonheterocystous cyanoprokaryote manifesting some unique morphological and physiological characteristics (Komarek and Lukavsky 1988). The strain is highly adaptive, typical for extreme desert habitats, a very promising producer of phycobiliproteins (Chaneva et al. 2007). It is still unexplored and only a few studies are known examining its antitumor and antioxidant properties (Iliev et al. 2006; Gardeva et al. 2014).

Coelastrella sp. BGV, a newly isolated, fast growing Bulgarian strain, (Draganova 2018; Toshkova-Yotova et al. 2019; Toshkova-Yotova et al. 2020), has also focused the attention of researchers due to its high bioactive and antitumor capacity.

The aim of that study was to perform a comparative analysis of the stress response of two microalgal strains, *Arthronema africanum* (Cyanoprokaryota) and *Coelastrella* sp. BGV (Chlorophyta), after heavy metal treatment. This would contribute these strains to find effective application in phycoremediation.

Methods

The microalgal strains were maintained as batch cultures at the Algal Culture Collection, Department of Experimental Algology, IFRG, BAS. The cyanoprokaryote *Arthronema africanum*, strain Lukavsky, 1981/01 (Komárek and Lukavský 1988),

CCALA (Tøeboò Collection of Autotrophic Organisms, Czech Republic) was cultivated at 28–30 °C, on the nutrition medium based on the Allen and Arnon (1955) and Zehnder (Staub 1961) media, modified by Chaneva et al. (2007).

The green alga *Coelastrella* sp. BGV, newly isolated Bulgarian strain (Dimitrova et al. 2017), was grown at 28 °C, on the Setlik nutrition medium (Setlik 1967) modified by Georgiev et al. (1978). Both strains were intensively cultivated in 200 ml vessels, under continuous illumination by white light, 150 µmol phot m^{-2} s^{-1}. Carbon source was provided by bubbling sterile 2% (v/v) CO_2 in air (100 l h^{-1}). The algal cultures were centrifuged at the end of the exponential phase of growth and the biomass was further re-suspended in a fresh nutrition medium at an inoculum 0.5 mg ml^{-1} DW (dry weight). The 10-day treatment was performed by adding to the medium 50 µM and 100 µM of Cu^{2+}, Cd^{2+} and Pb^{2+} (added as $CuSO_4$, $CdCl_2$ and $(CH_3COO)_2Pb$). These concentrations were chosen according to data received in our previous study (Marinova et al. 2018).

Growth and physiological changes of the algal cultures were determined on the 3rd, 7th and 10th day of the treatment. Algal growth was monitored by the changes of dry weight and total protein content (measured according to Lowry et al. 1951). Pigment content was determined spectrophotometrically after methanol extraction and calculated after McKinney (1941). Malone dialdehyde (MDA) content was determined according to Dhindsa et al. (1981). Superoxide dismutase (SOD) activity was measured after the method of Beauchamp and Fridovich (1971) and catalase (CAT) activity – after Aebi (1984). The content of Cu, Cd and Pb in dry algal biomass was analyzed by atomic absorption spectrophotometer Perkin-Elmer. The experimental data were averaged of triplicate measurements and only statistically significant results (as defined by a p-value < 0.05) were discussed.

Results

All heavy metals (Cu, Cd and Pb) that were studied inhibited *A. africanum* growth. The application of 100 µM Cu and 100 µM Cd led to a significant decrease in dry biomass, which was by 55%–57% lower than the control variant. In the initial stages of treatment, on the 3th day, 50 µM Cu stimulated, albeit slightly, the growth of *A. africanum* (Fig. 1). The green microalga *Coelastrella* sp. BGV also suffered the t toxic effect of heavy metals, however, less pronounced than in the cyanoprokaryote. The most significant influence was observed at 100 µM Cd and 100 µM Pb when the dry algal biomass was 30%–33% reduced on the 10th day. 50 µM Cu led to an increase of dry weight on the 3rd and 7th day. However, on the 10th day of the experiment, the accumulation of biomass slowed and remained 17% lower than the control (Fig. 2). The lowest protein content in *A. africanum* biomass was measured on the 10th day after 100 µM Cd and 100 µM Pb treatment. The least inhibition of protein biosynthesis was observed after Cu treatment – 8%–22% decrease (Table 1). Both Cd concentrations caused maximal reduction of protein biosynthesis in *Coelastrella* sp. (~36%). The effect of copper ions was the weakest and a decrease of about 5% was observed at 50 µM Cu (Table 1).
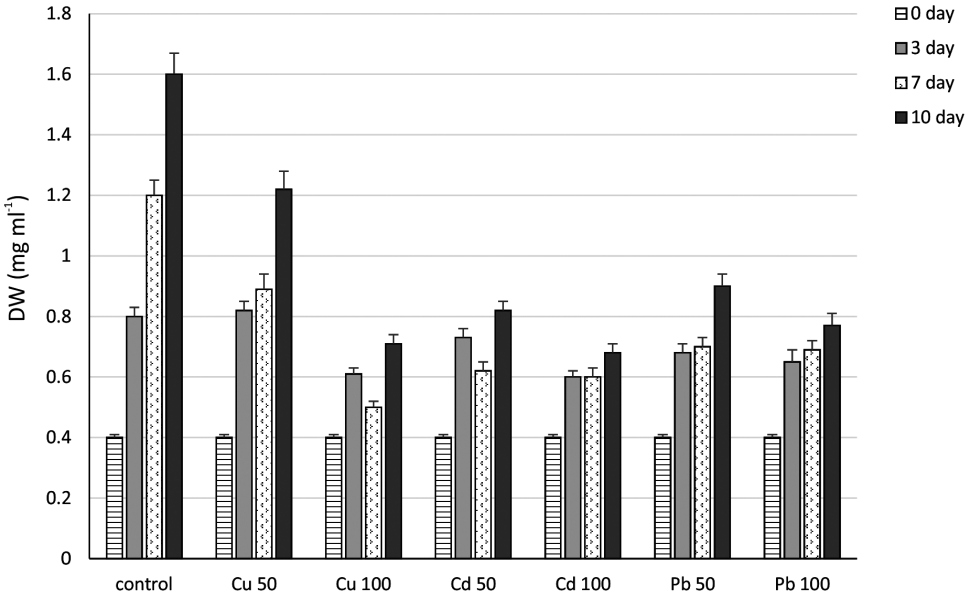
Figure 1. Influence of heavy metals (Cu, Cd, Pb) on the *Arthronema africanum* growth (DW, mg ml^{-1}), measured on the 3rd, 7th and 10th day after treatment by Cu, Cd and Pb (each metal added in concentrations 50 μM and 100 μM).
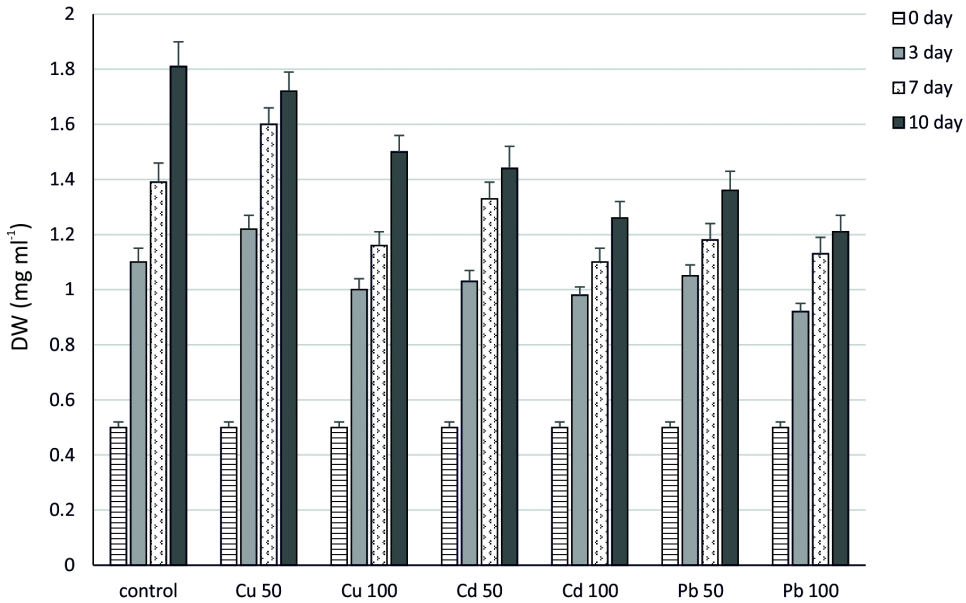
Figure 2. Influence of heavy metals (Cu, Cd, Pb) on the *Coelastrella* sp. BGV growth (DW, mg ml^{-1}), measured on the 3rd, 7th and 10th day after treatment by Cu, Cd and Pb (each metal added in concentrations 50 μM and 100 μM).

Table 1. Effect of heavy metals (Cu, Cd, Pb) on the protein and pigment content of *Arthronema africanum* and *Coelastrella* sp. BGV, 10th day.

Variant	Proteins (% DW)	Chlorophyll *a* (% DW)	Carotenoids (% DW)
		Arthronema africanum	
control	46.8 ± 1.8	1.71 ± 0.06	0.29 ± 0.01
Cu 50 μM	43.5 ± 1.9	1.59 ± 0.07	0.30 ± 0.01
Cu 100 μM	36.6 ± 1.4	0.97 ± 0.04	0.22 ± 0.01
Cd 50 μM	33.9 ± 1.4	0.81 ± 0.04	0.17 ± 0.01
Cd 100 μM	28.3 ± 1.3	0.62 ± 0.03	0.18 ± 0.01
Pb 50 μM	36.5 ± 1.6	1.04 ± 0.04	0.34 ± 0.01
Pb 100 μM	30.7 ± 1.3	0.65 ± 0.02	0.33 ± 0.01

Variant	Proteins (% DW)	Chlorophyll *a* (% DW)	Chlorophyll *b* (% DW)	Carotenoids (% DW)
		Coelastrella sp. BGV		
control	44.8 ± 1.6	1.57 ± 0.07	0.64 ± 0.03	0.28 ± 0.01
Cu 50 μM	42.9 ± 1.8	1.19 ± 0.05	0.49 ± 0.02	0.25 ± 0.01
Cu 100 μM	33.4 ± 1.3	1.23 ± 0.06	0.44 ± 0.02	0.35 ± 0.01
Cd 50 μM	29.0 ± 1.2	0.82 ± 0.04	0.34 ± 0.01	0.39 ± 0.02
Cd 100 μM	28.5 ± 1.1	0.63 ± 0.03	0.31 ± 0.01	0.54 ± 0.02
Pb 50 μM	37.1 ± 1.8	0.96 ± 0.04	0.38 ± 0.01	0.50 ± 0.02
Pb 100 μM	32.2 ± 1.5	0.87 ± 0.03	0.36 ± 0.01	0.51 ± 0.02

It was found a significant decrease of chlorophyll *a* biosynthesis in the *A. africanum* cells. The severe inhibitory effect was observed at 100 μM Cd and 100 μM Pb – a strong decrease by 62%–64%. The negative effect of Cu ions was the least pronounced (Table 1). Cd also caused a significant decrease in the carotenoid content of *A. africanum* – a decrease of about 40% compared to the control. In contrast, Cu ions enhanced the level of carotenoids – by 24% at 100 μM Cu (Table 1). *Coelastrella* sp. reacted by an extreme reduction of the amounts of chlorophyll *a* and chlorophyll *b* after Cd adding to the medium. Similar, though not so pronounced, was the effect of Pb. Both metals led to a decrease of about 60% of chlorophyll *a* content and 50% of chlorophyll *b*. Copper ions caused the least change in the chlorophyll content (Table 1). The addition of Cd and Pb to the nutrition medium led to a strong increase in the level of carotenoids in *Coelastrella* sp. After 100 μM Cd and 100 μM Pb treatment, a 93% and 82% increase in carotenoids was registered (Table 1).

Examining changes in the levels of malondialdehyde (MDA), one of the most commonly used markers for the degree of lipid peroxidation in cells, it was found that all studied heavy metals led to a sharp increase in MDA in *A. africanum*, especially Cd and Pb (Fig. 3A). As for *Coelastrella* sp., the strain showed a similar trend of MDA increase, although to a lesser extent – it was about 3 times higher in response to the addition of Cd and Pb (Fig. 3B).

SOD activity of *A. africanum* increased more than twice at both Cd concentrations. Less pronounced changes were observed under Cu and Pb treatment (Fig. 4A). The activity of SOD in *Coelastrella* sp. has been changed in different ways – the highest values were registered under Pb treatment (80% – 90% enhancement), as well as at 100 μM Cu. The enzyme activity remained lower than the control variant at 50 μM
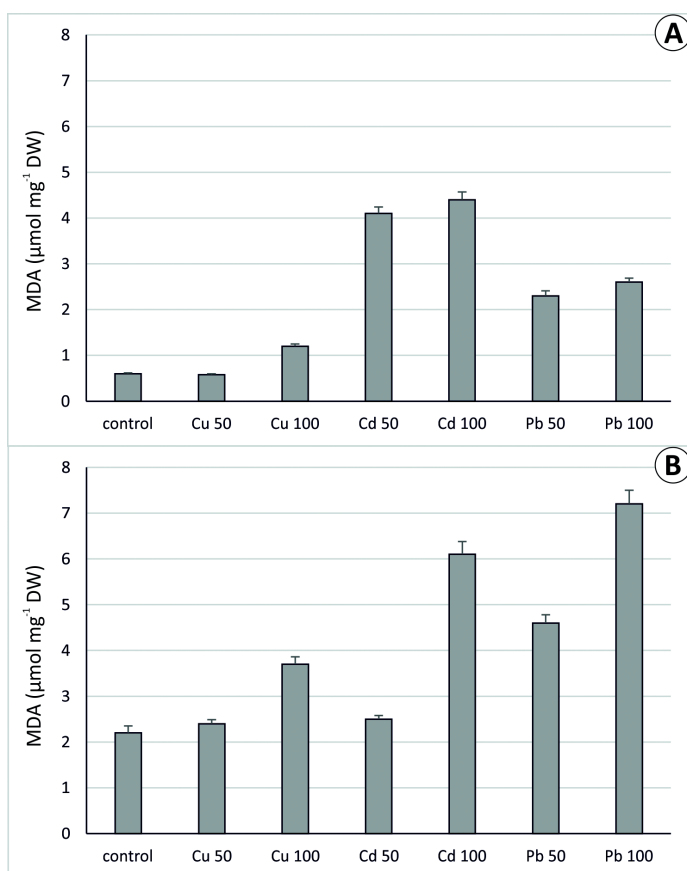
Figure 3. Changes of malondialdehyde content (MDA, µmol mg^{-1} DW) in the cells of *Arthronema africanum* – **A**, and *Coelastrella* sp. BGV– **B**, on the 10th day after treatment by Cu, Cd and Pb (each metal added in concentrations 50 µM and 100 µM).

Cu (Fig. 4B). It has been observed that the catalase activity varied differently compared to SOD. In the *A. africanum* cells, the extreme levels were measured at 100 µM Cd and 100 µM Pb, two times above the control. Copper ions had a lesser effect on the enzyme activity (Fig. 5A). Similar values were obtained for CAT activity in *Coelastrella* sp., which was most significantly increased at 100 µM Cd and 100 µM Pb, while Cu did not have such a strong effect (Fig. 5B).

Both experimental strains have been shown to accumulate large amounts of the heavy metals added to the medium. Algal cells appeared to be most willing to absorb Pb ions, followed by Cd and Cu. *Coelastrella* sp. showed a particularly high tendency to accumulate heavy metals in the biomass. The green microalgal strain expressed a much higher uptake capacity, compared to the cyanoprokaryote *A. africanum* (Table 2).
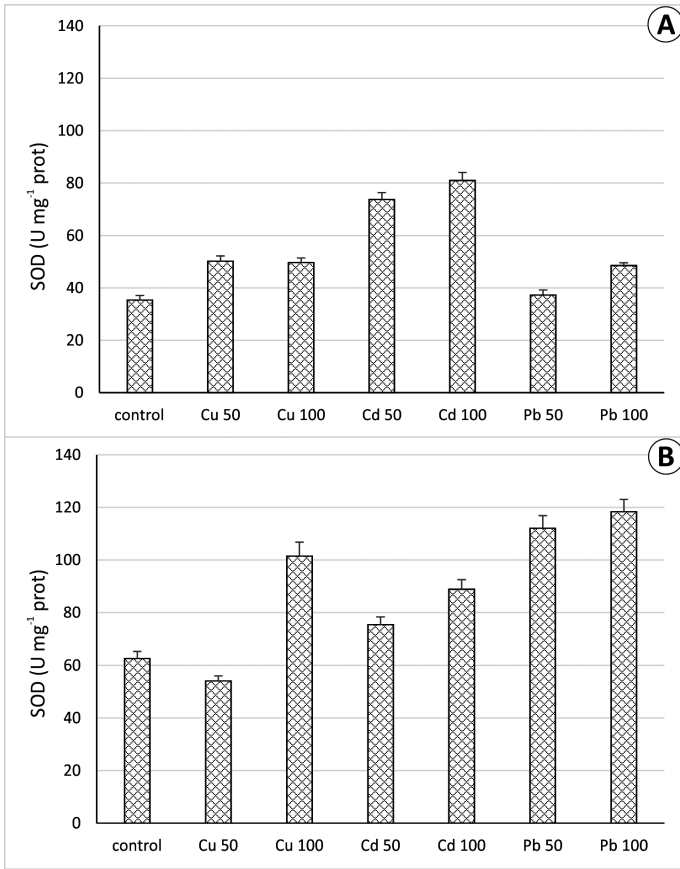
Figure 4. Changes of superoxide dismutase activity (SOD, U mg^{-1} prot.) in the cells of *Arthronema afri-canum* – **A** and *Coelastrella* sp. BGV – **B**, on the 10th day after treatment by Cu, Cd and Pb (each metal added in concentrations 50 μM and 100 μM).

Discussion

A detailed knowledge of heavy metals pollutants' action is necessary for the successful application of microalgae in the process of phytoremediation. Therefore, a screening of promising, fast-growing strains, able to absorb heavy metals with higher affinity, is required. In our experiments, it was found that treatment with Cd and Pb strongly in-hibited the growth of *Arthronema africanum* and *Coelastrella* sp. BGV, more essentially that one of *A. africanum*. (Figs 1, 2). Cd and Pb, applied at 100 μM concentration, affected the morphology and worsened the condition of the algal cultures.

It became clear that the effect of Cu was less pronounced and in the initial stages of the experiment 50 μM Cu had a certain stimulating effect on growth, especially
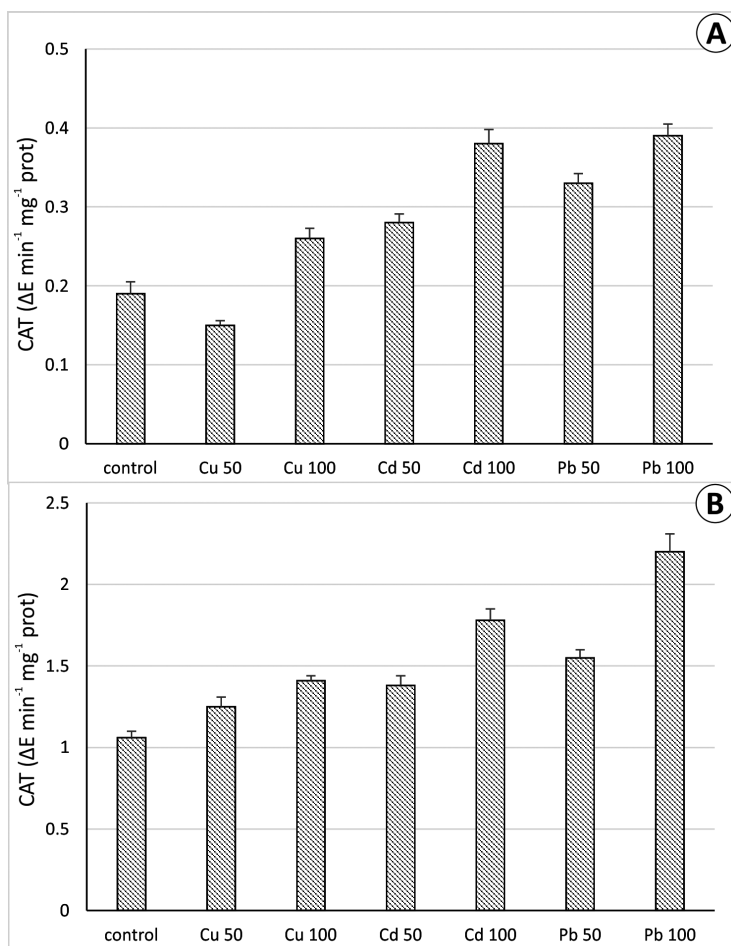
Figure 5. Changes of catalase activity (CAT, ΔE min^{-1} mg^{-1} prot.) in the cells of *Arthronema africanum* – **A** and *Coelastrella* sp. BGV – **B**, on the 10th day after treatment by Cu, Cd and Pb (each metal added in concentrations 50 µM and 100 µM).

Table 2. Accumulation of Cu, Cd and Pb (mg kg^{-1} DW) in the biomass of *Arthonema africanum* and *Coelastrella* sp. BGV, 10th day.

Variant	Cu (mg kg^{-1} DW)	Cd (mg kg^{-1} DW)	Pb (mg kg^{-1} DW)
	Arthronema africanum		
control	49.1	129.3	36.1
Cu 100 µM	349.8	106.8	16.9
Cd 100 µM	38.8	3258.3	13.7
Pb 100 µM	37.3	145.7	6431.3
	Coelastrella sp. BGV		
control	66.7	69.5	56.0
Cu 100 µM	1285.2	12.0	99.0
Cd 100 µM	59.5	5069.9	33.8
Pb 100 µM	75.5	29.5	12230.2

of *Coelastrella* sp. BGV (Fig. 2). The protein content of both strains was the least affected by heavy metal treatment compared to the other parameters we investigated (Table 1). It was reported (Marinova et al. 2018; Pham et al. 2020) that Cd and Pb strongly inhibited growth of green alga *Scenedesmus* sp. but the strain could efficiently remove Cd, Pb and Cu at low concentrations. The pigment composition was strongly influenced by Cd and Pb, which had a pronounced inhibitory effect on the chlorophyll content of both strains. The decrease of chlorophyll content might be a result of distortion of its structure or inhibition of pigment biosynthesis (Shen et al. 2021). However, a significant increase of carotenoids was observed, particularly well manifested in *Coelastrella* sp. BGV, which indicated their antioxidant role in stressful conditions provoked by adding Cd and Pb into the medium (Table 1). It was reported (Shen et al. 2021) that Cd ions were highly toxic to the cyanoprokaryote *Synechocystis* sp., by inhibiting its growth and pigment biosynthesis. Furthermore, the authors found a reduced carotenoid content under Cd treatment, in contrast to our study, in which the strong enhancement of carotenoids could be considered a protective reaction against oxidative stress caused by Cd and especially by Pb.

Heavy metals can damage membrane molecules, disturbing the homeostasis under the enhanced generation of reactive oxygen species (ROS) and increased lipid peroxidation (Ajayan and Selvaraju 2012). The level of MDA in the biomass of *Coelastrella* sp. increased significantly after application of 100 μM Cu and 100 μM Pb and that effect was particularly pronounced after Pb treatment. The stress response of *A. africanum* was much more intense and different – the largest increase in MDA levels was measured after Cd treatment followed by Pb (Fig. 3A, B).

Microalgae develop various antioxidant mechanisms – enzymatic and non-enzymatic, to alleviate oxidative damage caused by heavy metal stress (Zhang et al. 2019). Heavy metals treatment significantly increased SOD activity in both experimental strains. The activity of SOD of *A. africanum* was two times enhanced under Cd stress, while in *Coelastrella* sp. a similar increase in SOD was observed after the addition of Pb (Fig. 4A, B). Cd and Pb also led to a significant increase of CAT activity, but these changes did not strictly correspond to those of SOD and MDA levels (Fig. 5A, B). In *Coelastrella* sp., the most significant increase in CAT was observed after the addition of Pb in the medium, while in the cells of *A. africanum* Pb and Cd caused a similar increase of the activity. It is obvious that the enzymatic antioxidant protection of *A. africanum*, as a part of the anti-stress response, was not sufficient to overcome heavy metal toxicity, which was manifested by the severely inhibited growth of the strain. It is likely that in both strains the elimination of ROS involved certain additional protective mechanisms in addition to antioxidant enzymes, which should be studied in more detail.

The results concerning physiological and biochemical changes in *A. africanum* and *Coelastrella* sp. suggested that Cu influences the metabolic processes in the algal cells by a different mechanism, compared to Pb and Cd. That understanding was confirmed by the way both strains accumulated heavy metals from the nutrition medium. Both microalga showed a high absorption capacity for metal ions, especially for Pb, which accumulated in the biomass in much greater concentrations than copper and cadmium. The

degree of TM uptake was performed as follows Pb>Cd>Cu. (Table 2). *Coelastrella* sp., in particular, had a significantly higher ability for metal ion absorption, which did not affect her growth so strongly as *A. africanum*. Similar results were obtained in *Sc. incrassatulus* treated by 50 μM and 100 μM Cu, Cd and Pb (Goswami et al. 2014; Marinova et al. 2018). The green microalga manifested a very high absorption capacity, accumulating predominantly Cd from the environment. For that reason, we assumed that the investigated strains, and especially *Coelastrella* sp., could find real application in the treatment of water bodies contaminated with Cu, Pb and Cd.

Conclusion

The obtained results supported the understanding of microalgae as very reliable for the purposes of phytoremediation. In addition to the differences in their uptake capacity, it is possible that one definite heavy metal ion can interact specifically with a particular algal strain. Cd and Pb proved to be the most toxic for growth and pigment biosynthesis and provoked the strongest antioxidant response and enhanced MDA levels and activity of antioxidant enzymes in both investigated strains. The cyanoprokaryote *A. africanum* was much more sensitive to heavy metal stress, in contrast to *Coelastrella* sp. BGV, which showed a significantly higher absorption potential. We believe that after further research we could suggest *Coelastrella* sp. BGV as a suitable species for non-toxic and effective heavy metals' removal.

In conclusion, this study could contribute to expanding knowledge about the mechanisms of heavy metal stress in microalgae, as well as their future application for the needs of phytoremediation.

Acknowledgements

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RESEARCH ARTICLE

Assessment of PAHs accumulation in *Donax trunculus* (Linnaeus, 1758) (Bivalvia, Donacidae) from the Bulgarian Black Sea Coast

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Abstract

Anthropogenic pollution of marine ecosystems is one of the main sources of polycyclic aromatic hydrocarbons (PAHs). Marine bivalves are often used as bioindicators of environmental pollution due to their wide distribution and capability of xenobiotic bioaccumulation. The aim of the present study was to assess the presence of PAHs in soft tissues of wedge clams *Donax trunculus* (Linnaeus, 1758), collected from sublittoral sandy habitats at different locations off the Bulgarian Black Sea coast. Wedge clams from the different locations showed variations in the content of accumulated PAHs' compounds. The concentrations of PAHs were measured by gas chromatography system with mass spectrometry detection. The total PAHs content (sum of 13 PAHs' compounds) measured was in the range from 5.59 to 50.50 ng/g wet weight and was comparable with other European studies. The compounds phenanthrene and fluorene were most abundant in all analyzed samples. The results showed that low molecular weight (LMW) PAHs (2 and 3 aromatic rings) were predominant, accounting for 91% of the total PAHs levels, while high molecular weight (HMW) PAHs (4–5- and 6- rings) presence was 8.9% on average. The ratio LMW/HMW PAHs was higher than one, suggesting predominant pollution of petrogenic origin. The concentrations of benzo (a)pyrene did not exceed the limit set in EC Regulation although it was detected in 20% of the analyzed samples. In conclusion, maximum overall PAHs content was found in clams from Arkutino, while minimum PAHs content was present in samples from Elenite. The Sum PAH4 (sum of four polycyclic aromatic hydrocarbons: benzo[a]pyrene, chrisene, benzo[a]anthracene, and benzo[b]fluoranthene) in the

wedge clams for all localities studied was below legislation limits. Data from the present research can be used for assessing pollution levels in the marine environment and also risk of human exposure to PAHs using *D. trunculus* as bioindicator species.

Keywords

bioaccumulation, Bulgaria, coastal marine ecosystems, polycyclic aromatic hydrocarbons, wedge clam

Introduction

Pollution with polycyclic aromatic hydrocarbons (PAHs) is becoming a serious problem in marine ecosystems. PAHs are a group of organic compounds that consist of two, three or more condensed aromatic rings. They are highly persistent and widespread in the marine environment. PAHs are produced primarily from organic combustion and also from other anthropogenic activities and can enter into the marine environment in two main ways - by chronic pollution (associated with boat traffic) or by acute pollution due to oil spills (Kucuksezgin et al. 2020). Different marine bivalves (clams, oysters, mussels, scallops) have been recommended as suitable bioindicators of contamination in the sea due to their wide geographical distribution, filter feeding and rapid accumulation of toxic substances in their tissues (Suárez et al. 2013; Tlili and Mouneyrac 2019). Reliable data exist on the bioavailability and accumulation of marine environmental pollutants in bivalve tissues (such as PAHs, polychlorinated biphenyls, pesticides and heavy metals) and their toxic effects (Tanabe and Subramanian 2006; Georgieva et al. 2016; Lehtonen et al. 2019). However, the accumulation of organic pollutants depends not only on the physico-chemical characteristics of contaminants, but also on bivalve physiology and their lipid content (Barhoumi et al. 2016; Lehtonen et al. 2019). The potential of PAHs to cause adverse effects as endocrine- and reproductive disruption, genotoxicity and oxidative damage in marine organisms has been previously reported (Banni et al. 2010; Machado et al. 2014) and it was also demonstrated that acute benzo[a]pyrene exposure significantly depressed acetylcholinesterase (AChE) activity in gills and digestive gland of mussels (*Mytilus galloprovincialis*) (Banni et al. 2010).

The wedge clam (*D. trunculus*) is a species inhabiting fine sandy habitats of the upper infralittoral subzone and feeds by filtration on phytoplankton and suspended particulate matter. In the Bulgarian Black Sea coastal zone *D. trunculus* dominates usually between 1.0 and 6.5 m depth and is exposed to intense wave action and fluctuations of abiotic environmental factors (Gumus et al. 2020). Although local people do not traditionally consume wedge clams, *D. trunculus* are increasingly being collected with dredges for export due to the high prices on the foreign markets (Gumus et al. 2020). Data on the accumulation of PAHs in tissues of marine bivalve species is scarce, which determines the importance of research of PAHs' content accumulated in wedge clams inhabiting the Bulgarian Black Sea area.

The aim of the present study was to carry out the first comprehensive assessment of the levels of bioaccumulated PAHs in tissues of *D. trunculus* as an indicator of the pollution affecting the Bulgarian Black Sea coastal area and the possible risks for humans.

Materials and methods

Sampling and sample preparation

Wedge clams *D. trunculus* were collected manually or were obtained from commercial providers from their sublittoral sandy habitats along the Bulgarian Black Sea coast from May 2019 – September 2020 (Table 1). From each sampling location 2–3 kg of adult wedge clams of similar shell length (mean 2.1 ± 0.34 cm) were gathered, placed in plastic bags, kept in ice and transported to the laboratory. Soft tissues of the individual wedge clams were removed and 250 g samples were formed. The tissues were homogenized and stored at -20 °C until analysis.

Chemical analysis

Analytical procedures used for preparing soft tissue of mussels were described previously (Georgieva et al. 2016) with some modification. Ten grams from the homogenized soft tissue of clams were taken for extraction. Each sample was mixed with anhydrous sodium sulfate in a mortar and spiked with internal standards PCB 30 and PCB 204. The compounds were extracted with hexane/dichloromethane (2:1; v/v) in Soxhlet Extractor for 16 h at a rate of five cycles per hour. The extract was cleaned-up on a multilayer glass column filled with neutral and acid silica. PAH compounds were eluted with n-hexane followed by n-hexan/dichloromethane (4:1 v/v). The eluates were concentrated

Table 1. Locations and period of *D. trunculus* collection along the Bulgarian Black Sea coast with GPS coordinates, depth and distance from the shore.

Code	Location	Period	GPS coordinates	Depth [m]	Distance from the shore [m]
S1	Varna Bay	May 2019	43.2667°N, 28.0266°E	1.50–2.00	50–100
S2	Kranevo	July 2019	43.2667°N, 28.0266°E	3.00–3.50	50–100
S3	Shkorpilovtsi	July 2019	42.9603°N, 27.8970°E	3.00–3.50	20–50
S4	Ahtopol	July 2019	42.1022°N, 27.9328°E	1.50–2.00	20–50
S5	Varna Bay	October 2020	43.2365°N, 28.0160°E	1.50–2.00	50–100
S6	Shkorpilovtsi	March 2020	42.9603°N, 27.8970°E	1.50–2.00	20–50
S7	Ahtopol	March 2020	42.1140°N, 27.9243°E	1.50–2.00	20–50
S8	Sveti Vlas	March 2020	42.7090°N, 27.7595°E	3.00–3.50	10–20
S9	Slanchev Bryag	March 2020	42.6906°N, 27.7137°E	1.50–2.00	5–10
S10	Irakli	May 2020	42.7498°N, 27.8901°E	3.00–3.50	100–150
S11	Nessebar	June 2020	42.6559°N, 27.7171°E	1.50–2.00	50–100
S12	Arkutino	June 2020	42.3311°N, 27.7368°E	2.50–3.00	50–100
S13	Duni	June 2020	42.3684°N, 27.7094°E	1.50–2.00	5–10
S14	Sozopol	June 2020	42.4148°N, 27.7004°E	1.50–2.00	5–10
S15	Primorsko	June 2020	42.2530°N, 27.7533°E	1.50–2.00	50–100
S16	Cape Emine	August 2020	42.7018°N, 27.8343°E	2.50–3.50	200–250
S17	Tsarevo	September 2020	42.1666°N, 27.8533°E	2.50–3.00	50–100
S18	Elenite	September 2020	42.7025°N, 27.8130°E	3.50–4.00	200–250
S19	Primorsko	September 2020	42.2577°N, 27.7520°E	1.50–2.00	50–100
S20	Arkutino	September 2020	42.3282°N, 27.7496°E	3.00–3.50	100–150

to near dryness with a gentle stream of nitrogen and reconstituted in 0.5 cm³ in hexane. One microliter of extract was injected into the gas chromatography system in triplicate.

The quantitative analysis of PAHs was performed on gas chromatograph (GC/MS) GC FOCUS (Thermo Electron Corporation, USA) using POLARIS Q Ion Trap mass spectrometer (MS), equipped with an AI 3000 autosampler and splitless Injector. The experimental MS parameters, temperature of ion source and temperature of transfer line, were 220 °C and 250 °C, respectively. The PAHs experimental oven temperature was programed as follows: 40 °C (1 min), 40 °C/min to 130 °C (3 min), 12 °C/min to 180 °C, 7 °C/min to 280 °C, 10 °C/min to 310 °C with a final hold for 5.0 min. The separation of compounds was achieved with a TG-5 ms capillary column with a length of 30 m, 0.25 mm ID and a film thickness of 0.25 μm (Thermo Fisher Scientific). The carrier gas helium was used at a flow rate of 1 ml/min.

Quality control

Pure reference standard solution (EPA 525 PAH Mix B, 500 μg/mL) of each component in acetone (Supelco) was used for instrument calibration, quantification of compounds and recovery determination. Procedural blanks were analyzed between each 5 samples to monitor possible laboratory contamination.

In the prepared extracts thirteen PAHs compound were measured: acenaphthylene (ACL), anthracene (AN), benz[a]anthracene (BaA), benzo[b]fluoranthene (BbFA), benzo[k]fluoranthene (BkFA), benzo[ghi]perylene (BghiP), benzo[a]pyrene (BaP), chrysene (CHR), dibenzo[a,h]anthracene (DbahA), fluorene (FL), indeno[1,2,3-cd]pyrene (IP), phenanthrene (PHE), pyrene (PY). Each sample was analyzed three times and an average was taken.

Limits of detection (LOD) were estimated as 3 times the standard deviation, based on the low concentrations of the analytes in the sample. LOD varied for individual compounds from 0.075 to 0.294 ng/g wet weight (ww). For each PAHs were (ng/g ww): ACL 0.185, FL 0.128, PHE 0.092, AN 0.294, PY 0.173, BaA 0.219, CHR 0.185, BbFA 0.155, BkFA 0.178, BaP 0.295, IP 0.175, DBahA 0.199, and BghiP 0.075. The concentrations were reported as MEAN±SD of three measurements. Differences in means were determined by t-test ($p < 0.05$ considered significant).

Results

The mean concentrations of individual PAHs measured ranged from 0.05 (benzo[g,h,i]perylene) to 19.03 ng/g ww (phenanthrene) (Table 2). Acenaphthylene, anthracene, benzo[a]anthracene, indeno[1,2,3-cd]pyrene and dibenzo[a.h]anthracene showed levels below the instrumental detection limit in all samples (not present in Table 2). Four of the 13 target PAHs were found in almost all samples. BkFA and BghiP were detected in clams with very low values, close to the limit of quantitation (LOQ). Phenanthrene (PHE) predominated (from 3.24 to 48.22 ng/g) in all samples.

Table 2. Individual PAHs concentrations (ME ±SD ng/g ww) in *D. trunculus* from the representative localities off the Bulgarian Black Sea coast in different sampling periods (nd – not detected, PAHs: poly-cyclic aromatic carbons; BbFA: benzo[b]fluoranthene, BkFA: benzo[k]fluoranthene, BghiP: benzo[ghi] perylene, BaP: benzo[a]pyrene, CHR: chrysene, FL: fluorene, PHE: phenanthrene and PY: pyrene)*.

Site	Location	Period	FL	PHE	PY	CHR	BbFA	BkFA	BaP	BghiP	Sum PAH4	Sum PAH13
S1	Varna Bay	May-19	4.11	21.81	nd	0.92	nd	nd	nd	nd	0.92	26.84
S2	Kranevo	Jul-19	6.48	29.05	nd	nd	0.50	nd	0.54	nd	1.04	36.56
S3	Shkorpilovtsi	Jul-19	9.55	27.44	nd	nd	nd	0.46	nd	nd	0.00	37.44
S4	Ahtopol	Jul-19	0.43	3.24	nd	0.98	3.41	nd	nd	0.07	4.39	8.13
S5	Varna Bay	Oct-20	1.49	Nd	nd	nd	3.59	nd	2.34	0.07	5.93	7.49
S6	Shkorpilovtsi	Mar-20	4.57	18.10	nd	0.92	0.16	nd	nd	0.07	1.08	23.82
S7	Ahtopol	Mar-20	1.56	8.92	nd	0.90	1.36	nd	nd	nd	2.26	12.74
S8	Sveti Vlas	Mar-20	10.02	15.25	nd	0.53	0.30	0.54	nd	0.00	0.83	26.64
S9	Slanchev Bryag	Mar-20	1.97	8.17	nd	0.91	1.46	nd	1.08	nd	3.45	13.58
S10	Irakli	May-20	7.71	35.31	0.30	1.10	1.33	nd	nd	0.08	2.43	45.83
S11	Nessebar	Jun-20	14.21	31.19	nd	0.28	3.38	nd	nd	nd	3.66	49.07
S12	Arkutino	Jun-20	0.21	48.22	nd	0.56	0.21	nd	1.22	0.07	2.00	50.50
S13	Duni	Jun-20	21.00	22.68	nd	0.64	0.24	nd	nd	nd	0.89	44.57
S14	Sozopol	Jun-20	4.51	nd	nd	0.41	2.13	0.81	nd	nd	2.54	7.86
S15	Primorsko	Jun-20	6.12	7.17	nd	0.46	0.26	nd	nd	0.10	0.72	14.11
S16	Cape Emine	Aug-20	2.99	19.72	nd	0.47	1.18	0.42	nd	0.08	1.65	24.84
S17	Tsarevo	Sep-20	9.38	73.81	nd	nd	4.10	nd	nd	0.00	4.10	87.29
S18	Elenite	Sep-20	0.60	4.16	nd	nd	0.78	nd	nd	0.06	0.78	5.59
S19	Primorsko	Sep-20	nd	5.62	nd	nd	1.76	nd	nd	0.00	1.76	7.38
S20	Arkutino	Sep-20	0.63	20.38	nd	0.64	0.56	nd	nd	nd	1.21	22.21

*Acenaphthylene, anthracene, benzo[a]anthracene, indeno[1,2,3-cd]pyrene and dibenzo[a.h]anthracene showed levels below the instrumental detection limit in all samples and are not present in the Table.

The sum of 4 PAHs: benzo[a]pyrene, chrisene, benzo[a]anthracene, and benzo[b]fluoranthene (Sum PAH4) in the studied wedge clams from the Bulgarian Black Sea coastal zone varied in the range from 0.72 to 5.93 ng/g ww (Table 2). Relatively low Sum PAH4 levels were observed in wedge clams from 3 sampling locations (Shkorpilovtsi (S3), Primorsko (S15) and Elenite (S18)) which are situated far from highly urbanized and industrial areas off the Black Sea coast (Table 2). In contrast, the Sum PAH4 in *D. trunculus* from Varna Bay (S5) (industrial city and harbor area) was found to be higher (5.93 ng/g ww) (Table 2). The total PAHs levels (sum of 13 individual PAHs, Sum PAH13) in samples from Elenite (S18) (5.59 ng/g ww) and Tsarevo (S17) (87.29 ng/g ww) differed significantly ($p < 0.05$). Relatively high total PAH levels were observed also in wedge clam samples from Arkutino (S12), Nessebar (S11), Irakli (S10), and Duni (S13).

Among the four PAHs (benzo[a]pyrene, benz[a]anthracene, benzo[b]fluoranthene and chrysene), chrysene was the most abundant compound (Table 2). As a whole, chrysene and benzo (b)fluoranthene were detected in more than 60% of the samples, while benzo[a]pyrene, known to be the most carcinogenic, was found in only 4 samples. Chrysene was present in 67% of the wedge clam samples (from 0.41 to 1.10 ng/g ww) while benzo[a]pyrene was found in 20% of the analyzed samples with (mean 1.30 ng/g
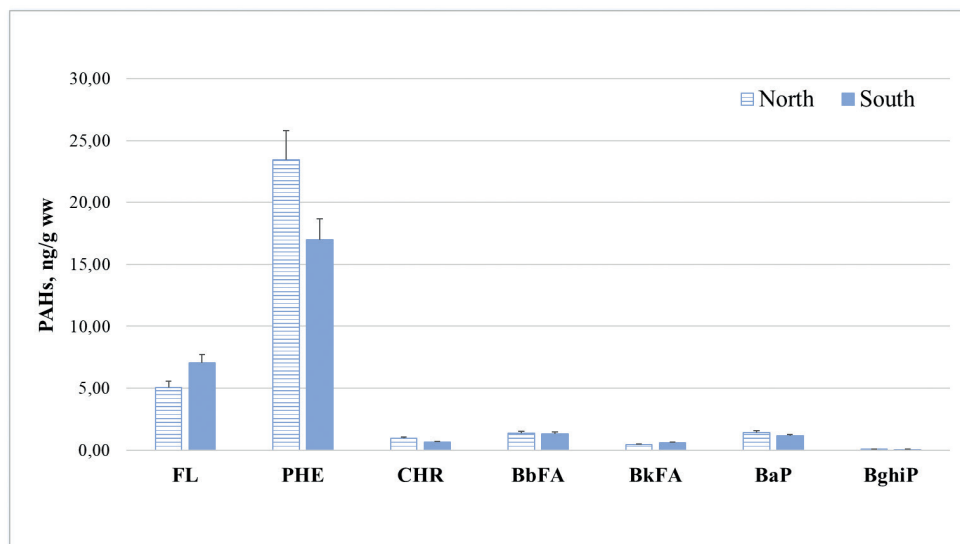
Figure 1. Individual levels of PAHs (ME±SD) in wedge clams (*D. trunculus*) sampled from the northern and southern locations of the Bulgarian Black Sea coast.

ww). Benzo[k]fluoranthene was found (mean 0.56 ng/g ww) only in wedge clams from Shkorpilovtsi (S3), Sveti Vlas (S8), Sozopol (S14) and Cape Emine (S16) (Table 2).

The mean individual levels of PAHs in *D. trunculus* sampled from the northern (north of Cape Emine) and southern (south of Cape Emine) locations of the Bulgarian Black Sea coast are presented in Fig. 1.

In general, the mean concentrations of CHR, BbFA, and BaP were higher in wedge clams from the northern coastal zone (Fig. 1). The concentration of PHE in wedge clam samples from locations in the northern part of the Black Sea coast was significantly higher (23.44 ng/g ww) than from the southern coast samples (17.00 ng/g ww). Only the mean concentration of FL was significantly higher in wedge clams from the southern coastal locations, compared to the northern ones.

The level of PAHs, accumulated in the studied wedge clams, displayed some seasonal variation (Fig. 2). The Sum PAH4 was significantly higher in autumn (difference significant for autumn 2019) and Sum PAH13 was significantly higher in summer compared to spring (2020) and autumn (2019 and 2020). The low molecular weight (LMW) PAHs accumulation was lower in autumn, whereas the accumulated high molecular weight (HMW) PAHs were higher in autumn (difference significant for autumn 2019).

Discussion

The results obtained in this study showed that in all wedge clams different PAHs were accumulated. This is a strong indication that seawater and sediments along the Bulgarian Black Sea coast are contaminated with organic pollutants. Similar findings for the Black Sea were
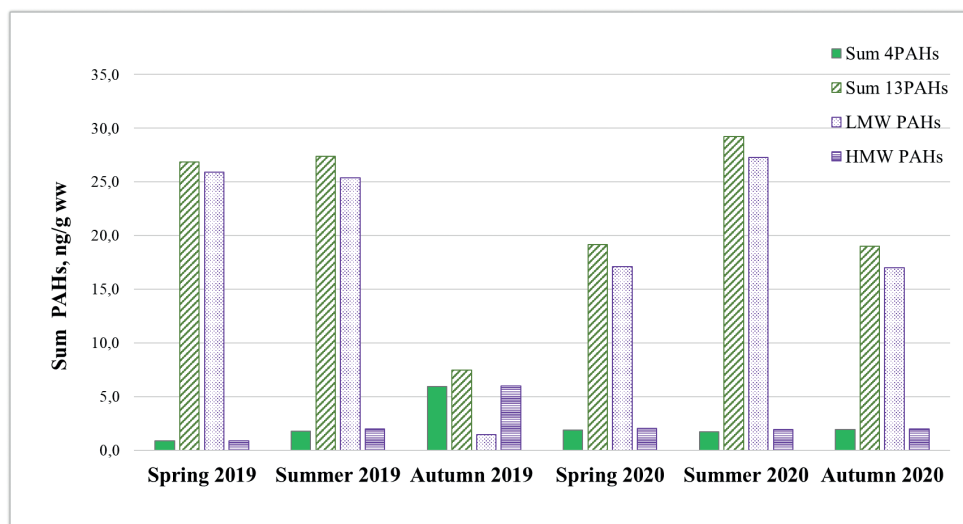
Figure 2. Total PAHs levels in wedge clams (*D. trunculus*) sampled in different seasons from the studied locations off the Bulgarian Black Sea coast.

reported earlier for bivalves from the Crimean Peninsula (Olenycz et al. 2015), Sevastopol Bay (Shchekaturina et al. 1995) and the Turkish coast (Güven and Coban 2012).

PAHs can originate from a variety of sources: LMW PAHs are defined as petrogenic compounds (resulting from spillage of diesel and fuel oil), and HMW PAHs as having pyrolitic origin (products of the incomplete combustion of organic matter) (Srogi 2007; Mercogliano et al. 2016). The molecular ratio of LMW and HMW hydrocarbons indicates the sources of PAHs. In our study the ratio LMW/HMW PAHs was higher than one (mean 17.61), suggesting that PAHs pollution of the Bulgarian Black Sea coastal region was predominantly of petrogenic origin. The pattern of accumulated composition of PAHs established by us was as follows: 91% were LMW PAHs with 3-rings (PHE and FL); 8.7% were PAHs with 4- and 5-rings -; < 1% were the PAHs with 6-rings.

In this study, the highest levels of Sum PAH13 were found in wedge clams from Tsarevo (S17), followed by Arkutino (S12) and Nessebar (S11). Given that the PAHs have mainly pyrolytic and petrogenic origin (Mercogliano et al. 2016), it seemed most likely that the high content of PAHs, present in wedge clams from these locations, resulted from local spills or currents that carried these pollutants. Relatively high levels of Sum PAH13 were also found in the wedge clams from the southern locations Duni (S13) and Arkutino (S12), but they were most probably the result of the oil spill in 2018 from the sunken ship SS Mopang near the coast of Sozopol (Panteleeva et al. 2021). This was further confirmed by the high ratio LMW/HMW PAHs established in *D. trunculus* from these locations, which strongly suggested a petrogenic source of the pollution. Although all the mentioned localities are situated in the southern part of the Bulgarian Black Sea coast, there were no significant differences present in the

accumulated PAHs in wedge clams from the northern and southern coastal regions. As an exception, statistically higher levels of accumulated phenanthrene were measured in wedge clams from the northern coastal areas. This could be associated with discharges from the Danube River, containing degraded petroleum and fresh oil which are reported as the major contributor of total hydrocarbons (Readman et al. 2002).

The high content of LMW PAHs in the studied wedge clams, strongly indicated that oil pollution was the major source of contamination. Phenanthrene has high lipophilicity that makes it readily absorbed from the gastrointestinal tract and transferred to the tissues (Ifegwu and Anyakora 2016). The highest benzo[a]pyrene contend was observed in wedge clams from Varna Bay (S5), indicating significant ecological pressure from the intensive maritime traffic and industrial activities in the area.

Most published data on PAHs accumulation concerned mussels (*M. galloprovincialis*) (Perugini et al. 2007; Mercogliano et al. 2016) and the only more comprehensive study of PAHs in wedge clams was from the Mediterranean Sea (Ferrante et al. 2018). The content of PAHs in *D. trunculus* found in our study (4.75 ng/g ww) was significantly lower than the PAHs' levels in the wedge clams reported from the Mediterranean Sea (247.4 ng/g ww) (Ferrante et al. 2018).

Admissible limit was set for Sum PAH4 in bivalve species by EC n.835 regulation (EC 2011). The established in our study values of Sum PAH4 in wedge clams were well below the regulation limit (35 ng/g ww). With regard to benzo[a]pyrene, which is considered to be highly carcinogenic according to the International Agency for Research on Cancer (IARC Group 1) (IARC 2010), we also did not establish values exceeding the admissible limits (6 ng/g ww) (EC 2011).

Conclusion

Results from this study showed the presence of accumulated PAHs in wedge clams from all the 15 locations along the Bulgarian Black Sea coast, which strongly indicated the presence of contamination with organic pollutants. The level of PAHs content in the wedge clams showed variations depending on local conditions. The maximum PAHs content was found in wedge clams from locations near to urbanized and industrial areas, or areas with highly intensive tourism. In general, there were no significant seasonal variations in the level of total PAHs, although some exceptions were observed. Our data did not show the presence of PAHs values exceeding admissible limits set by national and EC regulations. The data from the present research can be useful in further studies for assessing PAHs pollution levels and risks for human exposure using *D. trunculus* as bioindicator species.

Acknowledgements

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Saccharomyces cerevisiae yeast cells as a test system for assessing Zeocin toxicity

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Abstract

Having unique genetic machinery and a high degree of conservation with higher eukaryotes, the yeast *Saccharomyces cerevisiae* is recognised as a smart experimental system for studying the modes of chemical toxicity. The present study was undertaken to elucidate the changes in the intracellular redox homeostasis and key macromolecule structure following exposure to Zeocin. Cell populations of logarithmic, quiescent (Q) and non-quiescent (NQ) cells of *Saccharomyces cerevisiae* BY4741 were used as a model to examine the cytotoxic effect of this radiomimetic. The levels of endogenous ROS, oxidized lipids, carbonylated proteins, and glutathione were analysed after treatment with Zeocin (IC_{50}). An increase in ROS production and respectively increased oxidative stress was detected in all three types of cell populations, with the highest degree being observed in proliferating *S. cerevisiae* BY4741 cells. The stress response of both proliferating and stationary phase (Q and NQ) cells to Zeocin included an overexpression of glutathione. The quiescent cells also showed very low DNA susceptibility to high Zeocin concentration (100–300 µg/ml), presented as no induced double-strand breaks (DSBs) in the macromolecule. Based on our research it could be concluded that the cellular physiological state is a critical factor determining the resistance to environmental stress with Q cells being the most robust.

Keywords

Quiescence, stress response, yeast, Zeocin

Introduction

The worldwide use of chemicals, drugs and other pharmaceuticals is significant. However, they represent toxic pollutants and their presence in the environment seriously endangers human health. Pharmaceutical residues can interact with biological targets and thus exert their different toxic effects at very low concentrations. These often-irreversible interactions triggered serious damages in lipids, proteins, and DNA molecules (Farrugia and Balzan 2012). Our ability to predict toxicological outcomes of exposure to drugs as well as understanding the mechanisms underlying toxicity in biological systems are of significant importance for human health and safety. *Saccharomyces cerevisiae* is one of the best-explored models of eukaryotic cells for studying cellular mechanisms that occur under stress conditions. The main advantage of using yeasts in drug testing is the possibility to identify sensitive biomarkers and mechanisms of drug-mediated cell toxicity in the cases when the toxic compounds are still unknown. In addition, *S. cerevisiae* is currently the only system that allows evaluation of all targets in the cell, simultaneously and *in vivo* (Smith et al. 2010). However, most of the yeast-based toxicological studies involve the use of proliferating *Saccharomyces cerevisiae* cells (Gasch et al. 2000; Ericson et al. 2008; Dos Santos et al. 2012; Braconi et al. 2016). Since quiescence is the most common cellular state found on Earth, using *Saccharomyces cerevisiae* in quiescent state will be the more valuable tool for prediction of cellular response to exposure of environmental toxic compounds. Correspondingly, this study compares the toxic response of proliferating and stationary phase (Q and NQ) *S. cerevisiae* BY4741 cells to Zeocin. With its radiomimetic properties, this compound is known to induce single (Miné-Hattab and Rothstein 2013) and double-strand breaks (Chankova et al. 2007; Kopaskova et al. 2012; Todorova et al. 2015a) as well as basic substitutions in the DNA molecule (Guénolé et al. 2013). Moreover, it has been shown that Zeocin has pro-oxidative, mutagenic, and carcinogenic effects in *S. cerevisiae* (Todorova et al. 2015b). Taking this into consideration, we investigated the effect of Zeocin (IC_{50}) on the level of intracellular reactive oxygen species (ROS), total intracellular glutathione, oxidized lipids, and proteins as sensor molecules for measuring induced oxidative stress during drug toxicity. The DNA damaging potential of high concentrations Zeocin on proliferating, Q and NQ cells was also estimated.

Materials and methods

Microorganism and growth conditions

Yeast strain *Saccharomyces cerevisiae* BY4741 (*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) was used, obtained from the EUROSCARF Frankfurt collection (Germany). Yeast cells were grown on a liquid YPD medium at 30 °C on a reciprocal shaker (205 rpm) for 168 hours. Samples were withdrawn at exponential (24 hours) and late sta-

tionary phase (168 hours). The biomass was harvested by centrifugation at 5000 rpm for 10 min at 4 °C. The pellet was washed twice with distilled water and used for further analyses.

Isolation of quiescent (G$_{0/}$Q) and non-quiescent (NQ) cells

Isolation of Q and NQ *S. cerevisiae* BY4741 stationary phase yeast cells (168 h) was performed in Percoll density gradient according to the protocol described by Allen et al. (2006).

Zeocin treatment

IC$_{50}$ dose of Zeocin (Cayman Chemical Company, USA) was previously determined using a spot analysis (Daskalova et al. 2021). The toxic effect of IC$_{50}$ concentration on the level of intracellular damages was studied after exposure to 50 μg/ml Zeocin. The yeast cells were incubated with the stress-inducing agent for 60 min at room temperature, and then washed twice with distilled water and subjected to disintegration. Different concentrations of Zeocin (100, 200 and 300 μg/ml) were used in the experiments for double-strand breaks (DSBs) detection.

Disintegration mode

Washed biomass of unexposed and exposed to Zeocin log, Q and NQ cells was mechanically disrupted according to the previously described procedure (Daskalova et al. 2021).

Biochemical analyses

Protein content was measured by the method of Lowry et al. (1951). The concentration of reactive oxygen species (ROS) was determined by the nitroblue tetrazolium test (NBT) method described by Kostova et al. (2008). The levels of oxidized proteins were assessed following the methodology of Mesquita et al. (2014). Quantitative evaluation of oxidized lipids was done using the method described by Hodges et al. (1999). The concentration of intracellular glutathione was determined using the method of Zhang (2000).

Constant field gel electrophoresis (CFGE) for detection of double-strand breaks

CFGE was performed as described in Todorova et al. (2015b) and Todorova et al. (2019). Logarithmic, Q, and NQ cell suspensions were treated with different concentrations of Zeocin for 1 min on ice. Cells were centrifuged and included into agarose plugs at concentration 1×10^6 cells/ml. The agarose plugs were then placed in 1 ml of lysis solution (pH = 8) containing proteinase K at final concentration 1 mg/ml. After cell lysis, plugs were washed in Tris- EDTA (pH = 7.5) and inserted into a series of wells

in an agarose gel. Electrophoresis conditions were: 40 h at a constant field of 0.6 V/cm (20 V). The levels of induced double-strand breaks (DSBs) represented as a fraction of DNA released (FDR) from the wells were quantified by detecting the ethidium bromide fluorescence using the Gene Tool Analyser G:Box Syngene. To evaluate the repair capacity of cell populations 30 and 60 min recovery time was given after Zeocin treatment.

Data analysis

Used data represent the mean values with Standard error of the mean (±SEM) of three independent experiments. The statistical analysis was performed using MICROSOFT OFFICE 365 EXCEL 2020 software. Differences in means were analysed using Student's t test with independent measures. Differences were considered statistically significant at the $p < 0.05$ level.

Results

ROS levels

A comparative study on the cytotoxic effect of Zeocin in logarithmic, quiescent, and non-quiescent *S. cerevisiae* BY4741 cells has been conducted. After their exposure to 50 μg/ml Zeocin, the induced intracellular changes were determined based on the level of generated reactive oxygen species and their harmful oxidative effect on key cellular macromolecules - proteins and lipids. Results presented in Fig. 1 show the level of accumulated ROS in untreated and Zeocin-treated logarithmic, Q and NQ cells. They clearly indicate that the action of Zeocin leads to an increase in the concentration of toxic radicals in the three types of cells. ROS values measured in treated logarithmic (400 μM/ml) and NQ (125 μM/ml) cells were almost four times higher than those in untreated cells. The lowest increase in the level of ROS (1.5 times) after Zeocin treatment was observed for the quiescence cells.

Levels of oxidized proteins

Next, the presence of carbonyl groups after exposure to the toxic agent has been studied (Fig.2). Q and NQ cells of *S. cerevisiae* BY4741 exhibited differential responses to the action of Zeocin. The highest increase in the level of carbonyl groups has been observed in G_0 cells – about 3-fold. Logarithmically growing Zeocin-treated cells as well as non-quiescent cells showed a slight increase in the amount of carbonyl groups compared to the control ones.

Levels of oxidized lipids

The data obtained after the measurement of malonaldehyde equivalents in the three cell types showed that Zeocin could also impair the prooxidant balance and activate
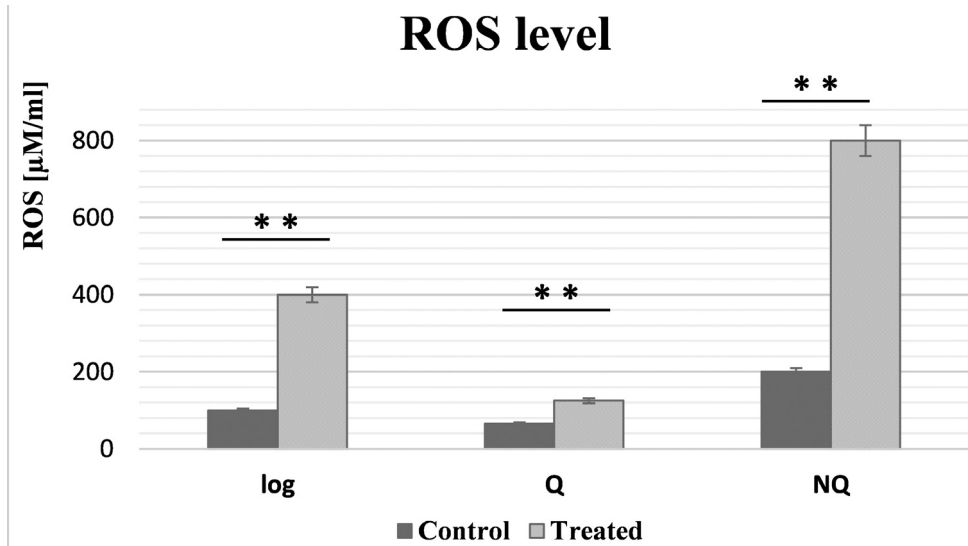
ROS level

Figure 1. Comparative analysis of ROS levels in untreated (control) and Zeocin-treated proliferating, Q and NQ cells of *S. cerevisiae* BY4741. Each value represents the mean ± SEM (n = 3). Significant differences (* $p < 0.05$; ** $p < 0.001$) are presented.

Carbonylated proteins
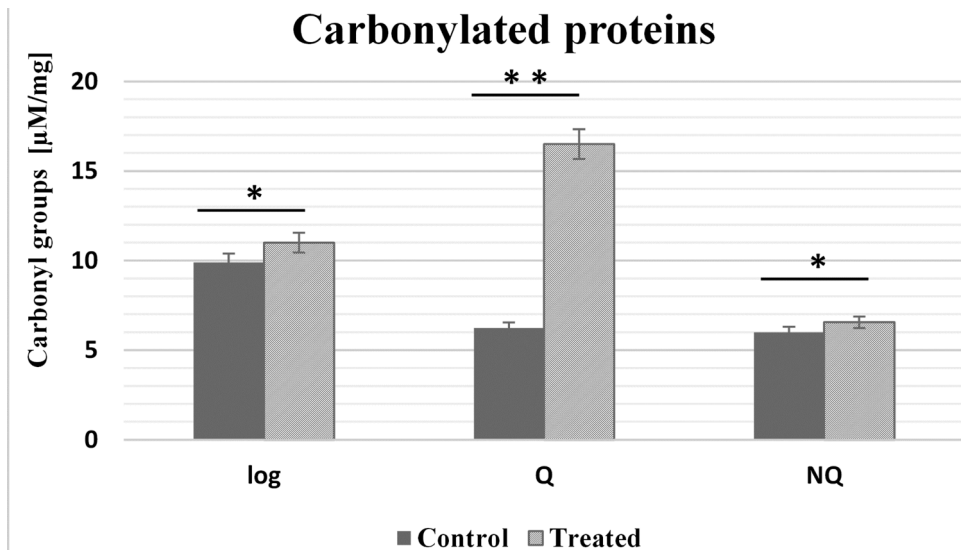
Figure 2. Comparative analysis of carbonylated proteins in untreated (contol) and Zeocin-treated logarithmic, Q and NQ cells of *S. cerevisiae* BY4741. Each value represents the mean ± SEM (n = 3). Significant differences (* $p < 0.05$; ** $p < 0.001$) are presented.

the process of lipid peroxidation (Fig. 3). The higher levels of oxidized lipids were observed in both Zeocin-treated proliferating and quiescent cells (1.88 and 1.4 nmol/mol, respectively). On the contrary, non-quiescent cells showed a very low level of lipid peroxidation whether or not they are treated with Zeocin.
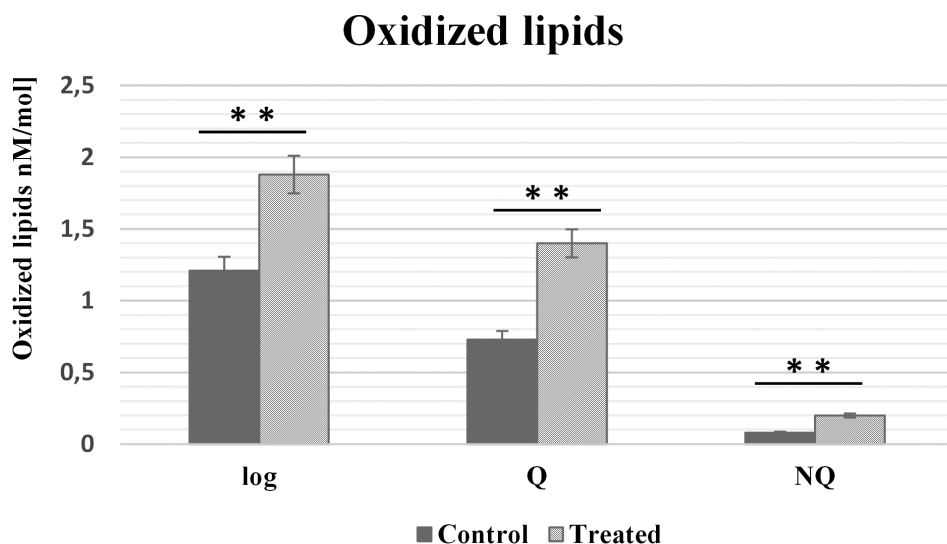
Oxidized lipids

Figure 3. Comparative analysis of oxidized lipids in untreated (control) and Zeocin-treated Log, Q and NQ cells of *S. cerevisiae* BY4741. Each value represents the mean ± SEM (n = 3). Significant differences (* $p < 0.05$; ** $p < 0.001$) are presented.

Intracellular glutathione levels

We also sought to investigate the effect of the radiomimetic Zeocin on the non-enzymatic defence mechanisms, in particular, glutathione. Obtained results revealed that the total levels of this tripeptide in all the treated yeast cell populations were higher compared to the control ones. The most significant increase was observed in proliferating cells - about 2.5 times (Fig. 4).

Spontaneous levels of double-strand breaks

The spontaneous DSB levels were found to depend on the growth phase. Around 1.5-fold higher DSBs levels were measured in non-quiescent cells compared to the logarithmic and quiescent ones (Fig. 5). No statistically significant difference was calculated between the DSB levels in logarithmic and quiescent cells.

Zeocin induced double-strand breaks

Furthermore, the growth phase was estimated as a very important factor for the DNA susceptibility of *S. cerevisiae* to Zeocin (Fig. 6). Approximately similar statistically significant response measured as DSB induction was measured in both logarithmic (Fig. 6 A, D) and non-quiescent cells (Fig. 6 C, D). The DSBs in logarithmic cells did not differ statistically from those in the non-quiescent cells. However, no significant effect of this radiomimetic has been observed in quiescent cells, regardless of the applied concentration (Fig. 6 B, D). The DSB levels were comparable with those in untreated cells.
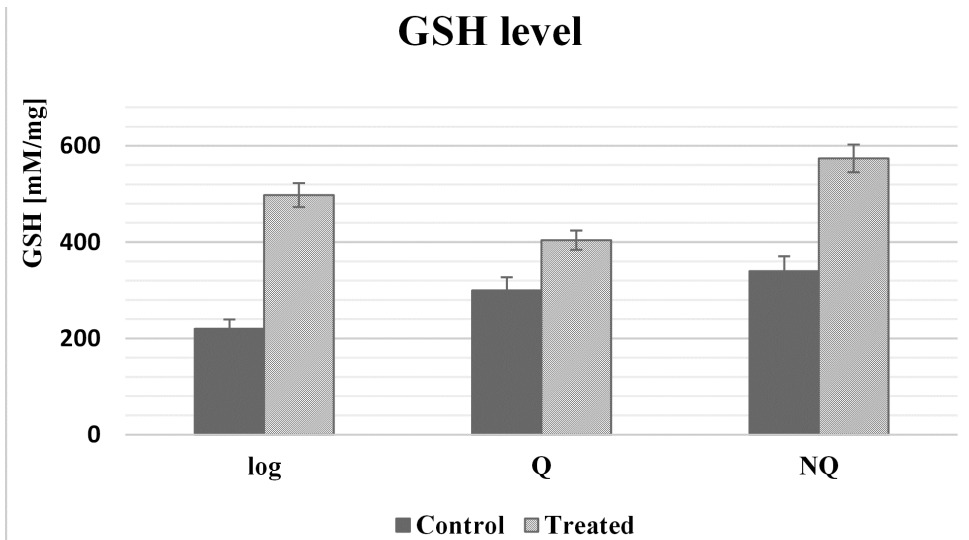
Figure 4. Comparative analysis of the amount of total glutathione in untreated (control) and Zeocin-treated Log, Q and NQ cell of *S. cerevisiae* BY4741. Each value represents the mean ± SEM (n = 3). Significant differences (* $p < 0.05$; ** $p < 0.001$) are presented.
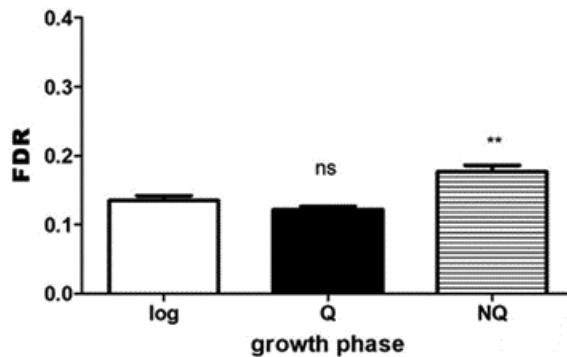
Figure 5. Spontaneous levels of DBSs depending on the growth phase. Error bars represent standard error of the mean from at least three independent experiments. Where no error bars are evident, they are equal of less than the symbols. Statistical significance of differences is indicated with an asterisk (** $p < 0.01$; ns $p > 0.05$).

Repair capacity depending on the growth phase

The best expressed repair capacity was calculated for the logarithmic cells when 60 min recovery time was given. On the other side, NQ cells were found unable to repair Zeocin induced DSBs despite the recovery time (Table 1). It was not possible to calculate repair capacity of Q cells, having in mind that no statistically significant increase of DSBs was measured after the treatment with different Zeocin concentrations.
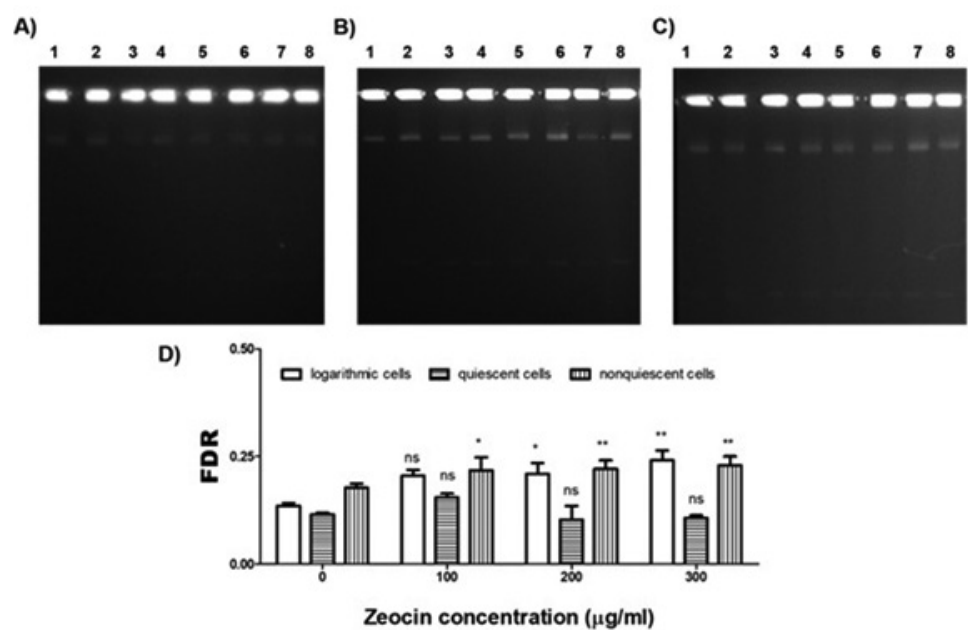
Figure 6. DSBs induced in *S. cerevisiae* BY4741, depending on growth phases after Zeocin treatment in a concentration range 100–300 μg/ml **A** cells in logarithmic phase **B** quiescent cells **C** non-quiescent cells. 1, 2 - control; 3, 4 - treatment with 100 μg/ml Zeocin; 5, 6 - treatment with 200 μg/ml Zeocin; 7, 8 - treatment with 300 μg/ml Zeocin **D** induction of DSBs after treatment with different concentrations of Zeocin calculated as FDR. The significance in the differences where ns $p > 0.05$, **$p < 0.01$. Where no error bars are evident, they are equal or less than the symbols.

Table 1. Repair capacity of logarithmic and non-quiescent cells calculated after the treatment with Zeocin at concentrations 100, 200 and 300 μg/ml with 30- and 60-min recovery time.

	Logarithmic		Non-quiescent	
	Recovery time (min)		Recovery time (min)	
Zeocin concentration (μg/ml)	30	60	30	60
100	1.25	1.41	0.84	0.87
200	2.41	1.86	1.20	1.02
300	2.29	3.21	0.99	0.88

Discussion

Currently, the yeast *Saccharomyces cerevisiae* remains one of the highly important experimental models in the field of toxicogenomics. Studying the biology of yeast cells, especially those in quiescence, could reveal the potential of this microorganism for investigating the cellular response to a particular environment. The evaluation of toxicological response and stress mechanisms in this microorganism could further be helpful in the clarification of equivalent mechanisms in higher eukaryotes. The environmental conditions can change dramatically, which includes progressive depletion of nutrients, rising ambient temperatures, or sudden contamination with

xenobiotics. Regardless of their nature, such changes in the environment invariably cause stress to the organisms, to which they must effectively adapt in order to survive. This stress is often associated with ROS accumulation (Avery 2011). Results obtained through this study confirmed that the exposure to Zeocin led to disturbance of the cellular redox balance and induced an increase in the levels of toxic oxygen radicals independently of the growth phase. The fraction of NQ cells probably rapidly lost the ability to divide and accumulated almost 4 times higher levels of ROS (Fig.1). Meanwhile, the stationary cells which have entered in G_0 cell cycle, showed significantly higher resilience to the effect of Zeocin. The treatment with this toxic chemical led to only a 2-fold increase in the levels of ROS, which was probably due to their specific morphological and physiological properties. It was well-known that *S. cerevisiae* quiescent cells are characterised by thicken cell walls, condensed chromosomes and increased termo- and osmotolerance (Gray et al. 2004). On the other hand, the NQ cells differ significantly, possessing genomic instability, being easily lysed, and providing nutrients for those cells entering G_0 (Aragon et al. 2008). As regards the logarithmic cells, they are characterised by higher metabolic activity, elevated ROS levels and respectively increased susceptibility to toxic compounds, in that case Zeocin (Cabral et al. 2003).

A direct indicator of the onset of the redox balance disturbance is the appearance of carbonyl groups in the proteins. Oxidative damage to proteins affects the processes maintaining the cellular homeostasis, which compromises their viability (Farrugia and Balzan 2012). In this respect as the Q-cells were characterised by very low metabolic levels and lower ability to sequestered damaged molecules, the observed elevated oxidative modifications in their proteins was not surprising. This might be also due to the coordinated toxic effects of Zeocin, and stress caused by the lack of nutrients in the environment. In NQ cells, the amount of carbonylated proteins after treatment with Zeocin was comparable to that in control cells - 6.56 and 6.06 μM/mg, respectively (Fig. 2). This indicated that here the appearance of oxidized proteins is rather a consequence of the physiological state of the cell population and is not directly resulting from the Zeocin-induced oxidative stress.

The measured higher intracellular concentration of malonaldehyde in Zeocin exposed cells further confirmed that one of the cytotoxic effects of this antibiotic was related to the induction of oxidative stress in the cell. It resulted in impairment of membrane functionality and permeability, probably causing the release of the intracellular content (Hodges et al. 1999). Obviously, when the metabolic activity of the cell is higher and the transport across the membrane - dynamic, the yeast cells are more vulnerable to the action of xenobiotics, including the Zeocin. This eventually explains the measured excess levels of oxidized lipids in the logarithmic cells comparing to the Q and NQ ones (Fig. 3).

To prevent the unbalanced accumulation of ROS and consecutive cellular damages, yeast cells react with specific induction of both non-enzymatic and enzymatic antioxidant defence mechanisms. Key molecule acting as first line of defence against oxidative injuries is the glutathione. That is why the total intracellular amount of this tripeptide is an important parameter for measuring the oxidative stress levels. In this study we confirmed that after exposure to IC_{50} Zeocin the three types of yeast populations showed

increased intracellular levels of glutathione ranging from 1.5-fold (for Q cells) to 2.5-fold (for logarithmic cells) (Fig. 4). Having in mind that in actively proliferating cells the rate of transcription and translation are the highest, it is not surprising that in these yeast populations the biosynthesis of glutathione was augmented with more than 100%. By contrast, in quiescent yeast cells, characterised by very low metabolic profile, the measured increase in glutathione is only about 30%. These findings also correlated with the observed tendency in the intracellular ROS levels of the studied yeasts. Non-quiescent cells had the highest measured concentrations of glutathione possibly related with the excess levels of intracellular ROS in general (Aragon at al. 2008).

Furthermore, this study aimed to assess the potential of Zeocin to induce DNA double-strand breaks (DSB) in dependence to the growth phase. The highest levels of spontaneous DSBs were observed in non-quiescent cell. Such results are not surprising, considering the fact that they represent the fraction of stationary cells having very low ability to reproduce and tendency to enter apoptosis. Such differences in the spontaneous DSB levels being dependent on the growth phase were also observed previously in other strains of *S. cerevisiae* (Todorova et al. 2015b, 2019). The statistically significant induction of DSB in logarithmic cells after the treatment with concentrations equal or higher than 200 μg/ml Zeocin is in a good correspondence with the one obtained on strain D7ts1 (Todorova et al. 2019). In late stationary cells significantly higher levels of DSBs were detected in comparison with those in exponential and early stationary cells. However, the entrance into G_0 cell cycle considerably affects the levels of DSB, rendering quiescent cells with a low susceptibility to DNA damages. When Zeocin is used as an inducing agent for DSB formation the tendency has been preserved. Most robust to its action were the cells already entered the G_0 state. A possible explanation for their resistance to this radiomimetic chemical could be again assigned to their specific cellular features. As mentioned above they are characterised by thickened cell walls, highly condensed chromosomes and, as a result, restricted ability of the Zeocin to penetrate the cell and reach its target molecule (Gray et al. 2004; Allen et al. 2006).

Conclusions

The comparative analysis of different yeast cell populations – logarithmic, quiescent, and non-quiescent – revealed that the cellular physiological state is a critical factor determining the resistance to xenobiotics. More robust to Zeocin exposure were the quiescent cells showing lower levels of ROS and DSBs. By contrast, the logarithmically grown yeasts are more susceptible to the action of this compound with observed higher damages in DNA, proteins and lipids.

Dataset deposition

The data underpinning the analysis reported in this paper are deposited at Figshare at https://figshare.com/s/e89e88bfc6f1247a4755.

Acknowledgement

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RESEARCH ARTICLE

BioRisk

Comparative study on the oxidative stress of commercially important fish species from localities with different ecological conditions along the Bulgarian Black Sea coast

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Abstract

The aim of the present study was to perform a pilot assessment and analysis of the oxidative stress (OS) level in four commercially important fish species (round goby, red mullet, sprat and horse mackerel) from different localities of the Bulgarian Black Sea coast. The fish were sampled during trawl selectivity experiments. The OS level in the fish was assessed by measuring lipid peroxidation (LPO), glutathione concentration (GSH), activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-S-transferase (GST), as well as acetylcholine esterase (AChE) in gills and liver. Round goby and red mullet caught in the Nessebar Bay showed clear signs of OS with the highest levels of LPO and GST activities, accompanied by the lowest AChE activities in both liver and gills. On the contrary, round goby caught near Maslen Nos (a region with good ecological conditions) were least affected by OS with low LPO and high GSH concentrations and SOD activity. There were no significant differences in the OS bioindicators of horse mackerel from the different localities. Sprat caught in Nessebar Bay, compared to those caught from the other localities, showed presence of OS indicated by lower GSH levels and relatively higher CAT, GPx and GST activities, accompanied by low AChE activity in gills. It can be concluded that round goby and red mullet were more vulnerable to OS induced by marine environmental factors than the horse mackerel and sprat. However, their antioxidant defense system allows them to tolerate and adapt to the environment of their habitats. Further studies are needed for the assessment of OS in important fish species in the Bulgarian part of the Black Sea.

Introduction

Fish are a major component of aquatic ecosystems and the good state and health of their populations is crucial for ecosystem stability. Marine fish populations are subjected to increasing environmental pollution and also to overfishing pressure (Daskalov 2003; Radu et al. 2011; Țoțoiu et al. 2018). At present, Black Sea fish species with significant economic and ecological importance, also in the Bulgarian part, are sprat, turbot, red mullet, bluefish, horse mackerel and round goby (Raykov et al. 2019). The assessment of the condition of the exploited fish species (Descriptor D3) in the Bulgarian Black Sea part showed that there are no species in "Good condition", and sprat, whiting, horse mackerel, mullet, turbot and others are even classified in "Bad" condition (Panayotova and Todorova 2017).

The sustainable management of marine fish stocks requires not only regulation of the fish populations, but also knowledge regarding the ability of the fish to respond and adapt to multiple environmental stressors (Hagger et al. 2006, 2008). A general reaction of marine organisms to environmental pressures (abiotic, biotic and anthropogenic) involves the activation of oxidative processes in their cells where the generated reactive oxygen species (ROS) play the role of signaling molecules that trigger the organisms' adaptative responses to environmental changes (Birnie-Gauvin et al. 2017). However, under prolonged or acute exposure, excess ROS generation cannot be compensated by the antioxidant defense system and provokes oxidative stress (OS) (Villarreal et al. 2014; Chowdhury and Saikia 2020). The OS in marine fish can be induced by multiple stress factors which were proved to adversely affect the growth, development and reproduction of fish species (Stoliar and Lushchak 2012; Vinagre et al. 2012; Dolci et al. 2013; Mozhdeganloo and Heidarpour 2014; Florescu et al. 2021). All these stressors (and related stresses) can result in community-scale effects (community structures) and may ultimately drive species coexistence (Steinberg 2012).

The aim of the present study was to provide a pilot assessment and analysis of the oxidative stress level of four commercially important fish species from representative localities of the Bulgarian Black Sea coastal area.

Materials and methods

Sampling

The fish species selected for this study were: the demersal round goby (*Neogobius melanostomus* Pallas, 1814), two benthopelagic species – red mullet (*Mullus barbatus* Linnaeus, 1758) and horse mackerel (*Trachurus mediterraneus* Steindachner, 1868), and

the pelagic species – sprat (*Sprattus sprattus* Linnaeus, 1758). The fish species were identified in accordance with BSFishList (2020). The fish individuals were randomly picked from several trawl selectivity experimental catches by pelagic Midwater otter trawl (7 × 7 mm mesh size of the codend) from 3 localities of the Bulgarian Black Sea aquatory (Table 1). On board, random samples of 9–12 fish individuals of every species were shock frozen and transported to the laboratory.

In the laboratory, fish individuals were measured with a caliper and weighed on scales with an accuracy of 0.01 g. The Fulton's condition factor (K) of the fish individuals was computed according to Nash et al. (2006): $K = 100*TW/TL^3$, where TW is the total body wet weight in grams and TL is the total length in cm; the factor 100 is used to bring K close to a value of one.

Tissue preparation

The fish were dissected and their liver and gills were extracted. The organs were homogenized in 0.1 M potassium phosphate buffer (pH 7.4) and centrifuged at 3000 g for 10 min to obtain a post-nuclear fraction, used for determination of lipid peroxidation and glutathione levels. A portion of post-nuclear fraction was re-centrifuged at 12000 g for 20 min at 4 °C for obtaining a post-mitochondrial supernatant, used for measurement of the antioxidant enzymes activities.

Measurement of oxidative stress biomarkers

The OS biomarkers were measured spectrophotometrically using commercially available kits: Lipid Peroxidation (MDA) Assay Kit MAK085, Glutathione Assay Kit CS0260, SOD Assay Kit-WST 19160, Catalase Assay Kit CAT100, Glutathione Peroxidase Cellular Activity Assay CGP1, and Glutathione-S-Transferase Assay Kit CS0410 (Sigma-Aldrich Co. LLC, USA). The manufacturer's working instructions were strictly followed.

Acetylcholinesterase (AChE) activity was assayed by the method of Ellman (Ellman et al. 1961). The reaction mixture contained 0.1 M K-PO4 buffer pH 8.0, 0.045 M acetylthiocholine iodide, 0.008 M 5,5´-Dithiobis(2-nitrobenzoic acid) (DTNB) and the appropriate amount of fish's tissue homogenate. The enzymatic hydrolization of acetylthiocholine produced thiocholine. The latter reacted with DTNB and yield a yellow colored product, 5-thio-2-nitrobenzoic acid with absorbance pick at 412 nm. The enzyme activity was calculated as U/mg protein. Protein concentration

Table 1. Trawling localities along the Bulgarian Black Sea coast with depth and bottom temperature.

Code	Trawling locality	Trawling start point N, E	Trawling end point N, E	Depth [m]	T_{bottom} [°C]
S1	Maslen Nos	42.312452°N, 28.054628°E	42.296292°N, 28.089651°E	37	7.7
S2	St. Oryahovo	42.993074°N, 27.951590°E	42.960442°N, 27.948072°E	23	7.9
S3	Nessebar	42.599613°N, 27.791345°E	42.619495°N, 27.826710°E	20	7.4

was measured according to Lowry et al. (1951) and was computed from a standard curve, obtained using bovine serum albumin (CAS Number: 9048-46-8, Merck KGaA, Darmstadt, Germany).

Statistical analyses

For statistical analyses the software package STATISTICA 10 (StatSoft Inc.) was used. The Kruskal–Wallis test was used to assess the significance of differences among the values of the OS biomarkers. Post hock comparisons between variables were made using Mann-Whitney test. Multidimensional Scaling (MDS) was carried out to detect patterns of similarities among the studied objects.

Results

Data from the measurements of the OS biomarkers in the liver and gills of the studied fish species from the selected localities of the Bulgarian Black sea coast are presented in Table 2. Our data showed that the measured OS bioindicators significantly varied among the fish species, the studied organs and the localities. In general, the two fish organs studied showed different susceptibility to oxidative changes, due to its unique composition and metabolic profile. Significantly higher lipid peroxidation (LPO) was found in the gills compared to the liver of the fish species, with the exception of the round goby, where no differences were observed (Table 2). The gills of all studied fish species had lower glutathione-S-transferase (GST) and higher acetylcholine esterase (AChE) activities than the liver. The activity of superoxide dismutase (SOD) in the liver was approximately twice as high as in the gills for all fish species studied, and the activity of catalase (CAT) was two to five times higher in the liver of round goby, red mullet and sprat, compared to gills. Glutathione peroxidase (GPx) activity was significantly higher in the gills than in the liver only in sprat. The glutathione (GSH) level in the gills of red mullet was significantly lower than in the liver, while the opposite was observed in sprat. In the round goby and horse mackerel no difference in GSH between the two organs was present (Table 2). The gills of the studied fish species were more affected by OS as a whole, showing high levels of LPO, with the exception of round goby. In the liver of the studied fish species lower activity of CAT, SOD (except for the fish species from Maslen Nos) and GST were present, compared to the gills (Table 2).

Significantly higher LPO and GST activities were established, together with the lowest AChE activity in both liver and gills of round gobies and mullets from Nessebar Bay, compared to those from Maslen Nos and Staro Oryahovo (Table 2), thus indicating clear signs of OS. Round gobies from Maslen Nos had the lowest level of OS as indicated by the lowest LPO levels and GST activities, and the highest concentrations of GSH and SOD activity, together with highest activity of AChE (Table 2). No significant differences were present in the measured OS biomarkers between mullets caught from Maslen Nos and from Staro Oryahovo. The OS markers in sprat from

Table 2. Values (ME±SD) of the measured OS biomarkers in gills and liver of the studied fish species from the different localities of the Black Sea coast (*statistical significance of differences at p<0.05: S^n indicate significant differences of the OS indicator in fish species between sites; letters (G, L) indicate significance of differences of the OS indicator between organs – G = gills and L = liver).

Site	Liver							Gills						
	LPO	GSH	SOD	CAT	GPx	GST	AChE	LPO	GSH	SOD	CAT	GPx	GST	AChE
Neogobius melanostomus														
S1	4.83	701	16.29	1.54	19.72	37.1	31.27	2.90	457	16.00	1.05	20.20	38.99	42.7
	±1.42 *s2.3	±134 *s2.3	±4.85 *s2.3	±0.02	±3.22	±12.1 *s3	±0.64 *s2.3L	±0.18 *s2.3	±99	±2.8 *s2.3	±0.02 *s3	±1.43 *s3	±0.87 *s2.3	±1.43 *s2.3G
S2	13.81	295	3.60	1.35	19.73	40.3	17.94	15.49	287	1.15	1.09	16.44	49.09	28.4
	±2.01 *s1	±7 *s1	±0.26 *s1	±0.08	±2.86	±1.8 *s3	±0.59 *s1L	±1.68 *s1	±39	±0.16 *s1	±0.15 *s3	±1.21	±1.22 *s1.3	±0.33 *s1G
S3	19.26	304	3.50	1.84	23.76	264.3	13.86	20.92	276	1.87	0.54	14.23	68.10	24.8
	±0.65 *s1	±23 *s1	±1.05 *s1	±0.03	±10.7	±40.6 *s1.2L	±1.06 *s1L	±1.06 *s1	±33	±0.38 *s1	±0.04 *s1.2	±1.84 *s1	±4.14 *s1.2	±0.85 *s1G
Site Mean	12.63	433	7.80	1.58	21.07	113.9	21.02	13.10	340	6.34	0.89	16.96	52.1	31.9
	±1.36	±54	±2.05 *G	±0.04 *G	±5.59	±18.2 *G	±0.76 *G	±0.63	±57	±1.13	±0.07 *L	±1.49	±2.08 *L	±0.87 *L
Mullus barbatus														
S1	0.64	692	58.13	15.08	10.94	55.45	54.64	2.77	294	20.88	1.77	19.46	9.77	292.4
	±0.12 *s3G	±217 *G	±3.75 *s3G	±2.12 *s3G	±3.24 *s3	±7.3 *s3	±12.0 *s3L	±0.86 *L	±51 *L	±5.18 *L	±0.33 *L	±5.07	±1.08 *L	±7.60 *L
S2	0.53	459	45.89	17.86	4.19	76.69	73.11	2.64	350	23.08	1.88	16.32	14.97	355.8
	±0.05 *s3G	±118	±8.27 *G	±3.65 *s3G	±1.51 *s3G	±10.4 *s3G	±10.9 *s3G	±0.19 *s3L	±27 *s3	±3.59 *L	±0.11 *L	±4.73 *L	±2.62 *s3L	±75.3 *s3L
S3	2.09	383	23.71	4.07	24.57	102.3	30.24	10.26	218	12.17	2.55	17.74	68.00	168.8
	±0.60 *s2.3G	±75 G	±12.4 *s1	±1.64 *s1.2	±3.08 *s1.2G	±4.72 *s1.2G	±3.40 *s1.2G	±1.25 *s1.2L	±12 *s2L	±7.16	±0.31	±0.07 *L	±1.91 *s1.2L	±1.9 *s2L
Site Mean	1.09	511	42.58	12.34	13.23	78.15	52.66	5.22	287	18.71	2.07	17.84	30.91	272.4
	±0.26 *G	±136 *G	±8.14 *G	±2.47 *G	±2.61	±7.49 *G	±8.79 *G	±0.77 *L	±30 *L	±5.31 *L	±0.25 *L	±3.29	±1.87 *L	±28.3 *L
Trachurus mediterraneus														
S1	2.93	335	14.80	1.30	46.51	79.55	51.92	21.90	393	7.64	1.04	31.49	34.84	214.4
	±1.48 *L	±68	±3.08	±0.72	±16.3	±18.2 *s3G	±20.3 *G	±0.39 *L	±21 *s3	±5.16	±0.27	±9.08	±10.5 *L	±60.4 *L
S2	2.09	249	14.37	0.60	36.09	59.07	44.05	22.32	368	7.57	1.16	27.36	35.68	199.6
	±0.58 *L	±8	±7.73	±0.17 *s3	±14.6	±11.0 *G	±13.5 *s3G	±0.43 *L	±49	±6.38	±0.33	±8.80	±1.55 *L	±28.7 *L
S3	3.57	227	9.64	1.73	34.37	48.43	66.00	24.41	246	4.79	1.31	28.48	39.17	203.3
	±1.25 *L	±18	±0.86 *G	±0.20 *s2	±5.42	±3.49 *s1G	±3.68 *s2G	±0.96 *L	±19 *s1	±0.95 *L	±0.24	±3.12	±0.89 *L	±7.81 L
Site Mean	2.86	270	12.94	1.21	38.99	62.35	53.99	22.8*	336	6.67	1.17	29.11	36.6	205
	±1.10 *L	±31	±3.89	±0.36	±12.1	±10.9 *G	±12.5 *L	±0.59	±30	±4.16	±0.28	±7.00	±4.31 *L	±32.3 *L
Sprattus sprattus														
S1	16.27	376	12.57	12.25	26.66	99.89	112.6	22.56	896	4.28	1.16	39.41	20.56	218.0
	±8.22	±8.63 *G	±3.28 *G	±1.97 *G	±11.2 *s3	±11.5 *s3G	±59.1 *s2G	±0.57	±343	±1.18 *L	±0.44 *L	±17.8 *L	±5.16 *s3L	±26.9 *L
S2	19.15	413	13.27	13.27	31.90	72.44	35.91	22.05	1044	6.73	2.01	49.49	24.87	228.9
	±4.15	±63 *G	±0.72 *G	±0.72+= *G	±11.6 *s3	±6.73 *s3G	±6.03 *s1G	±0.80	±41 *L	±1.0 *L8	±0.58 *L	±23.6 *s3L	±2.45 *L	±28.0 *L
S3	14.11	312	8.67	15.30	12.71	40.66	52.78	22.00	341	5.17	2.83	74.48	47.72	142.9
	±2.78 *G	±100	±3.61	±1.23 *G	±0.26 *s1.2G	±3.02 *s1.2	±6.20 *G	±0.8 *L2	±49	±0.90	±0.06 *L	±6.74 *s1L	±1.80 *s1.2	±41.9 *L
Site Mean	16.51	367	11.50	13.61	23.76	71.00	67.12	22.2	760	5.39	2.00	54.4	31.0	196
	±5.05	±57 *G	±2.54 *G	±1.3 *G1	±7.73	±7.09 *G	±23.8 *G	±0.73	±145	±1.05 *L	±0.36 *L	±16.1	±3.14 *L	±32.3 *L

*LPO was expressed as nmoles MDA/mg protein; GSH was expressed as ng/mg protein, all enzymes were expressed as U/mg protei

Nessebar Bay demonstrated significantly lower levels of GSH, higher activities of CAT, GPx and GST, along with significantly lower AChE activities in the gills, compared to those from Maslen Nos and Staro Oryahovo, thus indicating clear OS signs. No differences in the values of the studied OS biomarkers between sprat from Maslen Nos and Staro Oryahovo were found, indicating a relatively similar level of OS in the fish from these localities. There were no significant differences in the levels of the studied OS indicators in horse mackerel from the three studied localities.

In order to identify significant similarities (dissimilarities) between the values of the measured OS biomarkers MDS was applied (Fig. 1). The MDS clearly demonstrated the presence of differences in the OS level and the enzyme defense activation between the fish species studied (Fig. 1A). The red mullet differed significantly from the other studied species (separated at the right side of the two dimensional plane). The round goby was the other species with specific and different OS reaction (clearly separated from the other species in the two dimensional plane). Both the red mullet and the round goby have similar benthic live style and hence should have been subjected to similar environmental pressures resulting in OS. The level of OS in horse mackerel and sprat appeared more similar to each other than to the other two species (Fig. 1A).

The activity of the pro/antioxidative processes in the studied fish species showed some indicative similarities (and dissimilarities) (Fig. 1B). The different degree of activation of the fish antioxidant defense system, as a response to multiple environmental stressors, showed two lines of defense (including ancillary factors). The first one reflected the activation of the first line of defense by antioxidant enzymes, indicated by the activity of CAT and SOD (grouped together in the left part of the two dimensional plane). The second one was reflected by the grouping (although loose) of GSH, GPx and AChE at the right side of the factor plane, thus indicating the activation also of the detoxification defense system of the fish species as additional response to toxic pollutants.

As a general measure of fish condition and health, we used Fulton's condition factor (K) (Fig. 2). The results strongly indicated the presence of differences of the K
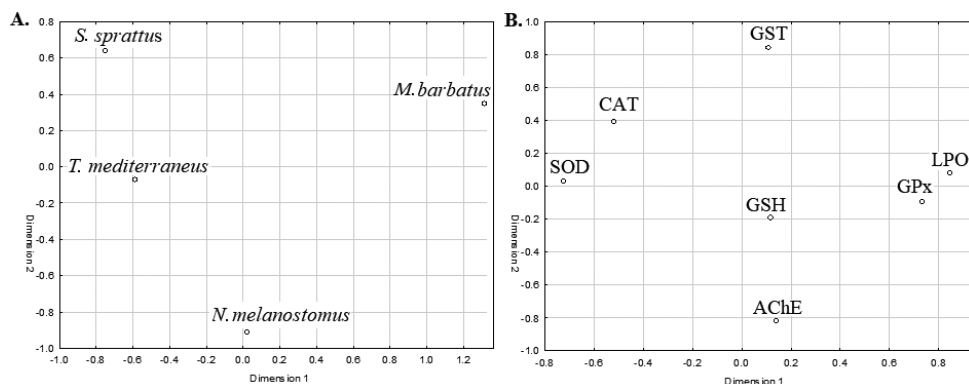
Figure 1. Multidimentional scaling of similarity (dissimilarity) between the studied fish species in their level of OS (**A**) and the induced activity of the pro/antioxidant processes (**B**).
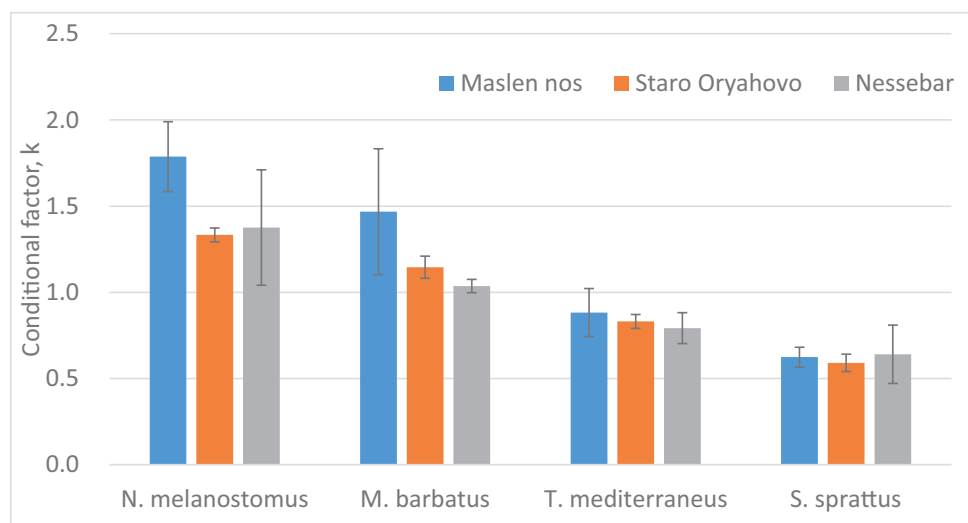
Figure 2. Fulton's condition factor (K) of studied fish species from the sampled localities of the Black Sea coastal zone (Mean±SD).

factor among fish species and also among the trawling localities. As a whole, the values of K for round goby and red mullet were significantly higher (also higher than 1) that those of the other species for all localities. The highest K factor values were present in round goby and red mullet from Maslen Nos. The K factor of sprat and horse mackerel was significantly lower (also less than 1) for all trawling localities studied.

Discussion

Changes in the balance of cell oxidative processes are a basic reaction of marine organisms to the impact of environmental pressure. The increasing contamination of the sea with metals, polycyclic aromatic carbohydrates and eutrophication leads to increase of LPO and alters the activity of antioxidant protection in the tissues of marine fish and other organisms (Regoli and Giuliani 2014).

In this study, we reported preliminary data on the OS level in the liver and gills of four commercially important fish species (sprat, horse mackerel, round goby and red mullet) from the Bulgarian Black Sea region. The first overall finding of our study was that in the gills and liver of fish different oxidative changes were present, most probably due to their unique composition and metabolic profile. Secondly, the studied fish species differed in the level of their OS and antioxidant systems activation, which depended on their life style and habitat (food preferences and habits, mobility and hence metabolism levels). The round goby and red mullet are classified as slow-swimming and carnivorous, while horse mackerel and sprat are fast-swimming and plankton feeders (Rudneva et al. 2010). The more active species compared to low-mobile forms

require higher oxygen consumption and hence, have a higher metabolic rate which determines higher ROS production and OS, and induction of different levels of antioxidant protection (Martinez-Alvarez et al. 2005; Filho 2007; Rudneva et al. 2010). In line with these findings were our results indicating significantly higher levels of LPO and GST in the gills of sprat and horse mackerel, i.e. the fish species with higher mobility. On the other hand, the benthic and suprabenthic, more sluggish, fish species (as round goby and red mullet), are assumed to live in more contaminated marine environments, because many pollutants accumulate in low water layers and bottom sediments. In support of this assumption, our data showed significantly higher OS levels in round goby and red mullet individuals from Nessebar bay (a bustling tourist center), compared to the individuals from Maslen Nos (protected area under the European ecological network NATURA). Furthermore, the extremely high GST values established, especially in the liver of the studied fish species from Nessebar Bay, indicated the presence of activated detoxification metabolism of xenobiotics, most probably entering the fish body from the sediment. This was confirmed by the performed MDS which showed activation of detoxification processes and decreased neurotransmission capacity (indicated by AChE activity). Our results corresponded with the observations of Rudneva et al. (2010), who also demonstrated higher activity of GST in the liver of slower swimming and sluggish fish species. It can be concluded that the studied by us round goby and red mullet appeared to be more vulnerable to OS induced by multiple marine environmental stressors than the horse mackerel and sprat. However, their antioxidant defense systems allowed them to tolerate and adapt to the changing environmental conditions of their habitats.

Our findings on the specific pro/antioxidative processes in the studied fish species were confirmed by values of the Fulton's condition factor (K). This biometric tool is used to indicate the general health and wellbeing of the fishes (Datta et al. 2013), as well as the quality of the marine environment. The value of K reflects the interactions between feeding conditions, parasitic infections and physiological nutritional/energy/ reserves and hence, OS levels cannot alone determine the K factor variations. In our study we found that the round goby and red mullet from all three localities were in good condition according to K (K>1), whereas the condition of the sprat and horse mackerel from all studied localities was not so good (K<1). These findings corresponded, as a whole, to the estimated OS levels in these fish species.

Conclusion

It can be concluded that round goby and red mullet are more vulnerable to OS induced by marine environmental factors than the horse mackerel and sprat. However, their antioxidant defense system allows them to tolerate and adapt to the environmental conditions of their habitats. Therefore, it could be assumed that demersal fish species are more convenient for monitoring the state of the marine environment and the risk for impairing the fitness of fish. Obviously, further studies are needed for more comprehensive assessment of OS in fish of economic importance in the Bulgarian part of the Black Sea.

Acknowledgements

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RESEARCH ARTICLE

BioRisk

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Cellular susceptibility and oxidative stress response to menadione of logarithmic, quiescent, and nonquiescent *Saccharomyces cerevisiae* cell populations

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Abstract

The aim of the present study was to compare cellular susceptibility and oxidative stress response of *S. cerevisiae* logarithmic (log), quiescent (Q), and non-quiescent (NQ) cell populations to menadione – a well-known inducer of oxidative stress. Three main approaches were used: microbiological – cell survival, molecular – constant field gel electrophoresis for detection of DNA double-strand breaks (DSB), and biochemical – measurement of reactive oxygen species (ROS) levels, oxidized proteins, lipid peroxidation, glutathione, superoxide dismutase (SOD) and catalase on *S. cerevisiae* haploid strain BY4741. The doses causing 20% (LD_{20}) and 50% (LD_{50}) lethality were calculated. The effect of menadione as a well-known oxidative stress inducer is compared in the log, Q, and NQ cells. Survival data reveal that Q cells are the most susceptible to menadione with LD_{50} corresponding to 9 μM menadione. On the other hand, dose-dependent DSB induction is found only in Q cells confirming the results shown above. No effect on DSBs levels is observed in log and NQ cells. Further, the oxidative stress response of the cell populations is clarified. Results show significantly higher levels of SOD and ROS in Q cells than in log cells after the treatment with 100 μM menadione. On the other side, higher induction of oxidized proteins, malondialdehyde, and glutathione is observed after menadione treatment of log cells. Our study provides evidence that *Saccharomyces cerevisiae* quiescent cells are the most

susceptible to the menadione action. It might be suggested that the DNA damaging and genotoxic action of menadione in *Saccharomyces cerevisiae* quiescent cells could be related to ROS production.

Introduction

Organisms have developed strategies to trigger a stress response when exposed to environmental challenges in order to restore cellular homeostasis (Tagkopoulos et al. 2008; Mitchell et al. 2009). The cellular stress response is thought to be universal and encompasses a range of cellular functions, including cell cycle control, repair of damaged proteins, stabilization and repair of DNA and chromatin, cell membrane repair, and more (Kültz 2005). In nature, cells may exist in a proliferative or non-proliferative state (Gangloff and Arcangioli 2017; Sun and Gresham 2021). The non-proliferative state includes quiescent or non-quiescent cells (Sun and Gresham 2021). As most of the cells in human tissues are non-dividing, quiescence is a major form of life (Gangloff and Arcangioli 2017).

Based on this understanding cellular quiescence is of great importance, especially since studies performed on quiescent cells are still scarce. Such studies in multicellular organisms are difficult because of the complexity of the signals that control them. One of the possible solutions is the application of quiescent yeast cells as it is believed that they function similarly to the mammalian and human cells and share similar mechanisms and the same set of genes involved in the quiescence (Gangloff and Arcangioli 2017; Daskalova et al. 2021a).

Saccharomyces cerevisiae is a widely used test system for studying oxidative stress and its related consequences. Results obtained on *S. cerevisiae* could be easily extrapolated at mammalian, including human level because of homology in genes and conservative functions of proteins (Foury 1997; Hartwell 2004; Wright et al. 2014). Thus, the application of quiescent cells may provide a suitable platform for studying the effect of various toxic compounds on mammalian and human cells.

The aim of the present study is to compare cellular susceptibility and oxidative stress response to menadione of *S. cerevisiae* logarithmic (log), quiescent (Q), and non-quiescent (NQ) cell populations.

Materials and methods

Saccharomyces cerevisiae strain BY4741

Saccharomyces cerevisiae BY4741 (*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) obtained from the EUROSCARF collection was used in the present work. The growth curve of *Saccharomyces cerevisiae* BY4741 on YEPD medium is provided as a Suppl. material 1: Fig. S1. Yeast cells were grown on a standard yeast extract-peptone-dextrose (YEPD) medium

at 30 °C, 204 rpm for 168 h. Yeast media were prepared as described by Sherman et al. (2001). The growth curve of the strain is provided as a Suppl. material 1: Fig. S1.

Samples were withdrawn at exponential (24 h) and late stationary phase (168 h). Quiescent (G_0) and non-quiescent cells were isolated from stationary phase yeast population (168 h) according to the protocol described by Allen et al. (2006). In brief, yeast biomass in stationary phase (OD540 = 200 (2×10^9 cells/ml) was layered on Percoll density gradient and after centrifugation at 400 g (60 min at 20 °C) two layers of cell fractions were formed – the denser one composed of Go (Q) cells (lower fraction) and a less dense fraction of NQ cells (upper fraction). Both fractions were separated and microscopically examined. Go cells were characteristically rounded with thickened cell walls, and no budding cells were observed – these morphological features are typical for the cells in Go state. For comparison, the stationary phase cell population of NQ cells (upper fraction) was heterogeneous – both budding, elliptical cells, and deformed, granular and non-budding cells were observed.

Cell survival

Cell suspensions with concentration 1×10^7 cells/ml were treated with various concentrations of menadione (2-methyl-l,4-naphthoquinone, synthetic form of vitamin K) in the range 1–200 μM for 60 min at 30 °C, 200 rpm. Cells were then centrifuged (825 g), the supernatant was removed and the pellet was resuspended in a liquid YEPD medium. Cells were plated on a solid YEPD medium and incubated at 30 °C for 3 days to evaluate the survival. Doses of lethality (LD_{20}, and LD_{50}) were calculated (Lidanski 1988) by the following formulae:

$$lgLD50 = lgA + (lgB-lgA)/((50-A)/(B-A))$$
$$lgLD20 = lgA + (lgB-lgA)/((20-A)/(B-A)),$$

where A – the closest smaller than 50 or 20%, respectively, lethality percentage; lgA- lg of the concentration corresponding to A; B – the closest higher than 50 or 20%, respectively, lethality percentage; lgB- lg of the concentration corresponding to B.

Cell-free extracts

Isolation of cell-free extracts from log, Q, and NQ cells was carried out according to the procedure described by Daskalova et al. (2021b) and were used for further biochemical analyses.

Constant field gel electrophoresis (CFGE)

CFGE for detection of DNA double-strand breaks (DSBs) was applied as described in Todorova et al. (2015, 2019). The levels of DSB induced presented as a mean fraction of DNA released (FDR) from the wells was quantified by measurement of ethidium bromide fluorescence using Gene Tool Analyser G: Box (Syngene) and calculated as described in Chankova et al. (2009).

Biochemical analysis

Oxidative stress markers assay

The redox state of logarithmic, quiescent, and non-quiescent yeast cells was assessed through measurement of intracellular levels of accumulated ROS (Kostova et al. 2008), levels of carbonylated proteins (Mesquita et al. 2014), and oxidized lipids (Hodges et al. 1999).

Glutathione measurement

The measurement of intracellular glutathione was carried out according to the procedure of Zhang (2000).

Enzymatic analysis

Superoxide dismutase (SOD) and catalase (CAT) enzyme activities were determined spectrophotometrically according to Beauchamp and Fridovich (1971) and Aebi (1984), respectively.

Protein content

Total intracellular protein was determined according to Lowry et al. (1951). As a standard, bovine serum albumin (Sigma St. Louis, MO, USA) was used.

Data analysis

The experiments were repeated at least three times from independently grown cultures. Data points in all the figures are mean values. Error bars represent standard errors of mean values. Where no error bars are evident, errors were equal to or less than the symbols. All the calculations were done with GraphPad Prism program, version 6.04 (San Diego, USA). The statistical analysis included the application of Student's *t-test* and One-way ANOVA followed by Bonferroni's *post hoc* test. $P < 0.05$ was accepted as the lowest level of statistical significance.

Results

Resistance to menadione measured as cell survival

Our first step was to determine the cell survival of the three cell populations after treatment with 100 µM menadione. Data revealed that the log cells are the most resistant to menadione action (Fig. 1A). Further experiments with log and Q cells were performed in order to determine the potential dose-response (Fig. 1B). A dose-dependent decrease in cell survival was obtained for both populations, better expressed in the Q cells.
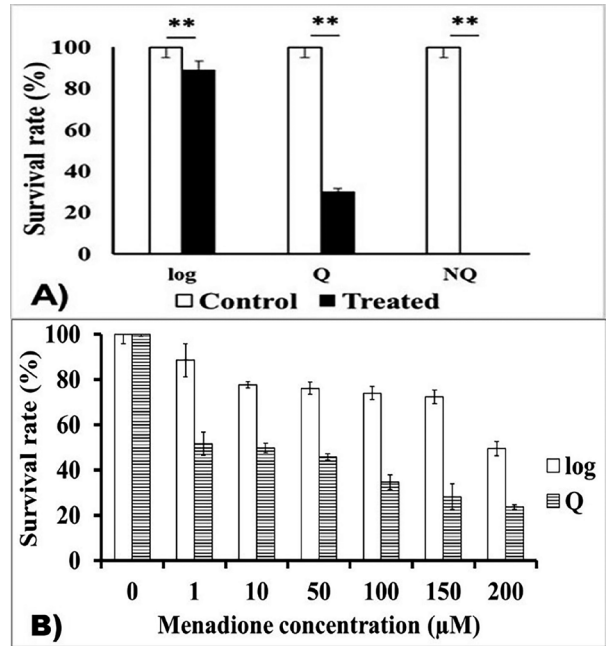
Figure 1. Cell survival after menadione treatment **A** effect of 100 μM menadione on log, Q, and NQ cell populations **B** effect of menadione in a concentrations' range of 1–200 μM on log and Q cells. Each value represents the mean ± SEM (Standard error of the mean) (n = 3).

Table 1. Levels of lethality calculated after menadione treatment.

Cell populations	LD_{20} (μM)	LD_{50} (μM)
Log	35	199
Quiescent	0.65	9

Two levels of lethality were calculated: LD_{20} and LD_{50} (Table 1).

Further, the levels of DSB induced were compared. Our results confirmed the ones obtained for cell survival. Dose-dependent DSB induction is measured only in quiescent cells (Fig. 2). The DSB levels measured after the treatment with 150 μM menadione were 1.5-fold higher than the spontaneous ones. No effect on DSBs levels is observed in log and NQ cells.

Further experiments were focused on studying the potential differences in the susceptibility based on various markers for oxidative stress – reactive oxygen species, oxidized proteins, malondialdehyde, intracellular glutathione, superoxide dismutase, and catalase.

Concentration of reactive oxygen species (ROS) after menadione treatment

The ROS measured in the three cell populations are presented in Fig. 3A. The levels measured in G_0 cells after menadione treatment are significantly higher – around 3-fold than those measured in the controls. There is a statistically significant but biologically insignificant effect on the ROS levels in log cells. This observation is in a good
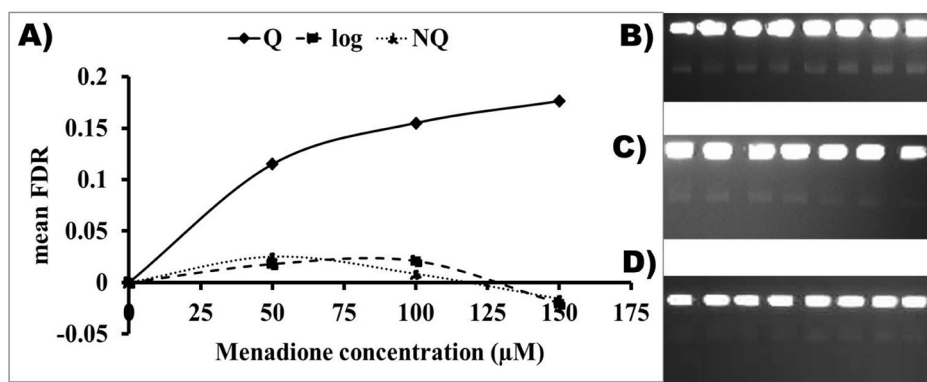
Figure 2. DSBs induced by various concentrations (50–150 μM) of menadione **A** induction of DSB presented as normalized FDR **B** Q cells **C** log cells **D** NQ cells.
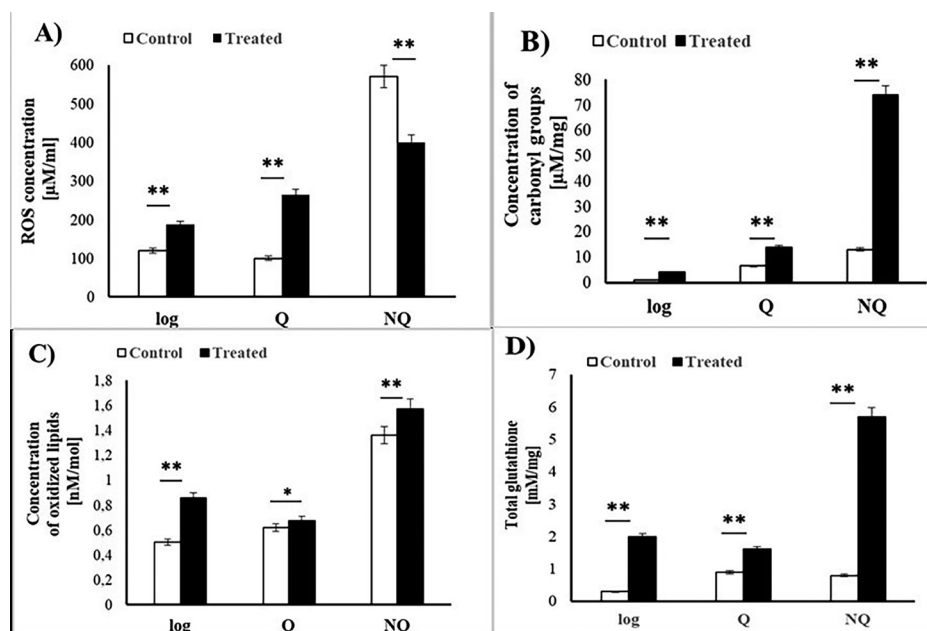
Figure 3. Comparative analysis of the levels of reactive oxygen species **A** oxidized proteins **B** malondialdehyde **C** and total glutathione **D** in *S. cerevisiae* logarithmic (log), quiescent (Q), and non-quiescent (NQ) cell populations after the treatment with menadione. Each value represents the mean ± SEM (Standard error of the mean) (n = 3). Significant differences (* $p < 0.05$; ** $p < 0.001$) are presented.

correlation with the cell survival and the DSBs induced in Q cells in comparison with those observed in log cells.

The constitutive levels of ROS, oxidized proteins, and MDA in NQ cells were significantly higher than those measured in log and Q cells. Treatment with 100 μM menadione resulted in significant induction of oxidized proteins and glutathione (Fig. 3B, D). Interestingly, the ROS levels measured in NQ cells were lower after the menadione treatment in comparison with the control levels (Fig. 3A).

Concentration of protein carbonyl groups

Data presented in fig. 3B provides information concerning the concentration of protein carbonyl groups. Comparing the constitutive levels, around 7-fold higher levels were measured in Q cells in comparison with the log ones. This could be explained as a result of the cells' starvation. Although, the highest quantity – 14 µM/mg was determined in Q cells the induction was only around 2-fold. Higher induction – around 6-fold was measured in the log cells.

Levels of malondialdehyde (MDA)

Concerning the MDA, comparatively equal constitutive levels were observed between Q and log cells (Fig. 3C). The NQ cells showed significantly higher MDA levels. As a result of the menadione treatment the most significant induction of MDA (around 2-fold) was measured in log cells ($p < 0.001$).

Concentration of glutathione

The GSH concentration in untreated Q cells was 3-fold higher than that in log cells. Interestingly, menadione treatment did not result in a significant induction of GSH compared to the untreated control. The GSH concentration was only 2-fold higher (Fig. 3D). At the same time, the treatment with 100 µM menadione resulted in 7-fold higher GSH levels in log cells.

Antioxidant enzyme (Superoxide dismutase and Catalase) activity after menadione treatment

Concerning the constitutive levels of the antioxidant enzymes superoxide dismutase and catalase, differences were obtained. The catalase levels were comparable in the three cell populations, while SOD was lower in Q cells than in the log and NQ cells (Fig. 4A, B).

Significant induction of SOD was observed in Q cells after the application of menadione (Fig. 4A). No effect was obtained concerning the catalase levels (Fig. 4B).
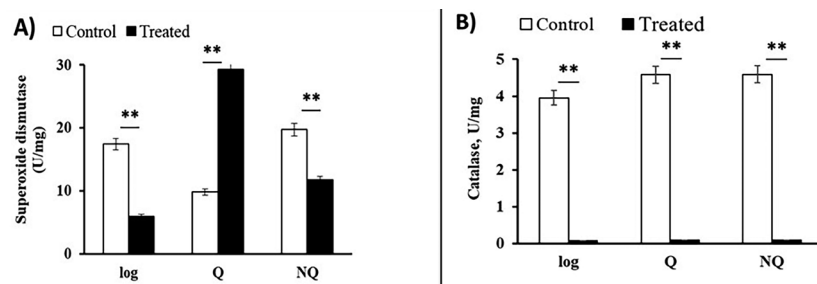
Figure 4. Comparative analysis of the response to menadione based on the enzymatic antioxidant system **A** superoxide dismutase and **B** catalase presented as units/mg. Each value represents the mean ± SEM (Standard error of the mean) (n=3). Significant differences (* $p < 0.05$; ** $p < 0.001$) are presented.

Discussion

Data presented here provide a comparative analysis of the cellular susceptibility and oxidative stress response to menadione of logarithmic, quiescent, and nonquiescent *Saccharomyces cerevisiae* cell populations. Differences in the cellular susceptibility are obtained depending on the endpoint used. Based on cell survival, DSBs induction, ROS, and SOD Q cells are more susceptible to menadione. On the other side, higher induction of oxidized proteins, MDA, and glutathione is observed following menadione treatment of log cells.

The measured increased ROS levels in Q cells correspond well with the decrease in cell survival and the well-expressed DSB induction. The cytotoxic mechanism of action of Menadione in G0 cells is stronger, probably due to lower metabolic activity and higher oxygen levels in the cells. This is in accordance with the report by Fabrizio and Longo (2008) that quiescent cells are characterized with lower energy consumption and ADP content, which may lead to increased intracellular oxygen levels and single-electron oxygen reducers. Such conditions may occur during the chronological aging of yeast cells. On the other hand, the decrease in ROS levels measured after the treatment with menadione of NQ cells could be explained by the higher percentage of cells in a terminal state and entering apoptosis (Davidson et al. 2011).

It is already reported that the toxicity of quinones including menadione in *S. cerevisiae* depends on the oxygen presence (Rodrigues-Pousada et al. 2004). This could be explained by their possible role as catalyzers in the ROS generation via redox-cycling activity. The cellular response to menadione has been shown to be associated with the induced synthesis of a large number of proteins, some of which are specific and are synthesized only upon exposure to this toxic agent (Flattery-O'Brien et al. 1993).

In the present work, log cells showed increased levels of oxidized proteins, MDA, and glutathione. This could be explained by their increased metabolic activity and a higher rate of protein synthesis (Daskalova et al. 2021b). Stress-induced toxic oxygen species, such as superoxide and hydroxyl radicals, damage biological membranes and other cellular macromolecules, leading to mutations, cancer, or cell death. A direct indicator of the onset of these processes is the appearance of carbonyl groups in proteins, as well as lipid peroxidation. In addition, the formation of ROS is inevitable under aerobic conditions due to the reactive nature of molecular oxygen. The action of these factors individually or jointly can lead to the appearance of oxidative stress – acute or chronic (Petrova and Kujumdzieva 2010). Oxidative processes that take place during oxidative stress may lead to reversible or irreversible functional changes in proteins, which are the main reason for cellular dysfunction. Protein changes are associated with the formation of carbonyl groups in them. Biochemical analyses have shown that carbonyl groups introduced into the side chains of specific amino acids in the active site of enzymes trigger the initial steps in the degradation of these proteins (von Herrath and Holzer 1985; Levine and Munro 2002; Grimsrud et al. 2008; Apoorva et al. 2020).

Lipid oxidation occurs through the interaction of ROS with fatty acids in the membrane lipid layer. This changes the functionality and permeability of biological membranes and also leads to other disorders. Cell death can be caused by the release

of cell contents as a result of these changes. Malonaldehyde is the end product of lipid oxidation. It accumulates in cells and is a highly reactive and toxic electrophilic compound that can form covalently bound products with different proteins. Its concentration in the cell is used as a biomarker to account for the influence of stress agents. In our work, the MDA levels remained similar in control and treated Q cells. One of the explanations could be the thicker cell wall (Daskalova et al. 2021b).

Glutathione plays an important role in protecting the cell against oxidative stress by protecting it from the toxic effects of ROS through its involvement in mechanisms for detoxification and regeneration of important cellular antioxidants (Valko et al. 2006). The antioxidant function of this tripeptide is directly related to the reduction state of the oxidized GSSG / reduced GSH glutathione pair. More than a few dozen genes have been identified whose transcription is affected by redox balance in the cell (Allen and Tresini 2000). It has been found that the GSH: GSSG ratio is of major importance for this regulation. The glutathione system serves as a cellular redox buffer and changes in GSH: GSSG balance can lead to oxidation of redox-sensitive cysteine residues in various proteins (Rahman 2005). Therefore, the increase in intracellular glutathione content may be one of the adaptive mechanisms to stress in the yeast *S. cerevisiae*. Glutathione is a compound with antioxidant and antielectrophilic activity, which suggests its role in the resistance of cells in a medium with menadione. The accumulation of oxidized glutathione in the cell is an important parameter for measuring the level of oxidative stress.

All enzymes in glutathione metabolism work in an integrated way, allowing the cell to adapt to different stress conditions (Hayes and Pulford 1995), with de novo glutathione synthesis being the most important mechanism for increasing levels of reduced GSH in response to oxidative stress (Rahman 2005). However, the oxidized/reduced glutathione pair (GSSG/GSH) ratio before and after treatment with menadione remained relatively constant in G0 cells. Controlled changes in GSSG / GSH contribute to the maintenance of cellular redox potential, which determines resistance to toxic effects. The stable GSSG / GSH ratio also indicates that in cells of *S. cerevisiae* BY4741 strain, menadione exhibits its toxicity through its redox-cyclic mechanism of action associated with the generation of reactive oxygen species rather than by interaction with reduced glutathione in the cell. In the second case, this would lead to the formation of menadione – S - glutathione conjugates, accompanied by a sharp decrease in the concentration of intracellular glutathione.

Our study provides evidence that *Saccharomyces cerevisiae* quiescent cells are the most susceptible to the menadione action. It might be suggested that the DNA damaging and genotoxic action of menadione in *Saccharomyces cerevisiae* quiescent cells could be related to ROS production.

Acknowledgements

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Supplementary material 1

Figure S1

Authors: Polya Galinova Marinovska, Teodora Ivanova Todorova, Krassimir Plamenov Boyadzhiev, Emiliya Ivanova Pisareva, Anna Atanasova Tomova, Petya Nikolaeva Parvanova, Maria Dimitrova, Stephka Georgieva Chankova, Ventsislava Yankova Petrova

Data type: jpg file

Explanation note: Fig. S1. Growth curve of Saccharomyces cerevisiae BY4741 and glucose assimilation in batch cultivation on YEPD media at 30 °C, 204 rpm for 168 h.

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RESEARCH ARTICLE

BioRisk

In vitro reconstitution of complexes of stress HliA protein with pigments

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Abstract

Proteins similar to Hli (high light inducible) proteins of cyanobacteria are present in all photosynthetic eukaryotes and are necessary for survival in various stressful conditions, although their exact function is not fully understood. In current study, the recombinant stress-induced protein HliA of cyanobacterium *Synechocystis* was isolated and characterised for the first time. The synthetic gene of HliA protein was created and cloned into plasmid for expression of recombinant protein with Hisx6-tag at the C-terminus in bacteria. Recombinant HliA protein of *Synechocystis* was isolated by metal-affinity chromatography. The HliA protein was reconstituted with chlorophyll a and carotenoids. Using circular dichroism spectroscopy, it was shown that chlorophyll a and carotenoids interact in vitro with the HliA protein. The binding of pigments to the HliA protein favours the protective function of this protein. Apparently, Hli proteins are involved in the coordinated delivery of pigments for the biogenesis of photosynthetic complexes, thereby reducing the risk of accumulation of phototoxic free chlorophyll molecules. Current results are important for understanding the processes of photoprotection in either cyanobacteria or algae and higher plants.

Keywords

Carotenoid, chlorophyll, cyanobacteria, high light inducible proteins, high light stress, *Synechocystis*

Introduction

Cyanobacteria are ubiquitous and inhabit almost all ecosystems, thanks to their effective adaptation to various conditions, including extreme ones. Therefore, these phototrophs are a promising model system for studying mechanisms of resistance and adaptation to various stresses, including one of the most common – light stress.

Under light stress, the photosynthetic apparatus is damaged due to the accumulation of both toxic free chlorophyll molecules and the formation of reactive oxygen species. For the normal functioning of photosynthetic organisms under conditions of light stress, numerous protective mechanisms have emerged in the course of evolution. Protective mechanisms involving light-induced stress proteins are of great importance. These include cyanobacterial Hlips (high light-inducing proteins) with a single transmembrane helix (Chidgey et al. 2014; Hey and Grimm 2020).

Hli proteins are necessary for cell survival under light stress and other stressful conditions, as well as for maintaining normal cell activity. It is assumed that Hli proteins take part in important processes, such as: regulation of chlorophyll biosynthesis; transport and binding of free chlorophyll molecules; quenching of singlet oxygen; assembly and repair of photosystem 2; non-photochemical dissipation of absorbed light energy (Komenda and Sobotka 2016). However, the full picture of the functioning of Hli proteins has not been fully studied.

Four Hli proteins (HliA, B, C and D) were found in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (Staleva et al. 2015). These proteins have been isolated as part of small complexes with other proteins and only recently, the first individual HliC protein from this family has been purified (Shukla et al. 2018). There is no such information about other representatives of Hli proteins. Of particular interest is the protein of this family – HliA, since it is important for the survival of *Synechosystis* cells under light stress (He et al. 2001). To protect cells from excessive light, binding of toxic free chlorophyll molecules, which cause photodestruction and oxidative stress, is necessary. The stress protein HliA can serve as such a key agent binding these molecules. Therefore, to understand this process, it is necessary to obtain a recombinant HliA protein and its subsequent complex characterisation and study of its interaction with chlorophyll *in vitro*. Isolation and characterisation of pure HliA protein has not yet been carried out. Until recently, it was not clear whether the pigments (chlorophyll a and beta-carotene) bind to the HliA protein.

The aim of the study was to isolate pure recombinant HliA protein and reconstruct the HliA protein complex with pigments (chlorophyll a and carotenoids).

Materials and methods

HliA gene expression and protein purification

Nucleotide sequence of the *hliA* gene of *Synechocystis* sp. PCC6803 (hereinafter *Synechocystis* is used) was obtained from CyanoBase (http://genome.annotation.jp/

cyanobase/Synechocystis/genes/ssl2542 ID ssl2542). This sequence was optimised for expression in bacteria and the synthetic gene was created. The gene was cloned into the pET28 vector in open reading frame with 6xHis at the C-terminus of resulting protein. *E.coli* BL(DE3) cells were transformed with obtained plasmid. HliA protein synthesis with His6 at the C-terminus (.....HGVIGWLNSL) 6xHis tag) was achieved by induction with IPTG. Recombinant protein was isolated from *E.coli* cells using metal-affinity chromatography on Ni-NTA Resin (Thermo-Fisher, USA) according to the manufacturer's recommendation. Protein was stored at 4 °C in the presence of 1 mM sodium azide. Protein concentration was determined spectrophotometrically at 280 nm, using extinction coefficients from ProtParam programmes in ExPASy for calculation (https://web.expasy.org/protparam/).

The reconstitution of complexes of HliA-6xHis protein and pigments

After metal-affinity chromatography, fractions containing HliA protein (0.5–1 mg/ml) were dialysed against the buffer: 15mM NaH_2PO_4 x $2H_2O$, 0.07M KF, 15mM urea, 0.1mM PMSF, pH 4.5 overnight at 4 °C and the protein concentration was measured spectrophotometrically using absorbance at 280 nm. Pigments (total chlorophyll a and carotenoids) were previously isolated from *Synechocystis* cells according to the method by Natali et al. (2014). The pigments were re-suspended in a buffer 15mM NaH_2PO_4 x $2H_2O$, 0.07M KF, 0.015M urea, 0.1mM PMSF, pH 4.5 and were sonicated on ice three times for 20 seconds with an interval of 10 seconds. Before the reconstitution, the HliA solution was subjected to short-term heating (95 °C) for 1 minute. Then the sample was transferred to ice and pigment extract was added in an equimolar ratio (1:1). After incubation on ice for 30 minutes, the sample was used for analysis.

Western blotting

Using SDS electrophoresis and subsequent Western blotting, the content (level) of recombinant HliA protein in *E.coli* cell lysate was evaluated (Akulinkina et al. 2015). Aliquots of HliA protein were separated in 12.5% SDS-polyacrylamide gel. The gel was stained by Coomassie R-250 or was used for immunodetection. The proteins were transferred from gel on to a nitrocellulose membrane (pore size of 45 mkm). Then the membrane with the transferred proteins was incubated for 1h at 4 °C in TBST buffer (50mM Tris-HCl, 200 mM NaCl, 0.1% Tween 20, pH 7.5), supplemented with 5% dry milk and then primary antibodies were added. We used polyclonal rabbit antibodies to HliA (1:4000) (Abcam, USA) or antibody in TBST buffer to 6X His-tag (1:5000) (ab213204, USA). The membrane was incubated with the antibodies overnight at 4 °C. The blots were rinsed and incubated with a secondary antibody – goat anti-rabbit IgG conjugated to horseradish peroxidase (1:2000) (Agrisera, Sweden). Immune complexes on a membrane were detected with the ECL fluorescent detection system (GE Healthcare England) and the signals were registered on X-ray film (Retina, Germany).

Fluorescence spectroscopy

Fluorescence measurements were made on the Spectrofluorophotometer RF-5301 PC (Shimadzu, Japan) at 20 °C. Tryptophan residues make a decisive contribution to protein fluorescence. HliA protein contains two tryptophan residues. Fluorescence spectra of HliA protein (0.5 mg/ml) along with reconstituted complexes of protein with pigments in buffer (15mM NaH_2PO_4 x $2H_2O$, 0.07M KF, 15mM urea, 0.1mM PMSF, pH 4.5) were excited at 295 nm (2 nm slit bandwidth) and were recorded in the range of 610–750 nm (5 nm slit bandwidth) at a speed of 200 nm/min.

Circular dichroism spectroscopy

The CD spectra of HliA protein (0.5 mg/ml) and reconstituted complexes of protein with pigments in buffer: 15mM NaH_2PO_4 x $2H_2O$, 0.07M KF, 15mM urea, 0.1mM PMSF, pH 4.5, were performed on a Chirascan CD dichrograph (Applied Photophysics, UK) equipped with a thermal insert at 13 °C. The CD spectra were recorded in the range of 190–260 nm and 400–740 nm at a speed of 20 nm/min in a 0.02 cm quartz cuvette. Then the signal of buffer was subtracted from the spectra, prescribing its spectrum under identical conditions.

Results

Isolation and purification of HliA-6xHis protein

Purified recombinant HliA protein of *Synechocystis* was isolated by metal-affinity chromatography for the first time (Fig. 1A). The isolated protein had the expected molecular weight 10 kDa (Fig. 1A). The protein was present mainly in monomeric form. Sometimes a small number of HliA protein dimers have been detected. The isolation of HliA protein was confirmed by Western blotting using antibodies to HliA (Fig. 1B).

Obtaining purified HliA protein is of interest due to the possibility of studying its spectral properties, as well as studying its functions and interaction *in vitro* with pigments. This would serve as a direct proof of the binding of the pigment by protein. Most analyses, with the exception of structural analysis using X-ray crystallography or NMR, do not require the removal of the His-tag. Its presence does not have a significant impact on the result of the analysis. The method of circular dichroism spectroscopy was used to characterise the secondary structure of the HliA protein. The data of CD spectroscopy of HliA protein were processed using the DichroWeb programme (http://dichroweb.cryst.bbk.ac.uk/). It should be noted that there are relatively few spectra of membrane proteins in the CD database, which is due to their low solubility.

The CD spectrum in the far UV of the HliA protein preparation shows the presence of a secondary structure (Fig. 2) and corresponded to the alpha-helical structure of the protein. According to calculations, the alpha helix segments make up 61%. The crystallographic structure of the HliA protein and other Hli proteins has not yet been studied.
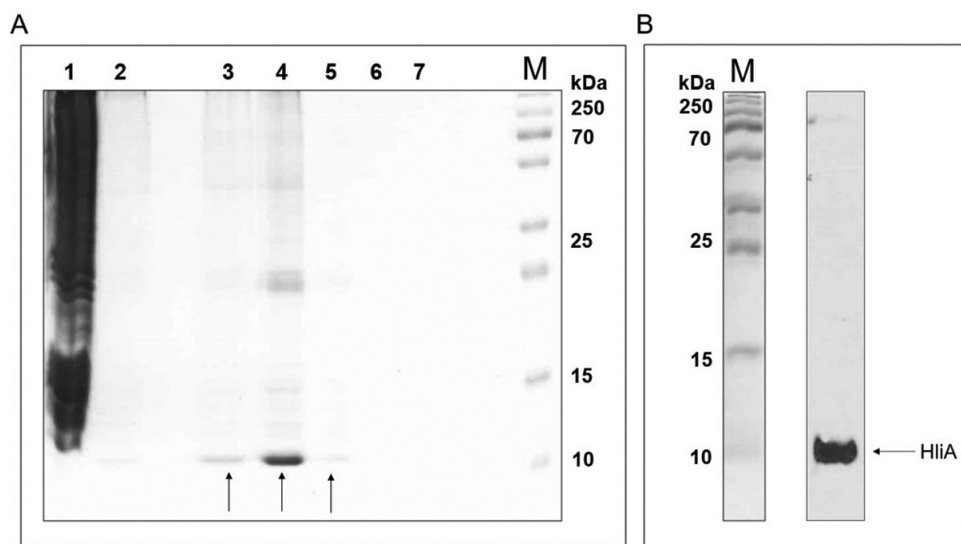
Figure 1. SDS-PAGE (**A**) and immunoblot analysis of the HliA eluate **A** fraction collected during His-tag affinity chromatography (separation) of HliA eluate were further separated by SDS-electrophoresis and were stained with Coomassie brilliant blue. The numbers at the top of the gel indicate elution fraction numbers from the column. The arrows show the location of the HliA protein in the gel **B** the gel was blotted and the HliA protein immunodetected.
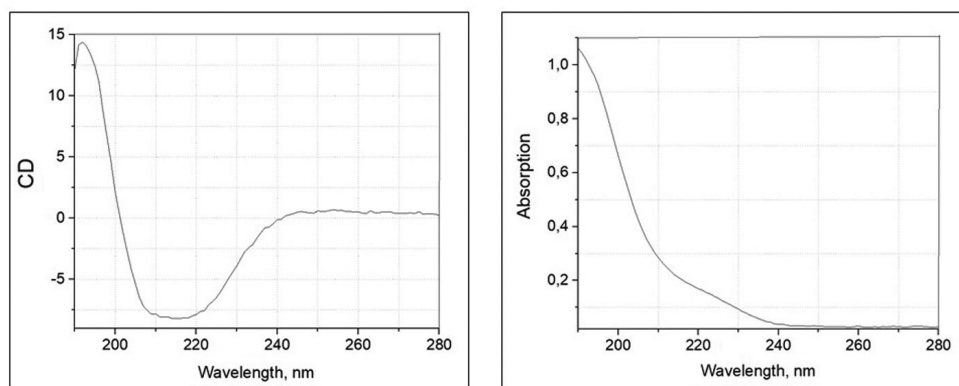
Figure 2. Circular dichroism **A** and absorption **B** spectra of the HliA protein.

Reconstitution of complexes of stress HliA protein with pigments

Pigments (chlorophyll a and carotenoids), isolated from *Synechocystis*, were used in HliA protein-binding experiments. It was planned to find out whether the HliA protein monomer has the ability to bind pigments.

Fluorescence spectra of HliA protein and HliA protein with pigments were studied (Fig. 3). As follows from the Figure, more intense fluorescence was shown for the sample of HliA protein with pigments then pure HliA protein. This may be due to the binding of the pigment to the formation of the complex.
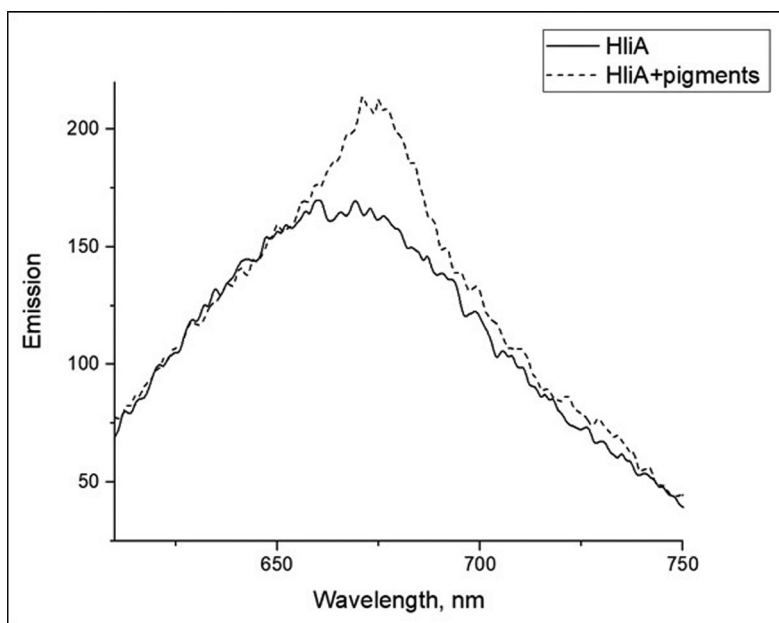
Figure 3. Fluorescence emission of reconstituted pigment-HliA protein (---) and HliA protein (—) samples.

Then the CD spectra of pigments (chlorophyll and carotenoids), HliA protein and their mixture were studied. A change in the CD spectra in the far red (640–740 nm) region of the HliA protein, incubated with pigments in comparison with the control pigments sample, was detected. This result indicates the interaction of chlorophyll with HliA protein. Small signal changes in the CD spectra in the 480–507 nm region were also detected, indicating the interaction of HliA protein with carotenoids (https://conf.icgbio.ru/plantgen2021/en/2021/06/06/detection-of-the-binding-the-stress-hlia-protein-synechocystis-sp-with-pigments/). Apparently, no more than 1–2 molecules of chlorophyll and carotenoids bind to the protein. Analysis of CD spectra of the HliA protein with pigments showed that the sample contained the monomeric chlorophyll. Thus, it has been shown experimentally that HliA protein molecules have the ability to bind pigments.

Discussion

In previously published papers, the HliA protein was studied in complex with other Hli proteins. In this work, the pure recombinant protein HliA *Synechocystis* was isolated for the first time. The pigment-binding capacity of the HliA protein was studied and the reconstruction of the HliA protein with pigments was carried out. CD spectroscopy has shown that chlorophyll a and carotenoids interact *in vitro* with the HliA protein.

The data obtained by us are consistent with the fact that Hli proteins contain conserved amino acid residues that are important for the binding of chlorophyll in

all proteins of light-harvesting complexes (Shukla et al. 2018). The HliA protein also contains a chlorophyll binding domain.

It is known that free chlorophyll molecules in a cell are dangerous for cells due to the threat of the formation of damaging reactive oxygen species. In this regard, the binding of pigments to the HliA protein revealed by us indicates the protective role of this protein. Apparently, Hli proteins can participate in the coordinated delivery of pigments in the biogenesis of photosynthetic complexes, reducing the risk of accumulation of phototoxic unrelated chlorophyll molecules (Shukla et al. 2018).

We found a smaller number of bound chlorophyll molecules (1–2) per protein than was hypothetically assumed (4–5 molecules). Perhaps this is due to the fact that a complex with other protein molecules is needed to bind more chlorophyll molecules. Apparently, the association of HliA protein with lipid components of the membrane is necessary for the binding of a large quantity of pigment molecules. It is possible that the binding of pigments depends on the formation of HliA/HliB heterodimers or HliA protein homodimers. It is assumed that Hli proteins are important for the re-utilisation of free chlorophyll molecules (Shukla et al. 2018).

The study of the constitution of the recombinant HliA protein with chlorophyll a and carotenoids *in vitro* allows us to expand knowledge about the photoprotective functions of light-induced Hli proteins. The results are important for understanding photoprotection processes in both cyanobacteria and algae and higher plants. Knowledge about the mechanism of functioning of stress proteins can also be used in the creation of artificial photosynthesis systems.

Conclusion

Pure recombinant protein HliA *Synechocystis* was isolated by Ni-affinity chromatography. The binding of pigments to HliA was shown, which confirms the protective function of this protein. Apparently, HliA proteins are involved in the coordinated delivery of pigments in the biogenesis of photosynthetic complexes, reducing the risk of accumulation of phototoxic free chlorophyll molecules. The results are important for understanding photoprotection processes in both cyanobacteria and algae and higher plants.

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REVIEW ARTICLE

BioRisk

Natural zeolites as detoxifiers and modifiers of the biological effects of lead and cadmium in small rodents: A review

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Abstract

The present investigation analyzes the literature about the toxicity of Cd and Pb in small rodents' organisms and the role of natural zeolites as modifiers of the biological effects. An array of ecotoxicological, morpho-physiological, hematological, genetic and biochemical methods as most representative are under discussion as a basic point for further exploration of biological effects in laboratory mice. The review of existing results demonstrated that there is abundant data on the sorption of lead and cadmium by modified natural zeolites in water and soils. Nevertheless, there is insufficient data on the ion exchange capacity and biological effects of this sorbent in living organisms, especially regarding Cd detoxification. On the basis of the current review, it is possible to conclude that future investigations in this field will elucidate the potential of the use of zeolites as successful detoxifiers against heavy metals and other toxic elements in living organisms.

Keywords

Cadmium, lead, natural zeolites, small mammals

Introduction

Pollution of the environment by different xenobiotics is a problem that still raises major concerns about the resilience of ecosystems. Despite serious measures having been taken, there is still the problem of removing toxicants already accumulated in the soil

and water. Their slow elimination and high rate of accumulation at higher levels of the food chains is expected to create problems in the next decades. Discovering suitable sorbents with detoxifying properties that reduce the content of heavy metals in nature will help to solve a number of problems related to the environment and the risk to public health. In recent years, natural zeolites are increasingly used in this field. There is a growing discussion of a direct positive effect of these exceptional crystalline minerals on improving the state of ecosystems and the quality of life.

Sorption properties of natural and modified zeolites and their application

Natural zeolites form one of the most unique groups among minerals, some of which, for example clinoptilolite, mordenite, and chabazite, have good potential due to their high sorption capacity and the presence of deposits with huge reserves in many countries. Bulgaria is one of the first countries in the world for the presence of quality clinoptilolites (Aleksiev and Djourova 1976; Aleksiev et al. 1997). They have a specific microporous crystal structure, permeated by channels and voids, which are formed by different numbers of SiO_4^{4-} and AlO_4^{5-}- tetrahedral rings. Skeletal charge-compensating cations (Na^+, K^+, Ca^{2+}, Mg^{2+} and Ba^{2+}) and coordinating water molecules are located in the channels and gaps. The structural configuration of the mineral predetermines its unique properties related to ion exchange and selective sorption (Mumpton 1999). It is possible to modify zeolite surfaces by adding specific surfactants, and thus to improve the ion exchange capacity to remove cations, anions, and different organic compounds (Apreutesei et al. 2008). This procedure is very important for enlarging the sorption capacity of these minerals.

The establishment of significant reserves of clinoptilolite in Bulgaria (Eastern Rhodopes) and their growing importance as an effective sorbent of heavy metals, determines the interest in their use as suitable detoxifiers of living organisms. Zeolitized tuffs are found in large areas in the Eastern Rhodopes and contain the largest deposits of zeolites in Bulgaria, formed during the Oligocene (Aleksiev and Djourova 1976; Jurova and Alexiev 1984; Jurova and Alexiev 1989; Djourova and Aleksiev 1990, 1995; Aleksiev et al. 1997; Raynov et al. 1997; Yanev and Ivanova 2010) (Fig. 1).

The study on zeolites' properties began in the 18th century, but they remained a curiosity for scientists and collectors until the middle of the past century, when their unique physicochemical features attracted the attention of many researchers. The absorption capacity of natural zeolites was first developed by Ames (1967) and Mercer et al. (1970), who demonstrated the effectiveness of clinoptilolite for extracting from municipal and agricultural waste streams and when materials with enhanced sorption capacity were necessary for nuclear waste management. In the past 60 years there has been an extraordinary development in zeolite science. Many investigations have been published on these promising silicate minerals, but none has been devoted specifically to natural zeolites, even though this theme may be of interest not only to earth
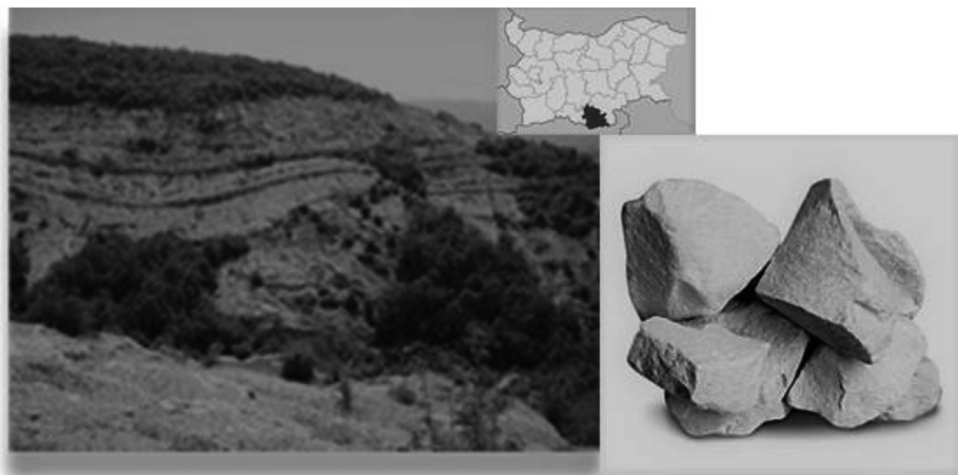
Figure 1. The largest deposits of zeolites in Bulgaria (Eastern Rhodopes).

scientists, but also to chemists and biologists, as the information obtained from natural samples complete and integrate the characterization of many zeolites.

Other areas of applications of natural zeolites are the purification of waters and the industrial and urban wastewaters (Boulinguez 2005). Natural zeolites were mainly investigated and applied as sorbents for ammonium ions from urban pollution, as well as for heavy metals (Pitcher et al. 2004) and dyes from industrial wastewaters. The removal of nutrient species (e.g. NH_4^+, $H_2PO_4^{2-}$/HPO_4), quantitative precipitation and recovery in slow-release fertilizer was also reported in the literature (Filippidis et al. 2008).

Nowadays natural zeolites have wide applications to limit the consequences of pollution and for purifying Cs- and Sr-isotopes from the nuclear industry and eventual accidents (Borai et al. 2009). Very effective zeolite pills have been prepared for humans to counteract Chernobyl fallout in Bulgaria (Filizova 1993), as well as for the workers in the nuclear power plant. The mechanism of action of zeolite is that the ion exchanges of ^{137}Cs and ^{90}Sr are carried out in the gastrointestinal tract and is eliminated by the excrements, thus minimizing assimilation into the blood system. The removal of heavy metals (e.g. Fe, Pb, Cd, Zn) from acidic mine drainage is another field of potential environmental applications of natural zeolitic materials (Xu et al. 2010).

Moreover, natural zeolites are used in agriculture to increase soil fertility through their useful adsorption and sorption, and cation-substituting catalytic properties. The most effective of the natural zeolites, is clinoptilolite. A lot of tests have been performed on rocks containing clinoptilolite on different types of soils, in different climatic conditions and geographical regions, in clear form or modified with mineral and organic fertilizers. The action of rocks containing clinoptilolite is diverse and affects both the characteristics of the soil and the growth, development and productivity of plants, as well as the quality of production. The use of clinoptilolite as a principal ingredient of

artificial soil was developed in Bulgaria in the late 1970s. A nutrient-treated substrate used for growing crops and the rooting of saplings in greenhouses produced greater development of root systems and larger yields of vegetables and fruits (Petrov et al. 1982).

Detoxification properties of natural zeolites and biological response of the organism

The use of natural clinoptilolites for animal nutrition began in Japan in the middle of the past century. Tests were initially made in Japan offering zeolite as a food additive to swine and poultry, and later to other animals. The experiments were successful. Researchers obtained good or excellent results with significant increases in productivity (Torii 1974, 1978; Mumpton 1978). Using clinoptilolite as a food additive could increase not only the animal production. Pigs fed with clinoptilolite gain significant weight and are more resistant to disease than pigs fed on a normal diet. Crucially they show regular digestion, increase in appetite, and the meat content increases due to the absence of needless fat (Veldman and Vanderaar 1997).

More of the biological applications of zeolites include ammonium removal from wastewaters and animal manure (Bernal and Lopez-Real 1993) air filtration, deodorants (Miner 1980) and soil fertilization (Mumpton 1999).

Clinoptilolite has incredible healing properties and has the power as mentioned above to remove from the body all kinds of toxins and poisonous substances, including radioactive particles, locking them in itself, so they can no longer be released.

Zeolite addition to cattle's fodder improves the caloric intake, digestion, appetite and the animal's body mass. Using clinoptilolite as a food additive to the diet of pigs and poultry also improves their body weight and leads to gains in the feed conversion ratios. Clinoptilolite binds some mycotoxins, absorbing toxins dangerous to animals' health. It also is useful for controlling toxins in animal food. This way the mineral decreases the mortality from digestive stress and alleviates the antibiotic needs. It is also able to absorb toxins produced by molds and microscopic parasites and thus improve the absorption of food by animals (Cooney et al. 1999).

One of the important questions is whether zeolites are able to adsorb vitamins and microelements which can potentially disturb physiological balance in organisms. By the way, Tomasevic-Canovic et al. (2000) found that amino acids and fat-soluble vitamins such as A, D and E do not get absorbed by clinoptilolite. Nevertheless, natural zeolites have some low affinity for binding vitamin B6 in vitro. This process closely depends on the crystalline and mineralogical structure of the zeolites. Investigations have demonstrated that, compared to all minerals with similar crystalline structure and functions, clinoptololite associates least with them.

Martin-Kleiner et al. (2001) report the effects of the natural clinoptilolite on hematopoiesis, serum electrolytes and other essential biochemical indicators of kidney and liver function in mice. The authors found a small increase in potassium levels in mice reared on a zeolite-rich diet but without any other changes in serum chemistry. Erythrocyte, hemoglobin and thrombocyte levels in peripheral blood were not

affected. There are studies that demonstrated that a natural clinoptilolite maintained the good condition of the mouse organism and prevented negative effects from the iron overload. When zeolites are given with iron in the form of intraperitoneal injections, mice showed near normal histology in comparison to mice injected with iron only. Such a pilot study examined potential possibilities to current treatments, and the results show positives in using zeolites in iron excess conditions (Fan et al. 2018).

Zeolite use in ecotoxicology and medicine is relatively recent, but its application in these areas has reached significant importance in the last few decades. Antioxidant and immunostimulatory properties of natural zeolites have been reported in different tumor types, and prove that zeolite acts as a serious immune system modulator (Pavelic et al. 2001, 2002). Montinaro at al. (2013) investigates the antioxidant properties of natural zeolites against oxidative stress, which is one of the main causes leading to the aging and degeneration process. The authors suggest zeolites as a novel potential adjuvant in counteracting oxidative stress and plaque accumulation in the context of neurodegenerative diseases.

Zeolite application as a food supplement in animal feeding is based on effects as a drug or detoxifier. These effects have been demonstrated in preliminary longtime investigations and critical assessments of its toxicological effects. Many studies demonstrate that zeolite, especially clinoptilolite addition in the feeding process, even for several months, showed no evidence of negative reactions or any pathological changes of animals' biochemical and hematological profiles. Topashka-Antcheva et al. (2012) tested the modified natural clinoptilolite KLS-10-MA to establish the LD_{50} of the used sorbent. The results show that all test group animals survived successfully up to the end of the experiment, with significant increases in body weight compared to the control group, and demonstrated a good activity and physiological condition. No symptoms of increased toxicity were recorded during the experiment. Any pharmacological effects were not established. Also, unusual behavior was not observed. The results on the toxicity of natural modified and nonmodified zeolites, and especially clinoptilolites, on the basis of their ion exchange capacity, have allowed research groups to investigate the detoxification possibilities of the mineral on animals and humans. Studies have been conducted in contaminated areas to eliminate some of the accumulated Pb in the body of hyperactive children. Clinoptilolite nanoparticles in the form of injection were applied. The idea was to minimize the effect of the lead on their central nervous system. It is important to note that these treatments have not been clinically approved yet (Delavarian et al. 2013). It was established that consumption of alcohol in parallel with clinoptilolite decreased the ethanol level in the blood in drinkers (Federico et al. 2015). Microencapsulated urease-zeolite sorbent was also investigated. The aim was to remove the urea from the organism during uremia (Cattaneo and Chang 1991). The established kinetic curves clearly showed the selective adsorption of zeolite for lead ions, as well as a significant removal of cadmium not only from wastewaters and soil but from the animal organism (Malion et al. 1992; Beltcheva et al. 2012).

On the basis of everything mentioned above, Beltcheva et al. (2012) and Topashka-Antcheva et al. (2012) provided a series of long term ecotoxicological experiments to test the levels of clinoptololite sorption capacity against Pb cations in the animal

organism. Laboratory white mice were used in 90-day variant experiments. Animals were divided into four experimental settings: with and without zeolite supplement as food additive, and with and without lead supplementation. The results showed that the sorbent reduced Pb concentrations in the body and different tissues and organs as follows – 84% in carcass, 89% in liver, 91% in kidneys, 77% in bones and the adsorbed quantity of Pb was excreted via the feces. A mathematical model for the bioaccumulation of lead in bones has been developed and the trends in animals fed with and without zeolite in the diet are presented. The absorption coefficient of Pb from the gastrointestinal mucosa was derived and a difference of about 4.5 times was found. In mice with zeolite, it is $\eta 03.53\%$ and in those without additives – $\eta 015\%$. The biological response of the organism was analyzed at the organ, tissue, cellular and subcellular levels. Changes in chromosome structure, mitotic index, erythrocyte shape and positive changes in animal body mass were recorded. The main results show that in animals fed with Pb and clinoptilolite as food additive, 2.3 times lower frequency of chromosomal aberrations, 2.5 times higher mitotic index and 1.5 times higher percentage of normal erythrocytes were observed, as well as 1.3 times increase in body weight compared to those exposed to Pb intoxication, but without the addition of a mineral sorbent.

It is well known that in regions with industries that seriously contaminate the environment with lead, high levels of cadmium in the environment are also reported. A future investigation aims to demonstrate that zeolite modifications in the direction of inset additional active sites of the inner mineral surface and adequate activation of the clinoptilolite sorbent will increase its sorption capacity compared to the natural material. This would increase the detoxification potential and reduce Cd and combinated Cd+Pb-induced oxidative stress and overall toxicity at the cellular and organism level.

Future prospects

The results of such types of investigations will have a significant theoretical contribution towards expanding our understanding of the ability of clinoptilolites to reduce the harmful biological effects of certain heavy metals, which are environmental pollutants. In the long run, such products may be used as low-cost detoxifiers in areas with a high level of anthropogenic pressure and contribute to solving important problems related to the environment, quality of life and health risk not only in nature but also in the human population.

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RESEARCH ARTICLE

BioRisk

Application of residual sludges from wastewater treatment technologies for construction of biofertiliser

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Abstract

To stimulate plant development in phytoremediation or in the cultivation of non-food crops in potentially contaminated soils, a biotechnologically created product could be applied. The aim of this study was to explore the possibility of creation of biofertiliser, based on activated sludge combined with bacterial strain with detoxifying and plant growth promoting properties. The presented study is focused on the effect of phenol in the following concentrations: 5 mg/l, 100 mg/l, 250 mg/l, 500 mg/l and 1000 mg/l on the metabolic activity of *Brevibacillus laterosporus BT271*. The gradual increased concentration of phenol was used to study the metabolic activity of mineralised activated sludge and *B. laterosporus BT271*. The CTC/DAPI staining showed high activity of the bacteria even at the highest concentration. The greatest amount of biomass was accumulated at 5 mg/l phenol (4.44×10^7 cells/ml). At this toxicant concentration, a total dehydrogenase activity of 5.72×10^{-4} μg H+/ml*min was found. Studies of the metabolic activity of microorganisms in experiments involving a combination of mineralised activated sludge, *B. laterosporus BT271* and phenol at three concentrations (5 mg/l, 250 mg/l and 1000 mg/l) showed the highest value for dehydrogenase activity in the variant with average phenolic concentration (up to 6.39×10^{-6} μg H+/ml*min. The results proved the detoxification potential of *B. laterosporus BT271* when different concentrations of phenol were present. The combination of a mineralised activated sludge and selected highly active biodegrading *B. laterosporus BT271* showed valuable properties of detoxification and metabolic activity and keep these potentials up to 1000mg/l phenol.

Keywords

Bioremediation, *Brevibacillus laterosporus BT271*, mineralised activated sludge, phenol

Introduction

Soils are a key resource for the existence and maintenance of normal human life and the planet. They are, therefore, the focus of the EU's Green Deal, adopted in 2019. According to this document and the European Commission's 'Towards Zero Pollution for Air, Water and Soil' action plan, Europe must have achieved zero soil pollution by 2050 (European Commission 2019, 2021). At the same time, in addition to the absence of pollution in the maintenance of soil fertility, the principles of the circular bio-economy need to be introduced (European Commission 2019). These ambitious goals will be difficult to achieve in the current economic and health crisis, combined with the lack of technology readiness in the various industries. Therefore, it is necessary to look for innovative solutions that allow environmentally-friendly treatment of soil resources and subsequent maintenance of fertility in them. One of the important directions in this subject is the creation of a less toxic environment. When it comes to soils, this involves the elimination of toxic substances, such as pesticides. Another aspect is the use of treated water in irrigation in agriculture. This again carries risks for the entry of toxic substances, antibiotics, steroid hormones and other xenobiotics into the soil. They must be degraded by the soil microflora or by the addition of biofertilisers. This requires a special focus on innovation activities related to the targeted management of detoxification processes in soils used for intensive or organic farming.

Activated sludge is formed during the treatment of domestic wastewater and, after stabilisation and decontamination, can be used for soil treatment (Gray 2010; Dimkov et al. 2017). They are alternatives to conventional fertilisers and offer a circular solution for solid waste from water treatment – stabilised sludge (Meuser 2013). These innovative and complex biofertilisers can be used for the reclamation of soils that are contaminated or damaged by human activity (Banov et al. 2020). The activated sludge is rich in both humic substances and micro-, macronutrients. It also contains microorganisms that are beneficial for the soil.

However, the bacteria in the activated sludge for recovery have low detoxification activity and are adapted mainly to the biodegradation of only trivial contaminants. In the case of soil contamination with organic pollutants, it is necessary to enhance the target detoxification activity by adding highly active biodegradants, which perform the biodegradation of toxic contaminants at a high rate and with a minimum degree of inhibition. This approach to bioaugmentation is used for contamination with petroleum products, pesticides, insecticides, pharmaceuticals, PFAS, antibiotics and other products, xenobiotic soil contaminants and residues in recycled water for irrigation (Hong et al. 2020; Li et al. 2021). In these bioaugmentation procedures for the detoxification activity of biological fertilisers, consortia (Varjani and Upasani 2019; Laothamteep et al. 2022) or individual bacterial cultures belonging to various genera can be used - *Pseudomonas* (Ramadass et al. 2018), *Enterobacter* (Koolivand et al. 2020), *Micrococcus* (Nwankwegu and Onwosi 2017), *Burkholderia* (Morya et al. 2020), *Bacillus* (Banerjee et al. 2019), *Shewanella* (Zou et al. 2019), *Xanthomonas* (Xu et al. 2018) or *Alcaligenes* (Haouas et al. 2021). Of particular interest are the bacteria of the genus *Brevibacillus*

(Arya and K. Sharma 2015). They have pronounced bioremediation properties against various organic and inorganic pollutants (Wei et al. 2019; Jebril, Boden and Braungardt 2021). These microorganisms are also of particular interest due to the possibility of their bioremediation detoxification properties to be combined in microbial preparations with the effect of protection against phytopathogens and enhancing plant growth (Ahmed et al. 2018). This study aims to explore the possibility of creating a biofertiliser, based on activated sludge combined with a bacterial culture from g. *Brevibacillus* with detoxifying and possible plant growth-promoting properties.

Materials and methods

Experimental design

The present study is focused on the exploration of the potential of a bacterial culture (*Brevibacillus laterosporus BT271*) for the creation of a biofertiliser with biodetoxifying properties. A combination of the highly-active bacteria with activated sludge that is conventionally used for the restoration of soil, will give added value to the future bioproduct in the trend of the circular solutions. A waste (the residual activated sludge) will be transformed into a valuable biotechnological product. It can be applied for the restoration of the damaged environment. The here-described experiments include the first stages of development of such a product. The *B. laterosporus BT271* metabolic activity in the presence of a model xenobiotic (phenol) was evaluated. The volume of the used 14th hour bacterial culture in the experiments at this stage was 4%. Nutrient broth was used. The microorganisms were cultured at 30 °C and aerated. Phenol was added once at zero hour in the following five concentrations: 5 mg/l, 100 mg/l, 250 mg/l, 500 mg/l and 1000 mg/l. Critical control points (CCPs) were set at 0 hour and 6th hour. The metabolic activity of the combined residual activated sludge (rAS) and *B. laterosporus BT271* was studied. Activated sludge weighing 0.5 g was used. Highly active biodegradant was added as fresh biomass, which is 10% of the weight of the activated sludge. The microorganism was cultured at 30 °C and aerated. Phenol was added once at zero hour in the following three concentrations: 5 mg/l, 250 mg/l and 1000 mg/l. CCPs were also set at 0 hour and 6th hour. The metabolic activity of the combined residual activated sludge (rAS) and *B. laterosporus BT271* was studied. The bacterial activity and the activity of the combination with rAS was estimated on the accumulation of the biomass, the total dehydrogenase activity of the microorganisms and the intoxication effects, determined with fluorescence and digital image analysis.

 Brevibacillus laterosporus BT271 – the bacterial culture was isolated from contaminated soil close to an oil refinery (Lukoil Neftochim Burgas, Bulgaria) by Prof. Yana Topalova (Topalova 2009). This bacterial culture was adapted to the biodegradation of aryl-containing xenobiotics (phenol, nitrophenols, pentachlorophenol in high concentration). The culture possesses a broad spectrum of biodegradation activity towards 30 different xenobiotics. The culture can survive in high percentage after lyophilisation and quickly recovered detoxification activity after re-hydration. The bacteria in our experi-

ments were kept as a lyophilised preparation prior to the experiments. They were cultivated in Nutrient agar (HiMedia, India). For the experiments for the intoxication effects, 18 h bacterial culture was used.

Activated sludge

The used activated sludge was residual activated sludge (rAS), treated with CaO (quicklime) and ready for utilisation in agriculture. The rAS was taken from the WWTP on Sofia City, Bulgaria.

Phenol

The model toxicant used in the study was phenol since its derivatives are major environmental pollutants. It was supplied by Fluka Analytical (Switzerland). Five phenol concentrations in the range 5–1000 mg/l were used in the experiments.

Methods

The accumulation of the biomass was monitored by the optical density at 430 nm. The total dehydrogenase activity was determined by the method of Lenhard (Lenhard et al. 1964).

CTC/DAPI-based analysis was applied for determination of the bacterial metabolic activity and detection of changes in the morphology of the cells. It is based on the use of two fluorescent dyes. CTC or 5-cyano-2,3-ditolyl tetrazolium chloride is a tetrazolium salt that has no fluorescent properties. In the living cells, it is reduced to CTC-formazan that emits a red fluorescence signal. In these experiments, it was used in 5 mM concentration. DAPI (4',6-diamidino-2-phenylindole) is a fluorescent dye that binds DNA and emits a blue fluorescent signal. In concentration 1 µg/ml, it can be used as a staining method for all the biomass in the samples. By using CTC and DAPI simultaneously, information about the live cells in the whole sample was obtained. Fluorescence images were taken by a "Leica" DM6 B microscope. They were further processed with the software DAIME (Daims et al. 2006). The share of the live cells, their intensity and size (or the area of the cells) were calculated with the programme by using custom threshold segmentation. All the analyses were performed in three independent repetitions.

Results

In the experiments performed, the accumulation of biomass in *B. laterosporus BT271* under optimal conditions for development was studied. The Nutrient medium provided a sufficient concentration of easily-degradable substrates and a lack of toxicants. The obtained results are illustrated in Fig. 1. The bacterial culture reaches its maximum number of cells at the 20th hour of its growth (3.25×10^7 cells/ml), after which it passes into a stationary phase of growth and a death phase. These data were also supported by the activity of dehydrogenase in the cells - the total dehydrogenase activity is highest
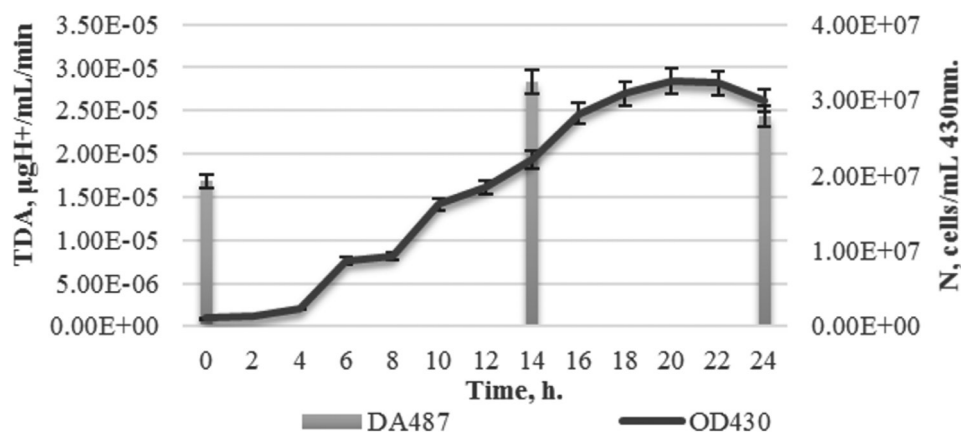
Figure 1. Biomass accumulation and total dehydrogenase activity (TDA) in *B. laterosporus BT271* (DA487 – dehydrogenase activity, measured at 487 nm; OD430 – optical density at 430 nm).

at the 14ᵗʰ hour (exponential growth phase). The value of this indicator exceeds TDA at the beginning of cultivation by 69% and by 16% at the 24ᵗʰ hour of the culturing.

In the further experiments for studying the detoxification properties of *B. laterosporus BT271*, cultures in the late exponential phase (18 hours) were used. They had the maximum number of bacterial cells with the most active enzymes.

In Fig. 2 A, the accumulation of biomass in *B. laterosporus BT271* in the presence of five phenol concentrations is presented. The presented data show that the bacterial culture degrades phenol in wide range of concentrations - from 5 mg/l to 1000 mg/l. The number of cells in the presence of phenol reached 4.44×10^7 cells/ml in the presence of 5 mg/l phenol. The largest increase occurred when the microbial count was recorded at low concentrations (16% for 5 mg/l and 13% for 100 mg/l). This is due to the lower inhibitory effect of the phenol in these concentrations. In contrast to the phenol experiments, in the cultivation of the bacterial culture in the absence of the phenol (control), a decrease of 3.25% was registered between the number of cells at 18 hours and 24 hours (Fig. 1).

A   **B**
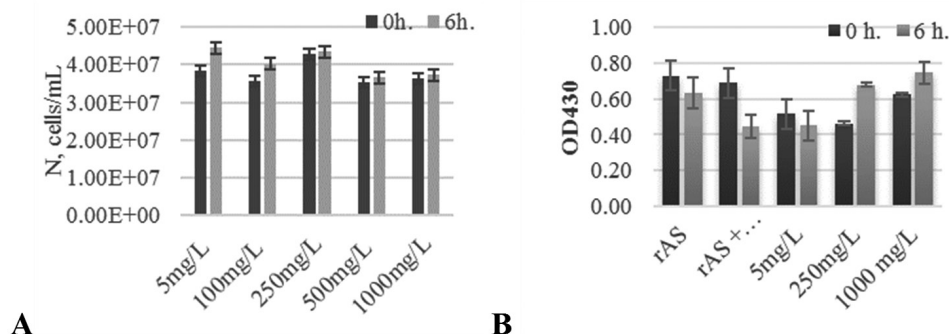
Figure 2. Change of the biomass accumulation in: **A** *B. laterosporus BT271* and **B** *B. laterosporus BT271* and rAS in presence of phenol in concentrations 5–1000 mg/l.

At the highest phenol concentrations, an increase in accumulated biomass was also found, although significantly less - 3.4% for 500 mg/l and 2.5% for 1000 mg/l phenol.

In addition to determining the biomass accumulated from *B. laterosporus BT271* in the presence of phenol, the change in absorbance when combining the bacterial culture with activated sludge for recovery was also investigated. This information can be used as an indirect indicator for the increase in biomass of the target detoxification culture. The obtained data are illustrated in Fig. 2B. At the high concentrations of the phenol, a significant increase was registered (average 34%). It is greatest at the addition of 250 mg/l phenol, but remains significant even at 1000 mg/l phenol.

The metabolic activity of the bacteria in the presence of phenol was determined, based on the total dehydrogenase activity. The results obtained showed that, in *B. laterosporus BT271*, there is a significant activation of metabolic processes in the presence of phenol (Fig. 3A). At the beginning of the experiment, the measured TDA was from 1.06×10^{-5} μg H⁺/ml*min to 1.06×10^{-4} μg H⁺/ml*min. After 6 hours of incubation, the values of this indicator increased from 7.58×10^{-5} μg H⁺/ml*min to 5.74×10^{-4} μg H⁺/ml*min. The highest increase was registered when 5 mg/l phenol was present - 7.3 times. It was lowest when 1000 mg/l phenol was applied - 2.7 times.

Fig. 3B represents the data on TDA for the combination of the highly active microorganisms *B. laterosporus BT271* with activated sludge for recovery. At the beginning of the experiment (0 h.), the values of this indicator were very low - they were at the lower limit of detection for this method. The only exception was the higher activity recorded in bacteria with the addition of 250 mg/l phenol. This is most likely due to the rapid activation of bacterial metabolism in the presence of this intermediate concentration of the toxicant.

After incubation for 6 hours, an increase in the activity of microorganisms was registered in all tested variants. This is from 30% (at 250 mg/l phenol) to 32 times (at the highest concentration of the toxicant). The effect recorded in the activated sludge controls was most likely due to the rehydration of the cells in the activated sludge and their incubation at the optimum temperature (30 °C).

The analysis of the metabolic activity, based on CTC, showed that, at most of the used concentrations, a slight decrease in the indicator was registered (on average by 14%)
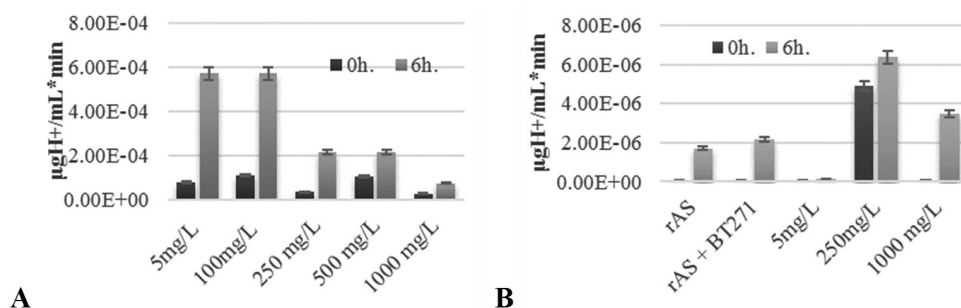
Figure 3. Total dehydrogenase activity in **A** *B. laterosporus BT271* and **B** *B. laterosporus BT271* and rAS in presence of phenol in concentrations 5–1000 mg/l.
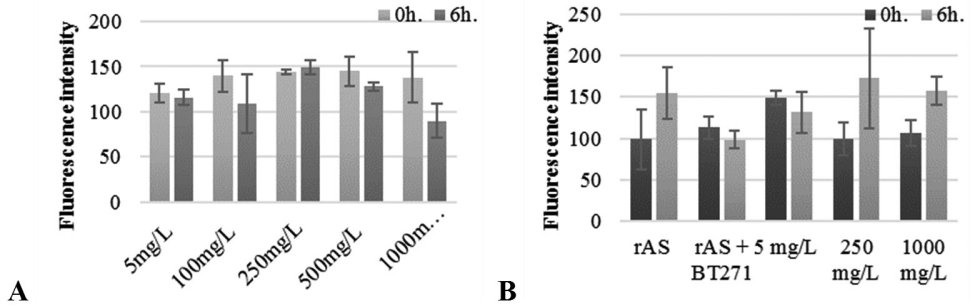
Figure 4. Fluorescence intensity in **A** *B. laterosporus BT271* and **B** *B. laterosporus BT271* and rAS after 6-hour incubation in presence of phenol.

(Fig. 5). The difference in the fluorescence intensity of CTC varies from 3% (at 5 mg/l phenol) to 35% (at 1000 mg/l). An increase in fluorescence intensity (by 4%) was found only at a phenol concentration of 250 mg/l. The differences in the results obtained with the TTC-based method are most likely related to the differences in methodology. The CTC method provides information directly about the individual cells in the samples. The information from the TTC method represents the activity of all bacteria in the sample. It is based on the extracted formazan, the concentration of which is determined spectrophotometrically. Additionally, in the TTC-TDA analysis, an easily degradable substrate is added (glucose). This activates the metabolism of the cells. The data from this analysis can be considered as information about the metabolic activity of certain microorganisms at the optimal concentration of the dehydrogenase substrate. On the other hand, the CTC method shows the metabolic activity of the cells directly under the conditions of the experiment, i.e. not to what extent their metabolic functions are preserved (as in the TTC-TDA method), but to what extent they are active in the conditions of intoxication.

Thus, it is clear that, in the presence of phenol in different concentrations, the bacterial culture *B. laterosporus BT271* had high metabolic activity, estimated as TDA, based on the reduction of TTC. However, under the conditions of the experiment, the activity of the culture decreased slightly after the 6-hour incubation with phenol. An exception was the concentration of 250 mg/l, at which higher fluorescence intensity was detected (Fig. 4A). In addition, it showed an increase in the average cell size (by 44%). At the same time, at the highest concentration used (1000 mg/l), there was a decrease in intensity by 34% and a decrease in cell area by 86% - an indication of intoxication changes in bacterial cells at this concentration.

In Fig. 6, fluorescent pictures of CTC staining of the residual activated sludge before and after the inclusion of *B. laterosporus BT271* bacteria are shown. The images represent the increased number of living cells after the addition of bacteria. The data obtained were confirmed by the fluorescence intensity after 6 hours of incubation. It is 73% higher in the presence of 250 mg/l phenol. The highest concentration of the toxicant increased the metabolic activity of the microorganisms by 48% (Fig. 4B). These results were also confirmed by the TTC method for the determination of TDA.
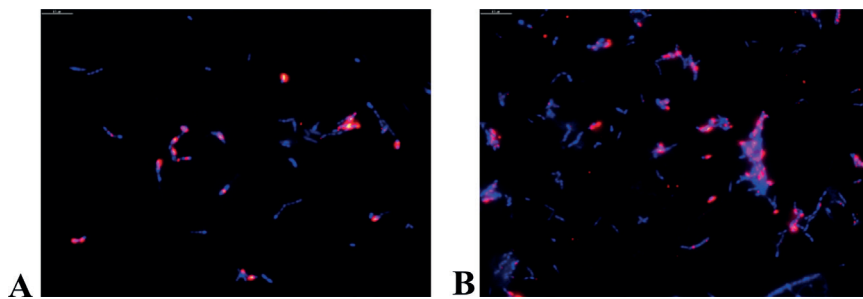
Figure 5. CTC/DAPI staining of *B. laterosporus BT271* in the presence of 5 mg/l phenol: **A** 0 h; **B** after 6 hours of incubation.

Figure 6. CTC staining demonstrating the bacterial abundance and metabolic activity in: **A** residual activated sludge; **B** residual activated sludge with added *B. laterosporus BT271*.

In both methods used to study the metabolic activity of microorganisms in the combination of activated sludge and *B. laterosporus BT271*, the lowest effect was recorded in the presence of 5 mg/l phenol.

The results of the CTC analysis also show an increase in the metabolic activity of the sludge after rehydration (the control with activated sludge only). In this experimental result, an increase in metabolic activity by 36% was found. The data obtained showed that, when *B. laterosporus BT271* was added to this sludge at the highest concentrations used (250 mg/l and 1000 mg/l), a significantly higher increase was achieved compared to the rehydrated sludge alone (61% on average). This is indicative of the high biodetoxification potential of the combination of activated sludge and *B. laterosporus BT271*.

Discussion

The data discussed in the Results section showed that the combination of activated sludge and bacterial culture of *Brevibacillus laterosporus BT271* has a high potential for use in biodetoxification procedures. These are required for environmental pollution with various pollutants. Soils have been identified by the EU as a resource that must be protected and purified because of their critical importance for "human health, the state of the economy and the production of food and new medicines" (European

Commission 2020). One of the most common techniques for soil decontamination (bioremediation) is bioaugmentation - the addition of highly active biodegradants to eliminate the respective contamination. Numerous bacterial genera have been identified as suitable for use in bioremediation procedures – *Bacillus* (Hsieh et al. 2020), *Pseudomonas* (Alhefeiti et al. 2021), *Shewanella* (Zou et al. 2019), *Xanthomonas* (Xu et al. 2018), *Alcaligenes* and *Brevibacillus* (Arya and K. Sharma 2015) and others.

The high potential for application of *Brevibacillus* bacteria was also confirmed in the experiments of the present study. *B. laterosporus BT271* increased in number despite the inhibitory effect of phenol applied in five increasing concentrations (5–1000 mg/l) (Fig. 2). At the lowest concentrations of the toxicant, its inhibitory effect was the weakest and the increase in the quantity of the detoxification culture was up to 16%. At the highest concentration (1000 mg/l), it was 3%. At the same time, during the cultivation on the Nutrient medium, a decrease of the biomass was registered at the 24th hour. The data obtained demonstrate that the culture can use the toxic phenol as a carbon source at all applied concentrations, which increases the number of cells, respectively the accumulation of the biomass of the detoxifying bacterial culture. Other authors also have established the applicability of *Brevibacillus* in the biodegradation and bioremediation of phenol and phenolic derivatives (Abu Talha et al. 2018; Wei et al. 2019).

In addition to specially-selected bacterial cultures, in practice, activated sludge from wastewater treatment plants is traditionally used as a biofertiliser. It is formed in the course of water purification after which it is subjected to stabilisation and decontamination and can be applied directly to soil enrichment in agricultural and bioremediation procedures (Bitton et al. 2016). Activated sludge is traditionally used for fertilisation because it is a natural source of nutrients (Abdollahinejad et al. 2020). When these sludges are enriched with an appropriate amount of biomass from microbial cultures with detoxifying properties, biofertiliser with combined valuable properties is obtained - for enrichment of the soil and soil fertility and simultaneous disposal of residual toxic contaminants in the soil.

The targeted combination of mineralised sludge and the detoxification culture of *B. laterosporus BT271* in the present study resulted in a very good result in terms of activity of the metabolic processes. As commented in the "Results", the combination with activated sludge and *B. laterosporus BT271* led to an increase in biomass accumulation by up to 47%. The low effect found in the controls and at 5 mg/l phenol is most likely due to the lack of sufficiently easily-degradable nutrient sources.

The presence of phenol in different concentrations has an effect, not only on the accumulation of biomass, but also on the activity of bacteria, both only on *B. laterosporus BT271* and in the combination of these microorganisms with the activated sludge. The rate of metabolic transformations increased from 2.7 to 7.3 times in the presence of phenol. When combining the highly-active microorganisms with activated sludge and the effect of phenol in a concentration of 1000 mg/l, it causes an increase in metabolic activity by 32 times. This result, as well as the rapid activation of metabolic processes and the high value of TDA at 250 mg/l phenol, are an indication of the prospects for the combination of activated sludge and *B. laterosporus BT271* for the creation of biofertiliser with valuable combined properties for soil detoxification and increase in soil fertility.

Fluorescence analysis showed that, although the individual *B. laterosporus BT271* cells decreased their activity after the addition of phenol, in the presence of activated sludge the total activity of the system increased by up to 73%. Thus, the use of preparation with *B. laterosporus BT271* and activated sludge in bioremediation activities could achieve a high rate of elimination of toxicants and, at the same time, would influence the soil fertility in a favourable direction in a way close to nature according to the rules of the circular economy and the principle "nature knows best".

Conclusion

The results, presented so far, were focused on the study of the prospects for the creation of biofertiliser with combined activated sludge and specially-selected microorganisms with detoxifying activity (*B. laterosporus BT271*). They showed that the accumulation of biomass and bacterial metabolic activity not only remain high, but also increased by 32 times in the presence of phenol in high concentrations (up to 1 g/l). This suggests that the application of the preparation with AS and *B. laterosporus BT271* will have accelerated biodegradation in areas contaminated with xenobiotics with a cyclic structure (phenol and phenolic derivatives). It will be possible to use the synergistic effect of the introduction of nutrients (in the form of activated sludge) and specially-selected biodegradants of xenobiotics (*B. laterosporus BT271*), which also have protective properties against plants. Further experiments will be continued in the direction of testing the preparation in conditions close to the real ones.

Acknowledgements

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RESEARCH ARTICLE

State of the antioxidant defense system in wedge clams from Bulgarian Black Sea as a measure of resistance to environmental impacts

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Abstract

Pollution and climate change can induce oxidative stress (OS) in aquatic organisms. Reduced activity or incoordination between antioxidant enzymes in marine bivalves may cause cellular impairment with effects on higher levels of ecological organization. The present study aims to assess the condition factor and the activity of antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-S-transferase (GST), and glutathione concentration (GSH) in soft tissues of *Donax trunculu*s (Linnaeus, 1758) as indicators of the state of marine ecosystems along the Bulgarian Black Sea coast. The wedge clams were sampled manually from different localities in June and in September. The activity of antioxidant enzymes and GSH concentrations varied both seasonally and among localities. Higher values of GSH, SOD and GPx were registered in wedge clams collected in autumn compared to those collected in summer. In wedge clams higher activity of the major phase II detoxification enzyme GST was observed in summer at localities with intensive tourism, suggesting an activation of the cell detoxification processes, presumably in response to increased environmental pressure. In conclusion, the observed presence of elevated enzyme activities suggested activation of the antioxidant protection system of the wedge clams in response to environmental pressure, indicating their ability to cope with induced OS and adapt to local conditions, and thus maintain ecosystem health.

Keywords

Introduction

Pollution of aquatic ecosystems with heavy metals (Bat et al. 2018), polycyclic aromatic hydrocarbons (PAHs) (Georgieva et al. 2016; Honda and Suzuki 2020), microplastics (Andrady 2011), and pesticides (Mojiri et al. 2020) is becoming a serious environmental problem. Pollutants enter the marine environment by industrial and municipal wastewater, agricultural activities, tourist flow and atmospheric inputs (Maranho et al. 2015). As a result, marine biotas are subjected to an increasing and complex mixture of chemicals. Marine organisms respond to the impact of these pollutants by activating different cellular pathways and defense systems aiming to overcome the negative consequences, which may affect higher levels of organization. Thus, stress responses are considered an ecological driving force and trigger of evolution (Steinberg 2012).

Oxidative stress (OS) is the result of the disturbance of cellular pro/antioxidant balance and is a general reaction of aerobic organisms to endogenous and exogenous stimuli, including environmental contamination. OS biomarkers are considered as early-warning indicators of pollution, proposed to be used in biomonitoring programs. The activation of the antioxidant defense system is a factor determining the survival of the organism in conditions of increased environmental pressure. Lower antioxidant enzymes' activities or lower concentrations of non-enzymatic antioxidants suggest higher susceptibility to stressors, and higher possibility of tissue damages in marine organisms (Steinberg 2012). Comparative studies showed that bivalves are characterized by a highly active antioxidant enzyme complex and a much greater pool of non-enzymatic antioxidants than vertebrates (Soldatov et al. 2007).

Wedge clams *Donax trunculu*s (Linnaeus, 1758) are the most common species inhabiting the shallow sandy sea bottom of the Black Sea, where most of the pollutants are deposited. Being filter-feeders they are capable of bioaccumulating and concentrating pollutants in their tissues. Furthermore, as a basic component of marine ecosystems, they are essential for maintaining ecosystem health.

This study aimed to assess the state and changes of the antioxidant defense system of wedge clams *D. trunculus* from representative localities along the Bulgarian Black Sea coastal area as a measure of their resistance and adaptation to variable environmental pressures.

Materials and methods

Sampling

Adult clams *D. trunculus* (length 23–35 mm) were sampled from their natural sandy habitats along the Bulgarian Black Sea coast (Fig. 1) in two seasons: summer (June) and autumn (September) of 2020. The samples were gathered manually or were obtained from commercial providers. The clams were stored at -80 °C until biochemical analyses.

Condition Index (CI) calculation

Before the biochemical analyses, the weight and length of random clam individuals from the same sites were measured with an analytical scale (accuracy of 0.01 g) and a caliper. The Condition index (CI) was computed according to Riascos et al. (2012) as CI=DW/SL*100, where DW is the dry whole soft tissue weight (g) and SL is shell length (mm).

Tissue preparation

On the day of the analyses, the clams were thawed, their soft tissues extracted and homogenized with 0.1 M K-PO$_4$ buffer, pH 7.4. The resulting homogenates were centrifuged, first at 3000 rpm for 10 min. to obtain a post-nuclear fraction to measure glutathione concentration and then at 12 000 rpm for 20 min. to obtain a post-mitochondrial supernatant where the activities of the antioxidant enzymes were assayed.

Biochemical analysis

The protein concentrations of the tissue post-nuclear and post mitochondrial supernatant were measured according to Lowry et al. (1951). The concentrations of total glutathione (GSH) were assayed using the method described by Rahman et al. (2006) and the results were expressed as ng GSH/mg protein. The antioxidant enzyme activities were determined as follow: catalase (CAT) activity by Aebi (1984); superoxide dismutase (SOD) activity by Peskin and Winterbourn (2017); glutathione peroxidase (GRx) and glutathione-S-transferase (GST) activities by using kits, Glutathione Peroxidase Cellular Activity Assay Kit (Cat. No CGP1) and GST Assay Kit (Cat. No

Code	Locality	N	E
S1	Shkorpilovtsi	42.9564	28.0345
S2	Slanchev Bryag	42.6902	27.7171
S3	Dyuni	42.3728	27.7163
S4	Primorsko	42.2773	27.7321
S5	Arkutino	42.3445	27.7322
S6	Ahtopol	42.1033	27.9259
S7	Kabakum	43.2656	28.0345
S8	Sv. Konstantin and Elena	43.2332	28.0111
S9	Tsarevo	42.1611	27.8656
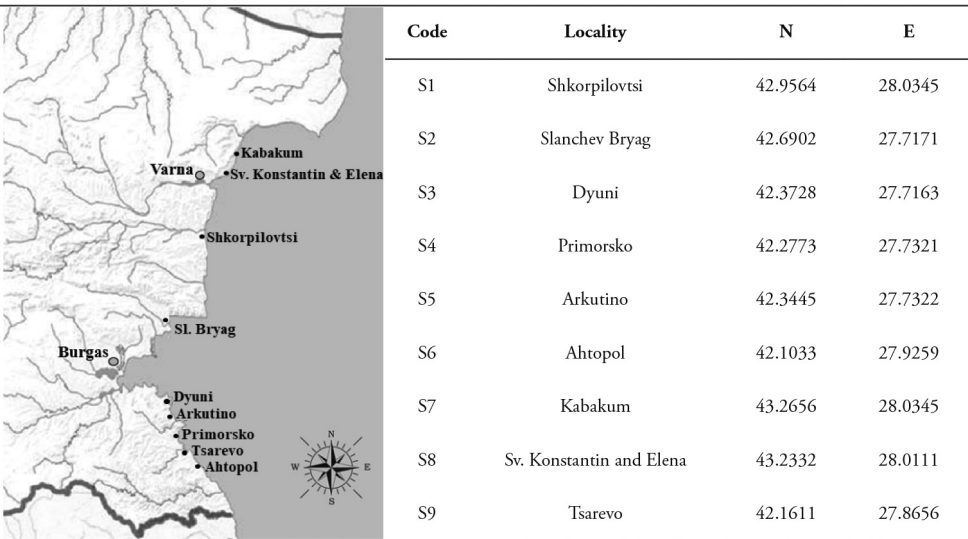
Figure 1. Sampling localities along the Bulgarian Black Sea coast with geographical coordinates.

CS0410), respectively, both purchased from Sigma-Aldrich Co. LLC (USA). The obtained values of all measured enzymes were expressed in U/mg protein.

Statistical analysis

Significance of differences of means between groups was determined using Student's t-statistic. Factorial ANOVA was applied to analyze meaningful factors of variable dependences. In order to identify meaningful predictors of dependent variables multiple regression analysis was carried out.

Results

In the samples gathered in summer, no statistically significant differences were observed in the GSH levels and SOD and CAT activities of the clams by localities (Table 1). However, significant differences between the clams were observed in their GPx and GST activities. The lowest GPx activity was measured in samples from Skorpilovtsi (S1). The highest GST activity was measured in samples from Slanchev Bryag (S2) and the lowest – from Ahtopol (S6) (Table 1), which resulted in marked statistical differences between them.

There were no significant differences in the measured OS biomarkers among localities in the autumn samples. The same pattern was also present in the summer samples. The only exceptions were the GPx and GST activities (Table 1). In autumn, statistically significant differences of GPx activities were observed between wedge clams from Arkutino (S5), Ahtopol (S6) and Kabakum (S7). There were also significant differences between clams from Kabakum (S7), where the lowest GPx was measured, and

Table 1. Assessment of OS biomarkers (mean±sd) in clams from selected sites along the Bulgarian Black Sea coast in summer and autumn with significance of the differences of indicators at the same site between seasons (*p<0.05; **p<0=001; ***p<0.0001) and between localities (†p<0.05; ††p<0.001; †††p<0.0001 as the S(n) indicates the particular locality).

Code	Indicators Locality	GSH (ng/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)	GST (U/mg protein)
			Summer			
S1	Shkorpilovtsi	369.33 ±80	3.24 ±1.3	2.56 ±0.51	0.99$^{†S2-6}$ ±0.4	110.85† ±32.5
S2	Slanchev Bryag	427.33 ±44.8	4.56 ±1.1	1.95 ±0.40	1.89^{††S1} ±0.5	164.20^{†S6} ±28.0
S3	Dyuni	331.00 ±14.8	3.84 ±1.0	2.48 0.63±	1.88^{††S1} ±1.1	112.03 ±38.5
S4	Primorsko	396.55 ±46.9	4.67*** ±0.8	2.03 ±0.27	2.22***;^{†††S1} ±0.8	149.94 ±37.2
S5	Arkutino	332.33 ±20.2	4.51*** ±0.6	2.39 ±0.33	2.02***;^{††S1} ±0.7	151.68 ±37.0
S6	Ahtopol	279.16** ±51.1	5.52 ±2.0	2.04 ±0.36	1.89^{††S1} ±0.5	85.83^{†S2} ±45.4
			Autumn			
S4	Primorsko	434.17 ±130.3	12w.06*** ±3.7	2.47 ±0.47	5.72*** ±1.8	133.97^{††S6,S7,S8} ±25.9
S5	Arkutino	357.98 ±124.5	10.30*** ±4.3	2.74 ±0.46	7.15***;^{†S6,S7} ±1.6	100.25^{†S6} ±24.0
S6	Ahtopol	396.08** ±49.8	9.58 ±3.6	2.01 ±0.35	3.11^{†S5} ±1.6	60.02^{††S4,S5,S9} ±15.1
S7	Kabakum	425.73 ±136.8	10.52 ±1.6	3.07 ±0.17	1.32$^{†S5, S9}$ ±0.5	43.78^{††S4} ±1.4
S8	Sv. Konstantin & Elena	378.80 ±139.2	7.41 ±1.0	2.95 ±1.07	4.23 ±1.23	61.24^{†S4} ±12.3
S9	Tsarevo	322.63 134.7±	11.37 ±2.0	2.32 ±0.66	6.22^{†S7} ±1.3	112.1^{††S6} ±23.1

those from Tsarevo where the activity of GPx was significantly higher (Table 1). GST activities measured in samples from Primorsko (S4) differed significantly from those in the samples from the localities Ahtopol (S6), Kabakum (S7) and Sv. Konstantin and Elena (S8). Statistically significant differences were also present between the clams from Ahtopol (S6) and Tsarevo (S9) (Table 1).

The measured antioxidant markers of clams were compared between the two seasons studied for the locations Primorsko (S4), Arkutino (S5), and Ahtopol (S6) (Table 1). No seasonal differences were present in the activities of CAT and GST. The level of GSH was raised in clams sampled in autumn from Ahtopol (S6). Similarly, the activities of SOD and GPx were higher in the autumn samples from Primorsko (S4) and Arkutino (S5), compared to those in summer.

In the present study, we calculated the CI of clams from the samples gathered in the two seasons from selected representative localities (Fig. 2).

The CI of wedge clams gathered in summer were much more similar among the studied localities, than in the wedge clams collected in autumn. In June, significantly lower CI values were found in samples from Slanchev Bryag (0.65±0.04) and Duni (0.75±0.06). In wedge clams from the autumn samples CI values showed higher dissimilarity among localities, than in the summer (Fig. 2). Significantly lower CI values were calculated for wedge clams in the autumn samples from Ahtopol (0.43±0.06)
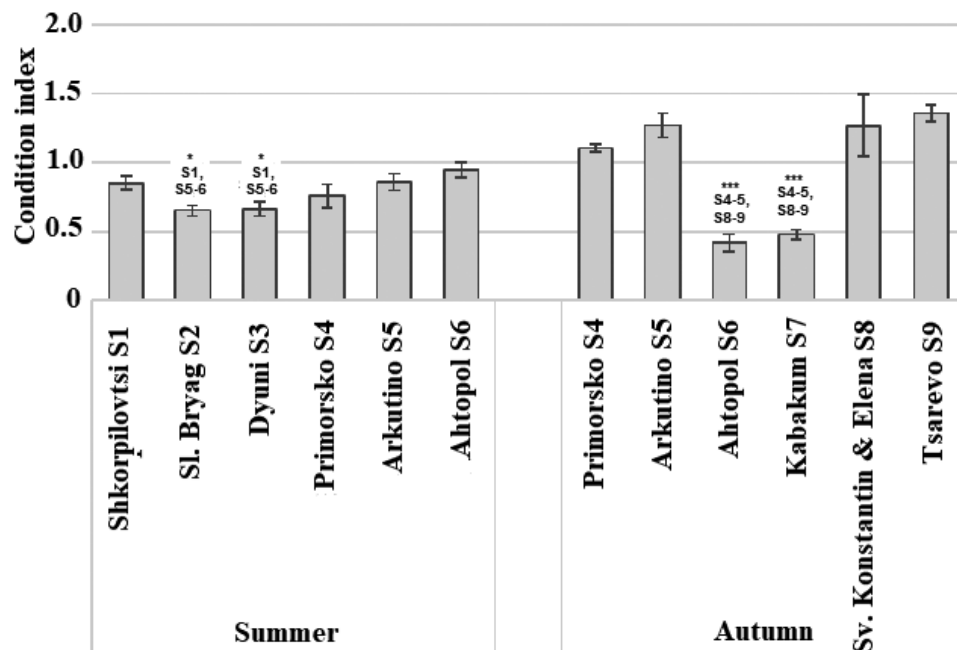
Figure 2. Calculated condition indexes (mean±SE) of wedge clams, gathered from localities along the Bulgarian Black Sea coast in summer and autumn with significance of the differences *p<0.05; ***p<0.0001; S(n) indicates the particular locality from which the difference was significant).

Table 2. Factorial ANOVA on dependence of wedge clams' Condition Index (CI) on the factors Season and Locality.

	Sum Squares	Degr. of Freedom	Mean SS	F	P
Intercept	0.52175	1	0.52171	1144.71	0.000000
Locality	0.01684	2	0.00842	18.47	0.000001
Season	0.00101	1	0.00101	2.22	0.140660
Locality*Season	0.02995	2	0.01497	32.86	0.000000
Error	0.02734	60	0.00045		

Table 3. Results of regression analysis of the Condition Index (CI) of wedge clams (dependent variable) on the five studied antioxidants as predictor variables.

	b	Std.Err. b	t (86)	p-value
Intercept	0.04764	0.01831	2.60166	0.01092
GSH	-0.00002	0.00002	-0.84815	0.39870
SOD	0.00048	0.00103	0.46778	0.64112
CAT	0.00977	0.00404	2.41578	0.01781
GPx	0.00017	0.00189	0.09154	0.92727
GST	0.00015	0.00007	2.01366	0.04717

and Kabakum (0.48±0.03). The CI values of the autumn samples from the remaining localities were significantly higher, even compared to the samples from all summer localities.

Factorial ANOVA was applied to analyse the overall dependence of CI (i.e. health status) of the wedge clams on the two factors Season and Locality (Table 2).

As can be seen from Table 2 the individual effect of Locality on CI was highly significant. The individual effect of Season alone was not significant. However, the joined effect of the two factors was highly significant, thus indicating that the state of the environment at a locality has a leading role in the condition and health status of the wedge clams, the seasonal effects being subordinate and specific for the particular locality.

In order to identify meaningful predictors of the CI of the wedge clams among the studied antioxidant biomarkers, multiple regression analysis was carried out (Table 3) where CI was set as the dependent variable and the GSH concentration and the activity of the antioxidant enzymes as predictor variables.

The obtained results (Table 3) indicated the presence of significant correlative relations between CI and the activity of CAT and GST. Thus, the two enzymes seem to be good predictor variables of the state and health of the wedge clams under changing environmental pressures.

Discussion

The results obtained in this study demonstrated the presence of significant variability in the pro/antioxidant processes in wedge clams sampled from representative locations of the Bulgarian Black Sea coast. The variations were most probably the result of

changes in the marine environmental conditions at the locations such as the geological characteristics of the area, proximity to large settlements and industrial sites, river inflows, etc. (Jenderedjian et al. 2007). In addition, seasonal variability was also present, which can depend on a large number of interrelated factors such as temperature, oxygen saturation, local hydrological cycle (which regulates productivity in an area), food availability, the metabolic status of the clams themselves, gonadal ripening and spawning (Soldatov et al. 2007; Nogueira et al. 2017), as well as the concentrations of metals (Reis et al. 2017) and PAHs (Koudryashova et al. 2019) in seawater. Data reported here indicated that glutathione-related enzymes GPx and GST were the mainly affected biomarkers. GSH itself, which is a co-substrate of both enzymes, did not show significant changes among localities. Although GSH plays a central role in antioxidant systems, this marker alone is not sufficient to indicate the OS rate due to the complex regulation of its homeostasis (Manduzio et al. 2005). Hence, changes in GSH level may be transient, and GSH should be measured in parallel with GPx and glutathione reductase, which are involved in its turnover, so that environmental stressors can be more precisely detected (Kulinskii and Kolesnichenko 2009). GPx, which is catalyzing the reduction of both hydrogen peroxide and organic hydroperoxides, provides an effective protection against oxidative damage and free radicals (Ighodaro and Akinloye 2018). Increased activity of GPx has been found in bivalves in response to marine pollution with heavy metals (Mansour et al. 2020) and PAHs (Moreira et al. 2020). In this study, increased GPx activities were found in clams only from Primorsko and Arkutino (both seasons), which was probably due to local environmental characteristics. This assumption was also supported by the finding that in the wedge clams from these locations an increased GST activity was measured, compared to the other locations. GST plays a principal role in the metabolism of xenobiotics by catalyzing their conjugation with GSH, making them more water-soluble and easily eliminated from the body. GST also plays a role in the protection against OS having peroxidase activity (Hayes et al. 2005). Furthermore, GST can regenerate S-thiolated proteins, which are the consequence of OS development in cells. Studies have reported increased activity and expression of various isoforms of GST in bivalves caused by xenobiotics such as PAHs, polychlorinated biphenyls, DDT, dioxins, metals and microplastics (Vidal-Liñán et al. 2010, 2014; O'Donovan et al. 2018). In our study, the highest GST activity among all samples was found in the soft tissue of clams collected from Slanchev Bryag in summer. This was most probably due to intensive touristic activities and pollution at this locality, which led to activation of phase II metabolism, and antioxidant defense system to overcome pollution. Furthermore, the wedge clams from this locality had low CI, which suggested decline in their physiological condition and proved the presence of significant biorisk for the marine biota. The activity of GST is known as a biomarker of exposure to pollutants since the enzyme has exhibited highly significant positive correlations with toxicants' amount and the use of GST as indicator is an advantage because of the regularity of its amendment and low seasonal variability (Vidal-Liñán et al. 2010). Indeed, in the present research we did not find seasonal changes in the activity of GST in the clams from one and the same locality. In our study, statistically

significant differences in summer and autumn were found for GSH, SOD and GPx for a number of localities (Table 1). In general, the activity of the measured enzymes as well as the concentration of GSH were higher in autumn. This was most probably due to the activation of the antioxidant defense system of wedge clams after the summer touristic flow in locations known for their high touristic activity.

In the present study, CI of clams was calculated in samples from selected localities in the two seasons, as an indicator of the physiological condition and health of individual wedge clams. The effects of the Locality and Season, as general factors, were assessed using Factorial ANOVA. The result indicated highly significant individual effect of Locality on CI, while the Season had not such effect. However, the joint effect of Locality and Season was highly significant. This result can be interpreted as a proof of the leading role of the state of the environment in a particular locality on the health of wedge clams, while seasonal effects seemed to be subordinate and dependent on the presence of different environmental factors and changes of a seasonal character at the particular locality. It was also proved that activity of the antioxidant enzymes CAT and GST was correlated with CI and they seemed to be good predictor variables of the presence of risks for the state and health of the wedge clams under variations of local environmental pressures.

Conclusion

The established variability in the antioxidant enzyme complex of *D. trunculus* clearly reflected the state of the particular environmental conditions in the studied locations of the Bulgarian Black Sea coast. The results indicated that the wedge clams demonstrated good and effective antioxidant defense, which was activated by increased environmental pressures at different locations, indicating their ability to cope with induced oxidative stress and adapt to local conditions, thus contributing to ecosystem health maintenance. Further work is obviously needed to prove the capacity of antioxidant enzymes as reliable bioindicators of ecosystem changes.

Acknowledgements

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RESEARCH ARTICLE

BioRisk

On the mode of action of *Origanum vulgare* spp. *hirtum* methanolic extract and essential oil on *Chlamydomonas reinhardtii*

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Abstract

Aim: To reveal whether methanolic extract and essential oil from *Origanum vulgare* subsp. *hirtum* in doses causing even low levels of mortality in aphids, would have harmful effects on plants-genotoxic, mutagenic and/or DNA damaging. **Materials and methods:** Aerial parts of *Origanum vulgare* ssp. *hirtum* from the *ex-situ* collection of IBER, BAS during flowering were collected. Extraction and isolation procedures, as well as GC/MS analysis of essential oil and methanolic extract were performed by standard protocols. The components were identified by comparing their relative retention times to the retention times of authentic standards, and with mass spectra with the NIST. **Test system:** *Chlamydomonas reinhardtii* strain 137 C+ (WT). **Endpoints:** "clonal" assay, the test of "visible mutations", constant field gel electrophoresis. **Statistics:** GraphPad Prism version 6.04 (San Diego, USA) and One-way Analysis of Variance ANOVA with multiple comparisons using the Tukey method. **Results:** A good correlation was observed between chemical composition of essential oil and methanolic extract, and their mode of action. Our genotoxic and double strand breaks results demonstrated mild genotoxic and statistically non-significant DNA damaging potential of methanolic extract and concentration-dependent well - expressed genotoxic and DNA damaging potential of essential oil. A good relationship between increased double strand breaks levels and decreased survival might be related to one of the main constituents of essential oil, suspected to be carvacrol. No mutagenic effect for ME and EO was found. **Conclusion:** Well-expressed toxic/genotoxic capacity of essential oil, as well as its capacity to damage DNA inducing double strand breaks, but the absence of mutagenic potential, could be considered as a good reason to recommend *Origanum vulgare* subsp. *hirtum* essential oil as a promising candidate for purposes of "green" technologies.

Introduction

For decades, the application of chemical / synthetic pesticides has been the most effective and common tool for weed, and pests control in agriculture. Unfortunately, their long-term use negatively affects the environment and biota, including human health (Gill and Garg 2014; Chu and Karr 2017; Böcker et al. 2019) due to the low biodegradability of most of them, and their ability to accumulate in the basic environmental matrices (Ali et al. 2019). On the other hand, many target organisms have increased their resistance to certain groups of pesticides that has provoked the development and release of new groups of chemical compounds (Heap 2021). As of 28 October 2021, the International Herbicide Resistant Weed website reported that 266 weed species (153 dicots and 113 monocots) were resistant to 164 different herbicides (Heap 2021). These alarming data "force" the scientific community to look for an environmentally-friendly solution for successful control of weed populations (Gnanavel 2015; Stankovic et al. 2020), and increased target specificity, as well as rapid degradation of the active substance (Cordeau et al. 2016).

Plants-based bioactive compounds with pesticide and/or herbicidal potential have been the focus of scientists for at least three decades (Balandrin and Klocke 1988; Gerwick and Sparks 2014; Fouad et al. 2015; Bona et al. 2016; Della Pepa et al. 2019; Elshafie et al. 2019; Gruľová et al. 2020) due to their chemical composition (Araniti et al. 2018; Jankowska et al. 2018; Lins et al. 2019).

Till now, the question of whether and how plants' essential oils or/and extracts could be effectively used for the purposes of "green agro chemistry" is ongoing. New information has been gathered about their insecticidal and inhibitory activity on seed germination and weed seedling growth (Matković et al. 2018; Nikolova and Berkov 2018; Stankovic et al. 2020), but information about their mutagenic, and/or DNA damaging effects are scarce (Karpouhtsis et al. 1998; Llana-Ruiz-Cabello et al. 2018).

This investigation was based on our previous finding that *Origanum vulgare* ssp. *hirtum* extracts and essential oil negatively affect *Myzus persicae* survival (Parvanova et al. 2020). Here, using *C. reinhardtii* as a plant test system, we aimed to reveal whether methanolic extract and essential oil of *Origanum vulgare* subsp. *hirtum* in doses causing even low levels of aphid's mortality would have harmful effects on plants – genotoxic, mutagenic, and/or DNA damaging.

C. reinhardtii was chosen because it is a robust model test-system in environmental mutagenesis (Chankova et al. 2006, 2013; Dimitrova et al. 2007, 2009, 2014; Chankova and Yurina 2012; Chen et al. 2012; Kopaskova et al. 2012; Jamers et al. 2013; Melegari et al. 2013; Aksmann et al. 2014; Angelova et al. 2014; Boenigk et al. 2014; Chalifour et al. 2014; Almeida et al. 2019; Xu et al. 2019; Todorova et al. 2020).

Materials and methods

Plant materials. Aerial parts of *Origanum vulgare* ssp. *hirtum* were collected during the flowering stage from the *ex-situ* collection of the Institute of Biodiversity and Ecosystem Research (IBER), http://www.iber.bas.bg/sites/default/files/projects/plantscollection/index.html.

Preparation of methanolic extract (ME). Air-dried, ground aerial parts of the species were extracted with methanol by classical maceration for 24 h. After filtration, the organic solvent was evaporated and the resulting crude extract was subjected to further analysis.

Isolation of essential oil (EO). The essential oil was extracted on a Clevenger apparatus by water distillation from 50 g dry plant material in a flask with 500 ml water for 2 h.

GC/MS analysis of EO and ME. For GC/MS analysis, 50 mg of methanolic extract was silylated with 50 μl of N, o-bis- (trimethylsilyl) trifluoroacetamide (BSTFA) in 50 μl of pyridine for 2 h at 50 °C. The spectra were recorded on a Thermo Scientific Focus GC combined with a Thermo Scientific DSQ mass detector as described previously (Berkov et al. 2021). The chromatographic conditions for EO analysis were described by Traykova et al. (2019). The quantities of the compounds have been expressed as the percentage area of the total peaks' area of the chromatogram. The components were identified by comparing their mass spectra and retention indices (RI) to known compounds from the literature, National Institute of Standards and Technology (NIST) and home-made MS databases.

Toxicity/Genotoxicity – a "clonal" assay, based on colony forming ability, was performed (Dimitrova et al. 2007). *C. reinhardtii* WT137C was cultivated at standard conditions – light of 70 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and t = 25 $\pm$ 3 °C to the end of the exponential and the beginning of the stationary phase. Concentrations of ME and EO – 250, 500, 750, 1000 ppm, as well as exposure time, were defined previously. Two negative controls – Sager-Granick liquid medium (SG) and 1000 ppm DMSO as a solvent, and one positive control – Nurelle D at the commercially- recommended concentration of 500 ppm for insects, were used. Both survival fraction (SF) (Bryant 1968) and three levels of lethality were calculated (Lidanski 1988).

Mutagenicity – test of "visible mutations", based on the changes in size, morphology, and pigmentation of surviving colonies, was applied. The method and calculations of a percentage of induced mutant colonies and index of mutagenicity (IM) were described by Dimitrova et al. (2007). When IM < 2.5 – no mutagenic effect is identified, when IM is in the range of 2.5 to 10, the mutagenic effect is mild, and when IM > 10, the mutagenic effect is moderate to strong.

DNA – the damaging potential of both ME and EO was evaluated by CFGE (Constant Field Gel Electrophoresis) (Chankova and Bryant 2002). The advantage of this method is well described by (Chankova et al. 2007). After electrophoresis ended, the gel was visualised in UV light and captured with a digital camera and the GeneSnap programme (SynGene). The images were analysed with GeneTools software (SynGene). The fraction of DNA released (FDR) from the wells was calculated according to Chankova et al. (2009).

Data analysis – All presented data are averages from at least three independent experiments. Statistical data processing was performed with GraphPad Prism version 6.04 (San Diego, USA) and One-way Analysis of Variance (ANOVA) with multiple comparisons, using the Tukey method to compare the results of different treatments.

Results

Chemical composition – carvacrol (74.34%), *p*-cymene (9.46%), *γ*-terpinene (10.66%), *α*-pinene (1.73%), *β*-pinene (1.34%) and carvacrol methyl ether (1.23%) were identified as the main components of essential oil. The other components were presented in quantities of less than 1%.

In the methanolic extract, various primary metabolites as fructose (10.32%), glucose (11.65%), sucrose (10.51%), organic acids – succinic (0.60%), malic (1.09%) and linolenic acid (0.78%) were found. Phenolic acids – 4(p)-hydroxybenzoic (0.53%) and vanillic (0.22%), rosmarinic acid (6.06%), terpenoids – carvacrol (15.67%), caryophyllene (0.40%), flavonoids – catechin (0.23%) 6-hydroxyflavone glycoside (1.49%) were identified as the main secondary metabolites.

Toxicity/Genotoxicity – as the first step of our investigation, we had to clarify two points: whether Nurelle D, chosen as a positive control at the recommended commercial dose for aphids control, would have a detrimental effect on the model plant cells and whether DMSO, as a solvent, would affect *C. reinhardtii* cells negatively. As seen in Fig. 1, no statistically significant decrease in the cells survival fraction after the treatment with DMSO and Nurelle D was found, compared to the negative control SG ($P < 0.05$).
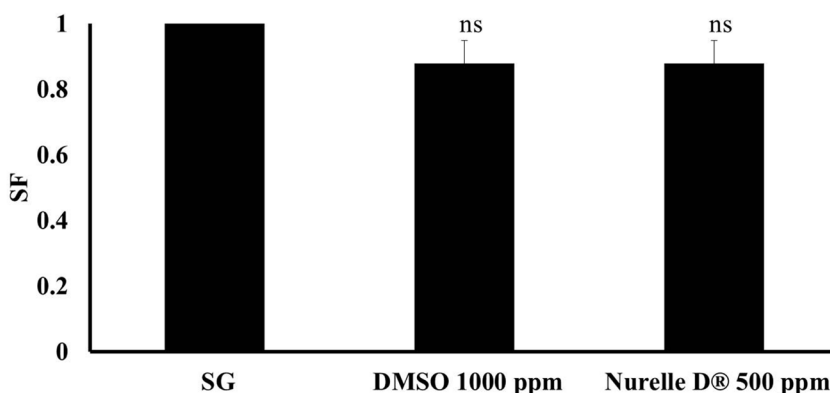
Figure 1. Cells survival fraction in negative and positive control samples of *C. reinhardtii* strain 137C. Mean values from at least three independent experiments. Error bars represent standard errors of mean values. The statistical significance of the differences is presented as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns – no significant difference.

Analysing curves in Fig. 2, statistically significant reduction of cell survival after the treatment with both highest concentrations of ME 750 ppm (SF = 0.25 ± 0.08) and 1000 ppm (SF = 0.00026 ± 0.08) was shown. In the range of 250 ppm and 500 ppm, some plateau was formed. Better expressed dose-effect was obtained for EO (Fig. 2) at a concentration of 250 ppm or above (SF = 0.51 ± 0.09; SF = 0.34 ± 0.11 and SF = 0.09 ± 0.02) (*** $p < 0.0001$).One-way ANOVA analysis reveals statistically significant differences between: SG vs. 250 ppm EO; SG vs. 500 ppm EO; SG vs. 750 ppm EO; SG vs. 1000 ppm EO; SG vs. 750 ppm ME; SG vs. 1000 ppm ME (***, $p < 0.001$).
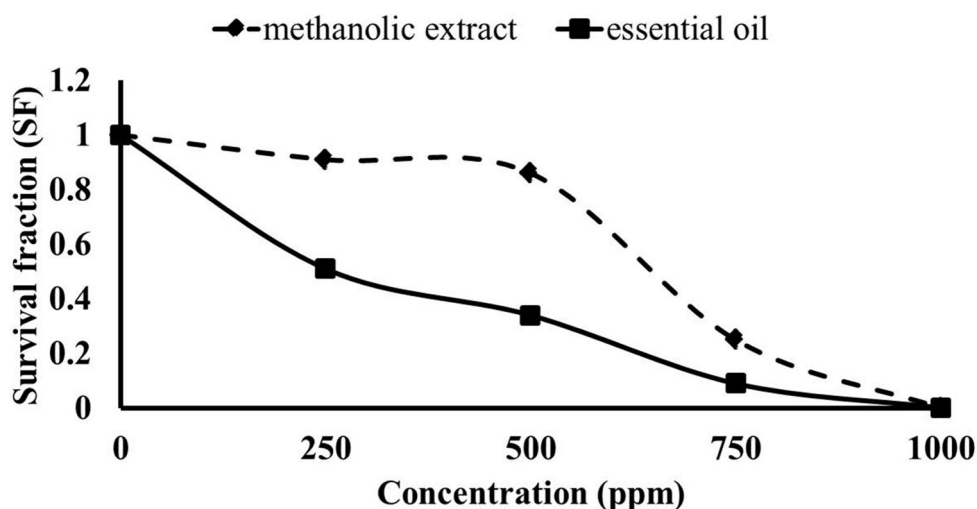
Figure 2. Cells survival fractions (*SF*) after the treatment with *Origanum vulgare* ME and EO. Mean data are from three independent experiments. Error bars represent standard errors of mean values. Where no error bars are evident, errors were equal to or smaller than the symbols.

Further, we had to calculate three levels of lethality – LD_{20}, LD_{50} and LD_{80} as a commonly-used approach for comparing the genotoxic potential of standard mutagens or other chemical/physical factors. The stronger genotoxic potential of essential oil is obvious by data shown in Table 1. Approximately two-fold lower EO concentrations can induce LD_{20}, LD_{50} and LD_{80} comparing with those of ME.

Table 1. LD_{20}, LD_{50} and LD_{80} in strain 137C, measured after the treatment with *Origanum vulgare* spp. *hirtum* ME and EO.

Origanum vulgare subsp. *hirtum*	LD_{20}	LD_{50}	LD_{80}
Methanolic extract [ppm]	523	634	810
Essential oil [ppm]	< 250	263	588

Mutagenicity – test of "visible mutations"

The next step in our investigation was to reveal whether both ME and EO of *Origanum vulgare* spp. *hirtum* possess some mutagenic potential on *C. reinhardtii*. The level of spontaneous "visible mutations" was 0.136%. Calculated IM clearly demonstrated an absence of mutagenic capacity for DMSO and Nurelle D. No mutagenic capacity of ME and EO was identified (IM < 2).

DNA-damaging potential of ME and EO

Our CFGE results show no DNA damaging capacity of ME. The levels of DSBs, measured after treatment with concentrations in the range of 250 – 1000 ppm, were approximately similar to the levels of spontaneously arisen DSB in the control sample (Fig. 3). One-way ANOVA analysis reveals statistically significant differences between: SG vs. 250 ppm EO; SG vs. 500 ppm EO; SG vs. 750 ppm EO; SG vs. 1000 ppm EO (***, $p < 0.001$); SG vs. 500 ppm ME (*, $p < 0.05$).

A quite different curve was drawn from the DSB levels measured after treatment with EO (Fig. 3). Around two-fold higher levels of DSBs were calculated compared to those in the negative control and the samples treated with methanolic extract. All differences, showing higher values compared to the negative control, were statistically significant. These results demonstrate a stronger DNA-damaging potential of oregano EO than that of ME under our experimental conditions. A well evident correlation was found between the potential of oregano EO to induce DSBs and lower SF measured as a colony-forming ability.
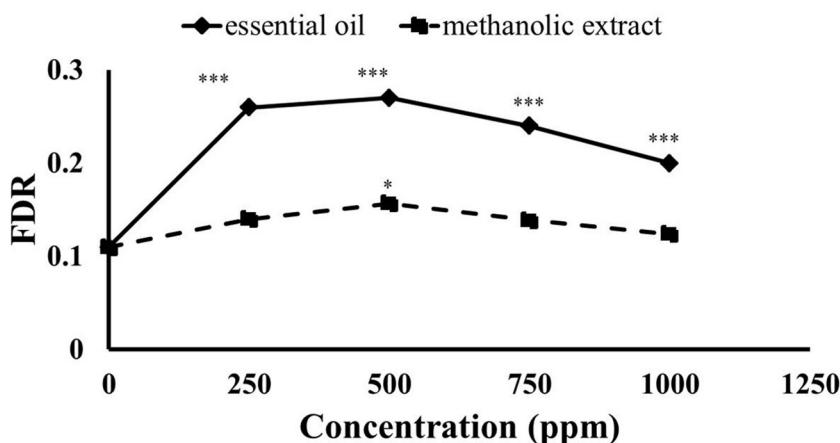
Figure 3. DSBs measured after treatment with different concentrations of ME and EO of *Origanum vulgare* spp. *hirtum*. Mean data are from three independent experiments. Error bars represent standard errors of mean values. Where no error bars are evident, errors are equal to or smaller than the symbols. The statistical significance of the differences is presented as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns – no significant difference.

Discussion

Previously, it was found by us that *Origanum vulgare* ssp. *hirtum* extracts and essential oil can cause *Myzus persicae* mortality depending on the concentrations applied. Here, an attempt was made to clarify whether ME and EO of *Origanum vulgare* subsp. *hirtum*, in doses causing even low levels of mortality in aphids, would have harmful effects – toxic/genotoxic, mutagenic and/or DNA damaging on *C. reinhardtii*, used as a plant test-system.

The better-pronounced capacity of EO vs. ME to decrease *C. reinhardtii* cell survival was demonstrated by comparing the concentrations inducing three levels of lethality – LD_{20}, LD_{50} and LD_{80}. It was calculated that EO is about 1.4–2-fold more genotoxic for algae cells than ME. Till now, a large spectrum of effects of oregano EO has been described – phytotoxic (Ibáñez and Blázquez 2017, 2020; Gruľová et al. 2020; Abd-ElGawad et al. 2021), antimicrobial (Karaday et al. 2020; Simirgiotis et al. 2020), antifungal (Puškárová et al. 2017; Saghrouchni et al. 2021); insecticidal (Alkan 2020), anti-plant pathogens (Raveau et al. 2020) etc. The data reported by us have further expanded this spectrum.

Information concerning DNA damaging or mutagenic potential of ME and EO are scarce. Llana-Ruiz-Cabello et al. (2018), using both MN test and comet assay (standard and enzyme-modified), have reported no increased levels of MN and DNA damage. Contrary to this study, our experiments revealed, for the first time, the DNA damaging capacity of oregano EO. It was clarified that the level of DSBs depends on the concentration applied. The good relationship between increased DSBs levels and decreased survival, described by us, might be linked to one of the main constituents of EO, likely to be carvacrol.

Both oregano ME and EO were shown to possess no mutagenic activity in *C. reinhardtii* test-system. Similar findings have been described previously by others (Adam et al. 1998; Karpouhtsis et al. 1998). No mutagenic effect on Aims test and no mutagenic or recombinogenic activity for ME and EO were found, using the Wing Somatic Mutation and Recombination Test (SMART) on *D. melanogaster*.

Conclusion

In this study, mild toxic/genotoxic and statistically non-significant DNA damaging potential of ME and concentration-dependent effects of EO were identified. The differences in the mode of action of EO and ME could be related to differences in their chemical composition. Further experiments are required in order to clarify the effect of main and minor constituents. Well-expressed toxic/genotoxic capacity of EO, as well as its capacity to damage DNA inducing DSBs, but the absence of mutagenic potential, could be considered as a good reason to recommend *Origanum vulgare* subsp. *hirtum* EO as a promising candidate for purposes of "green" technologies.

Acknowledgements

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RESEARCH ARTICLE

BioRisk

Polar and non-polar fraction from *Origanum vulgare* spp. *hirtum* methanolic extract – differences in their bioactivity on *Chlamydomonas reinhardtii* test system

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Abstract

Aim: To compare the bioactivity of both polar and non-polar fraction of *Origanum vulgare* spp. *hirtum* methanolic extract on *Chlamydomonas reinhardtii*.

Material and methods: The polar and non-polar fractions were derived from aerial parts of *Origanum vulgare* ssp. *hirtum*, collected during the flowering stage from the *ex-situ* collection of IBER-BAS. GC/MS analysis of both fractions was done following the standard protocol. The measured mass spectra were deconvoluted by the Automated Mass Spectral Deconvolution and Identification System (AMDIS), before comparison with the databases. *Chlamydomonas reinhardtii* 137C+ (WT) was used as a test system. Spot-test, cell survival fraction (SF), test of "visible mutations" and CFGE (for measurement of induced DNA double-strand breaks (DSBs)) were applied.

Results: The polar fraction did not possess genotoxic, mutagenic as well as DNA-damaging effect. The situation with the non-polar fraction was quite different. Even at the lowest concentration of 250 ppm, cell survival was decreased by 60% (SF = 0.41 ± 0.08). Treatment with concentrations equal to/or greater than 500 ppm resulted in around 100% lethality.
A mild mutagenic effect was obtained for the concentration of 250 ppm non-polar fraction (IM = 4.83 ± 0.004). Well-expressed and concentration-dependent induction of DSBs for even the strong DNA fragmentation was observed after the treatment with the non-polar fraction.

Conclusions: The different bioactivity of the two fractions correlated well with their different chemical composition. The polar fraction, rich in sugars, organic acids and flavonoid glycosides, did not possess

genotoxic and mutagenic potential. The strong genotoxic potential of the non-polar fraction might be related to carvacrol content (37.08%), which is not present in the composition of the polar fraction. To the best of our knowledge, this study provides the first information that the carvacrol-rich non-polar fraction of *Origanum vulgare* spp. *hirtum* methanolic extract possesses genotoxic, mutagenic and DNA damaging effect on some low eukaryotes, such as *C. reinhardtii*. Further experiments with carvacrol should be done in order to clarify the exact mechanism of action.

Introduction

Origanum vulgare L. (Lamiaceae) is a perennial herb native to the Mediterranean Region and western Eurasia (De Martino et al. 2009; Pezzani et al. 2017). Oregano has been widely applied not only as a flavouring herb, but also for medicinal purposes for centuries. Extensive data exist concerning its antioxidant, antimicrobial, anti-inflammatory and anti-cancer activity (reviewed in Pezzani et al. 2017). Together with these properties, current studies are focused on its promising application as a biopesticide (Ibáñez and Blázquez 2017; Alkan 2020; Gruľová et al. 2020; Abd-ElGawad et al. 2021). Most of the activities are contributed to the phenolic monoterpenes, thymol and carvacrol (De Santis et al. 2019). Our previous data already revealed that the methanolic extract with a quantity of carvacrol 15.67% is less toxic/genotoxic than the essential oil containing 74.34% carvacrol (unpublished data). Taking into account these data, our present hypothesis is that carvacrol-rich extract fractions would be more genotoxic than those without carvacrol.

The aim of the present work was to compare the bioactivity of polar and non-polar fractions of oregano methanolic extract on *Chlamydomonas reinhardtii*.

Materials and methods

Plant material and extraction procedure

Crude methanolic extract from aerial parts of *Origanum vulgare ssp. hirtum*, collected during the flowering stage from the *ex-situ* collection of IBER-BAS, was obtained by air-drying, extraction by classical maceration with methanol for 24 h, filtration and evaporation to dryness. Liquid/liquid extraction, using distilled water and chloroform, was used in order to separate the non-polar and polar compounds into two fractions.

GC/MS analysis

A total of 50 mg of each fraction was dissolved in 50 μl of pyridine. Then, 50 μl of N,O-bis-(trimethylsilyl)trifluoroacetamide were added and the samples were heated at

70 °C for 2 h. After cooling, the samples were diluted with 300 μl of chloroform and analysed using GC-MS. The GC–MS spectra were recorded on a Thermo Scientific Focus GC, coupled with a Thermo Scientific DSQ mass detector operating in EI mode at 70 eV. The chromatographic conditions have been described by Berkov et al. (2021). The metabolites were identified as TMSi derivatives by comparing their mass spectra and retention indices (RI) with online available plant-specific database. The measured mass spectra were deconvoluted by the Automated Mass Spectral Deconvolution and Identification System (AMDIS), before comparison with the databases. RI of the compounds were recorded with standard n-hydrocarbon calibration mixture (C9- C36) (Restek, Cat no. 31614, supplied by Teknokroma, Spain) using AMDIS 3.6 software.

Toxicity/Genotoxicity

The *C. reinhardtii* strain 137C (+) WT cell suspensions (1×10^6 cells/ml) at the end of the exponential and the beginning of the stationary phase were treated for 10 seconds with six concentrations of polar and non-polar fractions (250, 500, 750, 1000, 3000 and 5000 ppm). The concentrations and exposure time were the same as those previously defined for methanolic extract. Sager-Granick liquid medium (SG) and DMSO 1000 ppm were used as negative controls. As a positive control, 500 ppm Nurelle D was used following commercial recommendations for pest control. In order to calculate the survival fraction of cells (SF) (Bryant 1968), the "clonal" assay, based on colony-forming ability, was performed.

Mutagenicity

The mutagenic potential of polar and non-polar fractions was revealed by the test of "visible mutations". Changes in the size, morphology and pigmentation of surviving colonies were analysed (Shevchenko 1979). The calculations of percentage-induced mutant colonies and index of mutagenicity (IM) have been described in detail previously (Dimitrova et al. 2007).

Test for double-strand breaks (DSBs) induction in DNA

Constant Field Gel Electrophoresis (CFGE) procedure and its advantages for the evaluation of potential DNA damaging capacity of the fractions were described earlier by Chankova and Bryant (2002) and Chankova et al. (2005). The fraction of DNA released (FDR) from the wells was calculated according to the formula presented by Chankova et al (2009) and Dimova et al. (2009).

Data analysis

All data are mean values from at least three independent experiments. GraphPad Prism programme version 6.04 software (San Diego, USA) and one-way analysis of variance ANOVA were used.

Results

GC/MS analysis of polar and non-polar fraction of methanolic extract

Thirty-four compounds were identified in the studied fractions of *O. vulgare* ssp *hirtum* methanolic extract (Table 1). Mono-, di-, trisaccharides, flavonoids, organic and phenolic acids and sugar alcohols were found in the polar fraction. Sucrose (**25**) and 6-hydrohyflavon glycoside (**24**) were determined as main constituents. Monoterpenoids, fatty and triterpene acids, sterols and flavonoid aglycones were identified in the non-polar fraction with the main compound carvacrol (**3**) (37.08%).

Quantities are relative to the percentage of the area of all chromatogram peaks.

Toxicity/Genotoxicity of polar and non-polar fractions

As a first step, the toxic/genotoxic potential of the polar and non-polar fractions was compared using the 'spot' test and survival fraction (SF) assay.

Significant differences were obtained between polar and non-polar fractions with regard to these two endpoints. Spot test data illustrated no concentration-dependent effect of polar fraction – the spot's intensity was identical in all samples, including control samples. These results are informative for the absence of toxic/genotoxic capacity of the polar fraction in this concentrations range (Fig. 1). Quite different was the second picture when non-polar fraction was used – single colonies instead of a spot were formed after the treatment with the lowest tested concentration of 250 ppm and no survived colonies or spots formation were seen after the application of concentrations equal to/or greater than 500 ppm. These results could be attributed to the very strong toxic/genotoxic capacity of the non-polar fraction inducing cell death (Fig. 1, second row).

Our SF results corresponded well with the spot test information. None of the tested polar fraction concentrations decreased the cell survival. The results were comparable with those calculated for the control samples (Fig. 2). On the other side, well expressed toxic/genotoxic potential was found for the non-polar fraction. Even at the lowest concentration of 250 ppm, cell survival was decreased by 60% (Fig. 2). Treatment with concentrations equal to/or greater than 500 ppm resulted in around 100% lethality.

Mutagenicity

Further, the mutagenic potential was evaluated. Again, treatment with any of the tested polar fraction concentrations did not result in statistically significant induction of mutant colonies. Contrarily, 250 ppm of the non-polar fraction was found to possess a mild mutagenic effect (IM = 4.83 ± 0.004) (Table 2). Two types of "visible mutations" in *C. reinhardtii* strain 137C were obtained – low-size and pigmental, which are considered as a result of delayed cell division and point mutations in nuclear or chloroplast

Table 1. Metabolites in the polar and non-polar fraction of *O. vulgare* ssp *hirtum* methanolic extract identified by GC/MS.

Compounds	Retention time (RT)	Retention index (RI)	Polar fraction [%]	Non-polar fraction [%]
Glycerol (**1**)	4.48	1258	3.86	
Succinic acid (**2**)	4.90	1305	0.20	
Carvacrol (**3**)	5.18	1339		**37.08**
Fatty alcohol (**4**)	5.70	1389		1.65
Caryophyllene (**5**)	6.14	1492		1.95
Meso erythrol (**6**)	6.55	1496	0.64	
Pyroglytamic acid (**7**)	7.26	1512	0.6	
Caryophyllene oxide (**8**)	8.15	1517		2.33
4-Hydroxybenzoic acid (**9**)	8.45	1625	0.5	
Fatty acid (**10**)	8.50	1708		1.05
Arabitol (**11**)	9.65	1721	0.16	
Fructose 1 (**12**)	10.65	1800	0.6	
Fructose 2 (**13**)	11.24	1837	4.49	
Fructose 3 (**14**)	11.97	1855	1.66	
Glucose (**15**)	12.59	1882	7.14	
Monosaccharide 1 (**16**)	13.36	1918	0.57	
Monosaccharide 2 (**17**)	14.14	1967	5.47	
Hexadecanoic acid (**18**)	15.48	2041		1.99
Myo-inositol (**19**)	16.04	2080	6.22	
Caffeic acid (**20**)	17.02	2131	0.58	
Octadecatrienoic acid (**21**)	18.46	2211		3.74
Octadecanoic acid (**22**)	21.84	2238		0.56
Disaccharide 1(**23**)	23.17	2501	1.92	
6-OH flavone glycoside (**24**)	24.89	2603	**10.07**	
Sucrose (**25**)	25.18	2628	**26.28**	
Naringenin (flavanone) (**26**)	27.95	2778		0.76
Disaccharide 2 (**27**)	29.60	3202	2.66	
9-Octadecenoic acid (**28**)	29.83	3238		7.85
Taxifolin (flavanonols) (**29**)	30.00	3377	1.14	
ß-Sitosterol (**30**)	36.41	3389		1.69
Disaccharide 3 (**31**)	38.01	3618	1.5	
Rosmarinic acid (**32**)	38.69	3642	0.23	
Triterpene acid 1 (**33**)	42.82	3739		0.84
Triterpene acid 2 (**34**)	44.81	3786		1.61
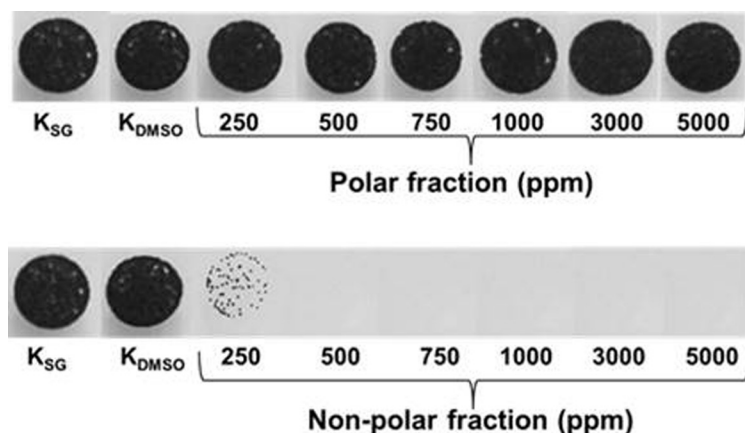
Figure 1. Spot test of *C. reinhardtii* 137C+ after treatment with different concentrations of polar and non-polar fractions of *O. vulgare* ssp *hirtum* methanolic extract.
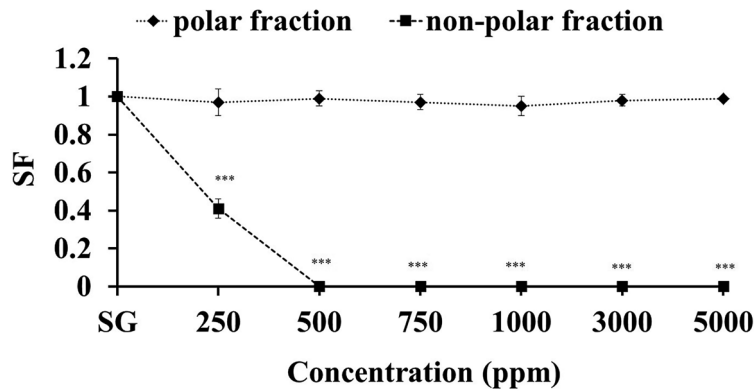
Figure 2. Cells survival fractions (*SF*) after the treatment with polar and non-polar fractions from *Origanum vulgare* methanolic extract. Mean data are from three independent experiments. Error bars represent standard errors of mean values. Where no error bars are evident, errors were equal to or less than the symbols. One-way ANOVA analysis reveals statistically significant differences between SG vs. all concentrations non-polar fraction (***P < 0.0001).

Table 2. Mutagenic activity of the polar and non-polar fraction of *Origanum vulgare* spp. *hirtum* methanolic extract in *C. reinhardtii* 137C. One-way ANOVA analysis reveals statistically significant differences between SG vs. all concentrations non-polar fraction (ns: non-significant; ***P < 0.0001).

Variants	SF	"Visible" mutations (%)	Mutagenic index (IM)
SG	1	0.098 ± 0.0002	
DMSO 1000 ppm	0.99 ± 0.001[ns]	0.117 ± 0.0002 [ns]	0.198
250 ppm polar fraction	0.97 ± 0.07 [ns]	0.120 ± 0.0003 [ns]	0.232
500 ppm polar fraction	0.99± 0.04 [ns]	0.110 ± 0.0002 [ns]	0.128
750 ppm polar fraction	0.97 ± 0.04 [ns]	0.119 ± 0.0005 [ns]	0.227
1000 ppm polar fraction	0.95 ± 0.05 [ns]	0.114 ± 0.0003 [ns]	0.169
2000 ppm polar fraction	0.98 ± 0.03 [ns]	0.131 ± 0.0005 [ns]	0.358
3000 ppm polar fraction	0.99 ± 0.002 [ns]	0.118 ± 0.0003 [ns]	0.210
250 ppm non-polar fraction	0.41 ± 0.08***	0.569 ± 0.001***	4.83

DNA, respectively. The absence of "visible mutations" after the treatment with a non-polar fraction in the concentration range of 500–5000 ppm could be explained with the 100% cell lethality.

DNA damaging potential

Our findings illustrated no DNA damaging capacity of the polar fraction – no statistically significant higher levels of DSBs were calculated after the application of the tested concentrations (Fig. 3). Well-expressed and concentration-dependent induction of DSBs for even the strong DNA fragmentation was observed after the treatment with a non-polar fraction (Fig 3). No statistically significant difference between DSBs levels measured after the treatment with 750 and 1000 ppm of the non-polar fraction was scored (p > 0.05).
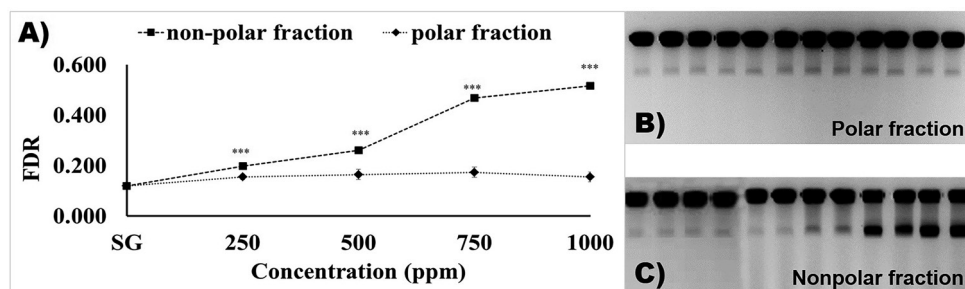
Figure 3. DSBs induced after the treatment with polar and non-polar fractions of *Origanum vulgare* spp. *hirtum* methanolic extract. Mean data are from three independent experiments. Error bars represent standard errors of mean values. Where no error bars are evident, errors are equal or less than the symbols. One-way ANOVA analysis reveals statistically significant differences between SG vs. all concentrations of the non-polar fraction (***P < 0.0001).

Discussion

The present work provides evidence for the differences in the bioactivity of polar and non-polar fractions isolated from *Origanum vulgare* spp. *hirtum* methanolic extract.

The polar fraction was shown to possess no toxic/genotoxic, mutagenic or DNA damaging potential on *Chlamydomonas reinhardtii* WT 137C.

Contrary to these results, the well-expressed genotoxic, mutagenic and DNA damaging potential of the non-polar fraction was revealed, depending on the concentration applied. SF data revealed a very strong decrease of cell survival in the range of 250 to 500 ppm, after that no effect of concentration was found. No survived colonies after the treatment with concentrations in the range 500–1000 ppm were scored. A concentration-dependent increasing of non-polar fraction induced DSB was found up to 750 ppm; after that, statistically not significant differences were calculated. Comparing the concentration-effect curves for the evaluation of SF and DSBs induced, we found that they are not completely similar. This confirmed our previous finding with Zeocin that other mechanisms in addition to DSB induction probably could be involved in the formation of cell death (Chankova et al. 2007).

Significant differences in the phytochemical content were obtained. The polar fraction was found rich in sucrose, while the main constituent of the non-polar one was carvacrol. Based on the reported results, it could be suggested that the strong genotoxic effect of the non-polar fraction could be attributed to the presence of carvacrol. Additionally, such difference could be explained with the previously reported data that essential oils and non-polar extracts are better absorbed into the algae cells than methanolic and polar extracts (Yi et al. 2011). Lipophilic compounds, such as carvacrol, may induce alterations to the cell membrane physico-chemical properties and thus allowing easier entry into the cells (Ben Arfa et al. 2006; Yi et al. 2011; Barani et al. 2015).

Based on this, it could be suggested that the carvacrol as a main constituent of the fraction could be responsible for the toxic/genotoxic and DNA damaging potential,

inducing DSB. At present, the available data in the literature reported the potential phytotoxicity of carvacrol, based mainly on results concerning seed germination, root length etc. (Kordali et al. 2008; Koiou et al. 2020; Zhou et al. 2021). De Assis Alves et al. (2018) provided evidence for the genotoxic properties of carvacrol on *Lactuca sativa* and *Sorghum bicolor*. Carvacrol has been classified with chronic aquatic toxicity according to CLP (Directive 1272/2008/EC) and European Chemicals Agency (ECHA) (https://echa.europa.eu/bg/registration-dossier/-/registered-dossier/23562/6/1).

Our study provides new data that the non-polar fraction at a concentration of 250 ppm possesses mild mutagenic activity by induction of mutations related to delayed cell division and point mutations in nuclear or chloroplast DNA. These results are in accordance with another study where carvacrol is reported to affect the plant cell cycle (Pinheiro et al. 2015). Additionally, this fraction is found to induce double-strand breaks in DNA. All of this provides evidence for the genotoxic potential of fractions with a high quantity of carvacrol.

Conclusion

Based on the reported data, it could be concluded that the different bioactivity of the two fractions correlates well with their different chemical composition. The polar fraction, rich in sugars, organic acids and flavonoid glycosides, did not possess genotoxic, DNA damaging and mutagenic potential.

To our present state of knowledge, this study provides the first information that the carvacrol-rich (37.08%) non-polar fraction of *Origanum vulgare* spp. *hirtum* methanolic extract possesses a genotoxic, mutagenic and DNA-damaging effect on some low eukaryotes, such as *C. reinhardtii*. Further experiments with carvacrol should be done in order to clarify the exact mechanism of action.

Acknowledgements

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RESEARCH ARTICLE

BioRisk

Screening of *Amorpha fruticosa* and *Ailanthus altissima* extracts for genotoxicity/ antigenotoxicity, mutagenicity/antimutagenicity and carcinogenicity/anticarcinogenicity

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Abstract

The aim of the present study was to evaluate the potential genotoxic/antigenotoxic, mutagenic/antimutagenic, and carcinogenic/anticarcinogenic effect of *Amorpha fruticosa* (AF) fruit, *Ailanthus altissima* bark hexane (AAEH) and methanol (AAEM) extracts on a model system Saccharomyces cerevisiae.

Plants were identified and extracted by Ekaterina Kozuharova. Three concentrations of each extract were tested – 10, 100 and 1000 µg/ml. In vitro pro-oxidant/antioxidant activities were evaluated by DPPH and DNA topology assay. The potential genotoxic/antigenotoxic, mutagenic/antimutagenic and carcinogenic/anticarcinogenic effects were revealed in vivo by: Zimmermman's test on Saccharomyces cerevisiae diploid strain D7ts1, and Ty1 retrotransposition test on S. cerevisiae haploid strain 551. Zeocin was used as a positive control.

Based on the in vitro antioxidant activity the extracts could be arranged as follows: AF>AAEM>AAEH. AAEH possessed moderate oxidative potential. No genotoxic and mutagenic capacity was obtained in vivo for extracts tested. The levels of total aberrants, convertants and revertants were comparable with the control ones. No Ty1 retrotransposition was induced by extracts treatment. Further, the extracts possessed well-expressed antigenotoxic, antimutagenic and anticarcinogenic activity. Significant reduction of the total aberrants, reverse point mutations and Ty1 retrotransposition was obtained. Only the AF extract was found to reduce the levels of zeocin-induced mitotic gene conversion.

The three extracts did not possess any genotoxic, mutagenic and carcinogenic effect on Saccharomyces cerevisiae. Based on their protective activity, they can be arranged as follows: AF>AAEM>AAEH which corresponds well with their phytochemical composition. Further experiments could provide more detailed information concerning the mode of action of extracts, as well as their main constituents.

Introduction

Invasive plant species are considered to be one of the main reasons for biodiversity loss (Luís et al. 2012; Weidlich et al. 2020; Dyderski and Jagodziński 2021). Their distribution to new areas has led to massive extinction of plant and animal species in the last few years (Panjković et al. 2021; Szumańska et al. 2021). Two alien plant species posing an increasing threat in Bulgaria are *Amorpha fruticosa* and *Ailanthus altissima.* Both are characterized by high tolerance to various habitat conditions and aggressive invasion due to the lack of suitable herbivores to control their populations (DAISIE 2009; Monaco 2014; Global Invasive Species Database 2019).

A. fruticosa L. (Fabaceae), known as false indigo, false indigo-bush, and bastard indigobush is a shrub native to North America (Wilbur 1975; USDA NRCS 2009). The plant has a high quantity of isoflavonoids, rotenoids and prenylated stilbenoids. Among the prenylated stilbenoids, the group of amorfrutins is quite diverse (Kozuharova et al. 2017).

A. altissima (Mill.) Swingle (Simaroubaceae), known as the tree of heaven is native in China. It was introduced in Europe and North America around the end of the 18th century (Luís et al. 2012; Andonova et al. 2021). It contains alkaloids, terpenoids and aliphatic volatiles (Kundu and Laskar 2010). The phytochemical composition is reviewed by Kožuharova et al. (2014). The phytochemical analysis of the bark reveals the presence of more than 221 compounds such as alkaloids, quassinoids, phenylpropanoids, triterpenoids, volatile oils, and other compounds (Li et al. 2021).

Both plants are used in traditional medicine. *A. altissima* is often applied for the treatment of asthma, epilepsy, spermatorrhea, bleeding, ascariasis, cold, gastric (dysentery) and ophthalmic diseases, etc. (Luís et al. 2012; Kozuharova et al. 2020; Li et al. 2021). The ethnobotanical application of *A. fruticosa* is related to the treatment of stomach pain, intestinal worms, eczema, neuralgia, and rheumatism (discussed in Kozuharova et al. 2017).

It the present work we hypothesized that *A. fruticosa* fruit extract and *A. altissima* bark extract would be safe and could decrease the zeocin-induced mutagenic, recombinogenic and carcinogenic effects on *Saccharomyces cerevisiae* model organism. As these plant species are very invasive growing almost unrestrictedly, they can provide abundant and cheap resources of bioactive compounds. Their pharmacological application

may lead to excessive harvesting and thus, a decrease in their populations as a strategy for the protection of native plant habitats. Both plants are promising candidates for the pharmacology. Even though, data in literature point out that the toxicity evaluation of the plant extracts is scarce (Kozuharova et al. 2017; Li et al. 2021).

Thus, the aim of the present study was to evaluate the potential genotoxic/antigenotoxic, mutagenic/antimutagenic and carcinogenic/anticarcinogenic effect of *A. fruticosa* fruit extract (AF) and *A. altissima* bark hexane (AAEH) and methanol (AAEM) extracts on *Saccharomyces cerevisiae*.

Materials and methods

Fruits of *Amorpha fruticosa* were collected in October 2018 from a location near Pasarel village, Sofia district. Stem bark of *Ajlanthus altissima* was collected in September 2018 from a location in Sofia, Bulgaria. The plant materials were dried at room temperature, then pulverized and sieved. The fruits of *A. fruticosa* were macerated with chloroform to remove the lipophilic compounds (both the fixed and the essential oils), and then the material was dried and extracted by percolation with 70% methanol. The solvent was evaporated on a rotary evaporator; then the extract was lyophilized and named AF. The stem bark of *A. altissima* was macerated with hexane to produce the lipophilic extract, which was dried *in vacuo* and named AAEH. The resulting defatted substance was percolated with 70% methanol to obtain the hydrophilic extract, which was concentrated, lyophilized and named AAEM.

DPPH radical scavenging activity

The DPPH assay, based on a color reduction of DPPH hydrate from purple to yellow, was applied as described in Todorova et al. (2015). The radical scavenging activity is presented as concentration inhibiting 50% of the DPPH radicals. Ascorbic acid was used as a standard.

DNA topology assay

DNA topology assay was applied according to Todorova et al. (2015). The transformation of supercoiled pBR322 DNA to a relaxed circular form was photographed with UV transillumination using G:BOX (Syngene). The relative quantity of supercoiled DNA was calculated using ImageJ software.

Treatment of Saccharomyces cerevisiae cells

Stock solutions of *A. altissima* hexane (AAEH) and methanol (AAEM) extracts dissolved in 0.1% Tween 20 and *A. fruticosa* (AF) extract dissolved in sterile MQ water were prepared prior to the experiments. Cell suspensions (1×10^7 cells/ml) to the end of the ex-

ponential and the beginning of stationary growth phase were pre-treated with three concentrations – 10, 100 and 1000 µg/ml of AAEH, AAEM and AF extracts for 30 min at optimal conditions (30 °C, 200 rpm). Cells were then washed and after that treated with 100 µg/ml Zeocin for 1 min. Single treatment with Zeocin was used as a positive control. After these procedures, cells were harvested, washed and prepared for further work.

Mutagenicity/antimutagenicity test

Zimmerman's test was applied on *Saccharomyces cerevisiae* strain D7ts1 as described in Todorova et al. (2015); Todorova et al. (2017). The following endpoints were evaluated: cell survival for genotoxic/antigenotoxic and mitotic gene conversion, reverse mutations and mitotic crossingover – for mutagenic/antimutagenic effects.

Carcinogenicity/anticarcinogenicity test

The Ty1 retrotransposition test applied for *in vivo* detection of carcinogenic effect was used as described by Pesheva et al. (2005) using *S. cerevisiae* strain 551 as a tester strain. A "fold increase" higher than two compared to the control, is considered as a positive response of the Ty1 transposition test.

Statistical analysis

The statistical analysis includes an application of One-way ANOVA with Bonferroni's *post hoc* test. P<0.05 was accepted as the lowest level of statistical significance. Concentrations inducing 50% inhibition of the cell growth (IC_{50} values) were calculated using non-linear regression analysis (GraphPad Prizm5 Software).

Results

Preliminary chemical analysis reveals differences among the chemical composition of the extracts: AF fruit extract is rich of flavonoids and stilbenoids (amorfrutins A and B) as it was described previously by (Kozuharova et al. 2017); flavonoids are typical for the extract of AAEM and terpenoids (sterols) for AAEH that corresponds well to the data already published by us (Kožuharova et al. 2014).

Antioxidant potential

Slight to moderate radical scavenging activity of the extracts in comparison with the standard control ascorbic acid was obtained. Based on DPPH assay (Fig. 1), the AF possesses the best radical scavenging activity calculated as IC_{50}=63.71 µg/mL, followed by the AAEM with IC_{50}=696.12 µg/mL. The AAEH show the lowest radical scavenging potential (IC_{50}= 1396.97 µg/mL). The IC_{50} of the ascorbic acid was calculated as 15.94 µg/mL.
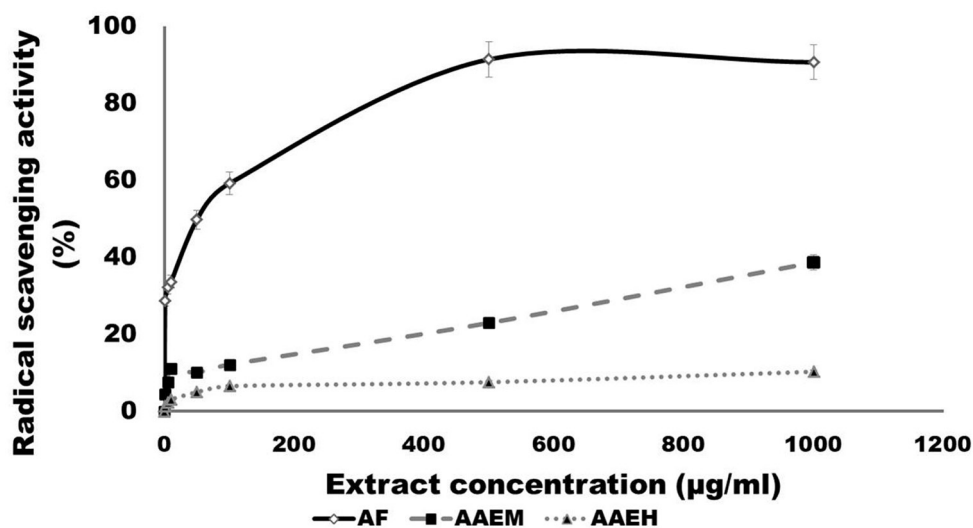
Figure 1. Radical scavenging activity (%) of *Amorpha fruticosa*, *Ailanthus altissima* methanolic and hexane extract. Data are presented mean values from at least three independent experiments.

To evaluate the oxidative potential of the extracts DNA topology assay was performed. This assay provides information not only about the oxidative/antioxidant but also on the DNA damaging/protective effect of the tested extracts. Based on the calculated relative quantity of supercoiled DNA the extracts could be arranged as follows: AF>AAEM>AAEH (Fig. 2). The hexane extract was shown to possess moderate oxidative potential.

Comparing antioxidant properties of the extracts, the moderate protection of AAEH was detected depending on the concentration – 500 and 1000 µg/mL. AF and AAEM did not show good antioxidant properties towards the hydroxyl anions (Fig. 3).
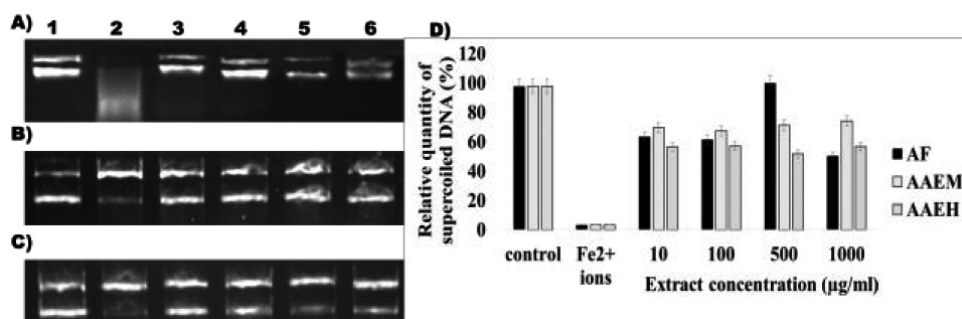
Figure 2. Agarose gel electrophoresis for studying possible DNA damaging effect. Agarose gel electrophoretic patterns of plasmid DNA treated with *A. fruticosa* L. **A** *Ailanthus altissima* methanolic **B** and hexane **C** extract in the absence of Fe^{3+} ions (0.08 mM): lane 1 – DNA control; lane 2 – Fe^{2+} ions control; lane 3 – 10 µg/mL extract; lane 4 – 100 µg/mL extract; lane 5 – 500 µg/mL extract; lane 6 – 1000 µg/mL extract **D** Densitometrical estimation of the relative quantity of supercoiled DNA.
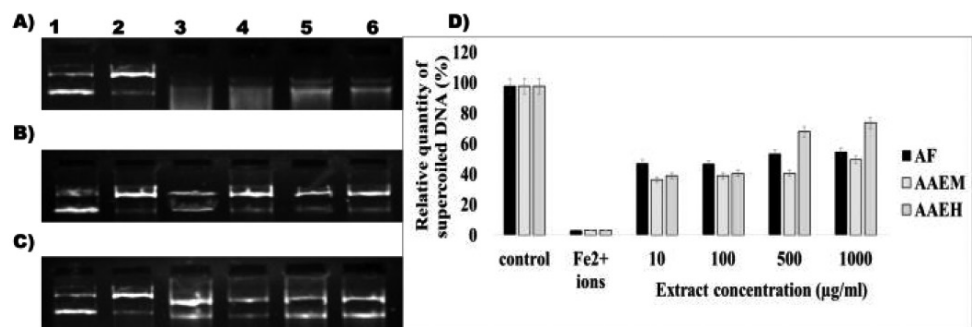
Figure 3. Agarose gel electrophoresis for studying possible DNA protective effect against Fe^{2+} ions. Agarose gel electrophoretic patterns of plasmid DNA treated with *A. fruticosa* L. **A** *Ailanthus altissima* methanolic **B** and hexane **C** extract in the presence of Fe^{3+} ions (0.08 mM): lane 1 – DNA control; lane 2 – Fe^{2+} ions control; lane 3 – Fe^{2+} ions and 10 µg/mL extract; lane 4 – Fe^{2+} ions and 100 µg/mL extract; lane 5 – Fe^{2+} ions and 500 µg/mL extract; lane 6 - Fe^{2+} ions and 1000 µg/mL extract **D** Densitometrical estimation of the relative quantity of supercoiled DNA.

Mutagenicity/antimutagenicity

The survival after the treatments was comparable with the negative control – untreated cells. No effect was obtained regarding the genetic events – convertant and revertant frequencies as well as total aberrants (Table 1). The three extracts did not possess genotoxic and mutagenic properties at the studied concentrations.

Concerning the antimutagenic properties, an increase in the cell survival in comparison with the positive control was measured after all the treatments. Significant reduction of the reverse mutations to levels comparable with that in un-

Table 1. Frequency of gene conversion in *trp5* locus, reversion in *ilv1-92* allele and mitotic crossing-over in *ade2* locus after single treatment of *S.cerevisiae* D7ts1 with 10, 100 and 1000 µg/ml AF, AAEM and AAEH. Zeocin was used as a positive control.

	Extract concentration (µg/ml)	Zeocin (µg/ml)	Survival (%)	Gene conversion/ 10^5 cells	Reversion/ 10^6 cells	Total aberrants (%)
	0	0	100	1.02±0.01	0.003±0.0002	0.437±0.021***
	0	100	32.99±2.75***	4.30±0.7***	0.038±0.0005***	2.331±0.667***
AF	10	0	99.62±1.21***	1.05±0.05***	0.002±0.00009***	0.576±0.150***
	100	0	99.05±3.93***	1.07±0.01***	0.002±0.0001***	0.741±0.163***
	1000	0	99.97±1.64***	1.07±0.07***	0.001±0.00004***	0.507±0.013***
AAEM	10	0	99.41±2.13***	1.15±0.05***	0.002±0.00005***	0.498±0.130***
	100	0	99.37±3.01***	1.13±0.03***	0.003±0.00005***	0.501±0.021***
	1000	0	98.31±1.59***	1.16±0.07***	0.002±0.00009***	0.542±0.043**
AAEH	10	0	96.47±1.98***	1.90±0.08***	0.004±0.00006***	0.602±0.046**
	100	0	89.11±2.04***	1.60±0.05***	0.004±0.00002***	0.689±0.016**
	1000	0	97.14±2.43***	2.02±0.04***	0.006±0.00001***	0.802±0.112**

Frequencies are means ± SEM, *n*=4. The significance of differences between positive control (Zeo) and treatment with various extracts' concentrations were calculated by ANOVA with a post-hoc test Bonferroni's Multiple Comparison Test (**P<0.01; ***P < 0.001).

Table 2. Frequency of gene conversion in *trp5* locus, reversion in *ilv1-92* allele and mitotic crossing-over in *ade2* locus after pre-treatment of *S. cerevisiae* D7ts1 with 10, 100 and 1000 µg/ml AF, AAEM or AAEH followed by treatment with 100 µg/ml Zeocin.

	Extract concentration (µg/ml)	Zeocin (µg/ml)	Survival (%)	Gene conversion/ 10^5 cells	Reversion/ 10^6 cells	Total aberrants (%)
	0	0	100	0.52±0.01	0.003±0.0002	0.437±0.021
	0	100	32.99±2.75***	4.30±0.70***	0.038±0.0005***	2.331±0.667***
AF	10	100	94.93±4.08***	0.75±0.50 ***	0.005±0.0009***	0.56±0.16**
	100	100	77.54±1.51***	0.81±0.10 ***	0.006±0.0002***	0.74±0.13**
	1000	100	71.01±9.07***	0.76±0.09 ***	0.005±0.0004***	0.50±0.03**
AAEM	10	100	87.68±1.46***	2.34±0.84 ns	0.021±0.0006***	1.51±0.035 ns
	100	100	76.83±2.41***	3.20±0.57 ns	0.005±0.00014***	0.57±0.056 **
	1000	100	58.06±2.11***	2.84±0.39 ns	0.006±0.00057***	0.76±0.03 **
AAEH	10	100	77.71±3.65***	6.93±0.79*	0.014±0.0035***	1.13±0.078 *
	100	100	71.55±4.59***	3.89±0.53 ns	0.031±0.0046 ns	0.92±0.09 **
	1000	100	84.60±1.24***	2.90±0.67 ns	0.032±0.0086 ns	0.85±0.05 **

Frequencies are means ± SEM, *n*=4. The significance of differences between positive control (Zeo) and treatment with various extracts' concentrations was calculated by ANOVA with a post-hoc test Bonferroni's Multiple Comparison Test (NS: nonsignificant; *$P<0.05$; (**$P<0.01$; ***$P < 0.001$).

treated control was obtained after the pre-treatment with AF and AAEM without concentration's effect. Around 2.5-fold lower levels were measured after pre-treatment with 10 µg/ml AAEH (Table 2). AF at all the concentrations decreased the zeocin-induced mitotic gene conversion. No effect on this genetic event was obtained after pre-treatment with AAEM, while a potentiation of the zeocin recombinogenicity was observed after pretreatment with 10 µg/ml AAEH. The percent of total aberrants after the pre-treatments was also lower than that measured after single zeocin treatment.

Carcinogenic/anticarcinogenic potential

Data revealed that single treatment with 1000 µg/ml AF possesses slight dose-dependent genotoxic effect, reducing the cell survival of strain 551 to 78% (Fig. 4A). None of the other extracts affected the cell survival. On the other side, pre-treatment results in around 2-fold increased cell survival for all the concentrations of the extract in comparison with the cell survival after single zeocin treatment (Fig. 4A). No dose-dependent enhancement of cell survival is observed. The Ty1 retrotransposition events are also found. Our results clearly indicate that none of the tested concentrations of AF, AAEM and AAEH can induce Ty1 retrotransposition in *Saccharomyces cerevisiae*. These data suggest no carcinogenic properties of the extracts.

Further, well expressed anti-carcinogenic activity, measured as a reduction of the transposition rate to levels comparable with the negative control is defined when pre-treatment with concentrations of AF and AAEH is applied (Fig. 4B). The only concentration that cannot protect cells from damaging action of Zeocin is the lowest concentration of AAEM – 10 µg/ml.
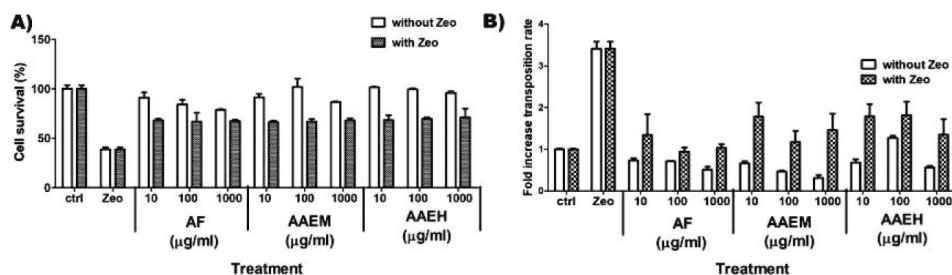
Figure 4. Cell survival **A** and Ty1 retrotransposition rates **B** of *Saccharomyces cerevisiae* strain 551 after treatment with 10, 100 and 1000 μg/ml AF, AAEM and AAEH with or without Zeocin. Where no error bars are evident, errors are equal or less than the symbols.

Discussion

Slight to moderate antioxidant activity *in vitro* of extracts is identified. Such activity of *Amorpha fruticosa* does not correspond to data published by Zheleva-Dimitrova (2013) and Ivanescu et al. (2019). The variation in the radical scavenging activity could be explained by different phytochemical composition based on the geographical origin of plant, variations in the plant extraction and the methodology, etc. Consistence between DNA topology assay results and those obtained by DPPH is found. Moderate oxidative potential leading to single-strand pDNA damage is found for hexane extract.

Our *in vivo* experiments were performed on *Saccharomyces cerevisiae*. *Saccharomyces cerevisiae* has been chosen as a model system for human cell due to the similarities in main stress response pathways (discussed in Todorova et al. 2015). Moreover, experiments on yeasts could be a valuable tool when taking into consideration the Directive 2010/63/EU. This directive is aiming to anchor firmly the „Principle of the Three Rs" – To Replace, Reduce and Refine" the use of animals for experimental and scientific purpose in the EU Member States. According to the Annex (47) there is a need to develop new methods alternative to animal testing and proposed to validation in the European Union Reference Laboratory for alternatives to animal testing (EURL EC-VAM) (http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam). Additionally, experiments on diploid and haploid yeast cells allow obtaining new fundamental information concerning the potential lethal effect of various chemical and physical agents and genetic instability (Evstratova et al. 2018).

The results obtained by us reveal that the three extracts do not affect the cell survival, the mitotic gene conversion in *trp5* locus, reversion in *ilv1-92* allele, mitotic crossing-over in *ade2* locus and Ty1 retrotransposition. From our point of knowledge this is the first finding that *Amorpha fruticosa* fruit extract and *Ailanthus altissima* bark extracts are not genotoxic, mutagenic and carcinogenic in our test-system.

Our research has been extended in order to evaluate the possible antigenotoxic, antimutagenic and anticarcinogenic potential of the extracts against the action of

the radiomimetic Zeocin. Zeocin was chosen as a damaging agent due to several reasons: it is a radiomimetic, member of the bleomycin family of antibiotics, that damages DNA in a way similar to that of ionizing radiation; possesses pro-oxidative capacity (Chankova et al. 2013; Todorova et al. 2015), mutagenic, and carcinogenic effect in *Saccharomyces cerevisiae* (Todorova et al. 2015), clastogenic, DNA damaging, and genotoxic effects in microalgae, higher plants, and human lymphocyte cell culture (Chankova et al. 2007; Dimova et al. 2009; Kopaskova et al. 2011; Gateva et al. 2015).

In this study it was demonstrated that pre-treatment with extracts could protect cells from the genotoxic action of Zeocin measured as cell survival. No relation between the cell survival and pre-treatment concentrations was identified. Concerning the antimutagenic capacity of extracts the specificity of their action was obvious. Significant reduction of the total aberrants was obtained after the treatment with the extracts. The only extract reducing the mitotic gene conversion was AF. No effect was observed after the pre-treatment with AAEM. A significant increase in the levels of this genetic event was measured when pre-treatment with 10 μg/ml AAEH which is in accordance with another study where sterols are reported to potentiate the activity of another member of the zeocin family – bleomycin (Hoffmann et al. 2011). Such differences in the activity could be related to the phytochemical content of the extracts. Isoflavonoids are the major constituents of AF extract while AAEH is characterized by the predominance of phytosterols. Flavonoids are already known to possess good antimutagenic properties. Based on the available literature and the present results it could be suggested that AF with flavonoinds as main constituents may protect yeast cells from zeocin-induced mitotic gene conversion and crossing over by activation of HR repair and modulation of chromatin structure.

On the other side, significant amelioration of the reverse point mutations and Ty1 retrotransposition was observed. It is well known that the antimutagenic and anticarcinogenic properties could be related to significant antioxidant activity or to activation of DNA repair processes. As in our *in vitro* experiments evidence was provided for mild to moderate antioxidant activity of extracts tested, it could be suggested that in this case the reduction of the genetic events is not related to the antioxidant potential. Having in mind that the reverse mutation frequency is used for measurement of error prone recombination (Mitchel and Morrison 1986), we could speculate that the potential mechanism of action of the extracts may be an activation of protective enzymes independent of those required for HR.

From our point of knowledge for the first time it was shown by us that *Amorpha fruticosa* fruit extract and *Ailanthus altissima* bark extracts possess no genotoxic, mutagenic and carcinogenic capacity on a model system *Saccharomyces cerevisiae*.

Based on their protective activity, they can be arranged as follows: AF>AAEM>AAEH that corresponds well with their phytochemical composition. Further experiments could provide more detailed information concerning the mode of action of extracts, as well as their main constituents.

Acknowledgements

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RESEARCH ARTICLE

BioRisk

Light and auxin treatments affect morphogenesis and polyphenolics productivity in *Artemisia alba* Turra cell aggregates in vitro

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Abstract

Artemisia alba Turra is an essential oil-bearing shrub, with a Euro-Mediterranean distribution widespread in the south-eastern parts of Europe. Phytochemical investigations have evidenced the presence of volatile mono- and sesquiterpene derivatives, as well as non-volatile sesquiterpenoids, flavonoids and phenolic acids contributing to the anti-inflammatory, antimicrobial, antioxidant and pro-apoptotic activity of different preparations, obtained from the plant. The current research aims at elucidation of the potential for biotechnological polyphenolic compounds productivity of non-differentiated cell lines of the plant. For this purpose, non-differentiated cell aggregates were initiated from either leaf or root explants of the sterile grown plant. They were cultivated either in the dark or at 16/8 h photoperiod in liquid media, supplemented with N^6-benzyladenine (BA) as auxin. The cytokinin effects of indole-3-butyric acid (IBA) and 1-naphthalene acetic acid (NAA) were compared. It was established that NAA supplementation was superior to IBA and light treatment – to dark growth conditions in terms of polyphenolics productivity. In addition, NAA supplementation led to better expressed compaction and larger size of the cell aggregates as compared with IBA. The results of the present experiment indicate that secondary metabolites productivity *in vitro* is a dynamic process closely related to the plant's growth and development and is in close relation to the interactions of the plant with its environmental conditions.

Introduction

The production, accumulation and translocation of secondary metabolites within the integral plant organism are determined by the presence and organisation of highly specialised anatomical structures. Thus, growth, development and morphogenesis of the plant individual play crucial roles for the production and accumulation of secondary metabolites. Since growth and development are dynamically related to the interactions of the plants with their surrounding environment, the latter plays a crucial role in determining the plants secondary metabolite profile. Plant cell tissue and organ culture represents the convenience of a controlled environment with the flexibility to alter selected factors related to *in vitro* cultivation and, thus, modify the production of desired secondary metabolites *in vitro* (Danova 2018).

Genus *Artemisia*, tribe Anthemideae, subtribe Artemisiinae is one of the largest in the Asteraceae family with more than 500 species and subspecies (Zhen et al. 2010). Representatives of the genus have been included in traditional medicinal preparations from centuries for treatment of conditions such as fever, high blood pressure, diabetes, gastrointestinal disorders, parasites etc. (Tu 2016; Zeb et al. 2018; Danova 2020). Scientific evidence for the pharmacological potential of *Artemisia* species is the presence of triterpenes, steroids, hydrocarbons, polyacetylenes, flavonoids, coumarins, mono- and sesquiterpenoids isolated from representatives of the genus (Maggio et al. 2012). Research has confirmed the cytotoxic, antihepatotoxic, anti-bacterial, antifungal and antioxidant properties of preparations of the species (Tan et al. 1998; Bora and Sharma 2011).

A. alba Turra (syn. *A. lobelii* All, *A. camphorata* Vill., *Artemisia biasolettiana* Vis, *A. suavis* Jord., *A. incanescens* Jord.) is an essential oil-bearing shrub natively distributed in south-eastern Europe (Radulović and Blagojević 2010; Biondi and Galdenzi 2012). Its decoction has been traditionally used in the Mediterranean Region as a tonic and stomach digestive (Rigat et al. 2007). Phytochemical studies on the volatile (camphor, 1,8–cineole and artemisia ketone dominating components of the essential oil) and non-volatile (kaempferol, luteolin and apigenin derivatives, rutin, oxygenated sesquiterpenoids, chlorogenic acid, dicaffeoylquinic acids, scopoletin, umbeliferone etc.) constituents of the species have been performed, giving the scientific grounds for the established anti-microbial, anti-inflammatory, antioxidant properties of the species (Stojanovic et al. 2000; Stalińska et al. 2005; Danova et al. 2020).

In previous research conducted on differentiated shoot cultures of the species, it was established that the development of the root system as a result of exogenous plant growth regulators treatment was decisive for the profile of the essential oils, derived from the aerial parts of *A. alba* Turra. Thus, plantlets with well-developed root system (in plant growth regulators-free media, as well as in media supplemented with 0.5 mg/l or 1.0 mg/l indole-3-butyric acid (IBA)) were characterised with over 2.5 times higher ratio of the mono/sesquiterpenoids content in the essential oil, as compared with plantlets with supressed rooting and callus formation at the explant base (in media where 0.5 mg/l or 1.0 mg/l IBA were combined with 0.2 mg/l benzyl adenine (BA)) (Danova et al. 2018).

Continuing the previous research on *Artemisia alba*'s biotechnological properties, we set as an aim to develop a fast-growing non-differentiated *in vitro* system of *Artemisia alba* Turra with high biosynthetic potential regarding the production of polyphenolics. A comparative analysis of the morphogenic and biosynthetic response of liquid cell-aggregate cultures derived from leaf and root explants with different photoperiod and different type of auxin (IBA or 1-Naphthylacetic acid, NAA), in the presence of the same cytokinin (BA) was conducted.

Materials and methods

Plant material

Shoot cultures were initiated through surface sterilisation of stem explants of field grown *A. alba* as previously described (Danova et al. 2012). Stock shoot cultures were kept on plant growth regulators (PGR) – free medium supplemented with the Murashige and Skoog (Murashige and Skoog 1962) macro- and micro-salts medium. Stock cultures were kept at a temperature of 25 $\pm$ 0.1 °C, 16/8 h photoperiod, light intensity 60 μmol m^{-2}s^{-1}, period of sub-cultivation 4 months.

Tissue culture experiment

For the needs of this research, leaf (A1) and root (A2) explants of the *in vitro* grown stock plants were used. Five leaf (A1) and root (A2) explants were inoculated into two types of liquid media with total volume 50 ml in Erlenmeyer flasks (Table 1). Media were prepared with macro- and micro-elements and vitamins according to Murashige and Skoog (1962), 30 g.l^{-1} sucrose and the cytokinin benzyladenine. We experimented with two types of auxin – indole-3-butyric acid and naphthylacetic acid as shown in Table 1. For each treatment, five separate culture vessels were placed in two types of light conditions – 16/8 photoperiod and in the dark. All flasks were placed on an orbital shaker, 100 rpm. That led to the formation of the eight experimental lines (Table 1). The biological experiment of non-differentiated cell aggregate culture induction from explants of the sterile grown plan was repeated in triplicate.

Table 1. The eight *A. alba* experimental variants.

		BA [mg.l^{-1}]	IBA [mg.l^{-1}]	NAA [mg.l^{-1}]	Photoperiod
1	A1 ER_3 hv	0.1	1.5	-	16/8
2	A2 ER_3 hv	0.1	1.5	-	16/8
3	A1 ER_3NAA hv	0.1	-	1.5	16/8
4	A2 ER_3NAA hv	0.1	-	1.5	16/8
5	A1 ER_3	0.1	1.5	-	Dark
6	A2 ER_3	0.1	1.5	-	Dark
7	A1 ER_3NAA	0.1	-	1.5	Dark
8	A2 ER_3NAA	0.1	-	1.5	Dark

Morphometric observations

The dynamics of the morphometric response of explants was tracked by stereomicroscopy using the Stereomicroscope Leica M60. Stereomicroscopy was performed 2 and 4 months after the initial induction of the cell lines. For the observations, plant material from at least two separate culture vessels was sampled.

Biochemical analyses

The quantitative content of malondialdehyde (Dhindsa et al. 1981) and H_2O_2 (Jessup et al. 1994) were assayed spectrophotometrically.

Phytochemical analyses

Spectrophotometric determination of the total content of phenolic (Singleton et al. 1999) and flavonoid compounds (Zhishen et al. 1999) was performed. For the analyses, plant material from at least four separate culture vessels was sampled. Measurements were performed in triplicate.

Statistical processing

The respective number of samples tested, measurements and biological repetitions have been mentioned at each method. The SEM values (standard error of the mean) are reflected in Figures. The means have been compared by t-test of unequal variances at $P < 0.05$. Unless otherwise stated, differences are considered statistically significant at $P \leq 0.05$.

Results and discussion

Impact of growth regulators and photoperiod on growth and development

Changes in the fresh/dry weight ratio, two and four months after induction of the suspension lines are shown in Figure 1. Biomass accumulation was influenced both by the type of initial explant (leaf or root) and the photoperiod. When comparing this parameter 2 and 4 months after initiation, the different capacity of dry biomass accumulation (expressed by a lower FW/DW ratio) in the different treatments is more noticeable. Two months after the initial induction, the FW/DW ratio of the eight lines has relatively similar values, the variants grown in the light tending to be more hydrated (expressed by a higher FW/DW ratio), compared to those grown in the dark (except A2 ER_3NAA).

In addition, a higher water accumulation tendency was observed in lines obtained from root (A2) vs. the ones obtained from leaf explant (A1). Two months after the in-

duction, no cell lines, being strongly productive in terms of dry biomass accumulation, could be observed, the FW/DW parameter being relatively similar in-between them.

However, after two consecutive passages of the suspension cultures (4 months after induction), cell lines grown in the light showed a significant drop in the FW/DW ratio, indicating the tendency of higher dry biomass accumulation.

Some of the cell lines grown in the dark (A1 ER_3, A1 ER_3NAA and A2 ER_3NAA) show a higher result compared to the ones grown in the light, indicating higher hydration, as the difference in leaf explants treated with IBA (A1 ER_3) is the most significant. This may be due to both reduced abilities to accumulate dry biomass and differences in the density of cell aggregates in cultures and the change in osmotic pressure in cells. The largest differences in the FW/DW ratio are observed in leaf explants grown in the dark A1 ER_3 and A1 ER_3NAA, which could be explained by the impaired photosynthetic ability due to lack of light (Fig. 1).

Stereomicroscopy of the eight plant lines 2 months after induction.

When performing the stereomicroscopy 2 months after induction, the presence of differentiated plant organs and tissues was still observed (Fig. 2). It was more pronounced in root (A2) vs. stem (A1) explants and in NAA treatments as compared with IBA ones. In the light-grown lines, intense green pigmentation was clearly observed, as compared with the ones grown in the dark, including both light-grown root lines, which is a visual indication of the formation of photosynthetic pigments. In the case of leaf explants with both auxins, callus formation was observed and, in the variant treated with IBA in light, it was less dense, lighter in colour and presence of antho-
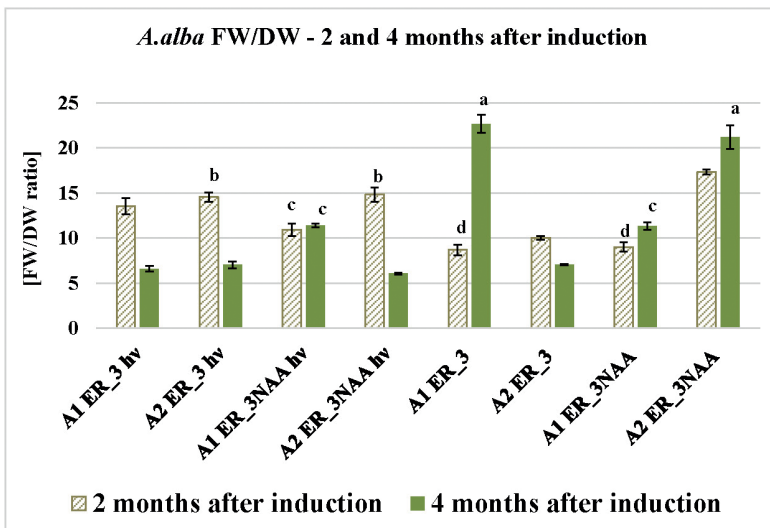
Figure 1. Changes in the FW/DW ratio 2 and 4 months after initial induction of the cell lines, respectively. Same letters denote statistically non-significant differences.
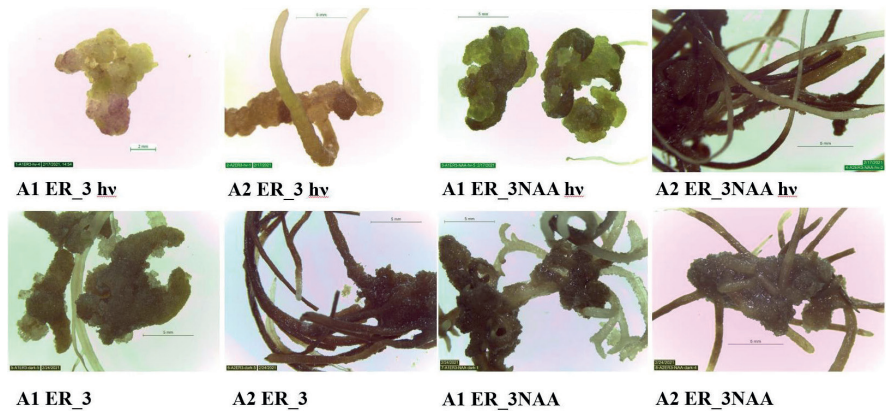
Figure 2. Stereomicroscopic imaging of the eight plant lines 2 months after initial line induction.

cyanin pigments was observed, in contrast to the variant treated with NAA, in which we observed intense green colouration, denser cell aggregates and relatively stronger organogenesis. The tendency for stronger organogenesis under the influence of NAA was maintained in the dark, as the leaf explant clearly showed many etiolated (due to lack of light) stems.

Stereomicroscopy of the eight cell lines 4 months after induction

When performing the same analysis 4 months after the initial induction (after two passages of the induced structures described earlier), the degree of differentiation decreased significantly, as indirect organogenesis was again observed more clearly in the lines obtained from root explants (A2) (Fig. 3). There were no significant differences in terms of indirect organogenesis when comparing the NAA and IBA treatments.
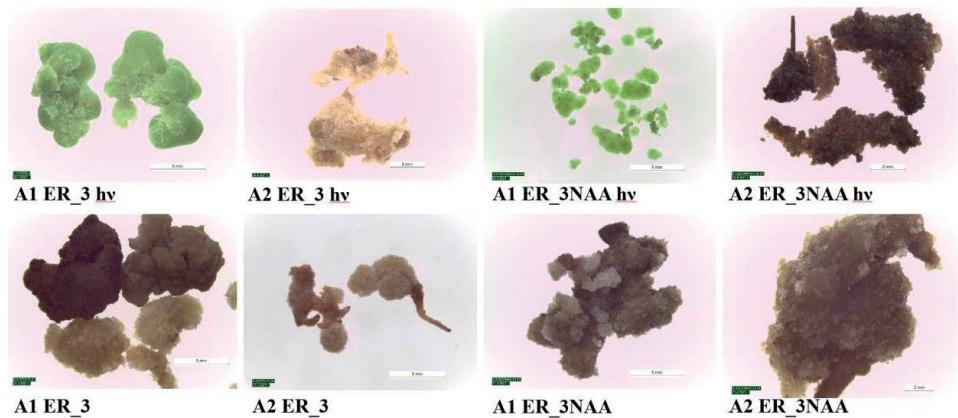
Figure 3. Stereomicroscopic imaging of the eight plant lines 4 months after initial line induction.

IBA-treated lines formed larger cell aggregates, which, however, were less dense, more brittle and looser. The cell aggregates obtained by NAA treatment were smaller in size, but significantly denser and more compact. In the case of aggregates, obtained from leaf explants in the light, we can assume the presence of photosynthesis to some extent, due to the preservation of the intense green colour. After stabilisation, the A2 lines, both the light and dark, formed aggregates with white to brownish colour, without green colouration.

Photographic characterszation of the eight cell lines 6 months after the initial induction

Six months after the initial induction and after 3 passages, all cultures were already completely de-differentiated (Fig. 4). The formation of cell aggregates was also observed in the eight cell lines and they were relatively larger in size in the lines induced by leaf (A1), as compared with the ones obtained from the root (A2) explant. In the lines obtained from leaf explant grown in the dark (A1 ER_3, A1 ER_3NAA), there was a gradual loss of colour in the de-differentiated cultures. A weak green staining was only preserved in the lines obtained from light-grown leaf explants (A1 ER_3 hv, A1 ER_3NAA hv), being weaker in the NAA treatment. Sporadic morphogenesis was only observed in the A2 lines, in support of the assumption that they were more difficult to bring to a fully de-differentiated culture.

MDA content in the eight experimental lines

Changes in malondialdehyde content in the eight plant lines are shown in Figure 5. In both light and dark, MDA is relatively high in leaf explants compared to root and the trend is more visible in variants grown in the presence of light.

Again, this may be due to the more active photosynthesis under illumination and the presence of a larger relative amount of photosynthetic tissue in the original stem
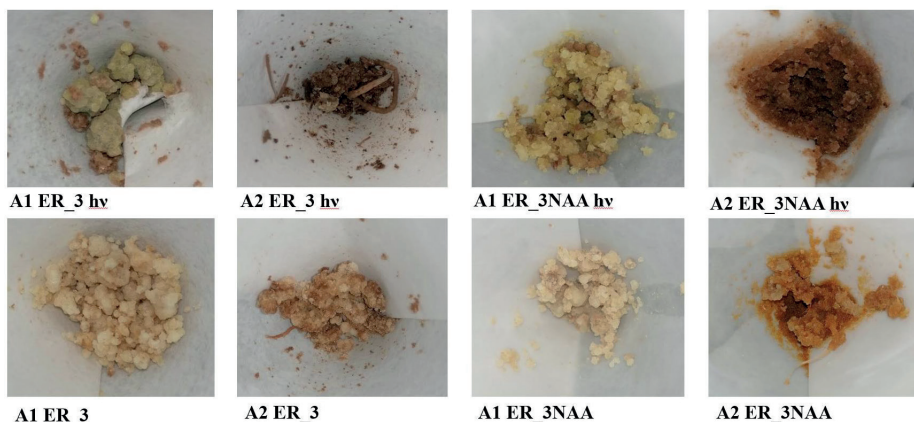
Figure 4. Photographic characterisation of the eight plant lines 4 months after initial line induction.
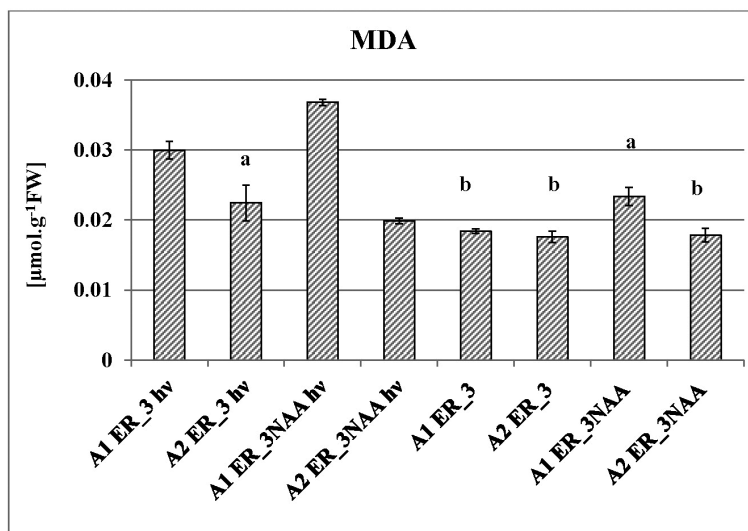
Figure 5. Changes in malondialdehyde ($\mu mol.g^{-1}$ FW) content in lines, induced from leaf and root of *A. alba* under the influence of different growth regulators and at different photoperiods. *p < 0.05.* Statistically insignificant differences in the average values of the measured parameters are indicated in identical letters.

explant. The drop of MDA levels in the absence of light may be due to inhibition of photosynthetic processes in A1 lines, respectively generation of less active forms of oxygen and lower lipid peroxidation. We can note that the trend observed in MDA was also maintained in the amount of H_2O_2 – light-grown variants, expressing significantly higher values than in the dark-grown ones, which can again be explained by the decrease in photosynthetic activity.

Hydrogen peroxide content in the eight experimental lines

Changes in hydrogen peroxide content in the eight plant lines are shown in Figure 6.

There was a slight decrease in the amount of hydrogen peroxide in the root explants treated with NAA compared to those treated with IBA.

The relations with the type of explant and the auxin applied was also clear – when using NAA, A2 lines always had lower levels of H_2O_2, respectively lower levels of oxidative stress, while, when using IBA, there was an inverse dependency – always A2 lines had higher levels of H_2O_2, regardless of the light treatment. When comparing the two parameters, we can note, as a tendency, that both MDA and H_2O_2 were higher in light-grown lines, probably due to the generation of ROS from the ongoing photosynthetic processes. However, in light-derived A2 lines explants, the lower MDA as compared to A1 lines did not correlate with higher H_2O_2 levels, indicating that, in the presence of light, the root tissue generated high H_2O_2 levels, which led to activation of the antioxidant protection of the cells and a correspondingly lower degree of lipid oxidation. In the case of lines obtained from leaf explants grown in the light, both parameters were high
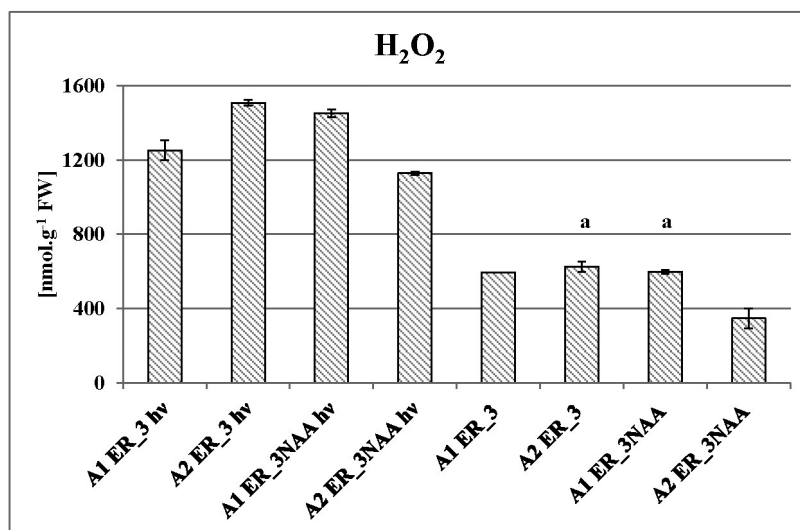
Figure 6. Changes in hydrogen peroxide (nmol.g^{-1} FW) content in lines, induced from leaf and root of *A. alba* under the influence of different growth regulators and at different photoperiods. *p < 0.05*. Statistically insignificant differences in the average values of the measured parameters are indicated in identical letters

and, in the case of lines obtained from leaf explants grown in the dark, they dropped. Probably the A1 lines, grown in the light, retain their photosynthetic ability to some extent and, accordingly, this leads to the generation of both MDA and H_2O_2, while, in the dark, this ability is limited and we see that, both morphologically, they completely lose their green pigmentation, together with the significant drop of MDA and H_2O_2, indicating lower levels of stress, less ROS and a lower degree of lipid peroxidation.

Content of phenols and flavonoids in the eight experimental lines

Phenolic and flavonoid compounds content in the eight plant lines is presented in Figure 7 and Figure 8, respectively.

Phenolic compounds are part of the plant's cellular response to various types of stress. Accordingly, we can assume that their content would increase with a source of stress, such as light.

The experiments show a dependence with a similar trend – the variants grown in the 16/8 photoperiod produced higher amounts of phenolic compounds than those grown in the dark (Fig. 7). There was also a tendency for the amount of phenolic compounds to be significantly higher in the root explants obtained lines, as compared to the leaf ones. Their amount was significantly higher (58% and 140%) in the root-derived lines treated with NAA, as compared to the root-derived explants treated with IBA. The trend that we observed in the levels of total phenolic compounds was analogical as far as flavonoids were concerned (Fig. 8). Again, we observed higher flavonoid levels in the light-grown lines, as compared to the dark-grown ones, which can be
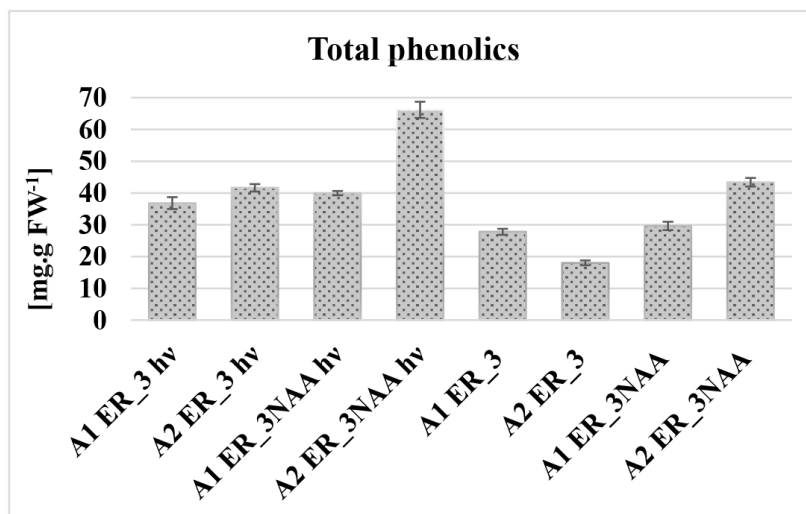
Figure 7. Phenolics content (mg. g^{-1} FW) in lines induced from leaf and root explants of *A. alba* under the influence of different growth regulators and at different photoperiods.
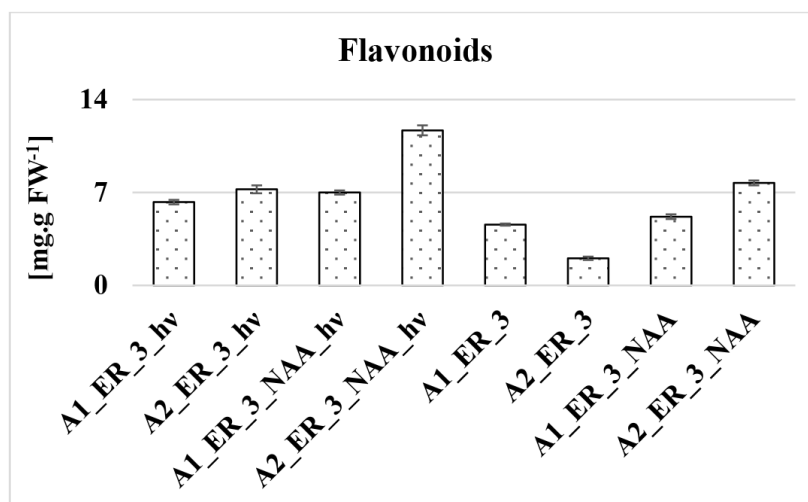
Figure 8. Flavonoid content (mg. g^{-1} FW) in lines induced from leaf and root explants of *A. alba* under the influence of different growth regulators and at different photoperiods.

explained by the higher photosynthetic activity. Thus, the observations on the degree of de-differentiation and its correlation with the levels of MDA, H_2O_2 and phenolic compounds showed that, in the lines initiated by root explant (A2), de-differentiation was much slower, a higher degree of tissue hyperhydricity in the early stages of development was observed and scarce root morphogenesis still occurred even after several consecutive passages. Nevertheless, root-derived cell aggregates of *A. alba* (with the

exception of A2_ER3) were shown to possess the highest biosynthetic capacity of phenolic and flavonoid compounds. Flavonoids have a wide range of applications in pharmacy, well known pharmacological properties, for example, activation or inhibition of specific enzymes, incl. cyclo-oxygenase, lipoxygenase, detoxification of carcinogens and other proven health benefits for humans and animals (Pollastro et al. 2018). They are thought to be involved in controlling the growth and differentiation of plant cells and tissues. Accordingly, higher levels of phenolic and flavonoid compounds of those lines may be related to their impaired ability to de-differentiate. Phenolic compounds have a high absorption capacity; however, evidence for their direct involvement in photosynthetic processes is not yet known (Agati et al. 2012).

Root explants treated with NAA and grown in the light (A2_ER3 hv) showed the best values of all four studied parameters (lipid peroxidation, oxidative stress, phenolic and flavonoid productivity). They were characterised by a strong hydration after the initial induction of the culture, which, however, decreased significantly after the second passage. Accordingly, this line could be distinguished as relatively more productive in this respect.

Artemisia alba Turra *in vitro* was shown to respond to the introduction in a de-differentiated state by activating both biochemical and physiological mechanisms, which were characterised by great dynamics.

The aim of this experiment was to establish the most competent cell lines in terms of biosynthesis of phenolics and flavonoids, experimenting with the presence or absence of light, the type of plant tissue used for induction of cell line (stem and root explants) and with the type of auxin added – IBA and NAA. Regarding the formation of cell aggregates and the differentiation of cultures, clear differences were observed according to the type of auxin used. Lines treated with IBA were easier to de-differentiate, forming bulky cell aggregates, which were, however, less dense. NAA-treated lines show more difficult de-differentiation, especially in those derived from root explants (A2), but after de-differentiation, they formed cell aggregates relatively smaller in size, denser and with a lower fresh/dry weight ratio, respectively, with better biomass accumulation.

In terms of stress markers, again light-grown cell lines showed elevated values compared to those grown in the dark, with the levels of MDA being significantly higher in leaf explants (A1), showing high levels of lipid peroxidation.

In terms of the amount of phenolic and flavonoid compounds, the root lines had a significantly higher synthetic capacity compared to those obtained from leaf explants. There was also a clear dependence on the type of auxin used and, again, NAA shows better results.

Conclusion

When we take into consideration everything said above, we can conclude that, despite the slower and more difficult de-differentiation, in terms of the synthesis of phenolic and flavonoid compounds, the most favourable parameters have been established for the lines obtained from root explants, when NAA was applied as auxin. A2 ER_3NAA_hv and A2 ER_3NAA stand out as the most productive lines for the

production of the target compounds. This may provide future guidance for the development of high-yielding root lines for the synthesis of secondary metabolites from the studied plant *Artemisia alba* Turra.

Acknowledgements

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Influence of proline and methyl jasmonate priming on in vitro seed germination and seedling development of *Chelidonium majus* L

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Abstract

Drought, salinization and heavy metal pollution of soils are main stress factors with an increasing impact on the deterioration of soil quality, yield and crop quality. Seed priming shows good results in improving seed germination, seedling growth and plant development. Proline (Pro) and metyl jasmonate (MeJA) show stimulating activity and help plants overcome stress. The study investigated the effect of Pro, MeJA and hydropriming on seeds sown on water agar supplemented with different concentrations of heavy metals (Cd, Pb, Zn) (HM), NaCl or Polyethylene glycol 6000 (PEG 6000). *Chelidonium majus* is a medicinal species which is grown as a crop in some parts of Europe. It is an ingredient in some remedies and is becoming an increasingly popular object of research regarding its biological activities. The low concentrations of all heavy metals applied increased the germination of all variants of seeds – control, hydroprimed and those which were Pro and MeJA primed. Seed priming with Pro and MeJA promoted high germination percentage of seeds germinated on water agar with NaCl. PEG 6000 at its higher concentration (5%) slightly increased the seed germination of all variants. The growth of roots and hypocotyls was inhibited by HM and NaCl. However, PEG 6000 slightly influenced their growth.

Keywords

Abiotic stress, drought, elicitors, Greater celandine, heavy metals, hydropriming, salt stress

Introduction

Drought, salt and heavy metal stress are among the main abiotic stresses which lead to biochemical, molecular, and morpho-physiological alterations, and as a result influence plant growth, development and metabolism (Bhanuprakash and Yogeesha 2016). Anthropogenic activities such as smelting, mining, overuse of pesticides and fertilizers, sewage sludge disposal directly pollute and worsen the environmental conditions through accumulation of heavy metals. Additionally, industrialization, environmental pollution and unwise water utilization lead to climate changes and the increasing consequences of this – drought and soil salinization (Ullah et al. 2018; Ghori et al. 2019). Drought, salinization and heavy metal pollution of soils have an adverse impact on the deterioration of yield, crop and soil quality and as an extreme case result in the loss of arable land. These stress factors decrease seed germination percentage, uniformity and speed of seed germination, deteriorate seedling growth, reduce root and shoot length. As these problems increase, the quest to create new varieties, ones which could be tolerant to the aforementioned factors, is becoming more and more popular. Such a technique is seed priming, which shows good results in improving seed germination, seedling growth and plant development, and in the process of crop establishment in general. Seed priming leads to partial hydration of seeds which have been soaked in a certain solution but without inducing radicle protrusion (Chen and Arora 2011). There are different methods of priming depending on the pre-sowing conditions in which seeds are set: hydro-, osmo-, halo-, thermopriming, priming using plant growth regulators, matrix priming, etc. Seed priming alleviates stress effects and promotes stress tolerance of crops which could allow them to grow even in adverse conditions. Seed priming reduces germination time and increases germination percentage and plant hardiness even under unfavorable conditions. Moreover, the benefit of this method is the low cost, easy applicability and effectiveness (Bhanuprakash and Yogeesha 2016). Seed germination and early stages of seedling growth are the most vulnerable phases in adverse conditions which could further slow down germination onset, and uniformity and crop growth could be poor with low production (Bhanuprakash and Yogeesha 2016). Seed priming seems to be one of the methods which could improve seed germination and plant growth performance under drought, salt and heavy metal stress (Roychoudhury et al. 2016). In hormonpriming, chemopriming, plant growth regulators and chemicals are used such as proline and methyl jasmonate (MeJA). They both are biosynthesized by plants in order to overcome stress. The amino acid proline (Pro) plays a crucial role in alleviating abiotic stress as an osmoprotectant, a metal chelator and an antioxidant through its accumulation in plants (Hayat et al. 2012; Aslam et al. 2017). Exogenous application of Pro on different plant species contributes to overcoming stress factors. Pro stimulated seed germination of *A. thaliana* (Hare et al. 2003) and *Triticum aestivum* seeds pre-soaked with Pro solutions influenced plant growth under drought stress (Kamran et al. 2009). Pro improved seed germination and seedling growth of rice under salt stress (Roy et al. 1993). The exogenous treatment of *Solanum nigrum* with Pro increased its tolerance as hyperaccumulator to cadmium (Xu et al. 2009). The exogenous usage of plant growth regulators has become recently more popular in order to increase plant tolerance to abiotic stress

(Farooq et al. 2016). MeJA is a phytohormone which acts as a signal in plants which are submitted to abiotic and biotic stress (Fujita et al. 2006). MeJA alleviated the inhibitory effects of Cd on *Solanum nigrum* and *Glycine max* (Keramat et al. 2009; Yan et al. 2015). It mitigated the adverse effects of drought on seed germination and seedling growth of *Oryza sativa* (Sheteiwy et al. 2018) and the effects of salinity stress on primed lemon seeds (Asadi and Jalilian 2021). Seed priming with MeJA increased germination percentage and early seedling growth of *Solanum melongena* (Ali et al. 2019).

Chelidonium majus L. is a medicinal species which has promising pharmacological properties and attracts scientific attention which leads to increasing research. The species is an ingredient of some conventional herbal remedies. Moreover, it is used as a homeopathic drug administered in cases of liver disorders and cancer in humans. The species has anti-inflammatory, anti-tumor, anti-microbial and anti-viral properties (Biswas and Khuda-Bukhsh 2002). *Ch. majus* is cultivated as a crop in several countries in Central Europe (Zielinska et al. 2018). As a crop culture the species will face the challenges of an increasingly changing and more unpleasant environment. The changing environment and increasing pharmacological interest in the species require that the species' hardiness to the growing negative influence of abiotic factors be studied. The aim of the study was to ascertain if seed priming with Pro and MeJA mitigates the adverse effect of heavy metals, salt and drought on seed germination and seedling growth of *Ch. majus* in water agar medium.

Materials

The seeds of *Ch. majus* originated from plants of the species grown in a native habitat in the village of Mramor, near Sofia, Bulgaria (42.7885°N, 23.2794°E). Two-year-old seeds in good condition with uniform size were subjected to three priming treatments: hydro-priming (priming with distilled water), Pro-priming (with 30mM proline), MeJA-priming (with 1 mg/l MeJA). Seed priming was done by soaking the seeds in distilled water, 30 mM Pro or 1 mg/l MeJA solutions for 24 hours at room temperature and then they were dried under a laminar air flow cabinet for an hour. The seeds were soaked in 70% ethanol for 2 minutes as a first step. Then they were sterilized in 0.1% $HgCl_2$ for 2 minutes and then washed three times in sterile distilled water. After that the seeds were cleansed for 10 minutes in NaClO (chlorine < 2.5%) half diluted with sterile distilled water. As a last step the seeds were triple rinsed with sterile distilled water (Doycheva in press). Sterilized seeds were sown in water agar medium. The medium consisted of distilled water solidified with 7 g/l plant agar (Duchefa, NL) and autoclaved at 121 °C for 20 minutes. The autoclaved water agar (5.5 pH) was supplemented with different compounds: $Pb(NO_3)_2$, $ZnSO_4 \cdot 7H_2O$, $CdCl_2 \cdot 2\frac{1}{2}H_2O$, which were added to create heavy metal stress. The chemical compounds were added in order to obtain certain concentrations of metal ions: 100; 150; 250 mg/l Pb^{2+} or Zn^{2+} ions and 1; 5 and 10 mg/l Cd^{2+}. NaCl and Polyethylene glycol 6000 (PEG 6000) were supplemented to the distilled water before adding the plant agar, and then

the medium was autoclaved. The following concentrations were applied: 50, 100, 150 mM NaCl, and 1% and 5% PEG 6000. The control variants were hydro-primed seeds sown on water agar (WA). The seeds were sown in sterilized plastic petri dishes (90 mm in diameter) with hardened medium supplemented with one of the various compounds. The petri dishes were enveloped with Parafilm. Seeds were put in the dark, first at 8 ± 2 °C for 7 days and then at 23 ± 2 °C for 2 weeks. After that seeds were set at 23 ± 2 °C with photoperiod of 16 h light/8 h dark (Doycheva in press).

Each treatment had 3 replications with 20 seeds in each. Radicle emergence was considered a sign of germination. Pro primed seedlings grown on WA + 5% PEG 6000 were contaminated, that is why the values for root and hypocotyl length are absent.

Germination percentage, mean root and hypocotyl length, tolerance index and phytotoxicity percentage were measured to determine the significance of priming on *Ch. majus* seeds to mitigate the adverse effects of stress factors (Rasafi et al. 2016). The closer a certain value of the tolerance index was to 1, the more phytotoxic the studied compound was.

Results were shown as mean values of the three replications with ± standard deviation (SD). Statistical significance was evaluated with Student's t-test at $p \le .05$. Values with different letters in the table and figures were significantly different. The statistical analyses were done using SigmaPlot v. 14.0

Results

Germination percentage, root and hypocotyl length of Pro and MeJA primed seeds germinated on WA supplemented with HM

Seed priming with Pro and MeJA slightly increased the germination compared to that of the seeds sown on water agar without HMs. The addition of Pb^{2+} to the medium raised the germination percentages in all sown seeds, no matter if they were primed with solutions of the elicitors (Pro and MeJA) or if they were just hydroprimed (Fig. 1). Surprisingly, higher Pb^{2+} concentrations resulted in higher germination percentages.

Seeds primed with MeJA had the highest germination percentages in all applied concentrations of Zn^{2+} (Fig. 2). In contrast, the increase in Zn^{2+} concentrations in the medium led to a decrease in the germination percentage of the hydroprimed seeds and those primed with Pro. However, the germination percentage of all primed seeds sown on WA + HM was higher than that of the Control (Fig. 2).

Cd^{2+} had the opposite effect to that of Zn^{2+} on the MeJA primed seeds. The higher Cd^{2+} concentrations were, the higher the reduction in the germination percentage of MeJA pre-soaked seeds was (Fig. 3). Thus, the germination percentage of the seeds primed with MeJA was highest at the lowest Cd^{2+} concentration (1 mg/l) and dropped to 64.71% at the highest Cd^{2+} concentration. The same trend was observed in the germination of Pro primed and hydroprimed seeds (Fig. 3).

HMs availability in the medium reduced the root and hypocotyl length (Table 1). That was most significantly observed in the seedlings grown in medium with Pb^{2+} and
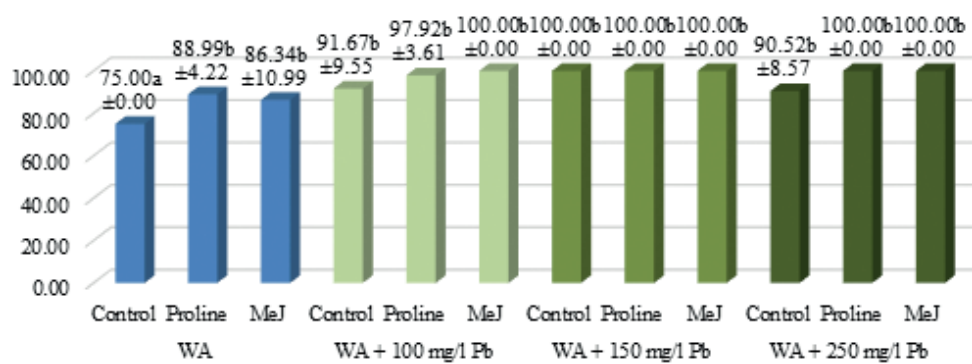
Figure 1. Germination percentage (%) of Pro and MeJA primed seeds germinated on WA + Pb.
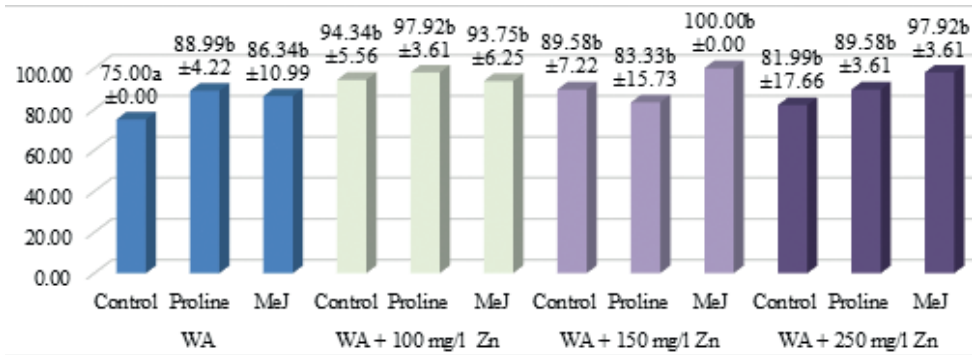
Figure 2. Germination percentage (%) of Pro and MeJA primed seeds germinated on WA + Zn.
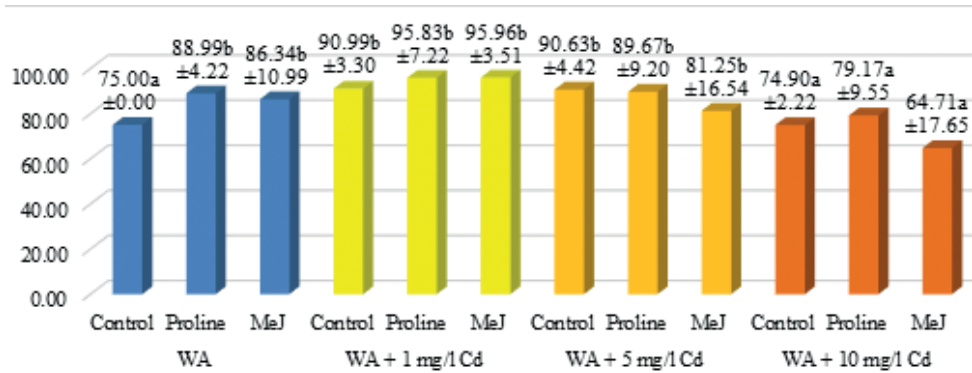
Figure 3. Germination percentage (%) of Pro and MeJA primed seeds germinated on WA+Cd.

Zn^{2+} and less so in those grown in the presence of Cd^{2+}. The radicles of seedlings on media with Pb^{2+} and Zn^{2+} only protruded the seed coat, turned black and perished. Seed priming didn't contribute to overcoming the inhibitory influence of the HM on the growth and development of the seedlings.

Table 1. The effect of HMs, NaCl and PEG 6000 on mean hypocotyl and root length of *Ch. majus* seedlings.

Media	Mean hypocotyl length (mm)			Mean root length (mm)		
	Control	Pro	MeJA	H_2O	Pro	MeJA
WA	29.65a±1.14	31.01a±2.85	30.47a±1.78	23.33a±2.23	21.56a±0.64	21.13a±4.05
Pb^{2+} 100 mg/l	19.53b±1.69	17.94b±2.13	17.76b±1.47	-	-	-
Pb^{2+} 150 mg/l	14.41c±1.06	13.05c±1.18	14.21c±1.85	-	-	-
Pb^{2+} 250 mg/l	11.72c±1.04	11.64c±1.29	11.51c±1.37	-	-	-
Zn^{2+} 100 mg/l	8.89c±1.63	9.60c±2.41	12.54c±2.30	-	-	-
Zn^{2+} 150 mg/l	7.40d±1.44	6.62d±0.79	7.39d±1.50	-	-	-
Zn^{2+} 250 mg/l	6.34d±0.98	5.40d±0.91	6.65d±0.79	-	-	-
Cd^{2+} 1 mg/l	19.74b±2.06	17.02b±0.47	13.18c±2.78	4.03b±0.24	3.54b±0.28	3.33b±0.53
Cd^{2+} 5 mg/l	11.23c±2.21	11.86c±2.67	11.36c±2.44	1.49c±0.22	1.63c±0.04	1.44c±0.31
Cd^{2+} 10 mg/l	12.56c±1.26	8.17c±2.33	11.70c±2.47	1.05c±0.06	1.00c±0.00	1.00c±0.00
WA + 50 mM NaCl	11.68b±2.52	10.56b±0.13	12.37b±3.15	5.70b±1.44	6.98b±0.83	7.23b±0.93
WA + 100 mM NaCl	5.39c±1.51	3.46c±0.47	5.18c±0.33	1.00c±0.00	1.00c±0.00	1.00c±0.00
WA + 150 mM NaCl	3.83c±1.27	3.45c±0.71	3.17c±0.31	0.00c±0.00	1.00c±0.00	1.00c±0.00
WA + 1% PEG 6000	27.25a±5.87	28.21a±3.63	23.41a±2.77	21.36a±6.30	27.32a±13.95	18.10a±7.88
WA + 5% PEG 6000	22.27a±4.92	N/A	21.08a±0.65	17.73a±6.17	N/A	20.86a±0.34

Table 2. The effect of HM, NaCl and PEG 6000 on tolerance index and phytotoxicity percentage of *Ch. majus*.

Media	Tolerance index			Phytotoxicity percentage (%)		
	H_2O	Pro	MeJA	H_2O	Pro	MeJA
WA	100.00d±0.00	93.06d±12.74	90.19d±8.77	0.00f±0.00	0.07f±0.13	0.1f±0.09
WA + 50 mM NaCl	22.79bc±9.29	28.34c±0.63	32.98c±0.26	0.77c±0.09	0.72c±0.01	0.67d±0.00
WA + 100 mM NaCl	4.31a±0.41	4.31a±0.41	4.31a±0.41	0.96b±0.00	0.96b±0.00	0.96b±0.00
WA + 150 mM NaCl	reduced	4.31a±0.41	4.31a±0.41	reduced	0.96b±0.00	0.96b±0.00
WA + 1% PEG 6000	103.99d±11.39	144.06d±33.78	68.92d±38.13	-0.04f±0.11	-0.44f±0.34	0.31f±0.38
WA + 5% PEG 6000	77.63d±33.86	N/A	89.90d±10.05	0.22f±0.34	N/A	0.10f±0.10
Cd^{2+} 1 mg/l	17.42b±2.67	15.13b±0.26	13.81b±1.76	0.83a±0.03	0.85a±0.00	0.86a±0.02
Cd^{2+} 5 mg/l	6.36a±0.33	6.96a±0.84	6.96a±0.55	0.94c±0.00	0.93c±0.01	0.93c±0.01
Cd^{2+} 10 mg/l	4.52a±0.71	4.31a±0.41	4.31a±0.41	0.95b±0.01	0.96b±0.00	0.96b±0.01

Pb2+ and Zn2+ were extremely phytotoxic for seedling development. Cd2+ also had very high phytotoxicity, but the roots didn't turn black and stop their growth at 1 mm at the lowest Cd2+ concentration. The tolerance index of the Pro and MeJA primed seeds was higher than that of the water pre-soaked seeds at 1 mg/l Cd2+. However, the tolerance index sharply dropped at 5 and 10 mg/l Cd2+ and the indices of elicitor primed seeds and hydroprimed seeds were almost equal (Table 2).

Effect of Pro and MeJA priming on germination and seedling growth in agar medium supplemented with NaCl

NaCl significantly reduced the germination of the hydroprimed seeds. Pro and MeJA enhanced the germination of seeds sown in the media with NaCl compared to the germination percentage of the hydroprimed seeds on the same media. However, the higher the NaCl concentration was, the lower the alleviating effect of Pro and MeJA was (Fig. 4).
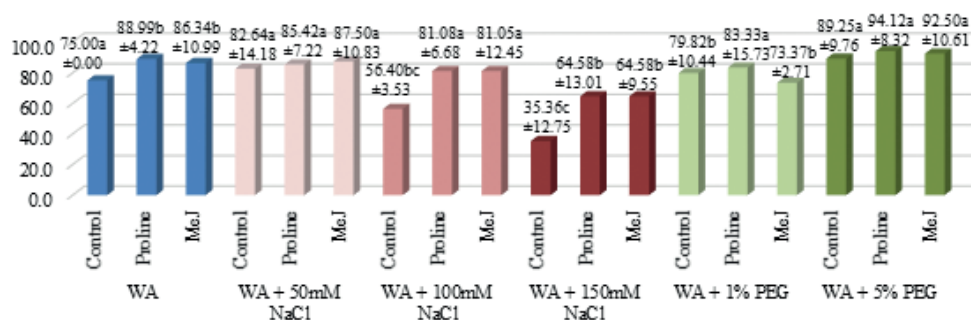
Figure 4. Germination percentage (%) of Pro and MeJA primed seeds germinated on WA+NaCl and WA+PEG.

NaCl also significantly reduced the length of roots and hypocotyls (Table 1). Seed priming with the elicitors didn't promote the reduction of the unfavorable influence of NaCl on root and hypocotyl growth. *Ch. majus* seeds had very low tolerance to NaCl at the higher applied concentrations. Above 50 mM NaCl the values of phytotoxicity and tolerance index were the same for the hydroprimed seeds and those primed with the elicitors (Table 2). However, at 150 mM NaCl the roots of the hydroprimed seeds were absolutely reduced.

Effect of Pro and MeJA priming on seed germination and seedling growth under drought conditions induced by PEG 6000 supplementation

Seed priming didn't influence significantly the germination percentage of seeds in medium supplemented with PEG 6000. The percentages of primed germinated seeds were close to or much higher than the Control value. The germination was more stimulated at 5% PEG 6000 (Fig. 4). The root length of Pro primed seeds on WA + 1% PEG 6000 was significantly higher than that of the Control (Table 1). The values of the other variants were close to that of the Control. PEG 6000 slightly reduced the length of hypocotyls and its effect enhanced with the increase of PEG concentration (Table 1). The Pro- and hydroprimed seeds had high tolerance to the lower concentration of PEG 6000 and there was no phytotoxicity (Table 2). The positive influence of MeJA priming occurred at the higher PEG concentration.

Discussion

The process of seed sprouting and especially the early stages of seedling growth are the most vulnerable stages of plant development. The stress conditions can reduce germination and retard its onset, which results in weak plant development and low productivity (Bhanuprakash and Yogeesha 2016). Plants acclimatize to the unfavorable environmental conditions through activation of complex internal mecha-

nisms with the final aim to respond to external factors in the most optimal way and to survive the stress conditions (Hossain et al. 2014). Thus, Pro accumulation occurs when plants are subjected to salt, drought and heavy metal stress and can be provoked by its exogenous application (Hayat et al. 2012). Similarly, exogenous application of MeJA elevates endogenous levels of Pro and MeJA which provides protection of plants under abiotic stress (Yan et al. 2015). Exogenous applications of MeJA – increased JA content and biosynthesis of secondary metabolites, which have a defensive role in the alleviation of abiotic and biotic stress in plants (Dar et al. 2015; Farooq et al. 2016).

The results showed that Pro and MeJA priming stimulated the seed germination on WA.

Overall, the germination of seeds on WA supplemented with Pb was high, which probably hid the Pro and MeJA effect. According to Parera and Cantliffe (1994) the beneficial influence of priming is more visible at adverse conditions than at favorable ones. The results of the study suggested that Pb^{2+} did not inhibit the germination of *Ch. majus* seeds. Yang et al. (2010) observed that the germination percentage of wheat seeds was increased after treatment with 1mM Pb. The seeds on WA with Zn^{2+} also had high germination percentage. However, the increase of Zn^{2+} concentration promoted MeJA mitigation of the inhibitory effect of Zn^{2+}. On the other hand, the higher Cd^{2+} concentration applied reduced the protective influence of MeJA.

In regard to Pro priming, the strengthening of the stress factor influence (the increase of HMs concentration) led to the reduction of the Pro effect. In the current study, the germination percentage of seeds on WA supplemented with HMs was high but HMs greatly inhibited the seedling development. This might be caused by the impermeability of the seed coat to some HM and the role of the seed coat for seed tolerance (Li et al. 2005; Akinci and Akinci 2010). The fact that seeds germinated at high HM concentrations but seedling growth was retarded at low concentrations indicated exactly that (Akinci and Akinci 2010). According to the research of Yang et al (2010) though the low Pb concentrations improved the germination of wheat seeds its higher concentrations inhibited the further growth of the seedlings. It was reported that HMs are more toxic to seedling growth than to their germination in *A. thaliana*, too (Li et al. 2005). HMs strongly inhibited the roots which could be explained with the fact that roots are the first contact organ with the stress factor in growth medium (Andresen and Küpper 2013). NaCl significantly inhibited the germination percentage because of perturbed water imbibition, hyperosmotic stress and aggravated stored food mobilization (Kalaji and Pietkiewicz 1993; Johnson and Puthur 2021). However, Pro and MeJA promoted higher percentage of germination. The treatment with NaCl revealed the beneficial effect of priming with the elicitors, whereas hydropriming did not decrease the adverse effect of salt stress. Pro and MeJA priming promoted the metabolic processes which occurred during the pre-germination period (Paparella et al. 2015). The negative effect of NaCl on seedling growth is because of high salt accumulation and osmotic stress, cytotoxic ion gathering, increased transpiration because of impaired stomatal closure. Seed germination and seedling growth were not influ-

enced negatively by drought stress to a great extent and MeJA effect was stronger at higher PEG concentration. MeJA alleviating effect was observed in the osmotic stress in *Oryza sativa* through a change in the physiology of the seedlings (Sheteiwy et al. 2018). Pro and MeJA degree of influence depend on the concentration applied, the stage of plant development and the way of application on plants (in the medium or leaf spraying) (Ashraf and Foolad 2007). Further studies are required in order to elucidate the influence of all previously mentioned factors on the overall mitigating effect of Pro and MeJA on stress factors.

Conclusion

The influence of Pro and MeJA priming on seed germination depended on the type of the stress factor applied. Thus, HM and PEG 6000 did not inhibit seed germination or inhibited it to a small extent, and Pro and MeJA priming effect remained hidden. However, NaCl decreased the germination percentage, – but elicitor priming compensated the adverse effect of NaCl. Seedling growth was influenced by the concentration and type of stress agent and by the priming treatment. Thus, Zn reduced seedling growth to the greatest extent. And Pro priming increased root length at 1% PEG 6000.

Acknowledgements

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Supplementary material 1

Figure S1
Authors: Iva Doycheva
Data type: docx file
Explanation note: Mean hypocotyl length (mm) of seedlings grew on WA+NaCl and WA + PEG 6000.
Link: https://doi.org/10.3897/biorisk.17.77465.suppl1

Supplementary material 2

Figure S2
Authors: Iva Doycheva
Data type: docx file
Explanation note: Mean hypocotyl length (mm) of seedlings grown on WA+Cd (mg/l).
Link: https://doi.org/10.3897/biorisk.17.77465.suppl2

Supplementary material 3

Figure S3
Authors: Iva Doycheva
Data type: docx file
Explanation note: Mean hypocotyl length (mm) of seedlings grown on WA+Pb (mg/l).
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Link: https://doi.org/10.3897/biorisk.17.77465.suppl3

Supplementary material 4

Figure S4
Authors: Iva Doycheva
Data type: docx file
Explanation note: Mean hypocotyl length (mm) of seedlings grown on WA+Zn (mg/l).
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Link: https://doi.org/10.3897/biorisk.17.77465.suppl4

Supplementary material 5

Figure S5
Authors: Iva Doycheva
Data type: docx file
Explanation note: Mean root length (mm) of seedlings grew on WA+NaCl and WA + PEG 6000.
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Link: https://doi.org/10.3897/biorisk.17.77465.suppl5

Supplementary material 6

Figure S6
Authors: Iva Doycheva
Data type: docx file
Explanation note: Mean root length (mm) of seedlings grown on WA+Cd (mg/l).
Link: https://doi.org/10.3897/biorisk.17.77465.suppl6

RESEARCH ARTICLE

Seasonal changes in the pro/antioxidant status of mussels *Mytilus galloprovincialis* (Lamarck, 1819) from Bulgarian Black Sea coastal habitats

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Abstract

The pro/antioxidant status of marine macrozoobentic organisms is being increasingly applied in environmental monitoring and conservation programs. The oxidative stress level in marine bivalves can provide valuable information not only on the health of the organisms and their populations, but also on the current state of habitats and ecosystems. The aim of the present study was to make the first comprehensive investigation of the seasonal changes in the antioxidant activity in different organs (gills, digestive gland and foot) of *M. galloprovincialis* from representative Bulgarian Black Sea coastal habitats. The lipid peroxidation and glutathione levels, as well as activities of the antioxidant enzymes catalase, superoxide dismutase, glutathion peroxidase, glutathion reductase, glucose-6-phosphate dehydrogenase and glutathione-S-transferase of the organs were measured spectrophotometrically. Our hypothesis was that enhanced environmental pressure during the summer season, induced by multiple factors (biogenic, abiogenic and anthropogenic) led to weakening of the antioxidant protection in mussels at the beginning of autumn. The reaction of the mussel organism to the multiple stress factors was specific for the target organ and the type of the biomarker. Significant differences were present in the activity of the antioxidant system in mussels from the northern and southern coastal locations. The seasonal changes in the pro/antioxidant status of mussels were primarily due to specific seasonal changes in factors concerning the marine environment at the concrete locality. Further research is obviously needed to confirm the present results and provide a more complete data of seasonal and spatial changes in the antioxidant defense system of mussels from the Bulgarian Black Sea coastal area and their implementation in biomonitoring programs.

Introduction

Mussels *M. galloprovincialis* are key components of Bulgarian Black Sea ecosystems (Petrova and Stoykov 2011). Being sedentary and sessile filter-feeders, they are used as suitable bioindicators for chemical contamination and the state of the marine environment, as a whole (Kamel et al. 2014; Faggio et al. 2018; Gürkan 2020).

Oxidative stress (OS) is a universal expression of the reaction of organisms to environmental impacts (Steinberg 2012) and its induction in marine bivalves can be used to assess the condition of the marine environment and the health of ecosystems. Contamination of the marine environment with metals, polycyclic aromatic hydrocarbons (PAH) and eutrophication can lead to an increase of cellular prooxidative processes such as lipid peroxidation, protein oxidation, oxidative DNA damages and to stimulation of the antioxidant protection of marine organisms (Gnatyshyna et al. 2014; Kamel et al. 2014). Thus, the assessment of the state of the marine environment requires not only measuring concentrations of pollutants, but also the level of cellular responses of marine bivalves to xenobiotics, overexploitation, and climatic changes (Gürkan 2020). It is considered that OS biomarkers can provide an early warning for deterioration of the environment by identification of cell biochemical changes before effects at the organism, population or community level take place (Lushchak 2011).

The OS response of mussels can vary broadly due to seasonal variations of temperature, hypoxia, metabolic status of the animals themselves, gonadal ripening, food availability, hydrological cycle, as well as metal and PAH concentrations in seawater (Mirzaei et al. 2016; Koudryashova et al. 2019). Depending on the local strength and duration of impacts, the antioxidant system of mussels can be activated or inhibited with consequences for the organism – adaptation or death, respectively. This, in turn, can affect higher levels of ecological hierarchy, i.e. population, communities and ecosystems.

The aim of the present study was to make the first comprehensive investigation of the seasonal changes in the pro/antioxidant status of different organs (gills, digestive gland and foot) of *M. galloprovincialis* from representative Bulgarian Black Sea coastal habitats.

Materials and methods

Sampling

The mussels were hand-gathered from sublittoral rocks and other hard substrate at 1–6 m depth in June and September (2017–2018) from 16 different sites along the Bulgarian Black Sea coast (Fig. 1). Mussel samples were placed in clean thermostable containers with seawater and transported to the laboratory where they were further processed.

Code	Location	N	E
N1	Kavarna (farm)	43.4038°	28.3753°
N2	Kaliakra	43.3719°	28.4587°
N3	St. Konstantin & Elena	43.2294°	28.0146°
N4	Kranevo	43.3229°	28.0669°
N5	Tulenovo	43.4909°	28.5853°
N6	Varna harbor	43.1920°	27.9212°
N7	Galata	43.1709°	27.9436°
S1	St. Ivan Island (farm)	42.4391°	27.6850°
S2	St. Ivan Island (east)	42.4379°	27.6965°
S3	St. Ivan Island (west)	42.4367°	27.6874°
S4	Chervenka cape	42.4380°	27.6557°
S5	Stomoplo bay	42.2897°	27.7649°
S6	Sozopol town (port)	42.4244°	27.6934°
S7	Sozopol town (coastal rocks)	42.4251°	27.7003°
S8	Kolokita cape	42.4092°	27.7296°
S9	St. Parashkeva	42.3205°	27.7720°

Figure 1. *M. galloprovincialis* sample locations along the Bulgarian Black Sea coast

Tissue preparation

Three mussel organs were studied separately. The mussels (n=8–10 for each site) were immediately dissected and the gills, foot and digestive gland were removed. Each individual organ was frozen in liquid nitrogen and stored at –80 °C prior to the biochemical assays. Afterwards, all organs were homogenized in 100 mM potassium phosphate buffer (pH 7.4) using Potter Elvehjem homogenizer fitted with a Teflon pestle (Thomas Scientific, USA). To receive a post nuclear fraction for determination of lipid peroxidation and glutathione levels, the homogenates were centrifuged for 10 min at 3000 g. A part of the fraction was re-centrifuged at 12 000 g for 20 min to obtain a post mitochondrial supernatant used for measurement of the antioxidant enzyme activities. All operations were performed at 4 °C.

Biochemical analysis

All tested antioxidant biomarkers were measured spectrophotometrically using commercially available kits, purchased by Sigma-Aldrich Co. LLC (USA): Lipid Peroxidation (MDA) Assay Kit MAK085, Glutathione Assay Kit CS0260, SOD Assay Kit-WST 19160, Catalase Assay Kit CAT100, Glutathione Peroxidase Cellular Activity Assay CGP1, Glutathione reductase Kit GRSA and Glutathione-S-Transferase Assay Kit CS0410. The manufacturer's working instructions were strictly followed. The protein concentration was measured according to Lowry et al. (1951) by using a standard curve of bovine serum albumin as standard.

Statistical analyses

Statistical analyses of raw data were carried out by ANOVA with Bonferroni post hoc test. Comparisons were made using Mann-Whitney test. Multidimensional Scaling (MDS) was applied to detect meaningful underlying dimensions and to explain the observed similarities and dissimilarities.

Results

The estimated values of the studied antioxidant indicators in the mussels' organs are presented in Tables 1–3. The tested biomarkers demonstrated significant variability among the studied organs, localities and the two seasons. Lipid peroxidation (LPO) did not show seasonal differences (Table 1–3). The only significant difference was the higher LPO in September than in June in the organs of mussels from St. Ivan Island (west). Mussels from this location were gathered in the vicinity of the quay and the high LPO values measured were probably due to the intense floating of boats or some kind of momentary pollution.

The seasonal patterns in the activities of the antioxidant enzymes are presented in Table 1–3. No statistically significant seasonal differences were present in the activity of superoxide dismutase (SOD) in the foot and gills (except the samples from Chervenka cape) of mussels from all studied locations. In the digestive gland, however, significant seasonal differences were found in all samples from the northern locations of the coastal area, while in the samples from the southern locations only in the digestive gland of mussels from St. Ivan Island (west) and Sozopol coastal rocks, differences were present. Significant seasonal differences in the catalase (CAT) activities in the foot of mussels were found in the samples from the following northern coastal locations: Kavarna mussel farm, Kaliakra and Kranevo, as well as in the mussels from the following southern coastal locations St. Ivan Island (west), Sozopol coastal rocks and cape Kolokita. At all these five locations, CAT activity in the foot was decreased in September compared to June. No seasonal differences in CAT activity were observed in the gills in any of the mussels from all the studied locations, except from Tulenovo. In the digestive gland, no seasonal differences were found between the mussels from all northern locations. In the southern locations, higher CAT values in mussels were measured in September compared to June.

The values of glutathione (GSH) and related enzymes demonstrated clear seasonal variations. GSH showed significant seasonal differences in the foot of mussels from the northern locations (Table 1). In samples from the southern locations, the only significant seasonal difference in GSH was found in the foot of mussels from Sozopol port and Stomoplo bay, where significantly lower GSH in September was found compared to June. Concerning gills, only at 3 locations (St. Konstantin and Elena, cape Chervenka and St. Parashkeva) statistically significant increase of GSH in September was present (Table 2). The most pronounced seasonal differences in GSH concentration were found in the di-

Table 1. Seasonal changes in oxidative stress biomarkers in foot of *Mytilus galloprovincialis* from Northern (N) and Southern (S) coastal areas of the Bulgarian Black Sea (mean±SD; *- significant differences: p≤0.05*; p≤0.01**; p≤0.001***June vs September).

	LPO (nM MDA/ mg prot)		GSH (ng/mg protein)		SOD (U/mg protein)		CAT (U/mg protein)		GPX (U/mg protein)		GR (U/mg protein)		G6PDH (U/mg protein)		GST (U/mg protein)	
Site	June	Sept	June	Sept	June	Sept	June	Sept	June	Sept	June	Sept	June	Sept	June	Sept
N1	1.27	0.95	1700	325	21.80	21.79	0.19	0.03	5.90	3.39	5.94	10.88	16.40	19.65	18.66	65.58
	±0.4	±0.2	±239***	±69	±7.4	±4.8	±0.12*	±0.01	±1.9	±0.1	±3.2	±3.2	±4.8	±5.9	±5.5*	±24.6
N2	1.41	0.96	2298	548	19.12	25.61	0.27	0.06	9.38	9.92	3.70	14.68	18.33	22.01	15.79	159.91
	±0.7	±0.2	±294***	±108	±2.9	±7.2	±0.06**	±0.01	±4.6	±4.7	±0.9*	±0.6	±5.7	±3.7	±1.4**	±58.6
N3	1.02	1.19	2171	506	25.53	13.72	0.25	0.13	8.42	4.26	1.78	17.45	31.61	7.18	16.56	74.62
	±0.3	±0.2	±457***	±32	±12.7	±2.8	±0.07	±0.05	±3.7	±0.8	±1.0**	±4.7	±10.60**	±1.04	±9.14*	±35.6
N4	1.52	1.12	2352	578	21.82	25.59	0.21	0.07	10.51	16.33	3.16	6.63	28.77	48.69	23.70	128.70
	±0.6	±0.2	±921***	±168	±5.1	±10.6	±0.06*	±0.02	±4.2	±1.9	±1.2	±0.2	±13.0	±7.5	±7.2**	±64.0
N5	1.37±	0.90	2364	794	24.23	24.84	0.36	0.25	8.83	5.38	1.74	14.75	29.40	32.11	32.88	227.70
	0.3	±0.2	±480***	±379	±6.2	±7.8	±0.15	±0.06	±1.96	±1.1	±0.9***	±0.6	±9.1	±1.3	±8.7***	±114.5
N6	1.40	1.89	1483	527	23.41	22.53	0.24	0.13	4.82	0.96	3.87	4.95	32.01	14.5	25.61	94.97
	±0.27	±0.38	±168**	±138	±8.7	±2.7	±0.07	±0.02	±0.7	±0.35	±1.6	±1.30	±7.5**	±3.0	±5.9*	±39.6
N7	1.23	1.81	1407	480	14.40	20.68	0.19	0.10	4.40	0.85	3.19	11.91	24.04	22.17	23.40	113.71
	±0.3	±0.3	±160**	±169	±3.6	±6.4	±0.07	±0.04	±2.0	±0.1	±2.0*	±7.1	±4.6	±7.28	±6.5*	±30.2
S1	1.41	1.43	1100	1561	25.72	11.30	0.40	0.26	3.87	7.81	3.45	4.3	15.03	18.35	19.50	105.06
	±0.8	±0.6	±311	±106	±5.0	±0.2	±0.31	±0.09	±2.3	±2.2	±0.7	±0.3	±3.8	±0.9	±7.9***	±28.3
S2	1.30	1.48	1410	740	28.51	20.89	0.71	0.22	2.66	7.97	3.63	3.35	13.63	14.55		
	±0.1	±0.5	±737	±695	±14.2	±4.9	±0.46	±0.09	±0.3*	±2.9	±0.6	±0.4	±0.47	±5.1		
S3	0.82	3.18	2762	1720	19.19	12.55	0.71	0.21	3.26	7.11	3.94	1.00	13.94	12.9		
	±0.1*	±0.8	±873	±983	±4.5	±1.1	±0.52	±0.14	±0.6*	±1.6	±1.3	±1.1	±0.0	±0.04		
S4	0.85	2.68	2410	1583	19.37	7.53	0.67	0.37	3.76	9.77	19.73	6.70	19.73	21.27		
	±0.1	±1.5	±899	±45	±9.4	±0.7	±0.51	±0.02	±0.3*	±0.7	±6.0*	±1.8	±4.9	±0.8		
S5	1.54	2.08	2419	1030	27.72	19.03	0.41	0.22	2.55	7.13	12.35	2.47	18.55	16.89	23.99	73.68
	±0.6	±0.8	±10**	±922	±8.7	±4.4	±0.53	±0.06	±0.9*	±2.9	±2.3*	±0.1	±5.0	±6.7	±6.4**	±23.4
S6	1.84	2.12	2018	415	34.91	20.51	0.30	0.53	3.60	5.19	12.17	2.62	18.96	19.20	25.76	73.06
	±0.5	±1.4	±824***	±318	±16.5	±7.5	±0.20	±0.16	±1.7	±1.6	±5.8*	±2.0	±5.5	±5.6	±7.1**	±19.6
S7	1.71	1.62	1501	586	33.9	16.89	1.03	0.23	2.71	4.46	16.87	3.23	16.87	17.47		74.57
	±0.5	±0.6	±131	±508	±13.0	±4.6	±0.4***	±0.16	±0.3	±1.9	±4.8*	±1.2	±3.9	±10.2		±24.9
S8	1.14	2.27	1544	921	21.25	21.84	1.05	0.26	2.20	9.38	16.28	3.80	19.61	16.68		94.01
	±0.4	±0.7	±69	±788	±1.1	±9.6	±0.5***	±0.14	±0.2*	±4.8	±9.5*	±0.9	±3.1	±4.8		±16.0
S9	1.42	2.42	1422	725	28.06	19.84	0.09	0.18	4.15	9.82	9.67	0.80	18.60	13.46	18.25	73.68
	±0.5	±0.8	±209	±355	±9.9	±7.1	±0.03	±0.04	±1.7**	±4.6	±1.7*	±0.2	±3.4	±2.6	±1.4***	±26.5

gestive gland. In almost all locations, the GSH concentration in the autumn samples was significantly higher. The only exceptions were in mussels from Kavarna in the northern coastal area, and Sozopol port and Sozopol coastal rocks in the southern area. The activity of glutathione peroxidase (GPx) in the mussels' foot from the northern locations did not show seasonal variations, except for the mussels from Varna harbor and Galata cape, where GPx activity was significantly decreased in autumn (Table 1). In the foot of mussels from the southern locations, statistically higher activity of the enzyme was found in the autumn samples, except for those from St. Ivan Island farm, Sozopol port and Sozopol costal rocks. The GPx activity in the gills of all mussels from the northern locations, collected in September, was lower in comparison with those from June (Table 2). In mussels from the southern locations the inverse dependence was observed – an overall higher activity of the enzyme in September compared to June. A similar pattern was found for GPx activity in the digestive gland, i.e. in the northern coastal samples the activity in autumn was lower than in sum-

Table 2. Seasonal changes in oxidative stress biomarkers in gills of *Mytilus galloprovincialis* from Northern (N) and Southern (S) coastal areas of the Bulgarian Black Sea (mean±SD; *-significant differences: p≤0.05*; p≤0.01**; p≤0.001***June vs September).

	LPO (nM MDA/ mg prot)		GSH (ng/mg protein)		SOD (U/mg protein)		CAT (U/mg protein)		GPX (U/mg protein)		GR (U/mg protein)		G6PDH (U/mg protein)		GST (U/mg protein)	
Site	June	Sept	June	Sept	June	Sept	June	Sept	June	Sept	June	Sept	June	Sept	June	Sept
N1	5.45	5.71	730	629	20.5	25.7	0.53	0.25	10.28	6.78	7.65	4.69	44.54	16.03	39.03	64.03
	±2.2	±3.7	±210	±196	±6.1	±9.6	±0.23	±0.12	±5.0	±2.45	±3.6	±2.7	±11.4 **	±6.0	±11.7	±25.2
N2	3.85	5.05	607	761	19.1	23.3	0.38	0.24	13.76	1.76	7.75	10.93	46.05	13.24	37.62	70.49
	±2.1	±2.9	±126	±206	±3.8	±8.0	±0.04	±0.30	±3.9*	±0.18	±2.7	±5.7	±4.1*	±3.2	±6.7	±25.6
N3	5.09	5.25	758	1218	20.4	18.9	0.44	0.47	16.79	1.79	7.00	7.99	66.73	10.60	40.79	65.72
	±2.7	±0.6	±154*	±237	±4.6	±1.7	±0.06	±0.04	±8.9***	±0.55	±3.6	±3.9	±13.2***	±1.2	±27.1	±13.5
N4	4.47	4.92	636	946	18.2	26.6	0.33	0.44	14.69	4.54	9.90	6.41	51.54	6.40	42.82	99.25
	±0.9	±1.2	±162	±220	±8.7	±16.2	±0.09	±0.29	±8.0*	±1.92	±0.4	±2.9	±13.9***	±2.2	±12.1	±45.1
N5	4.79	5.91	647	778	28.7	34.9	0.61	0.29	15.05	4.25	6.32	21.75	65.32	21.43	49.85	85.75
	±1.3	±1.5	±150	±251	±11.0	±13.4	±0.24*	±0.04	±7.5*	±0.79	±1.1**	±4.3	±18.7***	±7.1	±13.9	±12.3
N6	6.42	7.10	547	583	20.2	34.9	0.55	0.50	13.31	0.80	16.01	15.78	59.85	20.64	63.29	76.59
	±2.6	±1.8	±117	±201	±4.6	±7.3	±0.12	±0.18	±4.3**	±0.46	±2.4	±3.3	±17.7***	±5.8	±20.2	±20.1
N7	5.36	5.29	575	889	22.3	25.7	0.37	0.50	13.90	6.25	14.02	15.36	58.67	14.85	61.52	67.76
	±1.3	±1.3	±85	±257	±10.4	±9.8	±0.12	±0.25	±5.6	±1.89	±14.0	±3.5	±18.6***	±2.0	±20.7	±24.6
S1	4.33	2.18	730	1355	14.5	15.6	0.71	0.86	3.80	6.10	10.05	2.53	25.44	22.21	18.04	83.33
	±1.7	±1.1	±176	±256	±6.2	±1.1	±0.36	±0.2	±2.4	±1.56	±2.4*	±0.7	±8.4	±0.1	±3.8*	±45.6
S2	3.76	7.43	733	1230	21.6	18.3	1.09	0.69	2.08	5.04	12.17	4.92	22.17	20.66		
	±1.8	±2.7	±30	±308	±0.6	±6.4	±0.77	±0.22	±0.3	±2.01	±1.1*	±0.3	±0.9	±7.6		
S3	3.95	9.26	717	1085	25.8	5.3	0.58	0.95	2.40	6.11	9.93	1.25	19.93	18.38		
	±0.4*	±0.2	±94	±236	±1.3	±1.7	±0.1	±0.28	±0.2	±1.24	±3.2*	±0.3	±2.6	±1.6		
S4	2.74	4.09	590	2436	36.7	11.8	0.85	0.74	1.73	8.06	7.88	2.04	18.85	16.66		
	±0.4	±1.1	±91***	±387	±6.2*	±0.8	±0.59	±0.11	±0.2*	±0.14	±0.6*	±0.5	±1.61	±3.5		
S5	4.42	7.13	551	1140	19.0	15.5	0.65	0.66	2.16	6.16	5.36	4.9	32.85	23.73	11.33	60.02
	±1.4	±2.3	±146	±419	±12.0	±4.7	±0.27	±0.43	±0.8**	±3.58	±2.5	±2.6	±12.7	±5.9	±3.8*	±13.7
S6	7.61	6.89	407	798	24.5	20.2	0.69	0.78	3.24	4.43	11.3	4.6	39.24	27.82	14.64	118.33
	±2.6	±3.2	±134	±268	±20.9	±8.5	±0.13	±0.37	±0.9	±2.3	±5.7	±3.2	±21.8	±9.8	±3.8***	±31.7
S7	2.89	5.3	704	1053	31.3	16.6	0.69	0.61	1.46	4.35	8.67	13.87	18.67	22.28		103.08
	±0.1	±1.5	±68	±488	±6.9	±7.0	±0.06	±0.14	±0.3	±2.65	±2.7	±10.0	±2.2	±6.5		±42.3
S8	3.63	5.54	743	1277	28.5	13.8	0.93	0.45	1.53	5.52	8.07	2.71	18.07	33.50		63.57
	±1.6	±1.9	±124	±835	±4.2*	±4.5	±0.63	±0.27	±0.0*	±2.11	±3.9	±1.6	±3.2	±17.5		±34.6
S9	4.14	6.90	432	1483	11.0	15.1	0.45	0.52	3.22	4.16	13.76	9.67	39.08	22.96	14.46	64.49
	±1.2	±1.9	±59***	±706	±6.0	±5.5	±0.16	±0.27	±1.3	±1.98	±3.2	±7.9	±7.5	±7.6	±3.0*	±28.5

mer, and in the southern locations higher activity in June was present (Table 3). Significant seasonal differences were also observed in the activity of glutathione reductase (GR). In the foot of mussels sampled in September a significant increase compared to June was found for all northern coastal locations (except Kavarna farm and Varna harbor) (Table 1). In contrast, in the foot of the sampled mussels in September from the southern locations, a significant decrease in the enzyme activity was present, with the exception of the region of St. Ivan Island (St. Ivan Island mussel farm, St. Ivan Island east and St. Ivan Island west), where low GR values in both seasons were present. In gills, a statistically significant decrease in GR in autumn samples was present for all southern locations (Table 2). For GR in the digestive gland of the mussels a statistically significant decrease in the autumn was observed for locations from the southern coastal area with the exception of Sozopol port. A statistically significant higher activity of glutathione-S-transferase (GST) in the gills and digestive gland was observed in autumn for mussels from all southern coastal locations. GST activity was

Table 3. Seasonal changes in oxidative stress biomarkers in digestive gland of *Mytilus galloprovincialis* from Northern (N) and Southern (S) coastal areas of the Bulgarian Black Sea (mean±SD; *-significant differences: $p \leq 0.05$*; $p \leq 0.01$**; $p \leq 0.001$***June vs September).

	LPO (nM MDA/mg prot)		GSH (ng/mg protein)		SOD (U/mg protein)		CAT (U/mg protein)		GPX (U/mg protein)		GR (U/mg protein)		G6PDH (U/mg protein)		GST (U/mg protein)	
Site	June	Sept	June	Sept	June	Sept	June	Sept	June	Sept	June	Sept	June	Sept	June	Sept
N1	3.72	3.76	335.25	325.33	6.36	15.20	1.80	0.80	10.22	1.50	12.2	11.86	22.25	13.42	44.09	26.91
	±1.4	±1.92	±213.19	±69.78	±2.94 *	±2.84	±0.95	±0.56	±4.78***	±0.22	±6.92	±3.44	±6.10 *	±2.23	±5.17	±6.17
N2	2.34	2.95	207	548	6.29	20.13	0.77	0.77	13.17	1.82	7.04	7.58	18.16	16.40	40.97	43.20
	±0.9	±0.8	±28*	±108	±1.9***	±5.4	±0.27	±0.18	±2.7***	±0.60	±4.1	±4.31	±2.7	±3.5	±9.9	±12.8
N3	2.52	2.48	311	506	7.85	19.49	0.80	0.60	12.28	1.84	8.89	18.67	25.95	4.77	50.08	29.38
	±0.8	±0.4	±92*	±32	±5.9*	±7.5	±0.18	±0.39	±2.1***	±0.54	±3.2	±4.42	±12***	±0.6	±8.4	±5.8
N4	3.75	3.94	241	578	7.77	24.64	1.05	1.16	13.39	3.67	10.46	11.30	23.35	6.18	50.87	40.74
	±2.5	±0.9	±54*	±168	±2.6***	±2.4	±0.29	±0.43	±4.2***	±1.54	±4.6	±5.87	±4.3*	±0.5	±12.8	±24.0
N5	2.08	3.11	315	794	6.85	17.01	1.14	0.45	14.34	4.66	8.91	11.53	23.19	4.33	53.42	38.98
	±0.3	±1.0	±45***	±379	±5.8*	±7.7	±0.44	±0.26	±4.5***	±0.78	±1.8	±6.05	±7.8**	±1.0	±8.1	±11.9
N6	4.29	5.33	168	527	4.57	19.25	0.43	1.29	13.11	0.75	5.24	16.52	19.73	6.23	70.76	37.99
	±2.3	±1.7	±41*	±138	±3.3**	±6.1	±0.16	±0.44	±5.7***	±0.48	±1.4*	±4.12	±3.6*	±1.3	±7.7**	±3.5
N7	3.03	3.55	156	480	5.11	17.52	0.45	1.39	12.15	6.03	5.38	11.58	16.48	6.23	64.20	32.64
	±2.0	±0.7	±26*	±169	±4.3***	±4.6	±0.17	±0.80	±1.7*	±1.91	±2.4	±2.33	±4.1*	±2.0	±14.1**	±9.5
S1	2.13	2.89	173	626	8.01	5.66	0.82	1.19	3.86	6.36	19.17	4.38	17.01	10.59	5.51	26.12
	±0.9	±1.2	±55*	±275	±2.0	±2.8	±0.25	±0.2	±2.7	±0.84	±6.2***	±1.1	±4.6	±2.5	±2.7*	±4.9
S2	2.12	2.92	171	530	8.81	3.07	0.59	0.84	1.42	4.63	15.09	5.02	15.09	13.57		
	±0.9	±1.1	±14*	±289	±3.9	±1.2	±0.47	±0.23	±0.1*	±1.87	±0.4**	±1.66	±0.3	±2.9		
S3	2.38	7.62	135	511	7.49	3.06	0.14	1.04	1.19	6.25	14.38	3.3	14.38	6.88		
	±1.1*	±0.2	±46*	±50	±2.6*	±0.7	±0.04*	±0.33	±0.2*	±1.25	±1.6*	±0.42	±1.3	±0.6		
S4	3.51	3.94	203	700	8.3	4.55	0.18	0.87	1.26	6.75	16.12	6.35	16.12	17.91		
	±0.67	±1.5	±75*	±258	±2.6	±1.5	±0.07*	±0.03	±0.2*	±1.19	±1.0*	±4.9	±0.8	±3.3		
S5	3.12	3.69	186	584	4.12	3.65	0.25	0.77	1.48	3.36	15.99	3.99	17.43	21.88	10.19	45.08
	±0.6	±1.2	±49***	±234	±1.5	±1.5	±0.09	±0.57	±0.8*	±1.85	±3.4***	±2.09	±3.4	±11.1	±4.4**	±18.9
S6	3.6	4.25	144 ±30	286	7.68	4.83	1.06	1.21	1.88	3.26	15.46	11.16	19.19	20.11	8.89	43.95
	±1.1	±1.9		±100	±5.7	±1.0	±0.42	±0.34	±0.5	±1.55	±2.1	±4.88	±4.7	±12.6	±1.8**	±21.3
S7	2.18	3.31	188 ±48	369	12.7	3.79	0.31	1.06	1.68	2.66	17.78	7.82	14.45	17.79		33.08
	±1.0	±1.1		±222	±4.1*		±0.1 *		±0.1	±1.34	±3.7***	±4.08	±2.3			±12.1
S8	2.99	3.57	158	533	6.69	5.21	0.19	0.92	1.3 ±0.0	3.54	17.25	5.5	17.25	15.39		45.85
	±2.1	±1.3	±36*	±256	±1.5	±1.6	±0.07*	±0.26		±1.78	±4.0***	±2.19	±3.3	±5.4		±19.1
S9	3.07	3.62	197	633	3.64	4.01	0.33	1.18	1.68	3.75	16.08	6.40	15.64	16.34	4.98	38.34
	±1.6	±1.3	±53**	±261	±1.4	±2.4	±0.09*	±0.66	±0.5	±1.73	±1.7***	±2.17	±2.9	±5.9	±1.6**	±9.5

significantly higher in foot of the mussels gathered in September from all coastal locations (Table 1) and in the gills and digestive gland of the mussels from the southern locations.

Significant changes were observed in the activity of glucose-6-phosphate dehydrogenase (G6PDH) in all three examined organs, only in the mussels from the northern coastal locations. All samples from September had lower enzyme activities in the gills and digestive gland compared to those from June. In the autumn food samples, a statistically significant decrease was observed in the mussels from St. Konstantin & Elena and Varna harbor (Table 1).

Multidimensional scaling was used to reveal the presence of meaningful underlying dimensions in the data set and to explain the observed similarities and dissimilarities between the values of the measured OS markers and their seasonal and spatial variations (Fig. 2). In the space plane of the first two dimensions several groupings of OS biomarkers based on similarity and distance could be observed.
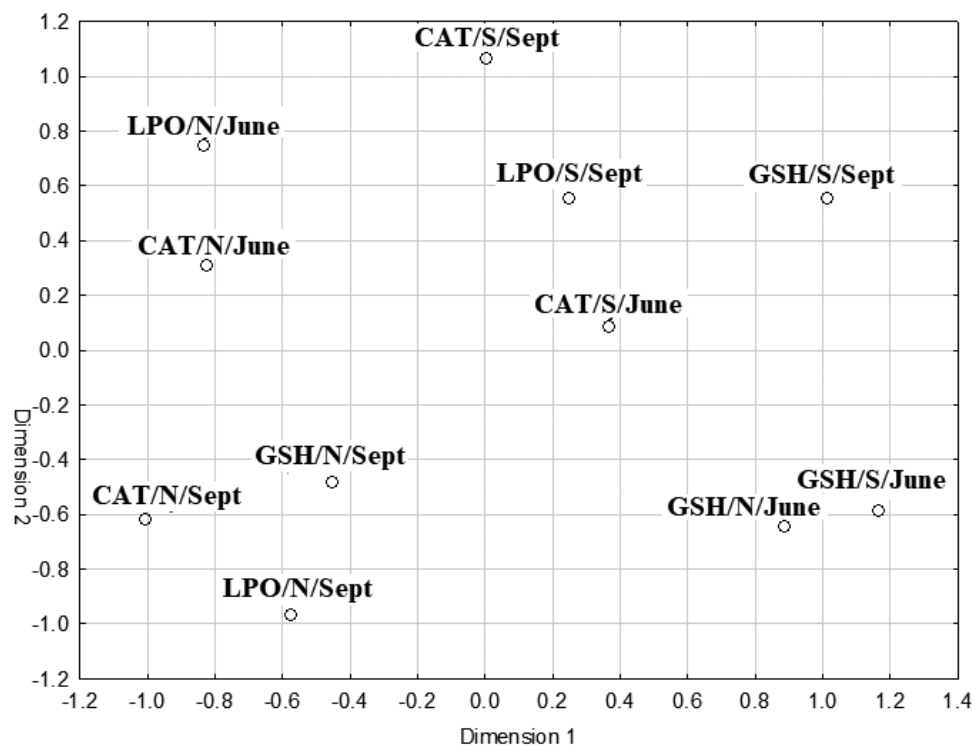
Figure 2. Two dimensional MDS plane of oxidative stress biomarkers in mussels – data for two seasons from the locations of the northern (N) and southern (S) Bulgarian Black Sea coastal areas

Along the horizontal axis (Dimension 1) two broad groupings according to similarity can be distinguished. On the left side of the dimensional plane, loose groups of pro/antioxidant markers in mussels from the northern locations of the coastal area were located and on the right side, those from the locations of the southern coastal area were situated. Along the vertical axis (Dimension 2) at the lower part of the dimensional plane samples with higher levels of GSH and related enzymes of the excited detoxification system were situated while on the upper side of the dimensional plane mussel samples with higher levels of LPO and activated CAT were located, indicating activated antioxidant system in mussels' cells.

Discussion

Data on the seasonal variation in cell oxidative status of *M. galloprovincialis* of the Bulgarian Black Sea part are scarce. Our results demonstrated for the first time the presence of extremely high variability in the intracellular oxidative processes and antioxidant defense system functioning in foot, gills and digestive gland of mussels together with well pronounced seasonal and spatial differences.

The main result obtained was the presence of significant differences in the antioxidative reaction of mussels from the northern and southern coastal regions. The observed significant variation in the activity of the antioxidant defense system of mussels strongly depended on the organs studied. This was due to the different composition and function of the studied organs. The most tangible seasonal changes were found in the digestive gland. Here, statistically significant seasonal differences were present in all studied biomarkers. The digestive gland constantly receives substances from the environment, incl. various xenobiotics, which makes it particularly susceptible to impacts (Livingstone 1981). The tissue specificity in the antioxidant response can be linked to the different bioaccumulation capacity and associated concentrations and retention times of xenobiotics (Benito et al. 2017).

Clear seasonal changes were observed in GSH concentration and in the activity of GSH-related enzymes. GSH can neutralize a broad variety of reactive oxygen species such as singlet oxygen, hydroxyl radical, superoxide, anion radical, hydrogen peroxide (Gostyukhina and Andreenko 2015). The major advantage of GSH as an antioxidant is its abundance in cells and the ability to be mobilized as soon as oxidative stress intensifies (Gostyukhina and Andreenko 2015). In addition to the ability of GSH to directly eliminate reactive oxygen species, it is also a co-substrate of GPx and GST. Specifically, GST plays a role in binding xenobiotics (Mannervik and Danielson 1988). Our results demonstrated a significant increase in GST activity in the autumn samples compared to those in early summer. This was most probably due to increased pollution pressure along the Bulgarian Black Sea coast from intensified touristic flow and municipal wastewater effluents during the summer months which activated the enzyme (Farcy et al. 2011). The changes in the activity of GPx were especially indicative – in September samples its activity was significantly reduced compared to June in the mussels from all the northern locations, while in the mussels from the southern locations its activity was significantly increased. Similar observations were found for GR. Concerning G6PDH in the gills and in the digestive gland, there was a significant decrease in all northern samples from September compared to June, while in the mussels from the southern coastal locations there were no significant differences. These differences were probably due to the presence of multiple stressor effects, related for example to the pollutant inflow by the Danube River (Dineva 2011; Doncheva et al. 2020). Results of the MDS analysis confirmed the presence of real dissimilarities in the pro/antioxidant status in mussels from the northern and southern locations. In addition, activation of the detoxification system of the mussels in response to local contamination of the marine environment was also proved.

Conclusion

The reaction of the mussel organism to various seasonal abiotic, biotic and anthropogenic stress factors was proved to be specific for the target organ and the type of biomarker reactions. It can be summarized that mussels *M. galloprovincialis* were

constantly exposed and responded to fluctuations of local conditions of their natural habitats of the Bulgarian Black Sea coast, i.e. specific seasonal changes in temperature, hypoxia, hydrological cycle and the metabolic status of the mussels themselves. This is a fact that should be considered in the interpretation of results and data from biomonitoring studies. Further research is obviously needed in order to confirm the present results and provide a complete picture of the observed relationships and dependences.

Acknowledgements

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DATA PAPER

Seasonal variations of the microflora of wedge clam *Donax trunculus* (Linnaeus, 1758) from the region of Arkutino (Bulgarian Black Sea aquatory)

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Abstract

The main goal of the present study was to investigate the impact of the state of the environment on the microbiota of the wedge clam *Donax trunculus* (Linnaeus, 1758) from the region of Arkutino (Bulgarian Black Sea aquatory). The species *Enterococcus hirae* was isolated during the summer (from May to August). The species *P. mendocina* prefers the warmer months and the species *P. alcaligenes* the colder ones. The temperature followed a course of decrease during the period September 2020 to January 2021, followed by a slow increase from February 2021. Comparing May 2020 with May 2021, it became evident that in 2021 the temperature was 1.5 °C lower. We can say that the number of the species *P. alcaligenes* was twice as high in May 2021. It is likely that this species preferred lower optimum temperatures and constant other parameters. For the species *Enterococcus hirae* such dependence was not observed - the number remained constant in May, but with increasing temperature the number of microorganisms decreased during the summer months. The species seemed to preferably develop at pH 7.78. The species *A. gyllenbergii* preferably grows at temperatures between 20.3–25.7 °C and the optimal temperature was 25.7 °C. For *C. farmeri* the optimum conditions were temperature 26.2 °C and pH 7.3. The species *E. vulneris* was probably related not only to the increase in water temperature, but also to the anthropogenic factor, as it was found only in July.

Introduction

Among marine bivalve species, the wedge clam *Donax trunculus* (Linnaeus, 1758) has one of the highest yields in the world. The habitat of this species is close to open sandy beaches, where it forms thick beds. It is found along the Atlantic-Mediterranean coast with the highest density at a depth from 0 to 3 m (Gaspar et al. 2002). The highest population density is reached at the surf. The species was found in the Mediterranean and the Black Sea from Senegal to the North Atlantic coast of France (Deval 2009). According to The State of World Fisheries and Aquaculture (2016) the annual production of *Donax* in Europe reaches 970–1353 tons. Both Donax species - *D. trunculus* and *D. variegatus* can be found along the Black Sea coast, but the population of *D. trunculus* (Fernández-Pérez et al. 2017) has a higher density. *D. trunculus* is commercially important in many countries like France, Italy, Turkey, Portugal and Spain as a food resource. In Galicia, *D. trunculus* is the bivalve with rising commercial value in the markets during recent years (42.37 €/kg in the year 2017), and its value has increased with the consequent increase in its fishing pressure (Fernández-Pérez et al. 2017). In Recent years have registered an increase in sites on the southern Black Sea coast where *D. trunculus* has been extracted; it is then mainly exported abroad on account of its high prices. Due to excessive collecting in some spots (including Bulgaria), the wild stocks are drastically depleted. Shellfish are exposed to diseases caused by various bacteria, which can also cause a mass extinction of species along the coast. It was detected, that the cause of outbreaks of diseases in bivalves is related to conditional pathogens, i.e. free-living pathogenic bacteria which, under favorable conditions, can cause diseases. This poses a serious risk to humans as consumers of clam species. Pathogenic bacteria can enter into the clams from seawater, from the microalgae they feed on, and as a result of anthropogenic pollution of the environment. The species *D. trunculus* is most often used as a bioindicator, as it is very sensitive to changes in the environment (Signorelli and Raven 2018). We have found no scientific publications on the microbiological status of wedge clams *D. trunculus* from the Bulgarian Black Sea aquatory and the impact of the state of the environment on it.

The main goal of the present study was to investigate the impact of the state of the marine environment on the microbiota of the wedge clams *D. trunculus* from the region of Arkutino (Bulgarian Black Sea aquatory).

Materials and methods

Place and duration of the study

The samples were collected from the region of Arkutino (exact field coordinates 42.3341 N, 27.7317 E: Datum WGS 84) from May 2019 until May 2021. The laboratory studies were conducted at the Department of Biology, University of Shumen, Bulgaria.

Sample collection

The wedge clams were harvested from the Bulgarian Black Sea aquatory. After collection of the three subsamples (each of about 1 kg), they were refrigerated (4 °C) and transported to the laboratory for further immediate analysis, without freezing the specimens.

In this study, we examined wedge clams of similar size, weight, and shape to ensure maximal uniformity in the applied methods (Duquesne et al. 2004). The average length of mussels used in the study was 2.2 ± 0.43 cm.

Physico-chemical analysis of the inhabited sea waters

During the mussel sampling, we measured in situ the temperature, total salinity (by using YSI Model 33 salinity meter), and pH (by using ATC Piccolo HI1280 pH-meter).

Microbiological analysis

Three subsamples (each of about 1 kg of wedge clams) were used for the microbiological analyses. The clams were scrubbed free of dirt, washed in hypochlorite solution (20 mg l^{-1}), rinsed with sterile distilled water, and shucked with a sterile knife. Tissue liquor samples (about 100 g) were homogenized (Maffei et al. 2009).

Fecal coliforms (FC) were enumerated through five tubes per dilution most probable number (MPN) series (Ignatova-Ivanova et. al. 2018). After 3 h at 37 °C plus 21 h at 44 °C, gas positive tubes were recorded for FC. From each FC gas positive tubes, 0.1 ml were transferred in tubes with 10 ml of Tryptone Water (Oxoid, Basingstoke, UK) and then incubated for 24 h at 44 °C. *E. coli* was enumerated by MacConkey agar (Merck, Darmstadt, Germany). The plates were incubated aerobically at 35–37 °C for 18–24 hours. *E. coli* grows matte dark pink to tile red, surrounded by an opaque area due to the precipitation of bile salts in this environment. *Pseudomonas* sp. was enumerated by Cetrimide Agar (Merck KGaA, 64271 Darmstadt, Germany).

Microbial identification databases for the "Biolog" systems

The microbial identification was performed by the Biolog Microbial Identification System (VIO45101AM). The isolated strains were screened on BL4021502 Tryptic Soy Agar (TCA), cultured for 24 hours at 37 °C, and then subjected to Gen III plaque identification to identify Gram-positive and Gram-negative aerobic bacteria. The microscopic pictures were made using stereomicroscope OPTIKA (Italy) with a Dino-Eye, Eyepiece camera with 5 megapixels. The photographs were performed by using a Canon EOS 60D camera. The GEN III MicroPlate test panel provides a standardized micromethod using 94 biochemical tests to profile and identify a broad range of Gram-negative and Gram-positive bacteria. Biolog's Microbial Identification Systems software (e.g. OmniLog Data Collection) is used to identify the bacterium from its phenotypic pattern in the GEN III MicroPlate. The BIOLOGIST system allows to quickly and accurately identify more than 2900 species of aerobic and anaerobic bac-

Table 1. The physico-chemical parameters of the marine water.

Region	Date	Depth	Temperature	pH	Salinity	Dissolved$_{o2}$
		M	[° C]	[pH]	[ppt]	[mg/l]
Arkutino	05.2020	2 to 4	24.4	7.78	12.2	7.9
Arkutino	06.2020	2 to 4	25.5	7.32	13.5	7.7
Arkutino	07.2020	2 to 4	27.2	8.26	13.5	7.8
Arkutino	08.2020	2 to 4	27.7	8.36	11.2	8.07
Arkutino	09.2020	2 to 4	26.2	8.20	11.2	8.1
Arkutino	10.2020	2 to 4	25.7	8.13	11.2	8.1
Arkutino	11.2020	2 to 4	22.5	7.2	12.78	6.9
Arkutino	12.2020	2 to 4	19.9	6.5	12.69	6.8
Arkutino	01.2021	2 to 4	19.8	6.5	12.71	6.9
Arkutino	02.2021	2 to 4	20.3	6.9	12.78	7.5
Arkutino	03.2021	2 to 4	22.5	7.3	12.99	7.7
Arkutino	04.2021	2 to 4	23.1	7.4	13.1	7.7
Arkutino	05.2021	2 to 4	22.9	7.70	13.3	7.6

teria, yeasts, and fungi. Biolog's advanced phenotypic technology provides valuable information on the properties of the strains, in addition to species-level identification. Biolog's carbon technology identifies the environment and pathogenic microorganisms by producing a characteristic pattern or "metabolic fingerprint" of discrete test reactions performed in a 96-well microplate. The culture suspensions are tested with a panel of pre-selected assays, then incubated, read and compared with extensive databases. https://www.biolog.com/products-portfolio-overview/microbial-identification/

Results

We conducted a physicochemical analysis of the sea waters. The results are summarized in Table 1. The data represents results from the measurement of 4 basic physicochemical parameters.

The dynamics of changes in the physicochemical parameter values are shown in Fig. 1.

From the data presented in Fig. 1, it is visible that the temperature follows a course of decrease from September 2020 to January 2021, followed by a slow increase from February. If we compare May 2020 with May 2021, is evident that the temperature in 2021 was 1.5 °C colder (Fig 1a). Comparing the results in Fig. 1 we can say that the number of the species *P. alcaligenes* was twice as high in May 2021. It is likely that this species preferred to grow at lower optimum temperatures and constant other parameters. For the species, *Enterococcus hirae* such dependence was not observed - the number of this strain remained constant in May, but with increasing temperature, the number of microorganisms decreased during the summer months. Only *Enterococcus* clarify preferably developed at pH 7.78 (Fig. 1b). For the species *A. gyllenbergii*, we can say that it preferably grew at temperatures between 20.3–25.7°C, and the optimal temperature was 25.7 °C. For *C. farmeri* the optimum temperature was 26.2 °C and the pH 7.3. The species *E. vulneris* was probably related not only to the increase in water temperature but also to the anthropogenic factor, as it was found only in July. The
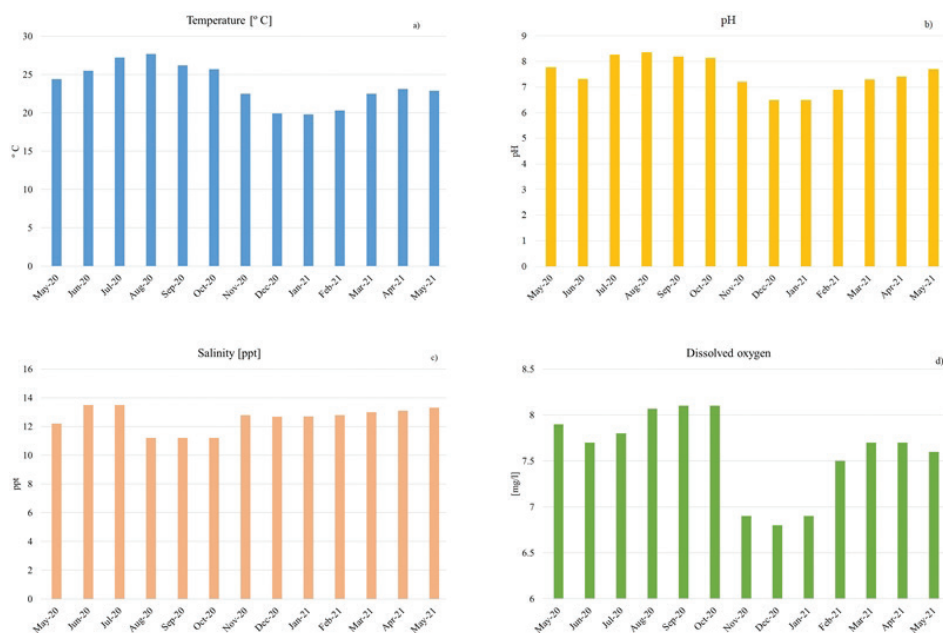
Figure 1. Dynamics in the change of physicochemical parameters of marine water.

Table 2. The number of bacterial cells in 1 ml on the different media.

Region/clam species	Pseudomonas agar	Cetrimid agar	Chromokult agar	MacConkey agar	Strain BIOLOG
Arkutino 17.05.2020/ *D. trunculus*	3.8×10^4		8.4×10^4		*Enterococcus hirae Pseudomonas mendocina*
Arkutino 20.06.2020/ *D. trunculus*			5.3×10^4	1.7×10^4	*Enterococcus hirae*
Arkutino 25.07.2020/ *D. trunculus*				1.6×10^7	*Escherichia vulneris*
Arkutino/ 25.08.20 *D. trunculus*			5.8×10^4		*Enterococcus hirae*
Arkutino/ 02.09.2020 *D. trunculus*			3.4×10^4		*Citrobacter farmeri*
Arkutino 17.10.2020/ *D. trunculus*				9.2×10^6	*Acinetobacter gyllenbergii*
Arkutino 18.11.2020/ *D. trunculus*	8.9×10^6				*Pseudomonas alcaligenes*
Arkutino 18.12.2020/ *D. trunculus*	1.34×10^7				*P. Pseudomonas alcaligenes*
Arkutino 18.01.2021/ *D. trunculus*	3.4×10^4				*P. Pseudomonas alcaligenes*
Arkutino 18.02.2021/ *D. trunculus*				4.4×10^4	*Acinetobacter gyllenbergii*
Arkutino 18.03.2021/ *D. trunculus*				8.4×10^5	*Acinetobacter gyllenbergii*
Arkutino 18.04.2021/ *D. trunculus*	3.2×10^4				*P. mendocina*
Arkutino 17.05.2021/ *D. trunculus*	7.4×10^4			7.3×10^3	*Pseudomonas mendocina Enterococcus hirae -*

lowest amount of dissolved oxygen was from November to January, when the species *P. alcaligenes* preferably develops (Fig. 1c).

The microorganisms isolated from *D. trunculus* were determined by the Biolog Microbial Identification System (Biologist VIO45101AM) to species level. After 24 h of cultivation on different media, the number of cells in 1 ml were obtained - data represented in Table 2 and Fig. 2.
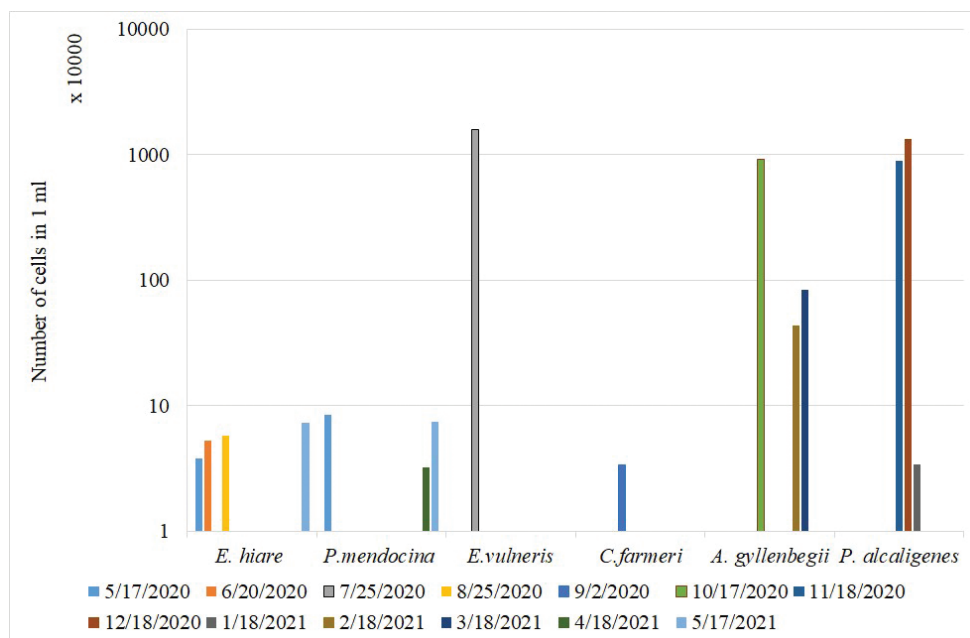
Figure 2. Dynamics of microorganism species, isolated from wedge clams for 2020–2021 period.

Fecal coliforms (FC) were represented by the species *Escherichia vulneris*, which was isolated only in July in a significant number of $1.6×10^7$ (Table 2; Fig. 2). The species *Enterococcus hirae* was isolated during the period from May to August (Table 2; Fig. 2). From genus, *Pseudomonas* (Table 2; Fig. 2) were detected *P. mendocina* (isolated in April and May) and *P. alcaligenes* (isolated in November, December and January). The species *Citrobacter farmeri* was isolated in September, and the species *Acinetobacter gyllenbergii* in February-March. The distribution dynamics of different types of microorganisms isolated from the wedge clam *D. trunculus* are represented in Fig. 2. Fig.1 shows that the species *Pseudomonas* preferably developed in the cooler months, although there was a variation within the genus, as well. The species *P. mendocina* preferred the warmer months, while the species *P. alcaligenes* preferred the colder ones.

Discussion

The microbiota found in marine organisms, and mussels in particular, can be considered in two respects - the so-called 'resident' microbiota, which is stable and unaffected by the environment, and the 'transitional', which depends on the environmental conditions. Specifically, our studies show that fecal coliforms represented by *E. vulneris*, which was isolated only in July, *Citrobacter farmeri*, which was isolated only in September, most likely belong to the transitional species. On the opposite, the species of *Pseudomonas* sp., *Enterococcus* sp. and *Acinetobacter gyllenbergii* are resident for the in-

vestigated mussels. Our results demonstrate an increase in the quantity of the coliforms in the region of Arkutino in July, when the quantity of the fecal coliforms is 190 times over the norms prescribed in Ordinance No. 4/20.10.2000 for the quality of fisheries water and the breeding of shellfish (the number of fecal coliforms in the inter-shell content should be less than 300 NVB). This can be harmful to human health following the consumption of mussels. Jorquera et al. (2001) suggested that the bivalve mollusks only present "transition" microbiota. In general, the resident microbiota performs various functions in mussels - it serves as food, a source of vitamins and growth factors, plays also a role in the defense mechanisms to prevent the colonization of bacterial pathogens or eliminate toxic substances (Prieur et al. 1990; Seguineau et al. 1996). On the other hand, the microbiota can also enter the mussels as a result of environmental pollution. According to data from the literature, many microorganisms belonging to different species – such as *Vibrio*, *Pseudomonas*, *Acinetobacter*, *Photobacterium*, *Moraxella*, *Aeromonas*, *Micrococcus*, and *Bacillus* (which are part of the bacterial population in the aquatic environment) may enter the mussels as a result of the diet by filtration. The first study analyzing the normal microbiota of a bivalve species was performed by Colwell and Liston (1960) with the Pacific oyster (*Crassostrea gigas*). The authors found a high proportion of Gram-negative bacteria (> 80%), with a predominance of the genera *Pseudomonas* and *Vibrio*, but also of *Flavobacterium* and *Achromobacter*. In general, the detected bacteria can be considered as typical psychrophilic marine bacteria, physiologically adapted to survive within the bivalve. In the literature, studies analyzing the diversity, distribution, and density of marine bacteria associated with bivalve mollusks are scarce. In fact, most studies have focused on characterizing bacteria with pathogenic potential for mollusks, or specifically for mussels. Thus, the genus *Vibrio* is widely studied, as it is known as one of the most important bacterial genera affecting the culture of bivalves. For example, *V. tapetis* has received special attention since it caused Brown Ring Disease (BRD), the bacterial etiology of which is described in adult clams. In addition, the disease caused by it is considered one of the main limiting factors for the culture of Manila clams (*Venerupis philippinarum*) (Europe Borrego et al. 1996), and was also detected in cultured clams in Korea (Park et al. 2006). Environmental parameters, such as variations in temperature and salinity, can affect the diversity of microorganisms and the environment as a physiological state of bivalves and its susceptibility to bacterial infections (Arias et al. 1999; Pujalte et al. 1999; Maugeri et al. 2000;Garnier et al. 2007). Evidence was found, that at lower water salinity BRD disease in mussels is much more severe (Reid et al. 2003). This assumption also correlates with our results. In the months of August, September, and October the lowest salinity of the seawater was reported in the region of Arkutino 11.2 ppt, which is also associated with the emergence of transitional species of *Citrobacter farmeri* and pathogenic coliforms (Table 2; Fig. 1). Romalde et al. (2012), found a wide variety of species of *Pseudomonas* sp, which make up about 52.8% of the microbiota in bivalves. These results completely correlate with the results obtained by us and are represented in Table 2. This fact shows that the genus *Pseudomonas* is one of the main groups of microbiota in mussels, although some seasonal variations can be observed, which are related to the

physicochemical parameters of the environment - temperature, pH and salinity. Our results indicate the need for deeper research on the microbiota of mollusks and on the pathogenic potential of marine bacteria, using both culture-dependent and molecular methods. To our knowledge, these are the first data of cultivated bacterial species in mussels from the Black Sea.

Conclusion

When studying the microbiota of populations of different species of bivalves, it is very important to know their sanitary status, as well as to determine the pathobiological basis of periodic outbreaks of diseases affecting these populations. Our results demonstrated the presence of bacterial species of genera *Pseudomonas*, *Enterococcus*, *Escherichia*, *Citrobacter*, and *Acinetobacer* in wedge clams *Donax trunculus* (Linnaeus, 1758). We found that the concentrations of *Escherichia vulneris* exceed 190 times the maximum available values according to Ordinance No. 4/20.10.2000. Inflated concentrations of coliforms in the summer attracted very special attention, indicating a seasonal worsening of the conditions of the seawater as a consequence of anthropogenic activity. We supposed that the pollution was very serious bearing in mind that the habitat of the wedge clams is in depth within the sand. The other important conclusion is the dominance of *Pseudomonas*, found in the mussels, which correspond to some seasonal variations related to the physicochemical parameters of the environment, such as - temperature, pH and salinity. With the worldwide increase in bivalve consumption, we would like to point out the possibility of the emergence of new diseases due to the interaction between the pathogen, the host, and the environment.

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RESEARCH ARTICLE

BioRisk

Sexually-manifested variations in pigmentation of *Boeckella poppei* (Copepoda, Calanoida) from Livingston Island (Maritime Antarctica)

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Abstract

Antarctic environments are exposed to high levels of ultraviolet radiation (UVR) that are often detrimental to their biota. Recent studies suggest that the genus *Boeckella* (Copepoda, Calanoida) has a high level of plasticity in terms of its reaction to UVR, which enables its wide distribution in various water bodies in Antarctica. *Boeckella poppei* is common in freshwater habitats of all three main biogeographic regions in Antarctica: sub-Antarctic islands, maritime and continental. We present for the first time a specific photoprotective response in populations of *B. poppei* from Livingston Island, Maritime Antarctica. In non-ovigerous females and in males, we observed uniform distribution of carotenoids in the body, while these pigments were almost entirely concentrated in the ovisacs of mature females. We consider this as a means of progeny protection from the teratogenic influence of the high level of UVR in Antarctic environments. Unequivocally, such adaptation would facilitate the expansion of *B. poppei* on the continent through colonisation and survival in shallow freshwater habitats. Given that the Antarctic environment is dynamically changing over the past decades and the accelerated retreat of permanent ice cover is a premise for the formation of shallow ponds, *B. poppei* could be a suitable indicator for reflecting the ongoing global environmental changes in Antarctica.

Keywords

Introduction

Solar radiation is an essential modulator of the functioning of natural ecosystems (Wetzel 2003). Ambient levels of biologically-damaging ultraviolet radiation (UVR) have been rising in aquatic systems in Polar Regions (Karentz and Bosch 2001; Perin and Lean 2004). During the 20th century, the release of anthropogenic contaminants increased the potential for photoactivated toxicity in these aquatic systems, triggering the organisms to develop mechanisms to minimise phototoxic damage (Diamond 2003).

The calanoid copepod *Boeckella poppei* (Mrázek, 1901) is widely distributed in freshwater habitats in the three main biogeographic regions in Antarctica: sub-Antarctic islands, maritime and continental Antarctica (Maturana et al. 2019). On Livingston Island, the species has been reported from the Hurd Peninsula (Pandourski and Apostolov 2004) and in most of the 15 studied lakes on the Byers Peninsula (Toro et al. 2007). During the last 25 years, owing to the environmental changes and the climate change-driven expansion of permanent ice-free habitats, *B. poppei* colonised many newly-formed temporary shallow water bodies in the north-west part of the Hurd Peninsula (Evtimova et al. 2021).

Boeckella poppei is known to have a wide plasticity to the specific polar environmental conditions, a result of various adaptations, including carotenoid pigmentation (Byron 1982) and photoprotection through the use of "sunscreen" compounds, for example, mycosporine-like amino acids (Rocco et al. 2002). Nevertheless, the population of *B. poppei* from Livingston Island demonstrates relatively high morphological variability and teratology (Pandourski and Chipev 1999; Pandourski and Evtimova 2009).

Here, we describe for the first time a sexually-manifested variation of pigmentation in *B. poppei*, inhabiting shallow temporary freshwater ponds on permafrost sediments.

Material and methods

We sampled two adjacent temporary turbid freshwater shallow ponds (with coordinates 62.63622°S, 60.35117°W and an altitude of 23 m a.s.l.) on 07.02.2020. The ponds were situated on permafrost sediments; dense and thick flocculation of microalgae and diatoms covered the ponds' bottom and edges (Fig. 1). The water transparency was very low due to colloidal inorganic particles, originating from glacier activity. Their depths did not exceed 10–12 cm, allowing solar radiation to penetrate to the bottom of the ponds. The bottom of the ponds was covered with a layer of fine inorganic particles and cobbles a few cm deep.

Basic physical and chemical characteristics of the water (Table1) were measured using hand-held oximeter Oxi 300i with DurOx 325 electrode and conductometer Cond 330i with KLE 325 electrode (WTW, Germany).

The specimens of *B. poppei* were collected with a hand-held net (50 μm mesh size) after intensive mixing of the water. They were transported alive to the laboratory of the Bulgarian Antarctic Base, immobilised in highly diluted ethanol and photographed.
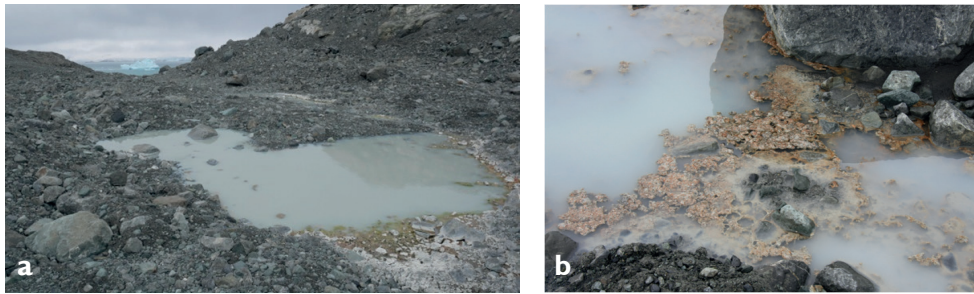
Figure 1. General view of pond 1 (**a**) and flocculation of microalgae and diatoms in pond 2 (**b**). Photo: I. Pandourski.

Table 1. Basic characteristics of the two ponds.

Pond	Area (m²)	Water temperature (°C)	Salinity (‰)	Oxygen (mg.dm⁻³)	(%)	Conductivity (µS.cm⁻¹)
1	10–12	7.6	0.00	8.4	74	82
2	5–6	9.1	0.00	9.5	81	131

Males, copepodites and females (ovigerous and non-ovigerous) were separated, based on the level of their morphological development and the morphological features typical for the species following published descriptions of the species (e.g. Bayly 1992). Body pigmentation was assessed visually in circa 100 individuals (males and females) from each pond. The basic morphometric characteristics of males and females in a population from Livingston Island are presented in Pandourski and Chipev (1999).

Results

We observed clearly manifested differences in body pigmentation of ovigerous females vs. adult males, non-ovigerous females and copepodites of *B. poppei*. The body of ovigerous females was depigmented, almost transparent, while their egg sacs were from red to dark orange. Ingested algae with a high concentration of pigments were clearly visible in their gut content (Fig. 2). Of the studied specimens, all mature females exhibited the described colouration. The body of males and non-ovigerous females was dark orange or red-coloured due to the even distribution of pigments (Fig. 3).

Discussion

We describe for the first time sexually-manifested pigmentation of *B. poppei*. Pigments were evenly distributed throughout the body of all specimens, except for mature ovigerous females, where they were concentrated in the egg sacs. Reddish colouration in copepods is caused by different carotenoids synthesised from the β-carotene

Figure 2. Depigmented, almost transparent body of mature females of *Boeckella poppei* with intensely pigmented ovisacs (above) and males with evenly pigmented bodies (below). Photo: L. Kenderov.
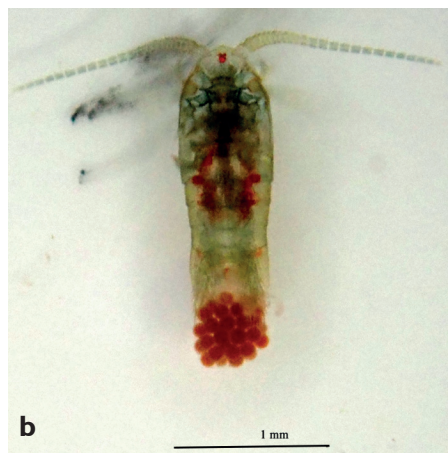
Figure 3. *Boeckella poppei*: two evenly pigmented males and one non-ovigerous female (**a**) and a mature female with almost transparent body and intensely pigmented ovisacs (**b**). Photo: L. Kenderov.

present in the algae used as a food source (Ringelberg 1980). One of the functions of this pigmentation is photoprotection. The synthesis of photoprotective compounds in the body of copepods is amongst the most important strategies to avoid the damaging effect of UVR in the Antarctic (Rocco et al. 2002) and high- latitude environments (Hansson 2004), as well as in alpine lakes (Tartarotti et al. 1999; Tartarotti et

al. 2017). Sommaruga (2010) established very high carotenoid concentrations (free astaxanthin) in calanoid copepods from clear fishless Himalayan alpine lakes. The concentrations of carotenoids in these copepods were inversely related to the lake depth refuge, while the lowest concentrations were found in copepods from a turbid glacier-fed lake.

The studied populations of *B. poppei* from Livingston Island inhabit small shallow turbid ponds. There the photoprotective strategy of migration to the deeper water layers was not possible as the depth of the ponds was only 5–12 cm and, despite the high turbidity of the water, the calanoids were likely exposed to the action of the UVR. In shallow ponds and lakes, the carotenoid pigments are known to play an important photoprotective role. According to Rocco et al. (2002), only the presence of photoprotection could be demonstrated in populations of *B. poppei* from the studied lakes on the Antarctic Peninsula, while the limited efficiency of enzymatic DNA repair mechanisms could be related to the low temperatures prevailing in Antarctic lakes.

In Antarctica, the life cycle of copepods in temporary ponds is controlled by the alteration between liquid water and ice and only few species can survive in these harsh conditions (Pociecha and Dumont 2008). Ovogenesis is one of the most sensitive stages in their life cycles as the damaging and teratogenic effect of UVR could cause morphological abnormalities incompatible with the survival of the individual. We consider the concentration of practically the whole amount of the accumulated pigments (as implied by the intensity of the colouration) into the ovisacs of females of *B. poppei* a specific strategy to protect progeny from the damaging UVR in the dynamically changing Antarctic environmental conditions over the past decades, enhancing the survival of individuals and expanding the range of this species.

Conclusions

The observed phenomenon of concentrating of carotenoid pigments in eggs of ovigerous females demonstrates the plasticity of *B. poppei* to survive in habitats exposed to high UVR. Further studies are needed to establish the type of the pigments and the mechanisms of their accumulation in the ovisacs. We suggest this pigmentation is a strategy for avoiding the teratogenic effects of UVR and for progeny protection. This, in turn, is facilitating the expansion of *B. poppei* in Antarctica through colonisation and survival in freshwater habitats, newly formed after the retreat of permanent ice cover. Thus, *B. poppei* could be a suitable indicator for reflecting the ongoing global environmental changes in Antarctica.

Acknowledgements

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RESEARCH ARTICLE

Organic vs conventional farming of oil-bearing rose: Effect on essential oil and antioxidant activity

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Abstract

The aim of this study was to establish whether the type of the agricultural system has any influence on the essential oil production and antioxidant activity of industrial cultivated *Rosa damascena* Mill. in the Rose valley, Bulgaria. Six private farms from Kazanlak (Rose) Valley, Southern Bulgaria were included in the study conducted in the period 2019–2020. The first three selected farms are designated within the conventional farming and the other three are certificated as organic farms. GC/FID and GC/MS analyses were performed; the contents of total polyphenols and flavonoids in the methanol extracts from rose petals were determined. Additionally, the antioxidant activity of rose extracts was evaluated by four reliable methods: 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), ferric reducing antioxidant power (FRAP), and cupric reducing antioxidant capacity (CUPRAC) assays. The impact of the agricultural system on the essential oil composition and antioxidant activity was evaluated by ANOVA statistical analysis. The results obtained showed that organic farming produced essential oil with a higher linalool and geraniol content and lower β-citronellol + nerol concentrations than conventional farming. It was found that organic farming production demonstrated a better antioxidant

activity evaluated by the three DPPH, ABTS, and CUPRAC methods according to the averaged data for two years, 806.82, 797.66 and 1534.40 mM TE/g dw versus 510.34, 521.94 and 917.48 mM TE/g dw for CF, respectively, with high statistical significance for the DPPH and ABTS analyses. Consequentially, the rose extracts from the organic farming accumulated more phenolic compounds that corresponded to the higher antioxidant potential of the organic roses.

Keywords

cv. Raduga, DPPH, *Rosa damascena* Mill., rose oil composition, total phenol

Introduction

Globally, the oil-bearing rose, for the production of essential oil, is industrially grown mainly in Bulgaria, Turkey, Iran, India, Pakistan, China, Morocco, Egypt, France, and Russia. However, Bulgaria is the leading producer of the main genotype oil-bearing rose (*Rosa damascena* Mill.) (Gunes 2005; Kovacheva et al. 2010; Ucar et al. 2017). According to Gunes (2005), 100 kg fresh rose flowers are required to produce approximately 10 g of essential oil. The commercial cultivation of roses in Bulgaria predominantly includes *R. damascena* Mill. *f. trigintipetala* Dieck. (Kazanlak rose) due to its high rose oil content and chemical composition (Chalova et al. 2017). The observations of recent decades revealed that the cultivar Raduga has spread widely in Bulgarian rose plantations (Kovacheva et al. 2010). It is a cross pollination result of *Rosa damascena* Mill. X *Rosa gallica* L., with high productiveness and essential oil content. Recently, interest in rose oil production has been growing not only due to its perfuming effects but also because of the wide range of biochemical reactions, such as an analgesic, hypnotic, antispasmodic, anti-inflammatory and anticonvulsant, which the rose flowers exhibit. (Baydar and Baydar 2013; Kumar et al. 2013; Mahboubi 2016) On the other hand, there is a real interest in organic crop production, particularly with aromatic and medical plants. Organic agriculture is an alternative cultivation model involving the agricultural practice without chemical additives. This cultivation method is designed to encourage respect towards the biological cycles of the production system to maintain and to increase soil fertility, to minimise any form of pollution, to avoid the use of synthetic fertilizers and pesticides, to maintain the genetic diversity, to consider the wide social and ecological impact of the food production system and to produce good quality foods in sufficient quantities. (Giuseppina et al. 2011). The choice of organic production of an essential oil crop in comparison with the conventional one is frequently registered as expensive and more challenging for agricultural producers, but the results are of particular importance for improved lifestyle, health, and longevity. Thus, the production of organic rose flowers and oil requires the application of new agricultural practices, which require additional investments and technologies in order for the production to be certified as organic and cost effective. One of the main challenges in rose oil organic cultivation is the fact that *R. damascena* exhibits low disease resist-

ance to major diseases and pests (Kovacheva et al. 2010). In this regard, the Integrated Pest Management (IPM) has a dominant role in organic rose oil cultivation. The most commonly applied IPM approaches include designing or redesigning the landscape, modifying the habitat to reduce the pest's resources and increase the habitats for natural predators, changing cultural methods such as cultivation, weeding, mulching as well as increasing inspections and tight monitoring of pest invasion (Chalova et al. 2017). Nunes and Miguel (2017) discussed the influence of several factors on the chemical composition of Damask rose oil and concluded that agricultural practices, important for essential oil productiveness and biochemical composition were mainly: method of propagation, time of the day of flower harvesting - air temperature, relative humidity, intensity of sunlight, flower stages, day period of harvesting, harvest procedures, time and level of pruning, storage of plant material, and method of distillation. Erdal and Munduz (2017) reported that the nutrient concentrations of the leaves from conventional gardens of *R. damascena* Mill were significantly higher than the organic ones, particularly for the nitrogen, manganese, and zinc content. Similarly, the flower nutrient concentrations of conventional gardens were higher for all examined nutrients, and the differences between organic and conventional gardens for the nitrogen, potassium, calcium, and iron concentrations were significant. The application of the anti-gibberellic, Paclobutrazol (PP333), combined with supplied nitrogen as NH_4-N and NO_3-N in appropriate amounts, and the micronutrients Mn^{2+}, Zn^{2+} and Cu^{2+} enhanced the flower bud formation and flowering as well as the rose oil yield with higher percentage of citronellol. Ucar et al (2017) reported the significant effect of different irrigation amounts and nitrogen doses 160 kg ha^{-1} and 240 kg ha^{-1} on the flower yield and rose oil yield for 2011 and 2012 (P < 0.01), but it was not established to be significant for the first year of investigation 2010. The effect of the cultivation practices on the fruit quality and antioxidant capacity was evaluated in many studies. (Wang et al. 2008) There is a small number of studies investigating the effects of agricultural practices on the secondary metabolites in medicinal and aromatic plants (Malik et al. 2011). Our primary investigations showed that the highest values of total phenols and flavonoids are found in the rosewater extract from organically grown plants: 47.09±2.89 mg GAE/g dry weight and 6.87±3.00 mg QE/g dry weight, respectively (Petkova et al. 2020). The highest radical scavenging activity was demonstrated by the extracts from organic plantations, while the metal-reducing assays showed higher antioxidant potential in the extracts from conventionally grown roses. Since many of the bioactive secondary metabolites produced by medical plants have ecological roles in promoting plant survival under a range of environmental conditions (Briskin 2000), Malik et al (2011) confirmed that the production of secondary metabolites within the medicinal and aromatic plants is under diverse physiological, biochemical, metabolic and genetic regulations and can be manipulated by alterations in the growing conditions. The aim of our study was to continue the investigation in that field in order to answer clearly whether the type of the agricultural system has any influence on essential oil production and the antioxidant activity of industrial cultivated *R. damascena* in the Rose valley.

Materials and methods

Location and site description

The field study was conducted in private farms, located in the northwest part of the Kazanlak Rose valley, Bulgaria, in the two-year period of 2019–2020. The valley is situated at 400–500 m altitude, in the middle of the country between the Balkan range in the north and Sredna Gora mountain to the south. The climate is continental, the winters are generally cold and wet, and the summers cooler than in other parts of Bulgaria. January is the coldest month of the year with a -1.1 °C average temperature. July is the warmest month in Kazanlak with an average temperature of 21.2 °C. Spring in Kazanlak has the feel of winter, with late snowfalls. The annual precipitation rates range from 500 to 650 mm in the Kazanlak valley, with heaviest precipitation between April and June (Sobotkova and Ross 2018). Data about the climatic conditions of the studied years were provided from the local meteorological station, situated in Kazanlak. The monthly distribution of the average temperatures and rainfalls, compared to the 100-year average rate are presented in Figs 1, 2, respectively.

The field study was conducted in six private farms, as three of the oil rose private plantations are certified as organic farms whereas they apply an organic agricultural system and the rest of them are designated within the conventional farming. The farms are located close to each other and the dominant soil type is fluvisols. Fluvisols (Deluvial soils) are formed by downhill creep, where the sorting of materials comes about through gravity. Creep is the slow movement of soil masses down slopes that are
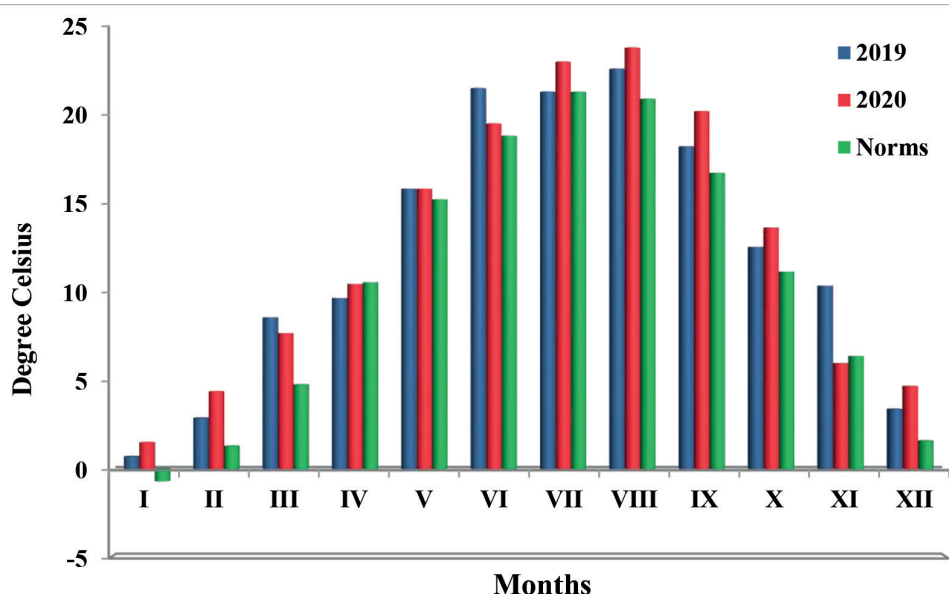

Figure 1. The average monthly temperatures (°C) for Kazanlak valley in 2019/2020.
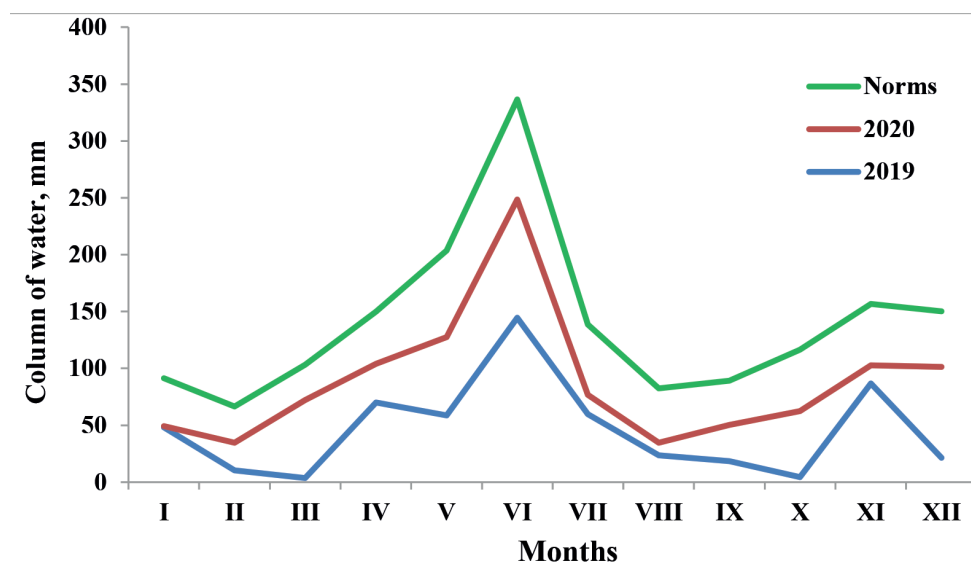
Figure 2. The average monthly rainfall (mm) for Kazanlak valley in 2019/2020.

usually steep. The process takes place in response to gravity where there is a pronounced water saturation (Shishkov and Kolev 2014). The soils in the region have ochric and nondiagnostic features, typical of fluvisols. The soil samples in all six arable areas were characterized by acid reaction, typical for that soil. The range of values of pH (H_2O) was between 4.20 and 6.10. The soil organic matter (OM) content varied between low and high content with values from 0.86 to 4.03%, where the mean OM, % value was 2.80. More detailed information about the soil characteristics was reported by Todorova et al. (2020).

Agricultural practices in the studied farms

Detailed characterization of the agricultural practices of the studied farms is presented in Table 1.

Farm 1 is characterized as typical conventional farming with soil tillage, mineral fertilization, foliar feeding with NPK + microelements during vegetation. The soil tillage included 3–4 hoeing with a cultivator between the rows.

Farms 2 and 3 are conventional with combined good agricultural practices, in our case - turf surface as mulching, drip irrigation, mineral fertilization, foliar feeding with NPK + microelements during vegetation.

Farm 4 is certified as organic farming with manure fertilization 2–3 t/dka (20–30 t/ha), applied every 3–4 years before vegetation. Before and after flowering – several times foliar application of organic fertilizer containing macro-, micronutrients, and amino acids. Only soil tillage was carried out to control weeds. The soil tillage included 3–4 hoeing with a cultivator or manually between the rows, with biopesticides application.

Table 1. Farming system, geographical data, variety with general agricultural practices in the studied farms.

Farm's number	Area	GPS coordinates	Variety	Agricultural practices	Drip irrigation
Conventional Farming (CF)					
01	Koprinka	42°38'10.74"N, 25°19'29.496"E	*R. × damascena f. trigintipetala* Dieck	Soil tillage, mineral fertilization, foliar feeding with NPK + microelements during vegetation, pesticides	-
02	Damascena 1	42°40'11.6"N, 25°11'50.1"E	*R. × damascena f. trigintipetala* Dieck	Turf surface as mulching, mineral fertilization, foliar feeding with NPK + microelements during vegetation, pesticides	yes
03	Damascena 2	42°40'6.60"N, 25°11'53.40"E	*R. × damascena f. trigintipetala* Dieck	Turf surface as mulching, mineral fertilization, foliar feeding with NPK + microelements during vegetation, pesticides	yes
Organic Farming (OF)					
04	Yasenovo	42°41'36.0"N, 25°16'39.8"E	*R. × damascena f. trigintipetala* Dieck	Soil tillage, manure application, bio pesticides	-
05	Asen	42°38'35.8"N, 25°10'30.0"E	*cv. Raduga Rosa damascena × Rosa gallica*	Turf surface as mulching, manure application, bio pesticides	yes
06	Skobelevo	42°40'16.5"N, 25°10'36.5"E	*cv. Raduga Rosa damascena × Rosa gallica*	Turf surface as mulching, manure application, bio pesticides	yes

Farms 5 and 6 are also certified as organic farming, with manure fertilization, foliar application of organic fertilizer containing macro-, micronutrients, and amino acids, with a turf surface as mulching between the rows and drip irrigation.

Plant material and sampling

We surveyed farms growing oil-bearing roses - *R. damascena* Mill. f. *trigintipetala* Dieck and cultivar Raduga [(*Rosa damascena* Mill. × *Rosa gallica* subsp. *Eryosyla Kell* var. *Austriaca* Br.) × *Rosa gallica* L.] (Nazarenko 1983). The harvest time for rose oil yield is early in the morning, between 6 and 11 a.m., to avoid temperature increase during the day, which negatively affects the yield and the quality of the rose oil (Chalova 2017). The samples of rose flowers were picked up in the morning (6–8 a.m.) within a day at the beginning of June 2019 and 2020. Three samples were randomly taken from each individual field, and each of them was collected from 40 different rose bushes. Similar experimental designs were presented by Vagn et al. (2000) for onion and peas and by Tuncay and Bostan (2010) for apricot under organic and conventional cultivation. Each sample of rose flowers (1000 g) was split into two parts. The first was used for distillation and essential oil production. The second sample of rose flowers was used for biochemical analysis.

Distillation and essential oil production

The essential oil content in the blossoms was determined after steam distillation in the Clevenger-type micro apparatus. The essential oil was measured to the graduated part of the apparatus in milliliters and was calculated as a percentage by volume (v/w). For higher accuracy, a relative density recalculation was made and was presented as a per-

centage by weight (w/w). After collection, the oil was treated with anhydrous Na_2SO_4 and stored in tightly closed vials at 4 °C till analysis. The analysis was performed on an Agilent 7820A GC System coupled with a flame ionization detector and 5977B MS detector. The protocol was made according to ISO 9842 for gas chromatographic analysis of rose oil. Two capillary columns: non-polar EconoCap™ EC™ (30 m × 0.32 mm ID, 0.25 μm film thickness) and polar HP-20M (50 m × 0.32 mm ID, 0.30 μm film thickness) were used. The first one was operated with an oven program from 80 °C (2.5 min held) to 320 °C at a rate of 10 °C/min; with 10 min held at the final temperature was applied. Hydrogen (99.999%) was used as a carrier gas at a constant flow rate of 20 ml/min. The split ratio was 1:10, the inlet temperature was set to 250 °C and the FID temperature was set to 300 °C. The non-polar column reveals a much richer spectrum of compounds and better presentation of paraffins, but it is not suitable for dividing the main terpene alcohols citronellol and nerol. They have very similar retention times and could not be split and calculated. For this reason the polar column was used for better separation. Due to the character of the HP-20M, the oven temperature program was the following: 65 °C for 0 min, then 2 °C/min to 220 °C for 10 min.

The GC/MS analysis was performed under all conditions, described above.

The ingredients were quantified by the area of FID peaks without any correction factor. The oil constituents were identified by their mass spectra, matching with the NIST and MS library, as well as whenever possible, the authentic substances were used.

Extracts preparation

Rose petals were subject to extraction in duplicate with 80% methanol in 1:15 solid to solvent ratio. The extraction was conducted in an ultrasonic bath (SIEL, Gabrovo, Bulgaria, 35 kHz, and 300 W) for 20 mins, at 65 °C. The combined extracts were used for further analysis.

Total phenolic contents

The total phenolic content was measured using a Folin-Ciocalteu reagent with slight modification (Ivanov et al. 2014).

The total flavonoids content

The total flavonoids content was analyzed using $Al(NO_3)_3$ reagent (Kivrak et al. 2009).

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The rose petal water extract (0.15 mL) was mixed with 2.85 mL 0.1 mM methanol solution DPPH. After 15 min at 37 °C, the reduction of absorbance was measured at 517 nm against methanol used as a blank sample (Ivanov et al. 2014).

2,2´-azino-bi's-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay

The rose petal extract (0.15 mL) was mixed with 2.85 ml of the ABTS solution. After 15 min at 37 °C in darkness, the absorbance was measured at 734 nm (Ivanov et al. 2014).

Ferric reducing antioxidant power (FRAP) assay

The assay was performed according to Benzie and Strain (1996).

Cupric reducing antioxidant capacity (CuPRAC) assay

The rose petal extract (0.1 mL) was mixed with 1 mL $CuCl_2.2H_2O$, 1 mL Neocuproine (7.5 mL in methanol), 1 mL 0.1 M ammonium acetate buffer and 1 mL distilled water. After 20 min at 50 °C, the absorbance was measured at 450 nm (Ivanov et al. 2014). All results for the antioxidant activity were expressed as mMTE/g.

Statistical analysis

The data were expressed as a mean ± standard deviation (SD) from three replicates for each sample. All the results from the determination of antioxidant activity were performed in triplicates and expressed as mM Trolox equivalents (mM TE) by dry weight. To establish the influence of the agricultural practices in the studied farms on the essential rose oil composition and antioxidant activity, ANOVA statistical analysis was performed. The significant differences were tested and the P values < 0.05 were considered statistically significant. The impact of the factors was evaluated via the Coefficients of determination R^2. The IBM SPSS Statistics 26.0, Copyright 1989, 2019 statistical package was used to process the data.

Results and discussion

Essential oil composition

The climate data for the 2019–2020 period showed that the temperatures were much higher than the average rates for the winter season (especially 2019). This is the dormancy period for the plants. During the spring months, the temperatures were normal and higher in June, particularly in 2019. Regarding precipitation, it is obvious that in both years they were below normal. The second year of the study is characterized as wetter with more rainfall than in 2019, especially during the spring.

The chemical composition of the essential oil of rose flowers under conventionally and organically grown roses is presented in Table 2. According to the averaged data for the two periods, the productiveness of essential oil, % obtained in conventional farming (CF) was 0.04% higher than the organic system (OF) - 0.03%, with insignificant

Table 2. Biochemical composition of the essential oil of rose flowers under conventionally and organically grown roses for the two years period of study.

Type of farming	Conventional farming - CF				Organic farming - OF				R^2
Biochemical component	min	max	average	SD	min	max	average	SD	
Rose oil,%	0.03	0.05	0.04 [ns]	0.01	0.02	0.05	0.03 [ns]	0.01	0.229
Ethanol	0.00	0.92	0.19 [ns]	0.36	0.01	0.92	0.21 [ns]	0.31	0.025
Linalool	0.38	0.71	0.54 [a]	0.11	0.52	0.93	0.61 [a]	0.22	0.569
cis –Rose oxide	0.15	0.27	0.22 [a]	0.05	0.00	0.27	0.16 [a]	0.10	0.521
trans –Rose oxide	0.08	0.14	0.12 [a]	0.02	0.00	0.14	0.08 [a]	0.05	0.532
Phenylethyl alcohol	0.33	1.33	0.83 [ns]	0.37	0.34	1.33	0.80 [ns]	0.34	0.000
β-Citronellol and Nerol	25.48	33.60	29.47 [a]	3.18	17.86	33.60	25.08 [a]	7.91	0.450
Geraniol	23.11	26.13	24.33 [a]	1.18	20.91	35.81	25.42 [a]	8.03	0.427
Eugenol	0.38	1.35	0.70 [ns]	0.35	0.00	1.35	0.56 [ns]	0.46	0.185
Methyleugenol	0.46	0.72	0.60 [ns]	0.10	0.00	1.32	0.50 [ns]	0.36	0.055
Heptadecane (C_{17})	0.85	2.47	1.96 [ns]	0.60	0.98	2.69	1.83 [ns]	0.68	0.000
Farnesol	0.19	3.37	1.51 [ns]	1.43	0.32	3.37	1.59 [ns]	1.32	0.003
Nonadecene ($C_{19:1}$) + Nonadecane (C_{19})	14.37	18.62	17.29 [a]	1.65	11.36	18.62	15.09 [a]	4.30	0.367
Eicosane (C_{20})	0.98	1.76	1.33 [ns]	0.27	0.52	1.77	1.11 [ns]	0.46	0.261
Heneicosane (C_{21})	4.56	8.56	6.00 [ns]	1.37	2.71	10.14	5.43 [ns]	2.26	0.053
Tricosane (C_{23})	0.90	2.06	1.36 [ns]	0.43	1.06	3.01	1.50 [ns]	0.70	0.139
Pentacosane (C_{25})	0.27	0.80	0.50 [ns]	0.19	0.36	1.16	0.56 [ns]	0.28	0.118
Heptacosane (C_{27})	0.16	0.64	0.38 [ns]	0.16	0.19	0.92	0.38 [ns]	0.21	0.013

a,a Same superscripts within the same row represent significant differences at the level of significance $P < 0.05$; ns – not significant differences ($P > 0.05$); R2 – Coefficients of determination based on observed means.

difference. Statistically significant differences were found between the average values of the main components of essential oil composition such as geraniol, β-citronellol + nerol, linalool, cis-rose oxide, trans-rose oxide, and nonadecene ($C_{19:1}$) + nonadecane (C_{19}). The content of geraniol, β-citronellol and neroll varied within the following range: geraniol (23.11–26.13%), β-citronellol and neroll (25.48–33.60%) for CF and (20.91–35.81%), (17.86–33.60%) for OF. (Table 2). The hydrocarbons are presented by aliphatic alkanes and alkenes. The major of them is nonadecane ($C_{19:1}$) + (C_{19}) with values between (14.37–18.62%) for CF and (11.36–18.62%) for OF, the next one is heneicosane ($C_{21}H_{44}$), varied from (4.56% to 8.56%) for CF, and (2.71 to 10.14%) for OF. The other saturated hydrocarbons (tricosane, pentacosane, and heptacosane) do not show a certain trend and move relatively within an equal range. The phenylpropane compounds are represented by phenylethyl alcohol, eugenol and methyleugenol. The first one is most abundant in a native flower odor, but in the essential oil, its quantity is minor (Dobreva 2011; Rusanov et al. 2011; Erbaş and Baydar 2016). Due to its high water solubility, this substance is carried off with the distillation waters. It is limited in the international standard with less than 3.5%. In our study, it varies within the narrow range from 0.33 to 1.33% for CF and 0.34 to 1.33% for OF. The methyleugenol is an odor contributor, but it is not desired above a certain concentration in the essential oils due to potential cancer and allergic effects on human health (Johnson et al. 2000). The rose oil contains methyleugenol in percentages up to 5.0%, especially if the rose flowers are fermented before processing. This compound is limited and subject to monitoring. For the two-year period, the levels are relatively low: from (0.46 to 0.72%) for CF and from (0.00–1.32%) for OF. The

results of the statistical analysis showed an influence on the essential oil composition in our study case. The coefficients of determination (R^2) vary within the range from 0.367 to 0.569, thus, the influence of the type of rose oil cultivation varied from 36.7 to 56.9%. A significant difference was not found for the other components of the biochemical composition of the essential oil, and the coefficients of determination (R^2) varied within very low limits. On the basis of the results obtained, the organic farming produced essential oil with higher linalool and geraniol content and lower β-citronellol and nerol concentrations, whereas the essential oil of CF is characterized by higher β-citronellol and nerol concentrations and lower linalool and geraniol content. Similar results were obtained by Kumar et al (2017), the authors reported more flower yield plant^{-1}, number of flowers plant,$^{-1}$ and flower yield ha^{-1} and a higher percentage of citronellol+nerol with an application of 120:40:90 kg NPK ha^{-1}. The authors discussed that the ratio citronellol+nerol/geraniol is higher for fertilized plots in comparison with manure plots. In our study the ratio citronellol+nerol/geraniol was also bigger 1.21 for CF than OF - 0,98.

Antioxidant activity of the rose flower

The total phenols, total flavonoids, and antioxidant activity in methanol extracts from conventionally and organically grown roses are presented in Table 3.

The phenolic compounds, even if not directly related to the food nutritional quality, have been receiving increasing attention as a result of their specific biological activ-

Table 3. The total phenols, total flavonoids and antioxidant activity in methanol extracts from conventionally and organically grown roses.

Compound, %	Region	Year	Total phenols, mg GAE/g dw	Total flavonoids, mg QE/g dw	Antioxidant activity, mM TE/g			
					DPPH	ABTS	FRAP	CUPRAC
Conventional farming	Koprinka	2019	49.01±0.22	11.07±0.23	524.32±39.89	522.74±65.06	3235.01±27.85	1713.83±147.19
	Koprinka	2020	40.60±13.30	9.38±1.12	596.61±128.1	544.50±111.18	955.50±333.31	456.79±52.22
	Damas 1	2019	41.14±0.23	11.49±1.44	509.57±34.58	571.33±96.56	2309.14±227.49	1128.14±77.45
	Damas 1	2020	39.50±7.3	11.13±1.30	639.64±127.62	578.43±77.21	1602.74±316.61	723.13±193.31
	Damas 2	2019	41.74±0.07	11.59±0.64	476.75±41.83	607.67±79.99	2744.97±189.04	1176.25±145.31
	Damas 2	2020	23.1±3.2	7.46±0.56	315.143±89.74	306.99±82.78	1062.2±326.452	306.76±44.27
	min		23.10	7.46	315.14	306.99	955.50	306.76
	max		49.01	11.59	639.64	607.67	3235.01	1713.83
	average		39.18 [ns]	10.35 [ns]	510.34 [a]	521.94 [a]	1984.93 [ns]	917.48 [ns]
	SD		8.58	1.63	112.77	109.26	927.65	523.16
Organic farming	Yasenovo	2019	63.45±0.05	12.36±0.50	1033.06±30.57	793.72±63.72	3141.96±29.02	2687.07±121.83
	Yasenovo	2020	36.30±4.9	8.89±2.27	592.86±64.63	604.33±78.95	1418.70±362.67	537.34±107.42
	Asen	2019	73.23±0.99	11.32±0.68	839.96±12.47	1033.31±84.04	746.41±90.16	2658.17±242.94
	Asen	2020	41.8±12.8	13.02±2.46	675.69±179.13	676.23±178.20	1301.9±609.73	687.46±169.86
	Skobelevo	2019	69.83±4.58	11.55±1.0	754.32±29.86	1026.13±21.60	721.49±44.06	1760.29±112.99
	Skobelevo	2020	41.3±3.3	13.03±1.56	945.04±326.25	652.21±45.17	3648.3±1951.29	876.06±107.71
	min		36.30	8.89	592.86	604.33	721.49	537.34
	max		73.23	13.03	1033.06	1033.31	3648.30	2687.07
	average		54.32 [ns]	11.70 [ns]	806.82 [a]	797.66 [a]	1829.79 [ns]	1534.40 [ns]
	SD		16.32	1.55	165.60	190.27	1255.27	978.51
	R^2		0.288	0.176	0.568	0.486	0.006	0.156

a,a Same superscripts within the same column represent significant differences at the level of significance $P < 0.05$; ns – not significant differences ($P > 0.05$); R2 – Coefficients of determination based on observed means.

ity. Higher values of the total phenols were found in the methanol extract of rose petals from OF between (36.30–73.23) mg GAE/g dw, with a mean value of 54.32 mg GAE/g dw and a lower concentration for CF between (23.10–49.01) mg GAE/g dw with a mean value of 39.18 mg GAE/g. The values of total flavonoids varied between 8.89 and 13.03 mg QE/g dw with a mean value of 11.70 mg QE/g dw for OF and (7.46–11.59) QE/g dw with a mean value of 10.35 QE/g dw for CF. An interaction was found between the impact of the agricultural system and annual conditions in the study period on the total phenol and total flavonoids content, but a statistically significant difference was not found with regards to the agricultural system. The antioxidant activity of the rose petal extracts was evaluated by four methods based on the different mechanisms (DPPH, ABTS, FRAP, and CUPRAC). It was found that organic farming production (Table 3) demonstrated the best radical scavenging activity evaluated by the three DPPH, ABTS, and CUPRAC methods according to the averaged data for two years, namely 806.82, 797.66 and 1534.40 mM TE/g dw for OF versus 510.34, 521.94 and 917.48 mM TE/g dw for CF with high statistical significance for the DPPH and ABTS analyses. The coefficient of determination (R^2) showed influence of the agricultural system type on the oil bearing rose cultivation with more than 0.49% on antioxidant activity in oil rose flower. The authors' team of Wang et al. (2008) also reported significantly higher total phenolics, total anthocyanins, and antioxidant activity in blueberry fruit grown from organic culture than fruit from conventional farming. The investigation of Taie et al. (2010) with basil plants also indicated that organic fertilization can yield a significant increase in antioxidant activity, anti-cancer activity, phenolics, and flavonoids of the culinary herbal plant.

The impact of climate conditions in 2019 and 2020, irrigation practice and the variety types on the essential oil composition and the antioxidant activity of rose petals was also studied. The results are presented in Table 4.

According to Dobreva and Angelova (2011), the greatest influence on the biosynthesis of the essential oil is attributed to temperature, humidity, and light intensity, whereas the air humidity is associated with an increased production of the rose flowers and rose oil content. Therefore, the second year 2020 would be more favorable for the growth of the oil-bearing rose and the production of essential oil in the study region. As can be seen from the data presented in Table 4, the productiveness of essential oil from the studied private farms was greater in 2020 by 0.04% than it had been in 2019, without a statistical significance. Results of the statistical analysis showed that climate in the period under study was a factor influencing essential oil composition. Statistically significant differences were found between the average values of the components such as farnesol, tricosane, pentacosane and heptacosane. The coefficients of determination (R^2) varied within the range from 0.35 to 0.97, thus, the influence of the climate conditions on these components varied from 35% to 97%. A statistically significant difference was also found with regards to the total phenols and the DPPH antioxidant activity, whereas the drier year of 2019 was more favorable for biosynthesis of second metabolites. This confirmed that the contents of polyphenolics and antioxidants in the rose petals (R^2 = 0.47; 0.65) are strongly influenced by the environment, e.g. the geographical and edaphic factors, as previously described by Ginova et al. (2013). Any impact of the ir-

rigation practice on the rose oil quality and biosynthesis of second metabolites was not found. According to Ucar et al (2017) irrigation, as an agronomic practice has a significant effect mainly on the oil bearing flower yield. A statistically significant difference of the essential oil composition was found between the R. *damascena* and cv. Raduga for the main components such as geraniol, β-citronellol and neroll and eugenol. The first thing that stands out is the relationship between the most abundant terpene alcohols – for Raduga the dominant is geraniol (32.36–35.81%) and lower for R. *damascena*, between (20.91–27.24%) whereas the coefficient of determination is $R^2 = 0.89$.

Table 4. Impact of the climate condition in 2019 and 2020 years, irrigation practice and variety on the rose oil composition and the antioxidant activity of rose petals.

Biochemical component of rose oil/rose flower	min	max	mean	SD	min	max	mean	SD	R^2
			2019 Year				2020 Year		
Essential oil, %	0.02	0.04	0.03 ns	0.01	0.03	0.05	0.04 ns	0.00	0.01
Farnesol	0.19	3.37	0.28 a	0.67	2.43	3.37	2.88 a	0.34	0.97
Tricosane (C_{23})	1.30	3.01	2.09 a	0.68	0.90	1.31	1.18 a	0.17	0.54
Pentacosane (C_{25})	0.45	1.16	0.77 a	0.30	0.27	0.53	0.42 a	0.98	0.44
Heptacosane (C_{27})	0.26	0.92	0.52 a	0.24	0.16	0.38	0.28 a	0.08	0.35
Total phenols, mg GAE/g dw	41.14	73.23	56.40 a	14.25	23.10	41.80	37.10 a	7.13	0.47
CUPRAC,mM TE/g	1128.14	2687.14	1853.96 a	686.34	306.76	876.06	597.92 a	204.66	0.65
			Irrigation				Non irrigation		
Methyleugenol	0.00	0.67	0.31 a	0.27	0.67	1.32	0.92 a	0.30	0.56
Heptadecane (C_{17})	1.76	2.69	2.22 a	0.29	0.85	2.36	1.40 a	0.68	0.47
			R. Damascena				cv. Raduga		
Linalool	0.38	0.83	0.57 a	0.14	0.81	0.93	0.87 a	0.56	0.62
cis –Rose oxide	0.15	0.27	0.22 a	0.43	0.00	0.04	0.02 a	0.21	0.88
trans –Rose oxide	0.08	0.14	0.12 a	0.23	0.00	0.03	0.01 a	0.02	0.87
β-Citronellol and Nerol	25.48	33.60	29.29 a	3.05	17.86	20.02	18.80 a	0.96	0.81
Geraniol	20.91	27.24	24.27 a	1.97	32.36	35.81	34.46 a	1.64	0.89
Methyleugenol	0.46	1.32	0.74 a	0.28	0.00	0.14	0.06 a	0.07	0.68
Eicosane (C_{20})	0.98	1.77	1.35 a	0.30	0.52	0.89	0.66 a	0.17	0.64
ABTS, mM TE/g	306.99	793.70	566.21 a	133.45	652.21	1033.31	846.97	211.27	0.45

a,a Same superscripts within the same column represent significant differences at the level of significance $P < 0.05$; R2 – Coefficients of determination based on observed means, SD-Standard deviation.

Conclusion

The conventional or organic agricultural type of system is a question of choice for every farmer, based on the benefits and challenges in the agricultural sector. In the medical plants cultivation, including oil bearing rose production, the choice of the system and the application of agricultural practices could have an enormous effect on the quality of cosmetic rose products and food supplements. Our results show that the application of combined eco-friendly agricultural practices in organic private farms in *R. damascena* cultivation gives a better quality of the rose flowers with higher values of antioxidant activity in comparison with the conventional agricultural system.

Acknowledgements

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RESEARCH ARTICLE

Evaluation of viral infection levels in intensive and organic poultry farming

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Abstract

Whereas early organic farming was mainly focused on plant production, in the last decade, the number of organically-managed poultry farms within the European Union has increased significantly. Similar to organic crop production, organic animal farming is based on the same principles: welfare-friendly, sustainable production and resource utilisation without or with very little addition of synthetic substances, such as antibiotics and antiparasitic treatments. These practices, as well as the access to wild animals, make the free-range poultry production systems predisposed to different viral diseases and, thus, associated with potentially higher public health risks or reduction in production quality. On the other hand, intensive farming amplifies the impact of viral diseases due to high density, low genetic diversity and elevated immunodeficiency. The aim of this analytical study is to compare free-range with intensive poultry systems and the occurrence of different viral diseases in these types of farms in the EU over the past decade. The research is based on official data from the statistical office of the European Union, as well as official data from the Member countries. The results were similar in each country and demonstrate that free-range production has a higher incidence of viral diseases with high zoonotical potential. This makes year-round surveillance absolutely necessary, as well as the need for implementation of additional criteria and requirements towards free-range systems.

Keywords

European Union, farming systems, free-range poultry, intensive farming, meat production

Introduction

In recent years, we have observed the characteristics, defined by Armelagos et al., of the Third transitional period in infectious diseases (Armelagos et al. 1991). This transitional period is characterised by the re-emergence of pathogens already considered eradicated or under control, as well as new pathogens (for example: HIV, SARS, MERS, SARS-CoV-2) and increasing antibiotic resistance. In addition to the recurrence of these viral diseases, some of them have significantly increased virulence (Barrett et al. 1998). According to other studies, human intervention and, in particular, the effects on the environment as a result of modern agricultural practices, are crucial for the emergence of these phenomena (Lebarbenchon et al. 2008).

The present study aims to trace the situation in the European Union with regard to the various poultry farming systems – both traditional and organic. Wild birds as a vector of viral diseases are also considered as a factor.

Demand for products produced by organic farmers has grown significantly over the last decade. The Council of the European Union defines organic farming as a system of food management and production that combines best practices in terms of environmental protection, high degree of biodiversity, protection of natural resources, application of high standards of animal welfare and a production method tailored to the preferences of some consumers for products produced using natural substances and processes (EC 2007).

On the other hand, intensive poultry farming, with a high density of animals in confined spaces, is a system with conditions significantly different from those of organic farming and wild birds. At the same time, the use of a number of drugs and modern vaccines in intensive care systems aims to protect animals from a number of pathogenic factors. In organic systems, significantly limited drug use would be a factor for allowing the easy spread of pathogens amongst herds, which, on the other hand, would be limited by significantly lower stocking densities compared to intensive systems. The introduction of specific requirements for organic production in terms of cultivation methods - regular control of certain infectious diseases, the requirement for indoor feeding etc. – is successful in terms of protection against a number of bacterial and parasitic diseases (Wierup et al. 2017) (Thapa et al. 2014). Despite these results, the presence of an outdoor area where birds spend most of the day poses a high health risk for some viral diseases (such as bird flu) compared to intensive care systems (Koch and Elbers 2006; Gonzales et al. 2013).

Based on these studies, it is difficult to determine whether organic systems pose a lower health risk to the population or a higher risk. At the same time, there is a significant increase in interest in organic farming in the European Union: doubling the size of organic farms compared to those with intensive production, more than 70% increase in the size of organic farming land within 10 years (EC 2019).

To date, analytical studies, based on data from Member States, have focused on an annual period and are published annually on the European Food Safety Authority (EFSA) website. Therefore, the aim of our study is to use and analyse data from a period

of time longer than 5 years. The advantage of using the results of the EU Member States is the collection and processing of data collected on the basis of unified criteria and through the use of uniform formats. In other words, EU Member States can present themselves as a country consisting of 27 districts. This, in contrast to previous studies, eliminates the problem of the lack of heterogeneity of input information - criteria and mechanisms of collection, all giving maximum statistical reliability of the processed data.

This study will examine the levels of infectious risk in traditional and organic systems in order to further clarify the presence of zoonotic risk in different systems, referring to the data recorded by individual Member States in the period 2012–2020.

Materials and methods

When selecting indicators in the design of this study, we focused on the following parameters: viral disease of zoonotic nature or having the potential for such, developed reliable methods of diagnosis, diseases subject to mandatory control and reporting by Member States and prevalence or establishment giving statistically significant results. West Nile Virus and Avian Influenza, considered as the two viral diseases in birds requiring mandatory reporting, were taken into account Council Directive of 30 November 2009 (EC 2009). The data for the registered cases are given in Table 1. For Avian Influenza, the count for registered cases is consolidated between High Pathogenic Avian Influenza (HPAI) and Low Pathogenic Avian Influenza (LPAI).

Referring to the indicated data and the p-values, both determining the use of data for Avian Influenza (AI) as a statistical indicator, give a better expression of the purpose of the present study.

Data collection and processing

This study is based on official data from the European Union Member States official reports. The parameters on which these reports are prepared and submitted are set in Directive 2010/367 (EC 2010). Data for the period from 2012 to 2018 are publicly available on the website of the European Food Safety Authority (EFSA) at: https://www.efsa.europa.eu/en/publications. Starting from the beginning of 2019, the data have been submitted in SSD2 format and can be found on the EFSA website Knowledge Junction community on ZENODO at: https://zenodo.org/. SSD2 format specifications are defined in (European Food Safety Authority 2019).

Table 1. Compulsory Notifiable Diseases cases registered in the European Union for year 2019. (European Food Safety Authority 2021; European Food Safety Authority 2020).

Compulsory Notifiable Diseases	Registered Cases
Avian Influenza (HPAI and LPAI)	1320
West Nile Virus	107

The data collected for the period 2012–2018, in the form of statistics from the annual reports themselves (available as PDF files at the above address) and datasets in SSD2 format with data for 2019–2020 were entered in separate databases. These datasets were processed with the software SPSS 28 (https://www.ibm.com/analytics/spss-statistics-software), with which the graphs in the study were produced.

For the purpose of the present study, the categories described in the EU Commission Decision of 25 June 2010 (EC 2010) are grouped into two categories, based on: access to open spaces, use of drug prophylaxis and treatment, similarities to provided care and give the following results:

- Conventional (Conv) consists of the following: laying hens, chicken breeders, turkey breeders, duck breeders, geese breeders, fattening turkeys, fattening ducks, fattening geese, farmed game birds and ratites (flightless birds);
- Category Free-range/Backyard (FrBy), consists of free-range laying hens, free-range broilers and backyard flocks.

Limitations

The data for this study were submitted by the relevant organisations from the Member States, according to an EU Commission Decision of 25 June 2010 (EC 2010). This could lead to differences in reported data, especially for wild birds, between databases: EFSA, Animal Disease Information System (ADNS) the World Animal Health Information Database (WAHID) or individual national surveillance databases.

The data for backyard flocks are combined with those for free-range. The reason is the proximity of the mechanisms and principles of poultry farming in both systems - by definition backyard flock is a flock of poultry raised in close to natural conditions and are not part of industrial production (not part of a registered farm) (Truscott et al. 2007; Sharkey et al. 2008).

Values for wild birds were collected by the "passive" method and for farm birds by the "passive" and "active" methods.

The results for Croatia, which joined the EU on 1 January 2013, are not included in the 2012 data.

Results

General overview

For the period between 2012 and 2020, a total of 316115 tests for HPAI and LPAI were performed within the territory of the European Union, of which 7511 (2.38%) are the total number of positive tests for HPAI and LPAI. Of the tests, 139821 (44.23%) were for birds in intensive farming systems, 73792 (23.34%) for birds in

organic systems or backyards and 102502 (32.43%) for wild birds. Of these tests, intensively reared animals tested positive in 1008 cases (13.42% of all positive results or 0.78% of Conv), 1222 cases (16.27% of all positive results or 1.65% of FrBy) in organic and backyard and 5281 cases in wild birds (70.31% of all positive results or 5.15% in these birds).

Member States report approximately constant proportionality in conventional systems. In organic and backyard systems, taking into account the statistics of a steady increase in the number of birds raised by these methods, the number of tests performed remains relatively low. (EC 2019). The summary data for the period covering 2012 to 2020 are presented in Table 2 and graphically in Fig. 1.

For the period 2012 to 2018, the tested conventional systems represent an average of 15.19% of the total. In the free-range birds, in the period 2012–2015, an average of 11.19% of the total number was tested and for 2015–2018, the percentage increased to 24.93%. Backyard flocks continue to occupy a very low part of the collected data, with an average ratio of 0.43% tested (Fig. 2).

Table 2. Annual representation of Avian Influenza sampled and positive results for the period 2012–2020.

|		2012	2013	2014	2015	2016	2017	2018	2019	2020
Conventional Farming	Total number of sampled	20726	19075	14402	16910	17804	12292	12350	14721	11541
	Total number of positive results	42	56	53	73	74	136	96	287	191
Free-range/Backyard	Total number of sampled	8779	6127	4419	4959	4957	4532	21498	9698	8823
	Total number of positive results	5	12	4	8	8	25	903	135	122
Wild Birds	Total number of sampled	6504	6563	5676	6730	6760	19325	12879	19097	18968
	Total number of positive results	65	26	30	75	71	1905	587	898	1624
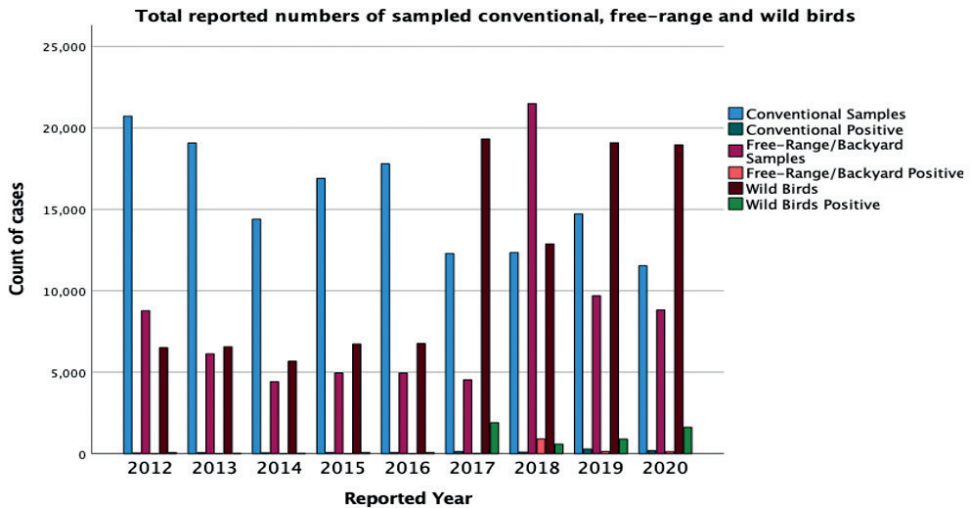
Figure 1. Graphical representation of the Avian Influenza samples and positive results in conventional systems, free-range and backyard farming and in wild birds between 2012 and 2020.
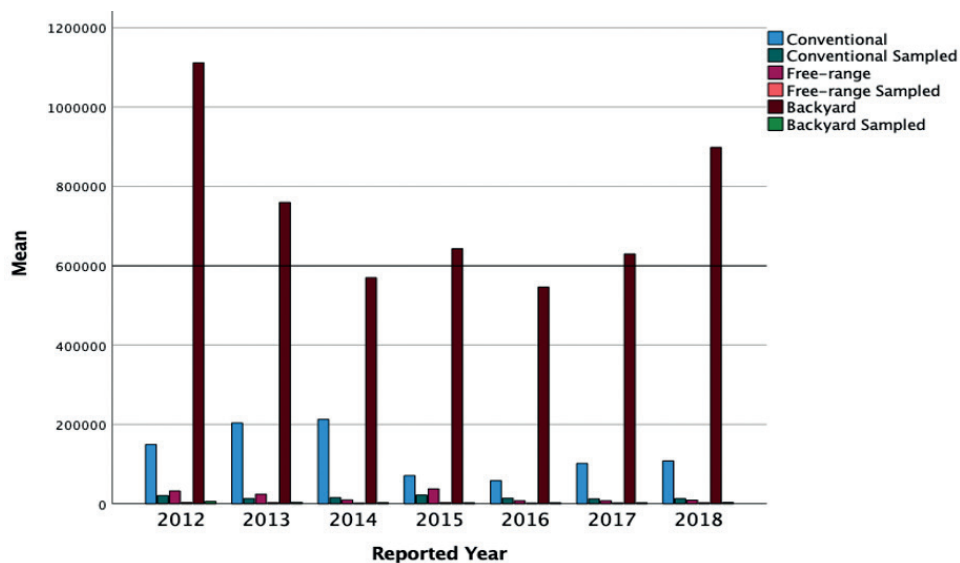
Figure 2. Graphical representation of the ratio between total and sampled for Conventional, Free-range and Backyard between 2012 and 2018.
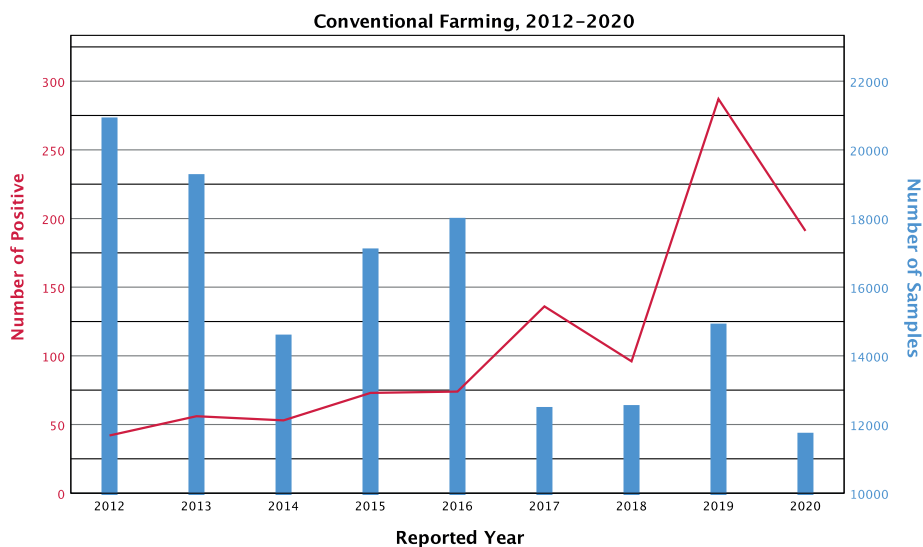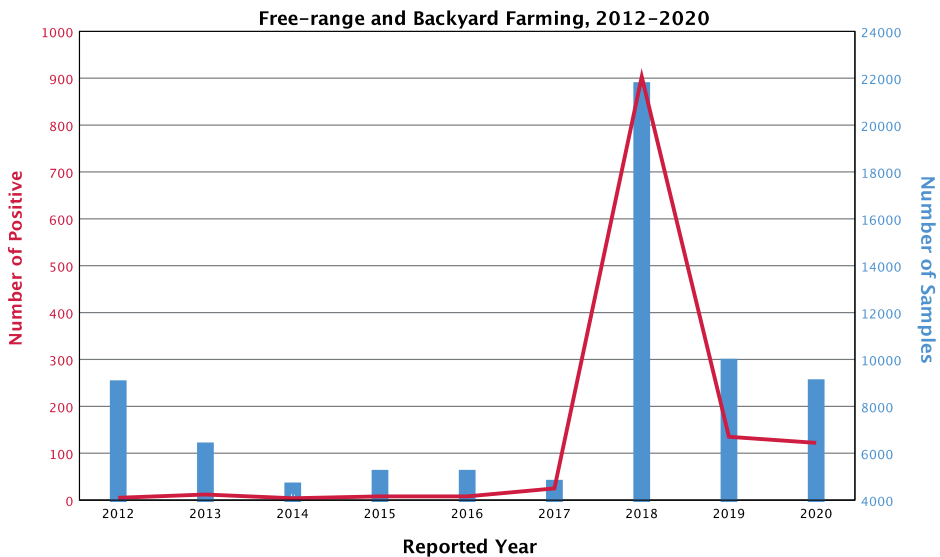

Figure 3. Bars represent the total number of samples and the red line the total number of positive results between 2012 and 2020.

Conventional farming

From the collected data for the intensive systems, compared to previous years, there is a gradual decrease in the number of tests at the end of the study period. In parallel, there is an increase in the number of tests ending with a positive result (Fig. 3). Detailed distribution is demonstrated in Table 2.

Free-range and backyard farming

The tests performed on birds in organic systems or backyard in the period 2012–2020 total 73792, representing 23.34% of the total number for all categories. In the positive tests, the ratio is close to that of the intensive systems: 16.27% in the organic, against 13.42% in the intensive (Fig. 4)

Figure 4. Bars represent the total number of samples and the red line the total number of positive results for free-range and backyard between 2012 and 2020.

Wild birds

From 2017, wild birds constitute the predominant part of the tested categories: 18.06% in 2012 to 48.23% in 2020. Positive results in wild birds have the highest value of the three categories and show an increasing trend. The minimum ratio between the birds tested by the passive method and those that gave a positive result for HPAI or LPAI is 27.66% (2013) and the maximum is 92.21% reported in 2017. There is a positive trend in the increase in the number of tested birds compared to previous periods (Fig. 5).

Discussion and conclusions

The number of consumers of organic products is constantly growing and is based on various motives, such as the belief in better taste, welfare of farm animals, low levels of unnecessary use of antibiotics and growth stimulants etc. This leads to a constant increase in the number of producers of organic products (EC 2019). The regulatory framework

for organic production in the European Union is defined in Council Regulation (EC) No 834/2007 (EC 2007). One of the questions from consumers of organic products is related to whether the risks to human health from zoonotic diseases transmitted by farm animals are higher than in conventional agricultural production. Despite the limited use of anti-infectious drugs, previous studies have shown no significant differences in the level of infectivity with potential human infections between the two types of agricultural production (Miranda et al. 2008; Young et al. 2009; Smith-Spangler et al. 2012).

Data from Member States for the period 2012 to 2020 show minimal differences between intensive and organic production in terms of HPAI or LPAI infectivity. These minima are probably due to the increased requirements for organic production, with the better health of animals on organic farms (Anderson 2011).

Wild birds continue to be a major source and reservoir of HPAI and LPAI. The annually increasing number of positive results in passive control in wild birds raises the requirement and need for the development of active control methods. Access to free-range and backyard open areas for most of the day creates an increased likelihood of farm animals coming into contact with wild birds. The lack of a high percentage of positive tests in Conv relative to FrBy may be the result of the insufficient number of tests in organic and backyard birds. The graphics in Figs 4, 5 show significant similarities between the levels of positive tests in free-range and backyard birds on the one hand and wild birds on the other. This determines the expected increase in the difference between organic and intensive cultivation methods with a future increase in the number of tests in the organic sector.
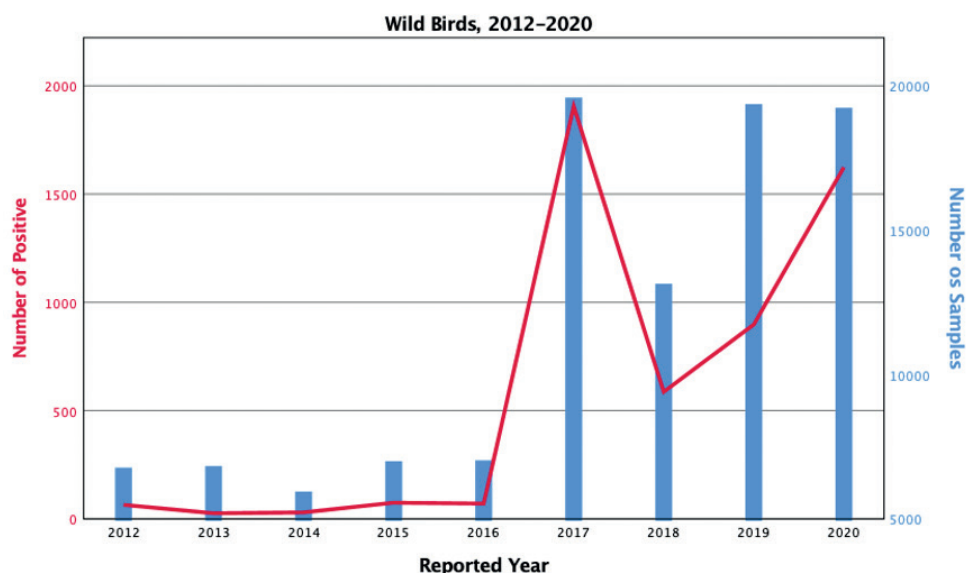

Figure 5. Bars represent the total number of samples and the red line the total number of positive results for wild birds between 2012 and 2020.

As a first of its kind analytical study, the observed trend in annual gradual increases in positive results in wild birds and the symmetry of related results in FrBy, necessitate the expansion of the present study by adding data from subsequent years and analysing trends, based on aggregated data.

The results were similar in each country and demonstrate that free-range production has an annually increasing number of incidences of viral diseases with high zoonotic potential. This makes year-round surveillance absolutely necessary, as well the need for implementation of additional criteria and requirements for free-range systems. Data collection and analysis need to continue in the future. The current study could be a starting point for similar studies in the coming years.

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RESEARCH ARTICLE

BioRisk

Pollinators of *Lavandula angustifolia* Mill., an important factor for optimal production of lavender essential oil

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Abstract

Lavender essential oil is widely used in pharmacy, perfumery and the food industry. It is one of the key essential oils in aromatherapy due to its valuable pharmacological properties. The producers of lavender essential oil are well aware that the greatest quantity of oil is obtained near the end of the inflorescence anthesis and that oil quantity is correlated with the pollination as unpollinated flowers drop down. In addition, it has been demonstrated that oil quality is also highest at the end of the flowering period, related to the gradual increase of monoterpenes (particularly the valuable linalool) and the decrease of sesquiterpenes during flower ontogenesis. The aim of this preliminary study was to measure the occurrence of spontaneous self-pollination in *Lavandula angustifolia* Mill. and to identify external pollinators. The field experiments were performed in a lavender plantation near Gorna Lipnitza Village, north Bulgaria and in the ex-situ lavender collection in the experimental plot of the Botanical Garden of Sofia University. It was revealed that spontaneous self-pollination did not occur in flowers from which external pollinators had been excluded. Exposed flowers were pollinated by polylectic insects, such as honeybees, several species of bumblebees and butterflies. Wild pollinators (particularly bumblebees) dominated over honeybees at both study sites. Our observations showed that all pollinators actively collected nectar. The pollen baskets of most bees were full, indicating the active consolidation of pollen adhering to the pollinators' bodies. Although lavender growers tend to place beehives in the fields for optimal essential oil production, it is also crucial to conserve wild pollinators.

Introduction

Lavender essential oil, extracted from *Lavandula angustifolia* Mill. (synonyms *L. vera* DC., *L. officinalis* Chaix), is of economic importance as it is widely used in pharmacy, perfumery and the food industry. It is one of the key essential oils in aromatherapy due to its valuable pharmacological effects (Clarke 2002, 2009; Lawless 2013; Buckle 2014; Salehi et al. 2018; Gallotte et al. 2020).

According to lavender essential oil producers, the greatest quantity of oil is obtained near the end of the inflorescence anthesis and the oil quantity correlates with the pollination as unpollinated flowers drop down (Pavlovi Food Industries Ltd. and S. Stanev, personal communication). In addition, it has been demonstrated that the increase in oil quality at the end of the flowering period is related to the gradual increase of monoterpenes (particularly the valuable linalool) and the decrease of sesquiterpenes during flower ontogenesis (Détár et al. 2021). Although more data are available about the pollination of *Lavandula latifolia* Medik. (Herrera 1987, 1988, 1989, 1990a, 1995, 2000, 2001, 2005, 2021), insect pollination of *L. angustifolia*, as a factor for the fruit-set, is surprisingly poorly studied (Benachour 2017; Gilpin et al. 2017). Bulgaria is one of the main lavender oil producers in the world, along with France, the UK, China and Spain (Zagorcheva et al. 2013; Stanev et al. 2016; Salehi et al. 2018). Despite this, there is no information about *L. angustifolia* pollination in Bulgaria. In addition, little is known about the occurrence of spontaneous self-pollination in the various cultivars of *L. angustifolia* (Romanenko and Buyukli 1980).

The aim of this preliminary study was to determine the occurrence of spontaneous self-pollination in *L. angustifolia* and to identify its insect pollinators.

Material and methods

There are six cultivars of the perennial shrub *L. angustifolia* grown in Bulgaria. They have differing flower yields and also differ in the yield, content and composition of their essential oils. For the unique quality and economic value of their oil to be retained, the different cultivars must be propagated vegetatively, not from seed. Cultivars are difficult to identify by morphological characters and molecular markers are needed (Stanev et al. 2016; Zagorcheva et al. 2020). Therefore, in this study, we specify the identity of this plant to species level only.

Study sites

The field experiments were performed in July (setting up pollinator exclusion experiments on selected flowers and conducting observations of pollinator activity) and September (collecting the pollinator-excluded flowers) of the year 2020. The location of the experiments was the experimental plot, ex situ collection of the Botanical Garden

Figure 1. *Lavandula angustifolia* inflorescence excluded from pollinators.

of Sofia University (GPS: 42°41'48.7"N, 23°20'02.3"E, 600 m above sea level) and a lavender plantation near Gorna Lipnitza Village, north Bulgaria (GPS: 43°19'45.1"N, 25°24'18.2"E, 180 m above sea level).

Spontaneous self-pollination test

At Gorna Lipnitza Village, the occurrence of spontaneous self-pollination in *L. angustifolia* was tested by excluding pollinators (Fig. 1) from 10 inflorescences (a total of 638 flowers). Additionally, a control was established: in order to test the effect of the pollinator exclusion on the fruit-set, a free-pollinated inflorescence was covered and its fruit-set was tested.

Pollinator composition and activity

A pollinator activity index, modified from Kozuharova and Firmage (2007), was calculated as the number of recorded pollinator visits to one inflorescence divided by the recording time in minutes, the result being multiplied by 60 minutes. The formula used is as follows: $PA = (N/T)*60$, where PA = pollinator activity index, N = number of recorded pollinator visits to one inflorescence, T = time of observation (minutes).

Observations were made for a total of 465 minutes over four days in the Botanical Garden of Sofia University and 293 minutes over four days in the lavender plantation near Gorna Lipnitza Village. The weather conditions during the observations were

similar and comparable. The pollinators were identified in the field or later in the laboratory from photos. Since it is not possible to distinguish with certainty between some *Anthidium* spp. and *Bombus* spp. in the field and from photos, we present them as undistinguished pairs. Their individual foraging behaviour was recorded and documented by pictures and videos taken with a Nikon D5100 camera.

Results

Spontaneous self-pollination test

At the experimental plot, the fruit-set of all flowers from which pollinators were excluded (N = 683) was 0% while that of the control (N = 64) was 85.9%. This strongly suggests an absence of spontaneous self-pollination.

Pollinator composition and activity

Botanical Garden ex-situ collection

During our study, the only visitors to lavender that we observed in the Botanical Garden in June – July 2020 were bees (Hymenoptera): *Anthidium manicatum* (Linnaeus, 1758), *Lasioglossum* spp. *Bombus pascuorum* (Scopoli, 1763), *Bombus terrestris* (Linnaeus, 1758) and/or *B. lucorum* (Linnaeus, 1761) and some unidentified bumblebees, *Apis mellifera* (Linnaeus, 1758) (Table 1). Interestingly, *Anthidium manicatum* (Linnaeus, 1758) and/or A. *florentinum* (Fabricius, 1775) and *Lasioglossum* spp. bees appeared only in June. Since these were not recorded subsequently, their average contribution was calculated to be low (Table 1). In July, pollinators were bumblebees and honeybees (Table 1).

Table 1. Pollinators of *Lavandula angustifolia* in the Botanical Garden of Sofia University.

		Observations in 2020				
Date		19.06.2020	14.07.2020	15.07.2020	16.07.2020	17.07.2020
Recording time		74 min	119 min	118 min	116 min	112 min
Temperature		26 °C	24 °C	26 °C	24 °C	25 °C
Clouds (scale 0–10)		1	0	0	2	1–6
Wind		1 m/s	1 m/s	1 m/s	2 m/s	3 m/s
Family	Species	Activity index (AI)				Average AI
		Order Hymenoptera				7.68
Megachilidae	*Anthidium manicatum/florentinum*	5.7	0.0	0.0	0.0	0.0	1.14
Halictidae	*Lasioglossum* spp.	1.6	0.0	0.0	0.0	0.0	0.32
Apidae	*Bombus pascuorum*	0.0	1.0	1.5	1.0	2.7	1.24
Apidae	*Bombus terrestris/lucorum* complex	0.0	3.0	2.0	2.6	3.2	2.16
Apidae	*Bombus* sp.	0.0	2.5	3.1	1.6	1.6	1.76
Apidae	*Bombus* sp. div.: total	0.0	6.6	6.6	5.2	7.0	5.08
Apidae	*Apis mellifera*	0.0	1.0	1.0	2.6	1.1	1.14

Table 2. Pollinators of *Lavandula angustifolia* in the lavender plantation near Gorna Lipnitza Village.

		Observations in 2020				
Date		8.07.2020	9.07. 2020	10.07. 2020	11.07. 2020	
Recording time		68 min	75 min	75 min	75 min	
Temperature		26 °C	24 °C	25 °C	27 °C	
Clouds (scale 0–10)		1	6	1–5	0	
Wind		1 m/s	4 m/s	2 m/s	1 m/s	
Family	Species	Activity index (AI)				Average AI
	Order Hymenoptera					12.3
Apidae	*Bombus pascuorum*	1.8	0.8	2.4	2.4	1.85
Apidae	*Bombus terrestris/lucorum* complex	2.6	0.8	0.8	1.6	1.45
Apidae	*Bombus niveatus*	1.8	3.2	3.2	2.4	2.65
Apidae	*Bombus* sp.	2.6	1.6	0.8	0.8	1.45
Apidae	*Bombus* sp. div.: total	8.8	6.4	7.2	7.2	7.4
Apidae	*Apis mellifera*	4.4	5.6	4.8	4.8	4.9
	Order Lepidoptera					0.43
Sphingidae	*Macroglossum stellatarum*	0	0	0	0.1	0.03
Lycaenidae	*Plebejus argus*	0.1	0.1	0.1	0	0.08
Pieridae	*Pieris rapae*	0.1	0.0	0.1	0	0.05
Pieridae	*Pontia edusa*	0	0.1	0.1	0.1	0.08
Papilionidae	*Iphiclides podalirius*	0	0.1	0	0.1	0.05
Nymphalidae	*Melanargia galathea*	0.2	0.1	0.3	0.1	0.15
Order Diptera						0.08
Bombyliidae	*Bombylius* sp.	0.1	0.1	0	0.1	0.08
	Order Coleoptera					0.05
Cantharidae	*Rhagonycha fulva*	0.1	0.1	0	0	0.05

Gorna Lipnitza Lavender Field

Bees (Hymenoptera) were the main lavender pollinators at the plantation near Gorna Lipnitza. Flower visits were dominated by bumblebees, followed by honeybees. However, some butterflies, moths, bee-flies and beetles were also recorded visiting the flowers as follow: Hymenoptera: *B. pascuorum*, *B. terrestris* and/or *B. lucorum*, *B. niveatus* Kriechbaumer, 1870 and some unidentified bumblebees, *A. mellifera*; Lepidoptera: *Macroglossum stellatarum* (Linnaeus, 1758), *Plebejus argus* (Linnaeus, 1758), *Pieris rapae* (Linnaeus, 1758), *Pontia edusa* (Fabricius, 1777), *Iphiclides odalirius* (Linnaeus,1758), *Melanargia galathea* (Linnaeus, 1758); Diptera – *Bombylius* sp.; Coleoptera – *Rhagonycha fulva* (Scopoli, 1763) (Table 2).

Discussion

Spontaneous self-pollination

Our experiments revealed a complete absence of spontaneous self-pollination in the studied *L. angustifolia* plants. This plant is known to have adaptations to avoid self-pollination, such as heterostyly and various systems of genetic self-incompatibility, as well as male sterility (Bujukli 1970). Other research states that, while *L. angustifolia* is

normally considered an allogamous species, autogamy is possible and the mode of pollination depends on the degree of heterozygosity of the plants involved (Romanenko and Buyukli 1980). Cytoplasmic male sterility (CMS) is reported for *L. angustifolia* and even used for selection purposes (Gostev et al. 1976). CMS plants do not produce and release functional pollen following a dysfunction in the respiratory cell metabolism of the anther tapetum during sporogenesis, promoting cross-pollination (Richards 1997). The number of fruits in the freely-pollinated control was less than the total number of flowers. Due to complex factors, not all ovules of *L. latifolia* mature to seeds (Herrera 1990b) and it is still unclear whether this is resource- or pollen-limited.

Pollinator composition and activity

Bumblebees dominated the pollinators at both experimental plots, followed by honeybees (Tables 1 and 2). The average activity index of bumblebees in the Botanical Garden was 5.08 and at the lavender plantation near Gorna Lipnitza, it was 7.4 (Tables 1 and 2). The average activity index of honeybees was 1.14 and 4.9, respectively (Tables 1 and 2). This is not surprising because beehives are common in the agricultural fields and the distance that honeybees can fly to their food resource is greater than that of wild pollinators, reaching 2–3 km (Steffan-Dewenter and Tscharntke 2000). Our results correspond to previous findings as it is shown that lavender (*Lavandula* spp.) flowers attract more bumblebees (*Bombus* spp.) than honeybees (*A. mellifera*) (Herrera 1990a, Balfour et al. 2013, Benachour 2017). Our research also confirms the conclusions of other studies that nectar is the main reward taken by bees (Herrera 1989; Balfour et al. 2013; Benachour 2017). Only a few bumblebees were observed to have full pollen baskets and they filled them mainly by consolidating the pollen that adhered to their bodies while flying from flower to flower.

The bumblebees, recorded in the study plots, comprise more than three species. Bumblebees are polylectic foragers, but differ in their requirements: some gather nectar, some pollen, while others may gather both (Goulson 2010). *Bombus terrestris* and *B. lucorum* visit a very wide variety of flowers, but if the corolla tube is deep, they often become nectar robbers. Although *B. pascuorum* visit a wide range of flowers, it is very fond of the flowers of legumes and Dead-nettles (Edwards and Jenner 2005). *B. niveatus* prefer Lamiaceae food plants (Aliyev et al. 2021). Bumblebees forage more efficiently because they have longer tongues and can process lavender flowers three times faster than honeybees (Balfour et al. 2013). In addition, bumblebees, in general and, in particular, the typically dominant *B. terrestris*, emerge as the most efficient pollinator of *L. angustifolia* as revealed by counting pollen grains transported on their bodies in comparison to five other bee visitors of this plant, including honeybees (Benachour 2017).

The butterflies and moths (order Lepidoptera) recorded in the study plots comprise six species belonging to five families.

Macroglossum stellatarum is a fast-flying nectarivorous hawkmoth (Sphingidae) which is abundant throughout Bulgaria. It is popularly known as the "Hummingbird Hawk-moth" owing to its spectacular feeding habits which involve hovering in mid-air

and using its long proboscis to probe flowers for nectar, without any other body parts making contact with the corolla (Goyret and Kelber 2012). Due to its nectar-gathering habits, *M. stellatarum* is noted as a major pollinator of plants with long corolla tubes (Láźaro and Santamaría 2016).

Plebejus argus, one of the most widespread and abundant species of Blue (Lycaenidae) in Bulgaria, is an obligate myrmecophilous species. The spatial distribution of the adult butterflies within a given habitat is primarily correlated with the occurrence of its mutualist host ant, *Lasius niger* (Jordano et al. 1992) and, to a far lesser extent, with the density of nectar sources (Seymour et al. 2003). While *Lavandula* is one of the favoured nectar sources for *P. argus* where available (Seymour et al. 2003), it is visited by the butterfly only if growing in proximity to the patches occupied by the host ant *L. niger*, the latter constraint thus determining the local and probably negligible importance of the butterfly as a pollinator of *Lavandula*.

The remaining four butterfly species, recorded in the study plots, are *P. rapae*, *P. edusa*, *I. podalirius* and *M. galathea*. Despite having rather different pre-imaginal development preferences, as adults, these four butterfly species share two ecological traits. Firstly, they all have a wide geographical and altitudinal distribution across Bulgaria (Buresch and Tuleschkow 1929; Abadjiev 2001), occurring in a broad range of open and semi-open habitats which notably include species-poor agricultural and ruderal landscapes (Z. Kolev, personal observations). Secondly and in keeping with their eurybiotic character, these four species are opportunistic feeders with regards to their use of flowering plants as a source of nectar. Thus, *P. rapae* has been shown to be able to utilise a broad range of native, as well as exotic, flowers with significantly different corolla depths in a complex, anthropogenic urban setting (Lazri and Barrows 1984). However, the value of that species as a pollinator has been questioned, as the study determined that, according to the small number of thereto attached pollen grains, the species may function to a limited degree as a pollinator, but to a much greater extent as a nectar thief (Lazri and Barrows 1984). As a general point, butterflies and moths are seen as far less important in terms of pollinating potential than are Hymenoptera: individuals of *P. rapae* have been shown (Lazri and Barrows 1984) to carry several orders of magnitude fewer pollen grains compared to, for example, honeybees, in which up to 15,000 pollen grains have been counted from a single individual (Kendall and Solomon 1973).

Conclusion

According to the results of the self-pollination test, the flowers of *L. angustifolia* appear to not spontaneously self-pollinate and require insect pollen vectors for their fruit/seed set to occur. Wild bees (particularly bumblebees), as well as other wild pollinators, are predominantly responsible for the pollination of this shrub, with bumblebees shown to be the most efficient pollinators (Balfour et al. 2013). Although lavender growers tend to place beehives in the fields for optimal essential oil production, it is also crucial to conserve wild pollinators.

Negative non-target effects of pesticides are apparent on pollinators and subsequent declines in these insects have been detected particularly in areas of more intensive agriculture (Söderman et al. 1997; Sepp et al. 2004; Biesmeijer et al. 2006; Rundlöf et al. 2008; Brown and Paxton 2009; Grixti et al. 2009; Potts et al. 2010; 2015, Goulson 2013; Goulson et al. 2013, 2015, 2018; Böhning-Gaese et al. 2019). This pollinator crisis thus demands action at many different geographic and political levels and actions for a variety of societal sectors are proposed by Forister et al. (2019). Such actions, however, must necessarily be informed by ongoing and expanding monitoring of insect pollinators. The latter is especially urgent in countries, such as Bulgaria, where such monitoring is yet to be adopted as an essential tool for science-based conservation and where baseline data on the status and population trends of wild pollinators are presently sorely lacking.

Acknowledgements

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RESEARCH ARTICLE

Plant products with acetylcholinesterase inhibitory activity for insect control

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Abstract

Acetylcholinesterase (AChE) inhibitors are widely used in Alzheimer's treatment, but they are also crucial for their action on organophosphorus insecticides. The latter exert their toxicity by inhibiting the AChE enzyme in insects, leading to their death. Amaryllidaceae alkaloids have been proven to be potent AChE inhibitors. In the present study methanolic extracts and essential oils being obtained from species of Asteraceae, Lamiaceae, Brassicaceae and Amaryllidaceae were evaluated in vitro for AChE inhibitory activity. Ellman's colourimetric method, with modifications, was used for AChE activity evaluation. According to the activity level, the tested plant products were divided into three categories. First: plant products with strong activity comparable to that of galanthamine; second: plant products with medium activity, with IC_{50} value about 1 mg/ml and the last group with low activity, with IC_{50} value greater than 1 mg/ml. Essential oils of *Origanum vulgare* subsp. *hirtum* Ietswaart., *Satureja pilosa* Vel., *Monarda fistulosa* L., *Thymus longedentatus* (Degen & Urum.) Ronniger and the methanolic extract of *Leucojum aestivum* L. showed the most potent activity and were referred to as the first group. Carvacrol was identified as the main component of the most active essential oils. In *L. aestivum* extract, galanthamine was found as the main alkaloid. The obtained results indicate that essential oils and alkaloid-rich plant extracts possess the strongest AChE inhibitory activity. This gives us a reason to recommend these plant products to be tested for insecticidal activity in the future.

Keywords

Acetylcholinesterase, alkaloids, carvacrol, essential oils, galanthamine

Introduction

The use of natural products as an alternative to synthetic insecticides is a priority for modern agriculture. At the root of the mechanism of action of organophosphorus insecticides is acetylcholinesterase inhibition (López et al. 2002; Lopez and Pascual-Villalobos 2010; Pang 2014; Mladenović et al. 2018). Amaryllidaceae alkaloids and terpenoids are secondary metabolites with proven high AChE inhibitory activity (López et al. 2002; Elgorashi et al. 2004; Wojtunik-Kulesza et al. 2017). Plant species of Amaryllidaceae, Lamiaceae and Asteraceae are rich in compounds from these chemical groups. Mono-, sesqui- and diterpenes, together with phenols, esters, oxides, ketones, alcohols and aldehydes are the main components of essential oils. Extensive studies on essential oils reveal that they possess various biological activities of great importance, such as toxic and repellent (insecticidal) activity (Isman 2000; Pascual-Villalobos and Ballesta-Acosta 2003; García et al. 2005).

In the present study, methanolic extracts and essential oils from species of Amaryllidaceae, Asteraceae, Brassicaceae and Lamiaceae were evaluated in vitro for AChE inhibitory activity.

Materials and methods

Plant material

Plant material was collected from natural localities of the studied species (*Artemisia santhonicum* L., *Artemisia lerchiana* L., *Micromeria dalmatica* Benth., *Thymus longedentatus* (Degen & Urum.) Ronniger, *Aurinia uechtritziana* (Bornm.) Cullen & T.R. Dudley, *Tanacetum parthenium* L., *Salvia forskaohlei* L., *Salvia sclarea* L., *Salvia aethiopis* L., *Thymus yankae* L., *Centaurea arenaria* M.Bieb. ex Willd., *Nepeta caria* L. and *Eupatorium perfoliatum* L.) or *ex situ* collections from the Institute of Biodiversity and Ecosystem Research (*Leucojum aestivum* L.) and the Institute of Roses and Aromatic Plants (*Origanum vulgare* subsp. *hirtum* Ietswaart., *Monarda fistulosa* L. and *Satureja pilosa* Vel.).

Extraction of plant material

Methanolic extracts. Air-dried powdered plant material (1 g) was extracted with methanol for 24 hours at room temperature. After filtration, the organic solution was evaporated and the dry extract stored at 4 °C until analysis.

Essential oils. The essential oil was extracted on a Clevenger apparatus by water distillation from 50 g of dry plant material in a flask with 500 ml water for 2 hours.

Acetylcholinesterase (AChE) inhibition assay

Acetylcholinesterase inhibitory activity of all samples was determined using Ellman's colorimetric method, as modified by López et al. (2002). The assay was performed in 96-

well microplates. Acetylthiocholine iodide (ATCI) in a solution with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was used as a substrate for AChE from *Electrophorus electricus* (Sigma-Aldrich, Germany). Methanolic extracts and essential oils from the studied plants with concentrations between 0.001 and 1000 μg/ml were tested.

AChE (50 μl) at a concentration of 0.25 U/ml was dissolved in phosphate buffer (8 mM K_2HPO_4, 2.3 mM NaH_2PO_4, 0.15 M NaCl, pH 7.5) and 50 μl of the sample dissolved in the same buffer were added to the wells. The plates were incubated for 30 minutes at room temperature before the addition of 100 μl of the substrate solution (0.04 M Na_2HPO_4, 0.2 mM DTNB, 0.24 mM ATCI, pH 7.5). The absorbance values were read on a microplate reader (BIOBASE, ELISA-EL10A, China) at 405 nm after 3 minutes. Enzyme activity was calculated as an inhibition percentage compared to an assay using a buffer without any inhibitor. Galanthamine was used as a positive control. The AChE inhibitory data were analysed in Microsoft Excel and the software package Prism 9 (Graph Pad Inc., San Diego, USA). The IC_{50} values were measured in triplicate and the results are presented as means.

GC/MS analysis

The most active samples were analysed for their bioactive components by GC/MS. The spectra were recorded on a Thermo GC, equipped with a Focus DSQ II mass detector, coupled to a HP-5MS capillary column (30 m length × 0.25 mm inner diameter × 0.25 μm film thickness). The chromatographic conditions for methanolic extracts and essential oils are described by Berkov et al. (2021) and by Traykova et al. (2019), respectively. The components were identified by comparing their mass spectra and retention indices (RI) with those of authentic standards and the National Institute of Standards and Technology (NIST) spectra library.

Results

Eighteen samples – 4 essential oils and 14 methanolic extracts of 17 plant species - were examined for AChE inhibitory activity by Ellman's colorimetric method, with modifications by López et al. (2002). The results are presented in Table 1.

According to the level of activity, the tested plant samples were divided into three groups. First group: plant samples with strong activity comparable to that of galanthamine (positive control: IC_{50} 4.03 μM = 0.0011 mg/ml), including the methanolic extract of *L. aestivum* and essential oils of *M. fistulosa*, *S. pilosa*, *O. vulgare* ssp *hirtum* and *T. longedentatus*; second group: plant products with moderate activity with IC_{50} value about 1 mg/ml, including the methanolic extracts of *S. aethiopsis*, *A. lerchiana*, *A. santhonicum*, *E.cannabinum*, *N. caria*, *M. dalmatica* and *O. vulgare* ssp *hirtum* and the last group with low activity with a IC_{50} value above 1 mg/ml; the third group comprises the rest of the samples which showed low activity. For *O. vulgare* ssp *hirtum*, it was shown that the essential oil is a stronger inhibitor of AChE than the methanolic extract.

Table 1. Acetylcholinesterase inhibitory activity of essential oils and methanolic extracts.

Plant species	Extract/EO	AChE activity IC$_{50}$ [mg/mL]
Amaryllidaceae		
Leucojum aestivum	MeOH	0.20
Asteraceae		
Artemisia lerchiana	MeOH	1.08
Artemisia santhonicum	MeOH	0.94
Centaurea arenaria	MeOH	> 1
Eupatorium cannabinum	MeOH	1.07
Tanacetum parthenium	MeOH	> 1
Brassicaceae		
Aurinia uechtritziana	MeOH	> 1
Lamiaceae		
Micromeria dalmatica	MeOH	1.16
Nepeta caria	MeOH	1.12
Origanum vulgare ssp *hirtum*	MeOH	0.73
Salvia aethiopis	MeOH	1.23
Salvia forskaohlei	MeOH	> 1
Salvia sclarea	MeOH	> 1
Thymus yankae	MeOH	> 1
Monarda fistulosa	EO	0.0042
Origanum vulgare ssp *hirtum*	EO	0.30
Satureja pilosa	EO	0.0069
Thymus longidentatus	EO	0.72
Galanthamine	Positive control	0.0011

The most active samples were analysed for their bioactive components by GC/MS. The essential oil profiles of the studied samples are presented in Table 2. Monoterpenoid phenols – isomers carvacrol and thymol - were identified as main components of the essential oils of *S. pilosa*, *O. vulgare* ssp. *hirtum* and *M. fistulosa*. *p*-Cymene (16.26%) and *γ*-terpinene (16.07%) were found as the next most abundant constituents of *O. vulgare* ssp. *hirtum*. In the essential oil profile of *M. fistulosa*, thymoquinone (25.41%) and *p*-cymene (21.82%) were present in significant amounts. Neral and geranial were identified as major components of the essential oil of *T. longedentatus* with 24.9% and 27.95%, respectively.

In *L. aestivum* methanolic extract, galanthamine was found as the main alkaloid. In the methanolic extract of *O. vulgare* ssp., *hirtum* carvacrol (15.67%) was also detected, but in a much smaller amount compared to the essential oil. Rosmarinic acid (6.06%), flavonoid glycosides (1.49%), malic acid (1.09%) and catechin (0.23%) were also identified as bioactive compounds.

Discussion

Four essential oils and 14 methanolic extracts were studied for AChE inhibitory activity. All studied essential oils showed significant activity and their profiles were determined by GC/MS. Isomers – carvacrol and thymol - were identified as the main

Table 2. Main compounds identified in the essential oils of studied species (*Mf*: *M. fistulosa*; *Ovh*: *O. vulgare* ssp *hirtum*; *Sp*: *S. pilosa*; *Tl*: T. *longedentatus*); Area (%).

Compounds	RI	Studied essential oils			
		Mf	*Ovh*	*Sp*	*Tl*
α-Thujene	930	4.72	4.66	–	–
α-Pinene	932	1.29	1.43	–	–
Sabinene	971	–	–	–	0.43
ß-Myrcene	988	–	–	4	–
p-Cymene	1025	21.82	16.26	2.97	–
trans-ß-Ocimene	1044	–	–	–	1.15
γ-Terpinene	1059	–	16.07	1.04	–
Camphor	1141	–	–	–	1.34
Terpinen-4-ol	1175	0.98	–	–	0.74
Neral	1227	–	–	–	24.9
Carvacrol methyl ether	1245	1.98	2.54	1.73	–
Thymoquinone	1250	25.41	–	–	0.22
Geranial	1264	–	–	–	27.96
Thymol	1290	19.75	–	30.58	–
Carvacrol	1299	12.24	51.18	50.54	0.19
Neryl acetate	1359	–	–	–	12.79
Caryophyllene	1466	0.69	1.43	–	–
Caryophyllene oxide	1590	–	–	1.18	–

components of the essential oils of *S. pilosa*, *M. fistulosa* and *O. vulgare* ssp. *hirtum*. Carvacrol is a compound with a previously demonstrated strong AChE inhibitory activity (Jukic et al. 2007) and probably determines the activity of the essential oils. The established profile of *O. vulgare* ssp. *hirtum* is in accordance with data reported in literature for the natural populations of the species in Bulgaria from East Rhodopes, Strumska Valley and cultivated areas (Konakchiev et al. 2004; Alekseeva et al. 2021; Baycheva and Dobreva 2021). The composition of *S. pilosa* and *M. fistulosa* corresponds to that reported by Semerdjieva et al. (2020) and Ghosh et al. (2020), respectively. Citral isomers neral and geranial were determined as main components of the essential oil profile of *T. longedentatus* (Aneva et al. 2019).

Essential oils of many plant species have been examined as an alternative to synthetic insecticides (Isman 2000; Pascual-Villalobos and Ballesta-Acosta 2003; García et al. 2005).

To the best of our knowledge, we report for the first time AChE activity of the essential oils of *O. vulgare* ssp. *hirtum*, *T. longedentatus S. pilosa* and *M. fistulosa*. The established strong inhibitory activity of the tested essential oils is a prerequisite for the presence of insecticidal activity. For *O. vulgare* ssp *hirtum*, it was shown that the essential oil is a stronger inhibitor of AChE than the methanolic extract. Assuming the activity is dependent on the presence of carvacrol, the difference in its content between the essential oil and the methanolic extract may also determine the difference in AChE activity.

As galanthamine is a classic example for a substance with potent AChE inhibitory activity (Sramek et al. 2000), it undoubtedly determines the activity of the methanolic extract of *L. aestivum*.

Conclusion

The obtained results indicate that the essential oils of *Monarda fistulosa*, *Satureja pilosa*, *Origanum vulgare* subsp. *hirtum* and *Thymus longedentatus* and the methanolic extract of *Leucojum aestivum* possess the strongest AChE inhibitory activity. GC/MS analysis proved the presence of bioactive compounds in these plant products. Thus, we recommend them to be tested for insecticidal activity in the future.

Acknowledgements

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RESEARCH ARTICLE

BioRisk

Monitoring bumblebee pollinator visits to the medicinal plant *Gentiana asclepiadea* L. (Gentianacese) – a comparison between the periods 1990–1994 and 2017–2020

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Abstract

Ever increasing data continue to indicate the decline of bumblebee populations. The key factors causing declines in their abundance and diversity are: 1) habitat destruction, 2) loss of floral resources, 3) emerging diseases, 4) increased use of pesticides (particularly neonicotinoids).

The aim of this study is to monitor bumblebee visits to *Gentiana asclepiadea* L, recording pollinator species, and taking measurements of seed set. This plant species is chosen for two reasons: 1) similar data is available from our previous research in the 1990's and 2) this montane plant species is supposed to be less exposed to hazards from pesticides and habitat destruction. Three study sites were chosen in Mt. Vitosha (SW Bulgaria) where natural populations of *G. asclepiadea* occur in 1990. The observations of bumblebee activity in the flowers of *G. asclepiadea* were conducted during the flowering seasons (August and September) of 2017 – 2020 at the same study sites and compared to the data obtained in the previous period (1990–1994). The free pollination fruit set was tested by monitoring of 100 *G. asclepiadea* flowers each year for development of fruit capsules. The seed set was tested by counting the matured seeds and non-fertilised ovules of 10 fruit capsules each year. A slight decline in bumblebee activity was recorded in 2017 – 2020 in comparison to 1990–1994. This is reflected in the fruit set and the seed set. Our data demonstrates that even in a mountain habitat, where there are fewer direct hazards to bumblebees, that pollination effectiveness has been suppressed over time. This corresponds to a research study which provides evidence that insect biomass fell by 76% in German nature reserves between 1989 and 2016.

Introduction

Pollinators are a crucial element of biodiversity, since the majority of plant species depend on them for reproduction by seed. However, ever increasing data indicate that pollinating insects, and in particular bumblebees, are in decline. The key factors causing declines in their abundance and diversity are: 1) habitat destruction; 2) loss of floral resources; 3) emerging diseases; 4) increased use of pesticides (particularly neonicotinoids) (Söderman et al. 1997; Sepp et al. 2004; Biesmeijer et al. 2006; Rundlöf et al. 2008; Brown and Paxton 2009; Grixti et al. 2009; Potts et al. 2010; Goulson 2013; Goulson et al. 2015, 2018; Becher et al. 2018). Beside other harm, pesticides reduce bumblebee colony initiation by reducing the number of queens, as well as the flight dynamics and endurance of workers and their activity as pollinators (Baron et al. 2017; Kenna et al. 2019).

For the conservation of wild pollinators, long-term monitoring is necessary (Naeem et al. 2020). There is a need for long-term monitoring especially in montane habitats to determine the role and impact of the different drivers of global change, since bumbles are shifting to higher elevations (Marshall et al. 2020). Bumblebee monitoring technique is based on bumblebee counts based on flower visits and standardised observation routes (Teräs 1976; Söderman et al. 1997; Sepp et al. 2004).

There is no long-term quantitative data about bumblebees in Bulgaria. An efficient approach is by monitoring pollinator visits to one or more plant species, ideally recording pollinator species and also taking seed set measurements (Goulson, personal communication). A good candidate is *Gentiana asclepiadea* L. for two reasons: 1) such data is available from our previous research in the 90's of the last century; 2) this montane plant species is supposed to be less exposed to the hazards such as pesticides and habitat destruction.

Gentiana asclepiadea is a perennial plant. The rhizome is more or less thick and branched. There are sterile and usually several fertile stems, which are straight, non-branched and 35-50 (80) cm tall. There are 1-3-5-merous flowers sitting in nodes, at the base of the leaves. They are of the funnel type. Their size varies between (35) 40-50 mm and the corolla lobes are 3-4 times shorter than the corolla tube (Tutin 1972; Kozuharov and Petrova 1982). The flowers of *G. asclepiadea* are not spontaneously self-pollinated. The main pollinators of this plant are several species of bumblebees and *Thricops* spp. flies (Kozuharova and Anchev 2004).

The aim of this case study is to monitor bumblebee visits to *G. asclepiadea*, recording pollinator species, and also taking seed set measurements.

Material and methods

Three study sites were chosen (Kozuharova and Anchev 2004) in Mt. Vitosha (SW Bulgaria) where natural populations of *G. asclepiadea* grow at altitudes between 1500–1900 m above sea level (Fig. 1). Study sites 1 and 2 were in open woodlands in the coniferous forest belt. Study site 3 was in the subalpine meadows just above the coniferous forest belt.

The field investigations were conducted during the flowering seasons (August and September) of 1990–1994 (Kozuharova and Anchev 2004) and then at the same study sites repeated in 2017 to 2020.

The field observations were conducted over 66 hours during 48 days in different meteorological conditions. The visiting bumblebees were identified in the field. Their visitation rate and behaviour in the flowers were recorded. Since the focus of this study was mainly on visitation rate, we refrained from further collection of specimens as this would affect the results. Although *Bombus wurflenii* (Radoszkowski, 1860) and *B. lapidarius* (L., 1758) belong to different subgenera (*Alpigenobombus* and *Melanobombus*) and ecologically, they are different (*B. lapidarius* is distributed everywhere, while

Figure 1. Study sites in Vitosha Mts., SW Bulgaria as follows: site 1 – 42°36'13.2"N, 23°15'04.0"E, site 2 – 42°36'20.5"N, 23°17'39.4"E, site 3 – 42°34'05.1"N, 23°17'52.6"E Study sites 1 and 2 were in open woodlands in the coniferous forest belt. Study site 3 was in the subalpine meadows just above the coniferous forest belt.

B. wurflenii is a montane species) it is hard to distinguish with certainty between these two species in the field as their colours are the same. Also, it is hard to distinguish *B. hortorum* (L., 1758) from *B. subterraneus* ssp. *latreillellus* (Kirby), and *B. lucorum* (L., 1758) from *B. terrestris* (L., 1758) in the field. Therefore, we preferred the approximate approach rather than irrelevant "precision" and in the result and discussion parts they appear as undistinguished pairs *B. wurflenii* and/or *B. lapidarius, B. hortorum* and/or *B. subterraneus, B. lucorum* and/or *B. terrestris*. A pollinator activity index was calculated as the quotient of the number of pollinators recorded and the minutes of observation multiplied by 60 minutes. The most numerous plants flowering simultaneously in the neighbourhood and their bumblebee visitors were recorded.

A Linear regression analysis of the total activity of bumblebees at each study site against the time/years of observation was applied to test the trend in the bumblebees' activity. The sequence of years of observations has a serious interruption (from 1994 to 2017) and thus the model does not allow prediction. It is just informative, rather qualitative than quantitative. Therefore, we refrained from Linear regression analysis of each bumblebee species' activity during the period of observations as well as the fruit and seed set.

The free pollination fruit set was tested by monitoring 100 flowers of *G. asclepiadea* each year for development of fruit capsules. The seed set was tested by counting the matured seeds and non-fertilised ovules of 10 fruit capsules. The damage inflicted by insect predators on the maturing seeds was not calculated for this research (see Kozuharova et al. 2018). Fruits that set were considered successful even if they were damaged subsequently. Seed set was evaluated using undamaged and undehisced opened fruits.

Results

Since the flowering period of the observed foraging plant is late summer and early autumn, we were observing the latter part of bumblebee colony activity. The species composition of the bumblebees visiting the flowers of *G. asclepiadea* remains basically the same over time. It is as follows: *Bombus pascuorum* (Scopoli, 1763), *B. wurflenii* /*B. lapidarius, B. pratorum* (L., 1758), *B. hortorum* /*B. subterraneus* ssp. *latrellellus, B. terrestris* and *B. lucorum*. The bumblebee activity varied over the years as well as within the study sites (Figs 2–5). A slight decline of bumblebees' activity is observed on the chart for the period 2017 – 2020 in comparison to 1990–1994 (Fig. 2).

Even though the linear regression model is just informative and not suitable for prediction, decreasing trends are observed at all three study sites (Figs 3–5).

Peculiarities which cannot be detailed in the charts are worth noting. In the first days of September 1991 high activity of *B. hortorum/B. subterraneus* males was recorded in the flowers of *G. asclepiadea*, at site 1, where they were feeding on nectar (Kozuharova and Anchev 2004, Figs 2, 3 and 7 A). The high activity of *B. wurflenii* in 2017 at site 2 was due to frequent visits of nectar robbing workers together with the less active nectar foragers (Figs 2, 4 and 7 B and C).
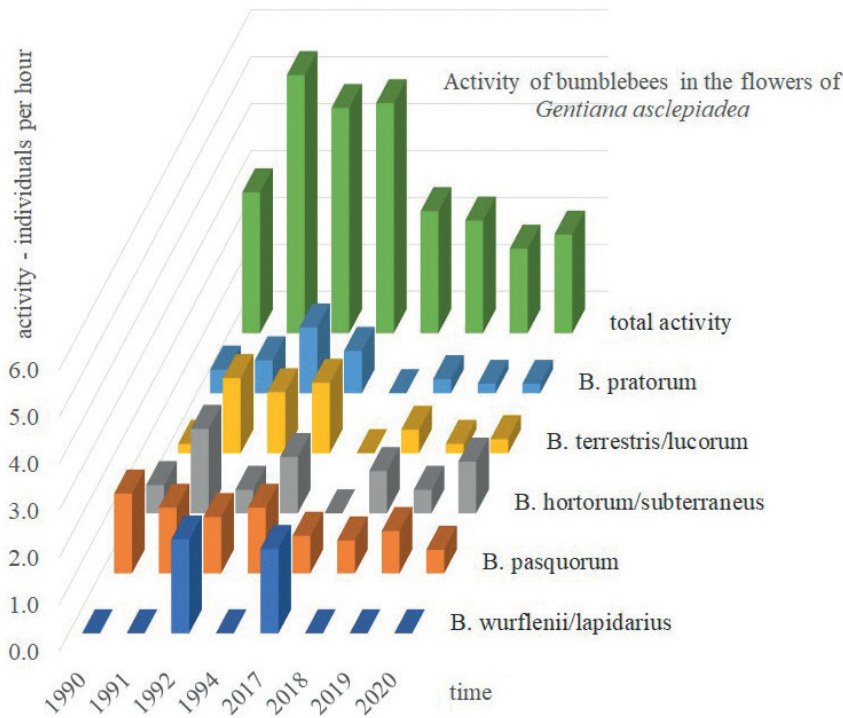
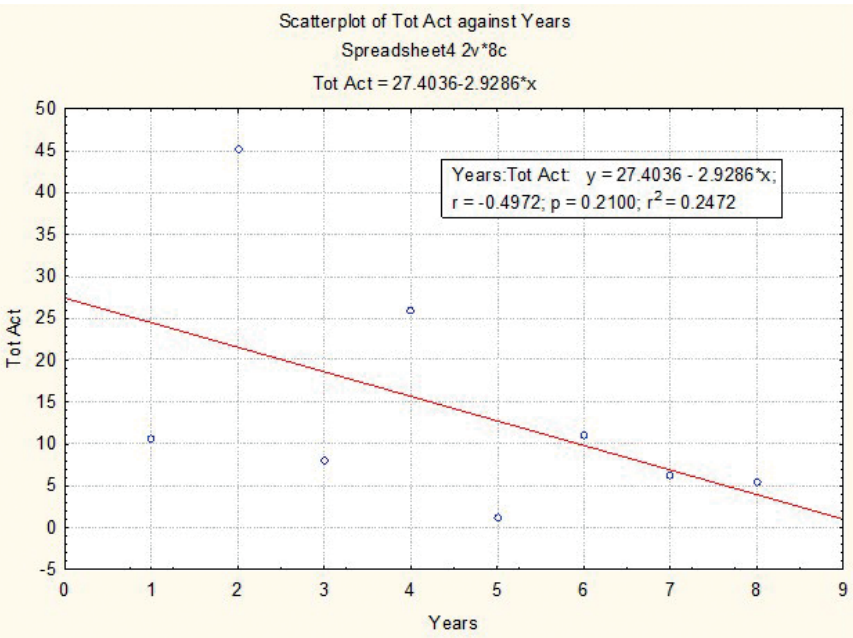**Figure 2.** Average annual activity of bumblebees in the flowers of *Gentiana asclepiadea*.

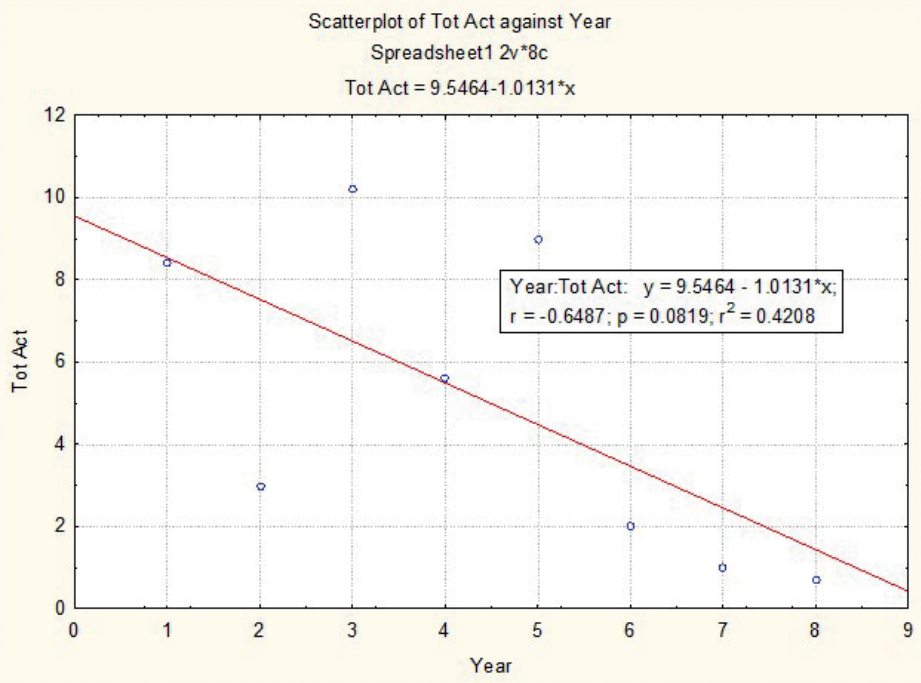**Figure 3.** Linear regression plot Tot Act = f (years) at study site 1.

Figure 4. Linear regression plot Tot Act = f (years) at study site 2.
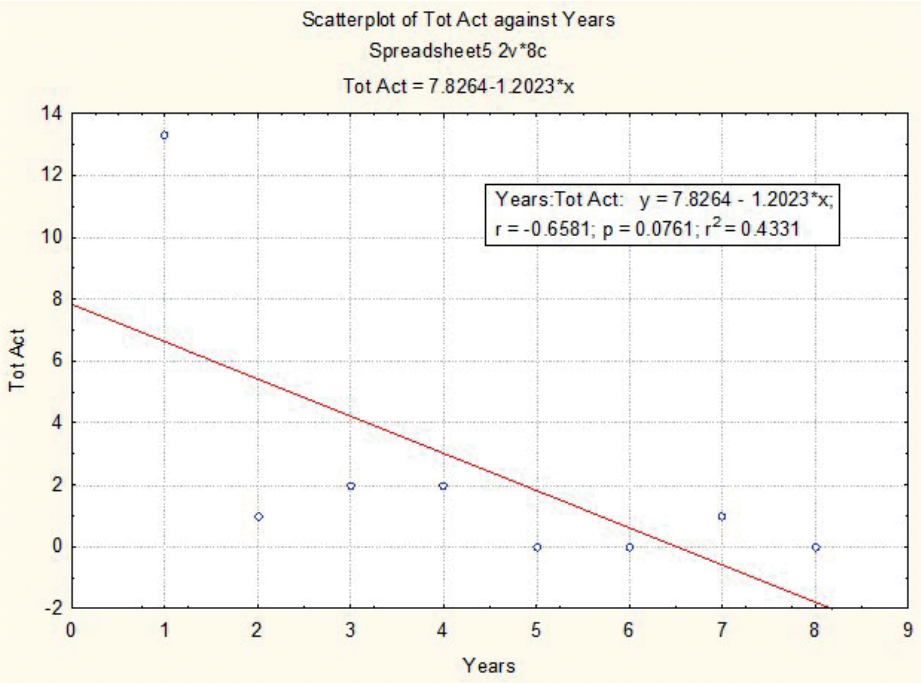

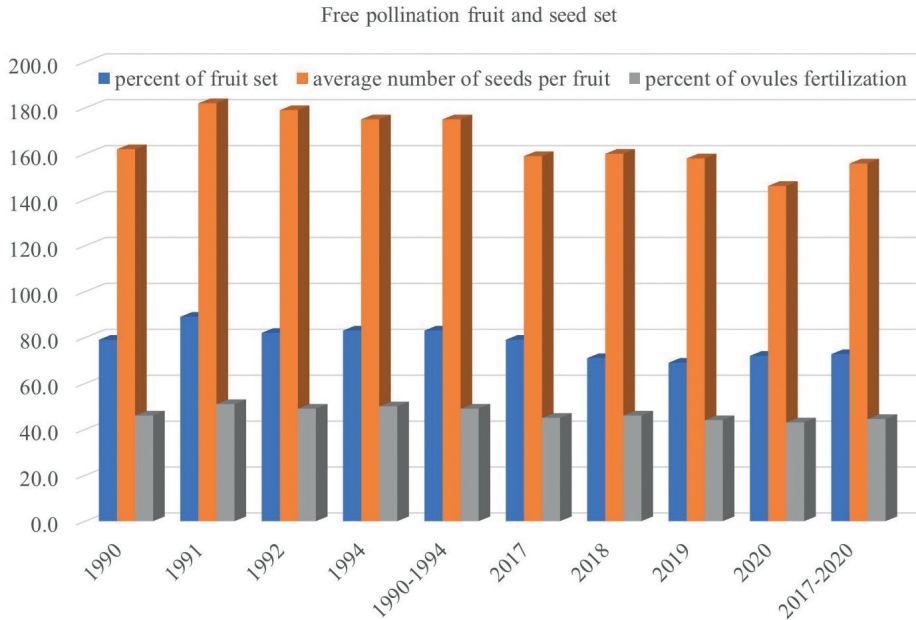**Figure 5.** Linear regression plot Tot Act = f (years) at study site 3.

Figure 6. Free pollination fruit and seed set of *Gentiana asclepiadea*.

The free pollination fruit set tested by counting fertilised fruits versus flowers that failed to set fruit, as well as the seed set tested by counting the matured seeds and non-fertilised ovules, are presented on Fig. 6. A slight decrease of the fruit and seed set was observed during the final four years.

Discussion

A fluctuation in bumblebees' activity within the years and the study sites was observed. Such fluctuation in bumblebees' activity seems to be a normal process depending on several factors including colony initiation, pathogens, parasitoids, predators, food resources and landscape etc. (Bowers 1985; Goulson 2010; Persson and Smith 2013). The fluctuation in the activity of bumblebees in the flowers of *G. asclepiadea* can be explained by the dynamics of colony formation and foraging range. According to Goulson (2010) it is a matter of chance whether a bumblebee colony will be established in the vicinity of the observed plant population and, if so, of which bumblebee species. However, a relatively small proportion of bumblebees seem to forage close to the nest and the foraging range of bumblebees can reach up to 10 km. Thus, they reduce intracolonial competition as well as probability for predation to their nests. Also, according to Goulson (2010), the failure rate of colonies seems to be very high, although data is sparse. Fewer than 30% of *B. pasquorum* colonies produce any new queens. And the success of *B. lucorum* colonies is even less – about 14% and all this depends on various factors (Goulson 2010).

Figure 7. A *Bombus hortorum/subterraneus collecting* nectar in 1991 at site 1 **B** *B. wurflenii/lapidarius* worker robbing nectar of *Gentiana asclepiadea* in 2017 at site 2 **C** *B. wurflenii/lapidarius* worker taking nectar from a male stage flower in 2017 at site 2 **D** *B. wurflenii/lapidarius* and pollinating *Epilobium angustifolium* at site 3.

The observed fluctuations in pollinator activity are related to the study sites where observations were performed. It is quite interesting that, in general, fewer bumblebees were recorded at study site 3 – subalpine meadows. At this site *Epilobium angustifolium* L. and *Solidago virgaurea* L. had large populations and these possibly over-competed for bumblebee pollinators. Bumblebees are known to favour *S. virgaurea* (Teräs, 1976). And *E. angustifolium* was observed to attract a lot *B. wurflenii/B. lapidarius* and *B.*

pratorum (Fig. 7D). However, the pollen of *E. angustifolium* was poorly represented in the pollen loads of *G. asclepiadea* (Kozuharova and Anchev 2004). At study sites 1 and 2, *Cirsium appendiculatum* Griseb. and *Prenantes purpurea* L. were competitors for bumblebees such as *B. pratorum*, *B. wuflenii*/*B. lapidarius*, *B. hortorum*/*B. subterraneus* ssp. *latreillellus* but these plants never made large patches like *S. virgaurea* and *E. angustifolium.* Unfortunately, our fruit and seed test materials were taken randomly, so we cannot confirm pollen limitation in relation to the study sites.

The high activity of *B. hortorum*/*B. subterraneus* ssp. *latreillellus* males feeding on nectar in the flowers of *G. asclepiadea* during the first days of September can be explained by their phenology (Goulson 2010). *Bombus pratorum* and *B. hortorum* nests last for about 14 weeks from founding, compared to about 25 weeks for the sympatric *B. pascuorum,* which in general means that no more workers are reared once the colony switches to producing reproductive individuals (Goulson 2010). The high activity of *B. wurflenii* in 2017 was due to frequent visits of nectar robbing workers (Fig. 7B.). *Bombus wurflenii* is known as a nectar robber (Utelli and Roy 2001, Goulson et al. 2013). Obviously, it has difficulties to reach the nectar of *G. asclepiadea* hidden in deep pockets. Nectar robbery – extracting nectar through holes made at the base of the corolla tube – has a wide spectrum of consequences for the plant, that ranges from negative, neutral, to positive according to life history traits of the interacting organisms and the ecological mechanisms involved (Rojas-Nossa et al. 2021). In the case of *G. asclepiadea* it can be regarded as neutral to slightly positive at the stage when the workers pollinate while trying to reach the nectar the normal way Fig. 7C). In any case these were not frequent visitors compared to *B. pasquorum, B. hortorum*/*B. subterraneus* subsp. *latreillellus* and *B. lucorum*/*B. terrestris.*

The slight decline of bumblebees' activity recorded in 2017–2020 in comparison to 1990–1994 reflected on the fruit set and the seed set. Our data demonstrate that even in a mountain habitat with fewer direct hazards for bumblebees a negative effect on pollinator activity over time is still detectable. Our results correspond to a research study which provides evidence that insect biomass fell by 76% on German nature reserves between 1989 and 2016 (Hallmann et al. 2017). There is no obvious explanation for the recorded decline of bumblebees in the flowers of *G. asclepiadea.* Some speculations could be offered. A large amount of the land in the foothills of the mountain which used to be meadows was integrated into Sofia's suburbs and urbanized with all the telecommunication infrastructure and car traffic. Also, in the last few years, adjacent agricultural land around Sofia is actively used for sunflower, oilseed rape and corn production. However, at this research stage we cannot say if these factors affect the bumblebees' colonizing habitats at higher altitude and about 15 km away from the urbanized area and 20 km away from the agricultural activity. Global warming is known to be a serious hazard for particular bumblebee species such as *Bombus monticola* (Smith, 1849) and *B. mucidus* (Gerstaecker, 1869) (Manino et al. 2007). Some bumblebees react to the climate change by relocation to higher altitudes (Marshall et al. 2020), however we do not have enough data for altitudinal assessment and thus no climate change conclusions can be done.

Acknowledgements

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RESEARCH ARTICLE

BioRisk

Pteromalid fauna (Hymenoptera, Pteromalidae) in oilseed rape (*Brassica napus* L.) fields in Bulgaria – species composition and perspectives for biological control

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Abstract

Parasitoid wasps belonging to the family Pteromalidae are widespread and abundant members of the insect communities in the temperate regions of the world. As many other chalcids do, pteromalids serve as natural enemies of the pests in various crops and play an important role in the biological control of these harmful insects. Here we present the results of a field study in Bulgaria which was focused on the diversity of family Pteromalidae in ten oilseed rape fields. All samples were collected by sweep netting on the border line or inside the crop field. A total of 93 pteromalid specimens belonging to 26 taxa were gathered. The most abundant genus was *Mesopolobus* – 67% of the sampled pteromalids. The most numerous species in the samples was *Mesopolobus morys* – a well-known key parasitoid of the cabbage seed weevil, *Ceutorhynchus obstrictus*, in Europe. One species – *Halticoptera patellana*, is recorded for the first time in Bulgarian fauna. Clearfield oilseed rape fields had relatively higher parasitoid abundance and richness than the fields treated by conventional technology. In the present work we discuss the overall species composition of Pteromalidae obtained from the studied areas and present our point of view on the perspectives for biological control of oilseed rape pests.

Keywords

Distribution ecology, major pests, parasitoids

Introduction

Oilseed rape (*Brassica napus* L.) (Brassicaceae) is one of the most important sources of both vegetable oil and oil extraction meal worldwide. The oilseed rape oil amounts to 11.7% of the total world consumption of vegetable oils, being exceeded only by the soybean and palm oil production. Nowadays, wild *B. napus* forms are unknown and that leads to an assumption that the species diverged relatively recently through cultivation of its parental species in geographically close areas (Friedt and Snowdon 2010).

Due to its negative influence on crop production, the insect pests of the oilseed rape have been well studied in Europe. Three groups of pest are considered to invade oilseed crop fields in Europe – major pests, minor pests and incidental pests (Alford 2003). Among the first group, six species are found to be most abundant and with a key significance for the growing of the winter rape, namely the pollen beetle, *Brassicogethes aeneus* (Fabricius) (Nitidulidae), the cabbage seed weevil, *Ceutorhynchus obstrictus* (Marsham), the cabbage stem weevil, *Ceutorhynchus pallidactylus* (Marsham) and the rape stem weevil, *Ceutorhynchus napi* (Gyllenhal) (Curculionidae), the brassica pod midge, *Dasineura brassicae* Winnertz (Cecidomyiidae), and the cabbage stem flea beetle *Psylliodes chrysocephala* (L.) (Chrysomelidae). Recent studies on these harmful species have revealed at least 12 hymenopterans, which have been considered as key parasitoids of the pests' larvae. They belong mainly to Ichneumonidae, Braconidae, Platygastridae, Pteromalidae and Eulophidae (Williams et al. 2005; Ulber et al. 2010). The total number of known egg and larval parasitoids is much higher, but most of them have wider host ranges and therefore various food sources, diminishing their impact on the oilseed rape pests. Besides the effects of hosts on the parasitoid diversity, the role of wildflower strips growing along field margins or within crops on the natural enemy populations in oilseed rape fields was recently studied (Hatt et al. 2018). This investigation revealed that the presence of flowering plants close to the crop fields positively affects the parasitoid abundance and increases the potential for biological control in these areas.

Nine pteromalid species are currently known to be associated with *B. napus* in Europe, mostly developing as parasitoids on *Ceutorhynchus* and *Dasineura* spp. (Herrström 1964; Kuhlmann and Mason 2002; Gibson et al. 2006a; Veromann et al. 2012; Noyes 2019). Among them five species have been reported from Bulgaria – *Mesopolobus morys* Walker, *Pachyneuron muscarum* L., *Pteromalus cerealellae* Ashmead, *Trichomalus nanus* Walker and *Trichomalus perfectus* Walker, but in papers not dealing with oilseed rape fields (Thompson 1958; Thuroczy 1990; Todorov 2013; Todorov et al. 2014).

According to Laufer et al. (2014) Clearfield is the combination of an imidazolinone-based herbicide and a corresponding plant, which is tolerant against the active ingredient of the herbicide. Cultivation of Clearfield oilseed rape aims towards a reliable control of broadleaf and grass weeds in post-emergence.

In respect to the insecticide treatment of *B. napus* pests, a number of chemical agents belonging mostly to carbamates, pyrethroids and organophosphates have been tested in the past and nowadays are usually used in crop fields (Alford et al. 1991; Murchie et al. 1999; Cook et al. 2004; Hansen 2004; Hansen 2008; Petraitienė et al.

2012). Besides the pests' mortality, chemical control may also have a negative effect on numerous beneficial insect species (Ruberson et al. 1998; Romeis et al. 2006; Karise et al. 2007; Wen et al. 2021). Conversely, the rate of parasitism on the pest larvae can reach a high percentage in rape not treated with insecticides (Murchie et al. 1997).

A suitable ecological structure within the agroecosystems obtained by suitable alternatives to the conventional agricultural systems provides resources such as food for adult natural enemies and influence their abundance and diversity (Landis et al. 2000; Möller et al. 2021).

The purpose of this study was to 1) obtain data about the biodiversity of Pteromalidae in oilseed rape fields in Bulgaria, and 2) assess the effect of the two production systems used, namely conventional and Clearfield technology, on the pteromalid assemblages.

Materials and methods

The field study was carried out in ten oilseed rape fields situated on the southern foots of Sarnena Gora Mountains and in the western and south-eastern part of the Thracian Lowland, Bulgaria (Fig. 1, Table 1), during late April and the second half of May, 2018. Details about the oilseed crops and management practices at the localities selected are shown in Table 2.
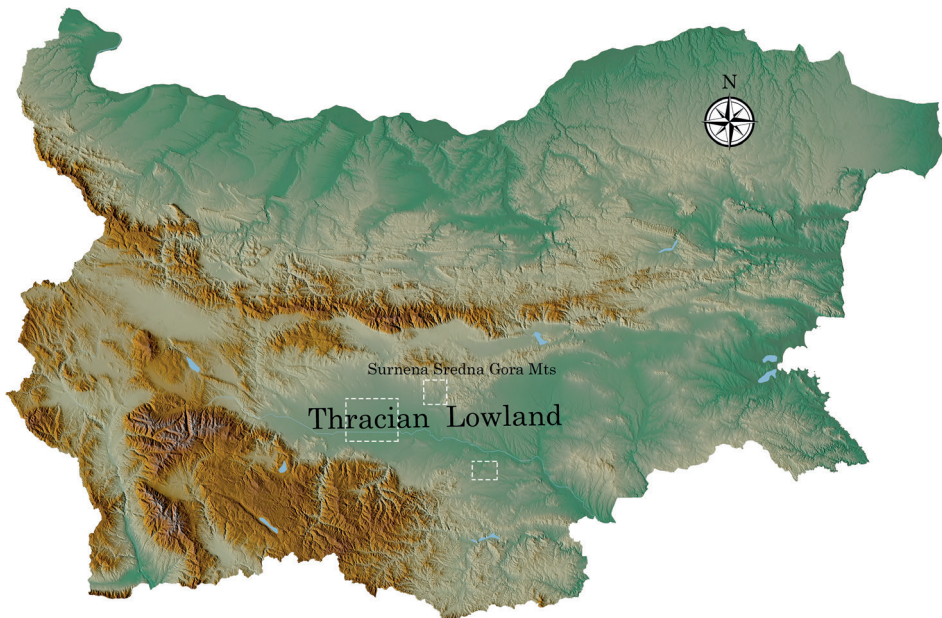
Figure 1. Approximate location of the studied areas (white rectangles) in Bulgaria.

Table 1. List of sampled crop fields in Bulgaria with exact geographic coordinates and names of the nearest villages. Abbreviations SG and TL mean Sarnena Gora Mts and Thracian Lowland, respectively.

Sampling field	Location	Nearest settlement	Coordinates	Altitude, m a.s.l.
Site 1	TL	Malak Chardak	42°16.73'N, 24°38.80'E	198
Site 2	SG	Zelenikovo	42°23.20'N, 25°02.85'E	281
Site 3	SG	Zelenikovo	42°22.75'N, 25°04.86'E	288
Site 4	TL	Stalevo	42°03.43'N, 25°23.85'E	171
Site 5	TL	Dobrich	42°01.41'N, 25°32.13'E	129
Site 6	TL	Momino selo	42°17.51'N, 24°52.83'E	175
Site 7	TL	Stryama	42°15.31'N, 24°50.86'E	174
Site 8	TL	Malak Chardak	42°16.66'N, 24°37.73'E	201
Site 9	TL	Kostievo	42°10.28'N, 24°36.78'E	175
Site 10	TL	Kostievo	42°09.66'N, 24°37.61'E	178

To assess the diversity of Pteromalidae, which could be potential parasitoids of oilseed rape pests in the crops, we used two classic sweep netting techniques: 1. sweeping with following catch of the target insects with an aspirator; 2. sweeping with immediate storage of all insects in vials of 70% ethanol. The first method was conducted by collecting three samples at one transect per site in the crop field. Each transect was 200 m long and samples were taken in the starting, middle and ending points, making 20 movements and walking 10 meters for every sample. The second method was conducted by walking a 100 m transect along the field margin. At every 20 m the insects were removed from the net. All samples were collected on sunny days, preferably in the morning between 09.30 and 11.30 a.m. or in the late afternoon (16.00 p.m. onwards). Collected material was stored in 70% ethanol, dehydrated with 99% ethanol and dried with HMDS following Heraty and Hawks (1998). Identification of the taxa was performed using the keys in Bouček (1963), Graham (1969), Bouček and Rasplus (1991), Mitroiu (2010), Klimmek and Baur (2018) and Gibson (2009). Nomenclature verification was performed following de Jong et al. (2014), Noyes (2019) and GBIF.org. (2021).

Results

A total of 93 pteromalid specimens were collected, from which 86 were identified. They belong to 15 valid species in 14 genera (Table 3). Nine species and two taxa identified at most to generic level were found in the samples conducted by the first method inside the crop fields. Nine species and nine taxa identified at most to generic level were caught by the second method along the field margin. Only three species were gathered by both of the collecting methods – *Macroglenes penetrans* (Kirby), *M. morys* (Walker) and *Pteromalus sequester* Walker (Table 3). Most species and also the taxa that were not identified to species level were represented by only one specimen in our material. The most abundant and widespread pteromalid was *M. morys*, averaging 60.2% of all collected specimens. It was followed by *Pteromalus semotus* (Walker) with 6.5% and *Pachyneuron*

Table 2. Details about oilseed crops and management practices at the localities selected.

Nearest settlement (site number) (technology)	Variety, company (*preceding crop*)	Insecticide, dose (area treated, date/period)	Herbicide, dose (area treated, date/period)	Fungicide/ fertilizer, growth regulator, dose (area treated, date/period)	Sowing date, dose, seed yield,
Malak chardak (1; 8) (conventional)	NA, Pioner (wheat)	Sherpa 100 EC 0.15 l/ha (twice during FBA)	Fusilade Forte 0.5 l/ha	NPK 20:20:20, 300 kg/ha (before sowing)	26.08.2017, 3 kg/ha 2640 kg/ha
Zelenikovo (2; 3) (Clearfield)	NA, Pioner (wheat, sunflower)	Proteus Bayer 0.45 l/ha (NA, FBA) Terraguard Plus EC (NA, FBA)	Cleranda® BASF 1.5 l/ha (NA)	NPK 20:20:20, 300 kg/ha (before sowing, 01.2018) AN, 300 kg/ha (01.2018, 04.2018)	01.09.2017, 3 kg/ha 5000 kg/ha
Stalevo (4) (conventional)	Vesuvio, Syngenta (wheat)	Cythrin Max, Agriphar 0.05 l/ha (110 ha, 15.11.2017) Cyperfor, 100 EC, 0.15 l/ha (110 ha, 02.04.2018, 14.04.2018)	Fusilade Forte 0.5 l/ha (110 ha, 01.11.2017)	Ferti Seeds, 100 ml/dka (before sowing) Toprex**, 0.3 l/ha (110 ha, 15.11.2017) VitaFer Bor, 0.5 l/ha (110 ha, 15.11.2017) Toprex**, 0.5 l/ha (110 ha, 02.04.2018) VitaFer Green, 1 l/ha (110 ha, 02.04.2018) VitaFer Bor, 2 l/ha (110 ha, 14.04.18) Urea, 200 kg/ha (01.2018) AN, 2000 kg/ha (04.2018)	10.09.2017 3 kg/ha 3000 kg/ha
Dobrich (5) (conventional)	Vesuvio, Syngenta (wheat)	Cythrin Max, Agriphar 0.05 l/ha (22.3 ha, NA) Cyperfor, 100 EC, 0.15 l/ha (22.3 ha, 02.04.2018, 14.04.2018)	Pantera 40 EC, 0.8 l/ha (12.5 ha, 01.11.2017) Fusilade Forte, 0.5 l/ha (19.8 ha)	Ferti Seeds, 1 l/ha (before sowing) Toprex**, 0.3 l/ha (22.3 ha, 15.11.2017) VitaFer Bor, 0.5 l/ha (22.3 ha, 15.11.2017) Toprex**, 0.5 l/ha (22.3 ha, 02.04.2018) VitaFer Green, 1 l/ha (22.3 ha, 02.04.2018) VitaFer Bor, 2 l/ha (22.3 ha, 14.04.2018) AN, 300 kg/ha (01.2018) AN, 200 kg/ha (04.2018)	14.09.2017 3 kg/ha 3000 kg/ha
Momino selo (6) (Clearfield)	NA, Pioner (wheat, sunflower)	Proteus Bayer 0.45 l/ha (NA, FBA)	Cleranda BASF 1.5 l/ha (NA)	NPK 20:20:20, 300 kg/ha (before sowing, 01.2018) AN, 300 kg/ha (01.2018, 04.2018)	05.09.2017 3 kg/ha 5200 kg/ha
Stryama (7) (Clearfield)	NA, Pioner (wheat, sunflower)	Proteus Bayer 0.45 l/ha (NA, FBA)	Cleranda BASF 1.5 l/ha (NA)	NPK 20:20:20, 300 kg/ha (before sowing, 01.2018) AN, 300 kg/ha (01.2018, 04.2018)	28.08.2017 3 kg/ha 5200 kg/ha
Kostievo (9; 10) (Clearfield)	Darko, Euralis (wheat, sunflower, maize)	Decis 100EC, 0.05 l/ha (NA, 03.2018) Proteus Bayer 0.45 l/ha (NA, FBA)	Cleranda BASF 1.5 l/ha (NA)	NPK 15:15:15 (before sowing) Folicur* Bayer, 0.5 l/ha (NA, 03.2018)	05.09.2017 3 kg/ha 2100 kg/ha

Ledend: NA information not available; * fungicide; ** growth regulator and fungicide; FBA the phase of flower bud appearance (butonization); AN ammonium nitrate.

aphidis (Bouche) with 4.3%. Crop fields with a relatively high presence of pteromalids, in terms of both the number of specimens and the number of species, were Momino selo (site 6 – 27 individuals, nine species) and Kostievo (site 9 – 19 ind., nine species). Sampling fields with the lowest presence of pteromalids were Malak Chardak (site 1 – 1 ind.) and Stryama (site 7 – 2 ind., one species). The number of specimens (total number: 76 ind.; mean number ± SE: 12.67 ± 4.16 ind.) captured in the crops managed by the Clearfield technology were higher than those in crops with conventional technology of oilseed rape production (total number: 22 ind.; mean number ± SE: 5.50 ± 3.23 ind.) (Fig. 2A). Similarly, the abundance of pteromalid taxa was higher in crops with Clearfield technology compared to the conventionally treated ones (Clearfield – total number: 30 taxa; mean number ± SE: 5.00 ± 1.39 taxa; conventional system: total number: 10 taxa; mean number ± SE: 2.50 ± 1.19 taxa) (Fig. 2B).
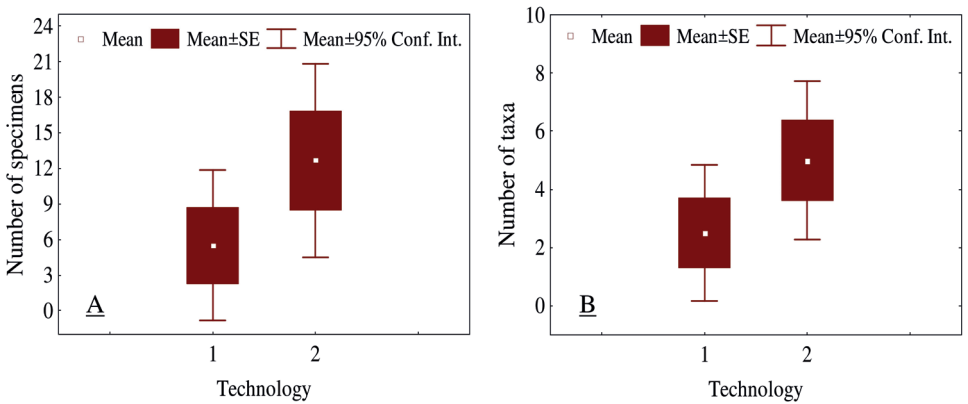
Figure 2. Box-plots showing species abundance **A** and taxonomic richness **B** between conventional (1) and Clearfield (2) technologies.

Table 3. List of the pteromalid taxa (ordered alphabetically) collected during the present study.

Taxa	Number of specimens	Collecting date	Sampling site (according to Table 1)	Presence in Clearfield sites	Presence in conventional sites
Chlorocytus cf *longiscapus*	1 (♀)	27.IV.	6	+	-
Chlorocytus cf *phalaridis*	1 (♀)	22.IV.	10	+	-
Chlorocytus cf *spicatus*	1 (♀)	24.IV.	6	+	-
Chlorocytus sp.	1 (♀)	30.V.	9	+	-
Chlorocytus spicatus (Walker)	1 (♀)	30.V.	6	+	-
Cyrtogaster vulgaris Walker	1 (♂)	16.V.	9	+	-
Halticoptera patellana (Dalman)	1 (♀)	19.IV.	2	+	-
Macroglenes penetrans (Kirby)	3 (♂)	27.IV.;16.V.	5, 9, 10	+	+
Mesopolobus incultus (Walker)	1 (♀)	21.IV.	6	+	-
Mesopolobus morys (Walker)	56 (55♀, 1♂)	19–27.IV.; 27–28.V.	2, 3, 4, 5, 6, 8, 9, 10	+	+
Mesopolobus sp.	1 ♀	28.V.	5	-	+
Pachyneuron aphidis (Bouche)	4 (3♀, 1♂)	28–30.V.	2, 9	+	-
Pachyneuron muscarum (Linnaeus)	1 (♀)	28.V.	2	+	-
Pteromalus cf *chlorospilus*	1 (♀)	14.V.	5	-	+
Pteromalus intermedius (Walker)	1 (♀)	19.IV.	3	+	-
Pteromalus puparum (Walker)	1 (♀)	22.IV.	9	+	-
Pteromalus semotus (Walker)	6 (♂)	14.V.; 27–30.V.	5, 6	+	+
Pteromalus sequester Walker	2 (♀)	22, 27.IV.	6, 10	+	-
Pteromalus sp. undet. J (Graham, 1969 – p. 496, 556)	1 (♀)	14.V.	4	-	+
Spalangia subpunctata Förster	1 (♂)	30.V.	9	+	-
Sphegigaster cf *intersita*	1 (♂)	27.V.	5	-	+
Sphegigaster stepicola Bouček	2 (♂)	27.V.	7	+	-
Spintherus dubius Ashmead	1 (♀)	22.IV.	10	+	-
Stenomalina cf *epistena*	1 (♀)	30.V.	1	-	+
Systasis sp.	1 (♂)	30.V.	6	+	-
Tritneptis cf *klugii*	1 (♂)	29.V.	5	-	+

Discussion

The use of an insect net is a well-known active method for collecting hymenopteran insects in vegetation. Weather, vegetation type and age, weight of net, type of mesh, and handler skill are some of the factors affecting net collections (Marshall et al. 1994).

Although a higher number of taxa was obtained by sweeping with immediate storage of the insects than collection of the target species captured in the net by aspirator, assessing relative sampling effort makes comparisons between collecting techniques problematic. As the purpose of this study was to investigate the diversity of Pteromalidae wasps in oilseed rape fields in Bulgaria, using a combination of sweep netting techniques we found a relatively high number of taxa. Ulber et al. (2010) reported 80 species of hymenopteran parasitoids of the pests of oilseed rape in Europe, including nine pteromalid species belonging to *Anisopteromalus* Ruschka, *Chlorocytus* Graham, *Pteromalus* Swederus (as *Habrocytus* Thomson), *Mesopolobus* Westwood, *Stenomalina* Ghesquière, *Trichomalus* Thomson and *Lyrcus* Walker (as *Zatropis* Crawford). These species are associated with *C. napi*, *C. obsctrictus*, *C. pallidactylus* or *P. chrysocephala*.

Mesopolobus morys was the only pteromalid species occurring in our samples, which was included in the list of species associated with oilseed rape pests in Europe by Ulber et al. (2010). It is very common and widely distributed and has been reported from various natural and agricultural habitats. *Mesopolobus morys* develops as a larval ectoparasitoid mostly on the curculionids belonging to *Ceutorhynchus* spp. (Rosen 1964; Kuhlmann and Mason 2002; Noyes 2019) and rarely on *Protapion apricans* (Herbst) and *Protapion trifolii* (Linnaeus) (OILB 1971). Being recognized as one of the three key parasitoids of the cabbage seed weevil, *C. obstrictus*, (Ulber et al. 2010), *M. morys* plays an important role in the biocontrol of this pest.

The rest of the identified taxa, which have been found during the investigation, can be divided into the following groups:

Parasitoid species potentially associated with hosts feeding on *B. napus* or relative Brassicaceae plants, growing around the crop fields

Pachyneuron aphidis (Bouche)

Widespread pteromalid species distributed all over the world. Similar to most species in this genus, *P. aphidis* is a polyphagous hyperparasitoid of many Aphididae or other plant sucking Hemiptera mostly through their Braconidae, Aphelinidae and Encyrtidae primary parasitoids (Gibson 2001; Noyes 2019). This life strategy is not beneficial for crop field production due to the negative effect of the hyperparasitism on the natural enemies of the plant pests. However, in the case with oilseed rape, *P. aphidis* is not of great importance. It is not commonly associated with these plants and has been reported from crucifers in only four papers (OILB 1971; Kamijo and Takada 1973; Haeselbarth 1985; Gibson 2001).

Pachyneuron muscarum (L.)

Widely distributed Palearctic species with similar life-history and hosts as *P. aphidis*. It is known from *B. napus* fields (Rosen 1964; Graham 1969; OILB 1971) and probably could affect more or less negatively its natural enemies, but does not attack any of the oilseed rape key parasitoids.

Pteromalus semotus (Walker)

Common and widely distributed Palearctic species, introduced in New Zealand for the purpose of the biological control of some Lepidoptera. It is known to attack *C. obstrictus* (as *C. assimilis*) on *Brassica oleracea* but this association seems to be incidental because only two individuals (1% of the total parasitoids) emerged from the host larvae (Dmoch and Sulgostowska 1986).

Parasitoid species biologically similar to other oilseed rape associated pteromalids

Chlorocytus spicatus (Walker)

This species has eight known host associations (Noyes 2019), but only one may be discussed in the light of our study. It is recorded by Vidal (1993) as primary parasitoid of one unidentified *Ceutorhynchus* sp. but not from *B. napus* or other Brassicacae host plant. Among the European *Chlorocytus*, only one – *Chlorocytus diversus* (Walker), has been found to attack some of the oilseed rape pests in Europe (cabbage seed weevil, *C. obstrictus*) (Ulber et al. 2010), but this pteromalid is not considered as a key species. Thus, *C. spicatus* appear to be of negligible or no importance for biocontrol in the *B. napus* crop fields.

Parasitoid species that have not been reported from any Brassicaceae-associated hosts and probably only use *B. napus* as a source of flower nectar or honeydew

Cyrtogaster vulgaris Walker

Well-known solitary pupal parasitoid of various dipteran hosts, mainly Agromyzidae, Chloropidae and Lonchopteridae, which has been reported to attack only one coleopteran species – *Bruchidius marginalis* (Fabricius) (Chrysomelidae), but probably as secondary parasitoid (Andriescu and Mitroiu 2001). According to its hosts that mostly develop on Asteraceae, Fabaceae, and Poaceae, *C. vulgaris* is not a common pteromalid species inside the oilseed rape fields, but certainly can be found in surrounding areas.

Halticoptera patellana (Dalman)

Cosmopolitan species associated mainly with flies belonging to Agromyzidae and Chloropidae (Peck 1963; Graham 1969; Herting 1978). New geographical record for the Bulgarian fauna.

Macroglenes penetrans (Kirby)

This pteromalid is a well-known natural biological agent of two cecidomyiid (Cecidomyiidae) wheat pests – the wheat fly, *Contarinia tritici* (Kirby), and the orange wheat

blossom midge, *Sitodiplosis mosellana* (Gehin). Common species in the grasslands and meadows but usually not numerous.

Mesopolobus incultus (Walker)

Primary and secondary parasitoid on some weevils belonging to *Apion* Herbst, *Protapion* Schilsky (Apionidae) or *Gymnetron* Schoenherr (Curculionidae), mostly associated with legumes (*Trifolium* sp.) (Fabaceae) and sometimes with plantains (*Plantago* sp.) (Plantaginaceae) (Graham 1969; Garrido Torres and Nieves-Aldrey 1992).

Pteromalus intermedius (Walker)

Not very common species known as primary parasitoid of some fruit flies (Tephritidae), mostly associated with Asteraceae and rarely with Chenopodiaceae, Lamiaceae and Tamaricaceae (Graham 1969; Garrido Torres and Nieves-Aldrey 1999; Askew et al. 2001).

Pteromalus puparum (Linnaeus)

Cosmopolitan species, which is known to attack a great number of hosts, mostly belonging to the butterfly families Nymphalidae, Papilionidae and Pieridae. It has not been recorded from *B. napus* in Europe. A study of Gibson et al. (2006b) based on voucher specimens of three species that McLeod (1953) listed as imported from Europe and released in British Columbia (USA), reported *P. puparum* as associated with *C. obstrictus*. However, the authors consider that the material likely represents an incorrect host association because of potential contamination of mass-reared seedpods by the diamondback moth, *Plutella xylostella* (L.) (Plutellidae).

Pteromalus sequester Walker

Cosmopolitan species, known as parasitoid mostly on coleopterans belonging to Apionidae, Bruchidae and Curculionidae associated with legumes (Noyes 2019).

Spalangia subpunctata Förster

This species belongs to the small subfamily Spalangiinae – specialized pupal parasitoids of dipteran hosts in manure piles or animal feces. Its presence in a sample from site 9R (Kostievo) can be explained with the presence of livestock herds feeding on the surrounding grasslands.

Sphegigaster stepicola Bouček

Rarely collected species in Bulgaria, with Palearctic range, known to attack larvae of a few Agromyzidae (Diptera) in grasses (Noyes 2019).

Spintherus dubius Ashmead

One of the most commonly collected pteromalid species in the natural or semi-natural grassland habitats in Bulgaria. *S. dubius* can be found almost everywhere from the sea level to the highly elevated mountainous meadows. It is associated mostly with *Apion* species on clovers (*Trifolium*) (Noyes 2019).

In order to interpret the biological potential of a certain parasitoid species controlling a certain pest one depends mostly on one's research experience but this should be confirmed by field or laboratory experiments. Such experiments, in most cases, are planned after a lot of theoretical assumptions in line with our current knowledge. Thus, the results presented here should be considered as a base for future studies, at least regarding some of the established parasitoids. According to the insecticides used in studied crops, a clear difference between Clearfield and conventional fields is presented (Table 2). Different chemical agents could be a possible reason for the difference in the number of specimens and the abundance of species in studied areas. However, comparative investigations between the effects of insecticides on beneficial insects in Clearfield vs conventional crops have not been conducted until now. More detailed laboratory and field studies about the parasitoid communities and their resistance to pesticide treatment in the discussed types of crops are necessary.

Conclusion

The most abundant species, *M. morys*, was also the only pteromalid species in our samples previously reported as oilseed rape associated. Its presence indicates a high biocontrol potential, at least against the cabbage seed weevil, *C. obstrictus*. For the present, no other species found in this study can be considered as useful in the biological control against the *B. napus* pests.

The pteromalid fauna established in the crops with Clearfield technology was more abundant according both to the number of specimens and number of taxa compared to the crops treated with conventional technology of oilseed rape production.

The high portion of unidentified taxa (12%), probably undescribed species, represents a typical picture for the natural fauna of Pteromalidae and shows our incomplete knowledge on these parasitoids.

Acknowledgements

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Influence of some environmental factors on the distribution of zooplankton complexes in Mandra Reservoir, in Southeastern Bulgaria

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Abstract

The aim of the present study was to trace the influence of some environmental factors (w.temperature, wind, transparency, depth) on the distribution of zooplankton communities in the system Reservoir Mandra and the ecotone zones formed at the confluence of rivers Fakiyska, Sredetska, Izvorska and Rusokastrenska. Four samplings were performed at seven sites between February 2020 and January 2021. After determining the species composition and abundance, the results were subjected to structural analysis and Canonical Correspondence Analysis (CCA). A total of 67 taxa were identified, constituting about 48% of the Rotifera group, 27% of Cladocera and 19% of the Copepoda and only 6% from Protozoa. The Shannon-Weaver index for individual species diversity was between 2.37 and 0.62. The positive and negative correlation of zooplankton distribution in CCA shows that the relative abundance of any species depends on specific environmental variables. Analysis showed that temperature and wind had the strongest impact on the distribution of zooplankton.

Introduction

The distribution of aquatic organisms in the environment is the result of influences of biotic and abiotic factors as well as of the interactions between the organisms in the different parts of the food webs (Menge and Sutherland 1976; Arnott and Vanni 1993; Harley 2003; Abdul et al. 2016; Carter et al. 2017). Many authors have discussed the influence of wind and other abiotic factors on holo-polymictic water basins (George and Edwards 1976; Karabin et al. 1997; Naidenov 1998; Pehlivanov et al. 2004; Güher et al. 2011; Traykov and Vladimirova 2015; Güher 2016; Ismail and Adnan 2016, Tyor et al. 2018; Hayee et al. 2021).

Shallow and deep lakes are affected differently by weather conditions and shallow polymictic fresh water ecosystems are particularly vulnerable to climate warming (Mooij et al. 2005, 2007; Tuvikene et al. 2011; Jeppesen et al. 2014; Haberman and Haldna 2017).

Zooplankton is not included in the European Union Water Framework Directive (Directive 2000/60/EC) as obligatory biological quality elements, despite it being considered a key component of pelagic food webs. Many authors such as Stemberger and Lazorchak (1994), Dodson et al. (2000, 2009), Pehlivanov et al. (2006), Imoobe and Adeyinka (2009), Caroni and Irvine (2010), Tisheva and Kozuharov (2013), Haberman and Haldna (2014) report that zooplankton can be used as a good indicator in assessing the trophic status of lakes.

Zooplankton is an integral part of aquatic ecosystems, playing a crucial role in connecting primary producers and higher trophic levels, such as fish. Zooplankton communities, on the other hand, are sensitive to changes in their resources and their predators and therefore reflect the balance of food web processes through body size distribution and taxonomic composition (Mills and Schiavone 1982; Carpenter et al. 1985; Hansson et al. 2007; Braun et al. 2021).

Mihailova-Neikova (1961) studies the food spectrum of fish in Lake Mandra. On the basis of this study is clear that the food of all fish species contain species from Copepoda, Cladocera, Rotifera groups and some chironomid larvae. It can be concluded that the zooplankton in Mandra Reservoir is a major trophic resource for both small and large fish.

Reservoir Mandra, situated in Southeastern Bulgaria, is part of the Mandra-Poda complex, which is a protected area under the two main environmental directives of the European Union – Directive 92/43 / EEC on the protection of natural habitats and of wild flora and fauna and Directive 2009/147 / EU Wildlife Conservation. The Via Pontica bird migration route passes over Mandra.

Earlier studies that were conducted on Mandra Reservoir (Kozuharov et al. 2021) have shown the high indicative ability of zooplankton to reflect the state of the ecosystem and water quality. The article traces the changes in zooplankton complexes due to the reconstruction of the coastal lake to the reservoir and the interrupted connection with the sea. Results indicate an acceleration of the eutrophication process in Mandra Reservoir. Some previous data that concern plankton in the reservoir have been given

by Michev and Stoyneva (2007). In the previous research about zooplankton in this reservoir (Kozuharov et al. 2021), we suggest that there might be a direct link between the distribution of zooplankton and certain environmental factors, in particular wind. To test this hypothesis, several field studies of Mandra Reservoir were conducted over a one-year period.

Materials and methods

Mandra Reservoir covers an area of 33 km^2 and the maximum depth reaches 7 m. The strong winds common to coastal lakes and reservoirs define Mandra as a holo-polymictic basin. The four sampling sessions (Feb 20, June 20, Sep 20, Jan 21) were performed between 1 February 2020 and 1 January 2021, during which qualitative and quantitative zooplankton samples were collected, as well as data on environmental factors. Our study is focused more on the dynamics in overlapping seasons when plankton comes under strong environmental pressure. The geographical coordinates of the sampling points (Fig. 1) were determined by using a Garmin Striker 5DV sonar with highly sensitive GPS. It was also used to measure the depth of the water body at the various stations, as well as the temperature. Transparency was measured by Secchi disc. The values for the wind speed for the period under investigation were taken from the information page of the National Institute of Meteorology and Hydrology in Bulgaria for the strength of the winds for the region on the respective day.

    24 quantitative and 24 qualitative samples were collected by using an Apstein plankton net 55 μm mesh size and via filtering of 100 dm^3 of water through the net. As the reservoir is shallow, in places between 1 and 2 meters (Table 2), it was not possible to use a Juday net for quantitative samples. Because of this reason, zooplankton samples, each of 100 dm3 of water, were collected from various spots around each station by means of a bucket and filtered through an Apstein plankton net. This method of directly filtering a certain amount of water through Apstein plankton net is widely used in the study of shallow holo-polymictic standing water bodies such as the studied reservoir and in ecotone river-reservoir zones (EN–15110: 2006; Kozuharov et al. 2007; Yakimov et al. 2016; Protasov et al. 2019). Samples, fixed in 4% formalin, were counted by using the method of V. Hensen modified by Dimoff (1959) and Naidenow (1981). This method includes the following operations, applied to each sample:

• Samples are brought to volume of 100 ml and mixed intensively until all organisms were distributed randomly in the sample volume.
• 5 or 10 ml of sample (depending upon zooplankton density) are taken and poured in the counting chamber of Dimov for count.
• All the organisms in this sample are counted through the use of stereomicroscope Leyca GZ6.
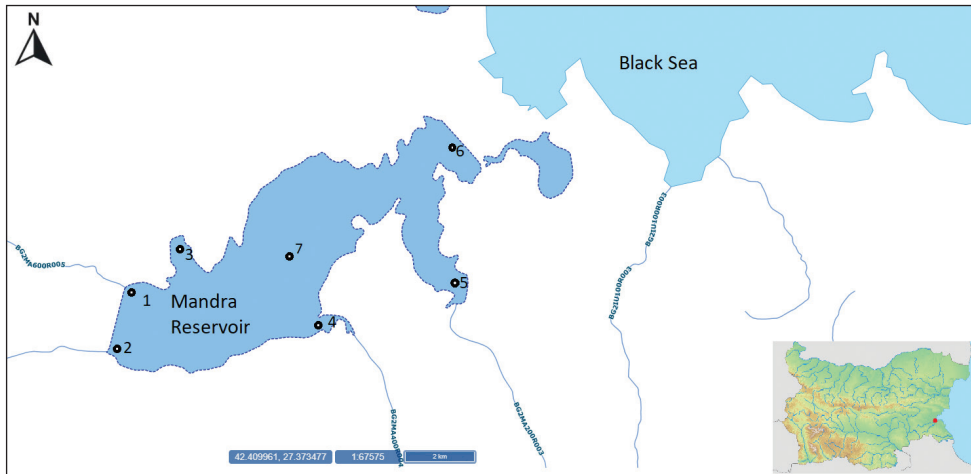• The data obtained are then expressed in terms of cubic meters.

Figure 1. Location of the sampling points on Mandra Reservoir. 1. 42°24.14'N, 27°19.26'E – the mouth of the Rusokastrenska River; 2. 42°23.19'N, 27°18.84'E – the mouth of the Sredetska River; 3. 42°24.68'N, 27°20.41'E – northern dike; 4. 42°23.57'N, 27°22.57'E – the mouth of the Fakiyska River; 5. 42°24.15'N, 27°26.06'E – the mouth of the Izvorska River; 6. 42°26.28'N, 27°26.11'E – dam; 7. 42°24.70'N, 27°22.65'E – central part.

We used three indicators that generally characterize the biological completeness of water through the parameters of the species structure of communities. These indicators are the Shannon-Weaver index for individual species diversity (H), Simpson's index of dominance (c) and the Pielou's evenness index (e) after Shannon and Weaver (1949), Pielou (1975). Margalef richness index was also used to express the degree of uniformity in the distribution of individuals among taxa in the study area. De Vries (1937) frequency of occurrence (pF), was calculated in %. A species with an encounter frequency pF ≥ 70% is considered permanent. Canonical Correspondence Analysis (CCA) was used in order to determine the influence of environmental variables on the abundance and distribution of zooplankton (Czerniawski et al. 2013; Abdul et al. 2016). In this analysis we used the species which are dominant in the abundance of zooplankton.

Results

A total of 67 taxa were identified during the laboratory processing of zooplankton samples. 10 of them were found in very low quantities only in qualitative samples. The list of taxa and their frequency of occurrence (pF) for the studied period are presented in Table 1.

The abundance observed in February and June is relatively low, compared to the other months (Fig. 2).

In February 2020, the highest numbers had Nauplius with 32 500 ind/m³, measured at sampling point 5. With a slightly lower number, but close in value, are Copepodites-Copepoda and *Asplanchna priodonta*. The maximum number of Copepodites-

Table 1. List of zooplankton species found in Reservoir Mandra and their values of pF – frequency of occurrence for the studied period.

Taxa	pF	Taxa	pF
Testacea		*Keratella hiemalis* Carlin, 1943	75.00
Difflugia sp. Leclerc, 1815	4.17	*Notholca squamula* (Müller, 1786)	8.33
Arcella catinus Penard, 1890	12.50	*Lepadella patella* (O. F. Müller, 1773)	4.17
Ciliatea		*Lepadella ovalis* (O.F. Müller, 1786)	8.33
Stentor polymorphus	4.17	*Asplanchna sieboldi* (Leydig, 1854)	50.00
Stentor roeseli Oken, 1815	4.17	*Asplanchna priodonta* Gosse, 1850	50.00
Rotifera		*Trichocerca* sp.	4.17
Pompholyx complanata Gosse, 1851	79.17	*Trichocerca similis* (Wierzejski, 1893)	33.33
Testudinella sp.	20.83	*Trichocerca cylindrica* (Imhof, 1891)	4.17
Testudinella truncata (Gosse, 1886)	12.50	*Trichocerca capucina* (Wierzejski & Zacharias, 1893)	25.00
Filinia longiseta/ Triarthra longiseta (Ehrenberg, 1834)	12.50	*Trichocerca pusilla* (Jennings, 1903)	4.17
Filinia terminalis (Plate, 1886)	8.33	*Synchaeta* sp. Ehrenberg, 1832	12.50
Lecane sp.	12.50	*Polyarthra* sp.	20.83
Lecane monostila (Harring & Myers, 1926)	4.17	*Polyarthra remata* Skorikov, 1896	62.50
Lecane luna (Müller, 1776)	4.17	*Polyarthra dolichoptera* Idelson, 1925	62.50
Epiphanes sp.	4.17	*Polyarthra vulgaris* Carlin, 1943	62.50
Euchlanis sp.	4.17	*Polyarthra minor* Voigt, 1904	16.67
Brachionus angularis Gosse, 1851	20.83	*Polyarthra major* Burckhardt, 1900	8.33
Brachionus calyciflorus Pallas, 1776	8.33	**Cladocera**	
Keratella cochlearis (Gosse, 1851)	100.00	*Diaphanosoma lacustris* Korjinek, 1981	33.33
Keratella tecta (Gosse, 1851)	75.00	*Bosmina longirostris* (O. F. Müller, 1776)	12.50
Keratella quadrata (Müller, 1786)	54.17	*Bosmina kessleri* Uljanin, 1874	54.17
Bosmina coregoni Baird, 1857	83.33	*Harpacticoida genus* sp. G. O. Sars, 1903	4.17
Daphnia cucullata G.O. Sars, 1862	58.33	*Cyclops* sp.	4.17
Daphnia galeata G. O. Sars, 1864	37.50	*Cyclops* c.f. *insignis*	8.33
Daphnia pulex (O.F. Müller, 1785)	4.17	*Tropocyclops prasinus* (Fischer, 1860)	12.50
Daphnia sp. *Juv.*	12.50	Copepodites-Copepoda	100.00
Ceriodaphnia quadrangula (O.F. Müller, 1785)	4.17	Nauplius	100.00
Simocephalus vetulus (O.F. Müller, 1776)	4.17		
Alona guttata Sars, 1862	8.33		
Alonella nana (Baird, 1850)	4.17		
Chydorus sp.	4.17		
Chydorus sphaericus (O.F. Müller, 1776)	79.17		
Chydorus latus G.O.Sars, 1862	4.17		
Chydorus sp. *Juv.*	4.17		
Pleuroxus sp. Baird, 1843	4.17		
Leptodora kindti (Focke, 1844)	8.33		
Copepoda			
Eudiaptomus gracilis (Sars, 1862)	50.00		
Cyclops strenuus Fischer, 1851	12.50		
Cyclops vicinus Uljanin, 1875	29.17		
Thermocyclops crassus (Fischer, 1853)	37.50		
Acanthocyclops sp.	4.17		
Acanthocyclops americanus (Marsh, 1893)	16.67		

Copepoda is 23 200 ind/m^3, also measured at sampling point 5, and for *A. priodonta*, respectively, 20 400 ind/m^3, measured at sampling point 7.

Dominant in number in June 2020 are three taxa, with maximum numbers as follows – Nauplius – 172 800 ind/m^3, at sampling point 3, *Chydorus sphaericus* – 102

Table 2. Hydrological values measured in Mandra Dam in the period 02.2020–01.2021.

date-sampling point	depth (m)	transparency Secchi (cm)	wind (m/s)	t (°C)
Feb 20-S4	1.10	50	6	7.7
Feb 20-S5	1.70	150	6	8.4
Feb 20-S6	3.00	130	6	7.5
Feb 20-S7	2.30	65	6	6.2
June 20-S1	1.50	40	0	26
June 20-S2	1.50	40	0	25
June 20-S3	1.80	45	0	22
June 20-S4	1.20	50	0	22
June 20-S5	1.50	60	0	22
June 20-S6	3.80	60	0	26
Sep 20-S1	1.50	30	4	20.38
Sep 20-S2	1.50	30	4	18.7
Sep 20-S3	1.80	35	4	19.8
Sep 20-S4	1.20	30	4	20.14
Sep 20-S5	1.50	35	4	20.17
Sep 20-S6	3.80	35	4	20.5
Sep 20-S7	3.20	30	4	20.35
Jan 21-S1	2.00	70	8	10.2
Jan 21-S2	1.50	80	8	10
Jan 21-S3	2.00	45	8	10.4
Jan 21-S4	2.60	65	8	10.15
Jan 21-S5	1.30	90	8	10.6
Jan 21-S6	3.70	70	8	9
Jan 21-S7	4.00	75	8	9.9
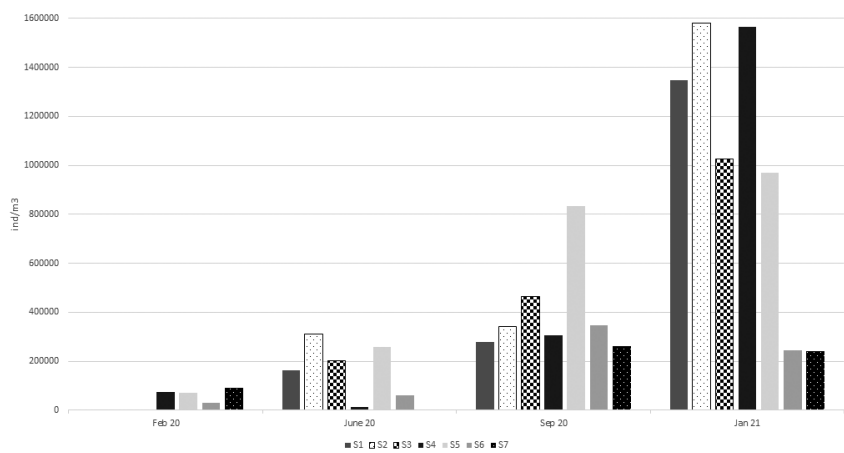
Figure 2. General zooplankton abundance in Mandra Reservoir for the studied period.

813 ind/m³, at sampling point 2, *Polyarthra vulgaris* – 72 500 ind/m³, measured at sampling point 5.

In September 2020, the highest numbers had *Keratella cochlearis* – 339 000 ind/m³, measured at sampling point 5, *Polyarthra vulgaris* – 156 000 ind/m³, at sampling point 5, Nauplius – 136 000 ind/m³, measured sampling point 3.
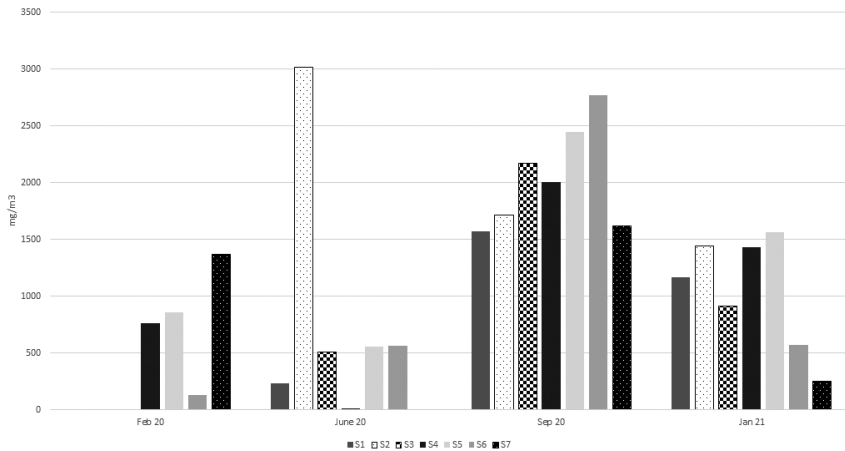
Figure 3. General zooplankton biomass in Mandra Reservoir for the studied period.

While in the other seasons the dominants are followed by other species with a slightly smaller value, in January the absolute dominant for the Mandra Reservoir is *K. cochlearis*. The maximum number of 1 282 000 ind/m³ was measured at S2.

The highest and the lowest biomass within the four samplings were measured in June (Fig. 3), respectively, at sampling point 2 with 3013 mg/m³ and at sampling point 4 with 8.4 mg/m³. The high biomass of station 2 is due to the high number of relatively large Cladocera *C. sphaericus*. This is euribiont, a species with a cosmopolitan distribution.

The ratio between the species composition of the different zooplankton groups during the four periods is shown in Fig. 4.
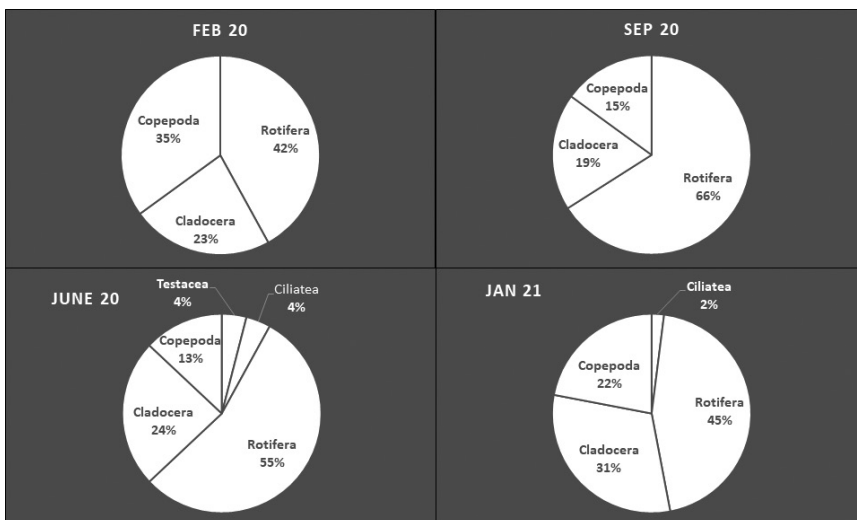
Figure 4. Percent species composition of different plankton groups (February 2020, June 2020, September 2020, January 2021).
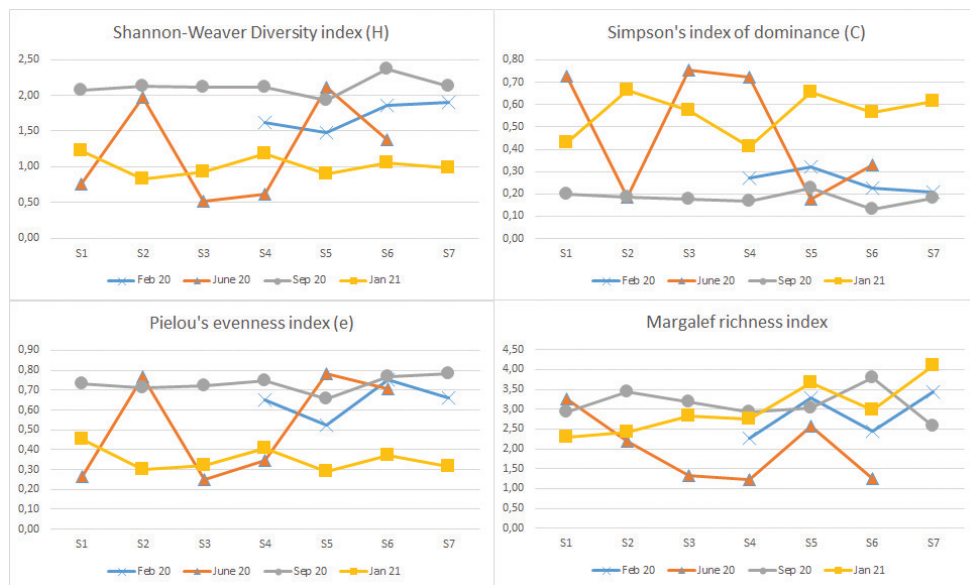
Results of Shannon-Weaver diversity index (H), Simpson's index of dominance (c), Pielou's evenness index (e) and Margalef richness index are shown in Fig. 5. It can be seen that the trends of all indices are relatively constant during the different periods except in June, when the values vary a lot.

For the study period, the Shannon-Weaver diversity index ranged between 0.52 at station 3 in June and 2.37 at station 6 in September. These are comparatively low values of the index. The degree of dominance index was always inversely proportional to the individual species diversity index. Its value was lowest at station 6 in September (0.13) and highest at station 3 in June (0.75). This was the period of higher abundance in the larval stages – Nauplius and Copepodites-Copepoda.

The Margalef richness index varies between 1.23 at station 4 in June and 4.09 at station 7 in January. In general, there is a relatively constant trend between stations for different periods, except for the June series. Then the index varies between 1.23 (station 4) and 3.26 (station 1). This trend is also observed in Pielou's evenness index. The maximum and minimum values were reported at the same time – June, at station 5 (0.78) and at station 3 (0.25). High values of Pielou's index are registered when and where abiotic factors often change and a species or group of species cannot be dominant.

The CCA (Fig. 6) of the samples and dominant zooplankton taxa abundance revealed that temperature, depth, transparency and wind correlated best with the first axis 1, which accounted for a total variance of 91.45%. It was positively correlated with depth, transparency and wind, but negatively correlated with temperature. Axis 2, showed 7.22% variation, and it was positively correlated with temperature, and negatively correlated with the other factors.

Figure 5. Shannon-Wiener diversity index (H), Simpson's index of dominance (c), Pielou's evenness index (e) and Margalef richness index after Shannon and Weaver (1949), Pielou (1975), (S1, S2, S3, S4, S5, S6, S7 – sampling points).
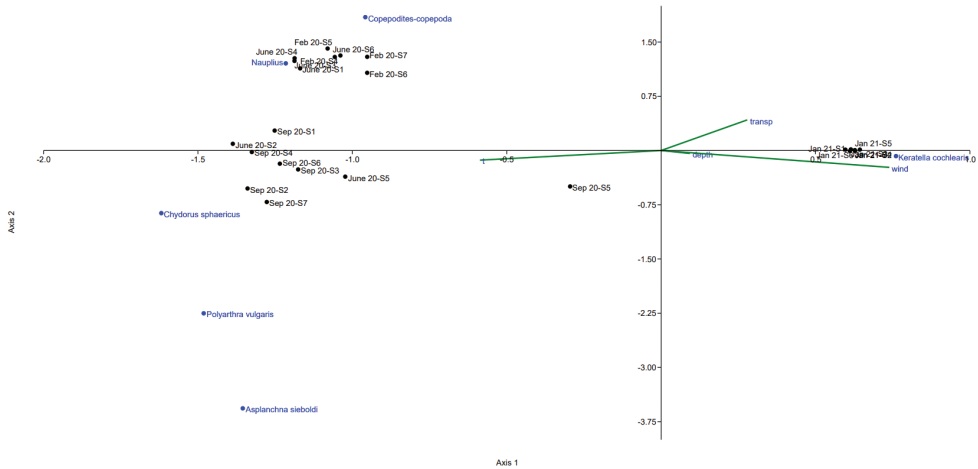
Figure 6. Canonical correspondence analysis (CCA) triplot for the ecological correlations between dominant zooplankton taxa in Mandra Reservoir and some environmental variables. (S1, S2, S3, S4, S5, S6, S7 –sampling points).

Discussion

Some species like *Keretella quadrata*, *Brachionus angularis*, *Trichocerca pusilla*, *Filinia longiseta* are considered indicative of advance processes of eutrophication (Imoobe and Adeyinka 2009). These 4 species were recorded in the species composition of Mandra Reservoir during our study.

The ratio between the groups of zooplankton taxa in different seasons and the predominance of the species diversity of organisms from the Rotifera type confirm the observations in the dominant complexes from a previous study (Kozuharov et al. 2021), which indicate the effects of eutrophication. Light rotifers *K. cochlearis* showed strong correlation (0.76) with wind in January. Nauplius and Copepodites of *Copepoda* are strongly influenced by the summer temperature when they are also the dominant group. The same tendency can be seen from the structural analysis. *Ch. sphaericus*, which is indicatory species of eutrophic waters, showed strong correlation with warm water in September.

The analysis also shows that depth is not essential for the distribution of zooplankton in this shallow polymictic basin. It showed weak positive correlation (0.09) with Axis 1.

According to one of the biocoenotic principles formulated by Thienemann (1931), for aquatic communities, as early as 1920, the more variable the abiotic conditions of a place, the richer in the species is the local community (Sladecek 1973).

The rare zooplankton taxa established only in qualitative samples could be called casual components. Their quantities are lower than the range of quantitative parameters of the samples. That means very rare components.

Probably the main reason for the comparatively low values of the Shannon – Weavers index is the stabile dominant species and complexes that have high quantitative values in the reservoir of zooplankton. The obtained high values of the index of dominance confirm that conclusion.

The significant differences in the values of the Simpson's index of dominance show that different conditions were observed in various parts of this comaratively large (in surface) reservoir during different samplings and seasons. Environmental factors have a great influence, but, on the other hand, the low diversity and richness values might be result of fish predation on site 3 and 4, moreover the observed data corresponded with lowest zooplankton biomass at the seasons (Fig. 3). The presence of small rotifers and the lack of large cladoceras and copepods might be an indirect sign of planktivorous fish pressure (Mihailova-Neikova 1961; Carpenter et al. 1985; Stemberger and Lazorchak 1994; Imoobe and Adeyinka 2009) and coincides right after the breeding period of the fish species. The results reveal the fish spawning and feeding key zones and should be used to increase the efficiency of the conservation measures in a protected area, the part of the European ecological network Natura 2000 and site of the "Via Pontica" bird migration route.

Winter and summer conditions show characteristics of two different water basins. Water basins in which Rotifera predominate go from mesotrophic to eutrophic. Large zooplankton organisms from the group Copepoda like *Cyclops strenuus* and *Eudiaptomus gracilis*, which have the highest biomass in winter, are typical indicators for mesotrophic conditions in the reservoir. As a whole, the conditions in the studied shallow artificial water body are very dynamic during different seasons, which determines the dynamics in the structure and the distribution of zooplankton complexes of the zooplankton in Mandra Reservoir.

Conclusions

Based on the results of our study and taking into account relevant data from numerous zooplankton studies, we can conclude that the zooplankton can be used as key indicator in the monitoring of shallow holo-polymictic water bodies such as Mandra Reservoir.

The results obtained for the calculated structural indices are normal for mesotrophic and eutrophic water basins. The obtained high values of the diversity index are determined by the more diverse habitat conditions along the reservoir and ecotone zones of the inflowing rivers. However, biotic interactions may have adverse impact on the formation of a community structure and should be the next step in our investigation.

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RESEARCH ARTICLE

Comparative determination of antimicrobial activity of the Balkan endemic species *Stachys thracica* Davidov during the process of ex situ conservation

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Abstract

Stachys thracica Davidov – Thracian woundwort is a Balkan endemic plant included in The Red Data Book of Bulgaria with conservational status "rare". The plants from genus *Stachys* have a long history of use to treat various diseases, inflammatory conditions, coughs, ulcers, genital tumors, and infected wounds. Due to its limited distribution the information on the biological activity and chemical composition of *S. thracica* is rather scarce. The aim of the present research is the comparative determination of the antimicrobial activity of methanolic extracts obtained from in situ wild, in vitro cultivated and ex vitro adapted *S. thracica* plants. The in vitro shoot culture of the Thracian woundwort was maintained in hormone-free MS medium under controlled environmental conditions. The methanolic extracts from in situ, in vitro cultivated and ex vitro adapted *S. thracica* plants were active mainly against Gram-negative bacteria. All three extracts showed equal activity against *Acinetobacter calcoaceticus*. The establishment of in vitro shoot culture and its subsequent adaptation in ex vitro conditions was an appropriate alternative approach for the ex situ conservation of *S. thracica* as well as for the study of its biological activity.

Keywords

Antimicrobial activity, in vitro cultivation, Thracian woundwort

Introduction

Genus *Stachys*. L, or woundworts, comprises more than 300 herbs and shrubs and is considered one of the largest genera from *Lamiaceae* family (Tomou et al. 2020). Most of the species are distributed in temperate and tropical regions of the world especially in the Mediterranean. There are 22 species from *Stachys* genus in Bulgaria, 5 of which are under the protection of the Bulgarian Biodiversity Law. The natural habitats of some of these species are located in some of the Bulgarian national parks and others are within localities included in NATURA 2000. The plants from genus *Stachys* have a long history of use in ethnomedicine for various diseases, coughs, ulcers, genital tumors, inflammatory conditions, and infected wounds (Tundis et al. 2007; Conforti et al. 2009; Goren et al. 2014).

It is reported that woundworts exhibit various biological effects such as antioxidant, antibacterial, anti-inflammatory, wound healing, cytotoxic, hepatoprotective properties (Khanavi et al. 2005; Vundać et al. 2007; Háznagy-Radnai et al. 2012; Tundis et al. 2014; Tomou et al. 2020). According to different phytochemical studies, the plants from the *Stachys* genus are sources mainly of phenylethanoid glycosides (Karioti et al. 2010; Delazar et al. 2011), iridoids (Murata et al. 2008; Tundis et al. 2014) and phenolic acids (Venditti et al. 2014).

Taking into account the research done so far, *Stachys* species may be considered a favourable subject for exploration and discovery of secondary metabolites with antimicrobial potential.

The inconsistent application of antibiotics poses a great risk of antibiotic resistance in most of the microbial species that cause human infections (Ventola et al. 2015). This creates an urgency for the research and discovery of alternative sources of antimicrobial agents.

Plants have been used by humanity since ancient times for the treatment of various bacterial infections even without scientific proof of their effectiveness. As a potential source of numerous biologically active substances, plant species have always been potential candidates for alternative agents with antimicrobial activity.

In recent years the antimicrobial potential of some *Stachys* species was a great point of interest among different research groups. Published data indicate that different polar extracts, as well as essential oils, show antimicrobial activity against human pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Dulger et al. 2005; Aleebrahim-Dehkordy et al. 2016; Cuce et al. 2016).

Stachys thracica Davidov (The Plant List) or Thracian woundwort is a Balkan endemic plant distributed in Bulgaria, Greece and Turkey. In Bulgaria, it is classified as "rare" and some of its localities are within Natura 2000 ecological network. The populations of the Thracian woundwort are comprised of a small number of individuals and are located in the Strandja Mountain, Black Sea coast and Sofia region. There is no available data on ex-situ conservation of the species and its chemical composition and biological activity are not well studied.

The aim of the present research is a comparative determination of the antimicrobial activity of methanolic extracts obtained from in situ wild, in vitro cultivated and ex vitro adapted *Stachys thracica* plants.

Materials and methods

Plant material

S. thracica Davidov plants grew in situ in their natural habitat near the village of Sinemorets, Tsarevo municipality, Bulgaria. A small set of samples from aerial parts of the plants in the period of active blooming (in June) and seeds (in September) were collected with the permission of the Ministry of Environment and Water of Bulgaria. A voucher specimen SO107847 was deposited in the Herbarium of Sofia University "St. Kliment Ohridski".

In vitro shoot culture from *S. thracica* was induced by sterilisation of seeds with 70% ethanol for 5 min. The sterilised seeds were placed on a germination medium containing water and agar (WA) and further, the sprouting seedlings were transferred on MS medium (Murashige and Skoog 1962) supplemented with 3% sucrose and 0.7% agar, without growth regulators. The in vitro collection was maintained under controlled environmental conditions (16 h light/8 h dark, 60 mmol/(m²s) photosynthetic photon flux density, Philips TLD-33, temperature 25 °C and 60–70% relative air humidity).

Ex vitro adaptation was performed in three stages with plants having well-developed root systems. At the first step, the regenerated plants were planted in pots and subjected to acclimation in a phytotron chamber for a period of one month. After that, they were transferred to a greenhouse for another month and at a final stage were planted on the experimental field of Sofia University "St. Kliment Ohridski".

Methanolic extracts preparation

Three grams (3 g) of finely powdered dry plant material from aboveground parts of in situ grown, in vitro cultivated and ex vitro adapted *S. thracica* were subjected to triple sonication extraction with 30 ml chloroform (Sigma-Aldrich, Spain) in ultrasonic bath for 10 minutes. In the next step, the dried biomass was extracted three times with methanol for 30 minutes. The final plant extract from each variant was concentrated through a vacuum evaporator (IKA, Germany) and dried to constant dry weight. The yields of extracts from in situ, in vitro cultivated and ex vitro adapted plants were 13.8%, 28.46% and 13.6% respectively. For the current study, each methanolic extract was dissolved in 5% DMSO.

Antimicrobial activity

Microbial strains

The methanolic extracts from in situ, in vitro cultivated and ex vitro adapted *S. thracica* plants were individually tested against seven Gram-negative microbial strains – *Pseudomonas aeruginosa* NBIMCC 3700, *Proteus mirabilis* NBIMCC 8690, *Proteus hauseri* NBIMCC 1393, *Enterobacter cloacea* NBIMCC 8570, *Acinetobacter calcoaceticus* NBIMCC 3730, *Escherichia coli* NBIMCC 8954, *Klebsiella pneumoniae* NBIMCC 3670

and three Gram-positive bacteria – *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermitis* NBIMCC 3360, *Enterococcus faecalis* NBIMCC 1093 microbial species and the yeast *Candida albicans* NBIMCC 74. The microbial specimens were purchased from The Bulgarian Collection for Industrial Microorganisms and Cell cultures (NB-CIMCC). The bacterial strains were cultured overnight at 37 °C on Muller-Hinton agar (MHA) and the yeast was cultured on Sabouraud Dextrose Agar (SDA).

Disk diffusion assay

An initial screening of the antimicrobial activity of the dried methanolic extracts from *S. thracica* was performed by agar disk diffusion method according to the guidelines of CLSI (Clinical and Laboratory Standards Institute). The dried extracts from in situ, in vitro and ex vitro adapted plants were dissolved in 5% DMSO to a final concentration of 200 mg/ml and filtered by 0.45 μm Millipore filters for sterilization. Briefly, 100 μl of each suspension containing 10^7 cell/ml was inoculated in 25 ml MHA for bacterial strains and SDA for the yest respectively. Sterile paper disks (6 mm diameter) were impregnated with the extracts (8 mg/disk) and allowed to dry under aseptic conditions before placing them on the inoculated agar. DMSO at concentration 5% was used as a negative control. The antibiotics tetracycline and amikacin were used as a positive control for the bacterial strains and nystatin for *C. albicans*. The samples were incubated at 37 °C for 24 hours and 48 hours for bacterial strains and *C. albicans* respectively. The antimicrobial activity of the extracts was related to the inhibition zones.

Micro-well dilution assay

Bacterial strains which were sensitive to the methanolic extracts in the disk diffusion assay were studied for their minimal inhibitory concentration (MIC) using the micro-well dilution assay (Wiegand 2008, EUCAST). For the experiment, the bacterial suspension was prepared in a liquid MH-Muller Hinton medium with a density of 0.5 on the McFarland scale, corresponding to 10^7 cells/ml. The 96-well plates were prepared by dispersing 50 μl MH broth in each well. The serial dilutions of each extract were prepared directly in the wells as the starting concentration was 64 mg/ml. Then, 50 μl of each well was transferred to the next and the final dilution of each extract was 2 mg/ml. Finally, 50 μl of the bacterial suspension was added to each well and the final volume of each well was 150 μl.

Prior to incubation, the absorbance of each microplate was measured using ELISA reader (Uscn Kit Inc., China) at λ=630 nm and this was considered the absorption at 0 h.

Results

In vitro multiplication and ex situ conservation of *S. thracica*

In vitro shoot culture from *S. thracica* was successfully induced by the sterilisation and subsequent germination of ripe dried seeds. The in vitro regenerated plants
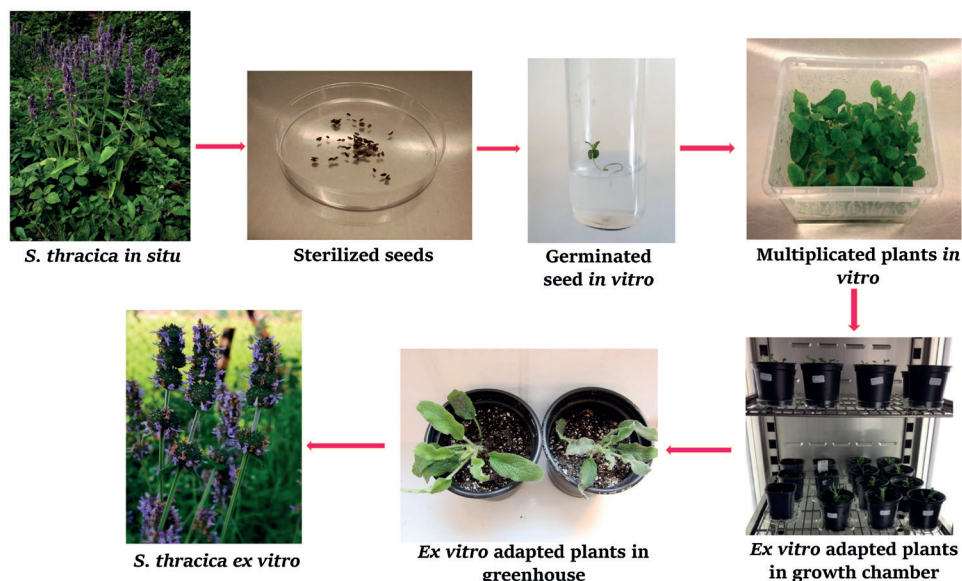
Figure 1. Ex situ conservation of *S. thracica.*

were further propagated and maintained on hormone-free MS medium and characterised with plentiful leaf biomass and well-developed roots. This allowed their further 3-stage acclimatisation – in a phytotron chamber, in a greenhouse and an experimental field. For the current research, the collection of ex vitro adapted *S. thracica* plants were successfully maintained on the experimental field with 83% survival rate (Fig. 1).

Antimicrobial activity

The antimicrobial activity of the methanolic extracts from in situ, in vitro cultivated and ex vitro adapted *S. thracica* plants was evaluated against 11 microorganisms that are frequently related to human infections and are typically present in infected wounds.

The results from the preliminary screening by the disk diffusion assay as well as the microdilution assay are presented in Table 1. All tested extracts show tendency to be active against Gram-negative bacterial strains rather than Gram-positive strains. Overall, the extracts of *S. thracica* showed activity against only 4 of the tested microbial strains and no dependency between the type of extract and its activity was observed (Fig. 2). The most sensitive microbial species appeared to be *A. calcoaceticus* as all three extracts showed bactericidal zones and MIC values of 8 mg/ml. The other bacterial strains that were sensitive to either of the extracts were *K. pneumoniae*, *P. mirabilis* and *E. faecalis*. The highest MIC value – 16 mg/ml and the smallest inhibitory zone – 7 mm were established against *K. pneumoniae*. Although the zones in *P. aeruginosa* were seen as bacteriostatic, no activity was detected in the microdilution assay.

Discussion

In vitro multiplication and ex vitro adaptation of *S. thracica*

The conservational status of the Thracian woundwort and its limited distribution enforced us to apply an alternative method which would allow simultaneously the conservation of the species and the determination of its biological activity. The in vitro micropropagation is a reliable method for ex-situ conservation of endemic and threatened plant species and it is successfully applied for the investigation of such, without disturbing their natural population and habitats.

For the current study we successfully initiated in vitro shoot cultures of *S. thracica* from sterilised ripe dried seeds. The in vitro culture is successfully grown on MS medium without the addition of plant growth regulators and the micropropagated plants are characterised with vigorous growth, plentiful leaf biomass and very well-developed root system. This in turn led to the successful ex vitro acclimation of the Thracian woundwort with 83% survival rate.

Table 1. Antimicrobial activity of methanolic extracts from in situ wild, in vitro cultivated and ex vitro adapted *Stachys thracica* plants.

| Test microorganisms | *Stachys thracica* methanolic extracts | | | | | | Antibiotics | | |
| | In situ | | In vitro | | Ex vitro | | Amicacine | Tetracycline | Nystatine |
	DD[a]	MIC[b]	DD[a]	MIC[b]	DD[a]	MIC[b]	DD[a]	DD[a]	NA
Acinetobacter calcoaceticus	9	8	8	8	7.5	8	11	12	NA
Enterobacter cloacea	-	-	-	-	-	-	8	21	NA
Proteus mirabilis	6*	-	8*	-	8*	8	13	8	NA
Proteus hauseri	-	NA	-	NA	-	NA	20	19	NA
Staphylococcus aureus	-	NA	-	NA	-	NA	20	28	NA
Staphylococcus epidermitis	-	NA	-	NA	-	NA	20	12	NA
Klebsiela pneumoniae	7	16	9	4	-	NA	15	29	NA
Pseudomonas aeruginosa	15*	-	12*	-	10*	-	25	12	NA
Escherichia coli	-	NA	-	NA	-	NA	12	22	NA
Enterococcus feacalis	-	NA	9*	4	-	NA	8	25	NA
Candida albicans	-	NA	-	NA	-	NA	NA	NA	18

(-) – no antimicrobial activity; (*) – bacteriostatic zone; aDD – disc diffusion method; Inhibition zones (mm); bMIC – minimal inhibitory concentration (mg/ml); NA – not tested.
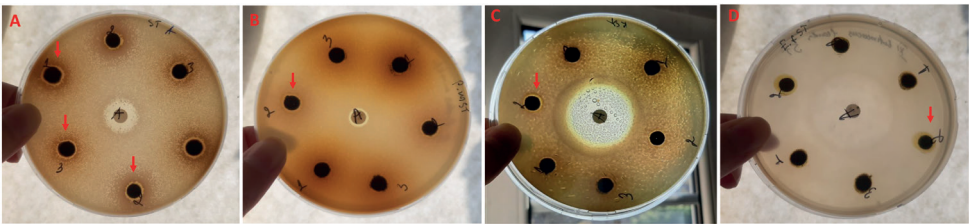
Figure 2. Antimicrobial activity of methanolic extracts from in situ [1], in vitro [2] and ex vitro [3] *S. thracica* plants measured by the disk diffusion assay **A** *A. calcoaceticus* **B** *P. mirabilis* **C** *K. pneumoniae* **D** *E. faecalis*.

Similar to our results, the successfully initiated in vitro culture from the Balkan endemic *S. maritima* was maintained on a hormone-free MS medium and the plants showed an excellent regeneration rate (Panayotova et al. 2008).

Antimicrobial activity

There is a high possibility that different growth conditions would affect the biological activity of plants extracts. *S. thracica* is a source of pharmacologically active secondary metabolites such as phenylethanoid glycosides (Bankova et al. 1999). To evaluate the changes in the antimicrobial activity of methanolic extracts from *S. thracica*, a comparison between the in situ grown, in vitro cultivated and ex vitro adapted plants was made.

The antimicrobial activity was evaluated against 10 bacterial strains and 1 yeast strain – *C. albicans*. The disk diffusion method was used for preliminary study of the antibacterial activity and the microdilution assay was applied afterwards for determination of MIC and verification of the results obtained by the initial screening. All the extracts were more active against Gram-negative bacteria which may be due to the different structure of the cell wall of gram-negative and gram-positive bacteria. We observed no visible trend in the antimicrobial activity of the methanolic extracts obtained from in situ, in vitro cultivated and ex vitro adapted plants with the exception that all the extracts were equally active against *A. calcoaceticus* showing inhibitory zones of 8 mm and MIC values – 8 mg/ml.

Typically, *A. calcoaceticus* is a soil bacterium but it is very frequently associated with infections within hospitals due to its ability to form a complex with another species – *Acinetobacter baumanii* and it is usually used in laboratory testing instead of *Acinetobacter baumanii* (Mancilla-Rojano et al. 2020).

Ebrahimabadi et al. (2010) reported that the polar fraction of *Stachys inflata* Benth. was active against only two microbial species which in parts overlaps with our results. In another study, Dulger et al. (2004) demonstrated that methanolic extracts from *Stachys* species were active against *E. coli*, *P. aeruginosa*, *S. aureus* and *B. cereus* but no activity was established against the tested yeast cultures – *C. albicans*, *K. fragilis* and *R. rubra*. Contrary to our results, Cüce et. al. (2017) reported that methanolic and hexane extracts from in situ and in vitro cultivated *S. annua* plants were active against *S. aureus* and the methanolic extract showed activity against *P. aeruginosa*.

Conclusions

The initiated in vitro culture from *S. thracica* was successfully maintained on hormone-free MS medium under controlled environmental conditions and the micropropagated plants continued to form plenty of biomass and well-developed roots. The methanolic extracts from the Thracian woundwort showed activity mostly against Gram-negative bacteria and the most sensitive bacterial strain was *A. calcoaceticus* against which all three different extracts exhibit equal antimicrobial activity. Further research on the

chemical profile would be necessary in order to reveal which compounds are responsible for the antimicrobial activity of *S. thracica*.

The established in vitro and ex vitro plant cultures serve as an effective alternative approach for the preservation of the rare *S. thracica* and at the same time represents a model system for the study of its biological activity and pharmacological potential.

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RESEARCH ARTICLE

Forests of Breznik municipality

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Abstract

The current study aims to uncover the forest habitat diversity of Breznik municipality, following the EUNIS Classification. Initial data was collected from the Ministry of Environment and Water and the Forestry Management Plans. Forest habitat polygons were spatially processed with the use of the ArcGIS 10.8.1 software package. Field studies were performed to add more detailed information to the analysis. The phytocoenoses of the forest habitats are dominated by *Quercus dalechampii*, *Q. frainetto*, *Fagus sylvatica*, *Carpinus betulus*. Some artificial plantations with *Pinus nigra* and *P. sylvestris* were also present, as well as with non-native species, such as *Robinia pseudacacia* and *Quercus rubra*. The results of this study could be used for more in-depth research of the Breznik municipality vegetation.

Keywords

EUNIS, GIS, habitats, vegetation

Introduction

Forests are among the most important ecosystems for humanity, since they are key providers of many ecosystem goods and services. To a large extent, human well-being relies on forest ecosystems for the provision of food, clean water and air, pollination, genetic resources, erosion prevention, and to sustain biodiversity, etc. (MEA 2005;

TEEB 2010; Powell et al. 2013). Interacting with forests provides Forest Cultural Eco-system Services which also helps in the improvement of human health and well-being (Dodev et al. 2020).

The study of forest ecosystem structure and function is important for understanding biodiversity–productivity relationships (Bohn and Huth 2017) and thus helps to cultivate the sustainable use of natural resources.

Forests in Bulgaria cover approx. 1/3 of its territory and provide about 85% of the water flow in the country (Raev et al. 2005; MOEW 2010). All Bulgarian forests are managed. According to their functions, forests are divided into three main types – forests for timber production, protective and recreation forests, and forests in protected areas (Stoeva et al. 2018). In 2010, the total area of protected native forests in Bulgaria was ca. 572 000 ha (FRA 2015).

Our current forest ecosystem knowledge at municipality level cannot adequately address any policymaker's needs for habitat monitoring and management. Only two papers were found dealing with the forest vegetation and reconstruction afforestation activities of Breznik municipality (Panova and Bondev 1985; Krastev 1990). The European Nature Information System (EUNIS) is an essential tool for implementing nature conservation activities like conducting habitat inventories, monitoring, and the management of protected areas, etc. (Chytrý et al. 2020). A habitat map will summarize the current distribution of forest habitat types in the municipality and will help in any ecological surveys and analysis.

The aim of this study is a complete investigation and mapping of forest habitats according to EUNIS classification on the territory of Breznik municipality.

Methods

Breznik municipality is located in the western part of the country. The territory falls mainly within 600–1000 m a.s.l. It covers an area of 404 km^2 and has diverse natural features, including the mountains of Zavalska, Lyubash, Viskyar, Erulska and Cherna Gora, as well as Breznik Valley. According to Zagorchev et al. (1990), the lithology includes conglomerates, sandstones, clays, marls, limestones, dolomitic limestones, dolomites, marls, shales and volcanics – hornblende andesites, trachyandesites, and andesitobasalts. Soils are predominantly Luvisols, Leptosols and Fluvisols, following Koinov et al. (1956). A part of the territory falls within the NATURA 2000 sites (Council Directive 92/43/EEC 1992) Lyubash BG0000624 (with 0.29 km^2) and Rebro BG0000314 (with 0.009 km^2).

One hundred and fifty relevés were collected in the field following the Braun-Blanquet approach (Braun-Blanquet 1965) and the vegetation type was verified with 135 field points additionally (Fig. 1) during the 2021 field season. Verification points present GPS coordinates of verified polygons. They are placed in the homogenous part of polygons. The GPS data was collected by Juno BS Trimble device. All the field data collected was laid over the most recent orthophoto images available. Mapping was

Figure 1. Field study in Breznik municipality. Each star marks 21 sample plots and verification points.

done using ArcGIS 10.8.1 software. The habitat map was created by polygon modifications, based on the available Forestry (from Forestry Management Plans), Habitat (from NATURA 2000 network in Bulgaria) and field survey data. The map scale was set at 1:5000. These modifications included merging, deletion, creation of new polygons and making changes in their boundaries.

The EUNIS habitat types were determined using the classification expert system EUNIS-ESy (Chytrý et al. 2020) integrated into JUICE 7.1 software (Tichý 2002). For all defined habitat groups were determined diagnostic, dominant and constant species following Chytrý et al. (2020). Every semi-natural habitat type was classified to alliance level according to Mucina et al. (2016). Associations were determined based on the expert knowledge and available literature sources for the country (Tzonev et al. 2006, 2019).

Results

All the studied forest types in Breznik municipality were related to 8 EUNIS habitat types. The EUNIS habitats map of the Breznik municipality is shown in Figure 2.

T1E *Carpinus* and *Quercus* mesic deciduous forest

Abiotic characteristic

This habitat type was widely distributed in the semi-mountainous areas of the municipality. It was found between 600 and 1000 m a.s.l. These forests were typically developed on northern and western slopes where the continental climatic conditions prevailed. Soils were averagely deep, Chromic Cambisols, Luvisols and Cambisols. The
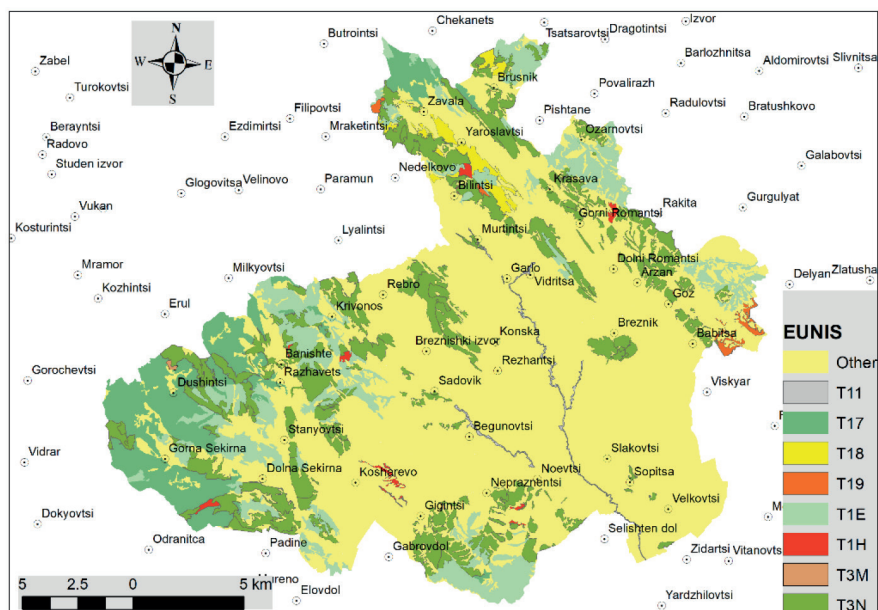
Figure 2. EUNIS forest habitat types in Breznik municipality.

bedrock types were from the magmatic, sedimentary and metamorphic rocks groups. This habitat type was presented by 60 polygons (map units) and covered a total area of 35.2 km². The polygon's area was in the range of 0.01–3.73 km².

Species composition and vegetation structure

Species-poor monodominant or mixed forests with a closed horizontal structure and total cover of 90–100%. The tree and shrub layers were well-developed. The herb layer was absent sometimes or present with a very low cover (5–8%). The tree layer was formed by *Carpinus betulus* or/and *Quercus petraea* agg. In the species composition, *F. sylvatica* was found in some stands with a cover of 20–30%. Other tree species were *Sorbus torminalis*, *S. aucuparia*, *Acer platanoides*, *Tilia platyphyllos*. The shrub layer was formed by the same species from the tree layer as well as *Chamaecytisus hirsutus*, *Ligustrum vulgare*, and *Corylus avellana*. The shrub layer had a cover of 10–50%. The herb layer was characterized by low species richness in the monodominant *C. betulus* forests and by a higher species richness in the mixed *Carpinus betulus-Quercus petraee* agg. and the monodominant *Q. petraea* agg. forests. The most frequent species in the herb layer were *Poa nemoralis*, *Festuca heterophylla*, *Luzula luzuloides*, *Melica uniflora*, *Mercurialis perennis*. These vegetation types were subjected to alliances Carpinion betuli and Fagion sylvaticae s.l., order Fagetalia sylvaticae and class Carpino-Fagetea.

T1H Broadleaved deciduous plantation of non-site native trees

Abiotic characteristic

This category had a scattered distribution. The terrains were slightly inclined (10–15°). These forests were planted in the last 50–70 years to stop erosion. Soils were shallow to moderately deep and the bedrock type was composed of volcanics, limestones, sandstones, and shales. This habitat type was presented by 11 polygons and covered a total area of 1.6 km². The polygon's area was in the range of 0.02–0.33 km².

Species composition and vegetation structure

These plantations were covered by the monodominant stands of *Robinia pseudoacacia* and *Quercus rubra*. The *Quercus rubra* stands especially have poor species composition. The total vegetation cover was 85–100%. Other common tree species found were *Quercus cerris*, *Q. frainetto*, *Q. petraea* agg. The shrub layer was formed from the same species mentioned above, accompanied by *Crataegus monogyna*, *Prunus cerasifera*, *L. vulgare*, *Cornus mas*, *Euonymus verrucosus*. The herb layer was characterized by poor species composition also, with the occurrence of *P. nemoralis*, *M. unuflora*, *F. heterophylla*, *Viola riviniana* agg. The *Robinia pseudoacacia* plantations were typical for eroded terrains and had a semi-open horizontal structure. These stands were formed by a low tree layer with a total cover of 80–90%. Other tree species found were *P. cerasifera*, *P. avium*, *Q. cerris*, *Q. frainetto*, *Q. petraea* agg. The shrub layer consisted of the same species, accompanied by *C. mas*, *Prunus spinosa*, *Rubus caesius*, *Rosa canina*, *C. monogyna*, *Clematis vitalba*, and *E. verrucosus*. It had coverage between 25 and 40%. The herb layer was well-developed and covered 80–100% for *R. pseudoacacia* forests and 50–65% for *Q. rubra* forests. In the *R. pseudoacacia* forests, *Bromus sterilis* was a dominant species and *Galium aparine* was subdominant. This vegetation was classified to association Bromo sterilis-Robinietum, alliance Balloto nigrae-Robinion pseudoacaciae, order Chelidonio-Robinietalia pseudoacaciae, class Robinietea.

T3M Coniferous plantation of non-site native trees

Abiotic characteristic

The artificial plantations dominated by *Pseudotsuga menziesii* were presented by just one polygon in Breznik municipality. The bedrock was composed of marls and shales. Soil type was Chromic Luvisols. The single locality of this habitat type covered an area of 0.11 km².

Species composition and vegetation structure

Species-poor plant community with a closed horizontal structure and canopy of 100%. The only dominant species in these stands was *Pseudotsuga menzeisii*. The strong shady

effect of the canopy limited the development of shrub and herb layers. In the shrub layer, some single individuals of *C. monogyna*, *R. caesius* and *Quercus* spp. were found. The herb layer barely covered 5–10% and was formed by the species *Geum urbanum*, *P. nemoralis*, *Aremonia agrimonoides*, *Veronica chamaedrys*, *M. uniflora*.

T3N *Coniferous plantation of site-native trees

Abiotic characteristic

This category was widely distributed in the northern, western, and southern parts of the municipality. It occurred on flat terrains and slopes (20–25°) with different expositions. These forests were planted in the 50–70s of the last century to battle erosion. The soils were shallow to averagely deep – Chromic Luvisols and Rendzic Leptosols. The bedrock was composed of magmatic, sedimentary, and metamorphic rocks. This habitat type was presented by 189 polygons and covered a total area of 64.9 km². The polygon's area was in the range of 0.003–2.91 km².

Species composition and vegetation structure

The artificial forests of *Pinus nigra* and *P. sylvestris* had a diverse species composition. The *Pinus sylvestris* forests had a closed horizontal structure with a well-developed tree layer. Other species found in this layer were *Q. petraea* agg., *Q. frainetto*, *Populus tremula*, *Acer pseudoplatanus*, *F. sylvatica*, *C. betulus*. Apart from these species, the shrub layer consisted of *R. caesius*, *R. canina*, *C. monogyna*, and *C. vitalba*. The herb layer had poor species composition and was presented by *G. urbanum*, *Aremonia agrimoides*, *P. nemoralis*, *Luzula luzulodies*, *F. heterophylla*, etc.

The artificial plantations of *P. nigra* were typical for eroded carbonate terrains with shallow soils. The latter ecological conditions were a barrier to the development of highly productive forests. The average tree height was 3–5 m. This vegetation type had a semi-open horizontal structure. Some polygons had experienced fires and intensive timber extraction. These places have turned into transitional woodland-shrub complexes. Typical species were *Q. cerris*, *Q. frainetto*, *Q. pubescens*, *Fraxinus ornus*, *C. mas*, *C. monogyna*, *P. spinosa*, *R. caesius*, *C. hirsutus* and *Carpinus orientalis*. The herb layer consisted mainly of *P. nemoralis*, *Dactylis glomerata*, *G. urbanum*, *Festuca dalmatica*, *M. uniflora*, *Buglossoides purpuleacea* and *Fragaria vesca*.

T11 Temperate *Salix* and *Populus* riparian forest

Abiotic characteristic

This habitat type was distributed along the riverbed of Konska River and its tributaries. Soils were averagely deep and the alluvial terraces were periodically flooded. This

vegetation was degraded, moreover, destroyed in some areas. The habitat type was presented by 9 polygons and covered a total area of 1.4 km². The polygon's area was in the range of 0.008–0.69 km².

Species composition and vegetation structure

Vegetation with a closed horizontal structure and a total cover of 95–100%. There were three well-developed layers. *Salix fragilis* and *Alnus glutinosa* were dominating the top one. Other common species were *P. tremula*, *R. pseudoacacia*, *Salix purpurea* and *C. betulus*. The shrub layer was formed by the same species, accompanied by *Cornus sanguinea*, *C. monogyna*, *P. spinosa*, *P. cerasifera*, *Rubus* spp., *C. vitalba*. The herb layer had different species richness along with the plots due to the tree layer shading effect. Common species were *Aegopodium podagraria*, *Ranunculus serbicus*, *Agrostis stolonifera* and *Lysimachia nummularia*. Invasive species, such as *R. pseudoacacia*, *Amorpha fruticosa*, *Erigeron annuus* and *Conyza canadensis* were typical as well.

T17 *Fagus* forest on non-acid soils

Abiotic characteristic

This habitat type was typical for the southwestern part of the study area. Terrains were slightly inclined (up to 10°) with variable exposition. Soils were shallow to moderately deep Cambisols. The bedrock types were limestones and dolomites. This habitat type was presented by 16 polygons and covered a total area of 32.3 km². The polygon's area was in the range of 0.1–19.55 km².

Species composition and vegetation structure

Species-rich communities with semi-open to closed horizontal structure and total cover of 85–100%. In the tree layer, the dominant species was *F. sylvatica*. Other tree species were *Q. petraea* agg., *C. betulus*, *S. aucuparia*, *S. torminalis*, *A. pseudoplatanus*, *Tilia cordata*. The shrub layer was well-developed and consisted of young trees from the aforementioned, as well as *C.monogyna*, *C. orientalis*, *Ulmus minor*, *L. vulgare*. The herb layer was species-rich and included some orchid species such as *Neotia nidus-avis*, *Dactylorhiza cordigera* and *Cephalantera longifolia*. This vegetation was classified to the alliance Cephalanthero-Fagion, order Fagetalia sylvaticae and class Carpino-Fagetea.

T18 *Fagus* forest on acid soils

Abiotic characteristic

This habitat was typical for the northwestern part of the territory, occurring in Viskyar Mountain from 600 to 1000 m a.s.l. The terrains were slightly inclined (up to 10°)

and the exposition was variable. The soils were shallow to moderately deep Cambisols. The bedrock types were magmatics and volcanics. This habitat type was presented by 9 polygons and covered a total area of 4.6 km². The polygon's area was in the range of 0.02–2.92 km².

Species composition and vegetation structure

Species-poor to moderately species-rich phytocoenoses with a closed horizontal structure and total cover 90–100%. There were three layers – tree, shrub and herb layers. The dominant species was *F. sylvatica*. The tree layer had a canopy of 75–100%. Other species found were *A. pseudoplatanus*, *Q. petraea* agg., *and C. betulus*. The shrub layer was formed by the same species from the tree layer as well as *Corylus avellana*, *Rubus hirtus*, *R. idaeus*. The herb layer within the stands with a high tree layer canopy (close to 100%) was patched and had low cover (5–15%). On the other hand, within stands that have been recently cut, the herb layer reached a cover of 30–70%. Species with higher cover and abundance found in the herb layer were *Galium odoratum*, *Festuca drymeja*, *Cardamine bulbifera*, *Lamiatsrum galeobdolon*. The cover of bryophytes was very low – about 2–5%. This vegetation was classified to associations Asperulo-Fagetum and Festuco drymejae-Fagetum, alliance Fagion sylvaticae s.l., order Fagetalia sylvaticae and class Carpino-Fagetea.

T19 Temperate and submediterranean thermophilous deciduous forest

Abiotic characteristic

This habitat type was widely distributed within the municipality's boundaries. It was found from 600 to 1000 m a.s.l. on flat and slightly inclined terrains up to 10°. The exposition was mainly eastern and southern. Soils were shallow to moderately deep with high skeletal composition, dry during the summer period. The main bedrock types were silicates and limestones. This habitat type was presented by 27 polygons and covered a total area of 7.5 km². The polygon's area was in the range of 0.008–0.76 km².

Species composition and vegetation structure

The vegetation had a semi-open to closed horizontal structure. The vertical structure consisted of 3 or 4 well-formed layers. There were tree, shrub, and herb layers, and in some stands, a separate layer formed by bryophytes and lichens above the ground. The tree layer had a canopy of 75–90% and dominant species were *Q. cerris*, *Q. frainetto* and *Q. pubescens*. In general, these tree species were codominant and formed mixed forests but some monodominant stands were found also. Other tree species were *F. ornus*, *Acer campestre*, *C. orientalis* and *S. torminalis*. The shrub layer had a cover between 10 and 60% and was formed by the same species found in the tree layer, as well as *C. monogyna*, *R. canina*, *P. spinosa*, *Euonymus europaeus*, *E. verrucosus*, *C. vitalba* and *Acer tataricum*. These communities had a well-developed and species-rich herb layer with highly abundant species such as

P. nemoralis, *F. heterophylla*, *Galium pseudoaristatum*, *M. uniflora*, *D. glomerata*, *Buglossoides purpurocaerulea*. The cover of bryophytes and lichens was mainly in the range of 5–15%. These vegetation types were subjected to alliances Quercion confertae and Quercion petraeo-cerridis, order Quercetalia pubescenti-petreae and class Quercetea pubescenti.

Discussion

The present research is among the few in the country that offer a map of all forest habitats in a single municipality on such a broad scale (1:5000). According to the EUNIS classification, a total of eight habitat types were established and mapped in Breznik municipality. They were presented by 322 polygons and covered a total area of 147.51 km². Forest ecosystem mapping is an essential part of the modeling and assessment of ecosystem condition and services (Glushkova et al. 2020).

The widest-spread forest habitat type in this municipality is the Coniferous plantation of site-native trees (T3N) covering an area of 64.9 km². It is followed by the *Carpinus* and *Quercus* mesic deciduous forest (T1E) with an area of 35.2 km², and the *Fagus* forest on non-acid soils (T17) with an area of 32.3 km². The least common forest in the municipality is the artificial plantation of *P. menziesii*. The artificial plantations occupy ca. 45% of the total forest area in the municipality. These forests come as a result of the intensive afforestation activities implemented during the 60–80s of the last century. Large areas of the native forests have been cleared in the past for pastures and have been used as such for decades. These activities have led to the development of soil erosion, especially in areas with shallow ones. Panova and Bondev (1985) also pointed out the large extent of forest destruction and that only remnants of natural forests exist on the eastern part of Viskyar Mountain. Nowadays, semi-natural forests are well-preserved in the southwestern part of the municipality where slopes are steep. Dominant species are *F. sylvatica*, *Q. cerris*, *Q. frainetto*, *Q. petraea* agg. and *C. betulus*. The *Quercus pubescens* forests have a restricted distribution on places primarily with southern and eastern expositions and higher solar insolation received.

Fieldwork observations in 2021 found that most of the forest habitats in the municipality are affected by negative processes and phenomena, and thus are not in a favorable condition. This is a direct result of human activities – cutting, afforestation with non-native and invasive species (*P. menziesii*, *R. pseudoacacia*) and pollution. Fires are common as well. There are natural events that also have a negative impact: erosion, pests and climate change, leading to droughts. Combined, the aforementioned processes change the structure and species composition of the natural forests. For instance, xerothermic oak forests are turning into shrublands or are mixing with *Pinus nigra*. Riverine forests of *S. fragilis* are damaged almost everywhere.

There is an urgent need to adopt measures to sustain natural forest regeneration. Plans and practices of the foresters have to be updated in order to reduce the pressure on forests, and curb forest degradation and species richness loss (Krastev 1990; Vacik et al. 2009; Zlatanov and Lexer 2009). Afforestation with non-native species has to be switched to native ones and the introduction of invasive species has to be halted.

Conclusions

The forest habitats of Breznik municipality are presented by 8 habitat types according to the EUNIS classification. Some 45.6% of the forests are artificial plantations, including non-native and invasive species. The latter also provide ecosystem services, such as pollination potential as being suitable habitats for wild pollinator insects – e.g. the stands of honey plant *R. pseudoacacia*.

The intensive and prolonged anthropogenic pressure like deforestation has led to the formation of coppice forests in some areas. Other forests have been intensively exploited for the last 30–40 years and turned into shrublands with pastures. On the whole, the forests in Breznik municipality could be evaluated as being in an unfavorable condition.

Acknowledgements

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RESEARCH ARTICLE

Pre-monitoring geochemical research of the river sediments in the area of Ada Tepe gold mining site (Eastern Rhodopes)

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Abstract

The article depicts the geochemical properties of the landscapes in the Ada Tepe gold mine area before its launching. The research is conducted by examining the heavy metals (Cu, Pb, Zn, Co, Cr, Mn and Ni) content in samples of river sediments in the local landscapes. The research aims to analyse the concentration of heavy metals before the launch of gold mining. The study implements the coefficient of Clarke concentration. The deviation from the background concentrations is a ratio between the element concentration in the collected environmental samples and the Clarke value of the element. The coefficient has a scale from 0 to a particular positive value, corresponding to the level of enrichment of the sample in comparison to the background Clarke value. The values corresponding to the Clarke concentration are equal to 1, the lower values are between 0 and 1 (dispersion) and any value higher than 1 is a case of concentration (enrichment). The obtained results display the researched territory as a natural background area. The content of heavy metals in the river sediments of the researched area (mg/kg, median value) by chemical elements is Cu (15), Zn (72), Pb (17), Mn (461), Ni (35), Co (8) and Cr (60). That is the reason it could be defined as not impacted by human activities and it is not influenced by natural geochemical anomalies. Heavy metals do not pollute the researched landscapes before mining. This outcome is obtained by the geochemical content of the investigated heavy metals in the river sediments.

Introduction

In 2016, the current research was conducted as an environmental pre-monitoring activity in the area of the Ada Tepe gold mining site (Eastern Rhodopes, Bulgaria). The mine itself started exploitation in 2018 after years of delay due to obstructions by local authorities and non-governmental organisations. The non-supporters of the project outlined possible environmental damage, such as contamination of rivers with heavy metals, loss of habitats, air pollution etc., that might be caused by the mine. As a result, the company Dundee Precious Metals Inc. reshaped its initial business plans to satisfy the demands of the local community for better environmental security.

In ancient times, the area of Ada Tepe was a well-known place for its metal deposits. People used to mine gold thousands of years ago. This activity caused local environmental changes such as deforestation, soil erosion and topographical transformations (Popov et al. 2015; Nikov 2017; Nikov et al. 2018). Probably, at that time, people impacted the geochemistry of the area as well.

The study is focused on the concentration of heavy metals (Cu, Pb, Co, Zn, Mn, Ni and Cr) in the river sediments of particular catchments as a geochemical indicator for the environmental status of the territory. The obtained results are a baseline for future monitoring and assessment of contamination with heavy metals and human impact.

Methods

The methodological base of this investigation was the system approach (Perelman 1975; Avesalomova 1987; Kabata-Pendias 2010; Kasimov 2013). We conducted a study through an analysis of the chemical elements'content in the river sediments. The sediments naturally absorb the substances transported by rivers and that is why any anomaly of chemical concentration could be easily detected. This approach allows the tracking of anomalies, either human activities or natural factors.

The chemical elements'content in the various rocks and soils does not match the Clarke value of the element (the average chemical element's content in the lithosphere). That is why the coefficient of Clarke concentration outlines the abundance or the lack of particular elements in rocks, soils and river sediments. It is a quantitative proportion between the chemical element's content in a natural component such as a rock, soil, water, plants and the Clarke value of the element.

In the current study, the deviation from the background concentrations is a ratio between the element concentration in the collected environmental samples of river sediments and the Clarke value of the element. The coefficient has a scale from 0 to a particular positive value, corresponding to the level of enrichment of the sample in comparison to the background Clarke value. The values corresponding to the Clarke concentration are equal to 1, the lower values are between 0 and 1 (a case of dispersion) and any value higher than 1 is a case of concentration (a case of enrichment).

The coefficient allows the implementation of a comparative analysis between particular areas. The current study compares the area of Ada Tepe to technogenic and natural territories in Europe and Bulgaria.

This study analysed 12 samples of river sediments for particular chemical elements (Cu, Pb, Zn, Ni, Co, Cr and Mn). The samples were collected by a standardised methodology. Every sample (500 g, grain size < 0.5 mm) was collected from the upper layer (0–5 cm) of the river-dried thalwegs during the summer when all intermittent rivers in the area dry out. We selected the locations by an analysis of spatial perspective and topographic accessibility. The locations allow interpreting the geochemical influence of the Ada Tepe itself and the influence of the tributaries that confluence the main river in that area.

The collected samples were analysed in the Geochemistry Laboratory of Sofia University. The sediments were dried, quartered, levigated in a porcelain cup, sifted through a 63 μm sieve, burned at 500 °C and dissolved by a mixture of acids ($HClO_4$, HF and HCl). Heavy metals content in the chemical solution was obtained by the method of atomic-absorption spectrometry (Perkin-Elmer 3030).

Results

River sediments are a geochemical indicator for the environmental status within a catchment. In recent years, many scientists have applied the basin approach in evaluating the natural processes and human impact on the environment (Kasimov and Penin 1991; Perelman and Kasimov 1999; Kotsev 2003; Kasimov 2013; Zhelev 2016; Nikolova 2020).

The bedrock, the topography, the vegetation and the climate determine the properties of the river sediments in the area of Ada Tepe. The impact of ancient people is still visible in the features of the landscapes with some rock niches and ancient mining topography changes. The precipitation rates (760 mm per year) directly affect the river sediments'accumulation, transportation and deposition. The precipitations are at a maximum in autumn and winter. They affect the slope of the streams and the formation of sediments in the riverbeds of Krumovitsa and its tributaries. Deforested landscapes in the area enable active bedrock weathering and enforce lateral erosion, a triggering factor for increasing the solid outflow in the rivers. The numerous gullies and ravines in the local topography enable the easy accumulation of sediments alongside the river banks. The local lithology is the primary natural factor that determines the geochemical content of the river sediments. Different types of rocks specifically predetermine the variation in the mechanical and chemical structure of the river sediments.

The results from the investigated locations (Fig. 1) are shown in Table 1. Four elements significantly vary in different locations: Mn, Ni, Cr and Zn. The obtained results allow a comparative spatial analysis between particular locations alongside the river course (absolute values) and the comparison with other areas and norms outside the catchment (median values).
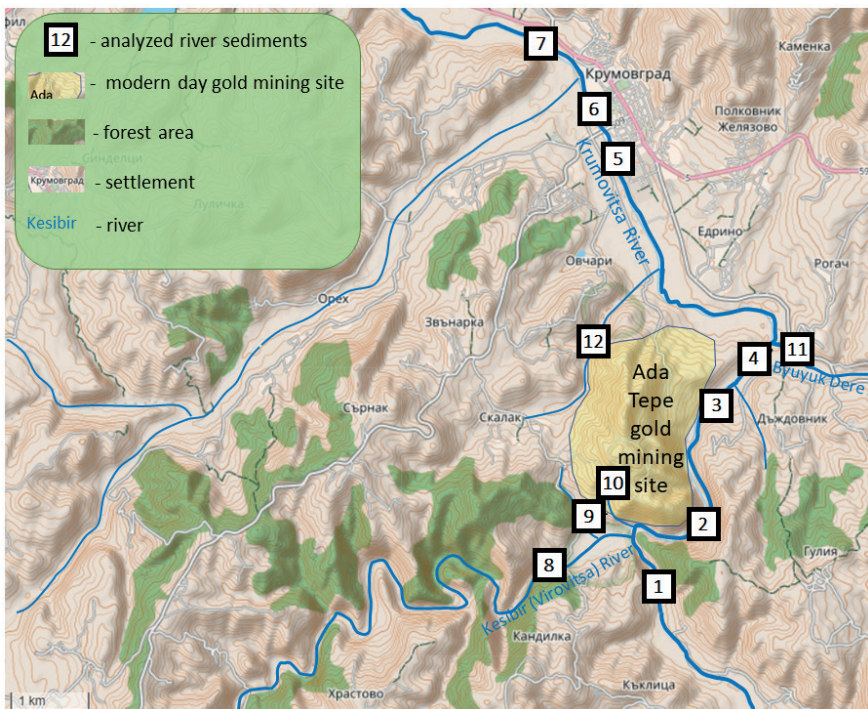
Figure 1. Locations of collecting samples of river sediments around Ada Tepe.

Table 1. Content of heavy metals in river sediments in the Ada Tepe Mining Site area (mg/kg).

No.	Location	Coordinates	Cu	Zn	Pb	Mn	Ni	Co	Cr
1	Krumovitsa River before the confluence of Kesibir River	41°25'18"N, 25°39'21"E	5	61	29	459	4	7	5
2	Krumovitsa River (a large meander south east of Ada Tepe)	41°25'30"N, 25°39'55"E	5	130	19	206	6	5	11
3	Krumovitsa River east of Ada Tepe (before the confluence of Kalach)	41°26'12"N, 25°39'56"E	24	46	10	553	55	16	95
4	Krumovitsa River before the confluence of Byuyuk Dere	41°26'34"N, 25°40'24"E	50	76	13	766	124	25	195
5	Krumovitsa River near the bridge between Ada Tepe and Krumovgrad	41°28'06"N, 25°39'03"E	42	80	10	609	94	21	149
6	Krumovitsa River in Krumovgrad	41°28'21"N, 25°38'54"E	45	68	15	92	53	7	88
7	Krumovitsa River north of Krumovgrad	41°28'47"N, 25°38'30"E	13	43	13	438	40	13	102
8	Kesibir River before the confluence in Krumovitsa River	41°25'14"N, 25°38'50"E	8	147	39	2000	12	4	16
9	A nameless left tributary of Kesibir River	41°25'27"N, 25°38'55"E	28	62	16	89	63	10	89
10	An intermittent stream – a small left tributary to Krumovitsa River	41°25'39"N, 25°39'09"E	6	141	21	250	4	3	7
11	Byuyuk Dere (Golemi Dol) – a right tributary of Krumovitsa River	41°26'39"N, 25°40'44"E	10	54	30	464	30	5	11
12	Kese Dere – a left tributary of Krumovitsa in Ada Tepe	41°26'29"N, 25°38'54"E	18	155	28	490	13	8	31
	Average		21	89	20	535	41	10	67
	Median		15	72	17	461	35	8	60
	Minimum value		5	43	10	89	4	3	5
	Maximum value		50	155	39	2000	124	25	195

Discussion

At first, the content of heavy metals of all sediments in the researched area (median value) is compared to other territories (Europe, Bulgaria), to lithological data (litho-

sphere, acidic metamorphic rocks in Bulgaria) and to adopted threshold environmental concentrations and predicted environmental concentrations (Table 2).

The data outline the similarities and differences between the Ada Tepe area and the compared references. A geochemical spectrum (Fig. 2) visualises the associations of chemical elements within the river sediments of the Ada Tepe area, Europe, natural background territories in Bulgaria and technogenic areas in Bulgaria. Several peculiarities are visible on the spectrum. Most of the investigated chemical elements are with a lower concentration in comparison to the river sediments of Europe. Only nickel has a higher value than Europe's: 35 mg/kg in the Ada Tepe area compared to 11 mg/kg in Europe. Comparing the research area to the natural background territories in Bulgaria (protected areas with minor human impact) outlines a similar situation. Only nickel has higher concentrations: 35 mg/kg in the Ada Tepe area and 28 mg/kg as a reference for a natural background territory in Bulgaria. The scale of this excess is relatively

Table 2. Comparative data for content of heavy metals (mg/kg) in the river sediments.

Area	Cu	Zn	Pb	Mn	Ni	Co	Cr
Lithosphere (Vinogradov 1962)	47	83	16	1000	58	18	83
River sediments in Europe (Salminen et al. 2005)	22	120	39	1120	11	35	93
River sediments in Bulgaria – natural background territories (Penin 2003)	45	94	25	777	28	17	64
River sediments in Bulgaria – industrial (technogenic) territories (Penin 2003)	217	155	102	972	35	37	74
River sediments in Ada Tepe Area (Median)	15	72	17	461	35	8	60
Soils in Ada Tepe Area (Median)	18	77	19	597	41	10	34
Acidic metamorphic rocks in Bulgaria – predominant in the Ada Tepe Area (Kuykin et al. 2001)	20	50	20	287	10	11	8
Threshold environmental concentrations (TEC) (MacDonald and Ingersoll 2002)	32	121	36	460	23	-	43
Predicted environmental concentrations (PEC) (MacDonald and Ingersoll 2002)	149	459	128	1100	49	-	111
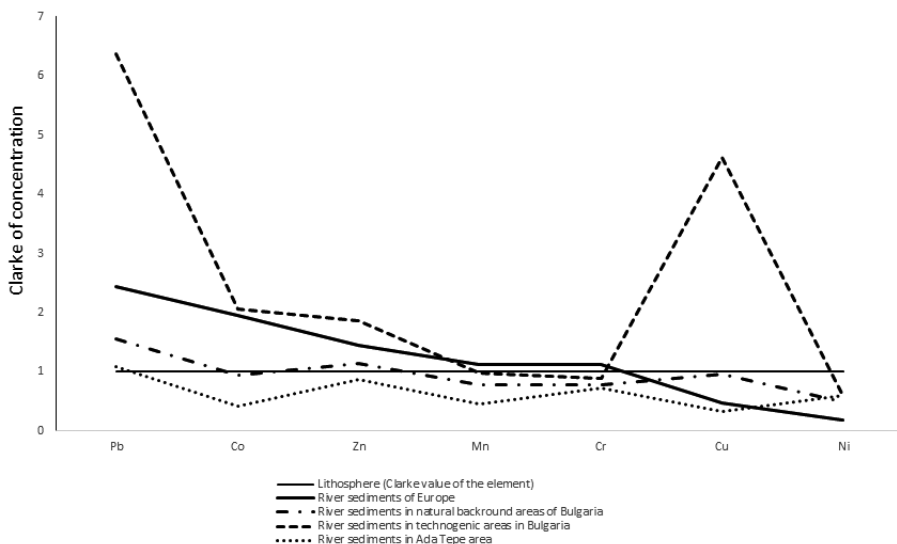
Figure 2. Heavy metals in river sediments in Europe, Bulgaria and Ada Tepe area, based on the Clarke concentration.

tiny and it demonstrates no anomaly. Nickel has coefficient values of Clarke concentration varying between 0.5 and 0.6 in the three comparable territories in Bulgaria. In the rivers of Europe, it is more dispersed and has smaller coefficient values (0.2). The obtained data certify no geochemical anomalies for this territory, either caused by natural factors or human impact. We published results based on investigated plant tissues about geochemical anomalies in the area (Penin and Zhelev 2020), with the same outcomes. The investigated heavy metals did not contaminate the catchments in the Ada Tepe area by 2016.

Another analysis compares the geochemical properties of the local rock formations of acidic metamorphic rocks in the area (Kuykin et al. 2001) and the investigated river sediments. The chemical elements, Zn, Mn and Cr, have higher concentrations in the river sediments than the rocks. The difference is relatively small and it correlates to local geochemical variants within the landscape.

We compared the river sediments of the Krumovitsa River (the main river in the catchment) in different sections of its course to clarify the spatial differentiation of chemical elements in the area (Fig. 3). The spectrum displays an association of chemical elements (Co, Cu, Ni and Cr) from the analysed sample, collected before the Ada Tepe area (Location 1, Fig. 1). Only lead has higher concentration (a Clarke concentration of 1.8) in this sample. The other chemical elements are dispersed in the sediments. Four of them (Co, Cu, Ni and Cr) have significantly lower concentrations than the results of the area where the Krumovitsa River passes near the foothill of Ada Tepe (Location 4, Fig. 1) and the downstream area after the river passes through the town of Krumovgrad (Location 7, Fig. 1). The results outline the local geochemical influence of Ada Tepe's metallogenic rock formations for chemical elements (Co, Cu, Ni and
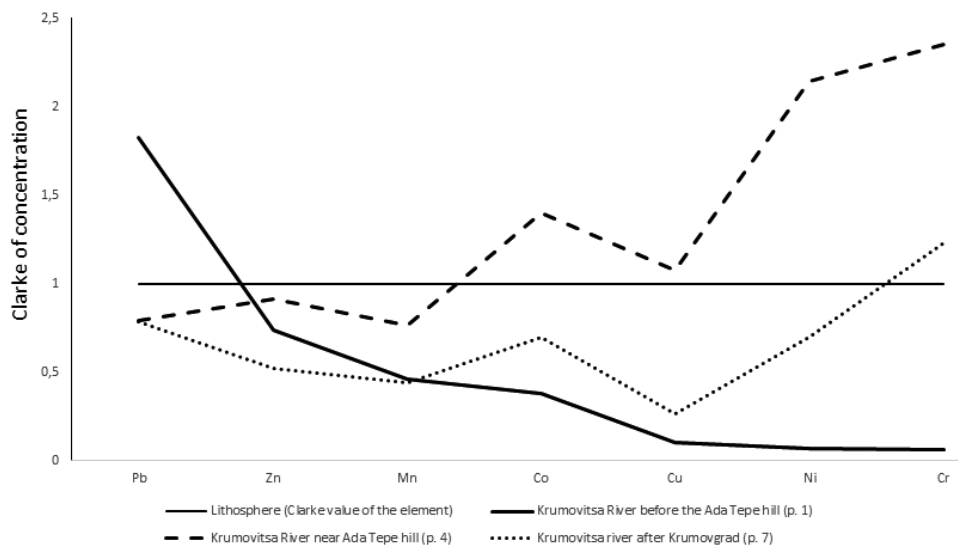
Figure 3. Heavy metals in the sediments of the Krumovitsa River, based on the Clarke concentration.

Cr). Six elements (Zn, Mn, Co, Cu, Ni and Cr) are with lower concentrations in the downstream area after the river passes through the town of Krumovgrad, compared to the area where the Krumovitsa River passes near the foothill of Ada Tepe. This circumstance proves that there is no technogenic geochemical anomaly in the river sediments caused by industrial or urban infrastructure.

Another more detailed analysis of the distribution of heavy metals in the river sediments alongside the Krumovitsa River focuses on seven locations (Fig. 4). The absolute values of chemical content in the sediments vary and there is one more specific sample (Location 4, Fig. 1) where there are increased concentrations of four chemical elements (Cu, Zn, Ni and Cr). This local anomaly is probably naturally determined and reflects the local geochemical influence of the ore-rich hill of Ada Tepe. The influence of Buyuyk Dere (one of the significant tributaries) explains the decrease of concentrations downstream. The values with a lower concentration of chemical elements in the tributary sediments (Location 11, Fig. 1) prove its impact as a natural disperser.

There are no legally adopted norms for recommended environmental concentrations in the river sediments of metals and metalloids in the European Union, although there are such norms in the United States of America. The Environmental Protection Agency (US EPA) institutionalises them. The norms adopted by the US EPA consider two levels of quality for the river sediments (MacDonald and Ingersoll 2002). The first is the threshold environmental concentration (TEC) and the second is the predicted environmental concentration (PEC). The threshold environmental concentration highlights the acceptable levels of metals and metalloids, while the predicted environmental concentration raises awareness for possible negative effects on the wildlife in the ecosystems where the river sediments are. There is a similar approach in
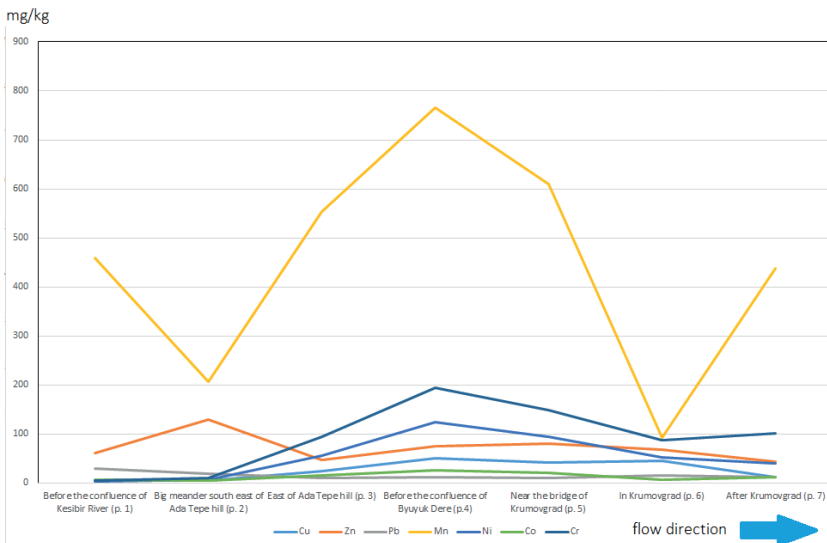
Figure 4. Quantity of microelements in the river sediments of the Krumovitsa River.

the Canadian Province of Ontario, where manganese and iron are chemical elements used for monitoring (Guidelines for Identifying, Assessing and Managing Contaminated Sediments in Ontario: An Integrated Approach 2008). Table 2 applies the norms for Mn from Ontario, Canada. The concentration of cobalt in river sediments does not have a standard value neither in the USA nor in Canada. Similar studies use the application of threshold environmental concentration and predicted environmental concentrations as a reference for a comparative analysis (Cholakova and Penin 2016). The obtained results from the Ada Tepe area are analysed under the perspective of threshold and predicted environmental concentrations for metals and metalloids. The results show that five of the investigated chemical elements (Cu, Zn, Mn, Co and Pb) do not exceed the threshold environmental concentrations. Nickel (35 mg/kg) and chromium (60 mg/kg) exceed the threshold environmental concentrations (23 mg/kg for Ni; 43 mg/kg for Cr). None of the investigated chemical elements exceeds the predicted environmental concentration.

Conclusion

The performed study highlights the environmental status of the Ada Tepe area before the start of the mining activity. There was no contamination with heavy metals of the investigated river sediments in the Krumovitsa River catchment up to 2016. The geochemical properties of the seven examined chemical elements (Cu, Zn, Pb, Mn, Cr, Co and Ni) within the local landscapes resemble a natural background territory with no traces for human impact. Local lithological specifics, but not anthropogenic activity, determine the geochemical properties of the river sediments in the catchment.

The study is a baseline for future research on the mining impact on landscapes and ecosystems. An extension of the list of investigated chemical elements is recommended to encompass the geochemical picture of the area. The effect of the ongoing mining activity in Ada Tepe must be a subject of regular independent environmental monitoring.

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RESEARCH ARTICLE

Development of accurate chemical thermodynamic database for geochemical storage of nuclear waste. Part II: Models for predicting solution properties and solid-liquid equilibrium in binary nitrate systems

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Abstract

The main purpose of this study is to develop new thermodynamic models for solution behavior and solid-liquid equilibrium in 10 nitrate binary systems of the type 2–1 ($Mg(NO_3)_2$-H_2O, $Ca(NO_3)_2$-H_2O, $Ba(NO_3)_2$-H_2O, $Sr(NO_3)_2$-H_2O, and $UO_2(NO_3)_2$-H_2O), 3–1 ($Cr(NO_3)_3$-H_2O, $Al(NO_3)_3$-H_2O, $La(NO_3)_3$-H_2O, $Lu(NO_3)_3$-H_2O), and 4–1 ($Th(NO_3)_4$-H_2O) from low to very high concentration at 25 °C. To construct models, we used different versions of standard molality-based Pitzer approach. To parameterize models, we used all available raw experimental osmotic coefficients data (φ) for whole concentration range of solutions, and up to supersaturation zone. The predictions of developed models are in excellent agreement with φ-data, and with recommendations on activity coefficients ($\gamma_{\pm}$) in binary solutions from low to very high concentration. The Deliquescence Relative Humidity (DRH), and thermodynamic solubility product (as $\ln K^{\circ}_{sp}$) of 12 nitrate solid phases, precipitating from saturated binary solutions have been calculated. The concentration-independent models for nitrate systems described in this study are of high importance for development of strategies and programs for nuclear waste geochemical storage.

Introduction

Computer models that predict solution behavior and solid-liquid-gas equilibria close to experimental accuracy have wide applicability. They can simulate the complex changes that occur in nature and can replicate conditions that are difficult or expensive to duplicate in the laboratory. Such models can be powerful predictive and interpretive tools to study the geochemistry of natural waters and mineral deposits, solve environmental problems and optimize industrial processes. However, development of comprehensive models for natural systems, with their complexity and sensitivity, is a very difficult, time consuming and challenging task. The specific interaction approach for describing electrolyte solutions to high concentration introduced by Pitzer (1973, 1991) represents a significant advance in physical chemistry that has facilitated the construction of accurate computer thermodynamic models. It was showed that this approach could be expanded to accurately calculate solubilities in complex brines, and to predict the behavior of natural and industrial fluids from very low to very high concentration at standard temperature of 25 °C (Harvie et al. 1984; Trendafelov et al. 1995a, 1995b; Christov 1996a, 1998, 1999, 2001, 2002a, 2002b, 2003a, 2003b, 2003c, 2004, 2005; Christov et al. 1998; Ojkova et al. 1999; Park et al. 2009; Kolev et al. 2013; Lach et al. 2018; Guignot et al. 2019; Donchev and Christov 2020; Lassin et al. 2020; Donchev et al. 2021; Tsenov et al. 2021), and from 0 to 290 °C (Petrenko and Pitzer 1997; Christov and Moller 2004ab; Moller et al. 2006, 2007; Lassin et al. 2015; Christov 1995, 1996b, 2005, 2007, 2009, 2012, 2020).

A long term safety assessment of a repository for radioactive waste requires evidence, that all relevant processes are known and understood, which might have a significant positive or negative impact on its safety. It has to be demonstrated, that the initiated chemical reactions don't lead to an un-due release of radionuclides into the environmental geo-, hydro-, and bio-sphere. One key parameter to assess the propagation of a radionuclide is its solubility in solutions interacting with the waste. Solubility estimations can either be based on experimental data determined at conditions close to those in the repository or on thermodynamic calculations. A so called "thermodynamic database" created from experimental data is the basis for thermodynamic model calculations. Since the disposal of radioactive waste is a task encompassing decades, the database is projected to operate on a long-term basis. Chemical models that predict equilibrium involving mineral, gas and aqueous phases over a broad range of solution compositions and temperatures are useful for studying the interactions between used nuclear fuel waste and its surroundings. The reliability of such predictions depends largely on the thermodynamic database. Waters of high salinity are not a typical of many geochemical environments which may be chosen as future nuclear waste repository sites. This suggests that an accurate description of highly saline waters should be required for modeling of chemical interactions in and around nuclear repositories. Currently, the most accurate description of saline waters uses the Pitzer ion interaction model. Extensive thermodynamic databases, which are based on the Pitzer ion interaction model was developed within the Yucca Mountain Project (YMTDB: data0.ypf.r2) (Sandia National Laboratories 2007), and Thereda project (THermodynamic REference DAtabase, THEREDA-Final Report) (Altmaier et al. 2011). Unfortunately,

many of introduced in YMTDB and in THEREDA databases Pitzer models are concentration restricted and cannot describe correctly the solid-liquid equilibrium in geochemical and industrial systems of interest for nuclear waste programs.

Nitrates are expected to play a significant role in the context of the underground geochemical repository of nuclear waste (Lach et al. 2018; Guignot et al. 2019; Donchev and Christov 2020; Lassin et al. 2020). More precisely, long-lived, intermediate-level radioactive wastes that are planned to be stored in deep clay formations are composed of dried sludge from effluent treatments that contain significant quantities of nitrate amongst other elements. They are enclosed in specific containers which are placed in underground cavities dug in a very low-permeable argillite host rock. "The storage safety analyses show that, despite the protection of the concrete or stainless steel-made external layers of the containers, the formation water of the host rock is likely to migrate and reach the waste during the disposal period" (Donchev and Christov 2020; Lassin et al. 2020). This would result in the potential dissolution of large amounts of nitrate and other elements, resulting in a highly saline, corrosive and oxidative media with a high reactivity towards the containment materials and their surroundings, including the host rock. Several options for the management of radioactive waste involve their preliminary leaching using nitric acid or nitrate salts to recover U and Pu, followed by the incorporation of the leaching residues into concrete or metal packages, which are then stored underground. For safety analysis purpose, the scenarios envisaged for these options assume that formation water returns to the storage compartments sometime after the end of the operating period Dossier ANDRA (2005). With a natural pH value slightly above 7 in clayey formations, pore water has to flow through basic concrete materials before being in contact with the acidic nuclear waste. A large range of pH leading to various chemical behaviors can thus be expected in the vicinity of the waste. At near-neutral to basic pH values, reactions of hydrolysis, complexation, and formation of solid phases can take place and control the fate of radionuclides (Wang et al. 2006; Lassin et al. 2020). Therefore, this reactivity must be characterized by development of not concentration restricted thermodynamic models, which accurately describe not only solution behavior at low molality, but also low and high molality solid-liquid phase equilibrium in nitrate systems (Donchev and Christov 2020). The experimental data presented in Rard et al. (1977, 2004), Rard and Spedding (1981), Maliutin et al. (2020), El Guendouzi and Marouani (2003), and accurate models reported in previous studies (Wang et al. 2006; Lach et al. 2018; Guignot et al. 2019; Donchev and Christov 2020; Lassin et al. 2020), and in the present work is a step towards this objective. It should be noted that THEREDA (Altmaier et al. 2011) do not include models for nitrate solutions and solids. The models introduced in YMTDB (Sandia National Laboratories 2007), including these for NO_3-systems are restricted up to 6 mol.kg^{-1}.

In our previous study (Donchev and Christov 2020) we reported very well validated accurate thermodynamic models based on Pitzer ion interactions approach for 7 nitrate binary systems of the type 1–1 (HNO_3-H_2O, $LiNO_3$-H_2O, $NaNO_3$-H_2O, KNO_3-H_2O, $RbNO_3$-H_2O, $CsNO_3$-H_2O, and NH_4NO_3-H_2O) from low to very high concentration at 25 °C. In this study we developed very well validated not concentration restricted thermodynamic models for solution behavior and solid-liquid equi-

librium in 10 nitrate binary systems of the type 2–1 ($Mg(NO_3)_2$-H_2O, $Ca(NO_3)_2$-H_2O, $Ba(NO_3)_2$-H_2O, $Sr(NO_3)_2$-H_2O, and $UO_2(NO_3)_2$-H_2O), 3–1 ($Cr(NO_3)_3$-H_2O, $Al(NO_3)_3$-H_2O, $La(NO_3)_3$-H_2O, $Lu(NO_3)_3$-H_2O), and 4–1 ($Th(NO_3)_4$-H_2O) from low to very high concentration at 25 °C. Models are developed on the basis of Pitzer ion interactions approach. The models for nitrate systems described in this study are of high importance, especially in development of strategies and programs for nuclear waste geochemical storage. These models are also of interest for industrial application, such as production and purification of nitrate compounds.

Methodology

The models for nitrate binary systems have been developed on the basis of Pitzer's semi-empirical equations (Pitzer 1973, 1991). Since the Pitzer's representation of the aqueous phase is based on the excess free energy, all the activity expressions are consistent, allowing different kinds of data (e.g., osmotic, emf, and solubility measurements) to be used in the parameter evaluations and other thermodynamic functions to be calculated (Christov and Moller 2004a, Christov and Moller 2004b; Christov 2007, 2009, 2012). Pitzer approach has found extensive use in the modeling of the thermodynamic properties of aqueous electrolyte solutions. Several extensive parameter databases have been reported. These include: 25 °C database of Pitzer and Mayorga (1973, 1974) (summarized also in Pitzer 1991); of Kim and Frederick (1988); YMTDB (Sandia National Laboratories 2007), and THEREDA (Altmaier et al. 2011). However, some of the models in all of these databases are concentration restricted, and do not include all minerals precipitating from saturated and supersaturated binary and mixed systems. The most widely used are databases of Chemical Modelling Group at UCSD (University California San Diego): at 25 °C (Harvie et al. 1984; Park et al. 2009), and T-variation (from 0 to 300 °C) (Christov and Moller 2004a, Christov and Moller 2004b; Moller et al. 2006, 2007; Christov 2009). Some of comprehensive minerals solubilitiy YMTDB (Sandia National Laboratories 2007), and THEREDA (Altmaier et al. 2011) databases also contain concentration restricted models for some low-, or high- concentration binary and mixed sub-systems with strong association reactions in unsaturated solutions. The concentration restricted sub-models are developed using experimental activity data in binary solutions, and solubility data in binary and high order systems up to maximum concentration (m(max)), which is much lower than concentration of saturated or supersaturated binary and mixed solutions (m(sat)). Such a restricted models predict minerals solubility, which is in pure agreement with experimental data.

The Pitzer's equations

According to Pitzer theory electrolytes are completely dissociated and in the solution there are only ions interacting with one to another (Pitzer 1973; Pitzer and Mayorga 1973). Two kinds of interactions are observed: (i) specific Coulomb interaction be-

tween distant ions of different signs, and (ii) nonspecific short-range interaction between two and three ions. The first kind of interaction is described by an equation of the type of the Debye-Hueckel equations. Short-range interactions in a binary system (MX(aq)) are determined by Pitzer using the binary parameters of ionic interactions ($\beta^{(0)}, \beta^{(1)}, C^{\varphi}$, and $\beta^{(2)}$). The Pitzer's equations (1 to 4) are described and widely discussed in the literature (Harvie et al. 1984; Moller et al. 2006, 2007; Christov and Moller 2004a, Christov and Moller 2004b; Christov 2005). Here only the expression for the activity coefficient of the interaction of cation (M) with other solutes, $\gamma_{(M^+)}$ is given:

$$\ln \gamma_M = z_M^2 F + \sum_a m_a \left(2B_{Ma}(I) + ZC_{Ma}\right) + \sum_c m_c \left(2\Phi_{Mc} + \sum_a m_a \psi_{Mca}\right) + \sum_a \sum_{a<a'} m_a m_{a'} \psi_{Mka'} + |z_M| \sum_c \sum_a m_c m_a C_{ca}$$
$$+ \sum_n m_n \left(2\lambda_{nM}\right) + \sum_n \sum_a m_n m_a \zeta_{naM} \tag{1}$$

Equation (1) is symmetric for anions. The subscripts c and a in eqn 1 refer to cations and anions, and m is their molality; z is the charge of the M^+ ion. B and Φ represent measurable combinations of the second virial coefficients; C and ψ represent measurable combinations of third virial coefficients. B and C are parameterized from single electrolyte data, and Φ and ψ are parameterized from mixed solution data. The function F is the sum of the Debye-Hueckel term,

$$-A^{\varphi} \left[\sqrt{I} / (1 + b\sqrt{I}) + (2/b) (\ln(1 + b\sqrt{I})) \right], \tag{2}$$

and terms with the derivatives of the second virial coefficients with respect to ionic strength (see Harvie et al. 1984). In Eq. (2), b is a universal empirical constant assigned to be equal to 1.2. A^{φ} (Debye-Hückel limiting law slope for the osmotic coefficient) is a function of temperature, density and the dielectric constant of water (Christov and Moller 2004b).

For the interaction of any cation M and any anion X in a binary system MX-H_2O, Pitzer assumes that in Eq. (1) B has the ionic strength dependent form:

$$B_{MX} = \beta^{(o)}_{MX} + \beta^{(1)}_{MX} g(\alpha_1 \sqrt{I}) + \tag{3}$$

$$+ \beta^{(2)}_{MX} g(\alpha_2 \sqrt{I}), \tag{3A}$$

where $g(x) = 2[1 - (1 + x)e^{-x}] / x^2$ with $x = \alpha_1\sqrt{I}$ or $\alpha_2\sqrt{I}$. α terms are function of electrolyte type and does not vary with concentration or temperature.

In Eq. 1, the Φ terms account for interactions between two ions i and j of like charges. In the expression for Φ,

$$\Phi_{ij} = \theta_{ij} + {}^E\theta_{ij}(I), \tag{4}$$

θ_{ij} is the only adjustable parameter. The ${}^E\theta_{ij}(I)$ term accounts for electrostatic unsymmetric mixing effects that depend only on the charges of ions i and j and the total ionic

strength. The ψ_{ijk} parameters are used for each triple ion interaction where the ions are not all of the same sign. Their inclusion is generally important for describing solubilities in concentrated multicomponent systems. Therefore, according to the basic Pitzer equations, at constant temperature and pressure, the solution model parameters to be evaluated are: 1) pure electrolyte $\beta^{(0)}$, $\beta^{(1)}$, and C^{φ} for each cation-anion pair; 2) mixing θ for each unlike cation-cation or anion-anion pair; 3) mixing ψ for each triple ion interaction where the ions are all not of the same sign.

Fluids commonly encountered in natural systems include dissolved neutral species (such as carbon dioxide ($CO_{2(aq)}$, $SiO_{2(aq)}$, and $Al(OH)_3°(aq)$). To account neutral specie interactions in aqueous solutions the UCSD Chemical Modelling Group included in their models additional terms to Pitzer equations, denoted as $\lambda_{N,X}$ or $\lambda_{N,A}$, and $\zeta_{N,A,X}$. (Eq. (1)) (Harvie et al. 1984; Moller et al. 2006, 2007).

The $\beta^{(2)}$ parameter (Eqn. 3A) for 2–2 type of electrolytes

Pitzer and Mayorga (1973) did not present analysis for any 2–2 (e.g. MgSO$_4$-H$_2$O) or higher {e.g. 3–2: Al$_2$(SO$_4$)$_3$-H$_2$O} electrolytes. Indeed, they found that three $\beta^{(0)}$, $\beta^{(1)}$, and C^{φ} parameters approach (see Eqns. 1 and 3) could not accurately fit the activity data for these types of solutions. For these electrolytes mean activity ($\gamma_{\pm}$) and osmotic (φ) coefficients drop very sharply in dilute solutions, while showing a very gradual increase, with a very wide minimum at intermediate concentration. Pitzer concluded that this behaviour is due to ion association reactions and that the standard approach with three evaluated solution parameters cannot reproduce this behaviour. This lead to a further (Pitzer and Mayorga 1974) modification to the original equations for the description of binary solutions: parameter $\beta^2(M,X)$, and an associated $\alpha_2\sqrt{I}$ term are added to the B$_{MX}$ expression (see Eqn. (3A)). Pitzer presented these parameterizations assuming that the form of the functions (i.e. 3 or 4 β and C$^{\varphi}$ values, as well as the values of the α terms) vary with electrolyte type. For binary electrolyte solutions in which either the cationic or anionic species are univalent (e.g. NaCl, Na$_2$SO$_4$, or MgCl$_2$), the standard Pitzer approach use 3 parameters (i.e. omit the $\beta^{(2)}$ term) and α_1 is equal to 2.0. For 2–2 type of electrolytes the model includes the $\beta^{(2)}$ parameter and α_1 equals to 1.4 and α_2 equals to 12. This approach provides accurate models for many 2–2 binary sulfate (Pitzer and Mayorga 1974; Christov 1999, 2003a) and selenate (Christov 2003a; Christov et al. 1998) electrolytes, giving excellent representation of activity data covering the entire concentration range from low molality up to saturation and beyond.

Inclusion of "standard Pitzer approach" $\beta^{(2)}$ parameter into a models for 1–1, 2–1, 3–1, 4–1, 1–2, 1–3, and 3–2 type of electrolytes

Some authors found that there are some restrictions limited the potential of the model to describe correctly activity and solubility properties in some binary electrolyte systems with minimum one univalent ion (see Petrenko and Pitzer 1997, 2012; Gruszkiewicz and Simonson 2005; Lach et al. 2018; Guignot et al. 2019; Lassin et al. 2020),

and of 3–2 type (see Christov, 2002ab, 2003b) at very high molality using classical 3 parameters ($\beta^{(0)}$, $\beta^{(1)}$, and C^{φ}) approach. According to discussion in Christov (2004, 2005, 2012) and in Lassin et al. (2015), there is one major factor which determined these restrictions: type of φ (osmotic coefficient) vs. m, or $\gamma_{\pm}$ (activity coefficient) vs. m dependences at high concentration. For all these systems φ vs. m, or $\gamma_{\pm}$ vs. m curves have a wide maximum at molality approaching molality of saturation: "LiCl(aq) type": see Lassin et al. (2015), "FeCl$_2$(aq) and FeCl$_3$(aq) type": see Christov (2003c, 2004); "HNO$_3$(aq) type": see Donchev and Christov (2020);"Al$_2$(SO$_4$)$_3$(aq), Cr$_2$(SO$_4$)$_3$(aq) type": see Christov (2002a, 2002b, 2003b).

To describe the high concentration solution behaviour of systems showing a "smooth" maximum on $\gamma_{\pm}$ vs. m dependence, and to account strong association reactions at high molality, Christov (1996a, 1998, 1999, 2001, 2005) used a very simple modelling technology: introducing into a model a fourth ion interaction parameter from basic Pitzer theory {$\beta^{(2)}$ in Eqn. (3A)}, and varying the values of α_1 and α_2 terms in Eqs. (3 and 3A))). The author also found that by variation of the values of α_1 and α_2 terms it is possible to vary the concentration range of binary solutions at which association reactions become more important and should be account by introducing $\beta^{(2)}$ parameter. According to Christov (2005), model which uses $\alpha_1 = 1.4$ and $\alpha_2 = 12$ accounts association only at low molality solutions (see also Christov and Moller (2004b) for Ca(OH)$_2$-H$_2$O model). According to previous studies of one of the authors (Christov) an approach with 4 ion interaction parameters ($\beta^{(0)}$,$\beta^{(1)}$, $\beta^{(2)}$,and C^{φ}), and accepting $\alpha_1 = 2$, and varying in α_2 values can be used for solutions for which ion association occurs in high molality region. This approach was used for binary electrolyte systems of different type: 1–1 type {such as HNO$_3$-H$_2$O, LiNO$_3$-H$_2$O (Donchev and Christov 2020), CsF -H$_2$O (Tsenov et al. 2021), and LiCl-H$_2$O (Lassin et al. 2015)}, 2–1 {such as NiCl$_2$-H$_2$O, CuCl$_2$-H$_2$O, MnCl$_2$-H$_2$O, CoCl$_2$-H$_2$O: (Christov 1996a, 1999); FeCl$_2$-H$_2$O: (Christov 2004); Ca(NO$_3$)$_2$-H$_2$O: (Lach et al. 2018); UO$_2$(NO$_3$)$_2$-H2O (Lassin et al. 2020)}, 1–2 {such as Na$_2$Cr$_2$O$_7$-H$_2$O: (Christov 2001); K$_2$Cr$_2$O$_7$-H$_2$O: (Christov 1998)}, 3–1 {such as FeCl$_3$-H$_2$O: (Christov 2004); Ln(NO$_3$)$_3$(aq): (Guignot et al. 2019)}, and 3–2 {such as Al$_2$(SO$_4$)$_3$-H$_2$O, Cr$_2$(SO$_4$)$_3$-H$_2$O, and Fe$_2$(SO$_4$)$_3$-H$_2$O: (Christov 2001, 2002a, 2003b, 2004, 2005)}. The resulting models reduce the sigma values of fit of experimental activity data, and extend the application range of models for binary systems to the highest molality, close or equal to molality of saturation {m(sat)}, and in case of data availability: up to supersaturation. For example, aqueous complexes free 4 parameters model for LiCl-H$_2$O system predicts LiCl.nH2O(s) solubilities from 0 to 200°C and up to 40 mol.kg^{-1} (Lassin et al. 2015). The resulting accurate 4 - parameters solution models are used directly to determine lnK°$_{sp}$ values of precipitated solid phases using solubility approach (Harvie et al. 1984; Christov 1995, Christov 1996a, Christov 1996b, Christov 2005, Christov 2012; Christov and Moller 2004a, Christov and Moller 2004b). Therefore, the developed not high molality restricted parameterization, were used without any changes for development of solid-liquid equilibrium models for high order systems. Thus, models for Al$_2$(SO$_4$)$_3$(aq) and Cr$_2$(SO$_4$)$_3$(aq) are used without additional adjustments to construct a model for multi-

component (Na+K+NH$_4$+Mg+Al+Cr+SO$_4$+H$_2$O) system (Christov, 2002ab, 2003b). Four parameters ($\beta^{(0)}$, $\beta^{(1)}$, $\beta^{(2)}$ and C$^\varphi$) models for NiCl$_2$(aq), CuCl$_2$(aq), MnCl$_2$(aq), and CoCl$_2$(aq) are used for construction of Na-K-Rb-Cs-Ni-Co-Cu-Mn-Cl-H$_2$O model (Christov 1996a, 1999). Four parameters models for FeCl$_2$(aq) and FeCl$_3$(aq) are directly used in development of high accuracy minerals solubility model for (Na +K+Mg+Fe(II)+Fe(III)+Cl+SO$_4$+H$_2$O) system (Christov 2004). A model for binary systems Na$_2$Cr$_2$O$_7$(aq), K$_2$Cr$_2$O$_7$(aq) was used without any changes to develop a comprehensive model for: (Na+K+Cl+SO$_4$+Cr$_2$O$_7$+H$_2$O) system (Christov 1998, 2001), and Ca(OH)$_2$(aq) model is used as s strong base for H+Na+K+Ca+OH+Cl+SO$_4$+H$_2$O model from from 0 to 300°C (Christov and Moller, 2004b).

Results and discussions

Model parameterization and validation of models for binary 2–1, 3–1, and 4–1 type nitrate systems

In this study we developed new thermodynamic models for solution behavior and solid-liquid equilibrium in 10 nitrate binary systems of the type 2–1 (Mg(NO$_3$)$_2$-H$_2$O, Ca(NO$_3$)$_2$-H$_2$O, Ba(NO$_3$)$_2$-H$_2$O, Sr(NO$_3$)$_2$-H$_2$O, and UO$_2$(NO$_3$)$_2$-H$_2$O), 3–1 (Cr(NO$_3$)$_3$-H$_2$O, Al(NO$_3$)$_3$-H$_2$O, La(NO$_3$)$_3$-H$_2$O, Lu(NO$_3$)$_3$-H$_2$O), and 4–1 (Th(NO$_3$)$_4$-H$_2$O) from low to very high concentration at 298.15 K. New sets of Pitzer ion interaction binary parameters are evaluated using available raw experimental osmotic coefficients (φ) data for whole molality range of solutions. Rard and co-authors (1977, 1981) reported an extensive experimental activity database for rare earth nitrate systems. Data of Rard and Spedding (1981) are used to parametrize the model for Lu(NO$_3$)$_3$-H$_2$O system. The φ vs. m data for remaining 9 nitrate solutions under study are given in Mikulin (1968), and Robinson and Stokes (1959). Reference φ vs. m data sets of Mikulin (1968), and Robinson and Stokes (1959) are in a good agreement. Data of Mikulin (1968) for La(NO$_3$)$_3$-H$_2$O are also in good agreement with the data of Rard (1987). However, the data of Mikulin (1968) cover the whole molality range of unsaturated and saturated solutions. In case of Ca(NO$_3$)$_2$-H$_2$O, UO$_2$(NO$_3$)$_2$-H$_2$O, and Th(NO$_3$)$_4$-H$_2$O systems, Mikulin also reported data for supersaturated solutions. In this study we parameterize the models using 1) all data of Mikulin (1968) for whole molalty range of unsaturated solutions from 0.1 m to m(max), 2) the data points at saturation (φ(sat)) (from Mikulin 1968), and 3) data for supersaturated Ca(NO$_3$)$_2$-H$_2$O, UO$_2$(NO$_3$)$_2$-H$_2$O, and Th(NO$_3$)$_4$-H$_2$O solutions (from Mikulin 1968), and 4) all experimental and recommended data of Rard and Spedding (1981) for Lu(NO$_3$)$_3$-H$_2$O system.

In parameterization we used the value of Debye-Hückel term (A$^\varphi$) equals to 0.39147 (Christov 2007, 2009, 2012). Following the parameterization scheme described in previous paragraph the model for all 10 binary nitrate solutions is parameterized using two different approaches: (I) standard for N-1 electrolytes (N = 2, 3, or 4) approach with 3 ion interaction binary parameters ($\beta^{(0)}$, $\beta^{(1)}$, and C$^\varphi$) and setting α_1 term equals

to 2, and $\alpha_2 = 0.0$, and (II) an extended approach with four Pitzer ion interaction binary parameters ($\beta^{(0)}$, $\beta^{(1)}$, $\beta^{(2)}$, and C^{φ}) and varying in the values of α_1 and α_2 terms. As a first step in parameterization we used classical 3 parameters approach (I) and evaluate binary parameters using all available raw φ data for whole molality range of solutions. As a next step, using the same φ data we re-parameterize the models on the basis of extended approach (II), and using three α – combinations: (IIa) $\alpha_1 = 2$ and $\alpha_2 = 1$, and (IIb) $\alpha_1 = 2$ and $\alpha_2 = -1$ (Christov 1996a, 1998, 1999, 2004, 2005) and (IIc) $\alpha_1 = 2$ and $\alpha_2 = 0.3$ (Guignot et al. 2019; Donchev and Christov 2020; Donchev et al. 2021). It was found that more combinations in "alfa" values do not improve the fit of data used in parameterization. The main criterion in the choice of established parameterization was the value of standard deviation (σ) of fit of used φ data, i.e. parameterization with the lowest sigma value is accepted. For definition of sigma (σ) see Christov and Moller (2004b), and Christov (2007, 2009, 2012). It was found that for 2 of studied systems Ba(NO$_3$)$_2$-H$_2$O, and Sr(NO$_3$)$_2$-H$_2$O, the approach (I) with 3 parameters ($\beta^{(0)}$, $\beta^{(1)}$, C^{φ}) give an acceptable agreement with the data. For these systems introducing into a model of fourth ($\beta^{(2)}$) parameter do not improve considerably the fit of data. For all other nitrate systems under study we construct a model on the basis of extended approach (II), and using different combinations of "alfa" values: for Lu(NO$_3$)$_3$-H$_2$O system. 1) $\alpha_1 = 2$ and $\alpha_2 = 0.3$ (approach IIc), and 2) $\alpha_1 = 2$ and $\alpha_2 = 1$ (approach IIa), and 3) $\alpha_1 = 2$ and $\alpha_2 = -1$ (approach IIb). The resulting models fits the data up to supersaturation zone (m(max) = 14.77 m in Ca(NO$_3$)$_2$-H$_2$O) with sigma values, which is much less than the sigma values of models of Pitzer and Mayorga (1973), and of Kim and Frederick (1988).

On next Figure 1 we present a comparison of osmotic coefficients in nitrate binary solutions 2–1 (Mg(NO$_3$)$_2$-H$_2$O, Ca(NO$_3$)$_2$-H$_2$O, Ba(NO$_3$)$_2$-H$_2$O, Sr(NO$_3$)$_2$-H$_2$O, and UO$_2$(NO$_3$)$_2$-H$_2$O), 3–1 (Cr(NO$_3$)$_3$-H$_2$O, Al(NO$_3$)$_3$-H$_2$O, La(NO$_3$)$_3$-H$_2$O, Lu(NO$_3$)$_3$-H$_2$O), and 4–1 (Th(NO$_3$)$_4$-H$_2$O) calculated by the accepted models developed here (heavy solid lines), and models developed by other authors (dashed lines and light solid lines: Pitzer and Mayorga (1973), Kim and Frederick (1988), Rard and Spedding (1981) (for Lu(NO$_3$)$_3$-H$_2$O system only), Rard et al. (2004) (for Mg(NO$_3$)$_2$-H$_2$O system only), and Wijesinghe and Rard (2005) (for Ca(NO$_3$)$_2$-H$_2$O system only). The recommended osmotic coefficients values given in literature at 25 °C are given on Fig. 1 by symbols. The vertical lines on the figures denote the molality of solutions saturated with corresponding nitrate solid phase (m(sat)), taken from Mikulin (1968). Excellent accepted new model (heavy solid line) – experiment (symbols) agreement has been obtained for all 10 systems and from low (see figures for Mg(NO$_3$)$_2$-H$_2$O, Ca(NO$_3$)$_2$-H$_2$O systems) and up to very high molality. As is shown Fig. 1, the new model for Ca(NO$_3$)$_2$-H$_2$O is in excellent agreement not only with the data at high molality ((m(max) = 14.77 m), but contrary to the models of Kim and Frederick (1988) and Wijesinghe and Rard (2005) also in low molality range. It should be noted that all reference models presented on Fig. 1 by dashed, dashed-dotted, and light solid lines (Kim and Frederick (1988), Pitzer and Mayorga (1973), Rard et al. (2004), Wijesinghe and Rard (2005), and Rard and Spedding (1981)) have been constructed on the basis of standard Pitzer approach with 3 interaction parameters. Therefore, these models
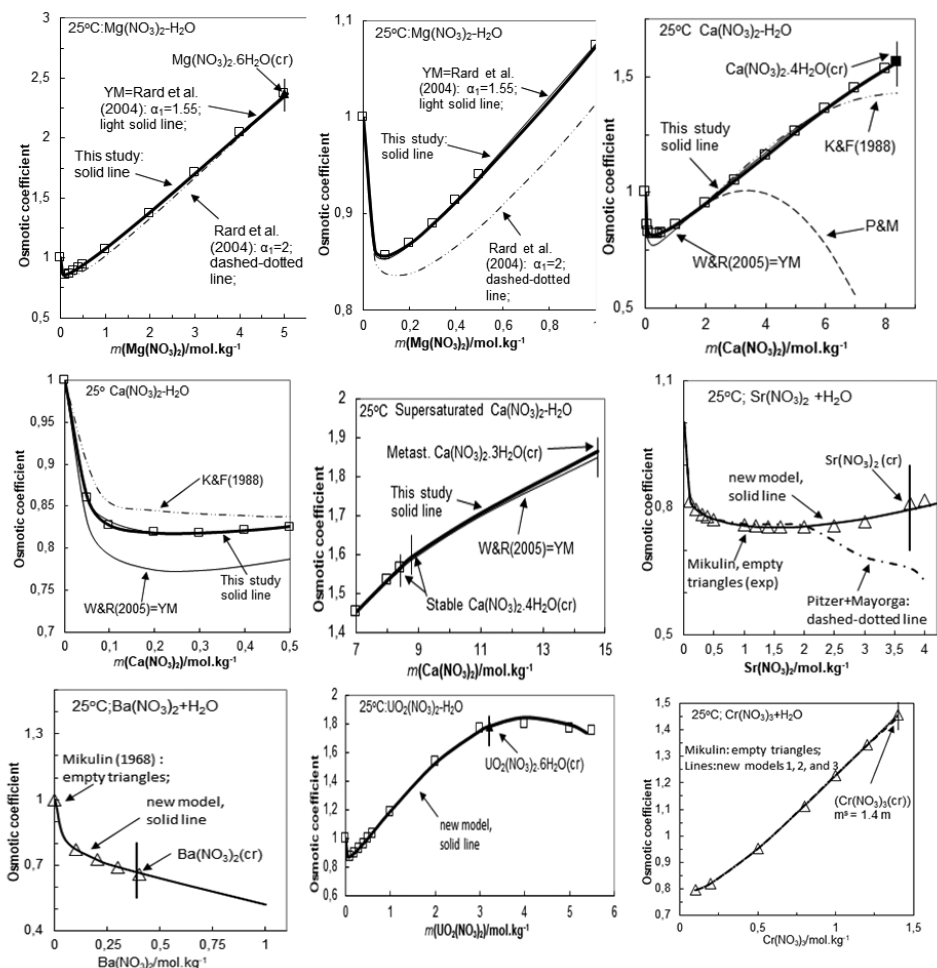
Figure 1. Comparison of model calculated (lines) osmotic coefficients (φ) of $Mg(NO_3)_2$, $Ca(NO_3)_2$, $Ba(NO_3)_2$, $Sr(NO_3)_2$, $UO_2(NO_3)_2$, $Cr(NO_3)_3$, $Al(NO_3)_3$, $La(NO_3)_3$, $Lu(NO_3)_3$, and $Th(NO_3)_4$ in binary solutions 2–1 ($Mg(NO_3)_2$-H_2O, $Ca(NO_3)_2$-H_2O, $Ba(NO_3)_2$-H_2O, $Sr(NO_3)_2$-H_2O, and $UO_2(NO_3)_2$-H_2O), 3–1 ($Cr(NO_3)_3$-H_2O, $Al(NO_3)_3$-H_2O, $La(NO_3)_3$-H_2O, $Lu(NO_3)_3$-H_2O), and 4–1 ($Th(NO_3)_4$-H_2O) against molality at T = 298.15 K, with recommendations in literature (symbols). For $Mg(NO_3)_2$-H_2O and $Ca(NO_3)_2$-H_2O systems an enlargement of the low molality corner is also given. Heavy solid lines represent the predictions of the developed in this study and accepted models. Dashed-dotted, dashed and light solid lines represent the predictions of the reference models of Kim and Frederick (1988), of Pitzer and Mayorga (1973), of Rard et al. 2004 (for $Mg(NO_3)_2$-H_2O), of Wijesinghe and Rard (2005) (for $Ca(NO_3)_2$-H_2O), and of Rard and Spedding (1981) (for $Lu(NO_3)_3$-H_2O). On Figures YM denotes YMTDB (Sandia National Laboratories 2007). For $Lu(NO_3)_3$-H_2O the experimental data and recommended data are taken from Rard et al. (1977) (open squares), and Rard and Spedding (1981) (crosses), respectively. For all other systems the experimental data of Mikulin (1968) are used (open squares and open triangles). The molality of stable and metastable (for $Ca(NO_3)_2$-H_2O) crystallization of solid nitrate phases (m(sat)) is given on all figures by vertical lines (see Table 1).
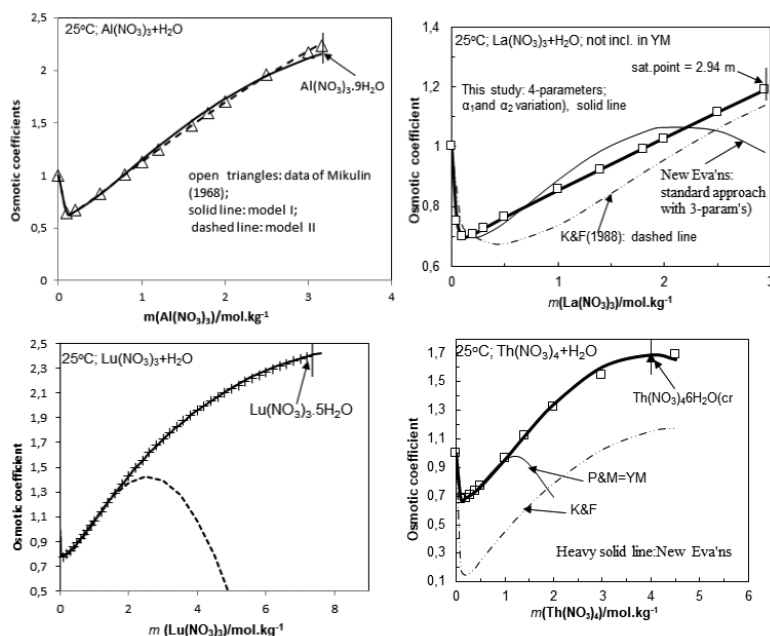
Figure 1. Continue.

cannot reproduce well the experimental data (Fig. 1). To illustrate this conclusion on Fig. 1 we give the predictions of two new models for $La(NO_3)_3$-H_2O system. As it is shown the 3 parameters model (light solid line) is in pure agreement with the data.

The models for all nitrate binary systems under study are also validated by comparison with recommendations given in literature (Rard and Spedding (1981) for $Lu(NO_3)_3$-H_2O); and Mikulin (1968)) (for all other 9 systems under study) on the mean activity coefficients ($\gamma_{\pm}$). These recommendations on $\gamma_{\pm}$ are model-dependent. Therefore, they are not used in parameterization process, and only to validate the resulting models. The comparisons between predictions of new developed models and reference recommendations, which are not given here, show an excellent agreement from low to very high concentrations.

Deliquescence relative humidity (DRH) calculations

Deliquescence of single inorganic salt or their mixture is a process of spontaneous solid-liquid phase change. It is a process in which a soluble solid substance sorbs water vapor from the air to form a thermodynamically stable saturated aqueous solution on the surface of the particle. It is occurring when relative humidity (RH) in the gas-phase environment is at, or above deliquescence relative humidity (DRH) of the salt, or mutual deliquescence relative humidity (MDRH) of a salt mixture. Within the solid-

liquid equilibrium model, relative humidity is related to water activity(a_w) (Clegg et al. 1998; Christov 2009, 2012; Donchev and Christov 2020) according to Eqn. (5):

$$a_w = P_w / P^\circ_w = RH/100, \tag{5}$$

where P_w and P°_w are the vapor pressure of the saturation solution and pure water, respectively, at given temperature. As a result, both DRH and MDRH of saturated surface solutions depend of temperature, the salt stoichiometry, and the solution composition. This process is of interest in many areas, such as heterogeneous chemistry of inorganic salts, corrosion of metals in wet atmosphere, in studies of chemistry of sea-type aerosol atmospheric system (Kolev et al. 2013), and especially in development of strategies and programs for nuclear waste geochemical storage. Because of very high complicity of experiments, the relative humidity DRH experimental data are sparse. Therefore, different sophisticated thermodynamic models have been proposed and developed to describe the deliquescence behavior of inorganic salts at wet conditions. In our previous studies it was showed that calculations based on not high concentration restricted Pitzer models can be used for accurate determinations of both DRH and MDRH of saturated solutions in a wide range of temperatures, and compositions (Christov 2009, 2012; Donchev and Christov 2020). On the basis of evaluated binary parameters ($\beta^{(0)}$, $\beta^{(1)}$, $\beta^{(2)}$, and C^φ) in this study we also determine water activity (a_w) and Deliquescence Relative Humidity (DRH (%)) (eqn. 5) of 12 solid phases crystallizing from saturated binary nitrate solutions [$Mg(NO_3)_2.6H_2O(s)$, $Ca(NO_3)_2.4H_2O(s)$, $Ca(NO_3)_2.3H_2O(s)$, $Ba(NO_3)_2(s)$, $Sr(NO_3)_2(s)$, $UO_2(NO_3)_2.6H_2O(s)$, $Al(NO_3)_3.9H_2O(s)$, $Cr(NO_3)_3(s)$, $La(NO_3)_3.6H_2O(s)$, $La(NO_3)_3(s)$ $Lu(NO_3)_3.5H_2O(s)$ and $Th(NO_3)_4.6H_2O(s)$]. Note that the widely used databases of Pitzer (1991), Pitzer and Mayorga (1973), Pitzer and Kim (1974) and Kim and Frederick (1988) do not consider solid phases. The results of calculations are given in Table 1. The model DRH predictions are in excellent agreement with the experimental data determined using isopiestic method, and given in Mikulin (1968). According to model calculations the solid-liquid phase change of $Ca(NO_3)_2.3H_2O(s)$, and $Lu(NO_3)_3.5H_2O(s)$ occurs at lowest relative humidity of environment. It can be concluded that the solid-liquid phase change of solid nitrates of Lanthanide metals is more activated in the presence of calcium in the nuclear storage environment.

Determination of thermodynamic solubility product (K°_{sp}) of precipitates

In this study we determine the thermodynamic solubility products (as K°_{sp}) of solid phases, precipitating from saturated nitrate binary solutions, s.a. anhydrous $Ba(NO_3)_2(s)$ and hydrate $Ca(NO_3)_2.3H_2O(s)$, precipitating in $Ba(NO_3)_2-H_2O$ and $Ca(NO_3)_2-H_2O$. The K°_{sp} have been determined on the basis of evaluated binary parameters and using experimental m(sat) solubility data, and using the following relationships (Christov 2005, 2007, 2009, 2012):

$$K^\circ sp\ (Ba(NO_3)_2) = 4 \cdot \gamma_{(\pm)}(sat)^3 \cdot m(sat)^3$$
$$K^\circ sp(Ca(NO_3)_2.3H_2O) = 4 \cdot \gamma_{(\pm)}(sat)^3 \cdot m(sat)^3 \cdot a_w(sat)^3 \tag{6}$$

Table 1. Comparison between model calculated and recommended values of the Deliquescence Relative Humidity [DRH (%) = a_w(sat) . 100; where a_w(sat) is activity of water at saturation] and of the logarithm of the thermodynamic solubility product (as $\ln K°_{sp}$) of nitrate solid phases crystallizing from saturated binary solutions at T = 25° C.

Salt composition	m(sat) (exp) (mol.kg⁻¹)	$\ln K°_{sp}$ This work calculated	$\ln K°_{sp}$ Reference data	DRH(%) This work calculated	DRH(%) Reference data [a]
$Mg(NO_3)_2.6H_2O$(cr)	5.06 [a]	7.0098	7.02 [b]	52.32	52.90
$Ca(NO_3)_2.4H_2O$(cr) (stable solid)	8.41 [a]	4.4362	4.53 [b]	49.07	49.10
$Ca(NO_3)_2.3H_2O$(cr) (metastable solid)	14.77 [a]	6.6449	5.34 [b] (m(sat) = 15.0 m)	22.52	-
$Ba(NO_3)_2$(cr)	0.39 [a]	-5.125	-	98.61	98.60
$Sr(NO_3)_2.4H_2O$ (cr)	3.76 [a]	0.0327	-	84.83	84.80
$UO_2(NO_3)_2.6H_2O$(cr)	3.21 [a]	5.3022	5.251 [c]	73.44	73.60
$Al(NO_3)_3.9H_2O$ (cr)	3.16 [a]	4.3081	-	59.88	60.20
$Cr(NO_3)_3$ (cr) [e]	1.4 [e]	1.2097	-	86.38	-
$La(NO_3)_3.6H_2O$ (cr) [d]	4.615 [d]	2.1599	2.97 [d]	62.38	-
$La(NO_3)_3$ (cr) [a]	2.94 [a]	1.4704	-	77.73	77.60
$Lu(NO_3)_3.5H_2O$ (cr)	6.815 [d]	10.7681	10.67	31.27	-
$Th(NO_3)_4.6H_2O$(cr)	4.00 [a]	4.4886	4.71 [c] (as $Th(NO_3)_4.5H_2O$)	54.46	55.0

Experimental data of Mikulin (1968); [b]Calculated values of Lach et al. (2018) [c]Calculated values of Lassin et al. (2020); [d] Values from Guignot et al. (2019); [e]Accepted m(sat) molality and stoichiometry of solid phase.

As a next step, using the accepted new developed parameterizations, and experimentally determined molalities (m(sat) of the saturated binary solutions (Mikulin 1968; Guignot et al. 2019; Lassin et al. 2020)) we calculate the logarithm of the thermodynamic solubility product (ln $K°_{sp}$) of twelve nitrate solid phases crystallizing from saturated binary nitrate solutions at 25 °C (Eqn. (6)). The model calculations are given in Table 1. With only 2 exceptions (for $Ca(NO_3)_2.3H_2O$(s) and $La(NO_3)_3.6H_2O$(s)) a good agreement has been obtained with calculations of Lach et al. (2018), Lassin et al. (2020), and Guignot et al. (2019) for all nitrate solids. The ln $K°_{sp}$ differences are mainly due on the 1) different m(sat) values used in calculations (see Eqn. (6)), and 2) different experimental data source with different m(max) values used in parameterization.

Summary and conclusions

In this study we developed new thermodynamic models for solution behavior and solid-liquid equilibrium in 10 nitrate binary systems of the type 2–1 ($Mg(NO_3)_2$-H_2O, $Ca(NO_3)_2$-H_2O, $Ba(NO_3)_2$-H_2O, $Sr(NO_3)_2$-H_2O, and $UO_2(NO_3)_2$-H_2O), 3–1 ($Cr(NO_3)_3$-H_2O, $Al(NO_3)_3$-H_2O, $La(NO_3)_3$-H_2O, $Lu(NO_3)_3$-H_2O), and 4–1 ($Th(NO_3)_4$-H_2O) from low to very high concentration at 25 °C. To parameterize models for binary systems we used all available raw experimental osmotic coefficients data (φ) for whole concentration range of solutions, and up to saturation point. Data for supersaturation zone, available for $Ca(NO_3)_2$-H_2O, $UO_2(NO_3)_2$-H_2O, and $Th(NO_3)_4$-H_2O systems, are also included in parameterization. To construct models, we used different versions of standard molality-based Pitzer approach. It was established that with only 2 exceptions ($Ba(NO_3)_2$-H_2O, and $UO_2(NO_3)_2$-H_2O) application of extended approach

with 4 parameters ($\beta^{(0)}$, $\beta^{(1)}$, $\beta^{(2)}$, and C^{φ}) and variation of α_2 term in fundamental Pitzer equations leads to the lowest values of standard model-experiment deviation. The predictions of new developed here models are in excellent agreement with experimental osmotic coefficients data (see Fig. 1), and with recommendations on activity coefficients (not given here) in binary solutions from low to very high concentration: up to 14.77 mol. kg^{-1} in $Ca(NO_3)_2$-H_2O. The Deliquescence Relative Humidity (DRH), and thermodynamic solubility product (as ln K°_{sp}) of of 12 solid phases crystallizing from saturated binary nitrate solutions [$Mg(NO_3)_2.6H_2O(s)$, $Ca(NO_3)_2.4H_2O(s)$, $Ca(NO_3)_2.3H_2O(s)$, $Ba(NO_3)_2(s)$, $Sr(NO_3)_2(s)$, $UO_2(NO_3)_2.6H_2O(s)$, $Al(NO_3)_3.9H_2O(s)$, $Cr(NO_3)_3(s)$, $La(NO_3)_3.6H_2O(s)$, $La(NO_3)_3(s)$ $Lu(NO_3)_3.5H_2O(s)$ and $Th(NO_3)_4.6H_2O(s)$] have been determined on the basis of evaluated binary parameters and using experimental m(sat) solubility data. Model predictions are in good agreement with available reference data. The accurate solid-liquid equilibrium models for nitrate systems described in this study are of high importance for development of strategies and programs for nuclear waste geochemical storage.

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Development of accurate chemical thermodynamic database for geochemical storage of nuclear waste. Part III: Models for predicting solution properties and solid-liquid equilibrium in cesium binary and mixed systems

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Abstract

The models described in this study are of high importance in the development of thermodynamic database needed for nuclear waste geochemical storage, as well as for technology for extracting cesium resources from saline waters. In this study we developed new, not concentration restricted thermodynamic models for solution behavior and solid-liquid equilibrium in $CsF-H_2O$, $CsOH-H_2O$ and $Cs_2SO_4-H_2O$ systems at 25 °C. To parameterize models, we used all available experimental osmotic coefficients data for whole concentration range of solutions, and up to saturation point. The new models are developed on the basis of Pitzer ion interactions approach. The predictions of new developed here models are in excellent agreement with experimental osmotic coefficients data (φ) in binary solutions from low to extremely high concentration (up to 21.8 mol.kg^{-1} for $CsOH-H_2O$, and up to 35.6 mol.kg^{-1} for $CsF-H_2O$). The previously developed by Christov, by Christov and co-authors, and by other authors Pitzer approach based thermodynamic models for five (5) cesium binary systems ($CsCl-H_2O$, $CsBr-H_2O$, $CsI-H_2O$, $CsNO_3-H_2O$, and $Cs_2SeO_4-H_2O$) are tested by comparison with experimental osmotic coefficients data and with recommendations on activity coefficients ($\gamma_\pm$) in binary solutions. The models which give the best agreement with (φ)-, and ($\gamma_\pm$)-data from low to high concentration, up to m(sat), are accepted as correct models, which can be used for solubility calculations in binary and mixed systems and determination of thermodynamic properties of precipitating cesium solid phases. The thermodynamic solubility products (ln K$^{o}_{sp}$), and the Deliquescence Relative Humidity (DRH) of solid phases, precipitating from saturated

cesium binary solutions (CsF(cr), CsCl(cr), CsBr(cr), CsI(cr), CsOH(cr), CsNO$_3$(cr), Cs$_2$SO$_4$(cr), and Cs$_2$SeO$_4$(cr)) have been determined on the basis of evaluated and accepted binary parameters and using experimental solubility data. The reported mixing parameters [θ(Cs,M^{2+}) and ψ(Cs,M^{2+},X)], evaluated by solubility approach for 15 cesium mixed ternary systems (CsCl-MgCl$_2$-H$_2$O, CsBr-MgBr$_2$-H$_2$O, CsCl-NiCl$_2$-H$_2$O, CsBr-NiBr$_2$-H$_2$O, CsCl-MnCl$_2$-H$_2$O, CsCl-CoCl$_2$-H$_2$O, CsCl-CuCl$_2$-H$_2$O, CsCl-CsBr-H$_2$O, CsCl-RbCl-H$_2$O, Cs$_2$SO$_4$-CoSO$_4$-H$_2$O, Cs$_2$SeO$_4$-CoSeO$_4$-H$_2$O, Cs$_2$SO$_4$-NiSO$_4$-H$_2$O, Cs$_2$SeO$_4$-NiSeO$_4$-H$_2$O, Cs$_2$SO$_4$-ZnSO$_4$-H$_2$O, and Cs$_2$SeO$_4$-ZnSeO$_4$-H$_2$O) are tabulated.

Keywords

Cesium binary and mixed systems, computer thermodynamic modeling, geochemical nuclear waste sequestration, Pitzer approach

Introduction

Radioactive waste is a by-product of the nuclear fuel cycle and the production of weapons and medical radioisotopes. As nuclear technologies become more widespread, so does the production of waste materials. In Europe, nuclear waste is classified into 1) high-level; 2) intermediate level; 3) low-level, and 4) transitional radioactive waste. The long-term storage of high-level waste is still experimental. Radiocesium isotopes, particularly ^{137}Cs, form part of the high-level nuclear waste group. Crucially, the storage of high-level waste in liquid form poses serious risks. On 29 September 1957 a liquid storage tank exploded at the Mayak facility (Chelyabinsk-40), contaminating more than 52,000 square kilometers with ^{137}Cs and [90]Sr (Kostyuchenko and Krestinina 1994). This is known as the Kyshtym accident and is the second most serious radiation disaster after Chernobyl at 1986. On 1987 a release of ^{137}Cs occurred as a result of improper disposal of radiotherapy source (Rosenthal et al. 1991). This is known as Goiânia accident in Brazil. According to Scharge et al. (2012) among the more common fission products from spent nuclear fuels, the radionuclide ^{137}Cs with half-lives of 30.17 years, is mostly critical for the design of the nuclear waste repository because of the intense γ and β radiation and the heat generated by the decay process, as well as the high solubilities of cesium halides. Thus, modelling the properties of cesium atoms in salts and solutions is a current, pertinent question in theoretical chemistry.

A long term safety assessment of a repository for radioactive waste requires evidence that all relevant processes are known and understood, which might have a significant positive or negative impact on its safety (Altmaier et al. 2011a, b; Lach et al. 2018; Donchev and Christov 2020; Donchev et al. 2021b). It has to be demonstrated, that the initiated chemical reactions don't lead to an un-due release of radionuclides into the environmental geo-, hydro-, and bio-sphere. One key parameter to assess the propagation of a radionuclide is its solubility in solutions interacting with the waste. Solubility estimations can either be based on experimental data determined at conditions close to those in the repository or on thermodynamic calculations. The thermodynamic database created from experimental data is the basis for thermodynamic model calculations.

Since the disposal of radioactive waste is a task encompassing decades, the database is projected to operate on a long-term basis. Chemical models that predict equilibrium involving mineral, gas and aqueous phases over a broad range of solution compositions and temperatures are useful for studying the interactions between used nuclear fuel waste and its surroundings. The reliability of such predictions depends largely on the thermodynamic database. An accurate description of highly concentrated waters should be required for modeling of chemical interactions in and around nuclear repositories. The modeling of dissolution and precipitation processes in concentrated solutions requires an adequate thermodynamic model for the prediction of activities and solubilities (Lach et al. 2018; Donchev and Christov 2020; Donchev et al. 2021b). This requirement is fulfilled by the ion interaction model of Pitzer (Pitzer 1973). Extensive thermodynamic databases, which are based on the Pitzer ion interaction model, were developed within the Yucca Mountain Project (YMTDB: data0.ypf.r2) (Sandia National Laboratories (2005, 2007), Thereda project (THermodynamic REference DAtabase, THEREDA-Final Report) (Altmaier et al. 2011a, b), and ANDRA project (Lach et al. 2018). However, the subject of long-term radioactive waste storage still has many questions left for scientists to solve. Unfortunately, many of the Pitzer models introduced in YMTDB and in THEREDA databases for cesium binary and mixed systems are concentration restricted and cannot describe correctly the solid-liquid equilibrium in geochemical and industrial systems of interest for nuclear waste programs.

This paper presents a comprehensive analysis and evaluation of existent thermodynamic database for cesium binary and mixed systems. It should be noted, that the thermodynamic properties, solubility isotherms and their simulation by thermodynamic model of the cesium binary and mixed brine type systems (s.a. CsX–MgX_2–H_2O (X =Cl,Br,I) ternary systems) are also of significant importance for extracting cesium resources from brine type solutions (Balarew et al. 1993; Christov et al. 1994; Christov 1995a, b, 1996a, 2005; Guo et al. 2017). According to Baranauskaite et al. (2021) carnalite type minerals of the type $MX.MgX_2.6H_2O$ (cr) (M=Li, K,NH_4,Rb,Cs) (Christov and Balarew 1995; Christov 2012; Lassin et al. 2015) "are interesting not only as natural sources of chemical compounds, but also they can be made use of in renewable thermochemical energy storage since their hydration reactions are exothermic".

In this study we developed new, not concentration restricted thermodynamic models for solution behavior and solid-liquid equilibrium in CsF-H_2O, $CsOH$-H_2O and Cs_2SO_4- H_2O systems at 25 °C. The new models are developed on the basis of Pitzer ion interactions approach. The previously developed by Christov (2003a, 2005), and Christov and co-authors (Balarew et al. 1993; Barkov et al. 2001; Donchev and Christov 2020) and by other authors (Pitzer and Mayorga 1973; Scharge et al. 2012; Palmer et al. 2002) Pitzer approach based thermodynamic models for five (5) cesium binary systems ($CsCl$-H_2O, $CsBr$- H_2O, CsI-H_2O, $CsNO_3$-H_2O, and Cs_2SeO_4- H_2O) are tested by comparison with experimental osmotic coefficients data and with recommendations on activity coefficients ($\gamma_\pm$) in binary solutions. The models that give the best agreement with (φ)-, and ($\gamma_\pm$) data from low to high concentration, up to m(sat),

are accepted as correct models, which can be used for solubility calculations in binary and mixed systems. We also summarized the previously established by the main author (C. Christov) solid-liquid equilibrium model for 15 cesium mixed ternary systems at 25 °C. The evaluated mixing parameters [$\theta(Cs,M^{2+})$ and $\psi(Cs,M^{2+},X)$], determined by solubility approach are tabulated.

Methodology

The models for cesium binary systems have been developed and tested on the basis of Pitzer's semi-empirical equations (Pitzer 1973). The specific interaction approach for describing electrolyte solutions to high concentration introduced by Pitzer (1973) represents a significant advance in physical chemistry that has facilitated the construction of accurate computer thermodynamic models. Pitzer approach has found extensive use in the modeling of the thermodynamic properties of aqueous electrolyte solutions. It was shown that this approach could be expanded to accurately calculate solubilities in binary and complex systems, and to predict the behavior of natural and industrial fluids from very low to very high concentration at standard temperature of 25 °C (Harvie et al. 1984; Christov et al. 1994, 1998; Christov 1995a, 1996a, 1998, 1999, 2002, 2003a, b, 2005, 2007, 2009, 2012, 2020; Barkov et al. 2001; Park et al. 2009; Lach et al. 2018; Donchev and Christov 2020; Donchev et al. 2021a, b), and from 0 to 290 °C (Christov and Moller 2004; Moller et al. 2006; Lassin et al. 2015). Several extensive parameter databases have been reported. These include: 25 °C database of Pitzer and Mayorga (1973, 1974), of Kim and Frederick (1988), the most widely used database of Chemical Modelling Group at UCSD [(University California San Diego) at 25 °C (Harvie et al. 1984; Park et al. 2009), and T-variation (from 0 to 300 °C) (Christov and Moller 2004; Moller et al. 2006; Christov 2009)], YMTDB (Sandia National Laboratories 2005, 2007), and THEREDA (2011a, b).

According to Pitzer theory, electrolytes are completely dissociated and in the solution there are only ions interacting with one another (Pitzer 1973; Pitzer and Mayorga 1973). Two kinds of interactions are observed: (i) specific Coulomb interaction between distant ions of different signs, and (ii) nonspecific short-range interaction between two and three ions. The first kind of interaction is described by an equation of the type of the Debye-Hueckel equations. Short-range interactions in a binary system (MX(aq)) are determined by Pitzer using the binary parameters of ionic interactions ($\beta^{(0)}, \beta^{(1)}, C^{\varphi}$). The Pitzer's equations are described and widely discussed in the literature (Harvie et al. 1984; Christov and Moller 2004; Christov 2005; Moller et al. 2006; Donchev et al. 2021b). Therefore, these equations are not given here. According to the basic Pitzer equations, at constant temperature and pressure, the solution model parameters to be evaluated for mixed ternary system are: 1) pure electrolyte $\beta^{(0)}$, $\beta^{(1)}$, and C^{φ} for each cation-anion pair; 2) mixing θ for each unlike cation-cation or anion-anion pair; 3) mixing ψ for each triple ion interaction where the ions are all not of the same sign (Christov 2003a, b, 2005; Donchev et al. 2021b).

Pitzer and Mayorga (1973) did not present analysis for any 2-2 (e.g. MgSO$_4$-H$_2$O) or higher {e.g. 3-2: Al$_2$(SO$_4$)$_3$-H$_2$O} electrolytes. In their next study (Pitzer and Mayorga 1974) modify the original equations for the description of 2-2 binary solutions: parameter $\beta^{(2)}$(M,X), and an associated α_2 term are added to the original expression. Pitzer presented these parameterizations assuming that the form of the functions (i.e. 3 or 4 β and C $^{\varphi}$ values, as well as the values of the α terms) vary with electrolyte type. For binary electrolyte solutions in which either the cationic or anionic species are univalent (e.g. NaCl, Na$_2$SO$_4$, or MgCl$_2$), the standard Pitzer approach use 3 parameters (i.e. omit the $\beta^{(2)}$ term) and α_1 is equal to 2.0. For 2-2 type of electrolytes the model includes the $\beta^{(2)}$ parameter and α_1 equals to 1.4 and α_2 equals to 12. This approach provides accurate models for many 2-2 binary sulfate (Pitzer and Mayorga 1974; Christov 1999, 2003a) and selenate (Christov et al. 1998; Barkov et al. 2001; Christov 2003a) electrolytes, giving excellent representation of activity data covering the entire concentration range from low molality up to saturation and beyond.

To describe the high concentration solution behaviour of systems showing a "smooth" maximum on $\gamma_{\pm}$ vs. m dependence, and to account for strong association reactions at high molality, Christov (1996b, 1998a, b, 1999, 2005) used a very simple modelling technology: introducing into a model a fourth ion interaction parameter from basic Pitzer theory {$\beta^{(2)}$ }, and varying the values of α_1 and α_2 terms (see Eqns. (3) and (3A) in Donchev et al. 2021b). According to previous studies of Christov, an approach with 4 ion interaction parameters ($\beta^{(0)}$,$\beta^{(1)}$, $\beta^{(2)}$,and C$^{\varphi}$), and accepting α_1 = 2, and varying in α_2 values can be used for solutions for which ion association occurs in high molality region. This approach was used for binary electrolyte systems of different type: 1-1 type {such as HNO$_3$-H$_2$O, LiNO$_3$-H$_2$O (Donchev and Christov 2020), and LiCl-H$_2$O (Lassin et al. 2015)}, 2-1 {such as NiCl$_2$-H$_2$O, CuCl$_2$-H$_2$O, MnCl$_2$-H$_2$O, CoCl$_2$-H$_2$O: (Christov and Petrenko 1996; Christov 1996b, 1999); Ca(NO$_3$)$_2$-H$_2$O: (Lach et al. 2018); 1-2 {such as K$_2$Cr$_2$O$_7$-H$_2$O: (Christov 1998)}, 3-1 {such as FeCl$_3$-H$_2$O: (Christov 2005), and 3-2 {such as Al$_2$(SO$_4$)$_3$-H$_2$O, Cr$_2$(SO$_4$)$_3$-H$_2$O, and Fe$_2$(SO$_4$)$_3$-H$_2$O: (Christov 2002, 2005)}. The resulting models reduce the sigma values of fit of experimental activity data, and extend the application range of models for binary systems to the highest molality, close or equal to molality of saturation {m(sat)}, and in case of data availability: up to supersaturation.

Results and discussions

Model parameterization for cesium binary CsF-H$_2$O, CsOH-H$_2$O and Cs$_2$SO$_4$-H$_2$O systems at 25 °C

In this study we developed new, not concentration restricted thermodynamic models for solution behavior and solid-liquid equilibrium in CsF-H$_2$O, CsOH-H$_2$O and Cs$_2$SO$_4$- H$_2$O systems at 25 °C. The new models are developed on the basis of Pitzer ion interactions approach. To parameterize models for cesium binary systems we used

all available experimental osmotic coefficients data for whole concentration range of solutions, and up to saturation point. Raw data at low molality from Hamer and Wu (1972) and Mikulin (1968), and extrapolated data from Mikulin (1968) are used to parameterize the model for CsF-H$_2$O system. The model for CsOH-H$_2$O has been constructed using low molality data from Hamer and Wu (1972) and Mikulin (1968), and osmotic coefficients data-point at saturation from Mikulin (1968). The new model for Cs$_2$SO$_4$- H$_2$O system has been developed using low molality data from Palmer at al. (2002) and Mikulin (1968), and extrapolated osmotic coefficients data-up to saturation from Mikulin (1968). To construct the models, we used different versions of standard molality-based Pitzer approach. It was established that for CsF-H$_2$O system application of extended approach with 4 parameters ($\beta^{(0)}$, $\beta^{(1)}$, $\beta^{(2)}$ and C$^\varphi$) and variation of α_1 and α_2 terms in fundamental Pitzer equations leads to the lowest values of standard model-experiment deviation. For CsOH-H$_2$O and Cs$_2$SO$_4$- H$_2$O system a standard approach with 3 interaction parameters was used. The predictions of new developed here models are in excellent agreement with experimental osmotic coefficients data (φ) in binary solutions from low to extremely high concentration (up to 21.8 mol. kg^{-1} for CsOH-H$_2$O, and up to 35.6 mol.kg^{-1} for CsF-H$_2$O) (see Fig. 1a, b, g, h, k). As it is shown on Fig. 1 for CsF-H$_2$O, CsOH-H$_2$O systems the new models are in pure agreement at high concentration with the low molality models of Pitzer and Mayorga (1973). For Cs$_2$SO$_4$- H$_2$O system the new model is again in pure agreement at high concentration with the low molality models of Palmer et al. (2002) and Scharge et al. (2012). New activity data are needed to validate the model for this binary.

Validation of models for cesium binary systems CsCl-H$_2$O, CsBr- H$_2$O, CsI-H$_2$O, CsNO$_3$-H$_2$O, and Cs$_2$SeO$_4$- H$_2$O

The previously developed by Christov (2003a, 2005), and Christov and co-authors (Balarew et al. 1993; Barkov et al. 2001; Donchev and Christov 2020) and by other authors (Pitzer and Mayorga 1973; Kim and Frederick 1988; Sharge et al. 2012) Pitzer approach based thermodynamic models for five (5) cesium binary systems (CsCl-H$_2$O, CsBr-H$_2$O, CsI-H$_2$O, CsNO$_3$-H$_2$O, and Cs$_2$SeO$_4$- H$_2$O) are tested in this study by comparison with experimental osmotic coefficients data and with recommendations on activity coefficients ($\gamma_\pm$) (for CsNO$_3$-H$_2$O) in binary solutions (Fig. 1). The models which give the best agreement with (φ)-, and ($\gamma_\pm$) - data from low to high concentration, up to m(sat), are accepted as correct models, which can be used for solubility calculations in binary and mixed systems and determination of thermodynamic characteristics of precipitating cesium solid phases. The following models are accepted as correct models: model of Balarew et al. (1993) and Christov et al. (1994) for CsCl-H$_2$O, and CsBr-H$_2$O systems (see heavy solid line on Fig. 1c,d,e); model of Pitzer and Mayorga (1973) for CsI-H$_2$O (see heavy solid line on Fig. 1f); model of Donchev and Christov (2020) for CsNO$_3$-H$_2$O (see heavy solid line on Fig. 1i), and the model of Barkov et al. (2001) for Cs$_2$SeO$_4$- H$_2$O system (see heavy solid line on Fig. 1j).
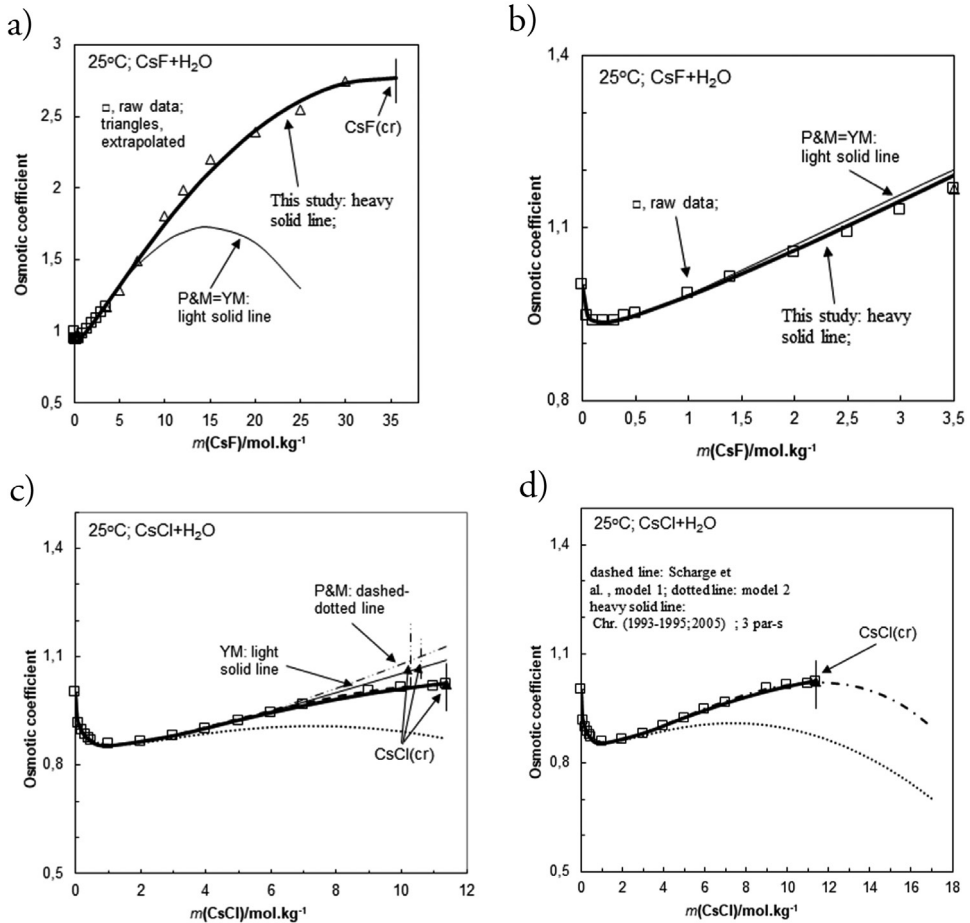
Figure 1. (**a**,**b**,**c**,**d**,**e**,**f**,**g**,**h**,**i**,**j**,**k**). Comparison of model calculated (lines) for activity coefficients (Fig.**i**) and for osmotic coefficients (φ) in cesium binary solutions (CsF-H_2O, CsCl-H_2O, CsBr- H_2O, CsI-H_2O, CsOH-H_2O, CsNO$_3$-H_2O, Cs$_2$SO$_4$- H_2O, and Cs$_2$SeO$_4$- H_2O) against molality at T = 298.15 K with recommendations in literature (symbols). For CsF-H_2O (Fig. **b**) and CsOH-H_2O (Fig. **h**) systems an enlargement of the low molality corner is also given. Heavy solid lines represent the predictions of the developed in this study (for CsF-H_2O, CsOH-H_2O, and Cs$_2$SO$_4$- H_2O systems) and previously reported and accepted models constructed by Christov and co-authors (Christov 2003a, 2005; Balarew et al. 1993; Barkov et al. 2001; Donchev and Christov 2020) and by Pitzer and Mayorga (1973) (for CsI-H_2O). Dashed-dotted, dashed and light solid lines represent the predictions of the reference models of Pitzer and Mayorga (1973) (as P&M on Fig. **a**,**b**,**c**,**f**, **g** and **h**), of Scharge et al. (2012) (for CsCl-H_2O and for Cs$_2$SO$_4$- H_2O (Fig. **c**, **d** and **k**)), and of Palmer et al. (2002) (for Cs$_2$SO$_4$- H_2O (Fig. **k**)) and of YMTB (given as YM on Fig. **c** and **g**) (Sandia National Laboratories (2005). Experimental data (symbols) are from Hamer and Wu (1972) (for 1-1 systems), Robinson and Stokes (1959), Mikulin (1968), Palmer et al. (2002) (for Cs$_2$SO$_4$- H_2O), Partanen (2010) (recommended values for CsI-H_2O) and from Barkov et al. (2001) (for Cs$_2$SeO$_4$- H_2O). The molality of stable crystallization of solid cesium phases is given on all figures by vertical lines (see Table 1 for m(sat) sources).

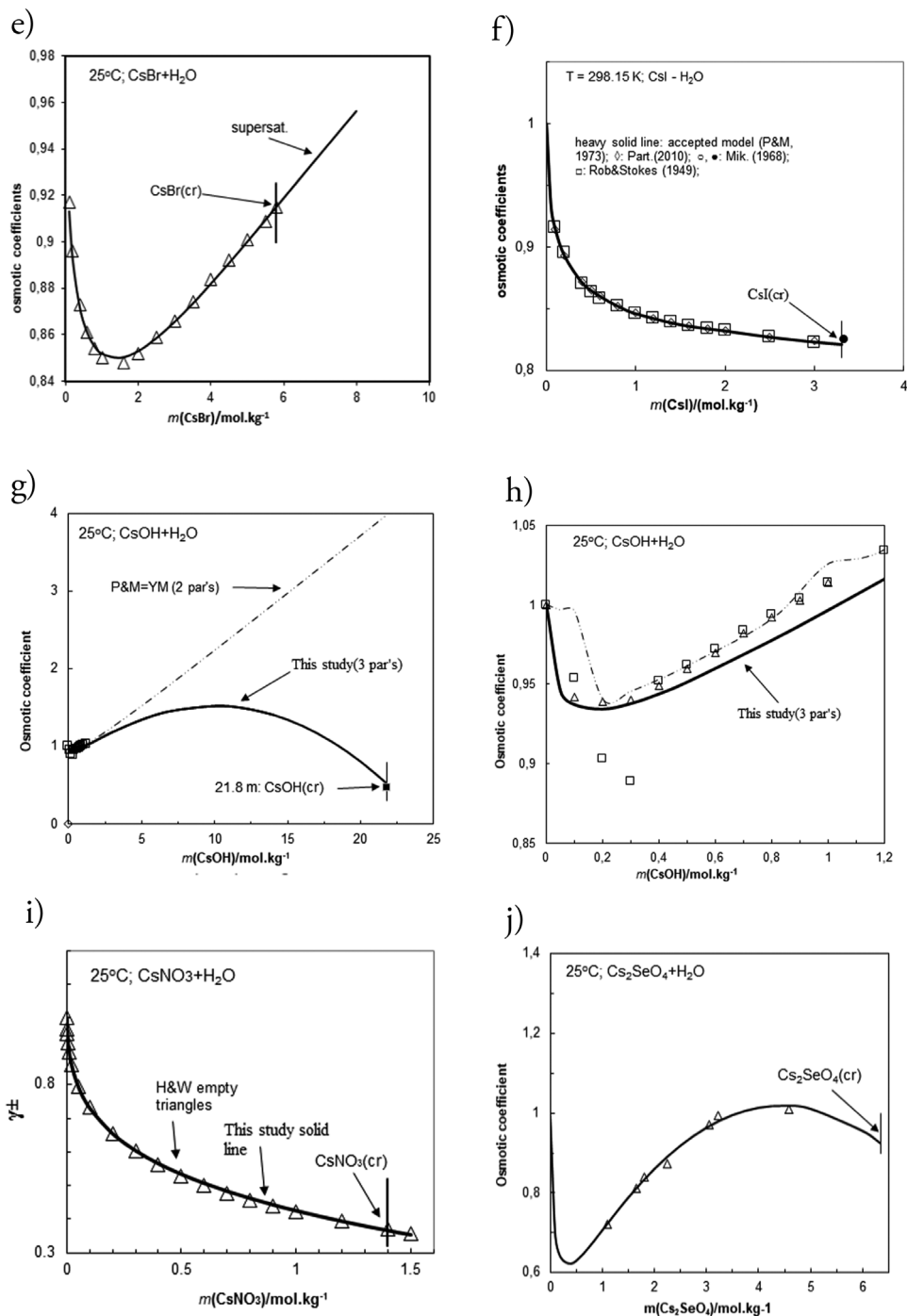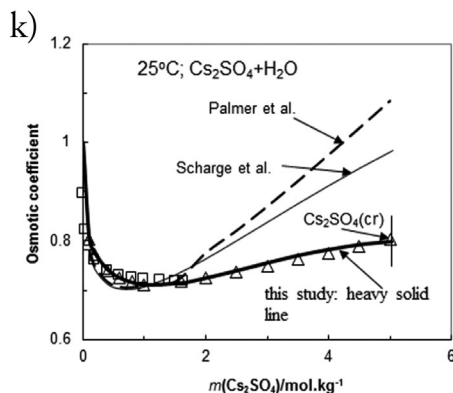**Figure 1.** Continued.

k)

Figure 1. Continued.

Deliquescence Relative Humidity (DRH (%)) and thermodynamic solubility product (ln K°$_{sp}$) of cesium solid phases

On the basis of evaluated previously and accepted models (see previous paragraph) and evaluated in this study binary parameters we determine water activity (a_w) and Deliquescence Relative Humidity (DRH (%)) of solid phases crystallizing from saturated binary solutions. According to Christov (2009, 2012), Donchev and Christov (2020) and Donchev et al. (2021b): DRH (%) = a_w (sat) × 100; where a_w (sat) is activity of water at saturation. The results of DRH calculations are given in Table 1. The DRH predictions of new and accepted models are in excellent agreement with the experimental data determined using isopiestic method, and given in Mikulin (1968). According to model calculations the solid-liquid phase change of CsF(s), occurs at extremely low relative humidity of environment. As a next step, using the accepted and new developed parameterizations, and experimentally determined molalities (m(sat)) of the saturated binary solutions (Mikulin 1968; Balarew et al. 1993; Barkov et al. 2001; Palmer et al. 2002) we calculate the logarithm of the thermodynamic solubility product (ln K°$_{sp}$) of cesium solid phases crystallizing from saturated binary solutions at 25 °C. The calculation approach is the same as in Christov (1995a, 1996a, 2005, 2009, 2012), in Donchev and Christov (2020), and in Donchev et al. (2021b). The model calculations are given in Table 1.

Models for cesium ternary systems

In previous studies of Christov (1996bc, 2003a, 2005) and Christov and co-authors (Balarew et al. 1993; Christov et al. 1994; Christov and Petrenko 1996; Barkov et al. 2001), a solid-liquid equilibrium Pitzer approach models for 15 cesium mixed ternary (CsCl-MgCl$_2$-H$_2$O, CsBr-MgBr$_2$-H$_2$O, CsCl-NiCl$_2$-H$_2$O, CsBr-NiBr$_2$-H$_2$O,

Table 1. Model calculated logarithm of the thermodynamic solubility product (as lnK^o_{sp}), and model calculated and recommended values of the Deliquescence Relative Humidity (DRH) of the of cesium solid phases crystallizing from saturated binary solutions at T = 25 °C.

Salt composition	m (sat) (exp) (mol.kg⁻¹)	Calculated lnK^o_{sp}	DRH(%)	
			Calculated	Experimental data[a]
CsF (cr)	35.6[a]	14.74	2.46	4.0
CsCl (cr)	11.37[b]	3.49	65.69	65.80
CsBr(cr)	5.79[b]	1.905	82.62	82.6
CsI(cr)	3.305[a]	0.675	90.71	90.60
CsOH(cr)	21.8[a]	6.067	66.57	-
CsNO₃(cr)	1.40[a]	-1.328[e]	96.54[e]	96.50
Cs₂SO₄(cr)	5.0[c]	0.9424[f]	80.60[f]	80.40
		1.971[g]	74.59[g]	
		1.486[h]	76.74[h]	
Cs₂SeO₄(cr)	6.34[d]	1.45	72.86	-

aExperimental data of Mikulin (1968); bExperimental data of Balarew et al. (1993) and Christov (2005); cExperimental data of Palmer et al. (2002) and Christov (2003,2005); dExperimental data of Barkov et al. (2001) and Christov (2003); eFrom Donchev and Christov (2020);
fCalculated using binary parameters determinate in this study (heavy solid line on Fig. 1k);
gCalculated using binary parameters from Palmer et al. (2002) (dashed line on Fig. 1k);
hCalculated using 4 parameters model of Sharge et al. (2012) (light solid line on Fig. 1k).

CsCl-MnCl₂-H₂O, CsCl-CoCl₂-H₂O, CsCl-CuCl₂-H₂O, CsCl-CsBr-H₂O, CsCl-RbCl-H₂O, Cs₂SO₄-CoSO₄-H₂O, Cs₂SeO₄-CoSeO₄-H₂O, Cs₂SO₄-NiSO₄-H₂O, Cs₂SeO₄-NiSeO₄-H₂O, Cs₂SO₄-ZnSO₄-H₂O, and Cs₂SeO₄-ZnSeO₄-H₂O) systems at 25 °C are reported. The validated here parameterization for binary systems CsCl-H₂O, CsBr- H₂O, and Cs₂SeO₄- H₂O have been used without adjustment to develop a model for mixed systems. The Pitzer mixing ion interaction parameters ($\theta(Cs,M^{2+})$ and $\psi(Cs,M^{2+},X)$ for the cesium common anion ternary systems have been evaluated on the basis of the experimental data on the compositions of the saturated ternary solutions, i.e. using " solubility approach" (Harvie et al. 1984; Christov 1995a, 1996a, b, 1998, 1999, 2005, 2012).

The values of evaluated mixing parameter are summarized in Table 2. The mixed solution models are developed using our own solubility data (Balarew et al. 1993; Barkov et al. 2001), or the reference data from Zdanovskii et al. (2003), and Silcock (1979). The choice of the mixing parameters is based on the minimum deviation of the logarithm of the solubility product (lnK^o_{sp}) for the whole crystallization curve of the component from its value for the binary solution. See Table 1 for lnK^o_{sp} values for cesium simple salts. In addition, the lnK^o_{sp} value for the cesium double salts crystallizing from the saturated ternary solutions has to be constant along the whole crystallization branch of the double salt. Since the parameters $\theta(M,M')$ take into account only the ionic interactions of the type M-M' in mixing solutions, their values have to be constant for the chloride, bromide, sulfate and selenate solutions with the same cations (M^+ and M^{2+}). Therefore, for common cation systems in constructing the mixing model, we keep the same value of $\theta(M,M')$, and only the $\psi(M,M',X)$ have been varied. In our θ and ψ evaluation the unsymmetrical mixing terms ($^E\theta$ and $^E\theta'$) have been

Table 2. Solutions mixing parameters [θ(Cs,M²⁺) and ψ(Cs,M²⁺,X)] evaluated on the basis of the *m* (sat) molality in cesium common anion ternary systems at 25 °C.

System	θ(Cs,M²⁺)	ψ(Cs,M²⁺,X)	Reference
CsCl-MgCl$_2$-H$_2$O	-0.1260	0.0000	Balarew et al. (1993)
CsBr-MgBr$_2$-H$_2$O	-0.1260	-0.0367	Balarew et al. (1993)
CsCl-MnCl$_2$-H$_2$O	0.00	0.00	Christov and Petrenko (1996)
CsCl-CoCl$_2$-H$_2$O	0.00	0.00	Christov and Petrenko (1996)
Cs$_2$SO$_4$-CoSO$_4$-H$_2$O[a]	(I) 0.00	(I)-0.09	Christov (2003a, 2005)
	(II) -0.05	(II) -0.04	
Cs$_2$SeO$_4$-CoSeO$_4$-H$_2$O[a]	(I) 0.00	(I) 0.04	Christov (2003a, 2005)
	(II) -0.05	(II) -0.02	
CsCl-NiCl$_2$-H$_2$O	-0.23	0.0000	Christov (1996b)
CsBr-NiBr$_2$-H$_2$O	-0.23	-0.0199	Christov (1996b)
Cs$_2$SeO$_4$-NiSO$_4$-H$_2$O[a]	(I) – 0.23	(I) 0.015	Christov (2003a, 2005)
	(II) -0.05	(II) -0.05	
Cs$_2$SeO$_4$-NiSeO$_4$-H$_2$O[a]	(I) – 0.23	(I) 0.015	Barkov et al. (2001)
	(II) -0.05	(II) -0.13	Christov (2003a, 2005)
Cs$_2$SO$_4$-ZnSO$_4$-H$_2$O	-0.05	-0.05	Christov (2003a)
Cs$_2$SeO$_4$-ZnSeO$_4$-H$_2$O	-0.05	-0.08	Christov (2003a)
CsCl-CuCl$_2$-H$_2$O	0.00	-0.050	Christov and Petrenko (1996)
CsCl-CsBr-H$_2$O[b]	-0.0001	0.00001	Christov (1996c, 2005)
CsCl-RbCl-H$_2$O[b]	0.00025	-0.00060	Christov et al. (1994)

[a] Two sets of mixing parameters (I and II) are evaluated in Christov (2003, 2005); [b]Mixing solution parameters calculated by using the Zdanovskii rule (Christov et al. (1994); Christov (1996c, 2005)).

included (2003a, b, 2005). Mixing solution parameters for systems with precipitation of solid solutions (CsCl-CsBr-H$_2$O and CsCl-RbCl-H$_2$O) calculated by using the Zdanovskii rule (Christov et al. 1994; Christov 1996c, 2005) are also given in Table 2.

Summary and conclusions

In this study we developed new, not concentration restricted thermodynamic models for solution behavior and solid-liquid equilibrium in CsF-H$_2$O, CsOH-H$_2$O and Cs$_2$SO$_4$- H$_2$O systems at 25 °C. To parameterize models for cesium binary systems we used all available experimental osmotic coefficients data for whole concentration range of solutions, and up to saturation point. The new models are developed on the basis of Pitzer ion interactions approach. To construct the models, we used different versions of standard molality-based Pitzer approach. It was established that for CsF-H$_2$O system application of extended approach with 4 parameters ($\beta^{(0)}$, $\beta^{(1)}$, $\beta^{(2)}$ and C^{φ}) and variation of α_1 and α_2 terms in fundamental Pitzer equations leads to the lowest values of standard model-experiment deviation. The predictions of new developed here models are in excellent agreement with experimental osmotic coefficients data (φ) in binary solutions from low to extremely high concentration (up to 21.8 mol.kg⁻¹ for CsOH-H$_2$O, and up to 35.6 mol.kg⁻¹ for CsF-H$_2$O). The previously developed Pitzer approach based thermodynamic models for five (5) cesium binary systems (CsCl-H$_2$O, CsBr- H$_2$O, CsI-H$_2$O,

$CsNO_3$-H_2O, and Cs_2SeO_4- H_2O) are tested by comparison with experimental osmotic coefficients data and with recommendations on activity coefficients ($\gamma_\pm$) in binary solutions. The models which give the best agreement with (φ)-, and ($\gamma_\pm$) data from low to high concentration, up to m(sat), are accepted as correct models, which can be used for solubility calculations in binary and mixed systems and determination of thermodynamic characteristics of cesium solid phases. The thermodynamic solubility products (ln K^o_{sp}), and the Deliquescence Relative Humidity (DRH) of solid phases, precipitating from saturated cesium binary solutions (CsF(cr), CsCl(cr), CsBr(cr), CsI(cr), CsOH(cr), $CsNO_3$(cr), Cs_2SO_4(cr), and Cs_2SeO_4(cr)) have been determined on the basis of evaluated binary parameters and using experimental solubility data. The previously established and validated here parameterization for binary systems CsCl-H_2O, CsBr- H_2O, Cs_2SO_4- H_2O, and Cs_2SeO_4- H_2O have been used without adjustment to develop a solid-liquid equilibrium model for 15 cesium mixed ternary (CsCl-$MgCl_2$-H_2O, CsBr-$MgBr_2$-H_2O, CsCl-$NiCl_2$-H_2O, CsBr-$NiBr_2$-H_2O, CsCl-$MnCl_2$-H_2O, CsCl-$CoCl_2$-H_2O, CsCl-$CuCl_2$-H_2O, CsCl-CsBr-H_2O, CsCl-RbCl-H_2O Cs_2SO_4-$CoSO_4$-H_2O, Cs_2SeO_4-$CoSeO_4$-H_2O, Cs_2SO_4-$NiSO_4$-H_2O, Cs_2SeO_4-$NiSeO_4$-H_2O, Cs_2SO_4-$ZnSO_4$-H_2O, and Cs_2SeO_4-$ZnSeO_4$-H_2O) systems at 25 °C. The evaluated previously mixing parameters [θ(Cs,M^{2+}) and ψ(Cs,M^{2+},X)], determined by solubility approach are tabulated. The models described in this study are of high importance in development of thermodynamic database needed for nuclear waste geochemical storage. The models are also of significant importance for extracting cesium resources from saline waters.

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