Actual problems of Ecology

Edited by
Stephka Chankova, Kalina Danova, Michaela Beltcheva,
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EDITORIAL

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International Seminar of Ecology – 2022

Kalina Danova[1]

1 *Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Acad. G. Bonchev 9 str, 1113 Sofia, Bulgaria*

Corresponding author: Kalina Danova (k_danova@abv.bg)

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Europe's first conference on the environment was held in 1970 in Menton, France. This event was organized by Alfred Hassler on behalf of the "Fellowship of Reconciliation" (a pacifist and civil rights organization, https://forusa.org/) together with other leading intellectuals. The forum conceived and issued the so-called Menton Message which was further signed by two thousand, two hundred scientists and displayed at the 1972 United Nations Conference on the Human Environment in Stockholm (Ivanova and Lele 2022). This statement was one of the earliest examples of a collective public alert by scientists demanding a societal change to cope with the environmental crisis and the nuclear weapons race that could lead to destruction of life on Earth. Numbering 3.5 billion people at the time of the Menton Message act, today, more than fifty years later, the World's population has reached an impressive count of 8 billion. The "Stockholm+50: a healthy planet for the prosperity of all – our responsibility, our opportunity" summit was held in June, 2022 (https://www.stockholm50.global/) as a historical legacy of the 1972 United Nations Conference on the Human Environment. Participants in the summit concluded that, despite the over five decades of efforts to restrain the damage of environmental threats, environmental challenges had even evolved deeper in their complexity and global impact. Humankind has reached the point of facing "a triple planetary crisis of climate change, biodiversity loss and pollution". These factors were recognized as a major prerequisite for hampering mankind's sustainable development, leading to poverty and the spread of diseases. The persistence of this crisis was cited as bringing our Planet to "tipping points beyond which there would be little chance of recovery" (Savisaar 2022).

The "Seminar of Ecology" is an annual event, held in Sofia, Bulgaria, since 2007 with the main purpose of alerting the Bulgarian scientific community about the latest ecological concerns arising in the country and suggesting scientifically based decisions to solve them. The first Seminar was co-organized by Section Biology at the Union of Scientists, Bulgaria and the Central Laboratory of General Ecology, Bulgarian Academy of Sciences (Chankova 2022). The fruitful communication of Bachelor, Master, PhD- students and young researchers with established scientists with lengthy expertise in the diverse aspects of ecology, has strengthened the traditions of the "Seminar of Ecology" as a regular meeting point and an annual scientific forum throughout the years. Outstanding oral presentations and poster contributions of young researchers have been selected and awarded at the event. Full-text contributions presented at the event have been peer-reviewed and published in the Proceedings Book. Selected full-text contributions of the last three years of the "Seminar of Ecology" have been published in the journals Ecologia Balkanica, BioRisk, and Phytologia Balcanica.

The event has also gained popularity beyond the borders of Bulgaria with researchers from abroad also presenting their latest results. In 2014 the event became the "Seminar of Ecology with international participation" and an "International Seminar of Ecology" in 2019.

The current special issue entitled "Actual problems of Ecology" consists of selected eleven full-text contributions presented at the "International Seminar of Ecology" in 2022. The event was held online on 29–30 September and was dedicated to "The International Year of Basic Sciences for Sustainable Development 2022". Contributions comprised four scientific topics.

In a plenary lecture in the first thematic topic "Biotic and abiotic impact on the living nature. Ecological risk. Bioremediation", Chankova et al. (2023) opened the discussion by focusing on the ecological, agricultural and medical aspects of genotype resistance to oxidative stress. The role of chaperone proteins in DNA repair, a topic not so widely discussed in literature, was addressed and systematized in the current special issue as a review paper. The session continued with two further lectures. The first was dedicated to the bacterial and fungal diversity in the long-term copper (Cu) contaminated soils of the highly industrialized zone of Topolnitsa-Pirdop valley where a number of mines and processing plants for copper and other non-ferrous metals long-term are found (Petkova et al. 2023). In their lecture Todorova et al. (2023) investigated the role of genotype for coping with $PbCl_2$ induced stress in model systems of *Chlamydomonas reinhardtii* strain 137C – wild type, *Saccharomyces cerevisiae* strain D7ts1 and *Pisum sativum* L. cultivar Ran1. The experiment allowed for elucidating the mechanism of oxidative stress induction caused by $PbCl_2$ on the DNA molecule and photosynthetic pigments. Three of the posters presented in the session of the first thematic topic of the Seminar and published in the special issue include the works of Georgieva et al. (2023a), Peteva et al. (2023) and Angelova et al. (2023). In the first of these contributions authors have evaluated the presence and characteristics of microplastic particles in waters from the Black Sea coast of Bulgaria (protected, aquaculture

and industrial areas were compared). Peteva et al. (2023) investigate the content of hydrophilic and lipophilic marine toxins in plankton samples taken in 2021 along the whole Bulgarian coastline, and have compared their results with those from the previous period. The third poster in the first scientific topic of the Seminar published as a full text studied the physiological response of human erythrocytes subjected to *in vitro* irradiation by magnetic field of high frequency (2.41 GHz) (Angelova et al. 2023). The second thematic session "Ecological agriculture. Ecological education" of the Seminar is represented by the oral presentation of Ilinkin et al. (2023) and two poster contributions by young researchers (Georgieva et al. 2023b; Kirov et al. 2023). In their study, Ilinkin et al. (2023) investigate the potential for *in vitro* propagation and *ex vitro* adaptation of *Tanacetum cinerariifolium* (Trevir.) Sch. Bip. (Dalmatian pyrethrum, Asteraceae). The study was motivated by the necessity to obtain a stable system for the vegetative propagation of the species, providing for a reliable and constant supply of elite planting material with a high capacity for pyrethrins production. Georgieva et al. (2023b) studied the effects of growth promoting rhizobacteria strain *Pseudomonas putida* 1046 on the germination of corn seeds. Kirov et al. (2023) investigated the accumulation of potentially harmful feed ingredients in hen eggs albumen with special attention to metal traces. The authors have presented the results of a comparative survey of metal content in egg albumen in industrial poultry farms with that of backyard and free-range hens. In the experiment, the contents of heavy metals were analyzed - Cr, Cu, Fe, Mn and Zn; Al, Cd, Ni and Pb, as well as B, Ba, Sr, Ca and Mg. The poster was awarded first place in the Young Researchers' Contest at the Seminar. The third thematic session "Biodiversity. Conservation biology" has been presented in the current issue by the work of Spasova et al. (2023) who study the plant communities and their utilization by mammals of two peat-shrub species habitats – those of *Spiraea salicifolia* and *Potentilla fruticosa* (boreal relicts among dominant coniferous forests) in the Rhodope Mountains. The fourth thematic session "Ecosystem research and services. Landscape ecology" comprises the oral presentation of Grigorov et al. (2023) and deals with the forest habitat diversity of Godech Municipality according to the EUNIS habitat classification.

The "Seminar of Ecology 2022" was the second consecutive forum, held online in the yearly history of the event. Nevertheless, the dynamics of discussions and scientific interest of the audience towards the research of the diverse range of presenting authors was well reflected.

Thus, issues of major concern regarding the impact of human activities on the vital characteristics of ecosystems of different types were brought forward. Alerts were raised that the threats of the "triple planetary crisis of climate change, biodiversity loss and pollution" have been evidenced to affect diverse living systems on the micro- and macroscale. The "Seminar of Ecology – 2023", entitled "Cutting Edge Research of Ecology" has been planned as a hybrid event to take place both face-to-face and online in Sofia, September 28th–29th with the prospect of providing the opportunity to deepen research already initiated, as well as to search for scientifically based solutions to the problems identified.

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REVIEW ARTICLE

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Does overproduction of chaperone proteins favour the repair of DNA injuries induced by oxidative stress? (Mini review)

Stephka G. Chankova[1], Nadezhda P. Yurina[2], Teodora I. Todorova[1], Petya N. Parvanova[1]

1 *Institute of Biodiversity and Ecosystems Research, Bulgarian Academy of Sciences, Sofia, Bulgaria* **2** *Bach Institute of Biochemistry, Research Center of Biotechnology of the Russian Academy of Sciences, Moscow, Russia*

Corresponding author: Nadezhda P. Yurina (nyurina@inbi.ras.ru, nadezhdayurina@hotmail.com)

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Abstract

Genotype resistance to oxidative stress, induced by various physical/chemical stimuli has been the focus of scientists for the last decades, with several aspects – ecological (the formation of the genetic elite of population), agricultural and medical (radio-chemotherapy).

Genotype resistance to oxidative stress is regarded as the integration of different morphological, physiological, biochemical, metabolic and genetic characteristics. Currently, it is supposed that the mechanisms involved in the formation of genotype resistance to oxidative stress are inter-correlated and inter-dependent, comprising changes in genes, proteins, enzymes, different metabolic pathways and/or biological networks. According to the present state of knowledge, various cellular targets, resulting in genotoxic stress, induction of DNA damage, mutations, genomic instability or apoptosis can trigger different signal transduction pathways, activating DNA repair, antioxidant and chaperone defence systems.

Till now, a lot of experimental data have been accumulated concerning the contribution of DNA repair to the formation of genotype resistance to oxidative stress. At the same time, genotype resistance of organisms is largely determined by the ability of molecular chaperones to maintain conformational homeostasis of proteins (folding – misfolding – refolding or aggregation – degradation). The role of chaperones in protein homeostasis and cell death, especially in apoptosis, is well discussed in literature, but much less is known about their function in DNA repair. In this regard, here we addressed the question of whether the overproduction of chaperone proteins contributes to the repair of DNA damage caused by oxidative stress.

Keywords
BER – base excision repair, DDR - DNA Damage Response, DSBs – double-strand breaks, HSPs – heat shock proteins, HSFs - heat shock transcription factors, oxidative stress

In this mini-review article, several items that we believe are of fundamental importance to the given topic have been highlighted.

The first one concerns the term genotype resistance - what exactly does this term mean?

The genotype resistance to oxidative stress is considered as an integration of different morphological (Lipiec et al. 2013; Rai and Agrawal 2017), physiological, biochemical, and metabolic (Badahur et al. 2011; Chankova and Yurina 2012; Lipiec et al. 2013; Marcińska et al. 2013; Wegener and Jansen 2013; Chankova et al. 2014; Rai and Agrawal 2017) and genetic characteristics (Dimova et al. 2008, 2009; Ahuja et al. 2010; Xu et al. 2011; Zinati et al. 2013; Dimitrova et. al. 2014; Todorova et al. 2015, 2019; Marinovska et al. 2022). Currently, it is believed that the mechanisms involved in the formation of genotype resistance to oxidative stress are inter-correlated and inter-dependent and include changes in genes, proteins, enzymes, different metabolic pathways or biological networks (Costantini et al. 2013; Gong and Miller 2019). According to the present state of knowledge, various cellular targets, resulting in genotoxic stress, induction of DNA damage, mutations, genomic instability or apoptosis can trigger different signal transduction pathways activating DNA repair, antioxidant and chaperone defence systems (Toulany 2019; Clementi et al. 2020; Kotob 2021).

The second one concerns the significance of genotype resistance for living organisms and their quality of life

sDuring the last decade, genotype resistance to oxidative stress, induced by various physical/chemical stimuli has been a focus of scientists in many aspects of science – ecological (the formation of the genetic elite of the population, adaptation in the target regions), medical (disease resistance, radio- chemotherapy), agronomics – tolerance/resistance to different abiotic and biotic environmental factors. The first ones who proposed the name “genetic elite” were Dobzhansky and Spassky in the far 1963 (Dobzhansky and Spassky 1963). They have understood “genetic elite” as “...genotypes whose fitness is greater than two standard deviations above the population mean. These elite lines have similar essential alleles for desirable end-use characteristics, agronomics, disease resistance and adaptation in the target region...”.

The third one is focused on the possible mechanisms involved in the formation of genotype resistance

Over the years, much data concerning the contribution of DNA repair, chaperone and antioxidant repair systems for the formation of genotype and induced resistance have been collected. Additionally, the contribution of other factors, such as high levels of constitutive and induced levels of SOD, SH-groups, the presence of cell wall, stability of ultra-structural compartments of cells, phases of the mitotic cycle, the energy provision of cells and others has been clarified (Chankova et al. 2000; Goldberg and Lehnert 2002; Marnett et al. 2003; Ramotar and Wang 2003; Schaue and McBride 2005; Bao et al. 2006; Chalmers 2007; Chankova and Yurina 2012, 2016; Wu et al. 2017).

DNA permanently is the main target of different damaging endogenous factors as a result of the work of cells metabolism machinery and exogenous factors – climate changes, ionising (IR) and non-ionising (UV) radiation, as well as various chemicals, drugs etc. This fact results in:

- The induction of different types of DNA damage as single-strand breaks (SSBs), double-strand breaks (DSBs), base and nucleotide modifications, as well as cross-links and dimers. The type of induced injuries depends on many factors (specific action of physical or chemical agent, species, tissue, age, experimental design, cell cycle and physiological state etc.) (Chankova et al. 2000, 2007, 2009; Sottile and Nadin 2018; Miteva et al. 2020; Penninckx et al. 2021).
- The induction of a complex system for recognition and activation of multiple defence systems, named DNA Damage Response mechanism (DDR) has evolved evolutionarily. DDR involves the detection of DNA injuries, DNA repair or apoptosis (Guénolé et al. 2013; Sottile and Nadin 2018; Gong and Miller 2019).

For some time, the question concerning the relationships between genotype resistance and the contribution of DNA susceptibility and/or efficiency of DSBs repair has been under discussion. Why was our attention focused on induced DSBs and their recovery?

Here, it is necessary to mention that DSBs are believed to be the most lethal for living organisms (Kelm et al. 2022). It was found by Khozooei et al. (2022) that non-repaired DSBs can induce cell death in radio-sensitised triple-negative breast cancer cells.

As it was described by Sottile and Nadin (2018) and Li et al. (2021), DNA repair mechanisms could be split into five categories: direct reversion of DNA damage, base excision repair (BER), mismatch repair (MMR), nucleotide excision repair (NER) and homologous recombination (HR) and non-homologous end joining (NHEJ) (Kciuk et al. 2020).

Quantification of radiation-induced DNA double-strand breaks is a good tool for the evaluation and prediction of cells/organisms' response to IR (Penninckx et al. 2021), especially in the case of occupational exposure (Kvitko et al. 2012), accidents and medical purposes.

In order to gain an insight into the mechanisms of genotype resistance, two main relationships should be clarified: the contribution of DNA susceptibility to this process and the contribution of DSBs repair capacity.

Currently, little is known about the possible role of DNA susceptibility, as well as DNA repair capacity in the formation of genotype resistance. Data in literature are very contradictory. Some of them have confirmed the crucial importance of DNA susceptibility for this process. For example, a significant correlation between initially induced levels of DSBs and cell radio-sensitivity of tumour cell lines has been reported by el Awady et al. (2003). Using constant-field gel electrophoresis (CFGE), the same correlation has been obtained by Saleh et al. (2012) concerning cells' sensitivity to cis-platin (CIS).

To clarify, the contribution of increased DNA repair capacity to the formation of genotype resistance is up-to-date because it relates to problems of radio-chemotherapy (Ghahe et al. 2021; Kelm et al. 2022). Data have been gathered, identifying that several factors including upregulation of DNA repair, especially DSBs and activation of DDR can promote tumour resistance to therapy, inducing an adaptive response. It was described by Ghahe et al. (2021) that increased glioblastoma cells' resistance to photodynamic therapy (PDT) is a result of accelerated repair of BER and DNA breaks, as well as DNA damage signalling. The finding that accelerated DNA repair essentially can contribute to the elevation of tumour resistance to the treatment provides a new perspective on treatment using DSBs repair targeted inhibitors (Kelm et al. 2022).

As was pointed out at the beginning of this mini-review, genotype resistance is of great concern to agriculture and the environment. Víquez-Zamora et al. (2022), by characterising the DNA repair capacity of the US inbred lines B73 and Mo17, as well as Central American maize landraces from Guatemala and Costa Rica, have directed this molecular approach for the breeding of more tolerant to DNA damaging environmental factors plants.

Our own results, using mutant strains or extremophile species of unicellular green algae, as well as *Saccharomyces cerevisiae* strains, demonstrated that differences in DSB's repair capacity are probably one of the main mechanisms involved in the formation of genotype resistance to chemical and physical factors. In Fig. 1, the DSBs' repair capacity of *Chlamydomonas reinhardtii* strains – 137C WT, CW15 cell-wall less with WT radio-resistance and H-3 –highly radio-resistant hybrid strain obtained by mating CW15 × AK-9-9 (mutant strain constructed by us using chemical mutagenesis approach) (Chankova et al. 2005; Dimova et al. 2009) are compared. The variation in repair capacity is clear. The most radio-resistant hybrid strain H-3 expresses several-fold higher potential to repair DSBs by accelerating DSBs' rejoining.

A similar picture was obtained in *Saccharomyces cerevisiae* strains (Fig. 2) The curves in Fig. 2 illustrate the relationship between DNA susceptibility, measured as the level of primary Zeocin-induced DNAs and repair capacity. A strain BY4741 that exhibits less DNA susceptibility, i.e. has a more resistant genotype to the radiomimetic Zeocin, is characterised by more effective DVR repair.

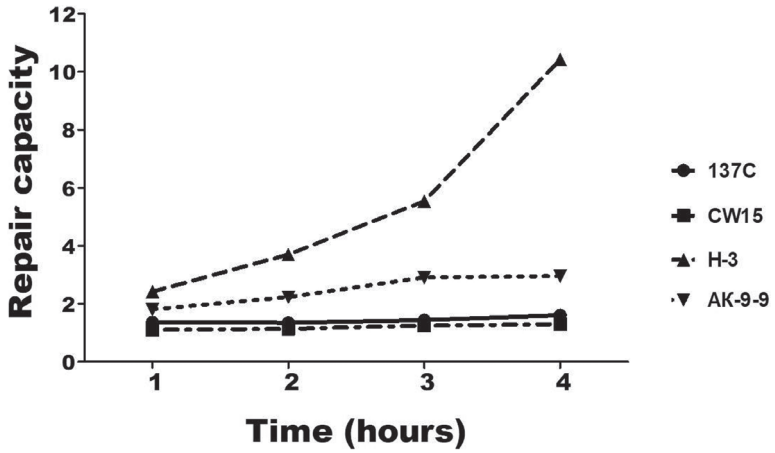


Figure 1. DSBs' repair capacity of WT 137C and CW15 and radio-resistant strains – H3 and AK-9-9 of *Chlamydomonas reinhardtii*.

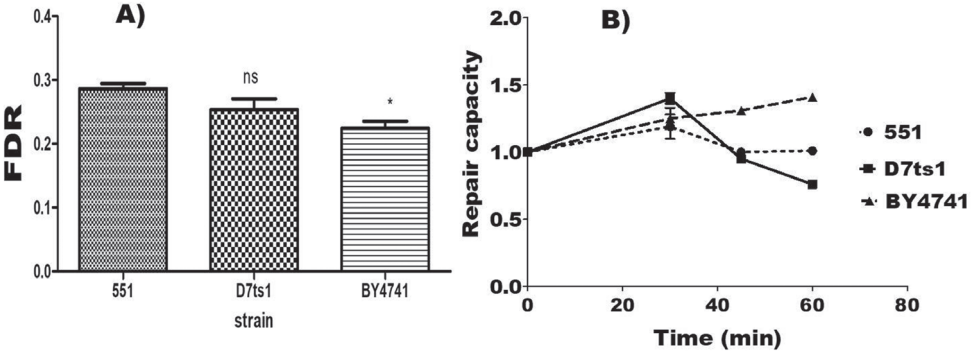


Figure 2. DNA susceptibility of *Saccharomyces cerevisiae* strains 551, D7ts1 and BY4741 measured as DSBs' induction (**A**) and DSBs' repair capacity (**B**). FDR – fraction damages released.

The differences between repair capacity of *Chlamydomonas reinhardtii* and *Saccharomyces cerevisiae* strains and extremophiles *Chlorella vulgaris* are probably amongst the mechanisms involved in the formation of cells' resistance to different inducers of oxidative stress through the acceleration of DSBs' repair rejoining (Dimitrova et al. 2014, 2022; Miteva et al. 2020; Marinovska et al. 2022). Using *Chlamydomonas reinhardtii* mutants – UVS-10 - *rec-* repair deficient and UVS-14 - mismatch repair deficient, the role of these two types of DNA repair for the formation of genotype resistance to different DNA damaging factors was confirmed (Dimitrova et al. 2022).

At the same time, high constitutive levels and overproduction of HSP70B were identified for more resistant *Chlamydomonas reinhardtii* strains and *Chlorella vulgaris* species after the induction of oxidative stress by various physical or chemical stressors (Chankova et al. 2013; Miteva et al. 2020)

About the contribution of the chaperone system in the formation of genotype resistance

Genotype stability of organisms is determined to a large extent by the ability of molecular chaperones to maintain conformational homeostasis of proteins (folding, improper folding, re-folding or aggregation - degradation). Heat shock proteins (HSPs) occupy one of the main places amongst biological protective reactions to oxidative stress (Chen et al. 2018). The role of chaperones in protein homeostasis and cell death, especially apoptosis, is widely described in literature, but much less is known about their function in DNA repair processes. In this regard, studies of the interdependence of the DNA double-strand break repair system and the activity of the chaperone system attract much attention (Sottile and Nadin 2018; Dubrez et al. 2020).

Heat shock proteins (HSPs) are found in all living organisms. Based on their molecular weight and cell functions, HSPs are classified into several families - small HSPs with mol. mass from 10 to 30 kDa and HSP40s, HSP60s, HSP70s, HSP90 and HSP100 (Al-Whaibi 2010; Ul Haq et al. 2019; Dubrez et al. 2020). It should be mentioned that the abbreviations of bacterial HSPs differ from those in eukaryotic cells as could be seen in Table 1.

Table 1. HSP of prokaryotic and eukaryotic cells.

Bacterial proteins	Eukaryotic proteins
Clp B	HSP100
Htp G	HSP90
Dna K	HSP70
GroEL	HSP60
Dna J	HSP40
Ibp A, Grp E	HSP20, HSP27
Gro ES	HSP10

Of particular interest are small sHSP, HSP70 and HSP90. Today, studies about their particular contribution to DNA damage sensing, signalling and repair are in a progress (Pennisi et al. 2015; Dubrez et al. 2020).

Below, HSP groups, related to the topic of the mini-review, are presented briefly.

Low-molecular-weight sHSP proteins are ancient proteins characterised by the presence of the main domain of α-crystalline. Under stressful conditions, sHSP prevents irreversible aggregation of unfolding proteins by integrating into the resulting protein aggregates. sHSP- containing aggregates have easier access to Hsp70 and ClpB/Hsp104 chaperones. These chaperones in ATP-dependent reactions secrete individual proteins from aggregates and contribute to their refolding into the native state (Rutgers et al. 2017).

The most numerous group of sHSP was found in higher plants and algae (19 in Arabidopsis, 23 in rice and 39 in poplar) than in Volvocales species (8 in *Chlamydomonas reinhardtii*, 7 in *Volvox carteri* and 6 in *Gonium pectorale*) (Rutgers et al. 2017). The more complex HSP system in plants compared to animals may be due to the sessile lifestyle that not allows them to avoid stressful conditions.

A comprehensive genome-wide analysis was used to identify and characterise the functional dynamics of the HSP20 gene family. Advances in whole genome sequencing have made it possible to detect all the suspected HSP genes, their duplication and their diversification. For example, this has allowed Hu et al (2021) to construct a phylogenetic tree of members of the HSP20 family showing by its example that a total of 33 HSP20 genes distributed across 13 chromosomes were identified from the genome.

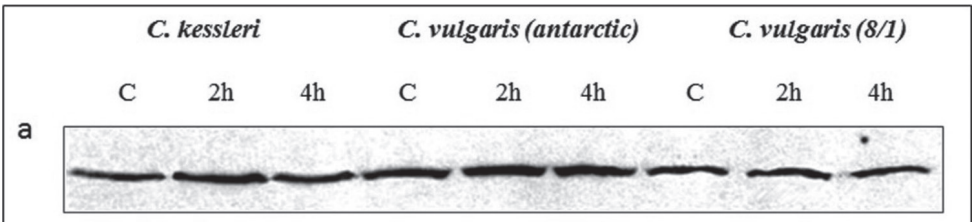
The expression levels of HSP20 genes were differentially induced by heat stress. The transcript level of six proteins was down-regulated by heat stress, while twelve were up-regulated by heat stress. The last proteins are very interesting because they could be used as heat tolerance candidate genes (Hu et al. 2021).

HSPs are pleiotropic proteins involved in a variety of biochemical processes and perform many important functions in eukaryotes, as well as contribute to enhanced stress tolerance/resistance. HSP70 is the most universally induced chaperone in response to various cellular stressors, such as UV radiation, gamma radiation and chemicals. The HSP70 chaperone network implements diverse housekeeping- and stress-related activities. The HSP70 chaperones participate in a wide range of cellular housekeeping functions - the folding of newly-synthesised proteins, the translocation of polypeptides into mitochondria, chloroplasts and the endoplasmic reticulum, the assembly and disassembly of protein complexes, regulation of protein activity, assisting in the HSP90 folding machinery and chaperonins (Rosenzweig et al. 2019). Stress-related activities of HSP70 are associated with preventing the aggregation of proteins, solubilising aggregated proteins, promoting the refolding of misfolded or unfolded proteins, cooperating with cellular degradation machinery, such as the ubiquitin-proteasome system, to clear aberrant proteins and protein aggregates (Rosenzweig et al. 2019).

Representatives of another chaperone family, HSP90, are localised in the cytosol in the absence of stress. The main function of HSP90 is to regulate protein metabolism, ensure protein stability and participate in intracellular protein transport. Usually, the chaperone HSP90 acts in combination with other chaperones, such as HSP70 (Dubrez et al. 2020). Several years ago, it was reported that cancer cells are characterised by over-expression of HSP90 chaperone and this poses a new challenge for cancer treatment (Pennisi et al. 2015).

HSP70B is a good marker of oxidative stress and stress state of cells

The synthesis of chaperones is induced and depends on abiotic and biotic stresses and, thus, the content of HSP can be a useful indicator of stress and stress reactions in various organisms. Previously, we have compared the heat stress response of two extremophiles - *Chlorella vulgaris* strain Antarctic, isolated from the soil of the Antarctic and 8/1 – thermophile, isolated from the hot spring Rupite in Bulgaria with those of *Chlorella keslerii* – mesophilic strain. Both higher constitutive levels and well-marked overproduction of HSP70B were obtained for *C. vulgaris* Antarctic strain – Fig. 3 (Chankova et al. 2013). It was also shown that the overproduction of HSP70 in this strain correlates with the increased resistance to UV - B irradiation and well-expressed



b

Algal species	Control	2 h after 42°C	4 h after 42°C
Chlorella vulgaris Antarctic	596	650	640
Chlorella kessleri	320	490	345
Chlorella vulgaris (8/1)	270	310	260

Figure 3. Comparison of the HSP70B level in *Chlorella* after heat stress (42 °C for 5 min) **a** western blot analysis of the HSP70B level in *Chlorella* cells incubated at heat-stress temperature **b** densitometry of the HSP70B contents; C - control sample.

photo-reactivation and dark repair (Miteva et al. 2020). Here, we can speculate that probably due to the higher constitutive level and well-marked overproduction of HSP70B, as well as effective DNA repair systems, this strain can survive in the extreme environment of Antarctica. Our finding contributes to the hypothesis of the conserved functional properties of HSP70B as a mechanism of thermo-tolerance in plants.

Heat Shock Transcription Factors (HSF) are the main regulators of HSPs

The expression of the HSP genes is mainly regulated by heat shock transcription factors (HSFs). HSFs are a group of evolutionarily conservative regulatory proteins present in all eukaryotes and regulating various responses to stress and biological processes in plants.

Plants have a more complex response to stress than yeast and animals, which may be due to their sessile nature. So, for example - the HSF family of plants contains 18-52 members, while in yeast and Drosophila, it is represented by single copies of HSF, in mammals - 4 HSFs (Andrasi et al. 2020; Tian et al. 2021). Despite significant variation in the number and sequence of HSFs, their structure and functions are highly conserved across plant species.

HSF contains a conserved DNA-binding domain at the N-end of the protein that recognises the DNA motif of 11 nucleotides: 5'-nGAAnnTTCn-3'. This motif is usually found in the promoter region of HSF-regulated genes (Tian et al. 2021).

Plants are simultaneously exposed to many types of stress (abiotic and biotic) that result in oxidative or secondary stress. Plants' response to heat stress is regulated by Heat shock transcription factors (HSFs), which bind to *cis*-acting elements known as HSE (heat shock elements).

Three domains have been identified in the HSFs structure: the DNA-binding domain, the oligomerisation domain and the C-terminal activation domain. Based on the differences in the composition of these domains, the HSFs of plants are divided into three classes: A, B and C which differ in their functions. Amongst HSFs, HSFA apparently plays a unique function as the main regulator of acquired thermal tolerance. Under normal conditions, HSFA activity is inactivated by HSP90. Under stress, this repression is reversed and HSF changes into the functional trimer state. This HSFA trimer then binds to heat shock elements (HSE) in the promoter region of the genes, transcription occurs and HSPs are synthesised (Fig. 4). HSFs regulate the down-stream HSPs, antioxidant enzyme genes, which help the plants to develop stress tolerance.

HSFs' class B and C factors have been scarcely studied and in fewer plant species. So, in contrast to the activity of class A HSFs, the class B HSFs factors lack the C-terminal activation domain and have a transcription repression domain at the C-terminus of the

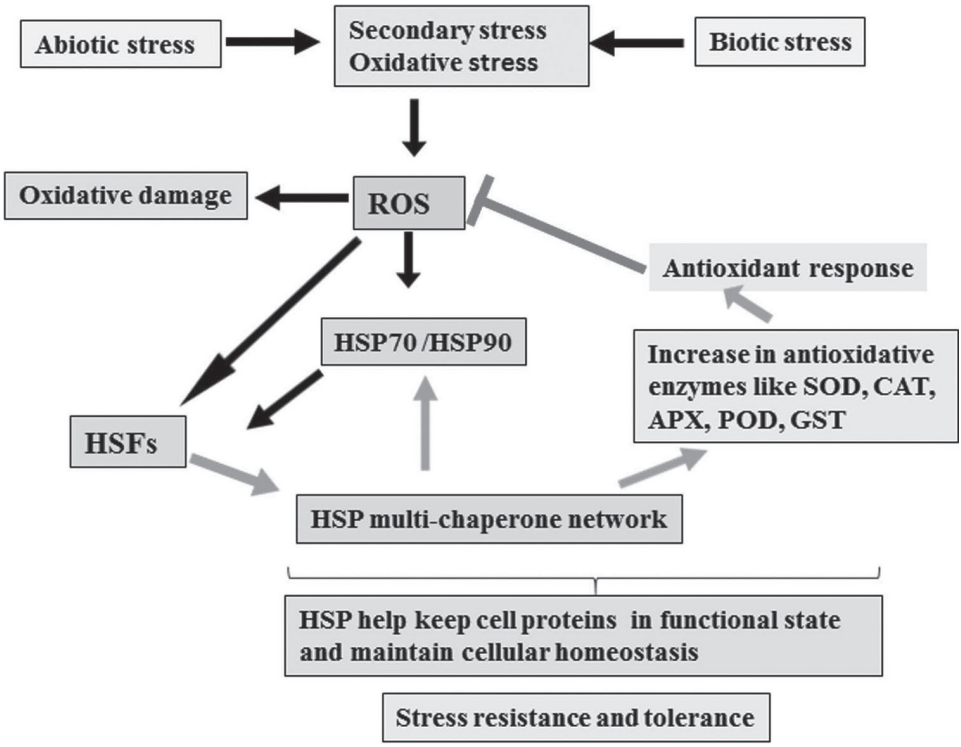


Figure 4. Scheme of the HSP transcriptional regulation, illustrating HSFs activation and their interaction with the other pathways to counter abiotic and biotic stress. ROS (Reactive oxygen species), HSF (Heat shock transcription factor), HSP (Heat shock protein), APX (Ascorbate peroxidase), GST (Glutathione-s-transferase), SOD (Superoxide dismutase), POD (Peroxidase), CAT (Catalase).

protein (Tian et al. 2021). The study of new HSF class B genes has shown that they play an important role in the response of plants to biotic and abiotic stresses (Peng et al. 2013).

Plants' heat shock proteins play a key role in ensuring plant resistance to stress through different mechanisms. They can use ROS as a signal to induce HSF and HSP biosynthesis (see Fig. 4), can increase the stability of membranes and can detoxify reactive oxygen species (ROS) and can positively regulate antioxidant enzyme systems (Ul Haq et al. 2019).

When plants are exposed to stress, the synthesis of normal proteins is decreased while the expression of stress genes is up-regulated and, as a result, the synthesis of HSPs is triggered. HSP gene expression positively regulates protective enzyme activities. So, for example, in Arabidopsis, over-expression of small HSP17.8 enhanced the SOD activity and, in tobacco, HSP16.9 increased the activities of peroxidase - POD, catalase – CAT and superoxide dismutase – SOD (Driedonks et al. 2015; Ul Haq et al. 2019).

HSF and HSP form a complex regulatory network in response to stress. With the rapid development of transcriptome sequencing technology and an increase in the volume of big data in publicly available databases, it has become possible to use networks of joint gene expression to study possible ways of regulating the stress response of the cell and protein-protein interactions (Tian et al. 2021).

On the possible relationship between HSP and DNA repair pathways

The potential role of heat-shock proteins in both cellular carcinogenesis and/or their contribution to DNA repair machinery has been under discussion over the last decade. This problem is closely related to mechanisms of carcinogenesis, as well as anti-cancer therapy and increased resistance of some tumours to medical treatment (Kang et al. 2015; Pennisi et al. 2015; Dubrez et al. 2020).

The HSP chaperoning system is associated with the reaction to DNA damage and can directly regulate the signalling pathways of DNA repair. In response to DNA damage, adaptive coordinated defence mechanisms are activated in cells. Depending on the nature of DNA damage, various DNA repair pathways will be involved. Damage affecting only one of the two DNA strands, such as single-stranded breaks (SSBs), is the most common type of damage. In mammals, there are several ways to repair single-stranded DNA breaks. The first pathway is base excision repair (BER). The second pathway is mismatch repair (MMR). The third pathway is the nucleotide excision repair system - NER (Sottile and Nadin 2018; Dubrez et al. 2020).

What is currently known about HSPs contribution to the regulation of SSB and DSBs repair? HSP70 cooperates with small HSP27 and HSP90 to reactivate misfolded substrates. Inducible HSP70 confers cell resistance against radiation and chemotherapeutic agents and facilitates DNA damage repair. HSP90 generally acts downstream of HSP70, during the later folding steps (Sottile and Nadin 2018; Dubrez et al. 2020). In the last years, HSP90 has been found in association with chromatin-bound proteins. Thus, HSP90 has emerged as a potent regulator of nuclear processes including DNA repair and transcription. Since HSP90 is overexpressed in a large variety of tumours, it is an attractive target for anti-cancer therapy.

Table 2. HSP chaperones regulate the repair of double-stranded DNA breaks.

Pathway	DNA lesions	DSB detection	DNA resection and exchange strands	DNA-polymerase/ Ligase
Non-homologous end-joining (NHEJ)	Ionising radiation, X-rays, chemicals	HSP27	HSP110, HSP90	DNA synthesis, incision and ligation
Homologous recombination (HR)	Ionising radiation, X-rays, chemicals	HSP27, HSP70, HSP90	HSP90	DNA synthesis, incision and ligation

As it was described previously, double-stranded DNA breaks (DSBs) could be repaired using two main repair mechanisms. The first one is named the non-homologous ends joining repair (NHEJ) and the second one is homologous recombination (HR) repair. As shown in Table 2, the non-homologous ends joining pathway can be regulated by the chaperones HSP27, HSP90 and HSP110, while the chaperones HSP27 and HSP90 regulate the homologous recombination pathway (Sottile and Nadin 2018; Dubrez et al. 2020). It has been shown that HSP90 is required for both repair mechanisms: NHEJ and HR (Steckleina et al. 2012).

It is assumed that the chaperone system is associated with the reaction of cells to DNA damage and can directly regulate the signalling pathways of DNA repair (Dubrez et al. 2020). It has been shown that HSP is rapidly induced when exposed to an agent that damages DNA. Additionally, it should be mentioned that HSPs were identified in DNA repair sites by confocal microscopy (Castro et al. 2015).

Recently, it has been shown that HSP110 can regulate DNA repair signalling pathways in mammals. It was found that, by blocking these chaperones, it is possible to elevate tumour cells' sensitivity to drugs. The HSP chaperoning system is associated with the reaction to DNA damage and can directly regulate the signalling pathways of DNA repair.

In conclusion, it could be summarised that several mechanisms are involved in the formation of genotype resistance:

- Up-regulation of DNA repair, especially DSBs and activation of DDR are of great importance for purposes of agriculture and medical treatment of cancer. The new finding that accelerated DNA repair essentially can contribute to the elevation of tumour resistance to medical treatment provides a new perspective on treatment using DSBs repair targeted inhibitors.
- Chaperones are not directly involved in DNA repair, but contribute to cell's/organisms' survival in stressful conditions due to their numerous interactions with proteins involved in DNA repair. Amongst the different HSPs, some of them - HSP27, HSP70, HSP90 and HSP110 are considered directly involved in the regulation of DNA repair.
- Having in mind the finding that hypersensitivity to anti-cancer therapy could be achieved by blocking the expression of HSP27, HSP70 or Hsp90 genes, new perspectives for cancer treatment are in progress.
- Over-expression of HSP70 genes in stressful conditions resulting in overproduction of HSP70B content can be used as an indicator of oxidative stress and

organisms' stress response. This finding is closely related to problems that have given rise to climate change and anthropogenesis and could be used for purposes of agriculture as well as for environmental impact assessment.

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Distribution of microbial abundance in long-term copper contaminated soils from Topolnitsa-Pirdop valley, Southern Bulgaria

Michaella Petkova[1], Nadezhda Nankova[1], Viktoriya Kancheva[2], Silvena Boteva[3], Anelia Kenarova[3], Galina Radeva[1]

1 *Department of Gene Regulation, Roumen Tsanev Institute of Molecular Biology, Bulgarian Academy of Sciences, "Acad. G. Bonchev" Str., Bl. 21, 1113 Sofia, Bulgaria* **2** *Department of Mechanization and Hydro Melioration, Institute of Soil Science, Agrotechnologies and Plant Protection "N. Poushkarov", Agricultural Academy, 7 "Shosse Bankya" Str., 1331 Sofia, Bulgaria* **3** *Department of Ecology and Environmental Protection, Faculty of Biology, Sofia University "St. Kliment Ohridski", 8 "Dragan Tsankov" Blvd, 1164 Sofia, Bulgaria*

Corresponding author: Galina Radeva (gradeva@bio21.bas.bg)

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Abstract

This study presents the distribution of bacterial and fungal abundances in long-term copper (Cu) contaminated soils in Topolnitsa-Pirdop valley – a highly industrialized zone with a number of mines and processing plants for copper and other non-ferrous metals. The bacterial (16S rRNA gene copies) and fungal (ITS rRNA gene copies) were estimated using quantitative PCR technique in five topsoils, differently Cu contaminated (ranging from 28.05 to 198.9 mg kg^{-1}). Bacterial abundance varied in a range of 1.68 × 10^{11} to 3.24 × 10^{11}16S rRNA genes, whereas fungi amounted from 1.95 × 10^{8} to 6.71 × 10^{8} ITS rRNA genes. Fungal and bacterial abundances were significantly (fungi) and insignificantly (bacteria) influenced by Cu contamination. The fungal/bacterial ratio related negatively with soil Cu, which shifted microbial communities' structure towards bacterial dominance. Since the ratio between bacteria and fungi are vital in explaining many soil functions, the calculated changes in this ratio indicated deterioration in soil quality, being of primary importance for plant production.

Keywords

ITS rRNA gene, microbial abundance, qPCR, soil contamination, 16S rRNA gene

Introduction

Heavy metals (HMs) are natural constituents of the environment, but intensive use for human purposes has altered their geochemical cycles and biochemical balance. The extraction and processing of mineral raw materials are one of the main sources of soil and water pollution with HMs and other toxic elements. In this respect, of particular interest are the open-pit mines and their generated waste materials, which cause serious environmental damage and significant changes to the landscapes of the impacted regions (Dabeva et al. 2012). Prolonged exposures to HMs in high concentrations are harmful to microorganisms, plants and animals. Furthermore, HMs can be absorbed by food crops, and entering into the food chain has proven to be a potential health hazard for plants and humans (Huang et al. 2018; Ali et al. 2019; Afonne and Ifediba 2020; Hasan et al. 2020). Microorganisms are highly diverse and ubiquitous in soil ecosystems and participate in a variety of key ecosystem functions such as nutrient cycling, structuring of soil aggregates and biomass production. A stable microbial community contributes essentially to stabilizing soil structure and maintaining soil ecosystem services (Bissett et al. 2013). Studies have shown that microorganisms can significantly promote the circulation of soil nutrients, maintain soil fertility, and improve crop health (Fierer 2017). The negative impacts of HMs on soil microbial communities have been reported in several studies. HMs have been shown to be harmful to soil microorganisms and may lead to significant changes in microbial diversity (Chodak et al. 2013; Zampieri et al. 2016), richness (Cui et al. 2018), abundance (Deng et al. 2015), metabolic activity (Chen et al. 2014; Hong et al. 2015; Zampieri et al. 2016; Fajardo et al. 2019) and structure of the communities (Cui et al. 2018; Feng et al. 2018; Zhang et al. 2018).

Copper is one of the most common soil contaminants (Griffiths and Philippot 2013) among heavy metals. Soils represent the largest sink of Cu, being released to the environment by anthropogenic activities, such as sewage irrigation, mining activities, municipal waste disposal, and intensive use of pesticides and herbicides (Smith 2009; Zhuang et al. 2009; Ballabio et al. 2018). A moderate level of Cu is regarded as a micronutrient and it is essential for microorganisms to carry out their metabolic activities (Giller et al. 2009). However, excessive inputs of Cu to soil ecosystems could persist for a long time after their introduction and cause toxic effects on soil microorganisms (Giller et al. 2009). Some authors found that Cu contamination had significant effects on bacterial abundance and diversity (Li et al. 2015), as well as fungal diversity (Zhang et al. 2022). Therefore, understanding the responses of soil microbial communities to Cu contamination is essential to counteract its negative effect on ecosystem functions and services.

The aim of this study was to assess the distribution of bacterial and fungal abundances in Cu long-term contaminated soils from the valley of Topolnitsa-Pirdop and its relationship with the local soil properties.

Materials and methods

Study area and soil sampling

Topolnitsa River and its tributaries (Medetska, Zlatishka, Pirdopska and Bunovska Rivers) were influenced by the ore industry, agriculture activities, and domestic wastewater discharge, which led to the deterioration of its ecological status according to the River Basin Management Plan (2016–2021) (East Aegean River Basin Directorate. MoEW). Three large metal mining and metal processing enterprises are located in this region- "Elatsite Med", "Dundee Precious Metals" and "Aurubis". Additionally, in the area, the open-pit "Medet" is located, and although the mine was closed in 1992, it is considered the main source of pollution in this area.

The study area is located in the Topolnitsa-Pirdop valley (Fig. 1). Topsoil samples (0–20 cm) with different Cu concentrations (ranging from 28.05 to 198.9 mg kg^{-1}) were collected in May 2021. Five sampling sites have been selected from a monitoring map (Kancheva et al. 2018), considering the sources of Cu pollution as follows: S1 – located in the valley of Medetska River at 710 m from the "Medet" mine tailing (42°39'12.9"N, 24°09'04.2"E), S2 – located in the valley of Topolnitsa River (after Zlatishka River) at 250 m from the "Medet" mine tailing (42°39'31.8"N, 24°08'26.6"E), S3 – located in the valley of Pirdopska River at 750 m from the "Medet" mine tailing (42°39'48.2"N, 24°08'11.3E), S4–located in the valley of Bunovska River at 2200 m from the mine tailings of "Elatsite-Med" A.D. (42°36'47.5"N, 24°00'44.8"E), and S5–located in the valley of Topolnitsa River-next to Muhovo village at 25–30 km straight from S4 and S1 (42°24'45.5"N, 23°59'54.4"E). Samples were taken in three replicates per site. All sampled soils were classified as Fluvisols (FAO 2015).

Soil physicochemical properties and copper concentration

Soil pH was potentiometrically measured in 0.1 M $CaCl_2$ soil suspension according to ISO 10390:2005. The soil organic carbon (SOC) was determined by sulfochromic oxidation according to BSS ISO 14235:2002. Nitrate (NO_3-N) and ammonium (NH_4-N) nitrogen were determined according to Keeney and Nelson (1982). Inorganic phosphate (P_2O_5) was determined by the method of Olsen (1982). Water content (WtC) was calculated after oven drying (105 °C). The concentration of Cu was measured by inductively coupled plasma-atomic emission spectrometry (ICP-AES) according to ISO 22036:2008.

DNA extraction

The genomic DNA was extracted from 0.5 g soil, using the E.Z.N.A soil DNA kit (Omega Bio-tek, USA) according to the manufacturer`s recommended protocol. The quality and quantity of the extracted DNA were subsequently assessed by Qubit 4 fluorometer (Invitrogen) and 1% agarose gel electrophoresis.

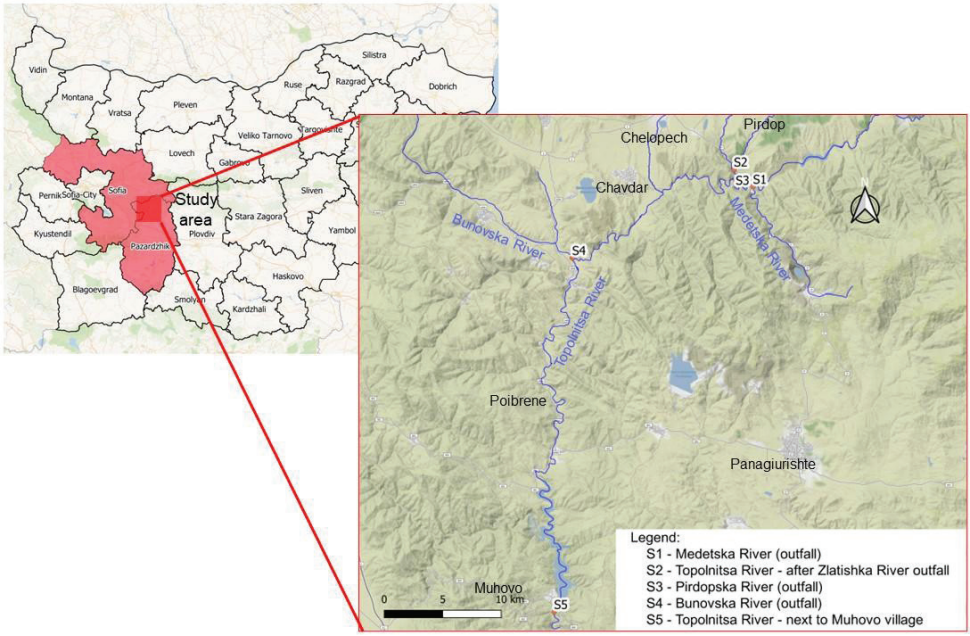

Figure 1. Map of the study area and sampling sites.

Real-time Q-PCR

Q-PCR analyses were performed using iTaqTM Universal SYBRGreen Supermix (BioRad) on Rotor Gene 6000 (Corbett Life Science) according to the manufacturer's protocols. Bacterial abundance was estimated using 0.4 μM of primers Eub338f (5'-ACTCCTACGGGAGGCAGCAG-3') and Eub518r (5'-ATTACCGCGGCTGCTGG-3') (Aleksova et al. 2020). The three-step thermal program was used at 95 °C for 5 min,45 cycles of denaturing at 95 °C for 10 s, annealing at 61 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 10 min. The plasmid DNA from the bacterial standard of Uncultured α – *proteobacterium* clone BuhD-104 (FM877535.1) was used to generate a linear calibration curve of the threshold cycle versus the gene copy numbers of four-point serial decimal dilution – 10^3, 10^5, 10^6 and 10^8. All measurements were run in triplicates. Data were expressed as gene copy numbers per gram of dry soil, and the specificity of amplicons was confirmed by melting curve and agarose gel electrophoresis. Amplification efficiency was estimated to be 109% (R^2=0.99). The results were processed via ROTOR-GENE Q SERIES, Software version 2.3.1.

The fungal abundance was estimated using 0.4 μM primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990). The following thermal program was used: 95 °C for 5 min, 45 cycles of 95 °C for 30 s, annealing 55 °C for 30 s, 72 °C for 60 s, and final extension at 72 °C for 10 min.

A four-point serial decimal dilution of a plasmid DNA of Uncultured *Basidiomycota* clone LS_Az0_D1_31 (MT785782.1) was used as a standard to generate a linear calibration curve of the threshold cycle versus a number of gene copies ranging from 10^2 to 10^5. All measurements were run in triplicates. Data were expressed as gene copy numbers per gram of dry soil. Amplification efficiency was estimated to be 92% (R^2=0.99). The results were analysed via ROTOR-GENE Q SERIES, Software version 2.3.1.

The relative ratio of fungi/bacteria was calculated as the ratio of the fungal gene copy numbers to bacterial gene copy numbers (Fierer et al. 2005).

Statistical analysis

Pearson correlation analysis was used to find the relationships between soil metrics. Hierarchical clustering (Algorithm: UPGMA; Similarity index: Bray-Curtis) was conducted to evaluate the distance between soil physical environments. Non-metric multidimensional scale (nMDS) ordination was applied to assess the similarity between abundance-weighted microbial communities. Statistical analyses were performed using the software PAST ($p<0.05$).

Results

Soil environmental variables

The values of studied soil properties are shown in Table 1. The soil pH was moderately acidic – pH 5.57 (S1 and S2) to 5.76 (S4). SOC ranged from 1.22 g kg^{-1} (S3) to 2.67 g kg^{-1} (S2). Concentrations of inorganic nitrate (except S3) and ammonium (except S2 and S4) ions and inorganic phosphates were relatively equally distributed among the soils.

Cu concentrations varied among the soils as follows: the concentrations in S1 and S3 exceeded the maximum permissible concentration (MPC) of 80 mg kg^{-1} under Bulgarian Regulation 3/2008 (2008) (http://eea.governement.bg/bg/legislation/soil). The most contaminated soils are located near the tailing of mine "Medet" in the valleys of the Medetska and Pirdopska Rivers. In the other soils, S2, S5 and S4, the concentrations of Cu were under or around MPC. In this regard, S5 can be assumed as a reference site.

Table 1. Soil physicochemical properties and Cu concentrations in the region of Topolnitsa River and its tributaries.

Soil sample	Soil physicochemical properties						
	pH	WtC (%)	SOC g kg^{-1}	NO_3-N mg kg^{-1}	NH_4-N mg kg^{-1}	P_2O_5 mg kg^{-1}	Cu mg kg^{-1}
S1	5.57	13.33	2.56	11.44	7.82	2.93	198.90
S3	5.60	11.67	1.22	0.65	8.32	2.37	110.00
S4	5.76	12.33	2.16	8.84	5.74	3.40	82.40
S2	5.57	12.33	2.67	11.12	3.07	3.47	67.40
S5	5.61	10.00	2.05	†ND	10.74	2.78	28.50

†ND – No data.

Pearson correlation analysis indicated a linear relationship (-0.61; p=0.015) between the level of soil Cu contamination and the distance to the source of pollution.

A cluster analysis of soil abiotic factors was conducted to visualize the similarity among the tested soils (Fig. 2).

Two main clusters were demonstrated: cluster I containing soil S5 with the lowest Cu concentration and cluster II, consisting of other soils sub-clustered according to the Cu threshold concentration of 100 mg kg^{-1}. This pattern of soil clustering identified the major role of Cu in structuring soil environments.

Soil bacterial abundance

Bacterial abundance varied in a relatively narrow range. The highest number was observed in S4 (3.24×10^{11}), and the lowest one in S5 (1.68×10^{11}) (Fig. 3). Pearson correlation analysis did not indicate a significant correlation (0.31; p=0.26) between bacterial abundance and soil Cu concentrations.

Soil fungal abundance

The highest fungal abundance was detected in uncontaminated soil S5 (6.71×10^{8}) and the lowest one in S3 (1.95×10^{8}) (Fig. 4). The fungal gene copy number variance among the sampling soils was higher (Coef. variance – 40.48) than that of bacterial gene copy number (Coef. variance – 25.07), indicating possible resistance of bacteria to Cu toxicity.

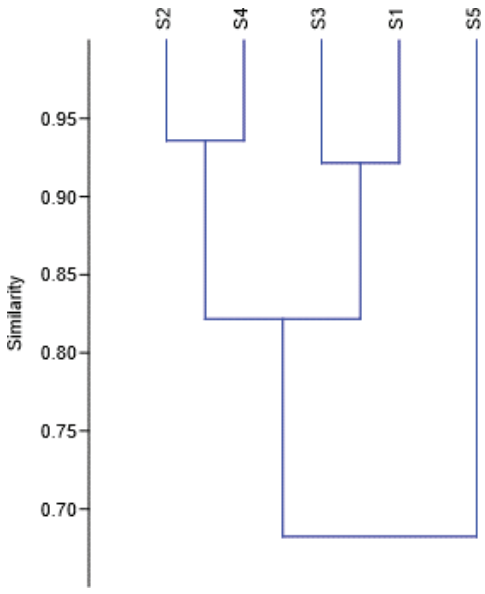

Figure 2. Cluster analysis of soil physical environments.

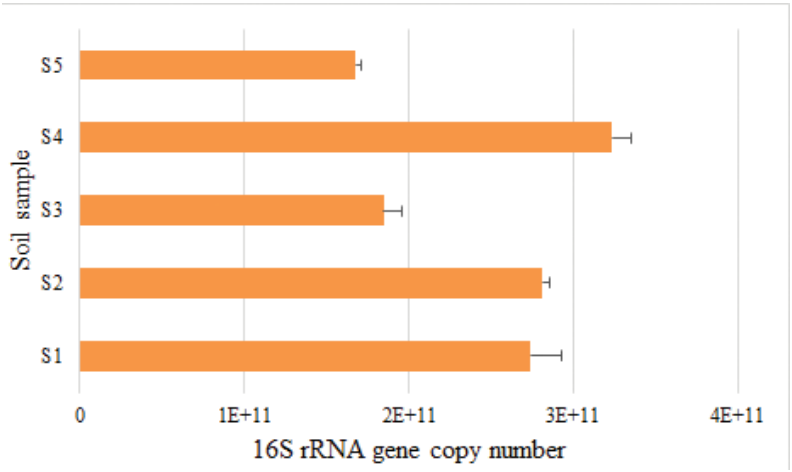

Figure 3. A bacterial abundance of 16S rRNA gene. Error bars are the standard deviations of the mean for the three replicates.

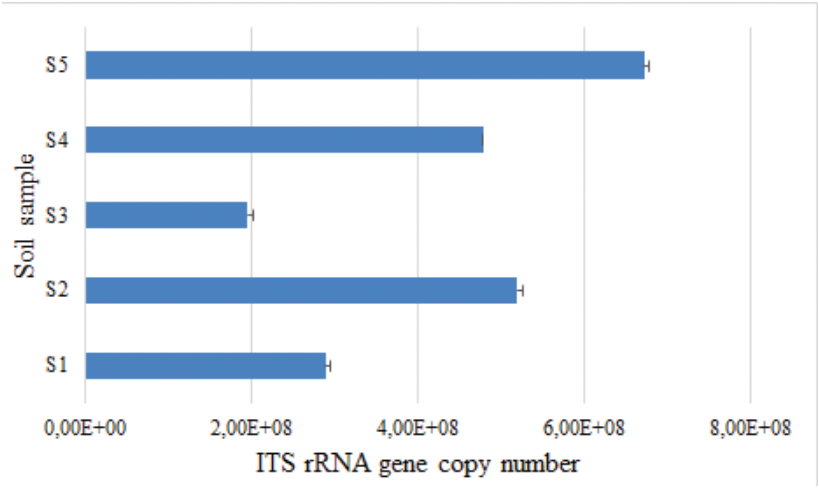

Figure 4. An abundance of ITS rRNA gene. Error bars are the standard deviations of the mean for the three replicates.

Pearson correlation analysis showed a significant negative correlation (-0.76; p=0.001) between fungal abundance and soil Cu contamination.

The highest relative fungal to bacterial abundance was observed in the uncontaminated soil S5 (3.98×10^{-3}), and the lowest ratio was recorded in the highly contaminated soil S3 (6.80×10^{-4}) (Fig. 5). Fungal to bacterial ratio related negatively with soil Cu (-0.66; p=0.007), probably as a result of the higher Cu impact on the fungal abundance.

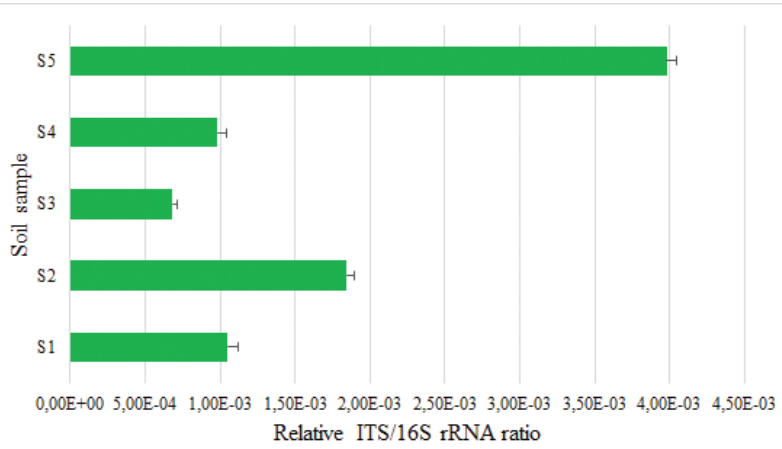

Figure 5. Relative fungal: bacterial ratio in the studied soils. Error bars are the standard deviations of the mean for the three replicates.

Relationships between microbial abundance, soil properties and Cu concentrations

Statistical non-metric multidimensional scaling (nMDS) was performed on patterns of fungal and bacterial abundances to assess soil similarities with respect to their microbiology (Fig. 6).

Plots separated the soils into four distinct abundance-weighted microbial communities The soils S2 and S4 grouped closely, indicating similar microbial abundances. The other soils were placed individually both away from the other and from the group S2 and S4. This distribution was probably due to differences in microbial abundance-weighted communities. Our results showed that the important factors influencing soil microbial abundance were soil Cu concentration and water content in S1, and soil organic carbon and phosphates concentrations in S2 and S4 (Fig. 6).

Discussion

The present study was focused on the distribution of microbial abundance as a response to the influence of Cu contamination in the area of Topolnitsa-Pirdop valley – a highly industrialized zone with a number of mines and processing plants for Cu and other non-ferrous metals.

The soil concentrations of the main contaminant Cu varied from 28.5 mg kg^{-1} to 189.9 mg kg^{-1}, being above MPC in three (S1, S3, and S4) of five tested soils. The mode of clustering highlighted the significant effects of Cu on soil environments, grouping soils in clusters or sub-clusters depending on the level of contamination. Additionally, soil pH (acidic) may influence soil microbial communities by primarily suppressing the bacterial diversity (Fierer and Jackson 2006) and increasing the mobility/toxicity (Wang et al. 2022) of HMs, including Cu. We expected also a high manifestation of Cu toxicity, taking into account the low levels of SOC (Chen et al. 2014).

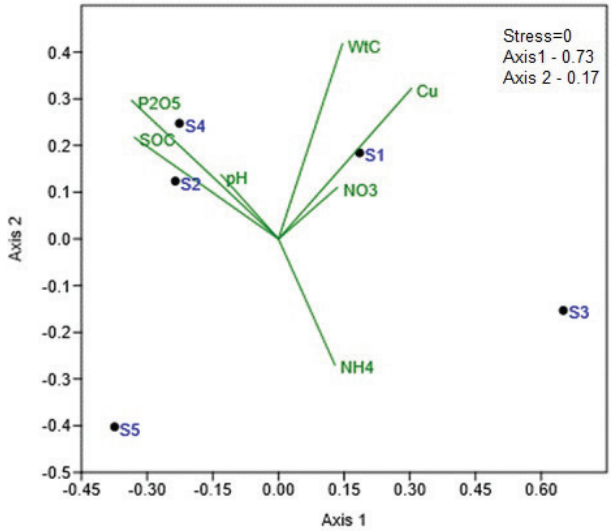

Figure 6. Microbial abundances in soils of Topolnitsa-Pirdop valley as indicated by non-metric multi-dimensional scaling plots.

The results showed high bacterial abundance in soils, ranging from 1.68×10^{11} to 3.24×10^{11} 16S rRNA gene copy numbers, being one-fold higher compared to the abundances estimated in our previous studies on mining activities in the area, where the soil Cu concentrations ranged from 53 mg kg^{-1} to 860 mg kg^{-1} (Aleksova et al. 2020; Palov et al. 2020; Nikolova et al. 2022). We suggested that this difference resulted from the ranges of soil Cu concentrations, but also microbial abundances might be edaphic- and climatic-dependent. In this respect, our results confirmed the findings of Yin et al. (2015), that long-term contamination with high Cu concentrations can decrease soil bacterial abundance. In our study, Pearson correlation analysis showed a lack of significant correlation between bacterial abundance and soil Cu concentration, probably due to the relatively low levels and long history of soil contamination. Hence, we could assume that bacteria developed mechanisms for adaptation to the newly created soil environments, and relatively low Cu concentrations can even stimulate their proliferation (positive correlation between Cu and relative bacterial abundance) (Zhao et al. 2019).

Similar to other authors (Chen et al. 2014), we found that the fungal abundance was sensitive to heavy metal contamination stress in the opposite to bacteria, proved by the significant and negative relationship between fungi and Cu. We suggested that the different sensitivity of bacteria and fungi to Cu resulted from their patterns of microbial- and HM- distributions among soil fractions. Several authors reported that bacteria dominated in silt and clay fractions, whereas fungi inhabited mainly the coarse sand fraction (Kandeler et al. 2000; Sessitsch et al. 2001; Poll et al. 2003). Using the Nemerow pollution index in their study, Chen et al. (2014) concluded that HMs (Cu, Pb, Zn) and their bioavailable forms were distributed in the order: coarse sand > clay > silt > fine sand. The similar mode of fungi and Cu distribution among soil fractions might be interpreted as a prerequisite for the higher Cu toxicity compared to bacteria.

The higher exerted Cu toxicity on fungi reflected also on the fungal/bacterial ratio, which decreased with increasing soil Cu contamination. According to some authors (Rajapaksha et al. 2004; Chen et al. 2014), the fungal/bacterial ratio is a sensitive indicator for soil health and its decrease in Cu-contaminated soils is a sign of deteriorated soil quality. Chen et al. (2014) reported a similar pattern of decreasing fungal/bacterial ratios under HM contamination in their study.

Although microbial communities were differently abundance-weighted (except that of S2 and S4) according to the ordination procedure nMDS, it demonstrated that only the highest soil Cu concentration (189.9 mg kg^{-1}) influenced significantly soil microbial communities (S1). The abundances of soil microorganisms from the other sampling sites were under the influence of local soil abiotic factors.

Conclusion

The present study showed that microbial abundance, especially fungal, was significantly affected by long-term Cu contamination of Fluvisols. The Cu shifted microbial communities' structure towards bacteria, suggesting that in this case, bacteria could be better at developing Cu resistance than fungi. Further studies should be implemented to clarify microbial functional responses to Cu.

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Genotype differences towards lead chloride harmful action

Teodora Ivanova Todorova[1], Petya Nikolaeva Parvanova[1], Krassimir Plamenov Boyadzhiev[1], Martin Dimitrov Dimitrov[1], Stephka Georgieva Chankova[1]

1 *Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 2 Gagarin Str, 1113, Sofia, Bulgaria*

Corresponding author: Teodora Ivanova Todorova (tedi_todorova@yahoo.com)

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Abstract

The aim of the study was to throw more light on the $PbCl_2$ mode of action (MoA) depending on the genotype by the application of three model organisms and microbiological, biochemical, and molecular approaches.

Three model systems – *Chlamydomonas reinhardtii* strain 137C – wild type (WT), *Saccharomyces cerevisiae* strain D7ts1, and *Pisum sativum* L. cultivar Ran1 and two experimental schemes – short- and long-term treatments were used. *C. reinhardtii* and *S. cerevisiae* cell suspensions (1×10^6 cells/ml) at the end of the exponential and the beginning of a stationary phase of growth were treated with various $PbCl_2$ concentrations (0.45–3.6 mM) for 2 hours. Lower $PbCl_2$ concentrations (0.03–0.22 mM) were also tested on *C. reinhardtii* 137C. Short-term treatment for up to 2 days with $PbCl_2$ concentrations in the range of 0.45–3.6 mM and long-term treatment for up to 10 days with concentrations in the range of 0.45–2.7 mM was performed on *P. sativum* L. seeds and plants, respectively. Long-term treatment with a $PbCl_2$ concentration of 3.6 mM was not tested because of the very strong toxic effect (plant death). The following endpoints were used – for *C. reinhardtii*: cell survival, "visible" mutations, DNA double-strand breaks (DSBs), malondialdehyde (MDA), intracellular peroxides (H_2O_2), and photosynthetic pigments; for *S. cerevisiae* – cell survival, gene conversion, reverse mutation, mitotic crossing-over, DSBs, superoxide anions, MDA and glutathione (GSH); *P. sativum* L. – germination and root length (short-term treatment), pro-oxidative markers – MDA, H_2O_2 and photosynthetic pigments (long-term treatment).

Genotype differences between *C. reinhardtii* (0.047 mM) and *S. cerevisiae* (1.66 mM) were observed by two endpoints: concentrations inducing 50% lethality (LD_{50}) and DSB induction. By contrast, no mutagenic effect was found for both unicellular test models. A slight toxic capacity of $PbCl_2$, measured as inhibition of *Pisum sativum* L. seed germination and around 20% root length reduction was revealed after the treatment with concentrations equal to or higher than 1.8 mM.

The variety of stress responses between the two plant test models was demonstrated by comparing MDA and H_2O_2. A dose-dependent increase in H_2O_2 levels and a minor increase of MDA levels (around 9–15%) were measured when *C. reinhardtii* cells were treated with concentrations in the range of LD_{20}–LD_{80} (0.03–0.11 mM). Analyzing the kinetics of MDA and H_2O_2 in pea leaves, the most pronounced effect of concentration was shown for 2.7 mM. A decrease in the photosynthetic pigments was detected in the two experimental designs – short-term on *C. reinhardtii* and long-term on *P. sativum* treatments. The pro-oxidative potential was also proven in *S. cerevisiae* based on increased levels of MDA and superoxide anions and decreased GSH.

New information is gained that $PbCl_2$ can affect the DNA molecule and photosynthetic pigments via induction of oxidative stress. Our study revealed that the magnitude of stress response towards $PbCl_2$ is genotype-specific. Our finding that *Chlamydomonas reinhardtii* is a sensitive test system towards $PbCl_2$ contributes to good strategies for revealing very low levels of contaminants present chronically in main environmental matrices.

This is the first report, as far as we know, affirming that $PbCl_2$ can induce DSBs in *Chlamydomonas reinhardtii* and *Saccharomyces cerevisiae*.

Keywords

Chlamydomonas reinhardtii, DNA damaging potential, lead chloride, mutagenicity, *Pisum sativum* L., pro-oxidative effect, *Saccharomyces cerevisiae*, toxicity/genotoxicity

Introduction

Currently, environmental pollution with various chemicals is considered as an important environmental problem provoked to a large extent by human activity (Zulfiqar et al. 2019). Due to this fact, strict legislation was created, controlling both manufacturing processes, the levels released into nature as well as maximum levels of certain contaminants in food, water, air, etc. (See Commission Regulation EC documents 2016/582, 2022).

Over the years significant contamination of the main ecological matrices with heavy metals such as cadmium, lead, chromium, copper, zinc, mercury, and arsenic has been reported by different authors (Schulin et al. 2007; Kabir et al. 2012; Satta et al. 2012). The increased levels of heavy metals as a result of human activity – industry, agriculture (irrigation with contaminated water, use of mineral fertilizers), waste burning, burning of fuel, road transport, etc. has caused concern about both the health of nature and man (Järup 2003; European Commission 2013; Tóth et al. 2016).

Heavy metals commonly present in the form of cations possess two main features: quick accumulation and slow release. Mercury, cadmium, and lead are considered substantial risk factors for the biota.

Lead (Pb) is considered the second most toxic metal after arsenic (As) as it is very toxic for all living organisms (ATSDR 2019; Zulfiqar et al. 2019). Sources of lead could be dust, old paint, different user products, leaded gasoline, batteries, smelting and refining processes, etc. (Can et al. 2008; Wani et al. 2015; Kumar et al. 2020).

The poisoning capacity of lead has been known since ancient times. In the last century, toxic effects of lead and its compounds were the focus of scientists. A lot of data were collected concerning lead's high accumulation in different body tissues and organs as well as its toxic capacity as a result of exposure to contaminated water, air, and food (Balali-Mood et al. 2021; Mohanta et al. 2022).

Currently, new experimental data were gathered suggesting lead's indirect mechanisms of genotoxicity – the production of free radicals, altered expression of DNA repair genes, and inhibition of DNA repair systems (García-Lestón et al. 2010; Hemmaphan and Bordeerat 2022).

For the first time, evidence was published by Liu et al. (2018) concerning the DNA damaging potential of lead by promoting oxidative stress as well as the methylation of DNA repair genes in human lymphoblastoid TK6 cells.

Everything written above demonstrated that lead and its compounds have been characterized as toxic/genotoxic in a variety of test systems but little is currently known about their mutagenic, clastogenic, carcinogenic capacity, and mode of action (MoA).

In short, at present, information concerning the potential pro-oxidative, mutagenic, and DNA damaging potential of lead and its compounds as well as its MoA, is scarce.

Here we aimed to try to compensate for this gap to some extent using three model organisms and different approaches – microbiological, biochemical, and molecular in order to shed more light on the MoA of $PbCl_2$ depending on the genotype.

This investigation was performed using three model organisms – unicellular green alga *Chlamydomonas reinhardtii*, *Saccharomyces cerevisiae*, and *Pisum sativum* L.

Unicellular green algae, including *C. reinhardtii*, are a robust model for plant cells in genetic, molecular, physiological, and eco-toxicological studies due to their advantages which have been well described previously (Chankova et al. 2000, 2005, 2007, 2014; Chankova and Bryant 2002; Dimitrova et al. 2007). On the other hand, results obtained in this model organism could be extrapolated to a variety of plant organisms (Merchant et al. 2007; Li et al. 2020).

S. cerevisiae is an extensively used model for studying the response of heavy metals due to the highly conservative mechanisms related to different stress response pathways as well as protein similarity with higher eukaryotes including humans (Rajakumar et al. 2020; Todorova et al. 2015a). The entirely sequenced *S. cerevisiae* genome reveals around 31% similarity to the human genome and thus results could be extrapolated at human level (Todorova et al. 2015b).

Pisum sativum L. (garden pea) is a classic model organism used in biochemical, physiological, and genetic studies of plants. In addition, it should be mentioned that *Pisum sativum* L. is one of the most important food items among legume crops (Galal et al. 2021). The variety Ran 1, is a widely popular agricultural crop in Bulgaria.

Materials and methods

Model systems, cultivation, and experimental schemes

Three model systems – *Chlamydomonas reinhardtii* strain 137C – wild type (WT), *Saccharomyces cerevisiae* strain D7ts1, and *Pisum sativum* L. cultivar Ran1, and two experimental schemes – short – and long-term treatments were used. $PbCl_2$ of analytical grade was purchased from Valerus LTD.

Short-term treatment

Chlamydomonas reinhardtii 137C was cultivated at standard conditions – light of 70 μmol/m^2.s and t = 25 ± 3 °C and *Saccharomyces cerevisiae* strain D7ts1 was cultivated at t = 30 °C, 200 rpm to the end of the exponential and the beginning of a stationary phase of growth. After that, cell suspensions with a density of 1×10^6 cells/ml were treated with various $PbCl_2$ concentrations (0.45, 0.9, 1.8, 2.7, and 3.6 mM) for 2 hours. Additionally, due to the very low cell survival of *C. reinhardtii* 137C after the treatment with this concentrations' range, lower concentrations were also tested – 0.03, 0.06, 0.11, and 0.22 mM.

Pisum sativum L. seeds were treated with $PbCl_2$ at concentrations of 0.45, 0.9, 1.8, 2.7, and 3.6 mM for 24 and 48 hours to evaluate both the germination and the root length.

Long-term treatment

Pea plants were grown on a Knop medium until they reached the third physiologically developed leaf (around 10 days) under controlled conditions in a growth chamber NUVE GC 400, light regime 16/8 day/night; a temperature of 24 ± 2 °C; humidity of 70 ± 5%. After that, the Knop medium of the plants was replaced with $PbCl_2$ solutions with various concentrations in the range of 0.45, 0.9, 1.8, and 2.7 mM for 10 days. Experiments for pro-oxidative potential were conducted in order to study the kinetics of these markers. Plants were grown for 2, 5, 7, and 10 days in a medium contaminated with different $PbCl_2$ concentrations written above; leaves' samples were subsequently collected and biochemical analyses were performed.

The genotoxic potential of $PbCl_2$ on *Chlamydomonas reinhardtii* was evaluated as described in Dimitrova et al. 2014. In short, a "clonal assay" was performed to evaluate the colony-forming ability of the strain after the treatment, counting macro-colonies' survival.

On *Saccharomyces cerevisiae*, the Zimmermann test was used (Zimmermann et al. 1984).

The survival fraction (SF) (Bryant 1968) as well as concentrations that can induce 20, 50, and 80% lethality was calculated (Lidanski 1988; Dimitrova et al. 2014). The toxicity was evaluated based on the inhibition of *Pisum sativum* L. germination and reduction of root length.

Oxidative stress markers

Intracellular malondialdehyde (MDA) was measured at 532 nm and 600 nm as described by Dhindsa et al. (1981). H_2O_2 content was measured at 390 nm (Heath and Packer 1968). Pigment contents were measured at 663, 645, and 452.5 nm (Arnon 1949; Dimitrova et al. 2007) on Ultrospec 2100 pro spectrophotometer (Amersham Biosciences). Total glutathione was measured according to Zhang (2000). Superoxide anions were evaluated as described in Stamenova et al. (2008).

Mutagenic action

A test of "visible mutant colonies" was applied to evaluate the mutagenic potential of $PbCl_2$ on the unicellular algae after that the Mutagenic Index was calculated as described by (Dimitrova et al. 2007). Changes in size, morphology, and pigmentation of surviving colonies were analyzed (Shevchenko 1979).

Zimmermann's test (Zimmermann et al. 1984) with *Saccharomyces cerevisiae* diploid strain D7ts1 (*MAT*a/*a ade2-119/ade2-40 trp5-27/trp5-12 ilv1-92/ilv1-92 ts1/ts1*) was applied as described before (Todorova et al. 2015b). The test provides simultaneous detection of cell survival, mitotic gene conversion at the *trp-5* locus, and reversion mutations in the *ilv1* locus.

DNA damaging potential

DNA double-strand breaks (DSBs) were measured by Constant field gel electrophoresis (CFGE) as described in Chankova and Bryant (2002), Chankova et al. (2005), and Todorova et al. (2015a, 2019).

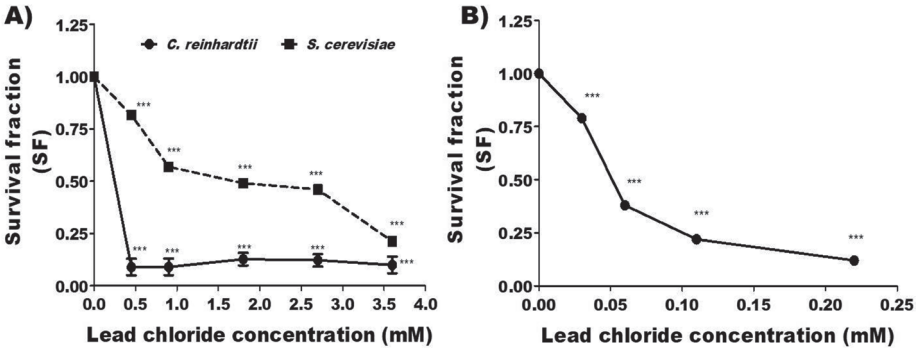
Statistical analysis

Data were analyzed using GraphPad Prism5 software (San Diego, USA) and the statistical analysis was done by one-way and two-way analysis of variances (ANOVA) followed by the Bonferroni posthoc multiple comparisons test. Linear correlation, using Pearson Product- Moment Correlation Coefficient analysis (PMCC, or r) and coefficient of determination (R^2) were determined. All the experiments were performed in triplicate. Data are presented as mean ± SEM (standard error of the mean).

Results

Genotoxicity in *Chlamydomonas reinhardtii* and *Saccharomyces cerevisiae*

Genotype-related differences in the response toward lead treatment were identified. Treatment with concentrations in the range of 0.45–3.6 mM $PbCl_2$ resulted



C)

	Chlamydomonas reinhardtii (WT)	*Saccharomyces cerevisiae* (D7ts1)
	Lead chloride concentration (mM)	
LD20	0.03	0.47
LD50	0.05	1.66
LD80	0.14	3.67

Figure 1. A Cell survival of *Chlamydomonas reinhardtii* (black circle) and *Saccharomyces cerevisiae* (black square) after the treatment with $PbCl_2$ concentrations in the range of 0.45–3.6 mM for 2 hours **B** cell survival of *Chlamydomonas reinhardtii* after the treatment with $PbCl_2$ concentrations in the range of 0.03–0.22 mM for 2 hours **C** three doses of lethality were calculated. Results are from at least three experiments with independently grown cell cultures and presented as mean ± SEM. Asterisks represent statistical significance (ns $P > 0.05$; *** $P < 0.001$). Where no error bars are evident, they are equal to or smaller than the values.

in a differential response of *Chlamydomonas reinhardtii* and *Saccharomyces cerevisiae* (Fig. 1A). Dose-dependent decrease of cell survival ($P<0.0001$) up to 25% was obtained for *S. cerevisiae* D7ts1 (Fig. 1A). All the concentrations tested resulted in around 10% cell survival of *C. reinhardtii* 137C. No effect of the concentration was calculated.

Further, lower $PbCl_2$ concentrations were tested in *C. reinhardtii* 137C (Fig. 1B). A dose-dependent decrease of survived colonies was obtained when concentrations in the range of 0.03–0.22 mM ($P < 0.0001$) were applied ($P < 0.0001$).

Based on the survival data, three doses of lethality were calculated (Fig. 1C). Data revealed that an around 30-fold lower dose can cause 50% lethality in *C. reinhardtii* in comparison with *S. cerevisiae*.

Toxicity in *Pisum sativum* L.

A slight toxic capacity of lead chloride on *P. sativum* L. was evaluated. Around 10% inhibition of seed germination (Fig. 2A) and around 20% root length reduction (Fig. 2B) were calculated after the treatment with $PbCl_2$ concentrations equal to or higher than 1.8 mM.

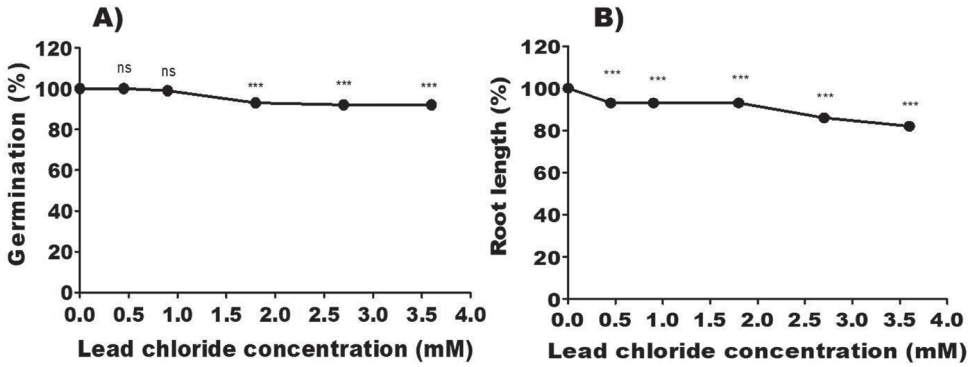


Figure 2. Toxic potential of various concentrations of $PbCl_2$ on *Pisum sativum* L. presented as percent germination (**A**) and root length (**B**). Results are from at least three experiments and presented as mean ± SEM. Asterisks represent statistical significance (ns $P > 0.05$; *** $P < 0.001$). Where no error bars are evident, they are equal to, or smaller than, the values.

Even though the decreases in both parameters were calculated as statistically significant to the corresponding control sample, they can hardly be regarded as significant from a biological point of view.

Pro-oxidative potential after short-term treatment with $PbCl_2$ on the unicellular model systems – *C. reinhardtii* and *S. cerevisiae*

The pro-oxidative potential of $PbCl_2$ was studied by several endpoints in the range of concentrations corresponding to LD_{20}, $LD_{50,}$ and LD_{80}.

A very minor increase in MDA levels (around 9–15%) (Fig. 3) was measured when *C. reinhardtii* cells were treated with concentrations in the range of LD_{20}–LD_{80} (0.03–0.11 mM) ($P < 0.001$).

Concerning the other pro-oxidative marker, dose-dependent statistically significant higher levels of intracellular peroxides were obtained (Fig. 3).

More than 3-fold higher levels of both MDA and H_2O_2 were induced after the treatment with concentrations that can cause over 80% cell lethality.

As a next step, the levels of photosynthetic pigments were evaluated (Fig. 3). A similar – around 50–60% decrease in chlorophyll *a* (chl *a*), chlorophyll *b*, and carotenoids were calculated without concentration dependence ($P < 0.0001$).

Concerning the other model system – *S. cerevisiae*, treatment with concentrations corresponding to LD_{20}, LD_{50}, and LD_{80} resulted in a statistically significant dose-dependent increase in the superoxide anions' levels (Table 1). The most pronounced effect – more than 2-fold higher levels of superoxide anions was calculated at LD_{80} ($P < 0.01$). Around a 2-fold increase with no effect of the concentration was obtained for MDA (Table 1). At the same time, a decrease in the total glutathione levels was measured for the same experimental conditions ($P < 0.0001$).

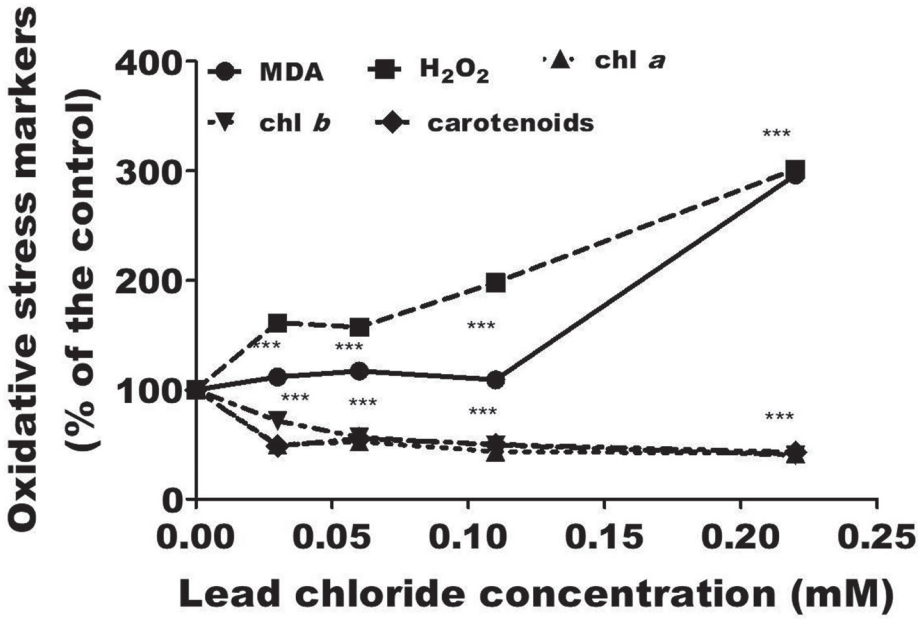


Figure 3. Oxidative stress induced by $PbCl_2$ at concentrations range of 0.03–0.22 mM in *Chlamydomonas reinhardtii*. Data are presented as mean ± SEM. All the results are from at least three independent experiments. Statistical significance was calculated among all the samples and to the controls ($P < 0.001$). Where no error bars are evident, they are equal to or smaller than the values.

Table 1. Oxidative stress markers in *Saccharomyces cerevisiae* after the treatment with various $PbCl_2$ concentrations for 2 hours.

	Superoxide anions (pM O2-/cell)	MDA (mM/g sample)	GSH (mmol GSH/g sample)
Control	0.404 ± 0.01	0.128 ± 0.039	0.003 ± 0.00006
LD_{20}	0.360 ± 0.04ns	0.260 ± 0.027*	0.002 ±0.00006***
LD_{50}	0.626 ± 0.03*	0.280 ± 0.012*	0.001 ±0.00006***
LD_{80}	0.965 ± 0.05***	0.296 ± 0.009**	0.001 ±0.00007***

Mean values were calculated from at least 3- experiments by independently grown cell cultures. Asterisks represent statistical significance between the control and the samples (ns $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Pro-oxidative potential after long-term treatment with $PbCl_2$ on *P. sativum* L.

No statistically significant differences were measured in the levels of MDA, intracellular peroxides, and the photosynthetic pigments (chl *a*, chl *b*, chl *a*/*b*, and carotenoids) in control samples grown without $PbCl_2$ for 2, 5, 7 and 10 days (data not shown).

The calculation of kinetics data shows that plants grown in an environment contaminated with different $PbCl_2$ concentrations for 2 days did not suffer at these experimental conditions. No statistically significant increase in MDA and intracellular peroxides was defined (data not shown).

Table 2. Kinetics of the oxidative stress markers MDA and intracellular H_2O_2 in *Pisum sativum* L. after long-term treatment (2, 5, 7, and 10 days) with various $PbCl_2$ concentrations.[1]

Concentrations	Marker	Days		
		5	7	10
Control	MDA	100	100	100
	H_2O_2	100	100	100
0.45 mM	MDA	140±10.87***	133±2.86***	125±2.66**
	H_2O_2	99±3.47 ns	96±9.80 ns	113±1.55 ns
0.9 mM	MDA	131±6.34***	123±1.63*	111±3.70 ns
	H_2O_2	93±2.55 ns	91±2.46 ns	100±3.32 ns
1.8 mM	MDA	121±7.64*	123±3.86*	127±4.77**
	H_2O_2	97±3.49 ns	104±10.02ns	128±8.65**
2.7 mM	MDA	129±9.88**	143±5.54***	166±7.53***
	H_2O_2	119±3.12 ns	129±9.85**	172±9.43***

[1] Results are calculated as a percent of the control. Data are presented as mean ± SEM. All the results are from at least three independent experiments. Asterisks represent statistical significance between the control and the treated samples (ns $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Treatment with $PbCl_2$ concentrations from 0.45 to 1.8 mM resulted in an approximately similar increase in both MDA and H_2O_2 levels (Table 2). No effect of exposure time was found. The most pronounced oxidative stress measured as increased MDA and H_2O_2 contents was calculated when plants were grown for 10 days in a contaminated with 2.7 mM $PbCl_2$ medium.

A similar relationship was defined concerning the other marker for oxidative stress induced by $PbCl_2$ and measured as levels of H_2O_2. The only statistically significant increase was calculated when plants were treated for 10 days with concentrations of 1.8 and 2.7 mM (Table 2).

Based on these results, it could be speculated that in our experimental conditions, $PbCl_2$ most probably induces lipid peroxidation in *Pisum sativum* L. plants.

Here, we have not discussed the effects obtained after the applications of the most favorable for *Pisum sativum* L. plants experimental conditions – the lowest concentration of 0.45 mM $PbCl_2$ and two days duration of plants growing at different $PbCl_2$ concentrations due to the fact that both factors have not affected in any way photosynthetic pigments contents (data not shown).

Concerning the other concentrations in the range of 0.9–2.7 mM, data revealed that the photosynthetic pigments are affected mostly by the concentration and not by the treatment time (Fig. 4A–C). The only time-dependent decrease (Fig. 4B) was detected for chl *b* and treatment with 0.9 mM ($P < 0.001$).

Our results have revealed the harmful potential of $PbCl_2$ on *P. sativum* L. plants grown for 2, 5, 7, and 10 days in a medium contaminated with various $PbCl_2$ concentrations. We found a statistically significant decrease in the levels of photosynthetic pigments we analyzed by around 40–60%, as well as no changes in the chl a/b ratio compared with those in the controls samples.

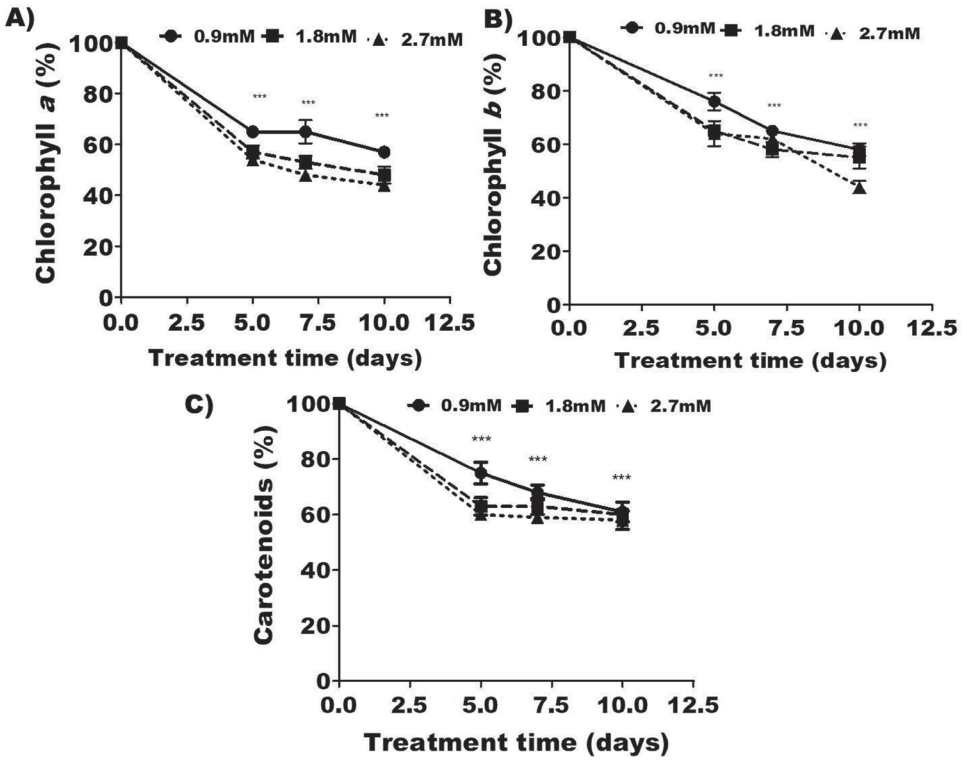


Figure 4. Kinetics of the photosynthetic pigments chlorophyll *a* (**A**), chlorophyll *b* (**B**), and carotenoids (**C**) in *Pisum sativum* L. treated with various $PbCl_2$ concentrations for different time (2, 5, 7, and 10 days). Results are calculated as a percent of the control. Data are presented as mean ± SEM from at least three independent experiments. Asterisks represent statistical significance between the control and the treated samples (*** $P < 0.001$). Where no error bars are evident, they are equal to, or smaller than. the values.

Our further steps were to clarify whether $PbCl_2$ would have some mutagenic and DNA damaging capacity on both unicellular test systems – *Chlamydomonas reinhardtii* strain 137C and *Saccharomyces cerevisiae* strain D7ts1 at our experimental conditions.

Mutagenic activity

No mutagenic potential of $PbCl_2$ was revealed on both unicellular organisms, despite the well-pronounced genotoxic effect. The mutagenic index in *Chlamydomonas reinhardtii* was calculated to be less than 2.5, indicating no mutagenic capacity of $PbCl_2$ in the tested concentration range. The mitotic gene conversion and reverse mutations in *Saccharomyces cerevisiae* were comparable with the control untreated cells (data not shown).

DNA damaging activity

To throw more light on the mode of action (MoA) of $PbCl_2$, its potential to induce double-strand breaks in DNA was evaluated on *C. reinhardtii* (Fig. 5A, C) and

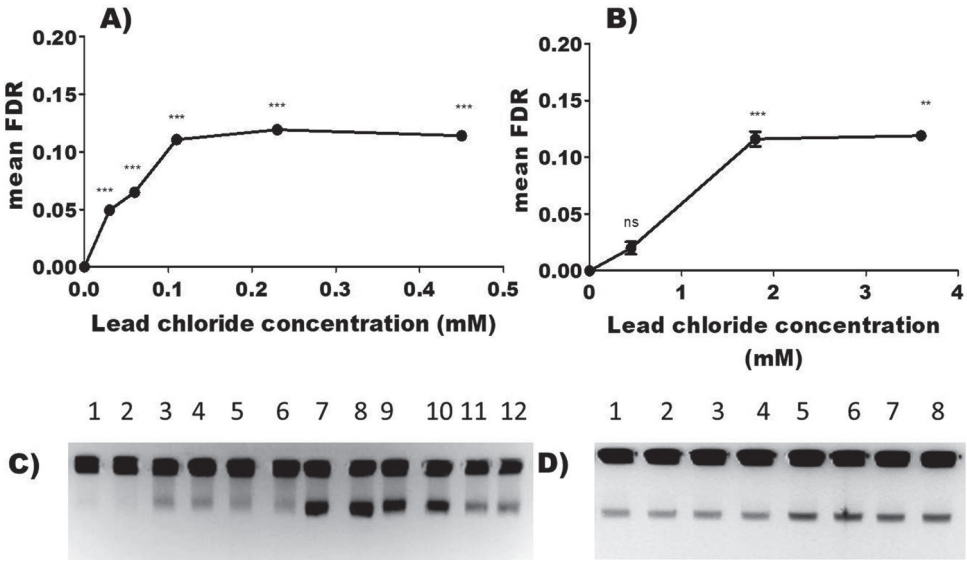


Figure 5. Levels of primary induced double-strand breaks in DNA of *Chlamydomonas reinhardtii* (**A, C** where 1,2 – control; 3, 4 – 0.03 mM; 5, 6 – 0.06 mM; 7, 8 – 0.11 mM; 9, 10 – 0.22 mM; 11, 12 – 0.45 mM) and *Saccharomyces cerevisiae* (**B, D** where 1, 2 – control; 3, 4 – 0.5 mM; 5, 6 – 1.7 mM; 7, 8 – 3.7 mM) after the treatment with various $PbCl_2$ concentrations. Values represent the mean fraction of DNA released (FDR). Data are presented as mean ± SEM. All the results are from at least three independent experiments (ns $P > 0.05$; ** $P < 0.01$; *** $P < 0.001$). Where no error bars are evident, they are equal to, or smaller than, the values.

S. cerevisiae (Fig. 5B, D). Our data is the first experimental evidence for the induction of DSBs after the treatment with $PbCl_2$. Again, genotype-related differences in the response to $PbCl_2$ were established.

A statistically significant dose-dependent DSBs increase was found after the application of $PbCl_2$ in concentrations up to LD_{80} – 0.11 mM for *C. reinhardtii* (Fig. 5A, C). No statistically significant difference was calculated between the effect of the treatment with 0.11 and 0.22 mM $PbCl_2$ – around 2-fold elevated DSBs levels. The treatment with a $PbCl_2$ concentration of 0.45 mM resulted in significant DNA degradation (Fig. 5C).

Interesting results were obtained when comparing the DSB induced by concentrations corresponding to the calculated LD. Concentrations corresponding to LD_{80} in both test systems resulted in a similar induction of DSBs – around 2-fold (Fig. 5A, B). Around 1.5-fold higher DSB levels in *C. reinhardtii* and more than 2-fold in *S. cerevisiae* were measured after the treatment with the respective LD_{50} concentrations. LD_{20} resulted in a small but statistically significant (P<0.001) increase in DSB levels for *C. reinhardtii* (Fig. 5A) and no effect on *S. cerevisiae* (Fig. 5B).

Comparing the concentration ranges, it should be pointed out that concentrations used in *Saccharomyces cerevisiae* experiments were approximately 10-fold higher than those used for *Chlamydomonas reinhardtii*.

In order to reveal whether a relationship exists among the pro-oxidative, DNA damaging, toxic/genotoxic, and mutagenic potential of lead chloride, correlation analysis was performed for both unicellular model systems.

Correlation analysis

In *Chlamydomonas reinhardtii* (Table 3), the decrease in cell survival (SF) was found to correspond strongly with an increase in the H_2O_2 ($P < 0.001$) and DSB ($P < 0.001$). A good correlation was calculated between higher levels of H_2O_2 and low levels of all the photosynthetic pigments (Table 3).

Based on this and the graphically presented changes in the markers studied (Fig. 6A), it could be speculated that the Pb-induced oxidative stress in terms of intracellular peroxides probably may participate in the induction of DSBs, resulting in a decrease in the cell survival.

Concerning the other unicellular organism – *Saccharomyces cerevisiae*, a statistically significant strong correlation was calculated between the decrease in cell survival and the induction of superoxide anions (R = -963, $P < 0.05$) (Fig. 6B). The increase in DSBs levels (Fig. 6C) was found to strongly correlate to the increase in MDA levels (R = 0.947, $P < 0.05$) and the decrease in GSH (R = -0.964, $P < 0.05$). The changes in the markers could be observed in Fig. 6B, C.

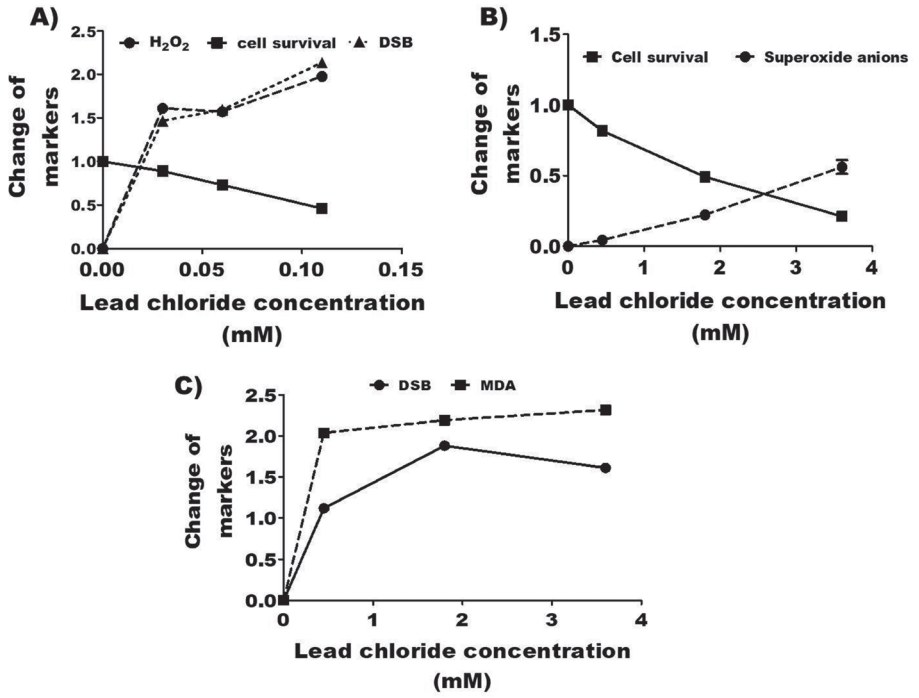


Figure 6. Marker's correlation in *C. reinhardtii* (**A**) and *S. cerevisiae* (**B**) after 2 hours treatment with PbCl2 in concentrations corresponding to LD_{20}, LD_{50} and LD_{80}.

Table 3. Correlation analysis among the studied endpoints in a model system *Chlamydomonas reinhardtii* after the treatment with $PbCl_2$.

	SF	DSB	MDA	H_2O_2	chl *a*	chl *b*	Car
SF		-0.942**	-0.717	-0.943**	0.763	0.944**	0.721
DSB			0.587	0.941**	-0.916*	-0.979**	-0.863
MDA				0.825	-0.561	-0.653	-0.646
H_2O_2					-0.888*	-0.953**	-0.889*
chl *a*						0.907*	0.985**
chl *b*							0.871
Car							

[1] Studied endpoints in the range of $PbCl_2$ concentrations 0.03–0.22 mM – MDA, H_2O_2, chl *a*, chl *b*, carotenoids (car), survival fraction (SF), and double-strand breaks (DSB). Values represent the R^2 for linear correlation. A correlation coefficient (R) higher than 0.900 denotes a strong positive correlation and higher than -0.900 – a strong negative correlation (*$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Discussion

Lead is a very toxic non-trace metal with well-proven both poisonous and genotoxic capacities (Oztetik 2021; Riyazuddin et al. 2022; Chmielowska-Bąk et al. 2022). Recently, it was reported that the bioactivity of lead and its compounds can be attributed to its pro-oxidative capacity (Devóz et al. 2021; Chmielowska-Bąk et al. 2022). As stated in the introduction, information concerning the potential pro-oxidative, mutagenic, and DNA-damaging potential of lead and its compounds as well as its MoA is scarce.

Our main aim was to supply new information about the MoA of $PbCl_2$. This aim has provoked us to focus our attention on two main items: the first relates to the evaluation of mutagenic and DNA-damaging capacity of $PbCl_2$ on two model test systems; the second was to analyze the possible contribution of oxidative stress in these biological events using various endpoints.

According to the WHO, a battery of test systems and endpoints evaluating different adverse effects at different levels is a good strategy for obtaining reliable information about MoA of different xenobiotics. Additionally, the application of several test systems provides more reliable information as some tested materials, such as chelating agents, heavy metals, and some surfactants with unusual physical and chemical properties, may cause practical and test system-specific difficulties, and compromise the outcome of the test by providing false-negative or -positive results (Turkez et al. 2017).

The advantages of the test systems were described briefly in the introduction. They were chosen based on the fact that each of them may provide information concerning different endpoints. Such a strategy may provide more detailed information concerning the MoA of xenobiotics. For the purpose of our study, this investigation has gone through several consecutive steps, using a complex of approaches – microbiological, biochemical, and molecular.

The first one was to evaluate the toxic/genotoxic capacity of tests-systems and the results obtained were compared with some experimental data of other authors.

The toxic capacity of $PbCl_2$ on *P. sativum* L. cultivar Ran1 was evaluated by two endpoints – inhibition of seed germination and reduction of root length. It can be said

that the minor toxic effect of $PbCl_2$ in our experimental scheme is not concentration-dependent, in spite of the fact that differences were statistically significant. Our observation confirms the one reported by Silva et al. (2017) that growth-related parameters could not be considered as the most sensitive and reliable endpoints to evaluate Pb toxicity. The slight toxicity of $PbCl_2$ on *Pisum sativum* L. presented as slightly inhibited germination and reduced root length have been reported by other authors and other plants such as *Parkinsonia aculeata* and *Pennisetum americanum* (Shaukat et al. 1999); *Ipomoea aquatica* Forsk (Ni'am and Yuniati 2021) and *Hordeum vulgare* L.) (Vasić et al. 2020). Ni'am and Yuniati (2021) speculate that the stronger effect on root length than germination could be due to the low permeability of seed testa to lead.

Based on the toxic/genotoxic results, additional data were provided concerning the sensitivity of *C. reinhardtii* strain 137C to $PbCl_2$ compared with *S. cerevisiae*. The high sensitivity of *C. reinhardtii* was previously reported for chlorpyrifos (Todorova et al. 2020). This finding is important because very sensitive genotypes are a very good tool for revealing very low levels of contaminants present chronically in main environmental matrices, and can result in long-term disturbance of biota.

Further, the pro-oxidative potential of $PbCl_2$ was confirmed in all the model systems used by us. The approach applied by us on the test systems covers a wide range of reactive oxygen species (ROS). It is well-known that the first ROS produced is the superoxide anion (Sullivan and Chandel 2014). A dose-dependent increase was calculated for the levels of superoxide anions in *S. cerevisiae* D7ts1, which is in accordance with such findings in *S. cerevisiae* haploid strains (Dimitrov et al. 2011; Sousa and Soares 2014) and *Pisum sativum* L. root cells (Malecka et al. 2001). It is well-known that the $O_2^{\cdot -}$ is easily converted to H_2O_2 by the enzyme mitochondrial superoxide dismutase (Sod2) (Herrero et al. 2008; Sousa and Soares 2014). Thus, we can assume that probably part of the superoxide anions could be converted to peroxides. As a next step, the levels of intracellular peroxides were studied. Data revealed that *P. sativum* L. and *C. reinhardtii* responded differently to $PbCl_2$-induced oxidative stress in terms of intracellular peroxides. While no effect was observed for most of the concentrations tested on *P. sativum* L., a dose-dependent increase was obtained for *C. reinhardtii*. It can be suggested that the genotype also plays a role in the response to the $PbCl_2$-induced oxidative stress.

The next marker for oxidative stress studied was MDA. Interestingly, the response significantly varies depending on the genotype. Our study provides evidence that although an increase in the MDA levels has been observed for all the model systems, the sensitivity of the marker depends on the genotype and the experimental conditions. In the unicellular organisms, MDA was not affected in a dose-dependent way, suggesting that it may not be the primary consequence of $PbCl_2$ treatment for 2 hours. Oppositely, in our long-term experiments on *P. sativum* L., the levels of MDA were found to be the most increased compared to the rest of the pro-oxidative stress markers. Contradictory data exist concerning the induction of lipid peroxidation by lead and its compounds. According to some authors, lead may play an indirect role in lipid peroxidation (Sivaprasad et al. 2004; Hasanein et al. 2017). Based on results in the

present work, it may be speculated that $PbCl_2$-induced lipid peroxidation depends on the experimental conditions.

The last marker studied was GSH. Its role in the antioxidant defense against various stressors is well documented (Perez et al. 2013). The results presented in this work also confirm those published by Perez et al. (2013) that $PbCl_2$ reduces the levels of GSH. The authors suggest that such a reduction could be explained by the high affinity of the GSH thiol group to Pb and thus the formation of Pb-GSH complexes in the cytosol, decreasing the level of GSH (Perez et al. 2013).

In addition to the well-known poisonous capacity of lead and lead compounds, our results confirmed and extended the current state of knowledge regarding their indirect mechanism of genotoxicity via induced oxidative stress. The specificity of induced radicals was found to depend on genotype and experimental conditions.

Further, the effect of $PbCl_2$ on the photosynthetic machinery was evaluated. Interestingly, all the photosynthetic pigments in *Chlamydomonas reinhardtii* and *Pisum sativum* L. decreased in a similar way by around 40–50% in both experimental schemes – short-term and long-term treatment without concentrations or time-dependence.

Contradictory data exist concerning the effect of lead on photosynthetic pigments. Some studies point out that lead treatment may result in a decrease in the chlorophyll content of *Phaseolus vulgaris* and *Lens culinaris* which may be attributed to the ability of lead to replace the magnesium (Mg) in the chlorophyll ring (Irfan et al. 2010; Rai et al. 2016) as well as in other plants (Ruley et al. 2006; Yang et al. 2015). Other authors have reported that Pb does not affect the photosynthetic pigments in *Scenedesmus acutus*, *Schroederia* sp. (Dong et al. 2022), and *Pisum sativum* (Rodriguez et al. 2015).

According to Zheng et al. (2020), the reduction of cell growth of *Chlamydomonas reinhardtii* could be due to the reduction of the chlorophyll content. The Pb-induced disturbance of photosynthesis in microalgae has been proposed as a major cause of cell death by Li et al. (2021). In our study, no statistically significant correlation was obtained between these markers. The SF was found to be negatively affected by H_2O_2 which confirms other findings that the metal-induced oxidative stress in *Chlamydomonas reinhardtii* is related to growth inhibition (Bertrand and Poirier 2005; Stoiber et al. 2013). It should be taken into account that the increase in H_2O_2 also correlates to the decrease in photosynthetic pigments.

Here we can speculate that $PbCl_2$ can affect DNA molecules and photosynthetic pigments via the induction of oxidative stress.

In the present work, an attempt was made to evaluate the potential mutagenic and DNA damaging potential of $PbCl_2$. No mutagenic effect was obtained for both unicellular organisms – *C. reinhardtii* and *S. cerevisiae* at our experimental schemes. Our results confirm those published by Francisco et al. (2018) that Pb does not possess a mutagenic effect on *Allium cepa* root meristematic cells. In the present work, two tests for mutagenic activity were applied each of them providing information concerning various types of genetic alternations. Our data provide indirect evidence that $PbCl_2$ toxicity at the tested concentrations may not be related to the induction of point mutations, impaired cell division; micro-chromosomal aberrations; altered cell

wall structure and composition, mitotic gene conversion and mitotic crossing-over (Zimmermann et al. 1984; Dimitrova et al. 2007).

To the best of our knowledge, the present work provides the first evidence that $PbCl_2$ induces DSBs in *Chlamydomonas reinhardtii* and *Saccharomyces cerevisiae*. It could be speculated that the Pb-induced oxidative stress could be the major mechanism for the induction of double-strand breaks in DNA which, in turn, may partially result in cell death.

Conclusion

Our study revealed that the magnitude of stress response towards $PbCl_2$ is genotype-specific. *Chlamydomonas reinhardtii* 137C is a more sensitive to $PbCl_2$ model than *Saccharomyces cerevisiae* D7ts1 and *Pisum sativum* L. cultivar Ran1. The approach applied by us provided additional information concerning the mode of action of $PbCl_2$. The toxic/genotoxic and DNA-damaging potential of $PbCl_2$ may be a result of the pro-oxidative effect. This is the first report, as far as we are aware, that lead chloride may induce DSB in *Chlamydomonas reinhardtii* and *Saccharomyces cerevisiae*. Our proposed approach – a battery of test systems and various endpoints could be considered a promising tool in the ecotoxicological assessment of various xenobiotics.

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Evaluation of abundance of microplastics in the Bulgarian coastal waters

Stanislava K. Georgieva[1], Zlatina V. Peteva[1], Mona D. Stancheva[1]

1 *Department of Chemistry, Medical University – Varna, Tsar Osvoboditel 84, 9002 Varna, Bulgaria*

Corresponding author: Stanislava K. Georgieva (stgeorgieva@mu-varna.bg)

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Abstract

Plastic pollution in seawaters is ubiquitous, but quantitative estimates on the floating microplastics in the Black Sea are still limited. Plastics may adsorb persistent environmental contaminants, thus representing a potential risk for marine organisms.

Aim: The aim of the study was evaluation of the presence and characteristics of microplastic particles (MPs) in waters from the Black Sea coast of Bulgaria.

Materials and methods: Samples of coastal waters were collected from March 2021 to April 2022 from different stations on the Black Sea coast, including protected, aquaculture and industrial areas. In order to determine the number of plastic particles, 23 samples were collected from the surface waters at depth of 1–3 m close to the Bulgarian shore. Samples were treated with H_2O_2, plastic particles were isolated by density separation and filtered over a membrane filter. Identification analysis of micro particles (< 5 mm) was performed visually by microscopy.

Main results: Results indicated widespread presence of microplastics in coastal waters. Mean MPs concentration was calculated 7.3 ± 4.9 pt/l. The comparison of the North, Varna and South sampling area showed that there is no significant difference in the abundance of plastic particles. The most dominant type forms were fibres followed by fragments. The most abundant size class of fragments was 101–500 μm Ferret diameter.

Conclusion: Further studies are needed in order to fill knowledge gap and to evaluate distribution of plastic particles in the Black Sea and their potential ecological risk.

Keywords
microplastics, sea waters, the Black Sea, Bulgaria

Introduction

Marine debris has been of concern to the scientific community for decades. The last report of EC 2020 on the implementation of the Marine Strategy Framework Directive (EC 2008) highlighted the main pressures affecting marine ecosystems: fishing, contaminants and marine litter (EC 2020). It was indicated that there are sizeable gaps in the data on litter on the seabed, in the surface layer and micro-litter and their effects on marine species (EC 2020). Plastic waste in the marine environment is classified into macro-, micro- and nanoplastics depending on their sizes. In recent years, microplastics (MPs; < 5 mm) have occurred in all geographical regions of the Oceans (Desforges et al. 2014), including the Arctic (Obbard et al. 2014; O'Donovan et al. 2018; Kanhai et al. 2020) and Antarctica (Lusher 2015). The occurrence of plastic particles was mostly determined in the last decade in marine water, sediment or biota samples all over the world (Cincinelli et al. 2017; Baini et al. 2018; Rios-Fuster et al. 2023).

The Black Sea is a semi-enclosed area and its coasts are subjected to high levels of marine litter pollution from rivers discharges (Danube, Dnieper etc.), harbours, industrial cities, fishery, tourism and agriculture in the region (Simeonova et al. 2010; Pojar et al. 2021; González-Fernández et al. 2022). Recent studies of microplastics in surface waters have been carried out along the Black Sea coast (Aytan et al 2016; Oztekin and Bat 2017; Berov and Klayn 2020; Pojar et al. 2021). Microplastics and nanoplastics (< 0.1 μm) pose a danger to marine organisms that can ingest them, causing physiological disorders and even death. Microplastics have the potential to adsorb persistent organic pollutants (POPs) from the marine environment, which may increase their detrimental impact once assimilated by the organisms (O'Donovan et al. 2018). Several studies identified that POPs residues (such as polychlorinated biphenyls (PCBs), PAHs and organochlorine pesticides (e.g. DDT, DDE) are present in the marine environment and biota of the Black Sea (Stancheva et al. 2017; Georgieva et al. 2016; Georgieva et al. 2022). These contaminants are lipophillic and could accumulate on the surface of plastics (Barnes et al. 2009; Cole et al. 2011). Ingestion of microplastics by marine organisms is a potential route for bioaccumulation of toxic chemicals in the food chain (Teuten et al. 2009).

Plastic pollution in the marine environment is ubiquitous, but the scientific data about the presence and characterisation of microplastics in surface waters from the Bulgarian coast of the Black Sea are still insufficient (Berov and Klayn 2020). The aim of the study was evaluation of the presence and characteristics of microplastic particles (MPs) in waters from the Black Sea coast of Bulgaria.

Methods

Sampling and sample preparation

The investigation of plastic particles in the coastal waters of the Bulgarian Black Sea targeted sampling areas with a high ecological importance, including protected, aquaculture and industrial areas such as the cities of Varna and Burgas (main harbours) and tourism resorts.

Samples of coastal waters (n = 23) were collected from March 2021 to April 2022 from different stations near the Black Sea coast. Surface waters at depth of 1 to 3 m were sampled aboard a vessel by a manta net. After sampling, the net haul concentrates were adjusted to water volume of 1.5 l using seawater, filtered by 20 μm mesh. In the laboratory, each sample was transferred into glass jars, using Milli-Q water for rinsing. A total of 23 samples were stored at 4 °C in darkness to minimise algal growth until analysis. The sampling plan, sampling preparation and microplastics' characterisation were conducted in accordance with Guidelines for the monitoring and assessment of plastic litter and microplastics in the ocean (GESAMP 2019).

The samples were transferred into individual pre-cleaned glass beakers and organic matter was digested using hydrogen peroxide. A total of 20 ml 30% hydrogen peroxide and 20 ml of 0.05 M ferrous sulphate ($FeSO_4$), were carefully added to 250 ml of water sample. The mixture was placed on a hot plate set to 60 °C (30 min) and the reaction was allowed to continue until all organic material disappeared (about 24 h). Plastic particles were isolated by density separation and filtered over a membrane filter (sterile MCE, 0.45 μm pore size, 47 mm diameter, FiltraTECH). Identification analysis of microparticles (< 5 mm) was performed visually by a technique under stereomicroscopy Primo Star (Zeiss, Jena).

Visual identification

For identification and characterisation of microplastics, membrane filters were examined using a Zeiss Primo Star microscope at 4× and 10× magnifications. All of the images were captured with a Zeiss Axiocam ERc 5 s microscope. All plastic items with a size range from 20 μm to 5 mm were taken into account and measured with micrometer ocular lens. Plastic particles were numbered and classified by size, characterised by colour (white, blue, red, yellow, black, transparent, green, other colours) and shape (spherical, fibre, filament, fragment, sheet, films) according to the MSFD guidelines (Hanke et al. 2013; GESAMP 2019). The criteria for visual identification, classification and characterisation by morphological types, size classes (Ferret diam.) and colour of plastics were based on the protocol by Hidalgo-Ruz et al. (2012) and Kovač Viršek et al. (2016).

Quality assurance

In order to prevent sample contamination, glass and stainless-steel materials were used for the laboratory work. During sample preparation and storage, all samples were

covered with aluminium foil. Each series of samples included procedure blank, using same the amounts of reagents and ultrapure Milli-Q water, which was digested in the same way as the other samples. All results were corrected with data from a parallel blank (Milli-Q water). Statistical significance of the differences in the mean values of particles in samples from the three regions were tested at $\alpha = 0.05$ with Student's t-Test, Excel for MS Office Professional Plus (Microsoft Corporation. 2018).

Results

Microplastic particles were identified by means of visual identification. The filters were visually examined and all potential microplastic particles ranging from 20 μm to 5 mm were registered and counted. The plastic particles were observed in 22 from a total of 23 samples (Table 1). Particle abundance was based on filtered water and the amount of identified plastic particles was present as particles per litre (pt/l). Mean concentrations of microplastics ranged from 1.3 pt/l (ME7) to 16.3 pt/l (ME60) (Table 1).

Table 1. Sampling sites and total concentrations of microplastics (mean and standard deviation), particles per litre (pt/l) in coastal waters of the Bulgarian Black Sea coast.

Sample No	Sampling season	Sampling site	GPS coordinates	Total concentration, pt/l
ME7	Winter 2021	Cape Kaliakra	43°19'24.5"N, 29°10'56.1"E	1.3 ± 0.9
ME10	Winter 2021	Varna Bay	43°12'45.9",N 28°16'13.9"E	2.3 ± 2.3
ME16	Spring 2021	Kavarna	43°23'36.9"N, 28°22'52.0"E	14.0 ± 2.0
ME22	Spring 2021	Burgas Bay	42°17'36.0"N, 27°17'41.2"E	2.3 ± 0.6
ME38	Summer 2021	Kamchia estuary region	43°01'23.5"N, 27°53'22.0"E	10.7 ± 1.5
ME49	Summer 2021	Ravda	42°38'14.3"N, 27°40'26.5"E	12.3 ± 1.5
ME60	Autumn 2021	Pasha dere, near Varna	43°07'09.8"N, 28°02'52.1"E	16.3 ± 1.2
ME61	Autumn 2021	Sts. Constantine and Elena	43°13'31.9"N, 28°02'12.3"E	4.3 ± 0.6
ME62	Autumn 2021	Golden Sands	43°16'52.5"N, 28°07'03.9"E	12.7 ± 0.3
ME65	Autumn 2021	Pomorie	42°33'19.4"N, 27°38'19.4"E	2.3 ± 0.6
ME67	Autumn 2021	Nesebar	42°39'58.6"N, 27°43'06.5"E	3.3 ± 0.6
ME77	Autumn 2021	Cape Kaliakra	43°21'01.7"N, 28°28'49.8"E	3.3 ± 0.6
ME78	Autumn 2021	Kavarna	43°23'49.9"N, 28°19'36.1"E	3.7 ± 0.6
ME79	Autumn 2021	Cape Emine	42°43'15.0"N, 27°55'26.1"E	4.3 ± 0.6
ME84	Winter 2021	Kamchia estuary region	43°01'23.5"N, 27°53'22.0"E	6.3 ± 0.6
ME87	Winter 2021	Varna Bay – Karantinata	43°10'28.3"N, 27°54'60.0"E	4.7 ± 0.6
ME90	Winter 2021	Varna Bay – 4th buna beach	43°12'42.2"N, 27°57'30.1"E	14.3 ± 0.6
ME93	Winter 2021	Lake Varna	43°11'36.8"N, 27°51'46.5"E	5.7 ± 0.6
ME102	Winter 2022	Durankulak	43°43'08.2"N, 28°35'09.8"E	12.0 ± 1.0
ME103	Winter 2022	Krapets	43°39'12.5"N, 28°36'10.1"E	12.7 ± 0.6
ME104	Winter 2022	Shabla	43°34'34.4"N, 28°38'33.8"E	nd
ME112	Spring 2022	Pomorie	42°33'19.4"N, 27°38'19.4"E	8.3 ± 1.2
ME113	Spring 2022	Nesebar	42°39'58.6"N, 27°43'06.5"E	10.0 ± 1.0

nd – not detected.

Discussion

Distribution of microplastics along the Bulgarian Black Sea coast

The results of our study indicated widespread presence of microplastics in coastal waters of the Bulgarian part of the Black Sea. The mean MPs concentration in seawater was calculated 7.3 ± 4.9 pt/l. A total of 23 samples of sea water were studied (Table 1) from the three samples regions North, Varna and South. The highest concentrations were found in samples ME60 (Pasha Dere, near Varna region) – 16.3 ± 1.2 pt/l, ME90 (Varna Bay) – 14.3 ± 0.6 pt/l and ME16 (Kavarna, North region, mussel farm) – 14.0 ± 2.0 pt/l. The lowest number of plastic particles was recorded at sampling points ME7, ME77 – 3.8 pt/l (Cape Kaliakra), ME65 and ME67 (Nesebar and Pomorie) – 2.3 and 3.3 pt/l (Autumn 2021).

Comparison of the North, Varna and South sampling areas showed that there is no significant difference in the abundance of plastic particles (7.8, 8.7 and 6.4 pt/l, respectively) – Fig.1. The large variation in results for each region is likely due to different sources of land-based plastic waste pollution (Simeonova et al. 2010; Simeonova et al. 2017).

Our findings showed the highest number of plastic particles in the surface water samples from the northern coast of the Bulgarian Black Sea (near Kavarna). Pojar et al. (2021) reported the highest plastic abundances occurred close to the mouth of the Danube River. The authors assumed that the plastic pollution might occur via the Danube River flows into the Black Sea. Close to Varna, the mean amount of microplastics in seawater samples was 8.6 ± 5.0 pt/l and several kilometers to the north in resort Sts. Constantine and Elena, the concentration decreased to 4.3 pt/l (ME61). The harbour city of Varna is one of the major ports in Bulgaria with industrial activity and high frequency of ships traffic. Surprisingly, in the South sampling area, the amount of

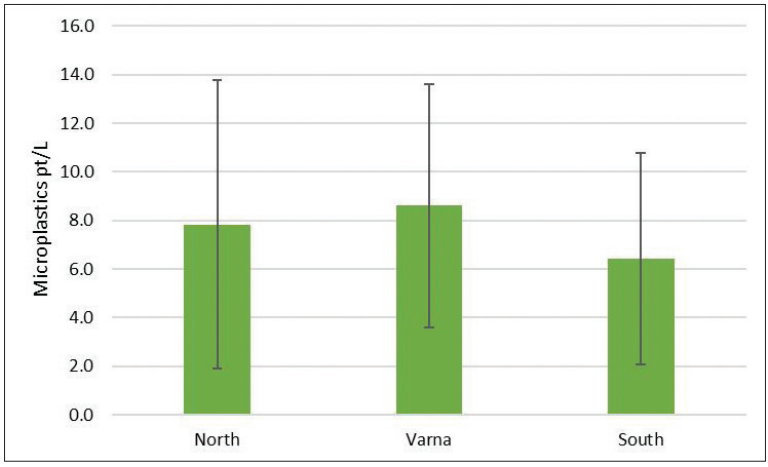

Figure 1. Distribution of microplastics along the Bulgarian Black Sea coast by sampling area.

plastics detected nearby urbanised areas Burgas Bay (2.3 ± 0.6 pt/l –ME22) was lower than in the environs of the protected areas (Ravda, Pomorie, Nesebar). The reason was the limited number of samples in this area and, thus, the results gives only first insights into the plastic pollution of south part of the Bulgarian coast of the Black Sea.

In a recent study, Berov and Klayn (2020) found that microplastic pollution was highest in the area of Cape Kaliakra. Our results showed relatively lower levels of abundance of microplastics in samples from Cape Kaliakra compared to other protected areas (ME102 and ME103) in the Northern region. Relatively high concentrations of macroplastics were found in front of the protected area in the Kamchia River Estuary (ME38 and ME84). The river is characterised with the largest catchment area and local sources are the possible emitters of plastic particles in this sampling site of the Black Sea coast. Similar findings were reported in a recent study by Berov and Klayn (2020). The MPs abundance in samples from protected areas along the Bulgarian coast was in the same ranges compared to nearshore regions close to the industrial cities of Varna and Burgas. This might be explained by the sea flows, seasonal influence, wind regime, sea surface currents and sea tides (Simeonova et al. 2017; Shapiro 2019; Pojar et al. 2021).

Higher concentrations of microplastic particles could be expected in the Northern region, due to the influence of freshwater inflows from the Danube River in the western Black Sea. The data showed variations in the amount of floating particles due to surface currents along the coast from north to south (Pojar et al. 2021) and the current regime of the Black Sea, generally described as counter-clockwise (Oguz et al. 1993).

Characterisation of plastics in coastal waters of the Black Sea, Bulgaria

A difference in plastic morphology is observed: in all samples, only fragments, fibres and films (secondary microplastics as a product of the degradation of macroplastics) were identified. Microparticles such as spherules, microbeads and granules (primary plastics) were not found in examined filtered samples.

The most dominant forms were fibres (73.3%), followed by fragments (23.4%) and other forms (3.2%) – Fig. 2. Fibres predominated in areas around ports and populated areas, because they originate mainly from wastewater, clothing and ropes, as well as shipping activities (Hidalgo-Ruz et al. 2012; Gewert et al. 2017).

Amongst the different colours found, transparent pieces were the most abundant (38%), followed by blue (29%) and black (26%) – Fig. 3. Other colours (red, yellow and others) were found in residual amounts and combined made up 7% of the entire sample. Several scientific studies reported a prevalence of black, grey and more brightly coloured particles (Cole et al. 2011, 2014; Kanhai et al. 2020). Further investigation of the possible sources of contamination could explain this higher concentration of the colourless and blue particles in the water samples from the Bulgarian Black Sea coast.

Analysis of the size distribution of microplastics in surface samples showed that the most abundant size class of fragments was 101–500 μm Ferret diameter in the whole dataset (Fig. 4). The small size of the particles poses a high risk to marine organisms

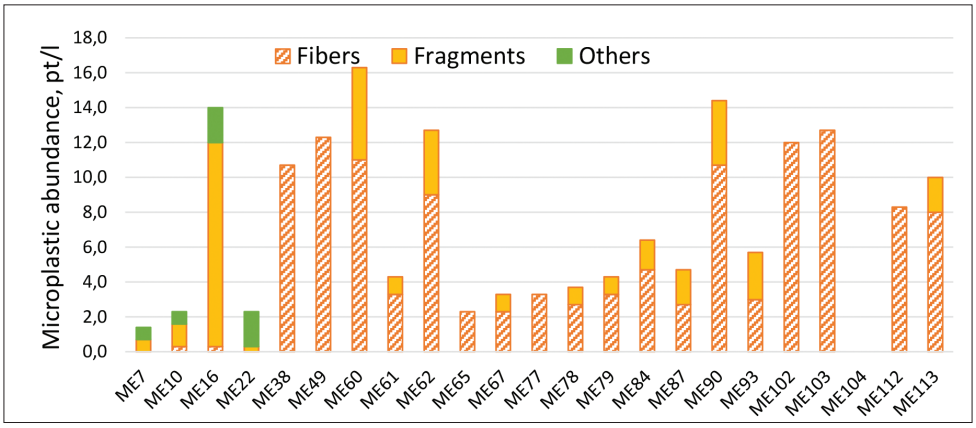

Figure 2. Distribution of MP particles by form types, particles per litre (pt/l) in coastal waters from different stations along the Bulgarian Black Sea coastline for the whole monitoring period.

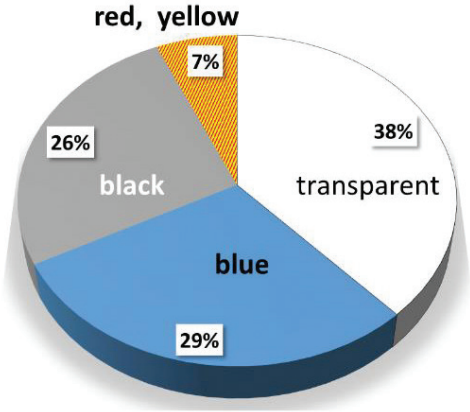

Figure 3. Composition of microplastic particles in seawater samples according to their colour classes.

which could accidentally ingest them during feeding (Teuten et al. 2009; O'Donovan et al. 2018). The items with sizes below 100 μm represented the minor portion of the total microplastics. A similar pattern was described in a recent study conducted in the Mediterranean Sea, Tuscany, Italy (Baini et al. 2018).

The mean concentration of microplastic particles (mean value 7.3 ± 4.9 pt/l) in coastal waters of Bulgaria was found comparable with data from other studies conducted in the Black Sea (Aytan et al. 2016; Berov and Klayn 2020), as well as with a previous study the Mediterranean Sea (Gündoğdu 2017). The results from the present study showed significantly higher abundance of microplastics than the microparticles contamination reported by Pojar et al. 2021 (average 7 pt/m^3) from the western Black Sea, Romania and by Scott et al. (2019) in UK coastal waters – 1.97 to 3.38 pt/m^3.

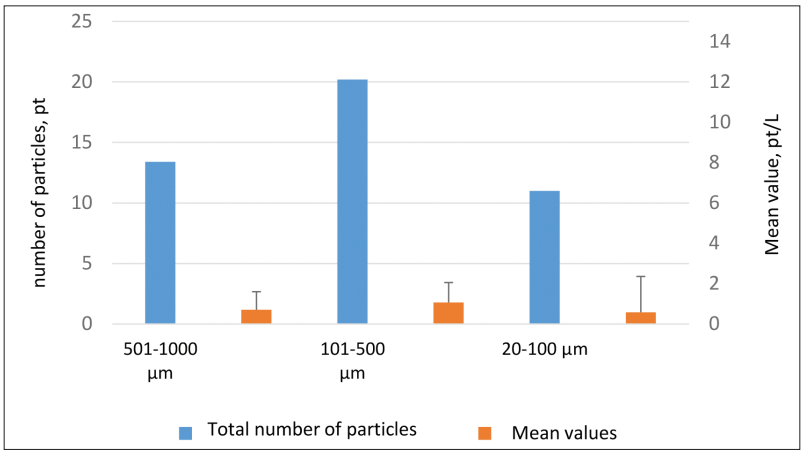

Figure 4. Size classes of MP particles (fragments and others*) in coastal waters (*fibres is not included).

Conclusion

With the aim of filling the gap and increasing the knowledge about plastic pollution, the present study provides data on the abundance and characteristics of microplastics in the surface waters along the Bulgarian Black Sea coast. The results showed that plastic pollution is ubiquitous as more plastic particles are present in the water samples from the Varna Bay and the northern Black Sea coast. The confirmation of the possible sources of pollution requires further analyses, for example, polymer composition by Pyrolysis GC/MS or FTIR spectrometry. Considering the limited number of samples and variation in results, we suggest to develop more a complex programme and continue monitoring the studied regions. There is an urgent need to coordinate monitoring methodologies regarding plastic pollution at national, regional and EU levels (EC 2020). Further studies are needed in order to evaluate the distribution of plastic particles in the Black Sea and their potential ecological risk.

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RESEARCH ARTICLE

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Occurrence of marine biotoxins on Bulgarian Black Sea coastal waters in 2021

Zlatina V. Peteva[1,2], Stanislava K. Georgieva[1], Bernd Knock[3], Thomas Max[3], Mona D. Stancheva[1], Simona Valkova[1]

1 *Medical University Varna, Tsar Osvoboditel 84, 9002 Varna, Bulgaria* **2** *Dobrudzha Technological College, Dobrotitsa 12, 9302 Dobrich, Bulgaria* **3** *Alfred Wegener Institut, Helmholtz Zentrum für Polar- und Meeresforschung, Chemische Ökologie, am Handelshafen 12, 27570 Bremerhaven, Germany*

Corresponding author: Zlatina V. Peteva (zlatina_peteva@mail.bg)

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Abstract

Marine biotoxins are produced by certain phytoplankton species and used to accumulate in filter-feeding marine organisms. The occurrence of marine biotoxins in all aquatic environments and latitudes is variable in time and space. Thus, it is an essentially natural phenomenon, but the occurrence of toxigenic phytoplankton cannot be completely avoided or eliminated. A serious concern appears if these substances accumulate at high levels in seafood. If it is consumed by mammals including humans, severe illness of consumers of intoxicated seafood may result. The aim of this study is to assess the presence of marine biotoxins in plankton samples taken in 2021 and to compare the determined levels with a previous period. Plankton samples (n = 21) were collected in 2021 along the whole Bulgarian coastline (Black Sea). The presence of hydrophilic (domoic acid (DA)) and lipophilic toxins (okadaic acid, dinophysis toxin – 1, dinophysis toxin -2, azaspiracid-1, goniodomin A, pectenotoxin-2 (PTX2), yessotoxin, spirolide-1 and gymnodimine A) was investigated via liquid chromatography – tandem mass spectrometry (LC-MS/MS). Results indicated the presence of only DA in three samples and PTX2 in two samples. The positive samples were sporadically distributed throughout the study period. During 2016–2019, LC-MS/MS analysis confirmed the presence of DA, PTX2, YTX, SPX-1 and GDA in plankton net samples collected from the same locations reported here. The matching toxins (DA and PTX2) were at comparable levels in both periods of investigation, thus lower than in other European waters where harmful algal blooms are registered. These results show the persistent appearance of some marine biotoxins in Bulgarian waters. Although levels were low in the monitored periods, a constant monitoring is required in order that toxic events by seafood consumption be avoided.

Keywords
Domoic acid, monitoring, pectenotoxins, the Black Sea

Introduction

Bulgarian Black Sea coastline comprises 432 km (Stanchev et al. 2013). It is important for recreational, touristic (Stoyanova et al. 2019; Ihtimanski et al. 2020; Nikolova et al. 2021) and commercial (Raykov and Nicheva 2018; Stancheva et al. 2022) activities. The Black Sea is a commercial seafood source, including shellfish and fish, as well as providing popular recreational fisheries (General Doctorate for Internal Policies 2011; EAFA 2020).

Black Sea mussel production and catchment has increased in recent years (EAFA 2021). Mussels have been documented to contain beneficial values of polyunsaturated fatty acid, proteins, vitamins etc. and, therefore, are a preferred food worldwide (Hyung et al. 2018; Carboni et al. 2019; Yaghubi et al. 2021).

Microalgae are the primary food source for mussels (Brown 2002; Pleissner et al. 2012), but some microalgal species are reported as toxic or harmful. These phytoplankton species tend to produce potent toxins that accumulate in filter feeders. Yearly, potential producers of marine toxins (Pseudo-nitzschia, Alexandrium, Dinophysis) are registered in Bulgarian coastal waters (Dzhembekova et al. 2021; Dzhembekova et al. 2022). Marine toxins are transferred through the food chain to the higher trophic levels and may cause severe illness in them. A wide range of symptoms, from dizziness, digestive (nausea and vomiting) to nervous complaints, are associated with human intoxication by biotoxins, characterising different and specific syndromes, called shellfish poisonings. The risk assessment of the occurrence of toxigenic phytoplankton is complicated by the fact that toxin levels of plankton samples do not always correlate with biomass and abundance of potentially toxigenic species.

The aim of this study is to evaluate the levels of marine biotoxins in plankton samples of the year 2021 collected from areas of different function and economic importance. Furthermore, the determined levels will be compared to marine toxins levels from previous periods of investigation.

Materials and methods

Sampling plan

Phytoplankton samples (n = 21) (Table 1) were hauled vertically from depths between one and five metres from the surface with a conical plankton net (20 μm mesh size, 40 cm outer diameter) along the Bulgarian coast in the period March-December 2021.

Table 1. Collected plankton samples.

№	Sample №	Sampling site (Coordinates)	Type of the region	Sampling date
1	**ME6**	North 43°32'348"N, 029°18'224"E	Intensive fishing activities	31.03.2021
2	**ME8**	South 42°26'296"N,027°41'360"E	Mussel farming site	25.05.2021
3	**ME9**	North 43°21'276"N, 028°27'053"E	Intensive fishing activities	31.03.2021
4	**ME15**	North 43°39'961"N, 029°39'793"E	Intensive fishing activities	13.04.2021
5	**ME17**	North 43°21'885"N, 028°20'528"E	Mussel farming site	13.04.2021
6	**ME38**	North 43°01'23.5"N, 27°53'22.0"E	Protected area	18.07.2021
7	**ME47**	North 43°24'14.3"N, 28°21'11.8"E	Mussel farming site	26.07.2021
8	**ME48**	North 43°23'58.9"N, 28°09'34.5"E	Intensive fishing activities	27.07.2021
9	**ME49**	South 42°38'14.3"N, 27°40'26.5"E	Area with anthropogenic activities	11.08.2021
10	**ME57**	North 43°07'09.8"N, 28°02'52.1"E	Protected area	08.10.2021
11	**ME58**	Varna 43°13'31.9"N, 28°02'12.3"E	Areas with anthropogenic activities	08.10.2021
12	**ME59**	Varna 43°16'52.5"N, 28°07'03.9"E	Areas with anthropogenic activities	08.10.2021
13	**ME66**	South 42°33'19.4"N, 27°38'19.4"E	Mussel farming site	1.11.2021
14	**ME68**	South 42°39'58.6"N, 27°43'06.5"E	Protected area	1.11.2021
15	**ME74**	North 43°21'01.7"N, 28°28'49.8"E	Protected area	4.11.2021
16	**ME75**	North 43°23'49.9"N, 28°19'36.1"E	Mussel farming site	4.11.2021
17	**ME76**	South 42°43'15.0"N, 27°55'26.1"E	Protected area	4.11.2021
18	**ME83**	South 43°01'23.5"N, 27°53'22.0"E	Protected area	29.11.2021
19	**ME86**	Varna 43°10'28.3"N, 27°54'60.0"E	Areas with anthropogenic activities	5.12.2021
20	**ME89**	Varna 43°12'42.2"N, 27°57'30.1"E	Areas with anthropogenic activities	5.12.2021
21	**ME92**	Varna 43°11'36.8"N, 27°51'46.5"E	Areas with anthropogenic activities	5.12.2021

Sampling sites close to mussel farming areas, areas used for harvesting of wild mussels (including areas of intensive fisheries and areas with anthropogenic activities), as well as protected areas, were included in the sampling plan.

Experimental plan

Immediately after sampling, net haul concentrates were adjusted to a defined volume of 500–1000 ml (depending on the net tow volume) using 20 µm filtered seawater. After centrifugation (4000 × g, 10 min at 10 °C), the supernatant was discarded. The cell pellets were stored in at -20 °C until further processing.

Plankton pallets were suspended washed with 1000 µl 100% methanol for domoic acid and lipophilic toxins extraction. The methanolic acid suspensions were than sonicated (40 Hz, 15 min) and centrifuged by 4000 x g for 10 min at 10 °C. The supernatant was filtered through syringe filters (0.45 µm pore size, ø25 mm, Minisart, Sartorius, Germany). Filtrates (1000 µl) were transferred into chromatographic vials and kept at -20 °C until further analysis.

The hydrophilic domoic acid (DA) and lipophilic toxins – goniodomin A (GDA), okadaic acid (OA), dinophysistoxins -1 and 2 (DTX1,2), pectenotoxins (PTX2, PTX2-sa, epi-PTX-sa), yessotoxins (YTX, OH-YTX), azaspiracid-1 (AZA1), spirolides (SPX1) and gymnodimine A (GYMA) were analysed according Krock et al. (2008) on

a LC-MS/MS system. It consists of liquid chromatograph (model 1100 LC, Agilent, Waldbronn, Germany) coupled to a triple quadrupole mass spectrometer (API 4000 QTrap, Sciex, Darmstadt, Germany), equipped with a Turbo Spray interface.

The quality control was performed by regular analysis of procedural blanks and certified reference material (National Research Council, Canada). Limits of detection (LOD) for lipophilic toxins and DA were determined based on 3:1 signal-to-noise ratio.

Calculations

Contents of the toxin are expressed as nanograms per net tow (ng/NT) in order to be compared with previous results and other literature data.

Results

In total, 21 plankton samples were collected in the studied period February-December 2021 along the Bulgarian coastline in accordance with sampling plan (Table 1).

With the aim to analyse for the presence of selected marine biotoxins, appropriate retention times and LODs were achieved (Table 2).

The huge efforts for the toxin profile revealed a scarce presence of marine biotoxins in the plankton samples. Amongst the investigated toxins – DA, GDA, OA, DTX1, DTX2, PTX2, PTX2-sa, epi-PTX-sa, YTX, OH-YTX, AZA1, SPX1) and GYMA, only DA and PTX2 were detected (Table 3).

Table 2. Investigated lipophilic toxins and domoic acid including associated standard solution concentrations, LODs, quantification transitions and retention times.

Marine toxins investigated	Concentration of standard solution pg/µl	LOD ng/NT	Quantification transition (*m/z*)	Retention time (min)
DA	100	4.93	312→266	7.17
OA	500	25.71	822→223	11.57
DTX2	500	36.59	822→223	11.87
DTX1	500	60.00	836→237	12.57
PTX2	100	3.73	876→213	12.14
PTX2-sa	–	–	894→213	11.70
Epi-PTX2-sa	–	–	894→213	11.90
GONA	412.5	30.56	786→607	12.67
YTX	1000	100.00	1176→981	13.00
OH-YTX	–	–	1176→981	11.70
AZA1	100	0.92	842→824	12.62
GYMA	500	1.50	508→490	10.33
SPX1	100	2.58	692→164	11.22

Table 3. Levels of detected toxins in plankton samples.

Sample №	DA, ng/NT	PTX2, ng/NT
ME6	170,94	< LOD
ME9	< LOD	9.23
ME15	138.48	< LOD
ME58	13.38	< LOD
ME76	< LOD	6.51

Results obtained in this study showed that only 14% of the samples were positive for DA and only 9% for PTX2. DA was present in spring and autumn samples from areas with intensive fishing and anthropogenic activities. Pectenotoxin-2 was detected in a spring and autumn sample from an area with anthropogenic activities and a protected region, respectively.

Comparison of the results with results obtained from previous studies in the same regions (Peteva et al. 2018; Peteva et al. 2020) showed that, in 2016, DA was detected in 57% of the samples, in 2017 in 41%, in 2018 – 17% of samples and in 2019, in none of the samples. In 2017 and 2018, plankton samples investigated were many more than in 2016 and in 2021. Comparison of the concentration range of the positive samples showed a great variability between the different periods of investigation (Fig. 1).

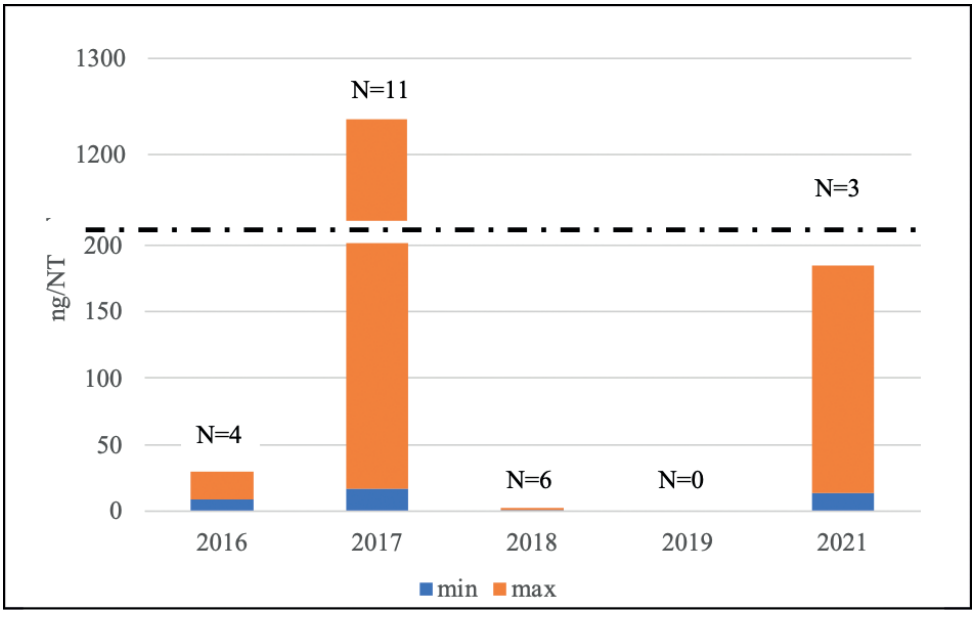

Figure 1. Comparison of DA levels with previous studies (n- indicates the number of positive samples).

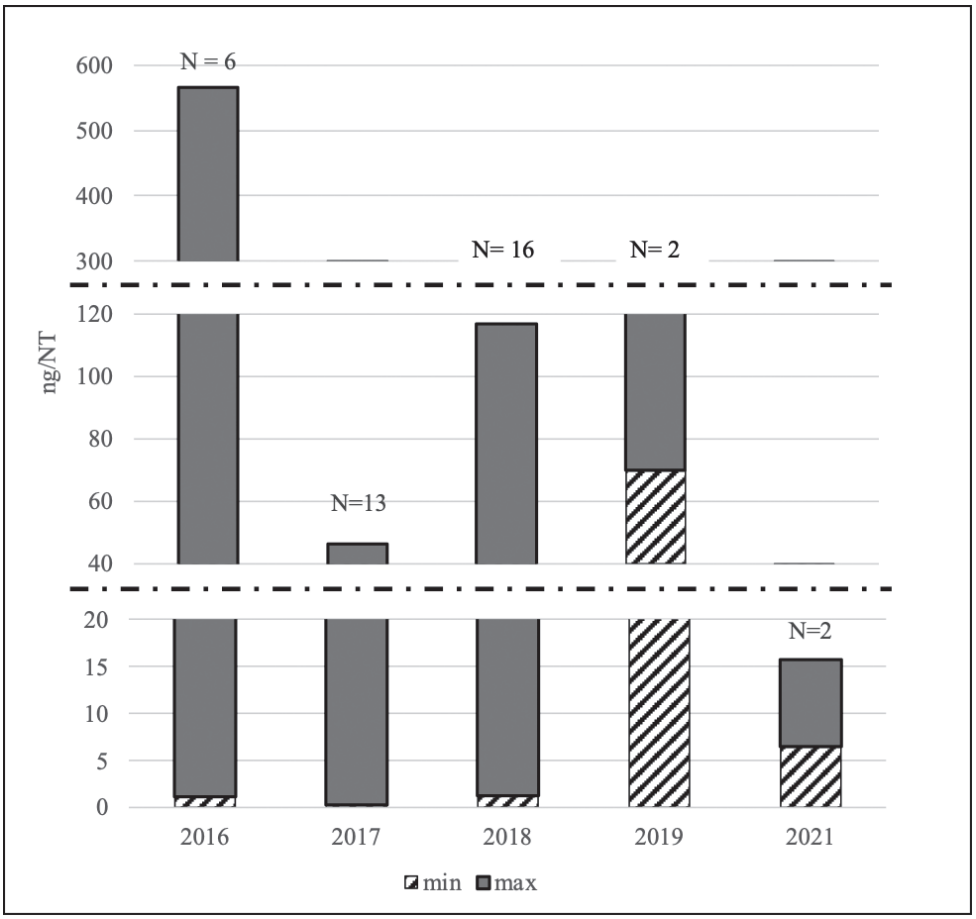

Figure 2. Comparison of PTX2 levels with previous studies.

The two PTX2 positive samples from 2021 represent 10% of all samples. Thus, in previous studies, the portion of positive samples was much higher – 86% in 2016, 48% in 2017, 47% in 2018 and in 2019 – 67% of the samples. Moreover, in this former period of time, the PTX2 concentration ranges are much wider (Fig. 2).

Discussion

The Bulgarian coast is important for the development of the economy and tourism in the country (Dimitrov and Rangelov 2018; Mooser et al. 2022).

Bulgaria is considered as a minor producer of seafood, responsible for 0.01 percent of world production and 0.4 percent of EU fishery and aquaculture products in terms of volume (EUMOFA 2020). Recent investigations showed a persistent value of the catch (for 2015–2020 – 8476 tonnes), as well as a visible peak in 2019

(Shivarov 2021). Fishing activities are performed almost throughout the whole year and along the whole coastline. Thus, it is known that fishing activities change the environmental parameters in the regions where they are undertaken (Stier et al. 2020; Gissi et al. 2021).

In Bulgarian mariculture farms, Mediterranean mussel (*Mytilus galloprovincialis* Lamarck) is dominant farmed species. The total marine aquaculture production of 2,531 t in 2018 consists mainly of this mollusc (Klisarova et al. 2020). Recent state reports showed that, since 2008, Bulgaria is one of the important suppliers of Mediterranean mussels in the Black Sea region. Nowadays, Bulgarian mussel farms produced over than 1.5% of the cultivated mussels in the world (Ministry of Agriculture and Food Bulgaria 2019).

A number of factors of natural and predominantly anthropogenic nature have a negative impact on the state of the environment of this region of the country (Kotsev and Prodanov 2020, Kotsev et al. 2021). Natural factors, superimposed in a number of cases by anthropogenic activity, are mainly abrasion, landslides, floods of the coast from the sea and climate change (Penchev 2019).

Anthropogenic activities and technological advances are commonly pointed out to justify the increasing occurrence, frequency and intensity of harmful algal blooms and the detection of new toxins or emergence of toxins in regions where they were previously not known (Costa 2019; Otero and Silva 2022). In this regard, investigation and comparison of the toxin profiles of plankton samples from locations of fishing and anthropogenic activities, as well as mussel farming sites, seem meaningful and informative.

Coastal protected areas in Bulgaria are established by national policy instruments/laws and EU Directives to protect a wide range of natural and cultural resources (Stancheva et al. 2016). In these areas, any catch and industrial activities are banned by the law (Ministry of Regional Development and Public Works 1998). Accordingly, protected areas are considered control sites in this study.

Quantitative and qualitative analysis of marine biotoxins was performed by applying liquid chromatography coupled with mass spectrometry which is acknowledged by the scientific community as one of the most powerful analytical tools able to identify multiple toxins (Visciano et al. 2016; Estevez et al. 2019). Results indicate that, in 33% of samples from the areas with anthropogenic activities and in 50% of the samples from the areas of intensive fishing, marine biotoxins were detected. No toxins were detected in the samples from the mussel farms and in one sample from a protected area. Further interpretation of the results would be possible if the investigation is repeated in a future period.

Interestingly, two other marine toxins were detected in previous periods – YTX and SPX1. Yesotoxins were registered in the samples from 2016–2018. The concentration range is very large – 0.001 – 1.959 ng/NT. In the present study, no YTXs were detected. The small number of positive samples in the previous period, as well as the absence hereby, is most likely due to the fact that yesotoxins are exotoxins. Once synthesised, they are rapidly released into the environment and, therefore, difficult

to determine in plankton samples (Hess and Aasen 2007). For example, Krock et al. (2013) also investigated the levels of lipophilic toxins along the German and Danish coasts, but yesotoxins were not determined.

Our previous investigation showed that SPX1 was registered in the samples from summer-autumn 2018 in a concentration range from 0.054–0.245 ng/NT. No spirolides were registered in this study. This result might be associated with low abundance or even absence of *A. ostenfeldii*, as SPX 1 production is associated with this species (Van Wagoner et al. 2011; Guinder et al. 2018).

This further reinforces the belief that toxin production by plankton is an unpredictable phenomenon (Kremp et al. 2019) and studies on it should be continued.

Conclusions

Results obtained in this paper including the values below LOD indicate that abundance of marine biotoxins is not alarming. This suggests that good quality of mussel meat might be expected. Monitoring of harmful phytoplankton composition and biotoxins should be continued in future, so it can provide the opportunity to react in good time in order to prevent negative consequences which can be caused by HABs and biotoxins.

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RESEARCH ARTICLE

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Alterations in membrane stability after *in vitro* exposure of human erythrocytes to 2.41 GHz electromagnetic field

Boyana Angelova[1], Gabriela Atanasova[2], Nikolay Atanasov[2], Momchil Paunov[1], Maria Gurmanova[1], Margarita Kouzmanova[1]

1 *Department of Biophysics and Radiobiology, Sofia University "St. Kliment Ohridski", 8 Dragan Tsankov Blvd., 1164, Sofia, Bulgaria* **2** *Department of Communication and Computer Engineering, South-West University "Neofit Rilski", 66 Ivan Mihaylov Str., 2700, Blagoevgrad, Bulgaria*

Corresponding author: Boyana Angelova (angelova_bd@uni-sofia.bg)

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Abstract

The growing use of wireless communication devices has been significantly increasing the level of high frequency electromagnetic fields (EMFs) in the environment, which raises a concern for possible deleterious effects on living organisms. Long lasting exposure to low-intensity EMFs can cause effects on the molecular and cellular level, and a number of morphological and physiological changes. The aim of this work was to investigate the effects of 2.41 GHz EMF emitted by wireless communication systems on human erythrocytes after *in vitro* irradiation. The amount of the hemoglobin released from the cells was measured as an indicator for membrane destabilization. Effects of different exposure times (20 min or 4 h) and time elapsed after exposure to 2.41 GHz pulsed or continuous EMFs with different intensities, emitted from a textile (0.213–0.238 V/m) or a dipole (5, 20, 40 and 180 V/m) antenna, were investigated. The obtained results showed that the low intensity EMF had no significant effect on the hemoglobin release from irradiated cells; even a slight tendency for membrane stabilization was noticed 3–4 hours after the end of 20-min exposure to 0.213–0.238 V/m, 2.41 GHz EMF. There was no difference in the effects of continuous and pulsed EMFs. Increased hemoglobin release was observed only during the 4-hour exposure to 180 V/m, 2.41 GHz continuous EMF. Under these conditions, the temperature of the cell suspension had been rising, so we compared the results obtained under EMF with the effects of conventional heating. Moreover, after 1-hour exposure to 180 V/m the released hemoglobin level was a bit higher than the control one but the difference disappears within an hour after terminating the irradiation. In conclusion, the *in vitro* exposure to 2.41 GHz EMF emitted by wireless communication devices with power density below the reference level for population exposure does not change the stability of the cell membrane of human erythrocytes.

Keywords
hemoglobin release, temperature effects, wearable textile antenna, wireless

Introduction

The growing use of wireless communication devices has been significantly increasing the level of high frequency electromagnetic fields (EMFs) in the environment, which raises a concern for possible deleterious effects on living organisms. Long lasting exposure to low-intensity EMFs may cause effects on the molecular and cellular level, and a number of morphological and physiological changes (Camara 2014). Of particular interest is their impact on children and adolescents, who are considered two of the most sensitive and affected groups because they will be exposed to EMFs for the longest time (Bodewein et al. 2022; Schmutz et al. 2022). In communication technology, industry and medicine one of the most commonly used EMF frequency bands is 2.4–2.5 GHz in the microwave range, which is sometimes considered a part of radiofrequency electromagnetic fields (RF EMFs).

Microwave EMF effects can be classified as thermal and non-thermal. Thermal effects are related to energy transfer during interaction between the field and the object, leading to an increase in temperature (Antonio and Deam 2007). The mechanisms of EMF action that are not directly related to temperature changes are not fully understood (Banik et al. 2003; Nguyen et al. 2016 and references therein; Ahortor et al. 2020; Zhao et al. 2021). The effects of EMF on humans can be divided into short-term, such as stress, fatigue, headaches; and long-term, including impaired embryonic development, reduced reproductive capacity, damage to brain tissue, heart problems, cancers, genetic disorders (Kaszuba-Zwoińska et al. 2015; Belyaev et al. 2016). It has been established that radiofrequency irradiation leads to an increase in the formation of free radicals and oxidative stress, which causes disruption of DNA and protein structure, as well as peroxidation of membrane lipids. Changes in gene expression and epigenetic and genetic alterations have been observed (Belpomme et al. 2018). Emerging electromagnetic hypersensitivity has been also receiving increasing attention.

Numerous studies on the effects of EMFs with different frequencies on biological objects with differing degrees of organization have been conducted. The obtained results are contradictory, probably due to differences in the applied irradiation conditions, the objects studied and the detection methods (Banik et al. 2003). The effects of 2.45 GHz microwaves on cell membranes were studied by determining the hemoglobin release and the osmotic resistance of human erythrocytes exposed to different power densities (0.025–10.0 mW/cm^2) at different irradiation times (Sajin et al. 2000). It was found that at low power densities (0.84 and 1.36 mW/cm^2), the degree of hemolysis increased quasi-linearly with exposure time, while at higher power densities (5 mW/cm^2), this trend reversed after the first 10 hours of irradiation – a protective effect against spontaneous hemolysis caused by blood aging was observed. The

osmotic resistance of exposed erythrocytes (5 mW/cm^2) increased with time, reaching a maximum at the end of irradiation (60 hours), while the osmotic resistance of control cells remained constant. Kouzmanova et al. (2007) found a decrease in the level of released hemoglobin over an hour after 20-minute exposure of erythrocyte suspensions to GSM 900 EMF probably as a result of cell membranes stabilization. Hassan et al. (2010) reported an increased rate of hemolysis after 2.45 GHz EMF irradiation of rats – 1 hour daily for 30 days. In the same study, the level of malondialdehyde, a marker of lipid peroxidation, was significantly increased, and the levels of antioxidant enzymes were significantly decreased. Exposure of male albino rats to EMF with a much lower frequency (50 Hz) caused conformational changes in hemoglobin molecules and significantly reduced serum testosterone, while degenerative changes in the testes were also registered (Salama et al. 2020). 60 Hz EMF exposure of mice was observed to significantly increase micronucleus frequency (Heredia-Rojas et al. 2018). However, a multi-generation study found no harmful effects of RF EMF (1966 MHz) on the fertility and development of mice (Sommer et al. 2009). In rats irradiated with 900 MHz EMF just once for 2 hours or for 4 days, 30 minutes each day, a significant increase in lipid peroxidation of their erythrocyte membranes was observed (Badzhinian et al. 2013). The results were obtained on the first and fifth days after exposure.

Results from *in vitro* experiments with human erythrocytes irradiated with 2.45 GHz EMF showed that short-term (20 minutes) exposure in the reactive near-field of wearable antenna at 6.3 mW input power had a stabilizing effect on the erythrocyte membrane, while long-term exposure (120 minutes) had a destabilizing effect (Atanasov et al. 2022).

Riffo et al. (2021) investigated the effect of EMFs within the frequency band between 1 and 5.9 GHz on yeast growth. A decrease in viability was reported at all applied frequencies. Using transmission electron microscopy, EMF has been found to disrupt the integrity of the cell membrane (membrane permeabilization). When different microorganisms were exposed to 2.45 GHz EMF, an increased permeability of the cell membrane to propidium iodide and dextran particles of various sizes was observed (Ahortor et al. 2020). Entry of propidium iodide was registered in microwave-treated *M. smegmatis* cells but not in cells conventionally heated to the temperature reached by irradiation. Release of DNA from the cells was also reported. 18 GHz EMF was found to induce permeabilization in bacterial and yeast cell membranes as the uptake of high molecular weight dextran (150 kDa) (Shamis et al. 2011) and silica nanoparticles (Nguyen et al. 2015, 2016) was investigated.

Exposure of red blood cells to 18 GHz EMF resulted in cell membrane permeabilization and nanosphere uptake with high efficiency (96% and 46% for 23.5 and 46.3 nm nanospheres, respectively), as demonstrated by scanning electron microscopy, confocal laser scanning microscopy and transmission electron microscopy (Nguyen et al. 2017). Exposure to 2.45 GHz EMF induced a stress response in the hippocampus of rats, evidenced by the presence of heat shock proteins (Yang et al. 2012). Exposure to high-frequency EMFs generated by base stations was associated with an increased risk of developing type 2 diabetes (Meo et al. 2015).

In this study, we investigated effects of EMF used in novel wireless technologies (such as body area or sensors networks, Internet of things, etc. communication systems) on human erythrocyte membranes during and after *in vitro* irradiation. Effects of different exposure periods (20 min or 1, 2, 3 and 4 h) and time elapsed after the exposure to 2.41 GHz pulsed or continuous EMFs differing in intensity emitted by textile (0.213–0.238 V/m) or dipole antenna (5, 20, 40 and 180 V/m) were examined. The amount of hemoglobin released from the cells was measured as an indicator of membrane destabilization.

Methods

Blood material and erythrocyte suspension preparation

The experiments were performed with human erythrocytes, isolated from whole blood drawn from clinically healthy donors (National Center for Transfusion Hematology, Sofia, Bulgaria). Two blood types were investigated: A+ and A–. The EMF treatment was applied between the 5th and 25th day after the drawing while the blood was stored at 10 °C in a refrigerator.

Whole blood samples were centrifuged first at 1500 rpm for 5 min (Eppendorf, Hamburg, Germany), after which the supernatant (blood plasma) and the white blood cells coating was removed and replaced with 0.9% NaCl (saline) solution. Then, the erythrocyte mass was washed twice again with saline, as the cell suspensions were centrifuged for 10 min at 2000 rpm. At the end, the washed erythrocyte mass was collected and its hematocrit was determined by centrifugation in 2–4 capillary tubes for 2 min (Yanetzki TH 11, Germany). The final erythrocyte suspension used in the experiments was obtained by dilution to a hematocrit of 40% with PBS (Sorensen's phosphate buffer – 0.9% NaCl, adjusted to pH 7.4 with Na_2HPO_4/KH_2PO_4). The EMF treatment was carried out in plastic cuvettes filled with 2 ml suspension and covered with Parafilm. Some of the cuvettes were left: as controls isolated from EMF in a metal box; in background irradiation; or in water bath at a temperature of 24, 32 or 38 °C.

Electromagnetic field exposure setups

Two exposure setups were developed to investigate the effects of RF EMF emitted from novel wireless technologies (such as body area or sensor networks, Internet of things, etc.) on human erythrocyte membranes. The first one was designed to test RF EMF exposures from wireless body area network devices. The RF EMF was generated with an XBee S1 RF module (Digi International Inc., Thief River Falls, MN, USA) connected to a microwave solid-state amplifier (CBA 9429, AMETEK CTS Europe GmbH, Kamen, Germany). The RF module was controlled by a personal computer to emit a Zigbee-like signal (1 ms between the packets) at 2.41 GHz. The signal was transmitted using a wearable textile polyester substrate antenna (Atanasova and Atanasov 2020),

connected via a 6 dB attenuator to the microwave solid-state amplifier, as shown in Fig. 1. The erythrocyte suspensions were placed in the far-field region of the antenna. The erythrocyte suspensions were exposed to EMF with power density 120–150 μW/m^2 and intensity 0.213–0.238 V/m, measured with a battery-operated E-field probe (HI-6006, ETS-Lindgren, Cedar Park, TX, USA). Since the two EMF parameters are proportional in the far-field region, only the intensity will be presented further. The effect of the pulsed EMF on erythrocyte membranes was compared to that of continuous EMF exposure. For that purpose, the erythrocytes were exposed to a 0.213–0.238 V/m continuous wave EMF at 2.41 GHz as well (see Fig. 1).

The second experimental setup was designed to test RF EMF with higher electric field intensity. The RF EMF was generated with a microwave generator (SMB100A, Rohde & Schwarz GmbH & Co. KG, Munich, Germany) connected to a microwave solid-state amplifier (FLG-50F, Frankonia, Heideck, Germany). The microwave generator was tuned to generate a pulse-modulated signal (pulse period 4.608 ms, pulse width 2.304 ms, 217 Hz) at 2.41 GHz. The signal was transmitted using a half-wave dipole metal antenna connected via a coaxial cable to the microwave solid-state amplifier. The erythrocyte suspensions were placed in the far-field region of the antenna on a Styrofoam in four positions differing in intensities: FF1 (180 V/m), FF2 (40 V/m), FF3 (20 V/m), and FF4 (5 V/m), as shown in Fig. 2. The electric field at each position was measured as in the first setup. Exposures were performed in a semi-anechoic chamber for both setups. The temperature of the samples during irradiation was monitored with an infrared thermal camera FLIR E5 (Teledyne FLIR, Wilsonville, OR, USA).

During the experiment the background control samples were placed in rooms adjacent to the semi-anechoic camera. The ambient EMF in those rooms was measured. Power density varied in the range of 36–72 μW/m^2 (0.116–0.164 V/m). The ambient EMF values were lower than those applied to erythrocyte suspensions in the semi-anechoic chamber.

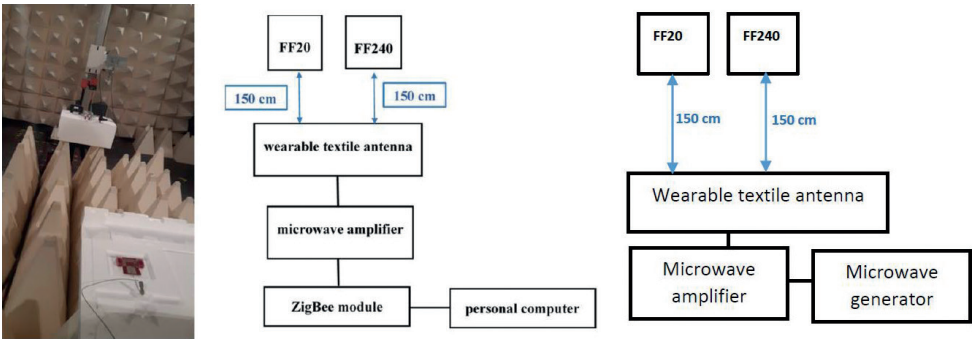

Figure 1. Experimental design 1. Two plastic cuvettes filled with 2 ml erythrocyte suspensions (hematocrit 40%) were located at 150 cm distance from the textile antenna (in the far field region) and irradiated for 20 (FF20) or 240 minutes (FF240) with pulsed or continuous EMF. Input power to the antenna was 450 mW, electric field intensity – 0.213–0.238 V/m. Left: photograph of the general setup; Center: scheme representing pulsed EMF setup; Right: scheme representing continuous EMF setup.

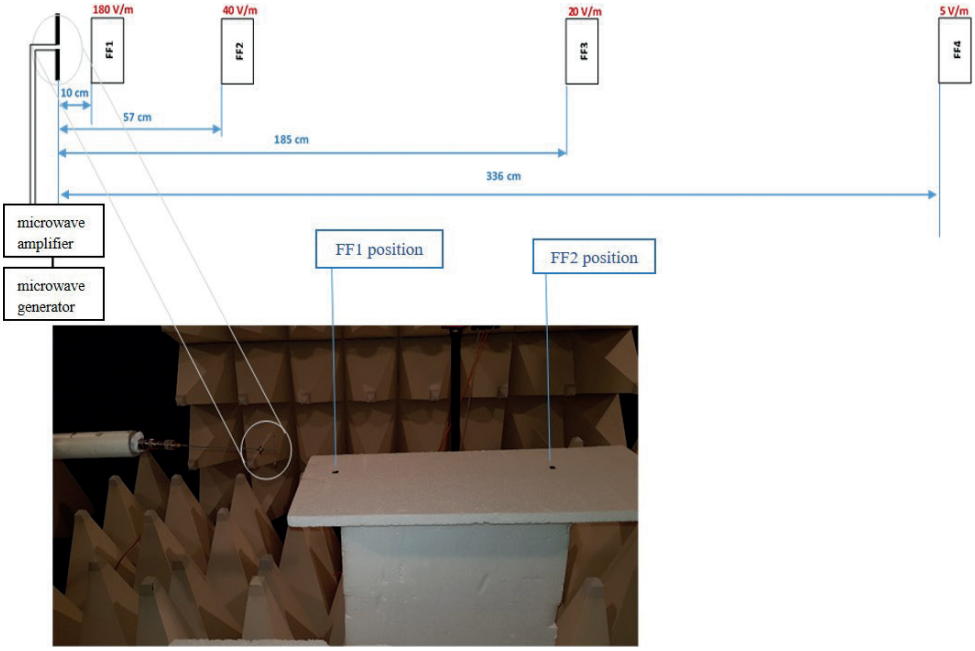

Figure 2. Experimental design 2. Four plastic cuvettes filled with 2 ml erythrocyte suspensions (hematocrit 40%) were located at different distances from the dipole antenna (in the far field region) at four intensities: FF1 (180 V/m), FF2 (40 V/m), FF3 (20 V/m), and FF4 (5 V/m), as shown on the scheme and the photograph. Input power to the antenna was 50 W.

Hemoglobin release measurement

The release of hemoglobin was estimated spectrophotometrically by measuring the absorbance at 413 nm (maximum for hemoglobin) of a supernatant solution (Spekol 11, Carl Zeiss Jena, Germany). The supernatant solution was prepared as 100 µl of the investigated erythrocyte suspension was added to 1.3 ml of PBS followed by centrifugation for 15 s at 12000 rpm. The concentration of the released hemoglobin in the 40% hematocrit experimental sample was calculated using the formula:

$$c = \frac{A \times V_2}{\varepsilon \times l \times V_1}$$

where c is hemoglobin concentration, µmol/l; A – absorbance; ε – molar extinction coefficient for hemoglobin at 413 nm (0.12 l.µmol^{-1}.cm^{-1}); l – optical path length through the spectrophotometrically measured sample (1 cm); V_1 – volume of added erythrocyte suspension (100 µl) and V_2 – final measured sample volume (1400 µl).

Statistics

The results presented in this study are average values ± standard errors calculated from 3–7 independent repetitions of each experimental variant.

Results

Exposure to the textile antenna

The hemoglobin release from erythrocytes in suspensions with hematocrit 40% was investigated for 5 hours (at 1-hour interval) after 20-min exposure to pulsed 2.41 GHz EMF with intensity 0.213–0.238 V/m (Fig. 3A). Simultaneously, the hemoglobin release in control untreated suspensions was measured. The control samples were placed in the semi-anechoic chamber during the exposure but were shielded from EMFs in a metal box. Thus both the control and the EMF-treated cells were at the same temperature during irradiation. No heating was registered for the EMF exposed samples. After the end of the treatment, both sample types were placed in a water bath at 24 °C. Both EMF-treated and control cells displayed a tendency for released hemoglobin increase with time passing after exposure but statistically significant changes were not registered even after 5 hours. No statistically significant differences in the quantity of hemoglobin between exposed and unexposed suspensions were observed.

Further, erythrocytes were treated with continuous EMF without changing the other irradiation parameters. The obtained results are presented in Fig. 3B. Again, no statistically significant difference in the released hemoglobin between control and treated samples was found due to the high variance (standard errors) of the values.

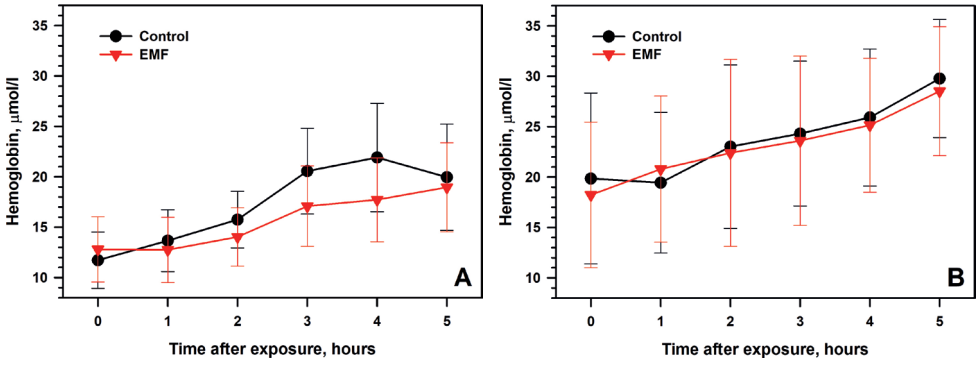

Figure 3. Hemoglobin release after 20-min irradiation of human erythrocyte suspensions with 2.41 GHz EMF. Source: textile antenna, intensity 0.213–0.238 V/m **A** pulsed EMF (1 ms between the pulses) applied **B** continuous wave EMF applied. Control: erythrocyte suspensions shielded from EMFs.

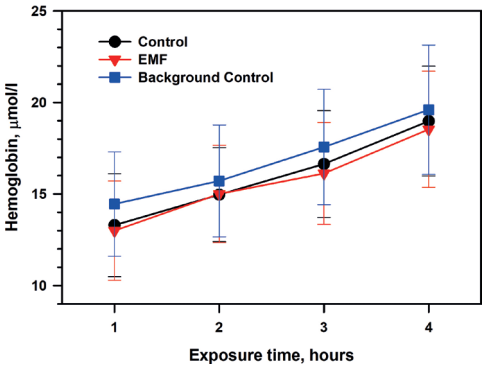

Figure 4. Hemoglobin release during 4-hour exposure of human erythrocyte suspensions with 2.41 GHz pulsed EMF. Source: textile antenna, intensity 0.213–0.238 V/m. Control: samples isolated from EMFs; Background Control: samples at 0.116–0.164 V/m background EMF.

Since no EMF effects were registered after 20-min irradiation from textile antenna and because the communication devices operate with EMF pulses, we continued our experiments with longer (4-hour) pulsed EMF exposures during which the hemoglobin release was measured every hour. Again, a control sample in a metal box was used. Moreover, two background controls were placed in two rooms during the experiment at 24–26 °C ambient temperature. The results from these two samples were averaged and presented as background control. No statistically significant differences between the control, background control, and EMF-treated samples were observed even after 4 hours (Fig. 4). Heating was not registered for the irradiated sample, so its temperature was the same as the control samples. However, in all the conducted (7) individual experiments a tendency for higher values in background control was observed which is also noticeable from the averaged data. During the experiments, intensity of 0.116–0.164 V/m was measured for the background EMF. For comparison, just after the end of the 20-min exposure at the same conditions the released hemoglobin was 12.8 µmol/l, and 4 hours after terminating the irradiation – 17.73 µmol/l, while after 4-hour continuous EMF treatment it was 18.54 µmol/l. Thus, at the applied EMF parameters the longer exposure did not affect strongly the integrity of the erythrocyte membranes *in vitro*.

Exposure to the dipole antenna

All the experiments conducted with the textile antenna show no effect of EMF on the stability of erythrocyte membranes. In search for effect, an antenna, allowing higher intensity emission, was used. The effect of 2.41 GHz EMF emitted by a half-wave dipole antenna with an output power of 50 W (pulse period: 4.608 ms, pulse width: 2.304 ms) on erythrocyte suspensions was investigated for 4 hours. Samples were placed in far-field at 4 positions from the antenna with different electric field intensities: 5, 20, 40 and 180 V/m. From Fig. 5A it is evident that the erythrocytes at the 5,

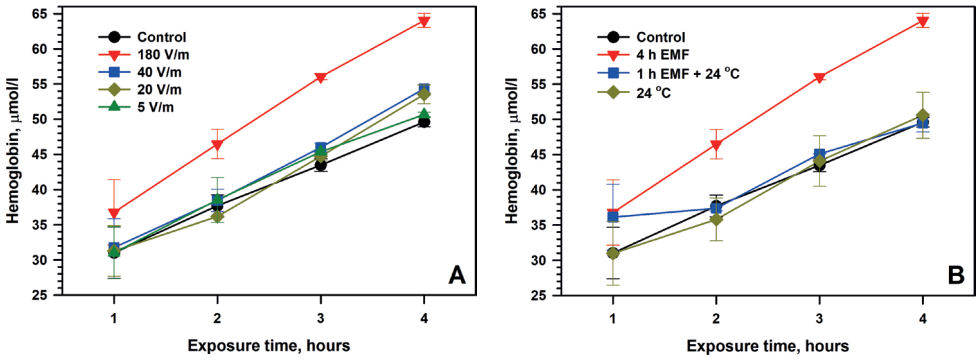

Figure 5. Hemoglobin release during 4-hour irradiation of human erythrocyte suspensions with 2.41 GHz pulsed EMF. Source: dipole antenna, pulse period: 4.608 ms, pulse width: 2.304 ms. **A** different intensities applied: 5, 20, 40 and 180 V/m **B** sample which 180 V/m EMF exposure was interrupted after 1 h, compared to 4-hours long uninterrupted 180 V/m treatment. Control: erythrocytes shielded from EMF; 24 °C: cells incubated in water bath at 24 °C for 4 hours.

20 and 40 V/m intensities released hemoglobin similarly to the control for the first 3 hours. Values of 20 and 40 V/m samples became higher than 5 V/m and control levels just after 4 hours. The highest intensity (180 V/m) caused the greatest hemoglobin release, as a significant difference compared to the control appeared at the second hour and continued throughout the exposure time.

A significant temperature increase to 32 °C was registered in the samples exposed to 180 V/m. Since it is known that the biological effects of EMFs are at least partially due to heating, the hemoglobin release after conventional heating was investigated. The concentration of the released hemoglobin after 4-hour incubation at 24, 32 and 38 °C in a water bath was 218±160, 311±235, 277±209 µmol/l, respectively. For the large standard errors, we could not determine a significant temperature-dependent change, just a tendency for heat-induced increase.

In addition, the stability of erythrocyte membranes after exposure to high-intensity EMF was examined and compared with membrane stability changes during the EMF action. Two cuvettes with erythrocyte suspensions were placed under irradiation with 180 V/m intensity EMF. After one hour, one of the samples was moved from the irradiation spot into a water bath at 24 °C while the other was left under treatment. A control sample in a metal box and a 24 °C incubated control were used. Released hemoglobin was measured simultaneously for all samples for 4 hours (Fig. 5B). Under the uninterrupted (4 h) EMF exposure, the quantity of released hemoglobin increased linearly with time. The hemoglobin amount in the interrupted treatment sample did not change during the first hour after removing the EMF irradiation, approaching control levels at the 2nd hour, but subsequently increased equally with control values over time. There were no differences in the released hemoglobin concentrations between the control sample in a metal box and 24 °C sample.

Discussion

The rapid development of wearable wireless sensor networks, and the fact that emitted EMFs may have impact not only on the people wearing such sensors, but also on the people around them, leads to an increased interest in the biological effects. In order to clarify possible effects of EMF exposure in the far-field region on cell membrane we conducted experiments with a small wearable textile antenna and with a dipole antenna.

The selectively permeable cell membrane allows the transport of some soluble substances across it and prevents the passage of others. Thus, the membrane is involved in the control of cell volume and integrity. When it comes to red blood cells, this is of great clinical importance. The process in which the integrity of the erythrocyte membrane is impaired and the intracellular protein hemoglobin is released into the environment is called hemolysis. It can result from normal cell aging or be induced by various biotic and abiotic factors (Goodhead and MacMillan 2017). The accumulation of free hemoglobin in the body can cause heart disease or kidney stones (Prastalo et al. 2003). Various literature data show that exposure of erythrocytes to EMF – both *in vitro* and *in vivo*, leads to a change in the stability of their cell membranes (Kiel and Erwin 1984; Kouzmanova et al. 2007).

Our results showed there was practically no change in the quantity of released hemoglobin for 5 hours after 20-min exposure of human erythrocytes to 0.213–0.238 V/m 2.41 GHz pulsed or continuous EMF emitted by the textile antenna. Slight variations between pulse-treated and control suspensions were observed at 3rd and 4th hour, hinting at possible tendency for membrane stabilization, similar to the results obtained by Kouzmanova et al. (2007). In both samples, the average hemoglobin values rose with the incubation time as to be expected because of cell degradation during storing erythrocytes at room temperature in absence of nutrients in the medium. However, the standard errors of the presented released hemoglobin concentrations are very large as a result of variation between the values measured in each replication of the experiment. That variance should be attributed mainly to the *in vitro* aging of the blood, respectively erythrocytes, i.e. the time elapsed between drawing the blood and conducting the experiment, as Kouzmanova et al. (2006) observed increasing level of hemolysis in erythrocytes from "aged" blood. Individual characteristics of blood donors should be also expected to introduce high variance.

A tendency for higher hemoglobin values in background control compared to the shielded control and 4-hour EMF exposed samples was noticed, which cannot be explained by the influence of 0.116–0.164 V/m background EMF. This intensity is lower than the experimentally applied 0.213–0.238 V/m. The intensity of the background EMF radiation varies throughout the day, depending mainly on the level of communication systems usage by the population. On the other hand, the background EMF may vary in frequency as well, and mild discrepancies between the temperature in the semi-anechoic chamber, where control and treated samples were placed, and the laboratories, where the background controls were placed, were pos-

sible to occur (in the range of 2 °C). The simultaneous action of all those factors may explain the observed results.

On the basis of the maximal levels of irradiation defined in IEEE Standard for Safety Levels with Respect to Human Exposure to Radio Frequency Electromagnetic Fields, 3 kHz to 300 GHz (2006), four positions were chosen in the dipole antenna exposure setup, ensuring EMF intensity values, significantly higher than those applied during the textile antenna experiment. The allowed maximal power density in controlled environment (an area in which workers are subject to control and accountability – in radio transmitters, installers of base stations, etc.) is 80.3 W/m^2, which corresponds to the intensity of 174 V/m. For the general population (people of all ages with different health statuses) a power density of 10 W/m^2 (61.4 V/m) is accepted as permissible. At the first sample position, the intensity was slightly higher than the maximally allowed for a controlled environment (180 V/m), while the other three positions had values (40, 20 and 5 V/m), resembling realistic cases of general population exposure.

There seemed to be slight alterations in the cell membrane permeability leading to a tiny increase of the released hemoglobin after 1-hour exposure to 180 V/m, but one hour after the end of irradiation, the membrane fully recovered. The properties of the biological membranes depend directly on the state of the membrane proteins. Upon their functioning, proteins undergo different conformational changes. They have many charged chemical groups, taking part in catalytic, regulatory, transport and aggregation processes, which can be influenced by EMF (Bucci et al. 2006). Such changes could result in the observed effects on hemoglobin release.

It is supposed that the thermally induced hemolysis includes 3 types of processes: 1) inactivation of vital enzymes and denaturation of structure proteins, 2) formation of lytic agents in the blood plasma and 3) melting of membrane lipids (Gershfeld and Murayama 1988). The structure of spectrin – a cytoskeletal protein, is not altered at temperatures under 45 °C, so the cytoskeleton impairment cannot be related to cell lysis under the examined experimental conditions. The highest temperature investigated (38 °C) is optimal for the functioning of the cellular enzymes. Hence their inactivation is not a possible explanation for the hemolysis. The temperature of the phase transition of the erythrocyte membrane from gel to liquid crystal is far below 37 °C so lipid melting is not possible to contribute to the observed hemoglobin release (Gershfeld and Murayama 1988). The activation energy of autohemolysis of red blood cells in the range 4–37 °C is significantly less than that at a temperature higher than 37 °C. This means that the change in the limiting stage of hemolysis occurs at 37 °C. Oxidative processes were found to be essential in autohemolysis in the range of 20–37 °C (Chernitskiĭ and Iamaĭkina 1996). Between 38 and 45 °C a process different from protein inactivation is responsible for hemolysis – mechanism based on the concept of the critical bilayer assembly temperature of cell membranes.

Our results could not differentiate thermal from non-thermal effects of EMF on hemolysis at 180 V/m *in vitro*. We plan future experiments to elucidate such differences, i.e. whether non-thermal effects exist at permissible EMF exposures and what are their mechanisms.

Conclusion

In vitro irradiation with 2.41 GHz EMF emitted from wireless communication devices with power density / electric field intensity below the reference level for the general population according to IEEE (2006) does not change the stability of the human erythrocyte cell membrane for up to 4 hours of exposure.

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RESEARCH ARTICLE

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In vitro clonal propagation of *Tanacetum cinerariifolium* and establishment of an *ex situ* collection of selected clones

Vladimir Ilinkin[1], Boryanka Traykova[1], Marina Stanilova[1]

1 *Institute of Biodiversity and Ecosystem Research – Bulgarian Academy of Sciences 23, Georgi Bonchev Str., Sofia, 1113, Bulgaria*

Corresponding author: Vladimir Ilinkin (vladimirilinkin@gmail.com)

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Abstract

Dalmatian pyrethrum *Tanacetum cinerariifolium* (Trevir.) Sch. Bip. (Asteraceae) is a perennial herb endemic to the eastern coast of the Adriatic Sea. The species is widely cultivated in many countries for its bioactive compounds pyrethrins, which are used as natural insecticides. Plants derived from seeds vary greatly in pyrethrin content; therefore, the vegetative propagation of high-quality individuals is very important for the establishment of agricultural pyrethrum crops. The present study deals with rapid *in vitro* multiplication of pyrethrum, *ex vitro* adaptation of selected clones and creation of an *ex situ* collection, as a first step towards introducing the species into agriculture in Bulgaria. Seeds from a private *ex situ* collection in Bulgaria and from a natural Croatian population were used as initial material for *in vitro* cultures initiation. Basal MS medium (Murashige and Skoog 1962) or MS supplemented with different concentrations of kinetin and indole-3-butyric acid were used for seed germination and multiplication of one-seed derived clones by consecutive subcultivations. The propagation effectiveness was evaluated as a number of new plants obtained per initial shoot. Considerable losses were noticed due to both endophytic contaminations and necrosis, especially on media supplemented with plant growth regulators. These problems were overcome by medium optimization: adding an antibiotic and modifying the medium to increase the calcium concentration using $CaCO_3$. In the best medium variant (basal MS + 200 mg/L Medaxone + 75 mg/L Ca) no more infected plants were observed, and the percentage of necrotic plants decreased threefold, which resulted in formation of 38.06±10.11 new plants per initial shoot for a period of 7 months. Three hundred and sixty plants were *ex vitro* adapted in a phytotron (88% surviving rate), then 16 plants from 4 selected clones were transferred to the *ex situ* collection and bloomed twice from the very first growing season (June and September). The number of the flower heads increased in the second year of field cultivation and an average of 328±138 capitula per plant were counted for the best clone. The first trials to establish a pilot plantation of pyrethrum are promising.

Keywords
Chrysanthemum cinerariaefolium, *ex vitro* adaptation, *in vitro* micropropagation, nutrient medium modification, plant acclimatization, pyrethrum

Introduction

Dalmatian pyrethrum *Tanacetum cinerariifolium* (Trevir.) Sch. Bip., also known by its synonyms *Pyrethrum cinerariaefolium* Trev., and *Chrysanthemum cinerariaefolium* Bocc (Pignatti 1982; Grdiša et al. 2009; Rahmatullah et al. 2010), is a perennial herb of Asteraceae family. It is endemic to the eastern coast of the Adriatic Sea, distributed from Italy to northern Albania and in the mountainous regions of Croatia, Bosnia and Herzegovina, and Montenegro (Staykov and Ilieva 1961; Nikolić and Rešetnik 2007; Grdiša et al. 2009; Grdiša et al. 2013; Šegota et al. 2016). The species was first introduced into agriculture in Armenia (Casida 1980), and is currently widely cultivated in many countries due to the pyrethrins it contains (Staykov and Ilieva 1961; Fulton et al. 2001a; Grdiša et al. 2009; Casida 2012). The use of pyrethrins as an insecticide is thought to have originated in Persia, where *Pyrethrum roseum* Bieb. and *Pyrethrum garneum* Bieb. were known long ago (Staykov and Ilieva 1961; Wainaina 1995). According to other authors, dried parts of *T. cinerariifolium* were used as insecticide in folk medicine in Croatia (Grdiša et al. 2009).

Natural pyrethrins can be characterized as an excellent means of combating insect pests (Jovetic 1994; Hammond 1996; Palmquist et al. 2012). They are very effective against a wide range of insects (Tattersfield et al. 1929; Arnaudov 1930; Richardson 1931; Greenhill 2007; Cai et al. 2010) acting as a contact insecticide (Grdiša 2009), and the risk of developing resistance is low.

Seed germination of *T. cinerariifolium* is not high, especially in natural populations (Singh and Sharma 1989; Fulton et al. 2001b). In our previous study on seeds gathered from an *ex situ* collection in Bulgaria, it was found that seeds quickly lost their vitality and their germination rate dropped dramatically after several years of storage (Ilinkin et al. 2020). Seeds can also be purchased from pyrethrum breeding programs (British Oxygen Company) with 80% germination rate (Fulton et al. 2001b). However, agricultural crops of *T. cinerariifolium* are usually established by vegetative propagation as plants derived from seeds vary greatly in pyrethrin content (Casida 1973; Ikahu and Ngugi 1988; Lindiro et al. 2013).

Access to high-quality plant material is the main limiting factor for the mass cultivation of *T. cinerariifolium*; therefore the development of an efficient *in vitro* protocol for micropropagation of selected high-yielding plants is a task for many researchers (Lindiro et al. 2013). Most studies have been based on agar-solidified media (Staba et al. 1984; Hussain et al. 1994; Keskitalo 1999) and rarely has liquid media been tested (Staba and Zito 1985; Keskitalo 1999). The characteristics of the culture medium can be a major factor in establishing a highly efficient *in vitro* culture. Some authors (Roest and Bokelmann 1973; Kaul et al. 1990; Hedayat et al. 2009) reported successful propagation of *Chrysanthemum*

species on MS medium (Murashige and Skoog 1962). According to Hedayat et al. (2009) the composition of MS medium is optimal for species belonging to the genus *Tanacetum*. These authors compared the results obtained on three media differing in their basic composition: MS, B5 (Gamborg et al. 1968), and SH (Schenk and Hildebrandt 1972) and noticed the highest weight of fresh biomass on MS medium. The addition of BAP in the medium stimulated the formation of multiple shoots and led to decrease of their length, which confirmed earlier results obtained for *Tanacetum vulgare* L. (Keskitalo 1999).

The aim of the present study was to establish suitable conditions for propagation of *T. cinerariifolium* by *in vitro* methods and to create an *ex situ* collection of selected clones, as a step towards introducing the species into agriculture in Bulgaria.

Materials and methods

Plant material

The initial studies on *in vitro* culture initiation were carried out with seeds of *T. cinerariifolium* taken from a private *ex situ* collection in the village of Bogdan, Bulgaria. Subsequently, seeds from a natural Dalmatian pyrethrum population were used for further experiments (MAPO2821 – Accession number from Croatian Plant Genetic Resources Database), kindly provided by Dr. Martina Grdiša.

Seed sterilization, nutrient media composition and culture room conditions

Seeds were disinfected after standard surface sterilization procedure: 1 min soaked into 70% ethanol, then 10 min into commercial bleach (Cl < 0.5%), and triple rinsed in distilled sterile water for 5, 10, and 15 min.

Seeds gathered from the collection of Bogdan village were germinated on three nutrient media on MS base (Murashige and Skoog 1962): medium MS free of plant growth regulators (PGRs), and two media supplemented with kinetin (Kin, Duchefa, NL) and indole-3-butyric acid (IBA, Duchefa, NL) in different concentrations: 1.0 mg/L Kin and 0.5 mg/L IBA (medium KI), or 0.2 mg/L Kin and 0.1 mg/L IBA (medium K_2I_1).

Seeds originating from the Croatian population were germinated on control MS medium (MS), three MS-based media supplemented with the antibiotic Medaxone (active compound ceftriaxone sodium) in concentrations 100 mg/L (medium MS100M), 200 mg/L (medium MS200M), and 300 mg/L (medium MS300M), and medium B5 (Gamborg et al. 1968) free of PGRs.

In consequence, medium MS200M supplemented with three concentrations of Ca (30, 75, and 120 mg/L) added as $CaCO_3$ were tested during subcultivations along with medium MS200M.

All media contained 30 g/L sucrose and were solidified with 6.5 g/L Plant agar (Duchefa, NL), they were autoclaved at 121 °C, under 1 atm, for 20 min, and then put into plastic containers with passive ventilation. Four sets of 100 seeds were used for each medium variant.

In addition, cultivation in temporary immersion system (TIS) was tested with shoots taken from *in vitro* culture with Croatia origin growing on agar-solidified MS medium MS200M. Six containers RITA were used, 10 shoots per container, with 200 ml liquid medium MS200M, flooding the shoots for 5 minutes 4 times a day.

Conditions in the culture room were: 16/8 h light/dark regime and temperature of 23±2 °C around the clock. Clones obtained by *in vitro* shoot multiplication were selected on the base of the number of surviving plants.

Culture subcultivation

Two months after the start of the experiments seedlings were cut to upper and lower parts and roots were removed, thus obtaining explants from stem segments. One-seed derived clones were obtained by several consecutive subcultivations on fresh medium with the same composition as the corresponding initial medium, in plastic containers: newly formed shoots were separated, and the longer ones were additionally cut to segments. Explants developed into new plantlets. Propagation coefficient (PC) was calculated as an average of *in vitro* plantlets obtained per explant. Subcultivations were performed at intervals of about two months for seedlings rising from the seeds gathered from Bogdan collection, and at intervals of three weeks for those originating from the Croatian population. Subcultivations on MS-modified media containing higher concentrations of Ca were done every month. Infected and necrotic plants were removed periodically during each subcultivation.

Ex vitro adaptation and outdoor acclimation

Three hundred and sixty plants belonging to four selected clones were obtained from seeds originating from the Croatian population germinated on medium MS200M and multiplied on medium MS200M supplemented with 75 mg/L Ca, were potted in soil mixture (Light mix Biobiss, France) and *ex vitro* adapted first in a growth chamber (POL-EKO Aparatura, Poland) for 6 weeks (under strict temperature and light control, and gradual decrease of the air humidity from 90% to 60%) and then in a room phytotron. Surviving plants were transferred to an unheated greenhouse and in October 2019 four plants per clone were acclimated outdoors in the *ex situ* collection of IBER, planted at a distance of 40 cm from each other and 40 cm between the rows. The numbers of both stem ramifications and flower heads per individual were assessed during the first and the second flowering in 2020. The numbers of the flower heads were compared between the clones and between the years 2020 and 2021.

Statistical analyses

Microsoft Excel (ver. 16.6) was used to calculate the regression equations, and ANOVA test (Microsoft Excel) to demonstrate statistical significance ($p < 0.01$) in the regressions shown. LSD Post Hoc (SPSS, version 26) test was used to verify statistically significant differences in seed germination ($p < 0.05$).

Results

In vitro cultivation on media supplemented with PGRs (initial seeds from Bogdan village)

Nineteen one-seed-derived *in vitro* clones of pyrethrum were multiplied on media MS, KI, and K_2I_1, up to eight subcultivations (Fig. 1). Explants formed one or more plantlets, and the presence of PGRs in the medium stimulated shoots formation and elongation; however, endophytic bacteria were noticed in some cultures, which caused necrosis and loss of entire clones. Obviously, the presence of Kin and IBA enhanced the microorganisms' multiplication as well, as 81.3% of the plantlets obtained on medium K_2I_1 and 65.0% of those obtained on medium KI died due to microbial contamination (Fig. 2), although losses were also high on the control MS medium. Propagation coefficients were calculated up to the third and the sixth subcultivations for media K_2I_1 and KI, respectively, because there were no more surviving plants on these media. It is noteworthy that with each subcultivation the number of plants dropping out of the experiment due to infections or necrosis increased. Both necrotic shoots and necrotic rooted *in vitro* plants were observed. The highest percentage of surviving plants was noticed on PGR-free MS medium, but after the eighth subcultivation, all plantlets were affected by necrosis or microbial contamination.

In vitro cultivation on media supplemented with antibiotic (initial seeds from Croatia)

The germinating rates of the seeds on the five tested media: basal MS or B5, and MS media supplemented with different concentrations of Medaxone, were similar (Fig. 3). The effect of the antibiotic was immediate, as at the time of the first subcultivation the number of the infected seedlings on medium MS100M was lower than on the control

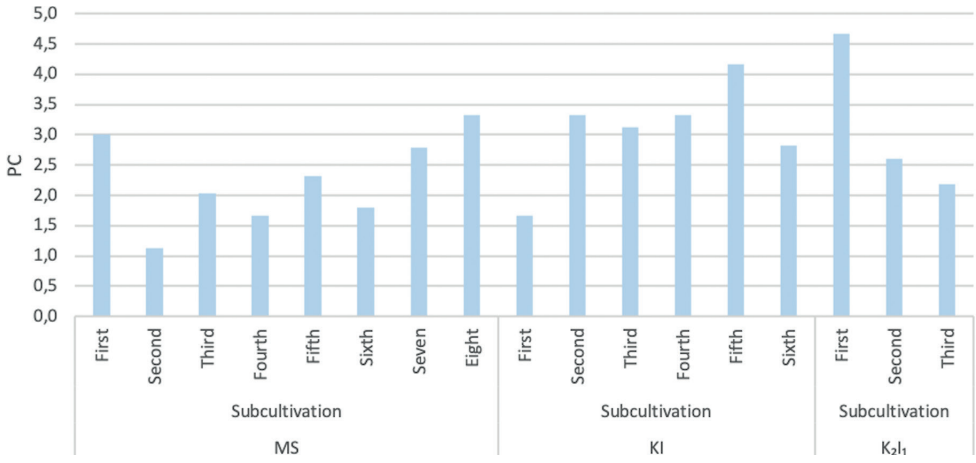

Figure 1. Effect of the plant growth regulators Kin and IBA on shoot multiplication rate.

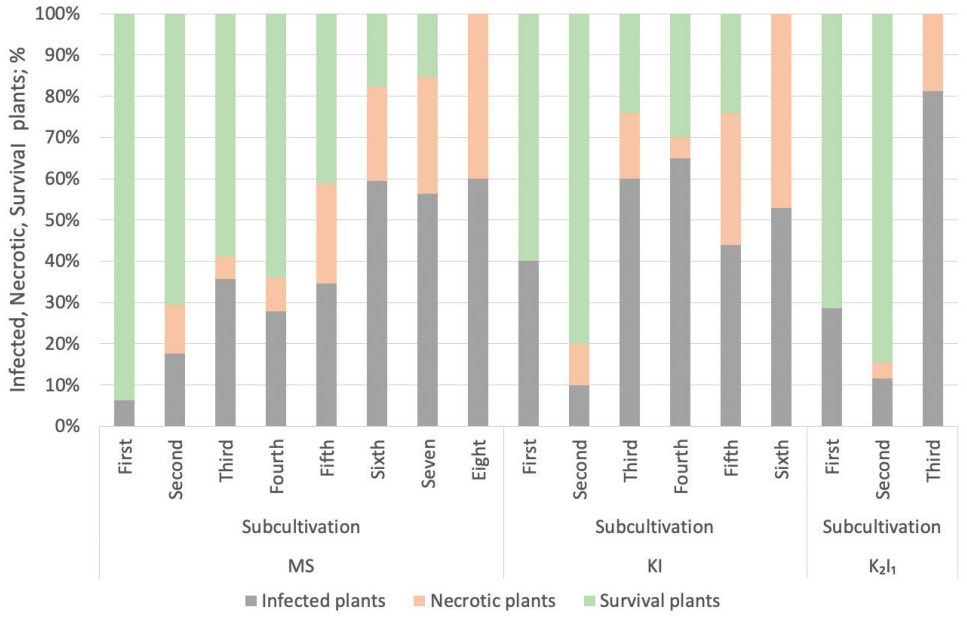

Figure 2. Effect of the PGRs Kin and IBA on plantlets survival in long-term *in vitro* culture.

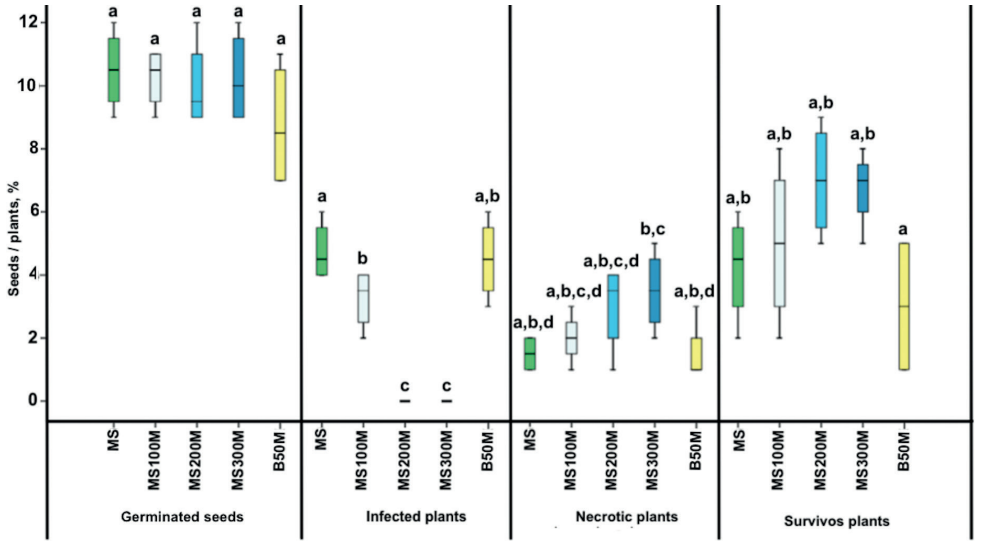

Figure 3. Seed germination and seedling survival evaluated as percentages from all tested seeds ($p < 0.05$).

MS medium ($p < 0.05$), while no infected seedlings were observed on the two media containing higher Medaxone concentrations (Fig. 3). The highest percentages of the surviving *in vitro* seedlings were on the same two media: MS200M and MS300M. However, with increasing antibiotic concentration, there was a clear trend towards an

increase in the percentage of necrotic plants, therefore medium MS200M was assessed as the best one for culture initiation, ensuring both bacteria elimination and the highest number of surviving seedlings.

Medium composition slightly influenced the time needed for seed germination, but there was no distinct peak of germination energy in any of the variants (Fig. 4) as the maximal percentage of germinating seeds in one day was 3%. Seeds began to germinate earliest on MS control medium (on day 12.5±1.7) but the duration of seeds' germination was the longest on this medium (15.5±2.5 days). Seed germination on all media containing antibiotic began about the 15th day and its duration was shorter (12.5±2.9 days for MS100M, 11.5±2.5 days for MS200M, and 11.5±0.6 days for MS300M). The longest time it took for seeds to start germinating was on B5 medium: 16.8±2.2, and the period of seed germination was 14.0±2.2 days.

In vitro subcultivation began with different numbers of seedlings on the tested media (between 12 and 28) due to the differences in their survival rates. Each seedling was separately multiplied during 8 consecutive subcultivations, which resulted in many one-seed-derived clones (Fig. 5A, B). The average propagation coefficients of all subcultivations are presented for each medium on Fig. 6. Cultures grown on antibiotic-supplemented media showed a tendency toward a slight decrease in the multiplication rates compared to that of the control culture on basal MS medium. Among the cultures grown on the media without antibiotic, the one on medium B5 had a lower PC, which was similar to that of the culture grown on MS100M containing Medaxone at the lowest concentration. The coefficient of variation of multiplication rate was higher in the antibiotic-free cases (8.82% for medium MS and 8.83% for medium B5) than that estimated for the media containing Medaxone (4.75% for MS100M, 6.08 for MS200M, and 6.22% for MS300M).

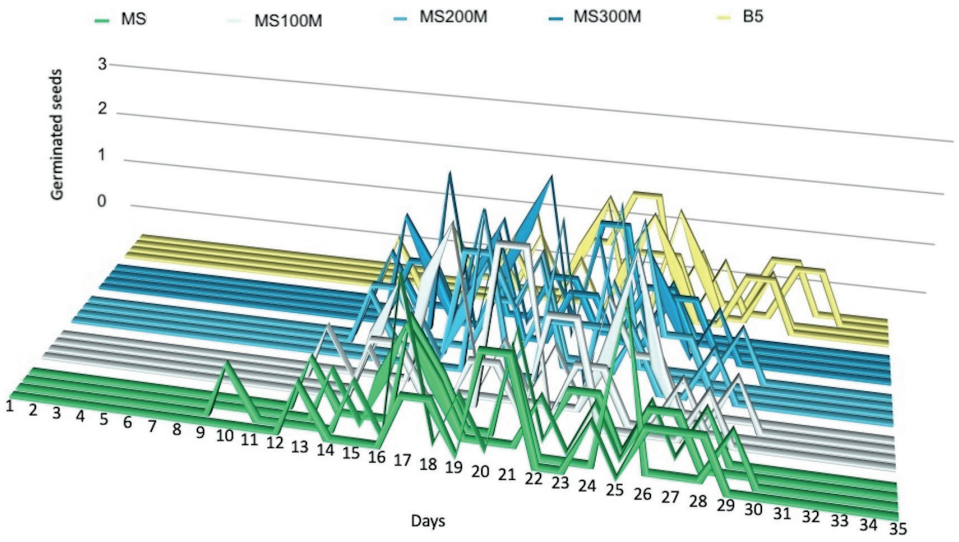

Figure 4. Effect of nutrient media composition on germination energy.

Figure 5. *In vitro* propagation of *T. cinerariifolium* plants (origin: Croatian natural population) **A** *in vitro* culture on agar-solidified medium **B** multiplication of *in vitro* clones **C** cultivation in TIS **D** *ex vitro* adaptation in a growth chamber **E** adaptation in a room phytotron **F** acclimated plants in the *ex situ* collection, first flowering in June 2020 **G** flowering in June 2021.

At every subcultivation, there were dropping out shoots. On MS and B5 antibiotic-free media only a few plantlets survived due to both bacterial infections and necrosis, the importance of which varied from one subcultivation to another (Fig. 7). At the last subcultivation, the number of surviving plantlets on MS and B5 media were 15 and 3, respectively, while on media supplemented with Medaxone their numbers were significantly higher: 58 on medium MS100M, 671 on MS200M, and 114 on MS300M, all of them well-shaped, rooted, and ready to be *ex vitro* adapted. Bacterial infection was completely eliminated in media containing 200 or 300 mg/L Medaxone; however, the highest antibiotic concentration seemed to inhibit shoot multiplication, as the number of *in vitro* plants at the 8th subcultivation was almost 6 times higher on the medium supplemented with 200 mg/L Medaxone. Medium MS200M was chosen for further optimization, as the number of necrotic plantlets remained high.

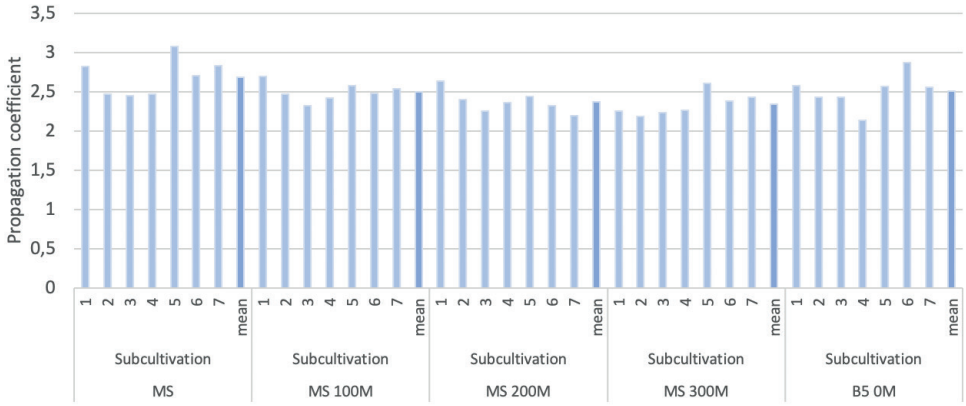

Figure 6. Effect of antibiotic concentration on the propagation rate of pyrethrum *in vitro* cultures.

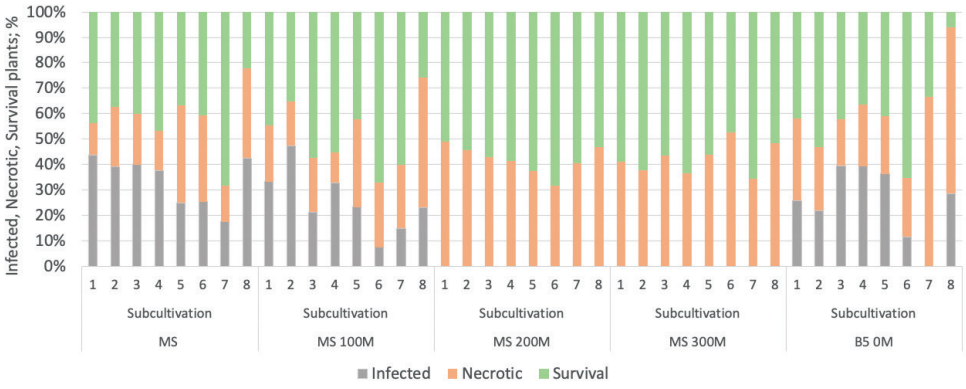

Figure 7. Effect of antibiotic concentration on survival of pyrethrum plants under long-term *in vitro* culture conditions.

Pyrethrum *in vitro* cultivation in TIS, RITA containers with liquid medium MS200M, did not improve the propagation coefficient of the clones tested. Plantlets formed long roots and grew faster, but the percentage of necrotic plants remained high (Fig. 5C). Agar-solidified medium turned out to be more suitable.

In vitro cultivation on MS modified medium, effect of Ca

The MS medium was modified by increasing the concentration of Ca, added as $CaCO_3$, and further optimization of medium composition was performed on MS supplemented with 200 mg/L Medaxone. The propagation coefficients of the four pyrethrum *in vitro* clones multiplied on the three media supplemented with different concentrations of Ca and on medium MS200M, remained similar, between 1.9 and 2.1 (Fig. 8).

However, the number of necrotic plants was significantly influenced (Fig. 9). The variation of PC decreased with the increase in the concentration of Ca supplemented: on medium MS200M with no added Ca it was 6.04; while at 30 mg/L Ca it was 5.40; at 75 mg/L Ca – 4.80, and at 120 mg/L Ca – 4.24. The lowest percentage of necrotic plantlets was noticed on medium containing 75 mg/L Ca, followed by that on medium with 120 mg/L Ca. However, with the increase of subcultivations, the percentage of necrotic plants on Ca-supplemented media increased, and in the case of the two higher concentrations these trends were statistically significant. At the highest concentration (120 mg/L Ca), this correlation was much more pronounced (Fig. 9), therefore the modified MS medium supplemented with 75 mg/L Ca and containing 200 mg/L Medaxone was selected as the best one for *T. cinerariifolium in vitro* multiplication. No more infected plants were observed, and the percentage of necrotic plants decreased threefold, which resulted in the formation of 38.06±10.11 new plants per initial shoot for a period of 7 months.

Ex vitro adaptation and outdoor acclimation

In vitro multiplied plantlets rooted spontaneously on all media tested (Fig. 5A). Several thousands *in vitro* plants were obtained in one year on the optimized medium. After 6 weeks of *ex vitro* adaptation in the growth chamber, 88% of the plants strengthened and reached about 10 cm in height (Fig. 5D). Almost all plants survived during the next steps of adaptation in the room phytotron and the greenhouse (Fig. 5E). Sixteen plants from 4 clones were transferred outdoors to the *ex situ* collection of IBER, where they grew and bloomed in June at the first season (Fig. 5F). Secondary flowering was also observed in autumn but with a smaller number of

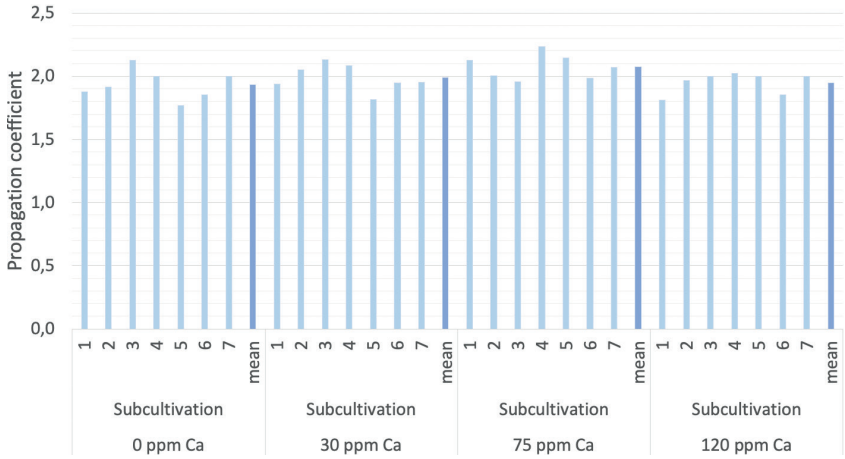

Figure 8. Effect of increasing calcium concentration in the medium on pyrethrum multiplication capacity *in vitro*.

capitula (Fig. 10). Differences in stem branching and in the number of capitula were observed not only between clones, but also between individuals within the same clone (Fig. 11). The uneven number of capitula of the plants from one and the same clone was most probably due to the faster growth of some individuals at the very beginning, which overshadowed the neighboring individuals. Plants of Clone 4 suffered from the shadow of a tree nearby, which led to delay of their growth and finally to their death. Plants of Clone 1 flowered slightly before those of the other clones (Fig. 12). Plants' growth continued and an average of 328±138 flower heads per individual plant formed in the second year for the best clone (Clone 3), which was 6 times more compared to the first year (Fig. 5G). Plants in the greenhouse also bloomed, but their growth was limited by the size of the pots. Some plants did not survive the next winter outdoors and were replaced by plants growing in the greenhouse. The following spring and summer were quite rainy, which had a bad effect on their development.

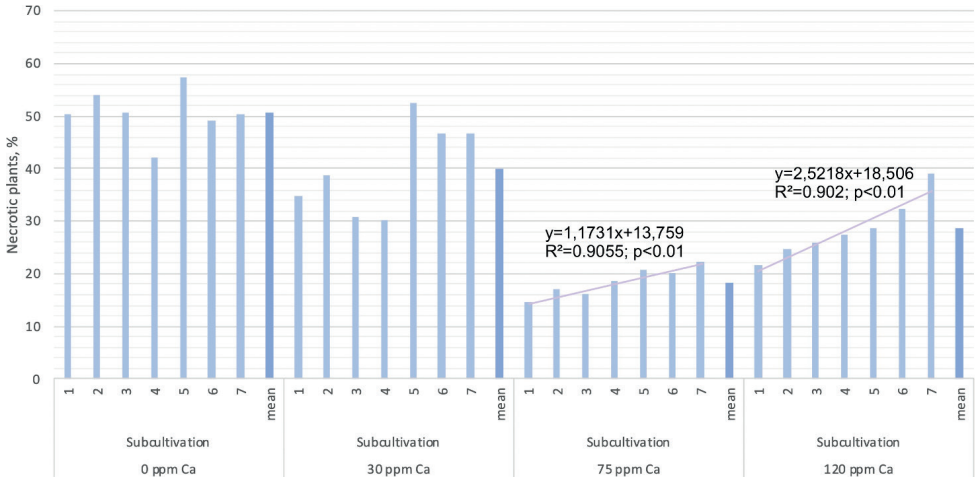

Figure 9. Effect of increasing calcium concentration in the medium on plant necrosis frequency ($p < 0.01$).

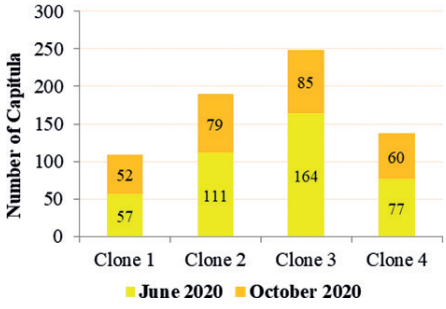

Figure 10. Primary and secondary flowering in the first year of cultivation in the *ex situ* collection.

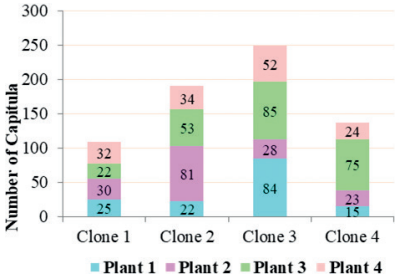

Figure 11. Total number of capitula in the first year of cultivation in the *ex situ* collection.

Figure 12. Clones during their secondary flowering in autumn 2020.

Discussion

For their normal growth and development, plants need macro salts and microelements, the optimal concentrations of which may depend on the species. Among media for *in vitro* plant propagation, MS and B5, as well as their modifications, with or without addition of PGRs, are most commonly used. Bacterial contamination could be critical for *in vitro* culture initiation despite disinfection of primary explants (Hennerty et al. 1987; Misaghi and Donndelinger 1990; Keskitalo 1999). Usually, the composition of media for plant cultures is not optimal for microorganisms or suppresses their development, so their presence in the culture can go unnoticed for a long time (Leifert and Waites 1992; Leifert et al. 1994; Isenegger et al. 2003). In general, bacterial infections appear soon after the cultures are transferred to fresh media, because during subcultivations the roots are removed and the plants are cut into segments, making them weaker, as in the case of our experiments. Antibiotics are habitually added to the medium to control bacterial contamination, but they can alter the morphogenesis of *in vitro* cultures and slow down their growth (Teng and Nicholson 1997; Eady and Lister 1998; Keskitalo et al. 1998; Teng and Teng 2000; Bergant et al. 2005). The effectiveness of the antibiotics varies depending on the plant species, and sometimes even on the species genotype (Keskitalo et al. 1998; Bergant et al. 2005).

In our opinion, necrosis observed *in vitro* in pyrethrum cultures was due to an insufficient amount of calcium in the most commonly used media. *T. cinerariifolium* is not pretentious regarding soil conditions (with the exception of waterlogged soils), but it develops remarkably well on calcareous soils (Hennigsberg 1941; Astadzhov et al. 1980). According to some authors, the natural populations of the species are on

carbonate soils and karst terrains (Sladonja et al. 2014). The physiological functions of calcium are related to cell division, normal functioning of cell membranes and activation of enzymatic reactions that positively affect photosynthesis. Calcification strengthens plant cell walls, thereby increasing plant resistance to infections (Gorbanov et al. 2005; George et. al. 2007). In addition, calcium manifests synergism with NO_3, i.e. facilitates its uptake and hinders the absorption of some elements such as K^+, Na^+, Mg^{2+}, Fe^{2+} and Zn^{2+}. Calcium deficiency in the presence of potassium and magnesium can lead to significant changes in the normal functioning and growth of roots and root hairs (Gorbanov et al. 2005). Another negative effect of this insufficiency can be the severe limitation of the shoot tips growth and even their death due to hyperhydricity, which can be avoided by frequent subcultivation (George et. al. 2007). According to some authors (Sha et al. 1985; Singha et al. 1990), the degree of changes may differ among different genotypes of the same species. On the other hand, excessively high carbonate content in soils and high pH of the soil solution can negatively affect plant development and production of pyrethrum flower heads (Sastry et al. 1988). In our experiments, the optimal result was obtained at the intermediate calcium concentration of the three variants tested. It is important to note that calcium is also essential for *in vitro* morphogenesis, especially when using PGRs such as auxins and cytokinins (George et al. 2007). This may explain the faster death of plants subcultured on media containing PGRs, without antibiotic and supplemental calcium. In *in vitro* cultures, the addition of calcium as $CaCl_2$ is not preferable, as it may even lead to a significant increase in chlorine content to levels of chlorine toxicity (George et al. 2007). On the other hand, addition of calcium as $CaCO_3$ and subsequent lowering of pH, which usually occurs in *in vitro* cultivation, could also lead to an increase in chlorine content. This may be a likely explanation for the presence of credible trends (at concentrations of 75 and 120 mg/L Ca) for an increase in necrotic plants with increasing cultivation time.

Some authors recommended for *in vitro* shoot rooting MS medium free of PGRs or B5 supplemented with 2 mg/L NAA (Rostiana and Seswita 2007; Hedayat et al. 2009). Information on *ex vitro* adaptation and outdoor acclimatization of regenerated pyrethrum plants is scarce. Survival of about 2/3 of the plants was reported, with the remark that the process can be greatly improved using a phytotron with tightly controlled environmental parameters (Catalano et al. 2011). Our results proved the positive effect of the strict control of the ambient conditions in the growth chamber during the first weeks of *ex vitro* adaptation, when *in vitro* plants are most vulnerable to changes in air humidity and temperature. Soil characteristics are of importance for seed germination and plant development. A pot experiment involving several different soil types proved that Rendzic Leptosol was the most suitable for pyrethrum seed germination (Ilinkin 2019). The soil mixture used for *ex vitro* adaptation in pots (Light mix Biobiss, France) was suitable for pyrethrum plants and they survived several years in the unheated greenhouse and flowered. The field plot conditions were less appropriate as the soil was loamy-sandy, poor in carbonates. Plants in the *ex situ* collection bloomed twice a year; however during the third growing season some of them remained small. Sunlight proved to be crucial for the survival and vigorous growth of *T. cinerariifolium* plants. Plants of

this species should be planted at a greater distance from each other to avoid their death. Wandahwa et al. (1996) also reported the importance of the edaphic conditions for successful cultivation of the species and capitula yield. These authors propagated selected clones vegetatively in soil for 3–4 months, and then seedlings were planted in a permanent place of cultivation at 60 cm inter-row spacing and 30 cm between plants in the row. Up to 10% of the plants died and were replaced with new seedlings to maintain the plantation. Planting on ridges was recommended by Kroll (1963) to provide better soil aeration and avoid waterlogging. In our case, the dead of some plants was probably due to the rainy spring and summer and the shadow of the neighboring tree.

Data on the influence of nitrogen, phosphorus and potassium fertilization reported for different pyrethrum growing countries are conflicting. Clone-specific responses to nitrogen and phosphorus fertilization were also noted (Ngugi and Ikahu 1989). Our results related to some features of the clones we selected, such as the number of capitula, are consistent with the observations of these authors and confirm the assumption that the study should be conducted at individual level.

Conclusion

A protocol for *in vitro* micropropagation of *Tanacetum cinerariifolium* has been established, starting with seeds, and several one-seed-derived clones have been obtained by multiple consecutive subcultivations. The optimization of the nutrient medium composition was of crucial importance for the successful *in vitro* cultivation. Shoot loss due to both endophytic bacteria and necrosis was overcome by adding an antibiotic and modifying the calcium concentration in the medium, according to the specific requirements of pyrethrum. The first attempts to establish a pilot plantation of pyrethrum are promising, as *ex vitro* adaptation of the plants was easy and the outdoor acclimated plants bloomed twice from the very first growing season. However, field cultivation conditions need to be improved, as pyrethrum plants are shade intolerant and direct sunlight is crucial for their survival. The number of flower heads increased during the second year of cultivation in the *ex situ* collection, and some differences were found between the clones tested. Flower heads were sampled from each individual for analysis of pyrethrin content. More field experiments are needed to select highly productive individuals in terms of both number of flower heads and concentration of pyrethrins. Selected individuals should be further *in vitro* propagated to produce seedlings for pyrethrum plantation.

Acknowledgements

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RESEARCH ARTICLE

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Study of the plant growth-promoting capacity of *Pseudomonas putida* 1046 in a model plant system

Gloria Georgieva[1,2], Trayana Nedeva[1],
Marina Badalova[2], Veronika Deleva[2], Valentin Savov[2]

1 *Department of General and Industrial Microbiology, Faculty of Biology, Sofia University St. Kliment Ohridski, 8 Dragan Tzankov Str., Sofia 1164, Bulgaria* **2** *ROMB LTD, 1 "Aboba" St. 1606, Sofia, Bulgaria*

Corresponding author: Gloria Georgieva (gloria.georgievaa@gmail.com)

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Abstract

Plant Growth Promoting Rhizobacteria (PGPR) represent a microbial community that exerts growth-promoting capabilities in plants by various mechanisms. Among the PGPR genera, *Pseudomonas* spp. deserves special attention. It is due to its characteristic traits, like the production of phytohormones and siderophores, solubilization of minerals and phosphates, and plant protection from biotic and abiotic stress. These PGPR properties depend on the microorganism and its plant counterpart. The use of microbial strains as bioinoculants must consider the physiological and economic aspects of the process, and the plant growth stimulating effect has to be checked and proved. This study aimed to explore the PGP capacity of *Pseudomonas putida* 1046 strain in a model plant system of the economically important corn culture (*Zea mays*). The effect of the strain's metabolic status on the plant germination capacity was evaluated. Bacterial cultures, grown 16 h and 48 h, were explored for the treatment of the corn seeds at three experimental concentrations: 0.1, 0.2, and 0.4%, and monitoring of their germination capacity through the growth indicators length of the radicle, length of the coleoptile, and the number of lateral roots. The data obtained outline the positive effect of *Pseudomonas putida* 1046 on the germination capacity of corn when applied at 0.2% concentration. The *in vitro* treatment of the model plants with 0.2% suspension resulted in a 22.87%–28.33% increase in the length of the radicle, a 35.96%–49.56% increase in the length of the coleoptile, and a 5.41–16.67% increase in the number of the lateral roots. High values of the vigour index (2125 for 16 h and 2721 for 48 h culture) were also registered. The strain's ability to produce siderophores of hydroximate type and exhibit phosphate solubilizing activity is proved. The optimal treatment parameters of the corn seeds comprise the application of 0.2% suspension of 16 h grown *Pseudomonas putida* 1046 strain for five days.

Keywords
biofertilizer, PGPR, rhizosphere microorganisms, *Zea mays*

Introduction

The soil microbial communities influence plant growth and development. The playground for this effect is the rhizosphere since the plant root system and the soil microorganisms interact there. This interaction is a complex process influenced by various environmental factors, such as temperature, humidity, pH, availability of nutrients, etc. (Mimmo et al. 2018). The plant-associated bacteria can execute beneficial or deleterious effects on plant growth (Dobbelaere et al. 2003). The soil bacteria that positively affect the plant are called Plant Growth Promoting Rhizobacteria (PGPR) (Kloepper and Schroth 1978). PGPR constitute a vital component of the rhizosphere. They support the development of the plant's root and shoot systems. PGPR positively affect plant morphogenesis, flowering, and photosynthesis efficiency (Hossain et al. 2017).

The need for environmentally friendly fertilizers or biofertilizers is constantly increasing nowadays (Meliani et al. 2017) on account of soil, water, and air contamination due to chemicals such as fertilizers and pesticides. A few alternatives to fertilizers that are environmentally friendly have been developed (Gupta et al. 2015). Thus, the impact of PGPR on agriculture steadily increases as they offer an attractive way to replace chemical products. Fertilizers containing microorganisms are an appropriate alternative to chemical ones since they are a natural component of healthy soil that enriches rather than pollutes it (Vessey 2003; Nadeem et al. 2016). Moreover, they produce growth-promoting substances in large quantities that influence the plant's morphology (Bhattacharyya and Jha 2012).

Many species belonging to g. *Pseudomonas* possess plant growth-promoting (PGP) characteristics that allow their application as bacterial fertilizers. They are abundantly present in the rhizosphere. *Pseudomonas* spp. are known for their well-established growth-promoting mechanisms. They encompass better root colonization, the production of enzymes, metabolites, phytohormones and siderophores, mineral and phosphate solubilization, and plant protection from biotic and abiotic stress (Nadeem et al. 2016).

The application of the microbial PGP properties depends on the microorganism and its plant counterpart. Corn (*Zea mays*) is one of the most important cereal crops in the world after wheat and rice. It is used as livestock feed, human food, and raw material for several industries (Kumar and Jhariya 2013). The corn growth, development, and yield are well-focused biotechnological targets, and the plant attributes, such as germination rate, drought tolerance, and yield components, are objects of profound research and technological interest. PGPR could be used for maximizing these parameters and applied as a potent tool in sustainable agriculture. The contribution of g. *Pseudomonas* representatives in the enhancement of some biochemical and agronomic

parameters of corn under normal conditions or exposure to various abiotic stress is well-recognized in the literature (Mubeen et al. 2021). However, the use of pseudomonads and their corn counterpart in biofertilization programs must consider the physiological and economic aspects of the process, and the plant growth stimulating effect has to be checked and proved.

This study aimed to explore the PGP capacity of bacteria belonging to *Pseudomonas putida* species in a model plant system of the economically important corn culture *Zea mays*.

Materials and methods

Bacterial strain and test plants

Pseudomonas putida 1046 strain was used in this study. It possesses the biochemical capacity to catabolize aromatic hydrocarbons and their derivatives. Corn seeds (Dekalb – DKC 5830 HD (Hybrid 101)) were used as a model plant test system. They are characterized by a fast initial development, high quality of the grain, tolerance to high sowing norms, and a response to high fertilizers and sowing norms. In some of the experiments, soybean seeds (*Glycine max* L.) were used.

Culture media and cultivation conditions

The experimental strain was maintained on Nutrient agar (BB-NCIPD Ltd., Bulgaria). Batch cultures in Nutrient broth were obtained after cultivation at 28 °C for 16 h or 48 h on a rotary shaker (220 rpm).

Biochemical analysis

Pseudomonas putida 1046 biochemical profile was established applying ApiZYM rapid systems for the detection of bacterial enzymes and Api 20 NE standardized system for the identification of non-fastidious, non-enteric Gram-negative rods. The tests performance was according to the methods described by Humble et al. (1977) and Donnelly (2006). Siderophore detection was performed following the method of Alexander and Zuberer (1991). The assay for phosphate solubilization was done according to Shahid et at. (2015).

PGP effect determination

The seed germination method was applied to study the PGP effect of *Pseudomonas putida* 1046 strain on corn (*Zea mays*) seeds. The corn seeds were subjected to surface sterilization before the germination test. Sodium hypochlorite (0.02%) solution was applied for 2 min., followed by intense rinsing with sterile distilled water. The seeds'

germination capacity was assessed through the growth parameters of the plant root system (Sharma et al. 2014). Water suspensions with a defined concentration of 0.1%, 0.2%, and 0.4% were prepared from the 16 h and 48 h test microorganisms containing 10^8 CFU/ml. The corn seeds were immersed in these suspensions and cultured for five days at 25 °C. The growth parameters: length of the radicle, length of the coleoptile, and the number of lateral roots were measured. The positive effect of the test strain on the germination capacity of the corn plants was calculated as a percentage of untreated control. The plant vigour index was also calculated using the following formula: Vigour index = (mean radicle length + mean coleoptile length) × % germination (Baki et al. 1973). To prove the relationship (and its strength) between *Pseudomonas putida* 1046, and the growth parameters of the plant root system the seed germination method was applied to another technical crop, soybean (*Glycine max* L.), at the same experimental conditions. Each treatment was performed following a completely randomized design with three replicates and 10 seeds/replicate.

Correlation analysis

Correlation analysis was performed by calculating the correlation coefficients with MS Excel software of two variables data sets – *Pseudomonas putida* 1046 suspension concentration (0% (control), 0.1%, and 0.2%) and the growth parameters of the plant root system (the indices length of the radicle, length of the coleoptile, the number of the lateral roots, and the vigour index).

Data analyses

All presented data are mean values of at least 3 individual experiments. The data were analysed by MS EXCEL build-in function, and the results were presented as means with standard deviations (n=3).

Results

Pseudomonas putida 1046 strain has been selected on the basis of preliminary biochemical analyses that indicate its potential plant growth-promoting capacity. The biochemical profile of the strain was established applying ApiZYM and Api 20 NE detection systems. The biochemical profile data presented in Table 1 showed activity of the enzymes alkaline phosphatase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase, indicative for PGP potential due to their involvement in the phosphate solubilization and mineralization. Additional phytohormones analysis showed the biosynthetic capacity of the test strain for the phytohormones gibberellic acid (GA3), indole acetic acid (IAA), and jasmonic acid (data not shown).

The effect of the strain metabolic status on the test plants' germination was evaluated in order to check the PGP capacity of *Pseudomonas putida* 1046 strain. The PGP

Table 1. Biochemical characterization of the *Pseudomonas putida* 1046 strain.

ApiZYM		Api 20NE	
Alkaline phosphatase	positive	NO_3 Reduction of nitrates to nitrites	negative
Esterase (C 4)	positive	Reduction of nitrates to nitrogen	positive
Esterase Lipase (C 8)	positive	TRP Indole production (tryptophan)	negative
Lipase (C 14)	negative	GLU Fermentation (glucose)	negative
Leucine arylamidase	positive	ADH Arginine dihydrolase	positive
Valine arylamidase	negative	URE Urease	negative
Cystine arylamidase	negative	ESC Hydrolysis (βglucosidase) (esculin)	negative
Trypsin	negative	GEL Hydrolysis (protease) (gelatin)	negative
α-chimotrypsin	negative	PNGP β-Galactosidase (paranitropheny l-ßDgalactopyranosidase)	negative
Acid phosphatase	positive	GLU Assimilation (glucose)	positive
Naphthol-AS-BI-phosphohydrolase	positive	ARA Assimilation (arabinose)	positive
α-galactosidase	negative	MNE Assimilation (mannose)	positive
β- galactosidase	negative	MAN Assimilation (mannitol)	positive
β-glucuronidase	negative	NAG Assimilation (N-acetylglucosamine)	positive
α-glucuronidase	negative	MAL Assimilation (maltose)	negative
β-glucosidase	negative	GNT Assimilation (potassium gluconate)	positive
N-acetyl-β-glucosaminidase	negative	CAP Assimilation (capric acid)	positive
α-mannosidase	negative	ADI Assimilation (adipic acid)	positive
α-fucosidase	negative	MLT Assimilation (malate)	positive
		CIT Assimilation (trisodium citrate)	positive
		PAC Assimilation (phenylacetic acid)	positive
		OX Cytochrome oxidase	positive

capacity of the bacterial strain was assessed in respect to its test plants. The seeds germination of the test plants was used as an assessment indicator through the quantitative measurement of three indices: length of the radicle, length of the coleoptile, and number of lateral roots. The corn seeds at three experimental concentrations (0.1, 0.2, and 0.4%) were treated with bacterial cultures, grown for 16 h and 48 h and monitored for their germination capacity. The length of the radicle, length of the coleoptile, and the number of lateral roots were used as growth indicators.

Length of the radicle

The values for the growth indicator length of the radicle are shown in Figs 1A, 2. They indicate a positive effect of the bacterial culture on the plant physiology, best represented during the treatment with 0.2% suspension. An increase of 22.87% for the 16 h culture and 28.33% for the 48 h one was observed. The values for the remaining two tested concentrations (0.1% and 0.4%) showed variations in the range of 1.7 to 9.9% compared to the untreated control.

Length of the coleoptile

The data for the length of the coleoptile show that all three tested concentrations exhibit a positive effect on its growth (Figs 1B, 2). The values indicated an increase in this

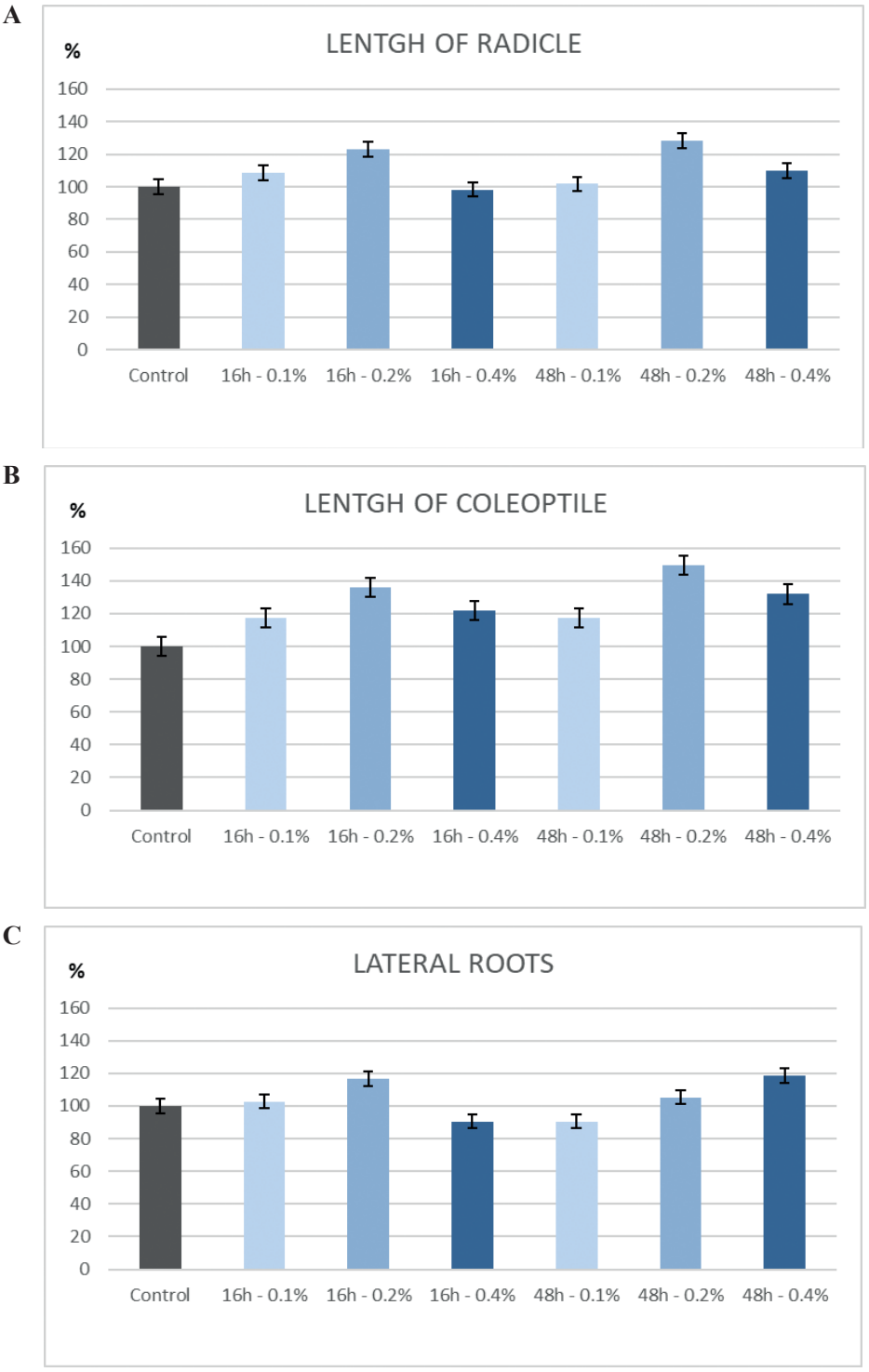

Figure 1. PGP effect of *Pseudomonas putida* 1046 on the germination of corn seeds evaluated through length of the radicle (**A**), length of the coleoptile (**B**), and the number of lateral roots (**C**).

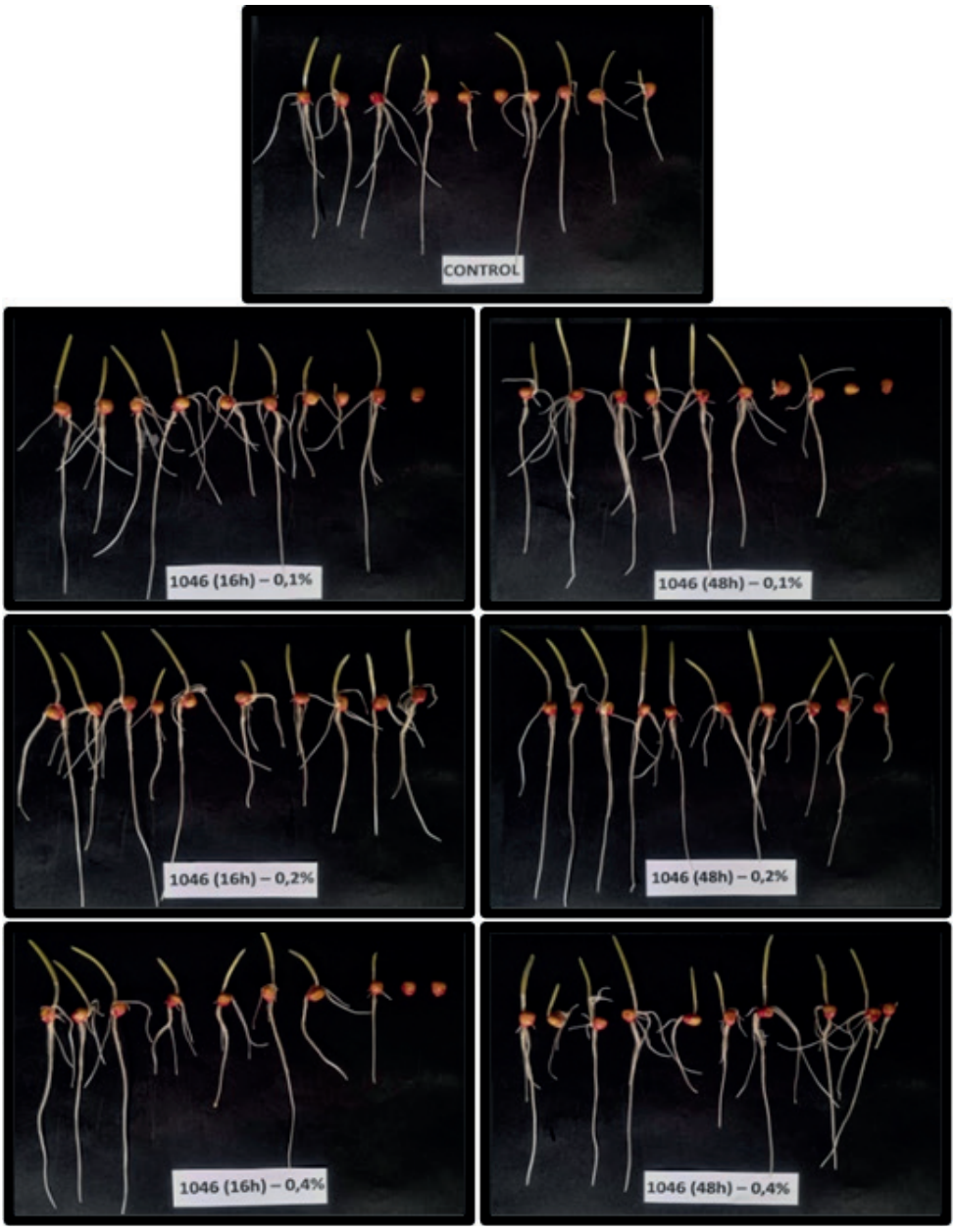

Figure 2. Visual representation of *Pseudomonas putida* 1046 PGP effect on corn seeds germination capacity.

growth parameter between 17.4% and 49.6%. The tendency for the best stimulatory effect of 0.2%, acknowledged for the length of the radicle, was also observed here. An increase of 35.96% for the 0.2% and 49.56% for 0.4% suspensions was registered, respectively. Comparing the physiological status of *P. putida* culture, it is evident that at 0.1%, the stimulatory effect was the same (11.7%) regardless of the culture age. With

the increase of the bacterial strain concentration (0.2% and 0.4%), the 48 h population stimulated the coleoptile development more efficiently (10% to 15%) compared to the 16 h one.

Number of the lateral roots

The third index (number of the lateral roots), used for assessment of the corn germination capacity, showed a relatively lower stimulatory effect (2.78%–18.60%) as compared to the previous two indicators. However, it is evident that the tendency for the highest positive results obtained by the 0.2% suspension of the bacterial strain is kept (Figs 1C, 2). The 16 h bacterial culture showed higher growth promotion of the lateral roots compared to the 48 h (16.67% vs. 5.41%).

Among the three tested bacterial concentrations, the 0.2% suspension was the right choice for the seeds' treatment. The 0.1% expressed no or mild positive effect on the number of lateral roots and the length of radicles, as shown in Figs 1, 2. Compared to the 0.2%, the highest tested concentration of 0.4% did not significantly affect any of the analysed indices.

Vigour index

The values for the vigour index are presented in Fig. 3. The corn plants were treated with 0.1%, 0.2%, and 0.4% suspensions of 16 h and 48 h *P. putida* culture and

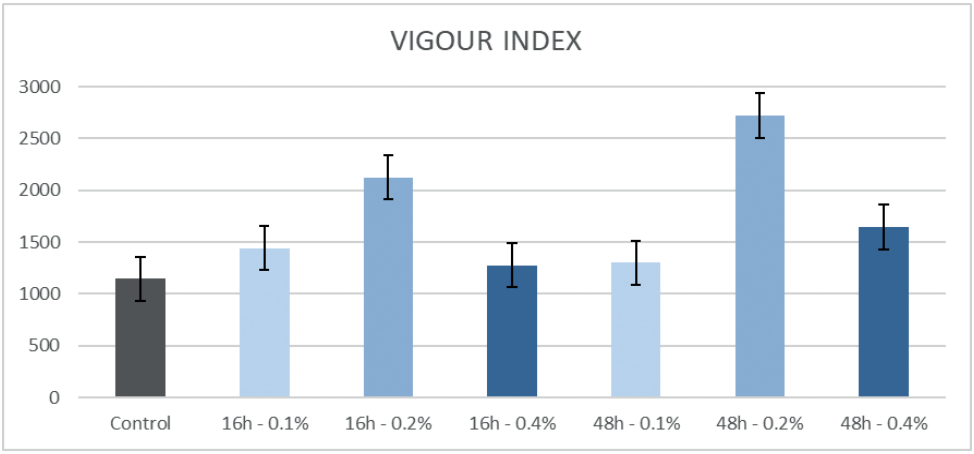

Figure 3. Effect of bacterial inoculation on corn vigour index after 5 days *in vitro* germination.

Table 2. Correlation coefficients for the relationship *Ps. putida* and plant root system growth parameters.

Time (h) / Index	Radicle	Coleoptile	Lateral roots	Vigour index
16	0,98927	0,99982059	0,95572399	0,975104
48	0,891681	0,99194071	0,36147266	0,90716648

evaluated five days after *in vitro* germination. The highest values of the vigour index were registered after treatment with 0.2% suspension of the bacterial strain: 2125 for 16 h and 2721 for 48 h culture.

Correlation analysis

Based on the experimental data for the plant growth indices evaluated, correlation analysis for the bacterial and plant data sets was performed as described in the section Materials and Methods. The results are presented in Table 2. They prove a positive relationship between the *Pseudomonas putida* 1046 and the growth parameters of the plant root system and the vigour index. In quantitative aspect, the correlation coefficient (r) can be evaluated as a very strong one since its values fall into the range 0.9–1.0 for the 16 h grown culture and 0.4–1.0 for the 48 h one.

Pseudomonas putida 1046 PGP effect on soybean

In order to confirm the PGP effect of *Pseudomonas putida* 1046 strain on the corn test plant, two different approaches were used: testing another plant model system and extended research on PGP characteristics of the bacterial strain.

Soybean (*Glycine max* L.) was exploited as a second model system. The crop has a global position as one of the most significant agri-cultures, a source of food, protein, and oil. The PGP effect of *Pseudomonas putida* 1046 was evaluated using two of the indices applied for the corn: length of the radicle and the epicotile (coleoptile). The tendency for enhanced germination due to the increased number of lateral roots and enlarged epicotile length, promoted by *Pseudomonas putida* 1046, is confirmed by the data depicted in Fig. 4A, B.

The ability of the model bacterial strain to produce siderophores and solubilize phosphates was examined by qualitative analyses. These activities were tested due to their promotional effect on plant growth: enhanced metal accumulation in plants and putative application for phytoremediation purposes of siderophores, and the growth-promoting ability attributed to phosphate solubilization. The data about these PGP characteristics are presented in Figs 5, 6. They indicate the production of siderophores of hydroxamate type (hallo coloured in yellow-orange) and the typical colourless hallo of the solubilized phosphates.

Discussion

Nowadays, extensive research is focused on PGPR as a versatile replacement of fertilizers, pesticides, and other agrochemicals for the promotion of plant growth. PGPR influence in a positive way (directly or indirectly) the soil structure and fertility, decomposition of organic matter and organic pollutants, solubilization of nutrients of mineral nature, production of plant growth regulators, stimulation of the root growth, and execution of biocontrol against soil and seed-borne pathogens. (Gupta et al.

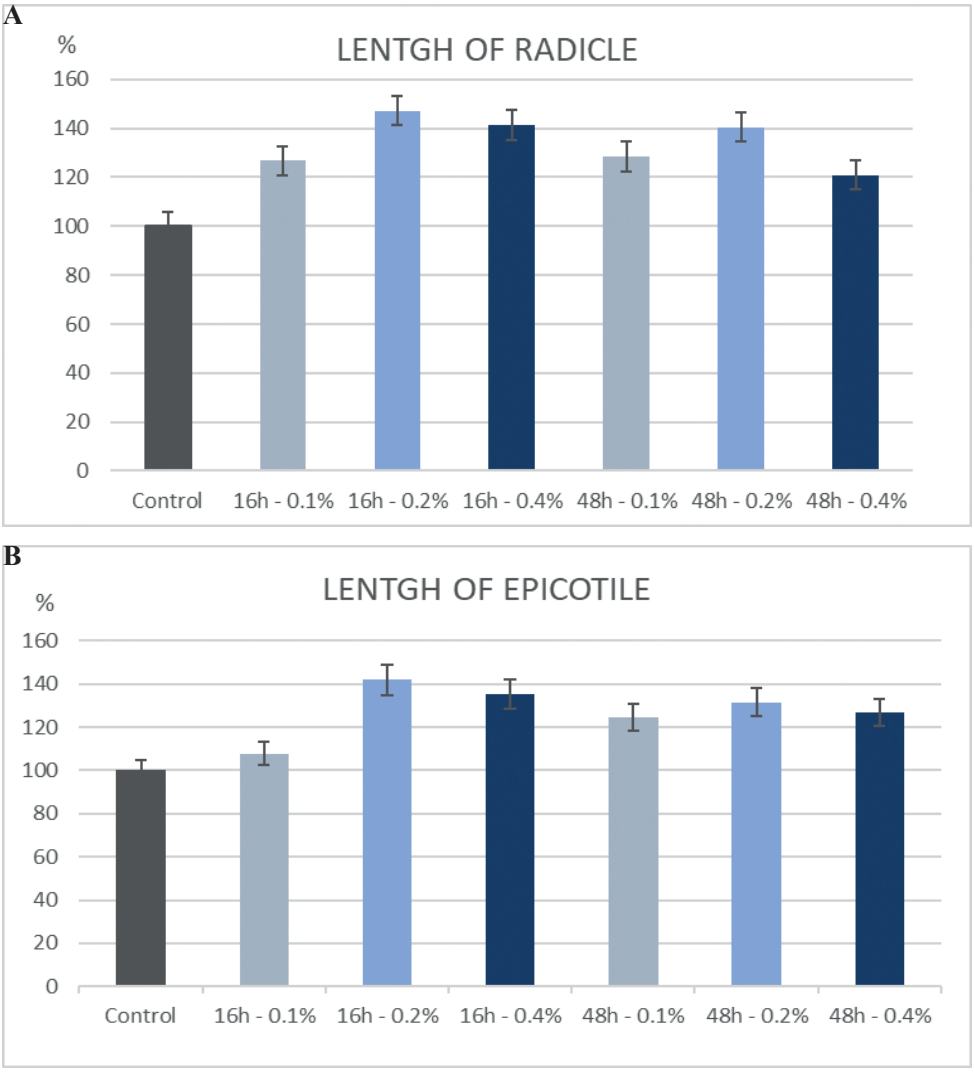

Figure 4. PGP effect of *Pseudomonas putida* 1046 on the germination of soybean seeds presented through the growth parameters length of radicle (**A**) and length of epicotile (**B**).

2015). Assessment of the plant growth-promoting potential of rhizobacteria requires a complex approach that includes, among others, the monitoring of plant germination capacity in test systems.

The data presented in the Results section outline the positive effect of *Pseudomonas putida* 1046 strain on the germination capacity of the technical crop *Zea mays*. Its biochemical profile speculated PGP potential, and the determined PGP traits confirmed it. The *in vitro* treatment of the model plants with bacterial strain improved their germination capacity. Gholami et al. (2009) reported similar results regarding improved seeds germination potential of corn. Other authors observed the same tendency for various crop cultures, such as canola (Glick et al. 1997), wheat (de

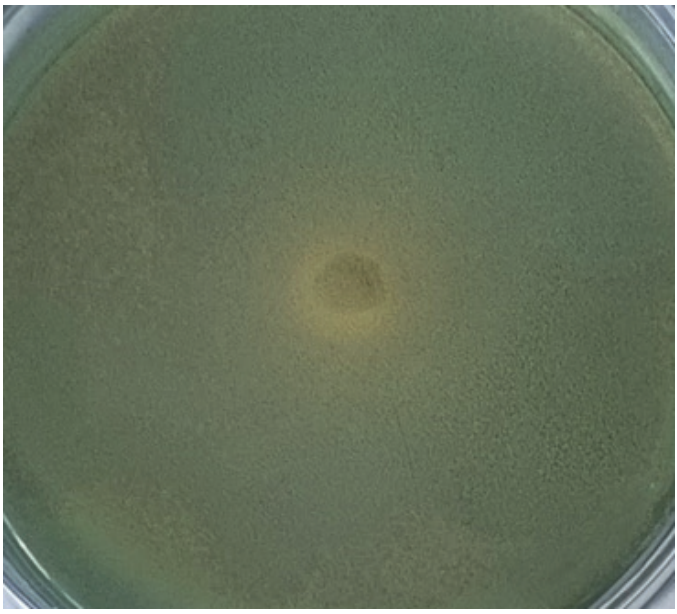

Figure 5. Siderophore production by *Pseudomonas putida* 1046.

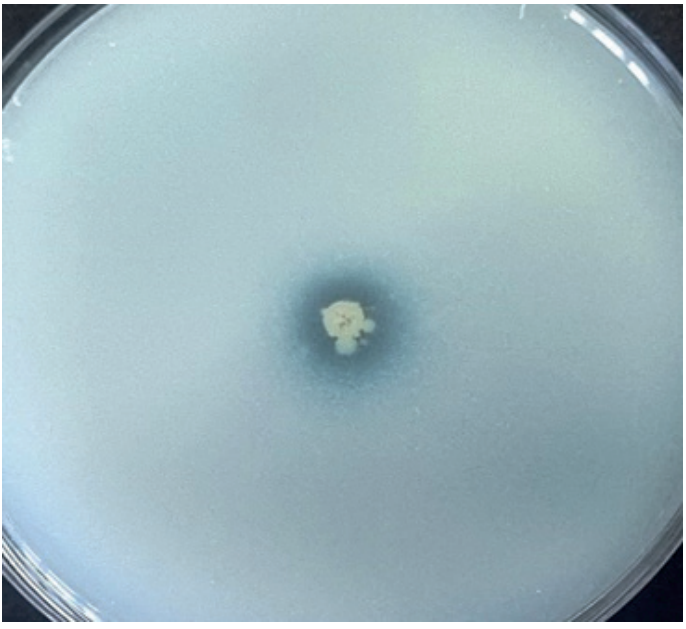

Figure 6. Phosphate solubilization activity of *Pseudomonas putida* 1046.

Freitas and Germida 1992), sunflower (Shaukat et al. 2006), and potato (Frommel et al. 1993), where significant enhancement of the seeds' emergence was registered – up to 100% of untreated control. It can be speculated that these findings are due to

the activity of hydrolytic enzymes that assure intensive substrate assimilation (e.g., α-amylases for the starch) and promote early germination. The enhanced hormones biosynthesis might trigger the activity of these hydrolytic enzymes. In addition, hormone dependency seems to increase the vigour index due to promoted synthesis of indoleacetic acid, the most abundant representative of auxins family of phytohormones. Its role in the root initiation and elongation and in differentiation and proliferation of plant tissues has been well documented (Meliani et al. 2017). Our results with soybean additionally enlarged the spectrum of the crop cultures subjectable to the PGP effect of *Pseudomonas putida*.

The physiological status of the culture is a parameter correlating with its biosynthetic capacity. The metabolically active 16 h culture expressed high values for the three tested indices and strong correlation coefficients of the bacterial and model plant data sets resulting in a better germination capacity. *Pseudomonas putida* strains and plants are in commensal relationships. The root exudates of the plant feed the bacteria, and the bacteria stimulate plant growth through the production of hormone precursors and antibiotics that suppress the pathogens' growth. It also supports nutrients immobilization (Molina et al. 2019). The data comparison for the 16 h and 48 h cultures indicated that the former executed its stimulatory function efficiently enough to result in high values for the all tested indices. The values for the length of the radicle and the coleoptile for the 0.2% suspension of the 48 h culture were slightly higher compared to those for the 16 h one (22.87% *vs.* 28.33%; and 35.96% *vs.* 45.96%). However, the shorter cultivation period (16 *vs.* 48 h) for the bacterial strain can compensate the difference of 5.5% to 10% since this is a defined technological advancement. Furthermore, the third index (the number of the lateral roots) is better presented in the 16 h compared to the 48 h bacterial culture (16.67% *vs.* 5.41). In fact, the formation of lateral roots contributes to the increase of the root surface reflecting the nutrients' assimilation.

Conclusions

The results demonstrate that the *Pseudomonas putida* 1046 strain possesses defined plant growth-promoting capacity. It enhances the process of corn germination due to the increased number of lateral roots and enlarged length of the coleoptile and the root. The optimal treatment parameters of the corn seeds, considering both the physiological and economic aspects of the process, comprise the application of 0.2% suspension of 16 h grown *Pseudomonas putida* 1046 strain for five days.

The positive germination capacity effect exerted by *Pseudomonas putida* 1046 strain contributes to improve our knowledge of the variety of approaches and mechanisms that are implicated in the plant growth promotion by these bacteria. However, greater understanding of the interaction between bacteria and host plants requires further study. From an economic point of view, in light of the possible application of *Pseudomonas putida* 1046 strain as a bioinoculant, the use of less concentrated suspension is more feasible and cost-effective.

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RESEARCH ARTICLE

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Comparative study of metal concentration determination in albumen of hen eggs originating from industrial poultry farms, backyard and free-range hens using ICP-OES technique

Plamen M. Kirov[1], Metody Karadjov[2],
Hristo K. Hristov[1], Radostina Alexandrova[1]

1 *Institute of Experimental Morphology, Pathology and Anthropology with Museum – Bulgarian Academy of Sciences, Acad. G. Bonchev Str., blk. 25, Sofia, Bulgaria* **2** *Geological Institute – Bulgarian Academy of Sciences, Acad. G. Bonchev Str., blk. 24, Sofia, Bulgaria*

Corresponding author: Plamen M. Kirov (plamen.vet@gmail.com)

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Abstract

There have been multiple types of research focusing on the relationship between feed ingredients and metal content in the egg white due to their role in human nutrition. The aim of the present study is to determine the metal concentration in hens' eggs and, in particular, to compare the metal concentration in egg albumen originating from industrial poultry farms with that of backyard and free-range hens. All samples were collected in Romania from five separate counties and 10 different farms, over a period of two weeks and, as a result, a total of 50 were collected, 10 from each housing system (batteries/cages, litter/soil, free-range, organic and backyard). The measurements of the metals were taken by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), with a wide range of elements reported. For the essential elements, we measured Cr, Cu, Fe, Mn and Zn; Al, Cd, Ni and Pb for the heavy metals and, in addition, we measured B, Ba, Sr, Ca and Mg. The present study revealed that the metals in eggs from free-range hens are richer in essential elements with mean concentrations as follows: 1.528 mg/kg for Fe, 3.278 mg/kg for Zn, 0.058 mg/kg for Mn and 1.362 mg/kg for Cu. We concluded that the egg quality is closely connected with the housing system and nutrition. Furthermore, the results demonstrate that eggs from backyard housing are no better than those from free-range hens in terms of essential metal composition. The heavy and non-essential metal contents, present in the albumen of all the examined eggs, were much lower than the maximum allowed concentration and, therefore, egg consumption does not pose any risk to human health.

Keywords
albumen, egg-white, food safety, farming systems, ICP-OES, poultry housing, poultry nutrition

Introduction

Products from the poultry industry are primary animal sources of proteins, microelements and vitamins for human nutrition. This is defined by the lower price of poultry products, compared to those from other animal sources, the easier way of production, good taste and provision of much of the recommended daily intake for microelements and vitamins (Vilà 1999; Youssef et al. 2014). During the last few years, the production of eggs in the European Union is in decline in some countries, while the demand from consumers is permanently increasing (European Parliament, Directorate General for Internal Policies 2010). Industrial development and lower environmental control in the past, pose risks for contamination of the hens' feed with different heavy metals and from there, the quality and microelement content of the eggs (Ternes and Leitsch 1997). Having knowledge of the eggs' mineral composition is required for different purposes: their nutritional value and percentage of daily requirements supplement, level of heavy metal contamination and proper poultry feeding and welfare. Contamination of poultry products could be a result of improper nutrition or dietary supplements for the laying hens. Feeds are often provided with the addition of essential supplements for the birds' growth: Cr, Cu, Fe, Mn and Zn: although heavy metals, such as Al, Cd, Ni and Pb have no important purpose for the hens' body functions, they may possess risks for human health (Li et al. 2005). Increased concentration of different metals in poultry products could be due to different sources: mainly from feed, but also from drinking water, litter, housing equipment and the living environment (Codling et al. 2008).

Despite the increasing interest in egg nutritional values for the human diet and higher expectations about organic and free-range production, the amount of information on the subject is still limited. The purpose of the present study was the analysis of the albumen of the eggs, resulting from different housing systems, using ISP-OES. The aims of our study were to establish baseline values for the metal concentration in eggs laid by hens from different production systems: conventional or intensive systems, organic and free-range and those raised in the backyard.

For simplification of the description of the housing systems, for the purpose of the study, we will use the term ecological when describing together backyard, organic and free-range and the term intensive farming, when describing soil/litter and cage housing systems.

Materials and methods

Study area and sample collection

Egg collection was undertaken during a 2-week period, between 1 and 25 February 2022. Eggs were collected from areas where the largest producers of commercially sold

Table 1. Location where collection was undertaken.

Code	Housing system	Area of collection
BkYd	Backyard	AR BH
0	Organic	DJ BH
1	Free-range	DJ DB
2	Soil	DB BH
3	Cages	DB BH

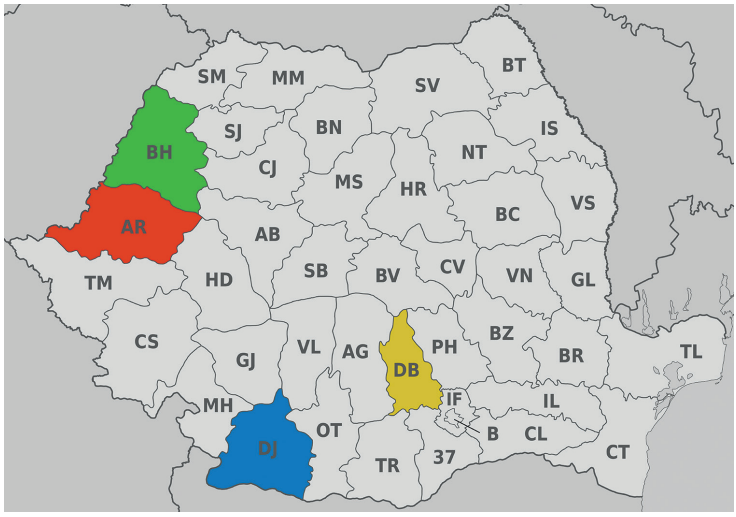

Figure 1. Map of Romania with areas of collection. (*TUBS, CC BY-SA 3.0*).

eggs in Romania are located. The areas from where the eggs were collected are shown in Table 1 and Fig. 1. A total of 50 samples were collected, 10 from each housing system. Forty eggs were collected from commercial producers and 10 from villages where people raise their hens in backyards for household consumption of produced eggs. All hens were fed with commercial feed with equal mineral content, composed of 2800 kcal/kg metabolisable energy, 18% crude protein, 0.72% cysteine+methionine, 0.9% lysine, 3.5% calcium and 0.35% phosphorus. Hens were also given mineral supplements on a regular basis. In addition, free-range, organic and backyard chickens had access to outdoor foraging areas. Backyard hens frequently received food leftovers from household meals with no supplementary vitamins and minerals. Organic and free-range poultry had access to yards with natural grass and soil for 5 to 6 hours per day. Backyard chickens had access to the yard and surrounding areas for more than 8 hours per day.

Sample preparation

The collected eggs were cracked and the egg white was separated from the yolk into ceramic containers. The samples were heated in ventilated ovens at 104 °C until no further weight loss was registered. All samples were evenly ground and homogenised.

The glassware used for analysis was firstly washed with detergent and rinsed, then filled with previously-prepared 6N nitric acid (HNO_3), left for 12 hours and, finally, rinsed with double-distilled water.

Using a digital analytical balance, five grams of each sample were measured and added to a glass beaker containing a 10 ml solution composed of 10 volumetric parts of concentrated HNO_3 (65%) and 3 volumetric parts of concentrated sulphuric acid (H_2SO_4). The beakers were left for 30 minutes during which time the initial reaction between the egg and the acid mixture abated. Next, the beakers were heated to 90 °C for 45 minutes, then the temperature was increased to 140 °C. During the boiling process, the volume in each container was maintained above 3 ml by the addition of concentrated HNO_3. The wet digestion process was complete at the time when the solution inside the beakers turned lighter and the released steam became white-coloured. For metal ions fixation, a mixture of 0.5% hydrochloric acid (HCl) and 2% HNO_3 was added to each solution. After filtration through ceramic filters, the samples were collected in sterile containers and double-distilled water was added until 25 ml final volume per sample was reached.

A blank sample was prepared in a similar way without the addition of the egg component.

ICP-OES

The analytical investigations were performed with a high-resolution radial viewing ICP-OES system - HORIBA JY ULTIMA 2 (Jobin Yvon, Longjumeau, France). The stock solutions of the elements of interest were prepared by using Merck mono – element standard solutions, traceable to SRM from NIST 1000 mg/l Certipur.

The ICP-OES working conditions are described in Table 2.

Table 2. CP-OES working conditions.

Parameter	Value
Rf generator power	1.0 kW
Plasma gas flowrate	12 l/min
Auxiliary gas flowrate	0 l/min
Sheath gas flowrate	0.2 l/min
Nebuliser gas flowrate	0.5 l/min
Nebuliser flowrate	2.0 bars
Sample uptake	0.8 ml/min
Argon humidifier	no
Injector tube diameter	3.0 mm

Statistical analysis

The collected data were subject to one-way variance analysis followed by the Tukey-HSD test for testing the effects of the husbandry system on the mineral content of eggs. Differences with $p < 0.05$ were considered statistically significant. All statistical analyses were performed using SPSS for Mac OS (IBM® SPSS® Statistics, version 23, IBM Corp.)

Results and discussion

The physical properties of the eggs showed no significant differences between the different housing systems. The weight of the eggs was 65.3 ± 3.0 g, albumen weight: intensive farming 36.8 ± 2.2 g, 36.2 ± 2.4 g for organic and free-range and 37.0 ± 2.8 g for backyard housing.

Essential elements

Table 3 identifies the detected values for the essential microelements. The higher values for essential microelements are closely dependent on the origin of the eggs – those from backyard, organic and free-range systems showed higher concentrations of essential elements, giving higher nutritional value to the eggs.

Chromium

Considered an essential element in the human diet, chromium functions in maintaining normal glucose tolerance primarily by regulating insulin action. It has been described that the presence of optimal amounts of biologically-active chromium resulted in much lower amounts of insulin being required. Glucose intolerance, related to insufficient dietary chromium, appears to be widespread (Anderson 1992). Although the mechanism of this action has not been clearly described, it has been proposed that chromium binds to an oligopeptide to form chromodulin, a low-molecular-weight, chromium-binding substance that binds to and activates the insulin receptor to promote insulin action (Ross et al. 2014).

In accordance with the US Institute of Medicine (2001), the recommended daily intake for chromium for an adult is 25–35 mcg/day (US Institute of Medicine, Food and Nutrition Board 2001). With the average content of chromium per egg of approx. 2 mcg/serving, a single egg provides 1% of the daily recommended dosage of the mineral.

In 2014, the European Food Safety Authority (EFSA) published a scientific opinion concluding that there is a lack of scientific evidence for chromium being an essential element and, therefore, setting chromium intake recommendations would be inappropriate (EFSA 2014).

As seen in Table 3, the medium value for chromium content is nearly double in the backyard, organic and free-range eggs in comparison with those from intensive farming: 0.05546 mg/kg vs. 0.02166 mg/kg.

Table 3. Trace concentrations of essential elements [mg/kg]. NDL – non-detectable levels.

Elements	Backyard	Organic	Free-range	Soil/Litter	Cages	min	max	Toxic level [mg/kg]
Cr	0.087377	0.033807	0.045204	0.0435248	0.0169195	0.0169195	0.087377	10
Cu	0.837978	1.361827	1.324966	1.0679699	0.6721668	0.6721668	1.361827	250
Fe	2.50444	1.528068	11.56562	0.901086	0.8495411	0.8495411	11.56562	4500
Mn	0.006639	0.057608	NDL	NDL	0.01324	0	0.057608	4000
Zn	1.088555	3.278107	1.5635	2.288861	0.8333671	0.8333671	3.278107	2000

Copper

In its role as an essential mineral, copper has an important role as a co-factor in some enzymes, related to the energy production in the body, synthesis of neurotransmitters and connective tissue, metabolism of iron and activation of neuropeptides (US Institute of Medicine, Food and Nutrition Board 2001; Li et al. 2005).

In accordance with the US Institute of Medicine (2001), the recommended daily intake of copper for an adult is 900–1300 mcg/day (US Institute of Medicine Food and Nutrition Board 2001). One egg contains approx. 10 mcg/serving, thus providing 1.1% of the recommended daily intake of copper.

In our results, the eggs from intensive systems have lower medium concentrations of copper 0.7100 mg/kg compared to 1.1749 mg/kg of those from ecological housing systems.

Iron

Iron is a highly important element in the human body. As part of the blood haemoglobin, it is required for oxygen transport and oxidative metabolism. It is also essential in the processes of cellular growth. Lack of nutritional iron can result in iron-deficient anaemia – a disorder leading to reduced performance and increased morbidity.

The current recommended daily intake for adults is between 10 and 15 mg/day (US Institute of Medicine, Food and Nutrition Board 2001). With their iron concentration, eggs could play an important role in human nutrition as a source of dietary iron. With their low energy values, eggs could be an easy choice for these requiring high iron and low-calorie nutrition. One egg provides approx. 10% of the daily intake of iron and less than 40% of the daily calories intake (for 3000 kcal diet).

In our study, the average iron content in ecological eggs was more than five times higher in comparison with the other housing systems: 5.1993 mg/kg vs. 0.8753 mg/kg. This is the result of the access that some of the hens have to iron-rich vegetation (*Urtica dioica*) and iron-rich soils.

Manganese

Manganese is an essential element with a trace presence in the human body. It acts as a co-factor in many enzymes through whose actions, manganese is involved in amino acid, cholesterol, glucose and carbohydrate metabolism and immune response (US Institute of Medicine Food and Nutrition Board 2001). With its lower concentration in different housing systems products, we cannot consider eggs as an important nutritional source for the mineral. Meeting the recommended daily intake of between 1.8 and 2.6 mg/day will require a high number of consumed eggs (US Institute of Medicine, Food and Nutrition Board 2001).

Our study showed that most of the eggs are with manganese concentrations below the concentrations detectable by ICP-OES.

Zinc

One of the most important elements in the human body, zinc is involved in the catalytic activities of hundreds of enzymes, plays a role in DNA and protein synthesis and assists with the proper functioning of the immune system (US Institute of Medicine Food and Nutrition Board 2001).

With recommended daily average intake for adults between 8 and 12 mg/day, eggs could be an easy choice for providing supplementary zinc with low calorie intake (US Institute of Medicine, Food and Nutrition Board 2001). From our study, the detected concentration of the element would provide approx. 5% of the daily intake from a single egg.

The zinc concentration in both types of housing systems had similar concentrations, with its being a little higher in the ecological systems.

Heavy metals

In addition to the essential microelements, we tested the yolk samples for the presence of some heavy metals. These metals have no biological functions and are considered contaminants in animal forages (EC 2002). Table 4 shows the levels of Al, Cd, Ni and Pb in the yolk obtained from different housing systems.

Aluminium

With high neurotoxicity for the human body, aluminium is considered a key player in the development of Alzheimer's Disease and embryotoxicity (BfR - Bundesinistitut für Risikobewertung 2014). Our study shows that, to reach the level of toxicity, an adult has to consume more than 80 eggs. Small differences between ecological and intensive farming products show that there is no contamination with aluminium in any of the egg groups.

Cadmium

An excess of cadmium in the human body could produce kidney and liver damage, enzyme inhibition, skin conditions and lung cancer. Specific for cadmium is its persistence in the organisms – the level of excretion is very low and can remain resident for years. For humans, the major pathway of exposure is smoking, followed by the consumption of contaminated food and water.

Table 4. Trace concentrations of heavy metal elements [mg/kg]. NDL – non-detectable levels.

Elements	Backyard	Organic	Free-range	Soil/Litter	Cages	min	max	Toxic level [mg/kg]
Al	0.980842	1.551223	1.14409	0.9189452	0.8907284	0.8907284	1.551223	100
Cd	0.019442	0.018028	0.033308	0.0407327	0.0518918	0.018028	0.051892	12
Ni	0.235058	0.094421	0.136302	0.1891993	0.1227397	0.0944213	0.235058	400
Pb	NDL	0.272676	NDL	0	0.1638184	0	0.272676	200

The higher concentrations of cadmium in the intensively-farmed eggs, compared to the ecological ones, can be explained by the higher use of cereal-rich feed in intensive housing systems – corn and wheat (Wang et al. 2017).

Nickel

For proper production of erythrocytes, small amounts of nickel are necessary. However, in excessive amounts, the metal can show some low levels of toxicity. Long-term exposure could result in reduced body weight, liver and heart damage and skin irritations. Short-term exposures are not known to result in any effect on the human body (EFSA 2015).

With similar concentrations of the element in eggs resulting from all the observed housing systems, we can conclude that nickel contamination is absent. The resultant presence demonstrates normal ranges of the element, delivered from feed sources – cereals and greens (Monika et al. 2019).

Lead

With its high toxicity, lead can affect almost every organ in the human body. In small children, lead poisoning produces arrested development, low IQ, CNS damage, mental impairment and hyperactivity. In people of all ages, it can produce anaemia, stomach and muscle weakness, brain damage and kidney failure. Most of the eggs in our research demonstrated lead concentration below detectable levels. Only in organic and cages production have we found concentrations with normal lead presence in forage with 1000–1300 times lower levels than the toxicity threshold.

Other minerals

Besides the essential and heavy metals, we obtained results for some other elements, as shown in Table 5.

Boron is an element naturally found in grains and mostly in leafy vegetables and greens. With an estimated daily requirement of 1–1.5 mg/day for an adult, eggs of any origin can be used as a good dietary source for the element (WHO 1996).

Higher concentrations of barium in ecologically-produced eggs result from the consumption of green vegetables by the hens when they have access to open fields. For

Table 5. Trace concentrations of other non-essential metal elements [mg/kg].

Elements	Backyard	Organic	Free-range	Soil/Litter	Cages	min	max	Toxic level [mg/kg]
B	1.451486	1.696924	1.839805	2.2440477	1.8133759	1.4514857	2.244048	
Ba	0.319478	0.158141	0.213257	0.1488283	0.0993431	0.0993431	0.319478	200
Sr	0.960984	0.524698	0.457679	0.5391281	0.4732974	0.4576793	0.960984	
Ca	125.3539	123.0285	138.6179	188.52904	145.91882	123.02849	188.529	
Mg	443.0058	489.142	496.7324	448.8178	473.33271	443.00577	496.7324	11200

backyard hens, the level of the element can be explained with the addition of lettuce and carrots to their diet.

A higher concentration of strontium in backyard eggs can be explained by richer concentrations of the element in the soils from the areas where the hens were housed. It is evident from other studies that the presence of strontium in eggs has the opposite correlation with calcium – calcium is lower in concentration in the eggs with higher strontium presence (Doberenz et al. 1969).

Magnesium is required for maintaining numerous body processes – muscle and nerve functions, heart rate, blood sugar and DNA production. With a daily recommended dosage of 310–420 mg per adult, eggs are a good source for a healthy diet (US Institute of Medicine, Food and Nutrition Board 1997). Our results demonstrated very slight differences between the eggs from all tested housing systems.

Discussion and conclusions

The eggs from ecologic and free-range systems showed that they are richer in essential elements Cr, Cu, Fe, Mn and Zn. However, the concentration of heavy metals, like Cd, was higher in intensive farming compared with the ecological systems. This may be due to contamination of the food composition used as hen feed or contamination from the surrounding equipment, used for the hens' housing.

The differences in egg quality from different housing systems suggests that consumers have the ability to improve their dietary intake by selecting eggs from ecological sources, as the ones with higher nutritional value. Furthermore, the results indicate the need for improving the husbandry practices and welfare in animal production with the direction pointing to ecological practices.

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The use of boreal relict shrub habitats of willow-leaf meadow sweet (*Spiraea salicifolia*) and shrubby cinquefoil (*Potentilla fruticosa*) in Western Rhodope Mts. by mammal species

Venislava Spasova[1], Daniela Simeonovska-Nikolova[1], Krastio Dimitrov[1], Gabriela Petrova[1], Kalina Pachedjieva[1], Rossen Tzonev[1]

1 *Sofia University, Faculty of Biology, Department of Ecology and Environmental Protection, Sofia, Bulgaria*

Corresponding author: Venislava Spasova (v_spasova@biofac.uni-sofia.bg)

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Abstract

Plant communities of two peat-shrub species – *Spiraea salicifolia* and *Potentilla fruticosa* were studied in the Rhodope Mts., with emphasis on their use as a resource for the mammals associated with them. These shrubs are boreal relicts among the dominant coniferous forests. The field surveys were conducted in the spring-autumn period of 2021–2022. In both study areas, the species composition of the mammals was studied by camera traps for medium and large mammals, and by Sherman live traps for small mammals. The species registered were roe deer (*Capreolus capreolus*), red fox (*Vulpes vulpes*), wild boar (*Sus scrofa*), pine marten (*Martes martes*), European hare (*Lepus europaeus*), red squirrel (*Sciurus vulgaris*), bank vole (*Myodes glareolus*) and yellow-necked mouse (*Apodemus flavicollis*). Having in mind that small mammals are vital prey base for avian and mammalian predators, it is not surprising that *M. glareolus* and *A. flavicollis* individuals were captured in the habitats that they probably use as shelters. The pine marten inhabits the forests by which the community of *P. fruticosa* is surrounded, but probably feeds on the rodents in the shrub. In this way, it probably provides it with an alternative to the forest food base and hunting ground. From the presented results, it seems that the *L. europaeus* uses *P. fruticosa* shrubs as food. Therefore, the plant communities of the two relict peat-shrub species studied probably provide shelter and food for the mammals. Their importance is established for at least one species of mammal with conservation significance at national and European level – *M. martes*. Therefore, it is necessary to continue and expand the future monitoring on mammal diversity of these relict communities.

Keywords

camera traps, endangered species, live traps, mammals, relict shrub habitats

Introduction

Relict species have an important position in Earth's biodiversity. Various studies are known on the relict nature of many boreal plant species such as *Parnassia palustris*, *Salix lapponum*, *Potentilla fruticosa*, etc. (Čarni and Matevski 2015; Horsak et al. 2015; Serafin et al. 2018). Due to their sedentary lifestyle and role in habitat foundation, relict plants have employed various strategies in adapting to their environment even under dramatic climatic changes such as glaciations. Their populations preserve ecological and evolutionary histories that can last thousands of years (Woolbright et al. 2014) but are also threatened by climatic changes, which narrow their range of distribution (Hampe and Jump 2011). Against this background, many studies have indicated climatic influences on mammal population dynamics, usually acting together with a complex set of biotic factors (Humphries 2009). With the changes in global temperatures and consequent changes to habitats, a clearer understanding of the relationship of mammals with the environment has become imperative. Hence, it is of particular interest what mammals inhabit the relict communities and how they use them. Determining habitat associations of mammal species and the environmental characteristics important for site occupancy is central to understanding species biology and community organization. The aim of the present study is to explore the use of two relict shrub habitats of willow-leaf meadow sweet (*Spiraea salicifolia*) and of shrubby cinquefoil (*Potentilla fruticosa*) in the region of Western Rhodope Mts as a resource for the mammals associated with them.

The two shrub habitats are assessed as critically endangered (Gussev and Vulchev 2015; Tzonev and Gussev 2015) at a national level. A particular feature of these shrub communities is the combination of standing water or high soil and air humidity with low temperatures characteristic of boreal and tundra biomes more broadly distributed in the northern latitudes. This, together with the richness of northern and boreal floristic elements and their complexity in terms of origin and evolution, significantly affects their habitat value as a refuge of relict plant, invertebrate and vertebrate species. Due to their glacial (boreal) relict origin, these habitats are threatened by climatic changes, which narrow their range of distribution relative to all boreal and glacial relicts (Hampe and Jump 2011). Many relict boreal plants, or even community types and animal communities related to them, growing along the southern edge of their range and/or at their altitudinal and climatic limits, are expected to be heralds of adverse climatic changes (Freeman et al. 2018; Beniston 2000; Fescenco et al. 2020). Global warming in mountain plant communities is accompanied not only by extreme heat waves, drought, and severe forest fires (Allen 1994), but also by invasion pressure from lower-elevation plant communities and species (Lenoir et al. 2008). Surveys of relict boreal plants or their communities growing near the extremes of their ecological tolerance could help to estimate the pressure of climate change on the ecosystems and promote better understanding and conservation of potential refuge areas for vulnerable to global warming species. In European mountains various studies have investigated the impact of recent climatic changes on mountain flora and vegetation (Dirnboock et al. 2011; Pauli et al. 2012; Cannone and Pignatti 2014; Steinbauer et al. 2018) as some expected

and unexpected results were found, such as: elevation upshifting or downshifting for different plant groups; increase of local species richness and decrease of rare or endemic boreal plants. The significance of boreal mires and shrubs related to them as endangered habitats for boreal relict plant and animal invertebrate species has already been studied in Bulgaria (Hajek et al. 2009, 2010; Langourov et al. 2018). However, there is still little information about their habitat use by mammals (Benedek et al. 2021). Establishing what species of mammals inhabit these communities will help to initiate effective monitoring programs in the context of climate change.

Materials and methods

Characteristics of *Spiraea salicifolia* and *Potentilla fruticosa* habitats

The investigated habitats are located and occupy areas in West Rhodope Mts., Southwest Bulgaria (Fig. 1). The communities in the studied sites are small and intra-zonal phenomenon, being surrounded on all sides by the boreal coniferous forests that dominate in Rhodope Mts. These forests are formed mainly by spruce (*Picea abies*), fir (*Abies alba*) and scots pine (*Pinus sylvestris*) (Bondev 1991). Both habitats form close and dense communities characterized by high soil moisture and represent parts of a huge natural complex of boreal peatlands and bogs in the zone.

The main characteristics of the two communities were based on their floristic composition and ecological features and were determined as follows:

P. fruticosa thickets are distributed in Beglika locality (41°50.11"N, 24°08.67"E), near Batak town at 1510–1530 m a.s.l., covering an area of about 1.2 ha (Tzonev and Gussev 2015) (Fig. 1). The soils are *Humic Cambosols* (Ninov 2002). The communities occupy mountain slopes with low inclination, between 2 and 5 degrees. They are dense and closed with high coverage of the dominant species. The total vegetation cover is 90–100% (Fig. 2). The cool and humid climate and substrate predetermine the development of mesophilic and cold-resistant plants. Almost 60 species and subspecies of vascular plants (excluding bryophytes) are part of the composition of the *P. fruticosa* community with most species in the families *Rosaceae*, *Poacea*, *Asteracea*, *Apiaceae* and *Fabaceae*, represented by 4–7 species each.

The communities of *S. salicifolia* occupy flattened river terraces along Dospatska River, (41°45.45"N, 23°59.00"E), near Dospat town, at 1210–1250 m a.s.l., covering an area of about 4.8 ha (Gussev and Vulchev 2015) (Fig. 1). The soils are *Fluvisols* with a thick humus layer and acidic reaction (Ninov 2002). *Spiraea salicifolia* forms dense, almost monodominant thickets along Dospatska River. The cover of the dominant species *S. salicifolia* is higher than 80% (Fig. 3) with herbaceous plants of low abundance. About 40 species and subspecies of vascular plants (excluding bryophytes) are part of the composition of the *S. salicifolia* community. The plant families with highest number of species are *Rosaceae*, *Poaceae*, *Fabaceae* which are also the biggest families in the general Bulgarian flora (Assyov and Petrova 2012).

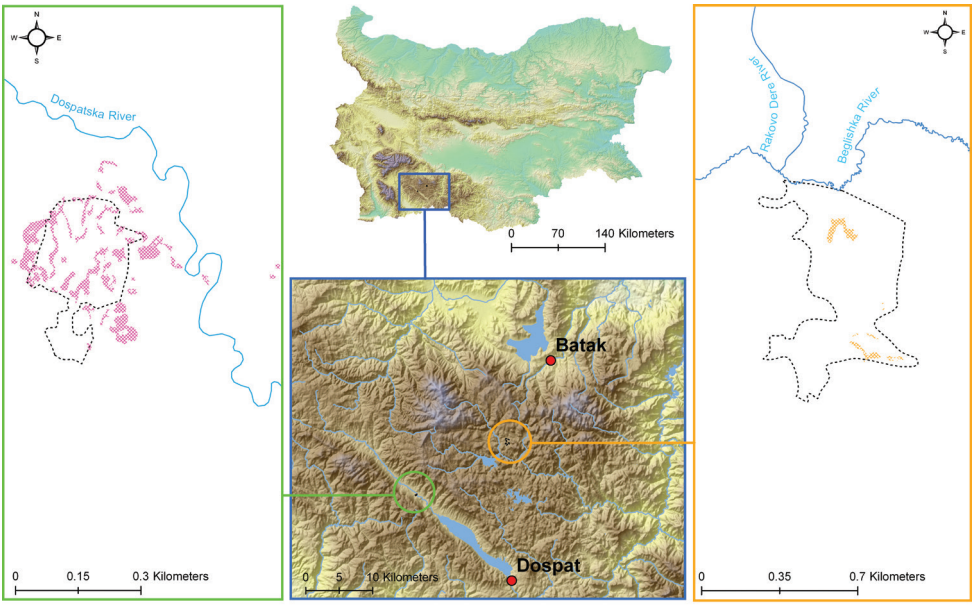

Figure 1. Map of distribution of the investigated habitats in West Rhodope Mts.

Figure 2. Rhodope thickets of *Potentilla fruticosa* – individual plant (**A**) and habitat (**B**).

The climate in these regions is typical of the boreal coniferous forest belt where both habitats are distributed. It is humid with mean annual temperature of 7.3 °C (Batak) and 8.1 °C (Dospat), and annual precipitation of 912 mm and 966 mm respectively (Fig. 4).

S. salicifolia and *P. fruticosa* are listed in Annex 3 of the Bulgarian Biodiversity Act. The territories occupied by their communities have been declared protected sites, "Chibutsite" and "Hrastoviden ochibolets" respectively, and are included in the Natura 2000 network (Fig. 1).

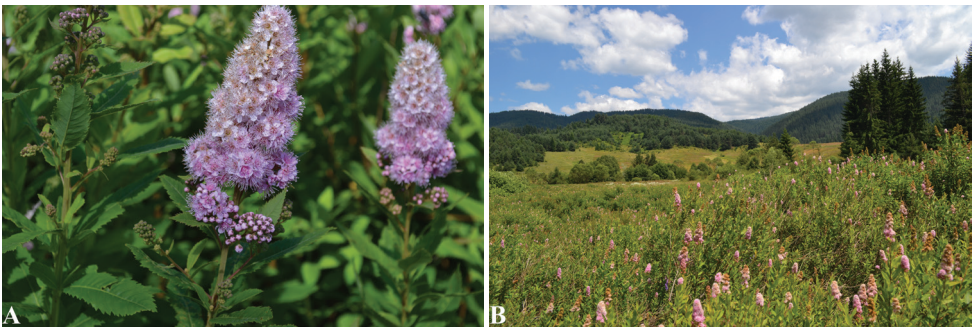

Figure 3. Rhodope thickets of *Spiraea salicifolia* – individual plants (**A**) and habitat (**B**).

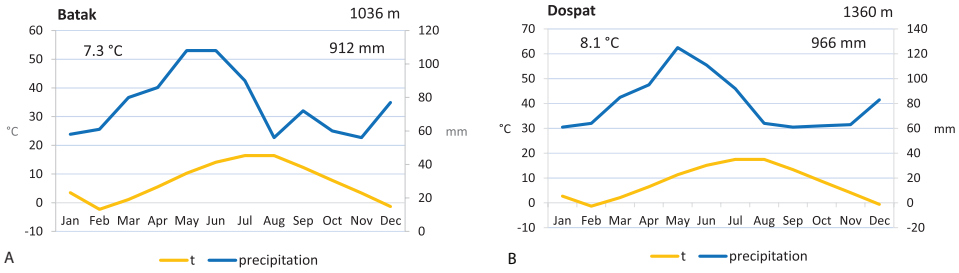

Figure 4. Climate diagrams for the towns **A** Batak (*P. fruticosa* habitat) and **B** Dospat (*S. salicifolia* habitat). The model is based on weather data collected for the period 1991–2021 and has a resolution of 0.1–0.25 grade (data source Climate-Data.org).

Mammals' data collection

The field surveys were conducted in the spring-autumn period of 2021 and 2022. In both habitats over areas of about 0.5 to 1 ha, the species composition was studied with camera traps (Moultrie M-40) for medium and large mammals, and by Sherman live traps (H. B. Sherman Traps, Inc., Tallahassee, Florida) for small mammals (Fig. 5).

A total of four camera traps, two at the edge of each community, were installed on trees at the height of about 50 cm. The effort was the same at the two habitats – 167 trap-nights average per habitat (142 for 2021 and 192 for 2022).

A total of forty Sherman live traps baited with oat nuts were set per habitat. The live traps were placed in lines at a distance of about 10 m from each other. To avoid animal disturbance the trapping was carried out for one night in each shrub habitat. All animals were released immediately after species determination at the place of capture. Relative abundance of small mammals was estimated on the base of the number of individuals captured per trap-night. Additionally, the presence of mammals was also registered by direct observation and traces of their activity.

Figure 5. Methods of mammal registration (**A**) camera trap (**B**) Sherman life trap.

Mammal identification

Species identification of mammals was made on the basis of external morphological features, body size, and ecology according to Popov and Sedefchev (2003). In Bulgaria, the wood mouse (*Apodemus sylvaticus*) and yellow-necked mouse (*Apodemus flavicollis*) do not differ in external morphological features. Using cranial measurements in a study on small mammal assemblages from North to South along the Bulgarian Black Sea coast, Popov (2000) established that *A. flavicollis* was better represented in the southern area where the forests were the prevailing vegetational type. In Central Western Bulgaria the majority of adult wood mice also belong to *A. flavicollis* (Minkova and Popov 2002) as well as in the Southwest part of the country (Popov 2015). This gives us reason to consider that, in all probability, the captured *Apodemus* individuals are *A. flavicollis*.

Ethical notes

The investigation conformed to the international requirements for ethical attitude towards the animals. All animals captured were released at the place of capture.

Results

In the *S. salicifolia* community, camera traps recorded the roe deer (*Capreolus capreolus*) and red fox (*Vulpes vulpes*), both nocturnally and diurnally (Table 1, Fig. 6).

Among the rodents, the red squirrel *Sciurus vulgaris* and *A. flavicollis* were photographed. Around the community, tracks of *C. capreolus*, *V. vulpes*, hedgehog (*Erinaceus concolor*), wild cat (*Felis silvestris*) and badger (*Meles meles*) were recorded as well. From the small mammals in the live traps the bank vole *M. glareolus* and the yellow-necked mouse *A. flavicollis* were captured (Fig. 7). Over the two years, the bank vole had a relative abundance 0.18–0.20 individuals per trap-night. The yellow-necked mouse was captured only in 2022 with a relative abundance of 0.20 individuals per trap-night.

In the community of *P. fruticosa* from the large and medium-sized mammals with the camera traps the roe deer *C. capreolus*, the wild boar *Sus scrofa*, *V. vulpes*, and the European hare (*Lepus europaeus*), as well as domestic horses (*Equus caballus*) were recorded. Two different individuals of pine marten (*Martes martes*) were recorded in April and May of 2021 and 2022 with the camera traps as well. The martens were registrated both during the night and the day (Fig. 8). From the small mammals, again the bank vole *M. glareolus* and the yellow-necked mouse *A. flavicollis* were captured. The bank vole was captured in both study years, while *A. flavicollis* was captured only in 2021. The relative abundance of the bank vole was 0.25 individuals per trap-night, and of the yellow-necked mouse 0.075 individuals per trap-night, respectively.

Table 1. Camera trap recordings of mammals in *S. salicifolia* and *P. fruticosa* habitats. Note: with * is indicated the presence of both day and night registrations.

	***S. salicifolia* habitat**		***P. fruticosa* habitat**	
Years	**2021**	**2022**	**2021**	**2022**
Recorded species	**Number of registrations**			
Capreolus capreolus	4	7*	3*	11*
Sus scrofa	–	–	–	2*
Vulpes vulpes	2	6*	1	3*
Martes martes	–	–	3*	10*
Lepus europaeus	1	–	4	12*
Sciurus vulgaris	–	2*	–	–
Apodemus flavicollis	–	8	–	–
Equus callabus	–	–	–	4

Discussion

The rodent species *A. flavicollis* and *M. glareolus* represented the small mammal community in our study. Their relative abundance is similar to those established by other authors in mountain regions (Benedek et al. 2021). According to Benedek et al. (2021) in most forests of Central and Eastern Europe *M. glareolus* and *A. flavicollis* are the dominant rodent species, with one or the other being more numerous depending on habitat conditions and geographic position. Our data are not sufficient to establish

Figure 6. Camera trap registration of *V. vulpes* in *S. salicifolia* habitat.

whether there is microhabitat segregation of the two rodent species in the studied habitats but, in other studies in mountanous areas, *A. flavicollis* seems to be more associated with the forest edge than *M. glareolus*, while the second species prefers areas within the forest with high tree and shrub cover (Hille and Mortelliti 2010). In our study *M. glareolus* was more numerous and captured in both habitats as well. Popov (2007) also found that in Bulgaria the *M. glareolus* is one of the rodent species with the highest abundance in mountainous regions. Based on its highest abundance in mountainous regions in Bulgaria we find that *M. glareolus* could be an indicator of climate change here. *Myodes glareolus* has been identified also as a key species in genetic studies for understanding the response of European fauna to climate change following the Last Glacial Maximum, being an example of a woodland mammal surviving in cryptic glacial refugia in Europe north of Mediterranean areas (Filipi et al. 2015).

Small mammals are also vital prey base for avian and mammalian predators. Therefore, it is not surprising that many small vertebrates prefer to forage under plant cover where it is more difficult for predators to detect them, avoiding areas with sparse cover or greater distances between shelters (Loggins et al. 2019). It is considered that the shrub cover is likely to have a strong influence on the species and communities of small mammals that rely on it for safety (Stephens and Anderson 2014). In this sense, the communities studied are very valuable for the small mammals found here, due to their location between open space and forest. Both habitats probably also serve as a shelter for roe deer, red fox and hare. These species were recorded both during the day and at night. From a camera trap video it seems that the hare is pulling and chewing a twig of the *P. fruticosa*. For herbivores, shrubs can be important food sources, but

Figure 7. Bank vole *M. glareolus* captured in *S. salicifolia* habitat.

are relatively sustainable to the grazing due to their deep root system, multiple stems, and height (Wikeem and Wikeem 2005). Besides, it is established that species such as birch-leaved spirea (*Spiraea lucida*; syn. *S. betulifolia*) are negligible in the diet of wild herbivores (Quinton 1984). However, the horses present in the area could be a threat to the shrubs due to trampling. Therefore, measures should be taken to monitor and control the number of domestic animals around the communities.

Figure 8. Camera trap registration of *M. martes* (**A**) and *L. europaeus* (**B**) in the *P. fruticosa* habitat.

In the community of *P. fruticosa* two pine marten were recorded. *Martes martes* is considered a habitat specialist mainly associated with forests (Clevenger 1994; Caryl et al. 2012; Lombardini et al. 2015). We assume that the pine marten inhabits the spruce forests surrounding the *P. fruticosa* community, but probably feeds on the rodents in the shrub community. In this way, it provides pine martens with an alternative to the forest food base and hunting ground. The *M. martes* is included in the Red Data Book of Bulgaria vol. II in the category of "Endangered" species (Spassov and Spiridonov 2015) and in the Biological Diversity Law – Appendix II and III. It is also included in the IUCN Red List, "Least Concern" category. The pine marten is a species that is

monitored within the National Biodiversity Monitoring System. The studies carried out in the two shrub communities provided data on its distribution at the national level and could be useful in clarifying its habitat specialization. However, more studies are needed to better understand the use of these boreal relict shrub habitats by mammals in the region. Like other authors such as Humphries (2009) we also think that it is necessary to continue and expand the examples of long-term monitoring of mammal diversity with more taxa and regions before it is too late.

Conclusion

The studied relict peat-shrub plant communities dominated by *S. salicifolia* and *P. fruticos*a in the Rhodope Mts. probably provide shelter and food for the mammals. Their importance is established for at least one species of mammal with conservation significance at national and European level – *M. martes*. The limited distribution of these communities makes it necessary to initiate a long-term program to monitor the impact of climate change on them and on their connection with the associated mammals.

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Forest habitats of Godech Municipality, Western Bulgaria

Borislav Grigorov[1], Nikolay Velev[2], Assen Assenov[1], Momchil Nazarov[2], Beloslava Genova[2], Kiril Vassilev[2]

1 *Faculty of Geology and Geography, Department of Landscape Ecology and Environmental Protection, Sofia University "St. Kliment Ohridski", Sofia, Bulgaria* **2** *Institute of Biodiversity and Ecosystem Research, Department of Plant and Fungal Diversity and Resources, Bulgarian Academy of Sciences, Sofia, Bulgaria*

Corresponding author: Borislav Grigorov (borislav.g.grigorov@gmail.com)

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Abstract

The current study aims at revealing the forest habitat diversity of Godech Municipality, according to the EUNIS habitat classification. Initial data was collected from the Ministry of Environment and Water and the Forestry Management Plans. Subsequently, 418 vegetation plots (relevés) and 3422 verification points were collected during the fieldwork seasons of 2019 and 2020. The research territory is situated in Western Bulgaria in close proximity to the country's border with the Republic of Serbia. Forests cover a total of 144.85 km^2. Their phytocoenoses are dominated by *Fagus sylvatica* L. (59.22 km^2), *Quercus cerris* L. (14.85 km^2), *Carpinus betulus* L. (4.94 km^2), *Quercus dalechampii* Ten. (2.39 km^2), *Q. frainetto* Ten. (2.99 km^2). There are plantations with *Pinus nigra* J. F. Arnold (20.87 km^2), *P. sylvestris* L. (16.06 km^2) and *Picea abies* H. Karst (11.65 km^2) also. Forests are experiencing some major threats, such as logging, pollution and fires.

Keywords

EUNIS, GIS, habitat mapping, syntaxa, vegetation and habitat diversity

Introduction

The role of forests in the functioning of our planet is indispensable. They hold one of the keys for the reduction of human's ecological footprint. Forests provide a vast array

of ecosystem services (Garcia-Nieto et al. 2013; Aznar-Sanchez et al. 2018; Acharya et al. 2019). The sustainability issue is also tied tightly to a forest's structure and functioning. Many forest habitats in Bulgaria are protected, but poorly managed in general. They are included in the Natura 2000 network and watched over by Council Directive 92/43/EEC of the European Union. Still more research is needed to unveil their full significance for our well-being. More awareness has to be raised for their overall protection, but not only on paper. Despite the lack of governmental acknowledgement of the importance of the forest ecosystem in Bulgaria, we still possess around 30% of forested territory. The authors share the view that forest habitats have to be mapped, complying with all current trends at the perspective of the EUNIS classification, striving for habitat monitoring and protection (Chytrý et al. 2020).

Forests in western Bulgaria have been investigated by a number of scientists (Yordanov 1924; Minchev 1938; Florov 1952; Garelkov 1973; Filipovich 1981; Filipovich and Antonov 1996; Dodev and Popov 2011). Tzonev et al. (2006, 2019), Tashev et al. (2010), Dimitrov and Petrova (2014) played their role in forest habitat research.

The present study represents a continuation of the habitat investigations in Western Bulgaria, based on the EUNIS classification (Grigorov et al. 2022a). Habitats in Godech and Dragoman Municipality have already been studied by Grigorov et al. (2021a, b, 2022a, b). The creation of a habitat map of forests in Godech Municipality, based on the EUNIS classification, will provide more data to policy makers and this is a main aim of the current work.

Methods

Godech Municipality covers ca. 375 km^2 (Fig. 1). The territory is situated in the western part of Bulgaria. It borders the municipalities of Berkovitsa and Varshets to the north, Svoge municipality on the east, Dragoman and Kostinbrod municipalities on the south. The orogenesis has led to the formation of the following mountain ranges: Berkovska Mountain, Ponor Mountain, Vidlich, Vuchibaba and Chepun Mountain. Godech Valley, also known as Zaburge, is located in-between. The highest peak is Srebarna (1931.3 m), situated in Berkovska Mountain. More than 50% of the territory has an elevation between 1000 and 1600 m above sea level. The geological features include carbonate rocks (limestones, dolomitic limestones, dolomites) – a prerequisite for development of the karstification process. Breccias, conglomerates and sandstones are present, as well. Intrusive rocks and alluvial deposits may also be found (Zagorchev et al. 1990). The main river is Nishava with its tributary – Arakul River. The wide variety of geographical conditions has led to the formation of different soil types, such as Cambisols, Luvisols, Alluvisols and vegetation communities.

The fieldwork seasons of 2019 and 2020 were used for collection of 418 relevés following the Braun-Blanquet approach (Braun-Blanquet 1965) and 3422 verification field points (Fig. 1). All data collected in the field was applied in order to build a precise habitat map of the area. All collected relevés contributed to the Balkan Vegetation

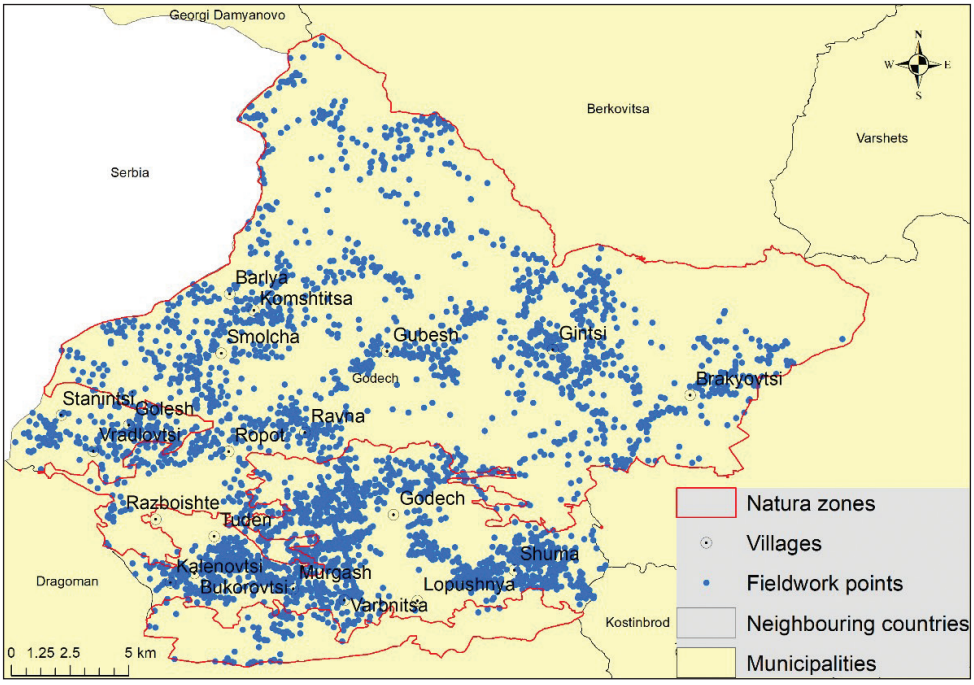

Figure 1. Forest vegetation relevés and verification points collected in Godech Municipality.

Database (Vassilev et al. 2020) and Balkan Dry Grassland Database (Vassilev et al. 2012). All relevés were plotted in homogenous areas of forest communities and were subsequently assigned to relevant habitat types. Habitat types were related subsequently to the revised version of the EUNIS system (Chytrý et al. 2020).

The EUNIS habitat types were determined with the help of the classification expert system EUNIS-ESy (Chytrý et al. 2020) integrated into JUICE 7.1 software (Tichý 2002). All defined habitat groups have had their diagnostic, dominant and constant species determined, 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).

Mapping was done using the ArcGIS 10.6 software package. Spatial data was collected in the field using GPS device Juno BS by Trimble and was later laid over the most recent orthophoto images available. The habitat map was created by the help of the "Intersect tool" by combining the layers, containing forestry data from Forestry Management Plans, as well as data about agricultural areas and habitat data from habitat mapping of NATURA 2000 in Bulgaria. Later, the "Cut polygon" tool was used in order to precisely modify the polygon geometry. All polygons were outlined manually using all the field collected data as well as the orthophoto images. The habitat map was elaborated in scale 1:5000.

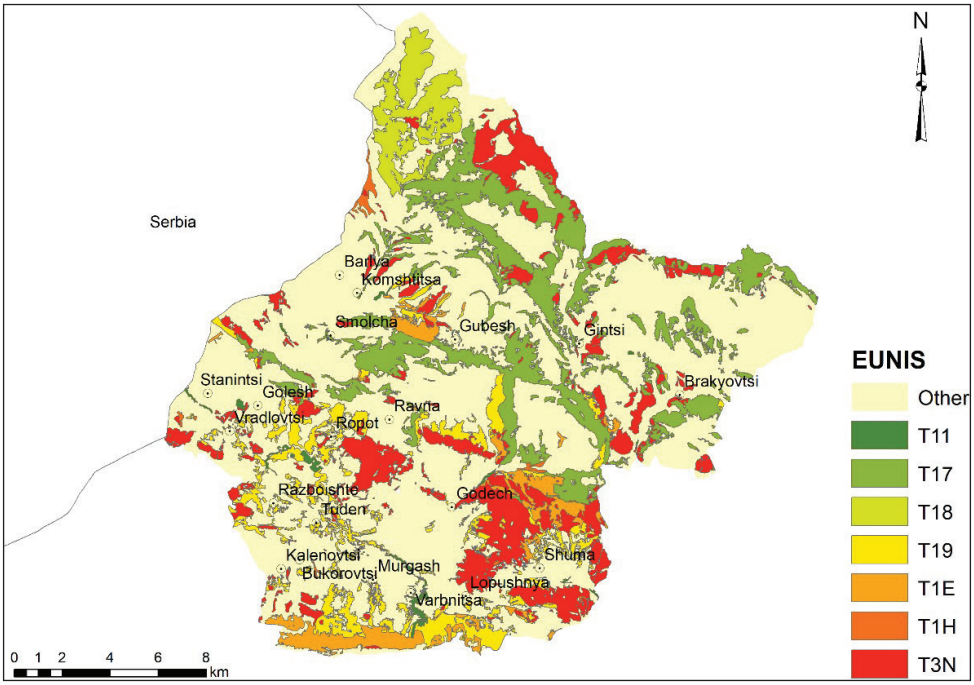

Figure 2. EUNIS forest habitat types in Godech Municipality.

Results

All studied forest types in Godech Municipality were related to 7 EUNIS habitat types (Fig. 2), which cover an area of 144.85 km^2.

T1E Carpinus and Quercus mesic deciduous forest

Abiotic characteristic: This habitat type covered an area of 12.20 km^2. It was located on slopes with humid conditions mainly in the mountains of Chepun, Vuchibaba and Berkovska Mountain predominantly in the hypsometric belts between 600 and 1000 m a.s.l. on slightly inclined slopes (10–15°) predominantly with northern and western components. Limestones, dolomites and marls were present. Soils were averagely deep. This habitat type was presented by 78 polygons (map units). The polygon's area was in the range of 0.0007–2.14 km^2.

Species composition and vegetation structure: T1E habitat type included monodominant or mixed forests with a closed horizontal structure and a total cover of 90–100%. *Carpinus betulus* L. or/and *Quercus petraea* agg. dominated the tree layer, which had a cover of 85–100%. *Fagus sylvatica* L., *Acer campestre* L., *Tilia platyphyllos* Scop., *Quercus cerris* L. and *Sorbus torminalis* Crantz were also present. The shrub layer had a cover of 20–40% and was formed by the same species of the tree layer as well as *Ligustrum vulgare* L. and *Corylus avellana* L. The herb layer also had poor species

composition and the most frequent species were *Festuca heterophylla* Lam., *Melica uniflora* Retz., *Poa nemoralis* L., *Aremonia agrimonoides* (L.) DC. Its cover was about 45–65%. The habitat type falls within class *Carpino-Fagetea*, order *Fagetalia sylvaticae* and the alliances *Carpinion betuli* and *Fagion sylvaticae* s.l. This vegetation represents 9170 *Galio-Carpinetum* oak-hornbeam forests, according to the Habitat Directive.

T1H Broadleaved deciduous plantation of non-site native trees

Abiotic characteristic: This habitat included plantations dispersed throughout the municipality and covered a territory of 1.21 km^2, mainly in Berkovska Mountain at 600–1000 m a.s.l. on slopes with various distribution. The bedrock types were represented mainly by limestone and dolomites and the soils were shallow to moderately deep. This habitat type was presented by 12 polygons. The polygon's area was in the range of 0.00000001–0.70 km^2.

Species composition and vegetation structure: Two tree species dominated the plantations: *Robinia pseudoacacia* L. and *Quercus rubra* L. The total vegetation cover in the studied polygons was 85–95%. *Prunus cerasifera* Ehrh., *Pyrus pyraster* (L.) Burgsd., *Malus domestica* Borkh., *Carpinus betulus* L., *Acer campestre* L., *Quercus cerris* L., *Q. frainetto* Ten., *Q. petraea* agg. were also present in the tree layer. The shrub layer was well-developed and had a cover of 25–50% and was formed by the same species of tree layer as well as *Crataegus monogyna* Jacq., *Prunus spinosa* L., *Euonymus verrucosus* Scopoli, *Rosa canina* L., *Rubus caesius* L., *Fraxinus ornus* L., *Carpinus orientalis* Mill. and *Cornus mas* L. The herb layer of *Robinia pseudoacacia* L. plantations was well-developed with cover 90–100%. *Bromus sterilis* L. was the dominant species. Other common species were *Galium aparine* L., *Myrrhoides nodosa* (L.) Cannon, *Chelidonium majus* L. *Quercus rubra* L. forests had a very poor species composition and the herb layer had a very low cover (up to 10–15%). *Robinia pseudoacacia* L. plantations belong to association *Bromo sterilis-Robinietum*, alliance *Balloto nigrae-Robinion pseudoacaciae*, order *Chelidonio-Robinietalia pseudoacaciae* and class *Robinietea*.

T3N *Coniferous plantation of site-native trees

Abiotic characteristic: This habitat type was distributed in all parts of the municipality and included planted coniferous forests at the hypsometric belts 200–600, 600–1000 and 1000–1600 m a.s.l. on slopes with various distribution. It covered an area of 41.97 km^2. Sedimentary and magmatic rocks were at the basis of shallow to averagely deep Chromic Luvisols and Rendzic Leptosols. This habitat type was presented by 246 polygons. The polygon's area was in the range of 0.0009–5.24 km^2.

Species composition and vegetation structure: The main tree species were *Pinus sylvestris* L., *P. nigra* J. F. Arnold and *Picea abies* H. Karst. The tree layer was well-developed with a cover of 85–100%. The horizontal vegetation structure was closed in the *Pinus sylvestris* L. stands and semi-open in the *Pinus nigra* J. F. Arnold stands. Other typical tree species were: *Fagus sylvatica* L., *Quercus* spp., *Acer pseudoplatanus* L.

The shrub layer of *Pinus nigra* J. F. Arnold and *P. sylvestris* L. plantations included *Rosa canina* L., *Rubus* spp., *Crataegus monogyna* Jacq., *Prunus spinosa* L. *Picea abies* H. Karst plantations, which were found at a higher altitude, included species such as *Vaccinium myrtillus* L., *V. vitis-ideae* L., *Chamaecytisus hirsutus* L., *Juniperus sibirica* Burgsd. The cover of the shrub layer was 60–70%. The herb layer was well-developed for the *Pinus nigra* J. F. Arnold plantations and had a cover of 50–70%. Some species from the neighboring habitats such as *Poa nemoralis* L., *Festuca dalmatica* (Hack.) K. Richt., *F. heterophylla* Lam., *Geum urbanum* L., *Melica uniflora* Retz., *Fragaria viridis* Weston, etc., were also discovered. *Pinus sylvestris* L. and *Picea abies* H. Karst plantations, where the tree and shrub layers form strong shady effect, had a herb layer with a lower total cover – 10–40%. The species composition was poorer and the most frequent species were *Luzula luzuloides* (Lam.) Dandy & Wilmott, *L. sylvatica* (Huds.) Gaudin, *Poa nemoralis* L., *Geum urbanum* L.

T11 Temperate Salix and Populus riparian forest

Abiotic characteristic: This habitat type was discovered along the riverbeds of Nishava, Glutnitsa, Zli dol and Shumska Rivers at lower altitudes on flat terrains. It covered an area of 1.8 km^2. The alluvial deposits, mainly on carbonate rocks, have been a prerequisite for the formation of typical averagely deep alluvisols. The habitat type was presented by 29 polygons. The polygon's area was in the range of 0.0005–0.63 km^2.

Species composition and vegetation structure: The vegetation had a closed horizontal structure with a total cover of 95–100%. The tree layer (cover about 85–95%) was dominated by *Salix fragilis* L. and *Alnus glutinosa* Gaertn., mixed with *Populus tremula* L. and *Salix purpurea* L. in some sites. The shrub layer had a cover of 40–60% and was formed by the same species, as the tree layer, but also included *Cornus sanguinea* L., *Prunus spinosa* L., *Rosa canina* L., *Rubus caesius* L., *Sambucus nigra* L. The herb layer was well-developed with a cover of 30–75%. Typical herb species were *Aegopodium podagraria* L., *Agrostis stolonifera* L., *Urtica dioica* L., *Lysimachia nummularia* L., *etc*. Invasive species, such as *R. pseudoacacia* L., *Amorpha fruticosa* L., *Erigeron annuus* (L.) Pers. and *Conyza canadensis* L. were typical as well. This vegetation represents the habitat type of 91E0*Alluvial forests with *Alnus glutinosa* and *Fraxinus excelsior* (*Alno-Padion*, *Alnion incanae*, *Salicion albae*), included in the Habitat Directive.

T17 Fagus forest on non-acid soils

Abiotic characteristic: This habitat type had the widest distribution and covered an area of 51.9 km^2 in the hypsometric belts between 600 and 1000 m a.s.l and 1000–1600 m a.s.l. It was common for slightly inclined slopes (up to 20–25°) with various expositions. It included mainly sedimentary rocks and shallow to moderately deep Cambisols. It was presented by 211 polygons. The polygon's area was in the range of 0.0002–7.54 km^2.

Species composition and vegetation structure: The horizontal vegetation structure was closed with a total cover of 90–100%. The tree layer had a cover of 90–100%.

The dominant species was *Fagus sylvatica* L., accompanied in some stands by *Quercus petraea* agg., *Carpinus betulus* L., *Sorbus* spp., *Tilia* spp. The shrub layer included species from the tree layer as well as *Crataegus monogyna* Jacq., *Corylus avellana* L., *Chamaecytisus hirsutus* L., *Ligustrum vulgare* L., *Carpinus orientalis* Mill. Its cover was 20–35%. The herb layer was species-rich with cover 15–65%. The most frequent species were *Aremonia agrimonoides* (L.) DC, *Mercurialis perennis* L., *Helleborus odorus* L., *Polygonatum odoratum* (Mill.) Druce and some orchid species such as *Dactylorhiza cordigera* (Fr.) Soo, *Cephalantera longifolia* (L.) Fritsch, *Neotia nidus-avis* (L.) Rich. This vegetation was classified to class *Carpino-Fagetea*, order *Fagetalia sylvaticae*, alliance *Cephalanthero-Fagion*. This vegetation represents the habitat type 9150 Medio-European limestone beech forests of the *Cephalanthero-Fagion*, according to the Habitat Directive.

T18 Fagus forest on acid soils

Abiotic characteristic: This habitat covered an area of 12.07 km^2 and was presented by 2 polygons only, located in Berkovska Mountain in the hypsometric belts between 600 and 1000 m a.s.l and 1000–1600 m a.s.l. The terrains were slightly inclined (up to 15°) and the exposition was variable. Soils were shallow to moderately deep and were from the Cambisols group. The bedrock consisted mainly of granodiorites, granites, conglomerates, sandstones, siltstones and limestones.

Species composition and vegetation structure: The phytocoenoses had a closed horizontal structure and total cover 90–100%. *Fagus sylvatica* L. dominated in the tree layer, which had a cover between 75% and 100%. Other tree species were *Acer pseudoplatanus* L., *Quercus petraea* agg. and *Carpinus betulus* L. The shrub layer had a low cover (10–25%) and was formed from the same species as in the tree layer along with *Rubus hirtus* Waldst. & Kit., *Corylus avellana* L. The herb layer was well-developed with a cover of 60–80%. There were stands with a cover of only 10%. Species with higher cover and abundance were *Galium odoratum* (L.) Scop., *Cardamine bulbifera* (L.) Crantz, *Mercurialis perennis* L., *Melica uniflora* Retz., *Luzula luzuloides* (Lam.) Dandy & Wilmott. This vegetation was classified to class *Carpino-Fagetea*, order *Fagetalia sylvaticae*, alliance *Fagion sylvaticae* s.l., associations *Asperulo-Fagetum* and *Festuco drymejae-Fagetum*. This vegetation represents the habitat type 9130 *Asperulo-Fagetum* beech forests, according to the Habitat Directive.

T19 Temperate and submediterranean thermophilous deciduous forest

Abiotic characteristic: This habitat was found on slopes with eastern and southern exposition mainly in the mountains of Vidlich, Vuchibaba and Ponor mainly in the hypsometric belts between 600 and 1000 m a.s.l. on slightly inclined slopes (10–15°) with predominantly eastern and southern components. Soils were shallow to moderately deep, overlaying mainly carbonates. This habitat type was presented by 212 polygons and covered an area of 23.70 km^2. The polygon's area was between 0.001 km^2 and 1.17 km^2.

Species composition and vegetation structure: The vegetation had a semi-open to closed horizontal structure with a tree layer cover of 75–90%. *Quercus cerris* L.,

Q. frainetto Ten. and *Q. pubescens* Willd. were the dominants. They formed mixed stands with *Fraxinus ornus* L., *Carpinus orientalis* Mill., *Ulmus minor* Mill. and *Sorbus torminalis* Crantz. The shrub layer reached 60% cover and included *Crataegus monogyna* Jacq., *Rosa canina* L., *Euonymus verrucosus* Scopoli, *Syringa vulgaris* L., *Chamaecytisus hirsutus* L. The herb layer was species-rich with cover in the range of 60–80%, including many herb and grass species such as *Poa nemoralis* L., *Festuca heterophylla* Lam., *Dactylis glomerata* L., *Galium pseudoaristatum* Schur, *Aremonia agrimonoides* (L.) DC, *Helleborus odorus* L., etc. These vegetation types were classified to class *Quercetea pubescenti*, order *Quercetalia pubescenti-petreae* and alliances *Quercion confertae* and *Quercion petraeo-cerridis*. This vegetation represents the habitat 91M0 Pannonian-Balkanic turkey oak-sessile oak forests and 91H0 *Pannonian woods with *Quercus pubescens*, according to the Habitat Directive.

Discussion

Fagus sylvatica L. forests dominate the forest landscape of Godech Municipality with the two EUNIS types, covering 64.6 km^2 in total, following the typical pattern, started in the Holocene, discussed by Filipovich and Antonov (1996). *Fagus sylvatica* L. participated in the last development phase of the forest vegetation in Stara Planina Mountain. These forests started their expansion 2–3 centuries before the beginning of the New Era due to climate and, more recently, anthropogenic factors. The forest belt in Western Stara Planina Mountain, dominated by *Carpinus betulus* L., once had a larger territorial extend and the same conclusion can be drawn for the coniferous species, mainly from the genera of *Picea* and *Abies* (Filipovich 1981). *Fagus sylvatica*'s timber has been used as a building material for many years. Cleared territories could experience restoration of *Fagus sylvatica* L. forests in areas free of weeds, such as *Urtica* spp., *Pteridium aquilinum* (L.) Kuhn., thriving in direct sunlight. This is an example of interspecies competition. Territories that are more prone to indirect sunlight cannot be invaded by weeds and *Fagus sylvatica* L. thrives there (Florov 1952). The restoration of *Fagus sylvatica* L. forests is more difficult on slopes with southern exposition and stony soils. Forests are restored better at 1000–1300 (1400) m a.s.l. (Garelkov 1973). According to Kumchev (1986) coniferous forests in Stara Planina Mountain will expand their territories by 2080 from 16 389 ha in 1980 to 26 941 ha. Broadleaved forest will decrease from 85 144 ha in 1980 to 74 592 ha by 2080.

The forests of Godech Municipality are presented by seven habitat types. They cover 144.85 km^2 in total, an area divided into 790 polygons, leading to a high rate of vegetation fragmentation. The habitat type with widest distribution is the T17 *Fagus* forest on non-acid soils (51.9 km^2), compared to the T3N Coniferous plantation of site-native trees that dominated the territory of Breznik Municipality (Grigorov et al. 2022a). Godech Municipality is dominated by beech forests which are typical for Bulgaria. Despite the fact that the artificial coniferous plantations are not the most widespread habitat type, they still cover 41.97 km^2, taking them into second place.

On the opposite side is the habitat type of T1H Broadleaved deciduous plantation of non-site native trees, which covers only 1.21 km^2.

There are several major threats to be addressed. Unfortunately, forest degradation, destruction and loss due to logging, fires, pollution, pest invasions, erosion etc., are typical for the study area. Some forest habitat types (T1E, T19) are turning into shrublands, while others (T11) are experiencing almost total damage. A whole new package of measures has to be adopted quickly to stop the negative effects, aiming at forest regeneration, afforestation with native species and ceasing of invasive alien species introduction.

Conclusion

The present study established 7 forest habitat types in Godech Municipality, according to the EUNIS classification. It represents a continuation of the habitat research of this scientific team in Western Bulgaria. It revealed some of the typical forest problems in Bulgaria – forest degradation due to natural and anthropogenic factors. The mapping in a 1:5000 scale proved once again to be desirable for analysis making. More research is needed to reveal the full picture of the forests' condition in the western parts of Bulgaria. The results of the current study may be used as a basis for further investigations on this matter.

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