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The resistance of biohumus microbiome to cobalt and nickel compounds

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Heavy metals, particularly cobalt and nickel, are highly toxic and widely distributed in ecosystems. Sometimes, their concentration in natural ecosystems can increase sharply due to anthropogenic activities. Metal-resistant microorganisms are considered to be promising for their detoxification. The purpose of the work was to study the sustainability of microorganisms derived from the biohumus in the presence of toxic cobalt and nickel compounds as well as determine the maximum limit concentration of Co^{2+} and Ni^{2+} for them. The biohumus served as a model natural ecosystem free from heavy metals where microorganisms were not adapted to them. The resistance of microorganisms was determined by cultivation in the medium with a gradient of simultaneous Co^{2+} and Ni^{2+} from 0 to 1000 mg/L. The quantification of Co^{2+} and Ni^{2+}-resistant microorganisms in the biohumus was determined by counting the number of colony forming units on nutrient agar. Using a Niton XL5 Plus manual XRF analyzer, it was determined that in metal missile fragments the concentration of cobalt ranged from 73 ± 22 to 589 ± 34 mg/kg, the concentration of nickel was 110 ± 15–577 ± 21 mg/kg. Cobalt was not detected in all soil samples. Nickel compounds were detected in two samples of the affected soil up to 408 ± 8 mg/kg and 36 ± 4 mg/kg in soil without shell explosions. On the example of the microorganisms of the biohumus, we confirmed that natural ecosystems contain microorganisms resistant to toxic Co^{2+} and Ni^{2+} compounds in high concentrations. The concentrations of simultaneous Co^{2+} and Ni^{2+} of 100 and 200 mg/L were established not to affect the growth of microorganisms, and the number of CFUs was $(6.2 \pm 0.2)\times10^5$ and $(6.1 \pm 0.2)\times10^5$ CFU/g. The maximum permissible concentration of simultaneous Co^{2+} and Ni^{2+} for the biohumus microbiome was 700 mg/L and the number of CFUs was $(5.0 \pm 0.1)\times10^2$ CFU/g after a month of cultivation. Moreover, microorganisms can adapt and maintain sustainable growth even after the increase in the concentration of metals from 500 to 2500 mg/L as well as to provide the detoxification of divalent metals by transforming into insoluble non-toxic sulfides.

Pollution of natural ecosystems with heavy metal is a serious concern because of the negative consequences it causes worldwide[1]. Pollution takes place due to natural processes such as volcanic eruptions, leaching of metals from rocks[2] as well as man-made activity including mining, industry, shelling, etc[3]. Natural emissions of metals, as well as emissions due to human industrial activities, can be predicted and prevented by planning economic activities in the areas of ores and installing treatment facilities[4]. However, in the situations of military actions, which are relevant in different regions of the planet in our time, spontaneous contamination of soil with heavy metals occurs due to shelling. Soils affected by military actions are reported to be contaminated with toxic metals and metalloids, organic and inorganic explosives, oxidizable secondary explosives, and their metallic derivatives[5]. In this regard, there is an urgent need to study the effect of such contamination on microorganisms and the mechanisms to reduce the negative consequences[6].

Cobalt[7] and nickel[8] are widespread among such pollutant metals. Cobalt and nickel production has been growing significantly in the last decade as global demand for them increases[9]. They are present in batteries, solar panels, electronics, car parts, shells, etc[10]. Due to physical and chemical properties, cobalt is used for ore smelting, alloy production, and the production of electrical and electronic devices[11]. Nickel, in turn, is used in

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modern metallurgy in a wide range of metallurgical processes, such as the production of alloys, electroplating, in the production of nickel-cadmium batteries, and as a catalyst in the chemical and food industries[12,13].

Cobalt is an important trace element for plants, animals and humans at concentrations of 5–40 µg/day. It plays an important role in biochemical reactions necessary for life; in particular, it is a component of the coenzyme cobalamin (vitamin B-12)[14]. Cobalt deficiency leads to such diseases as skin peeling, loss of appetite, anemia, and brittle bones[9]. Higher concentrations of cobalt are harmful to human health, causing asthma problems, liver damage, heart failure, and even cancer[15]. The most frequent symptoms of a high accumulation of cobalt in plants are a decrease in growth and the appearance of necrosis, as well as a violation of the assimilation of nutrients[16]. Nickel is an important trace element necessary for the normal growth of many types of microorganisms and plants[17]. It is an integral part of several enzymes, such as glyoxalase and urease, required for nitrogen metabolism in higher plants[18]. In humans, nickel in high concentrations can cause allergies, DNA damage, neurological disorders, cardiovascular and kidney diseases, pulmonary fibrosis, lung and nasal cancer[19,20].

The resistance of microorganisms to cobalt and nickel is being often considered a related phenomenon. This may be due to the fact that resistance to these two metals is associated with the functioning of the same genes, for example, the genes belonging to the resistance nodulation family which encodes proton-driven antiporters[21]. Therefore, the study of the resistance of microorganisms to cobalt and nickel simultaneously is justified due to the interconnectedness of the genetic mechanisms of resistance of microbial cells.

Chemical or physical methods are mainly used to purify the environment from heavy metals[22], including adsorption, ion exchange, membrane filtration, and chemical precipitation[23,24]. However, such methods are not effective enough considering the scale of pollution and are also expensive[25]. A promising way to detoxify metal pollutants is the use of diversified microbiomes resistant to high concentrations of metals[26]. Metal-resistant bacteria most often belong to the genera *Bacillus*, *Arthrobacter*, *Pseudomonas*, *Ralstonia*, *Stenotrophomonas*, *Desulfovibrio* and demonstrate a high ability to precipitate various heavy metals in the form of insoluble non-toxic compounds[27,28]. Metal-resistant microorganisms are known to be spread in various ecosystems and possess a great capacity to survive and interact with metals. Thus, microorganisms are cheap and effective biotechnological means for remediating metal-contaminated soil[29,30].

Therefore, the purpose of our study was to study the sustainability of microorganisms originated from the biohumus in the presence of toxic cobalt and nickel compounds as well as determine the maximum limit concentration of Co^{2+} and Ni^{2+} for them.

Results

Investigation of the contamination of the soil affected by shelling with cobalt and nickel

Military actions possess hazard to the environment via the spread of shell and rocket fragments, bullet residues and other metal-containing materials in soil. Corroding in soil, they are a source of contamination of territories with heavy metals. The investigation of three soil samples from the craters created by a shell explosion near Hostomel showed the following results (Table 1).

Compounds of cobalt were not detected in the places of shells hit. It is also natural that they were not found either in soil not exposed to shelling or in biohumus. On the other hand, in metal missile fragments, the concentration of cobalt ranged from 73 ± 22 to 589 ± 34 mg/kg. Nickel compounds were detected in two samples of the affected soil up to 408 ± 8 mg/kg. Some small amount (36 ± 4 mg/kg) was also found in soil without shell explosions confirming the spread of metal in ecosystems. The amount of nickel in missile fragments was 110 ± 15–577 ± 21 mg/kg. Thus, it can be assumed that nickel compounds could enter the soil directly when shells exploded, whereas this was not detected for cobalt. However, projectile fragments located in the soil pose a threat to the environment as sources of toxic metals that can be mobilized due to the metabolic activity of microorganisms and plants. Understanding the amount of metal compounds that can enter the soil during shelling, as well as when shell fragments are found there, provides a range of studies on the resistance of soil microorganisms to metals.It is especially relevant to study the limits of resistance of microorganisms not adapted to heavy metals to determine the adaptation potential of soil ecosystems and pathways for the detoxification of metals in soil. Though, it is important to study microbial community of the affected sites, current research was aimed to study the response of the microbiome from the non-affected site (biohumus) to understand the level of resistance of microorganisms which have never been exposed to toxic metals. These results will give a baseline for

No	Sample description	Place of sampling	Concentration, mg/kg	
			Ni(II)	Co(II)
1	Regosol from a crater 5.0 m in diameter and 1.5 m deep, field	Hostomel, Ukraine	< LOD	< LOD[1]
2	Regosol from a crater 4.0 m in diameter and 2.0 m deep, field		303 ± 9	< LOD
3	Retisol from a crater 16.0 m in diameter and 6.0 m deep, forest		408 ± 8	< LOD
4	Chernozem not affected by shelling, field		36 ± 4	< LOD
5	Metal missile fragments		110 ± 15	589 ± 34
6	Metal missile fragments		577 ± 21	73 ± 22
7	Biohumus after the fermentation of plant residues	Opole, Poland	< LOD	< LOD

Table 1. The concentration of cobalt and nickel in samples. [1]< LOD – represents the value lower than limits of detection (limits of detection in the soil of Ni(II) – 11 mg/kg, of Co(II) – 5 mg/kg.

the resistance of non-adapted microorganisms that can be used as a guide when studying microorganisms from the affected regions. Therefore, the following research deals with the study of the microorganisms of biohumus.

Determination of the maximum limit concentration of metals for microorganisms from biohumus

Biohumus obtained after fermentation of plant residues is the main component of fertile soil. It consists of lignocellulose residues, which are important structure-forming components of the soil, and is also the source of soil microbiome, which provides access to nutrients and growth stimulants for plants[31]. The presence of metal-resistant microorganisms in the biohumus microbiome is an important feature providing the stability of functioning of the whole soil ecosystem in the presence of heavy metals.

Therefore, the microbiome of biohumus was studied to determine metal resistance and the possible pathways of metal detoxification. The concentrations of simultaneous Co^{2+} and Ni^{2+} of 100 and 200 mg/L were established to not affect the growth of microorganisms (Fig. 1). Active growth was observed already after the first day of cultivation. The number of CFUs of metal-resistant microorganisms, isolated from biohumus on an agarized nutrient medium at a concentration 100 and 200 mg/L of Co^{2+} and Ni^{2+}, was $(6.2 \pm 0.2)\times10^5$ and $(6.1 \pm 0.2)\times10^5$ CFU/g. At a concentration 300 mg/L of Co^{2+} and Ni^{2+}, the delay in colony growth was only one day compared to the control without metals. The number of CFUs was $(3.4 \pm 0.1)\times10^5$ CFU/g after 30 days of cultivation. At 400 and 500 mg/L of Co^{2+} and Ni^{2+}, the delay in the growth of colonies was 7 and 11 days, respectively. The amount of microorganisms was $(3.0 \pm 0.2)\times10^5$ and $(1.1 \pm 0.1)\times10^5$ CFU/g after 30 days. At 600 mg/L of metals, the microbiome growth delay was 25 days, and the number of colonies was $(9.4 \pm 0.1)\times10^3$ CFU/g. The maximum permissible concentration of simultaneous Co^{2+} and Ni^{2+} for the biohumus microbiome was 700 mg/L (concentration of each metal 350 mg/L). At the same time, the growth of microorganisms was delayed for one month, and the number of colonies was $(5.0 \pm 0.1)\times10^2$ CFU/g. At a summarized metal concentration above 700 mg/L, only fungi growth was observed. The growth of biohumus microorganisms did not provide a pH change in the agarized nutrient medium. That was evidenced by the absence of change in the color of the BTB indicator.

Obtaining of the active microbiome from the biohumus

The line of the active microbiome was obtained from the biohumus in PNB. The growth activity was confirmed via the discoloration of sodium resazurin used as the Eh indicator on the second day of cultivation. By the 17th day of cultivation, the pH of the culture liquid was 8.7. The metabolic activity of microorganisms expressed in the alkalization of the medium is important to provide metal detoxification via the precipitation of divalent heavy metals, such as Co^{2+} and Ni^{2+} in solutions. The dynamics of the pH changes during the cultivation of the active biohumus microbiome are presented in Fig. 2.

Obtained microbiome was sown on an agarized peptone nutrient medium with metal concentrations of 400 and 700 mg/L of simultaneously added Co^{2+} and Ni^{2+} and cultivated at 25 °C for seven days. The growth was already observed on the second day of cultivation in the variant with 400 mg/L of metals. Within seven days, the medium became alkaline, as evidenced by the change of BTB color from yellow-green to blue in the growth zone of bacterial colonies (Fig. 3a). At 700 mg/L of Co^{2+} and Ni^{2+}, the growth started on the seventh day.

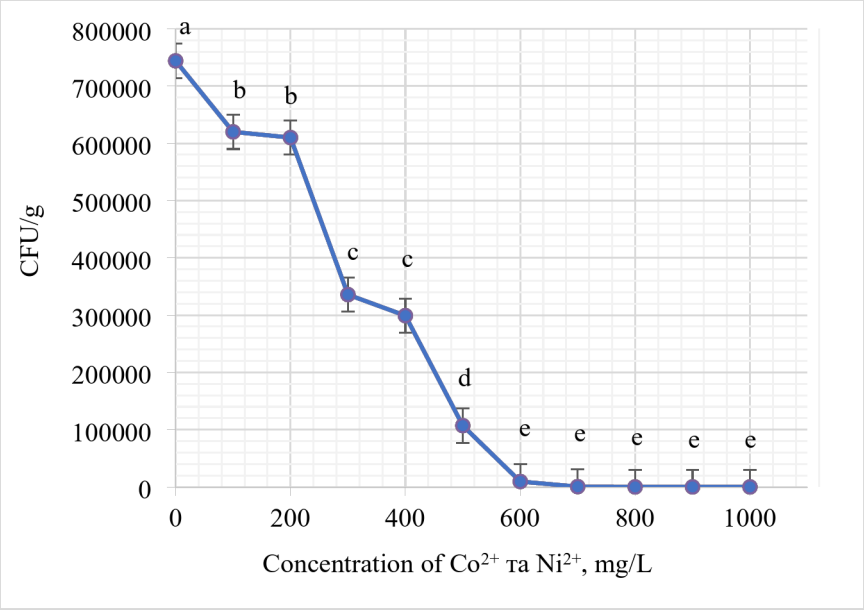

Fig. 1. Dependence of the amount of CFU of metal-resistant microorganisms of the biohumus and the concentration of simultaneous Co^{2+} and Ni^{2+} after 30 days of cultivation. Letters a, b, c, d, e show the statistical difference between the data set, $p < 0.05$ with Bonferroni correction.

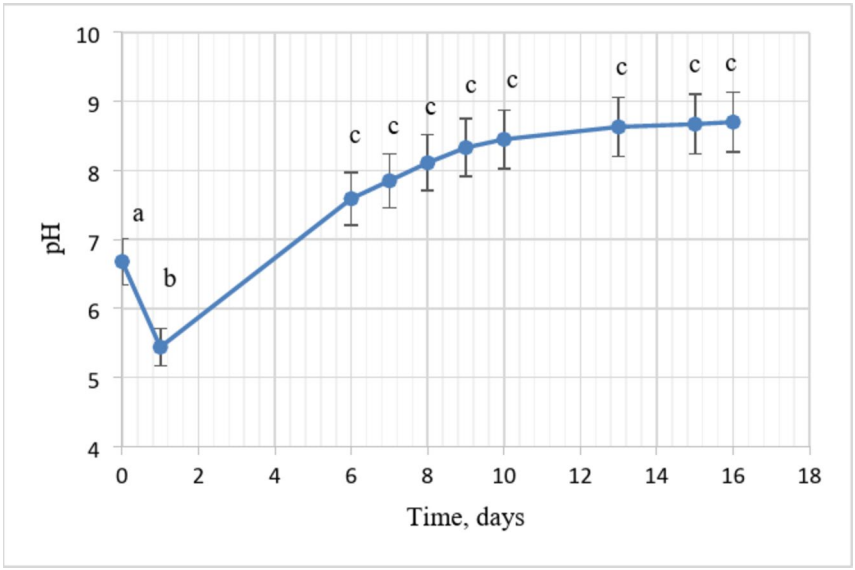

Fig. 2. The dynamics of pH changes during the cultivation of the active biohumus microbiome. Letters a, b, c show the statistical difference between the data set, $p < 0.05$ with Bonferroni correction.

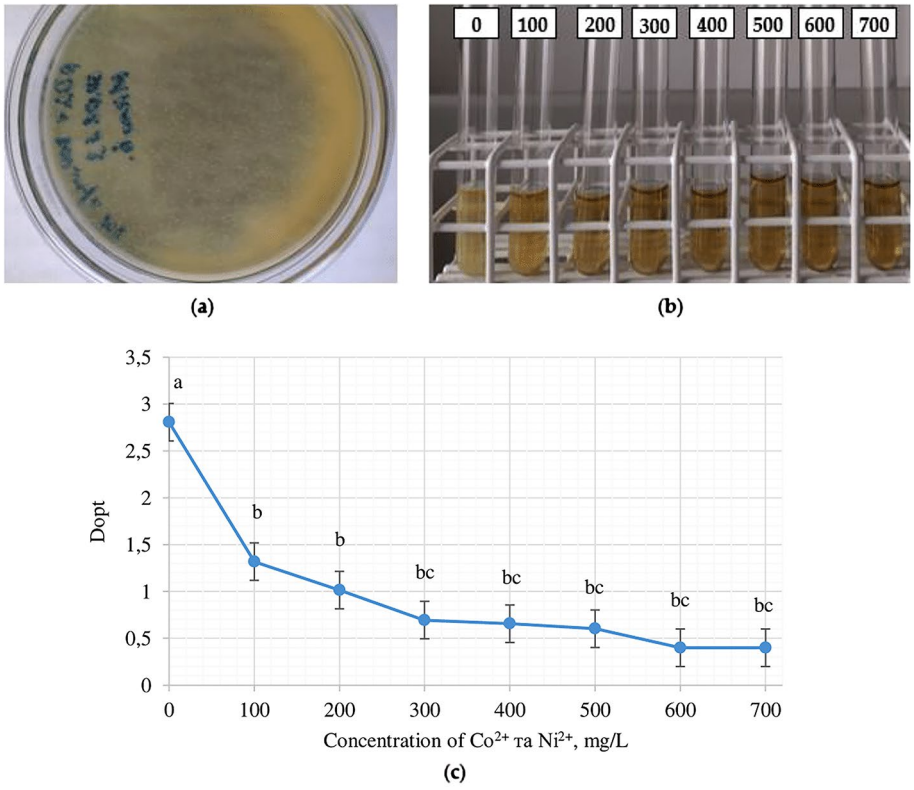

Fig. 3. Growth of metal-resistant microorganisms of the biohumus. (**a**) Change in the color of the medium during the growth of the metal-resistant microbiome at concentration of 400 mg/L simultaneously Co^{2+} and Ni^{2+} (7th day of cultivation); (**b**) Growth of metal-resistant microorganisms on a liquid nutrient medium (1st day of cultivation) with the concentration range Co^{2+} and Ni^{2+} from 0 to 700 mg/L; (**c**) The dependence of the growth of metal-resistant microorganisms of the biohumus on PNB with Co^{2+} and Ni^{2+} after 15 days of cultivation. Letters a, b, c show the statistical difference between the data set, $p < 0.05$ with Bonferroni correction.

Microorganisms grown in the presence of 700 mg/L of metals on an agar medium were tested to be resistant to metals in the liquid medium (Fig. 3b). The results were recorded on the basis of turbidity of the nutrient medium due to bacterial growth.

The growth of microorganisms in PNB with a concentration range of Co^{2+} and Ni^{2+} metals 0–700 mg/L was determined by the optical density of the culture liquid photocolorimetrically (λ = 540 nm). The concentration of 100 mg/L of Co^{2+} and Ni^{2+} more than twice suppresses the growth of metal-resistant culture compared to the control without metals. At concentrations of 600 and 700 mg/L, 7-fold growth inhibition was observed. The use of a liquid medium was shown to be a more unfavorable factor than an agarized one, which is associated with the constant influence of Co^{2+} and Ni^{2+} on the cell. The results are presented in Fig. 3c.

The adaptation of biohumus microorganisms resistant to high concentrations of Co2+ and Ni2+

The adaptation of biohumus microorganisms to high concentration of Co^{2+} and Ni^{2+} was carried out by sowing cultures onto a PNA with a gradual increase in the concentration of metals in steps of 200 mg/L. In this way, it was possible to obtain cultures growing at a simultaneous concentration of Co^{2+} and Ni^{2+} of 2500 mg/L (the concentration of each of the metals is 1250 mg/L) (Fig. 4a).

The obtained metal-resistant microorganisms were tested for the ability to reduce the redox potential and alkalinize the medium. The obtained microbiome was found to cause alkalization of PNA (Fig. 4b), but do not reduce the redox potential (Fig. 4c).

The growth of metal-resistant cultures from a Petri plates with a simultaneous Co^{2+} and Ni^{2+} concentrations of 2000 mg/L on a PNB with a concentration range of metals Co^{2+} and Ni^{2+} 500–2000 mg/L was tested. The results were recorded on the basis of the turbidity of the nutrient medium due to bacterial growth and on the basis of the delay in the color change to neutral red as a pH indicator (Fig. 5a).

It is shown that the more toxic liquid metal-containing medium has a lower inhibitory effect on microorganisms adapted to high concentrations of metals than on non-adapted ones. Thus, at a concentration of 2000 mg/L, inhibition occurs only 1.5 times compared to the control without metals (Fig. 5b).

Thus, the military actions possess hazard to the environment both in the moment of shells hitting the soil and exploding, which results in direct exposure to heavy metal compounds, as well as subsequently long-term contamination of soil with metals releasing from missile fragments due to the activity of microorganisms and plants. Therefore, it is very important to investigate the range of resistance of non-adapted soil microorganisms to toxic metals, as well as their adaptive potential and possible pathways for metal detoxification. Such study was undertaken in this work.

Discussion

Fighting inevitably has a negative impact, altering the environment, damaging natural ecosystems and destroying human habitats[32]. There is pollution of both the atmosphere[33], water environments[34] and soil[35]. Bombing, trenching and tunneling are likely to cause soil degradation and changes in landscape morphology. This is especially important because Ukraine has some of the most fertile soils in the world (chernozem), which means it will affect food production[36]. Metal missile fragments of various sizes constitute a significant part of the polluting factors. After the explosion, such particles fly over a large area and decompose in the

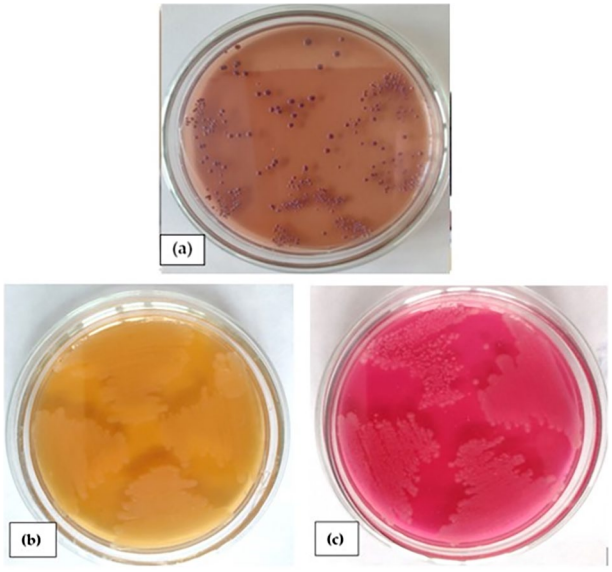

Fig. 4. Metal-resistant microorganisms of biohumus: (**a**) Growth of metal-resistant cultures from biohumus microbiome at a simultaneous Co^{2+} and Ni^{2+} concentrations of 2500 mg/L. Growth of metal-resistant microorganisms (**b**) using neutral red (alkalization of the medium with a change in color from pink to yellow); (**c**) using resazurin (growth without color change – no decrease in Eh).

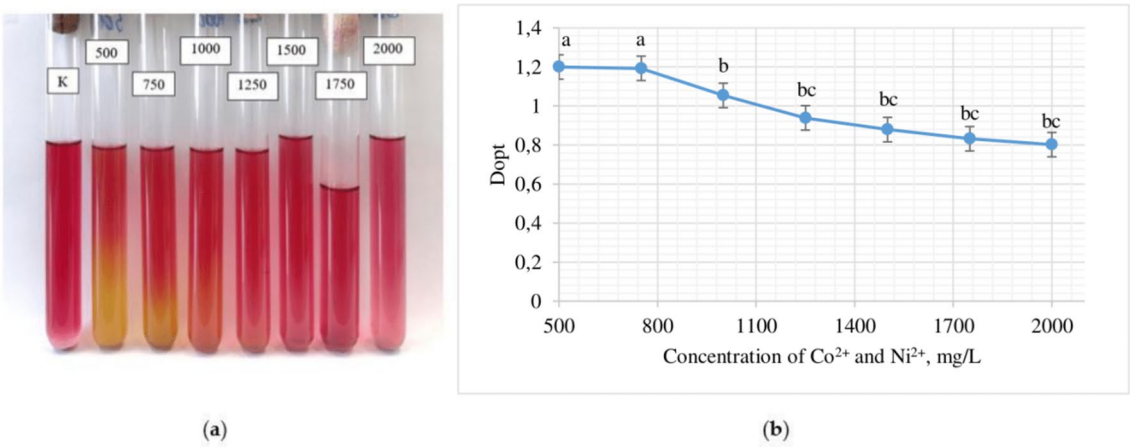

Fig. 5. Determination of the resistance of microorganisms to metals by cultivation in a liquid peptone medium with a gradient of Co^{2+} and Ni^{2+} from 500 to 2000 mg/L. (**a**) Visualization of the growth delay of the biohumus microbiome depending on the concentration of heavy metals (2nd day of cultivation). (**b**) The dependence of the growth of metal-resistant microorganisms of biohumus on the concentration of metals in the liquid nutrient medium on the 10th day of cultivation based on optical density. Letters a, b, c show the statistical difference between the data set, $p < 0.05$ with Bonferroni correction.

soil for a considerable period of time. This causes a long-term cumulative toxic effect on the environment[37]. Soils damaged by bombing and shelling contain large amounts of heavy metals and arsenic that are harmful to plants, animals, and humans, especially through the migration of these substances into groundwater and food chains[38,39]. Excessive concentration of heavy metals disrupts cation exchange, which negatively affects plants and microorganisms, reducing their ability to provide the necessary nutrients for growth and development[38]. The fastest spreading metals in the soil are: iron (Fe), zinc (Zn), cadmium (Cd), antimony (Sb); the slowest: arsenic (As), lead (Pb), copper (Cu), nickel (Ni), mercury (Hg), manganese (Mn), vanadium (V). The rate of diffusion of barium, cobalt, arsenic, lead, mercury, manganese, strontium and titanium does not depend on the type and mechanical parameters of the soil. It is known that loams and chernozems are more prone to the accumulation of heavy metals and iron than sandy soils[39].

The problem of pollution of ecosystems with heavy metals needs an immediate solution[40,41]. We propose the use of biological methods based on the use of metal-resistant microorganisms. The advantage of this method is that the sources of microbial biomass are unlimited. It can be any natural and artificial ecosystems. In particular, we investigated such an option as biohumus. Also, microorganisms isolated from such ecosystems can be adapted to higher concentrations of metals. Thus, we adapted the obtained microorganisms to 2500 mg/L of Co^{2+} and Ni^{2+} (the concentration of each of the metals is 1250 mg/L). Moreover, their viability in a liquid medium, which is more toxic, at simultaneous Co^{2+} and Ni^{2+} concentrations up to 2000 mg/L is also shown. All these factors are important prerequisites for the creation of new ecological biotechnologies of soil remediation and polymetallic wastewater treatment.

According to the literature, it is known that some metal-resistant microorganisms have been previously isolated[42,43]. However, in them it was about the isolation of microorganisms from environments already polluted with large concentrations of heavy metals[44–46]. Thus, the *Apiotrichum loubieri* M12 strain was isolated from the sediments of an abandoned gold mine located in San Luis (Argentina). It is able to transport and remove Cu^{2+} from liquid culture media, with an efficiency of 30–35% at an initial concentration of 40 mg/L Cu^{2+} for 48 h. The presence of Cu^{2+} induces differential expression of intracellular proteins involved in various metabolic processes in *Apiotrichum loubieri* M12. A specific response to the metal was detected in cell-free supernatants in which copper-binding proteins were identified. This microorganism reacts with copper not only by regulating intracellular protein expression, but also by regulating protein expression in the extracellular space[47]. Fifteen cultivated HMRB strains belonging to the genera *Bacillus*, *Shewanella*, *Lysinibacillus* and *Acinetobacter* were isolated from sludge samples of an electroplating treatment plant (Dungguan, China). Their maximum tolerance concentrations for Cu^{2+}, Ni^{2+}, Mn^{2+}, Co^{2+} and $Cr_2O_7^{2-}$ were 40 mM, 10 mM, 200 mM, 40 mM, and 10 mM, respectively[48]. Twelve fungal strains belonging to the genera of *Aspergillus*, *Penicillium*, *Fusarium*, *Cunninghamella*, *Simplicillium*, *Trichoderma*, *Rhizomucor*, *Cladosporium*, and *Hypocrea* were identified from Hindustan Paper Corporation (HPC), Assam. A further screening approach to assess their tolerance to heavy metals showed that most isolates were tolerant to environments containing various concentrations of trace metals Ni^{2+}, Cu^{2+}, Zn^{2+} and Cd^{2+} (0.1, 0.5, 2.0, 4.0 mM)[49]. Mine soils in southeastern Idaho, USA, contaminated with Se^{4+} and Se^{6+} were also investigated. The concentration of Se in the soil was 30 mg/kg. It was determined that as a result of contamination with this trace element, communities of Se-tolerant microorganisms were formed. *Actinobacteria*, *Gemmatimonadetes*, and *Ascomycota* predominated among them[50].

Many researchers suggest using genetically modified microorganisms to neutralize heavy metals[51–53]. In particular, the use of microorganisms of the genera and species *Flavobacterium*, *Pseudomonas*, *Bacillus*, *Arthrobacter*, *Corynebacterium*, *Methosinus*, *Rhodococcus*, *Mycobacterium*, *Stereum hirsutum*, *Nocardia*,

Fig. 6. A crater 16.0 m in diameter and 6.0 m deep in sandy-clay soil (50°69'89.9 N 30°34'39.8E) (Hostomel, Kyiv region, Ukraine).

Methanogens, *Aspergilus niger*, *Pleurotus ostreatus*, *Rhizopus arrhizus*, *Azotobacter*, *Alcaligenes*, *Phormidium valderium*, *Ganoderma applantus* is suggested[54,55]. However, in our opinion, this significantly complicates and increases the cost of heavy metal disposal technology.

Thus, the contamination of soil with heavy metals due to military actions is very relevant and important issue. The pollution of soil was shown to be able to take place both via direct explosion of shells as well as the release of metals form missile fragments left in soil. The accumulation of toxic metals possess hazard to the environment. Therefore, the study of the adaptation potential of soil microorganisms and the pathways of metals detoxification are required.

Conclusion

The study of samples from the sites affected by shelling in Kyiv region has shown that cobalt compounds were not detected in soil while nickel was in the range of 36 ± 4–408 ± 8 mg/kg. Missile fragments contained both cobalt and nickel compounds. Therefore, the investigation of the adaptation potential of soil microorganisms is necessary to get insight into the patterns of microbial interaction with metals as well as for the prospects for the application in environmental biotechnologies. The research showed the capacity of non-adapted microorganisms of the biohumus to grow at the concentration of Co^{2+} and Ni^{2+} up to 2500 mg/L. Moreover, their metabolic activity to alkalinize medium can be useful for various metals detoxification. The soil environment alkalization by microorganisms can be used as the fast and safe pathway to immobilize toxic soluble metal compounds in the form of insoluble and, therefore, non-toxic ones. This approach can be used to immobilize toxic metals and exclude them from circulation in soil.

Methods

Determination of the concentration of cobalt and nickel in the soil affected by shelling

To study if there is hazard of the contamination of soil with cobalt and nickel as the result of shelling, soil samples were collected form the affected sites. Three samples of soil were collected directly from different craters created by a shell explosion. In particular, 1 – regosol from a crater 5.0 m in diameter and 1.5 m deep, field (50°63'64.8"N 30°26'77.4"E), 2 – regosol from a crater 4.0 m in diameter and 2.0 m deep, field (50°63'38.2"N 30°26'85.6"E), 3 – retisol soil from a crater 16.0 m in diameter and 6.0 m deep, forest (50°69'89.9 N 30°34'39.8E). One sample of chernozem was also taken from a field not affected by shelling (50°38'11.3"N 30°16'03.9"E). The soil type is determined according to International soil classification system[56]. Metal missile fragments were taken from funnels 1 and 2 for analysis. These sites are located near Hostomel, a settlement in Kyiv Region, Ukraine, where active military actions took place in spring 2022 (Fig. 6). The evaluation of the concentration of cobalt and nickel compounds in samples was carried out via the Niton XL5 Plus handheld XRF analyzer (Thermo Scientific, Waltham, MA USA). Also, their content was analyzed in two samples of metal missile fragments. Such a set of researched options made it possible to draw conclusions about the possibility of soil contamination with heavy metals, in particular cobalt and nickel, as a result of their impact with fragments of various types of rockets.

As the control sample without metal contamination, the biohumus (Opole, Poland) was used. Biohumus was obtained by composting plant residues, in particular grass and food waste. It is a free-flowing fine granular mass of dark brown color.

Obtaining of the active microbiome of the biohumus

Since humus is the main soil-forming component, the biohumus obtained after the fermentation of plant residues was used as a source of an active microbiome non-adapted to heavy metals. It served as a model to study the resistance of microorganisms and their ability to maintain sustainable grows in the presence of heavy metals. To obtain a highly active microbiome with an increased number of metabolically active microorganisms, 150 mL of peptone nutrient broth (PNB) (BioMaxima S.A., Poland) was poured into the conical flask with a volume of 200 mL. Sodium resazurin solution added to the medium was used as an Eh indicator (discoloration at – 100…–150 mV). The biohumus (5 g) was added to the medium and cultivated at 25 °C for 16 days. Such parameters as pH, and redox potential (Eh, mV) were controlled[57]. They were measured potentiometrically via pH/conductivity meter CPC-411, electrode pH EPS-1, and electrode redox ERS-2 (Elmetron, Zabrze, Poland), respectively.

Preparation of cobalt and nickel solutions

A sterile solution of simultaneous Co^{2+} and Ni^{2+} ($CoCl_2 \times 6H_2O$ and $NiSO_4 \times 6H_2O$) with a summarized concentration of 50,000 mg/L (the individual concentration of each of the metals was 25,000 mg/L) was previously prepared. To prepare 100 mL of cobalt and nickel with a concentration of 50,000 mg/L, 10.08 g of $CoCl_2 \times 6H_2O$ and 11.15 g of $NiSO_4 \times 6H_2O$ were dissolved in distilled water.

Microbiological study of the biohumus

Metal-resistant microorganisms from the studied soil samples were isolated by Koch's method[58]. For this, a sterile agarized peptone nutrient medium (BioMaxima S.A., Poland) with the addition of 3 g/L cysteine was used. A solution of Co^{2+} and Ni^{2+} metals was added to the nutrient medium to a final total concentration of 0 to 1000 mg/L (a step in the concentration series was 100 mg/L). Microorganisms were isolated from tenfold dilutions of the soil suspension. To prepare the inoculum, a weight of 1 g of the biohumus was added to 9 mL of sterile 0.8% NaCl and suspended for 15 min, periodically shaking. 100 µL of prepared inoculum, 10 mL of nutrient medium, and metals solution were added to sterile test tubes to final concentrations of 0-1000 mg/L in steps of 100 mg/L to create a concentration series. As a pH indicator, a 0.1% solution of bromothymol blue (BTB) (transition interval pH: 6 (yellow color) – 7.6 (blue color)) was added to the medium at the rate of 2% of the volume of the medium. This mixture was poured into Petri plates and cultivated at 25 °C for 30 days.

Investigation of the adaptational limits of non-adapted microorganisms of biohumus to high concentrations of metals

The study of the adaptation potential of the biohumus microorganisms to the high concentrations of the simultaneous presence of Co^{2+} and Ni^{2+} was carried out both on solid and liquid nutrient media.

The growth on solid medium was studied via successive passages of bacteria on the media with a gradual increase in the concentration of metals[59]. The obtained active microbiome was sown on the peptone nutrient agar (PNA) (BioMaxima S.A., Poland) with concentrations of 400 and 700 mg/L simultaneously Co^{2+} and Ni^{2+} (concentration of each metal 200 and 350 mg/L, respectively). The cultivation took place at 25 °C for 7 days. a 0.1% solution of bromothymol blue was used as a pH indicator. 0.1% solution of bromothymol blue was added to the medium to concentration of 2%. Every next passage was conducted on the PNA with the gradually increased concentration of Co^{2+} and Ni^{2+} with a step of 200 mg/L. Cultivation took place for 3–5 days at 25 °C.

The obtained metal-resistant microbiome was tested for the ability to reduce the redox potential and alkalinize the medium to study its potential to precipitate soluble Co^{2+} and Ni^{2+} to insoluble forms. For this, sowing was carried out on agarized peptone nutrient medium in two variants: 1 – using resazurin (Eh indicator) and 2 – using neutral red (pH indicator, 6.8 (pink) – 8.4 (yellow)). Microorganisms were sown with a stroke and cultivated for 3 days at 25 °C.

The growth in liquid medium was investigated as follows. The cultures previously grown on Petri plates with a concentration of 700 mg/L, were tested on a liquid nutrient medium with a concentration range of simultaneously present Co^{2+} and Ni^{2+} from 0 to 700 mg/L. To do this, cultures from a Petri plate were transferred with a loop into a test tube with a liquid nutrient medium and left for a day in a thermostat at a temperature of 25 °C to obtain a stock culture. 5 mL of liquid peptone nutrient medium was poured into sterile test tubes and a solution of Co^{2+} and Ni^{2+} metals was added to final concentrations of 0–700 mg/L. The prepared inoculum in the amount of 0.75 mL (15% of the volume of the medium) was added to the ready nutrient medium. Cultivation was carried out for 15 days at a temperature of 25 °C. The results were recorded based on the turbidity of the nutrient medium due to bacterial growth. In addition, growth was determined by the optical density of the culture liquid photocolorimetrically ($\lambda = 540$ nm).

The growth of metal-resistant cultures from a Petri plate was also tested on a liquid nutrient medium with a concentration range of simultaneously Co^{2+} and Ni^{2+} 500–2000 mg/L. To do this, cultures from a Petri plate were transferred with a loop into a test tube with a liquid nutrient medium and left for a day in a thermostat at a temperature of 25 °C to obtain a stock culture. Portions of 10 mL of liquid peptone nutrient medium were poured into sterile test tubes and a solution of Co^{2+} and Ni^{2+} metals was added to obtain final concentrations of 500–2000 mg/L in steps of 250 mg/L. The prepared inoculum in the amount of 1.5 mL (15% of the volume of the medium) was added to the prepared nutrient media. Cultivation was carried out for 10 days at a temperature of 25 °C. The results were recorded based on the turbidity of the nutrient medium due to bacterial growth and the delay in the color change to neutral red as a pH indicator. In addition, growth was determined by the optical density of the culture liquid photocolorimetrically ($\lambda = 540$ nm).

Data analysis

The number of colony-forming units (CFU) of metal-resistant microorganisms, isolated from the biohumus on an agarized nutrient medium with a gradient of Co^{2+} and Ni^{2+} from 0 to 1000 mg/L, in 1 mL of suspension was calculated according to the formula:

$$M = a \cdot 10^n / V, \tag{1}$$

where: M – the number of cells in 1 mL; a – average number of colonies; 10^n – dilution factor; V is the volume of the inoculum, mL.

The number of microorganisms in the samples was calculated into the total number of microorganisms per 1 g of completely dry sample according to the formula:

$$X = a \times k, \tag{2}$$

where: X – the number of colony-forming units in 1 g of completely dry sample; a – the number of cells in 1 mL of the suspension of the studied microorganisms; k – the humidity coefficient of the sample.

The humidity coefficient was determined by the formula:

$$k = 100/(100 - H), \tag{3}$$

where: H – the moisture content of the sample.

The moisture content (H) in the samples was determined by the weight method. The samples were dried in a drying cabinet at a temperature of 105 °C to a constant weight. Humidity was determined by the formula:

$$H = A/(m - -A) \cdot 100\%, \tag{4}$$

where: A – the weight of evaporated moisture; m is the weight of the sample[58].

Each experiment was repeated in three replicates. Data analysis was carried out using Microsoft Excel 2013. Means and standard deviations (SDs) were determined with a 95% confidence level. Values were presented as mean ± SD. The level of significance of differences between the data sets was determined via the one-way ANOVA test with the post-hoc test (Bonferroni correction).

Data availability

All data generated or analysed during this study are included in this published article, however, row datasets used and/or analysed during the current study are available from the corresponding author (O.T.) on reasonable request.

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References

1. Briffa, J., Sinagra, E. & Blundell, R. Heavy metal pollution in the environment and their toxicological effects on humans. *Heliyon* **6**, e04691 (2020).
2. Hama Aziz, K. H. et al. Heavy metal pollution in the aquatic environment: efficient and low-cost removal approaches to eliminate their toxicity: a review. *RSC Adv.* **13**, 17595–17610 (2023).
3. Candeias, C., Ávila, P., Coelho, P. & Teixeira, J. P. Mining Activities: Health Impacts. In *Encyclopedia of Environmental Health*, 415–435 (Elsevier, 2019).
4. Panda, C., Pujary, A., Pradhan, N., Jena, T. & Mallick, S. K. Environmental impact evaluation of a proposed steel and cement complex in the waste management scenario. *Environ. Qual. Mgmt.* **32**, 139–149 (2022).
5. Tauqeer, H. M. et al. Environmental concerns associated with explosives (HMX, TNT, and RDX), heavy metals and metalloids from shooting range soils: prevailing issues, leading management practices, and future perspectives. In *Handbook of Bioremediation*, 569–590 (Elsevier, 2021).
6. Sharma, P. et al. Role of microbes in bioaccumulation of heavy metals in municipal solid waste: impacts on plant and human being. *Environ. Pollut.* **305**, 119248 (2022).
7. Saleh, H. M. et al. Potential of the submerged plant *Myriophyllum spicatum* for treatment of aquatic environments contaminated with stable or radioactive cobalt and cesium. *Prog. Nucl. Energy*. **118**, 103147 (2020).
8. Kumar, V. & Dwivedi, S. K. A review on accessible techniques for removal of hexavalent chromium and divalent nickel from industrial wastewater: recent research and future outlook. *J. Clean. Prod.* **295**, 126229 (2021).
9. Mahey, S., Kumar, R., Sharma, M., Kumar, V. & Bhardwaj, R. A critical review on toxicity of cobalt and its bioremediation strategies. *SN Appl. Sci.* **2**, 1279 (2020).
10. Tahir, M. U. et al. Modeling and evaluation of nickel manganese cobalt based Li-ion storage for stationary applications. *J. Energy Storage*. **36**, 102346 (2021).
11. Yao, Z., Li, J., Xie, H. & Yu, C. Review on Remediation technologies of Soil contaminated by Heavy metals. *Procedia Environ. Sci.* **16**, 722–729 (2012).
12. Genchi, G., Carocci, A., Lauria, G., Sinicropi, M. S. & Catalano, A. Nickel: Human Health and Environmental Toxicology. *IJERPH* **17**, 679 (2020).
13. Abdelwaheb, M., Jebali, K., Dhaouadi, H. & Dridi-Dhaouadi, S. Adsorption of nitrate, phosphate, nickel and lead on soils: risk of groundwater contamination. *Ecotoxicol. Environ. Saf.* **179**, 182–187 (2019).
14. Pourret, O. & Faucon, M. P. Cobalt. In *Encyclopedia of Geochemistry* (ed. White, W. M.), 1–3 (Springer International Publishing, 2016).
15. Abbas, M. N., Al-Tameemi, I. M., Hasan, M. B. & Al-Madhhachi A.-S. T. Chemical removal of cobalt and lithium in contaminated soils using promoted white eggshells with different catalysts. *S. Afr. J. Chem. Eng.* **35**, 23–32 (2021).
16. Kosiorek, M. & Wyszkowski, M. Remediation of cobalt-contaminated soil using manure, Clay, Charcoal, Zeolite, Calcium Oxide, Main Crop (*Hordeum vulgare* L.), and after-crop (*Synapis Alba* L). *Minerals* **10**, 429 (2020).

17. Buxton, S. et al. *Concise Rev. Nickel Hum. Health Toxicol. Ecotoxicol. Inorganics* **7**, 89 (2019).
18. Shahzad, B. et al. Nickel; whether toxic or essential for plants and environment - A review. *Plant Physiol. Biochem.* **132**, 641–651 (2018).
19. Kumar, A. et al. Nickel in terrestrial biota: Comprehensive review on contamination, toxicity, tolerance and its remediation approaches. *Chemosphere* **275**, 129996 (2021).
20. Harasim, P., Filipek, T. & Harasim, P. Nickel in the environment. *J. Elem.* (2015).
21. Kloosterman, T. G., Van Der Kooi-Pol, M. M. & Bijlsma, J. J. E. Kuipers O. P. The novel transcriptional regulator SczA mediates protection against Zn^{2+} stress by activation of the Zn^{2+}-resistance gene czcD in *Streptococcus pneumoniae*. *Mol. Microbiol.* **65**, 1049–1063 (2007).
22. Bolisetty, S., Peydayesh, M. & Mezzenga, R. Sustainable technologies for water purification from heavy metals: review and analysis. *Chem. Soc. Rev.* **48**, 463–487 (2019).
23. Qasem, N. A. A., Mohammed, R. H. & Lawal, D. U. Removal of heavy metal ions from wastewater: a comprehensive and critical review. *npj Clean. Water*. **4**, 36 (2021).
24. Saleh, T. A., Mustaqeem, M. & Khaled, M. Water treatment technologies in removing heavy metal ions from wastewater: a review. *Environ. Nanatechnol. Monit. Manage.* **17**, 100617 (2022).
25. Vardhan, K. H., Kumar, P. S. & Panda, R. C. A review on heavy metal pollution, toxicity and remedial measures: current trends and future perspectives. *J. Mol. Liq.* **290**, 111197 (2019).
26. Wang, P. Isolation and characterization of heavy metal-resistant bacterias capable of removing cr(VI). *Pol. J. Environ. Stud.* **24**, 339–345 (2015).
27. Margaryan, A. Diversity and application of heavy-metal resistant microbes. in Microbes in Microbial Communities (eds Singh, R. P., Manchanda, G., Bhattacharjee, K. & Panosyan, H.) 153–174 (Springer Singapore, 2021).
28. Joutey, N. T., Sayel, H., Bahafid, W. & El Ghachtouli, N. Mechanisms of hexavalent chromium resistance and removal by microorganisms. in Reviews of Environmental Contamination and Toxicology Volume 233 (ed Whitacre, D. M.) vol. 233 45–69 (Springer International Publishing, Cham, (2015).
29. Mishra, S. & Bharagava, R. N. Toxic and genotoxic effects of hexavalent chromium in environment and its bioremediation strategies. *J. Environ. Sci. Health Part. C.* **34**, 1–32 (2016).
30. Bukhari, D. A. & Rehman, A. Metal-resistant bacteria as a green bioresource for arsenic remediation in wastewaters. *Curr. Opin. Green. Sustainable Chem.* **40**, 100785 (2023).
31. Gneush, A., Zholobova, I., Petenko, A., Gorkovenko, N. & Yurina, N. The technology of producing biohumus and the study of its qualitative indicators. *KLS* (2021).
32. Paranjak, R. P., Gutyj, B. V., Lytvyn, N. A. & Didorenko Yu. O. problems of environmental protection as an aspect of military confrontation. *SMLNUVMBA* **25**, 200–207 (2023).
33. Goncharenko, A. V. & Sipakov, R. V. The formation of atmospheric air pollution emissions from separate explosions as a result of military operations. *esanr* **45**, 111–120 (2023).
34. Stelmakh, V., Melniichuk, M., Melnyk, O. & Tokarchuk, I. Hydro-ecological state of Ukrainian Water bodies under the influence of military actions. *Rocznik Ochrona Środowiska*. **25**, 174–187 (2023).
35. Drobitko, A. & Alakbarov, A. Soil restoration after mine clearance. *Int. J. Environ. Stud.* **80**, 394–398 (2023).
36. Pereira, P., Bašić, F., Bogunovic, I. & Barcelo, D. Russian-ukrainian war impacts the total environment. *Sci. Total Environ.* **837**, 155865 (2022).
37. Petrushka, K. & Petrushka, I. Influence of heavy metals oxides on the pollution of the soil environment as a consequence of military actions. *EP* **8**, 87–93 (2023).
38. Biyashev, B., Drobitko, A., Markova, N., Bondar, A. & Pismenniy, O. Chemical analysis of the state of Ukrainian soils in the combat zone. *Int. J. Environ. Stud.*, 1–9 (2023).
39. Trokhymenko, G. et al. Assessment of iron and heavy metals accumulation in the soils of the combat zone. *EEJET* **5**, 6–16 (2023).
40. Havryliuk, O. A. et al. Bioremoval of hazardous cobalt, nickel, chromium, copper and cadmium compounds from contaminated soil by *Nicotiana tabacum* plants and associated microbiome. *Biosys Divers.* **29**, 88–93 (2021).
41. Havryliuk, O. et al. Bioremediation of copper- and chromium-contaminated soils using *Agrostis capillaris* L., *Festuca pratensis* Huds., and *Poa pratensis* L. mixture of lawn grasses. *Land* **11**, 623 (2022).
42. Rahman, Z. An overview on heavy metal resistant microorganisms for simultaneous treatment of multiple chemical pollutants at co-contaminated sites, and their multipurpose application. *J. Hazard. Mater.* **396**, 122682 (2020).
43. Sridevi, V., Raghuram, M. & Ravisankar, D. Isolation and screening of Heavy Metal resistant microorganisms from Industrial Soil. *J. Pure Appl. Microbiol.* **12**, 1667–1674 (2018).
44. Kanekar, P. P., Kanekar, S. P. & Metallophilic Metal-Resistant, and metal-tolerant microorganisms. In Diversity and Biotechnology of Extremophilic Microorganisms from India (eds Kanekar, P. P. & Kanekar, S. P.) 187–213 (Springer Nature Singapore, Singapore, (2022).
45. Bonilla, J. O., Callegari, E. A., Paez, M. D., Gil, R. A. & Villegas, L. B. Characterization of copper stress response in Fusarium tricinctum M6: a metal-resistant microorganism isolated from an acid mine drainage-affected environment. *J. Hazard. Mater.* **412**, 125216 (2021).
46. Papale, M. et al. Heavy-metal resistant microorganisms in sediments from submarine canyons and the adjacent continental slope in the northeastern Ligurian margin (Western Mediterranean Sea). *Prog. Oceanogr.* **168**, 155–168 (2018).
47. Bonilla, J. O. et al. Characterization of copper stress response in Fusarium Tricinctum M6: a metal-resistant microorganism isolated from an Acid Mine drainage-affected environment. *J. Hazard. Mater.* **412**, 125216 (2021).
48. Cai, X. et al. Microbial characterization of heavy metal resistant bacterial strains isolated from an electroplating wastewater treatment plant. *Ecotoxicol. Environ. Saf.* **181**, 472–480 (2019).
49. Chanda, D., Sharma, G. D., Jha, D. K. & Hijri, M. Tolerance of microorganisms in Soil contaminated with Trace metals: an overview. in Recent Advances in Applied Microbiology (ed Shukla, P.) 165–193 (Springer Singapore, Singapore, (2017).
50. Rosenfeld, C. E., James, B. R. & Santelli, C. M. Persistent bacterial and Fungal Community shifts exhibited in selenium-contaminated reclaimed mine soils. *Appl. Environ. Microbiol.* **84**, e01394–e01318 (2018).
51. Saravanan, A., Kumar, P. S., Ramesh, B. & Srinivasan, S. Removal of toxic heavy metals using genetically engineered microbes: molecular tools, risk assessment and management strategies. *Chemosphere* **298**, 134341 (2022).
52. Maqsood, Q., Hussain, N., Mumtaz, M., Bilal, M. & Iqbal, H. M. N. Novel strategies and advancement in reducing heavy metals from the contaminated environment. *Arch. Microbiol.* **204**, 478 (2022).
53. Zehra, A., Meena, M., Swapnil, P., Raytekar, N. A. & Upadhyay, R. S. Sustainable approaches to remove Heavy metals from Water. in Microbial Biotechnology: Basic Research and Applications (eds Singh, J., Vyas, A., Wang, S. & Prasad, R.) 127–146 (Springer Singapore, Singapore, (2020).
54. Verma, S. & Kuila, A. Bioremediation of heavy metals by microbial process. *Environ. Technol. Innov.* **14**, 100369 (2019).
55. Ayangbenro, A. & Babalola, O. A New Strategy for Heavy Metal Polluted environments: a review of Microbial Biosorbents. *IJERPH* **14**, 94 (2017).
56. International Union of Soil Sciences (IUSS) & Working Group, I. U. S. S. WRB. International soil classification system for naming soils and creating legends for soil maps. 4th edition. *World Reference Base for Soil Resources* (2022).
57. Tashyrev, O. & Prekrasna, I. Express Method for Redox Potential and pH measuring in microbial cultures. *Int. J. Bioautomation*. **18**, 217–230 (2014).

58. Havryliuk, O. A. et al. Metal-resistant microorganisms of tap water: theoretical justification and biotechnological application. *Probl. Ecol. Biotechnol.* 1–2 (2021).
59. Soni, P., Pradhan, P. K. & Sood, N. Development, characterization and in vitro applications of a thymus cell line from *Pangasianodon Hypophthalmus* (Sauvage 1878). *Indian J. Anim. Sci.* **93** (2023).

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Author contributions
O.T. and E.M. developed the concept and the set up of the experiment; I.B., E.M., K.M., A.Ś., D.P., O.H., V.H., and O.T. conducted the experimental work; O.T., V.H., I.B., and E.M. conducted data analysis; I.B. and V.H. prepared the manuscript with help from all authors; E.M. and O.T.edited the manuscript. All authors reviewed the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

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