

Compost and Beneficial *Pseudomonas* Populations Promote Enzyme Activity, Amino Acids, and Polymers Utilization Patterns in Heavy Metal Contaminated Soils

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Abstract

PGPR-assisted phytoremediation is a biotechnology offering opportunities to improve the state of contaminated soil ecosystems. The present study has investigated the potential of six beneficial isolates tolerant to Cd, Pb, and Zn and identified through 16S rDNA sequential analysis as *Bacillus cereus*, *Pseudomonas putida*, and *Pseudomonas fluorescens* to stimulate spinach growth and soil microbial community development in heavy metal-contaminated soil with or without the addition of compost. We found improved plant growth in all treatments with compost, but especially applying consortiums of isolates. Microbial enzyme activities and basal soil respiration were significantly higher in the rhizosphere and in amended treatments than in non-rhizosphere and control. The microbial community-level physiological profiles in the rhizosphere studied with the Ecoplates® of Biolog™ system showed increased metabolic potential in treatments with compost and beneficial bacteria. Principal component analysis revealed domination of amino acids and polymers utilizing communities in compost and bacterial consortium amended soils, carbohydrates and amino acids in compost and single strains application, and the lowest representation of amines and amides and phenolic compounds utilizers. Thus, may conclude that the use of quality organic amendments together with beneficial soil bacterial populations is a suitable and promising approach for the phytostabilization of soils contaminated with heavy metals.

Keywords: compost; spinach; PGPR; CLPP; soil enzymes; heavy metals

Резюме

PGPR – асистираната фиторемедиация е биотехнология, която предлага възможности за подобряване на състоянието на замърсените почвени екосистеми. Настоящото проучване изследва потенциала на шест полезни изолата, толерантни към Cd, Pb и Zn и идентифицирани чрез 16S rDNA анализ като *Bacillus cereus*, *Pseudomonas putida* и *Pseudomonas fluorescens*, за стимулиране на растежа на спанак и развитието на почвените микробни съобщества в замърсена с тежки метали почва със или без добавяне на компост. Установихме подобрен растеж на растенията при всички варианти с внесен компост, особено при прилагане на консорциуми от изолати. Микробните ензимни активности и базовото почвено дишане бяха значително по-високи в ризосферата и във вариантите с добавки в сравнение с неризосферата и контролния вариант. Физиологичните профили на ниво микробни съобщества в ризосферата, изследвани със системата Ecoplates® на Biolog™, показаха повишен метаболитен потенциал при третиране с компост и полезни бактерии. Анализът на основните компоненти разкри доминиране на съобществата използващи аминокиселини и полимери във вариантите с компост и бактериални консорциуми. Съобществата, усвояващи въглехидрати и аминокиселини имаха преимущество при използване на компост и самостоятелни щамове, докато най-слабо беше наличието на съобщества, усвояващи амините, амидите и фенолните съединения. В тази връзка можем да заключим, че използването на качествени органични добавки заедно с полезни почвени бактериални популации е подходящ и обещаващ подход за фитостабилизиране на почви, замърсени с тежки метали.

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Introduction

Soils contaminated with heavy metals are often found in contemporary landscapes due to naturally occurring processes or human activities. Agricultural lands also suffer heavy metal contamination that may cause food and feed safety uncertainties (Singh *et al.*, 2011). Diverse bio/phytoremediation research strategies were applied to improve soil properties and alleviate the harmful heavy metals' effects on plants and soil biota (Kidd *et al.*, 2009). Besides, the problem is multi-component and needs complex tools to protect the environment and mitigate the toxic effects on living beings.

Composting is an environmentally friendly solution to convert agricultural and garden biowastes into valuable products desirable as a soil amendment in crop growing and landscaping (Shilev *et al.*, 2007). Incorporating good quality compost in heavy metal contaminated soil has different positive effects on soil and plants, such as increment of organic content, regulation of physicochemical properties, and improvement of plant nutrition, among others (Anwar *et al.*, 2017; Zafar-ul-Hye *et al.*, 2020). Researchers also reported the influence of organic content on heavy metals' availability in the soils as an essential condition to manage their toxicity (Lacalle *et al.*, 2023). In this regard, authors found a significant decrease if the Zn and Cu bioavailability in soil amended with activated carbon compost or rhamnolipid compost. Thus, the shoot concentration of pakchoi decreased considerably (Sun *et al.*, 2023). On the other hand, Tang and co-authors reported the ambiguous effect of organic amendments (compost, biochar, and a combination of them) expressed as a significant decrease of Cd and Zn availability in soils but a slight increase of As and Cu (Tang *et al.*, 2020).

Plant-associated microbial communities are crucial for plant nutrition and health (Caldwell, 2005; Kidd *et al.*, 2009). Each population occupied a critical niche vital for the community's survival (Falkowski *et al.*, 2008). Heavy metals are known to affect soil microorganisms in general negatively and their enzyme activity in particular (Hinojosa *et al.*, 2003; Hinojosa *et al.*, 2004). Despite this, compost incorporation mitigates most of the negative consequences of shaping microbial community structure by organic supplements and introducing allochthonous communities. Combined with the root exudates contribution, the system functioning is completed, albeit quite complex. Lin and collaborators (2021) suggested that heavy metal pollution reduces plant growth and alters soil microbial com-

munities. Growing *Trifolium repens* L. could mitigate the toxic effect of pollution by changing microbial community structure. The secondary consequences included increased availability of N, P, and K, but also stimulated enzyme activities. Like other authors, they concluded that root-associated communities might regulate plant growth and elemental uptake (Shilev, 2013; Lin *et al.*, 2021).

The described above is very important because the adapted and well-developed microbial communities are at the heart of soil health, playing a crucial role in organic decomposition and humus formation, biochemical cycling, and nutrient availability (Shilev *et al.*, 2019). Researchers have been informed about the controversial effects of organic amendments on enzyme activities. They found increased activity by adding compost and inhibited enzymes with biochar. However, diverse effects were observed when combinations of both were applied. Thus, the bioavailability of zinc, arsenic, cadmium, and copper influenced the activity of dehydrogenase, catalase, and urease (Tang *et al.*, 2020). In other cases, increased soil Pb and different toxic elements concentrations decreased urease activity and raised β -glucosidase, while dehydrogenase activity was unaffected. The incorporation of organic amendments was not unambiguous, showing increased dehydrogenase activity with compost and β -glucosidase with biochar but a reduced abundance of microbial communities in both cases (Kaurin *et al.*, 2018). As the development of microbial communities is mainly related to the availability of nutrients, the patterns of substrate utilization are characteristic of them and reflect changes in the soil. In this regard, in the study of biodegradation of organic contaminants, Koner *et al.* (2022) observed a positive correlation among L-arginine, phenylethylamine, and γ -hydroxybutyric acid utilization patterns and chloroalkane and chloroalkene degradation pathways. In another study, compost and biochar had controversial effects regarding community-level physiological profiling (CLPP) in trace elements contaminated soils. Researchers found that biochar increased the metabolic potential of microbial communities the most, with a higher impact on carboxylic acids and polymers. These amendments also strongly stimulated the soil dehydrogenase, β -glucosidase, and urease activities (Abou Jaoude *et al.*, 2020). Plants play an essential role in modeling microbial communities. Thus, *Cynara cardunculus* and *Brassica juncea* plants were found to increase significantly microbial functional diversity in metal-contaminated soils and sub-

strate utilization. Similarly, *Festuca arundinacea* shaped the substrate preferences of soil microbial communities, stimulating some utilized carbohydrates and decreasing those of the carboxylic acids (Wasilkowski *et al.*, 2019).

From the literature review is clear that there are many factors affecting plant growth and ecosystem functioning in heavy metal-contaminated soil. The consequences of organic amendment incorporation and its interrelations with soil and microbial properties are poorly understood because of the diverse amendment species. In addition, plant species and the introduction of external populations are new variables in the puzzle soil-microbe-plant system. In the present study, we set out to elucidate how the combination of compost and selected bacterial populations supports the growth of spinach plants and soil microbial communities in soil contaminated with heavy metals.

Materials and Methods

Isolation and characterization of plant growth-promoting bacteria

Previously we isolated bacteria from the rhizosphere of the following native plant species: *Lamium amplexycoule* (Lamiaceae), *Capsella bursa pastoris* (Brassicaceae), *Geranium molle* (Geraniaceae), *Valerianella* sp. (Valerianaceae), and *Cirsium* sp. (Asteraceae), from the contaminated area of KCM – Plovdiv. The isolates were selected based on their tolerance to Cd, Pb, Zn, and their ability to solubilize phosphates and produce indole acetic acid and siderophores using proven methods.

Molecular identification of the isolates by 16 rRNA sequence analysis

The six selected isolates were identified based on 16S rRNA sequencing analysis using standard methods of bacterial growth, DNA isolation, and quality assurance. The species identity of the iso-

lated bacteria was determined by PCR amplification of the 16S rRNA gene and sequencing of the PCR products. 1492R (ACCTTGTTACGACTT) and 27F (AGAGTTT-GATCMTGGCTCAG) were used to sequence part of the bacterial genome (Yuichi *et al.*, 2003).

Samples were sent for sequencing to Microsynth AG, Switzerland. The resulting sequences were analyzed with BLAST (www.ncbi.nlm.nih.gov/BLAST/), ClustalW (www.ebi.ac.uk/clustalw), and BioEdit software products and compared to nucleotide sequences in the Genbank database (www.ncbi.nlm.nih.gov). Evolutionary history was prepared using the maximum likelihood method based on the total time model (Yuichi *et al.*, 2003). The analysis includes six nucleotide sequences. There was a total of 242 positions in the final data set (Neip *et al.*, 2020).

Plant growth experiment

Heavy metal-contaminated soil was taken from a polluted region. Air contamination was spread decades ago from the non-ferrous metal's smelter in the Plovdiv region. Biovet Peshtera AD supplied the compost. It was produced by plant residues and fungal mycelium from the biological production of feed additives. Soil and compost characteristics are presented in Table 1. The soil and compost were mixed in a 75:25 ratio (v:v) and left for stabilization for 15 days. Ten treatments were arranged as presented in Table 2. Each treatment consisted of four 3000 cm³ pots. Commercially available spinach seeds (*Spinacea oleracea* L.) from a variety of Matador were sown in each pot. After germination, four plants per pot were left. Before sowing, some seeds were left for 2 h in Petri dishes with corresponding bacterial suspension in 0.85% NaCl solution with a cell density of 1 x 10⁵ colony-forming units (cfu) per milliliter.

Table 1. Elemental content in contaminated soil and compost on a dry-weight basis

Parameter	Unit	Compost	Contaminated soil	MPC	IC
Nitrogen – available	mg.kg ⁻¹	603.32 ± 12.08	16.52 ± 0.70	-	-
Phosphorus – available	mg.kg ⁻¹	2511.16 ± 69.16	31.71 ± 1.21	-	-
Total nitrogen	g.kg ⁻¹	22.34 ± 0.91	1.34 ± 0.10	-	-
Total phosphorus	g.kg ⁻¹	9.11 ± 0.14	0.35 ± 0.02	-	-
Organic carbon	g.kg ⁻¹	322.74 ± 11.14	9.81 ± 0.38	-	-
Cadmium	mg.kg ⁻¹	0	21.31 ± 1.61	3.0	12
Lead	mg.kg ⁻¹	0.75 ± 0.08	519.12 ± 18.19	120	500
Zinc	mg.kg ⁻¹	26.11 ± 1.93	424.19 ± 31.32	400	900

Data shows mean and standard deviation (n=3). MPC – maximum permissible concentration at pH >7.4; IC – intervention concentrations

Each isolate was grown separately in a liquid nutrient medium till the late logarithmic phase. The cells were centrifuged ($3500 \times g$, 4°C , 12 min), and the pellets were washed first in MgCl_2 solution, followed by washing in distilled water. Two weeks after plant germination, they were watered with bacterial isolate suspension at a concentration of 10^7 cfu ml^{-1} . The treatments without isolates were watered with 0.85% without bacteria. Consortia were prepared by mixing the corresponding isolates (Table 2). In parallel, the pots were duplicated, repeating all ten treatments with the addition of compost and bacterial isolates, but no seeds were sown. These pots were used to study the effect of spinach roots on microbial activity. The experiment was carried out in September for 60 days in an open greenhouse, maintaining water content at 55-60% of WHC.

Table 2. Treatments used in the study (C – compost).

Number	Treatments
1	Soil
2	Soil + C + strains 32', 32B, 41', 41B
3	Soil + C + strains 32', 32B, 41', 44'
4	Soil + C + strains 32', 32B, 44', 44B
5	Soil + C + strain 32'
6	Soil + C + strain 32B
7	Soil + C + strain 41'
8	Soil + C + strain 41B
9	Soil + C + strain 44'
10	Soil + C

Determination of heavy metals in plant tissue

The heavy metal concentration in spinach plants was determined through wet mineralization (Lozano-Rodríguez *et al.*, 1995). The measurements were performed by Agilent 4210 MP-AES.

Microbiological characteristics

The enzyme dehydrogenase was assessed through the method described by Alef (1995). The activity of β -glucosidase was estimated using the method of Tabatabai (Tabatabai, 1982) modified by Alef and Nannipieri (Alef and Nannipieri, 1995). In addition, basal soil respiration was carried out through the method described by Alef (1995).

Community-level physiological profiling using BiologTM Ecoplates[®]

The soil microbial communities' functional diversity was assessed by Ecoplate[®], Biolog Inc., CA, USA (Garland and Mills, 1991). The inoculation of Ecoplates was performed with 150 μl

followed by incubation at 25°C . The absorbance of each well was measured on BiologTM MicroStation[®] reader at 590 nm and 750 nm wavelength on 24 h basis for 120 h. The obtained data were normalized, and the carbon sources were grouped by their nature as amino acids (AA), amines and amides (A), polymers (P), carbohydrates (CH), carboxylic acids (CA), and phenolic compounds (PC) (Insam and Goberna, 2008).

The average well color development (AWCD) was assessed every 24 hours through the following formula:

$$\text{AWCD} = \sum (C - B) / n$$

where C is the optical density (OD) in the well, B is the blank well reading, and n is the total number of carbon sources. The AWCD represents the overall community performance through the diverse carbon-source utilization capacity.

To continue the abovementioned, we applied Shannon (H') and Pielou evenness (E) indexes to estimate community homogeneity description. The Shannon diversity index was calculated as:

$$H' = - \sum \left[\left(\frac{n}{N} \right) \times \left(\frac{\ln n}{N} \right) \right]$$

where H' is the Shannon diversity index, and n is the number of wells with a positive reading ($\text{OD} > 0.25$) to the total number of carbon sources.

The Pielou evenness index (E) comes from the Shannon index and represents a ratio of H' to the value of the substrate richness (S, number of wells with OD over 0.25):

$$E = \frac{H'}{\ln S};$$

Statistical data processing

The data processed were subjected to analysis of variance (ANOVA) using Statistics v.18 for Windows. The least significant difference (LSD) at $p \leq 0.05$ and $p \leq 0.01$ was applied to distinguish the mean of treatments. Comparative hierarchical cluster analysis using the method of Euclidean distance between groups is applied. Results are presented after calculating the mean and standard deviation using Microsoft Excel v.14. The correlations between the accumulation of heavy metals (Pb, Cd, and Zn), dry weight of spinach plants, β -glucosidase activity, dehydrogenase activity, and soil respiration were calculated based on the average values of the traits.

Results and Discussion

As a result of the initial steps of isolation and selection of rhizosphere isolates, six strains were

Table 3. Match of isolates to the registered sequences and numbers in the NCBI Genbank, and their correspondence to species identification and probability

Isolate	Sequences	Latin name	Numbers
41'	SUB11494244 seq1	<i>B. cereus</i>	ON557708
32'	SUB11494244 seq2	<i>P. putida</i>	ON557709
44'	SUB11494244 seq3	<i>P. putida</i>	ON557710
32B	SUB11494244 seq4	<i>P. fluorescens</i>	ON557711
44B	SUB11494244 seq5	<i>P. fluorescens</i>	ON557712
41B	SUB11494244 seq6	<i>P. fluorescens</i>	ON557713

selected to proceed with the molecular identification (specific results are not presented).

Molecular identification of isolates

The identification of all six isolates was successful. The nucleotide sequences of the isolates were registered in the NCBI gene bank under the corresponding numbers (Table 3).

One strain is from the genera *Bacillus* sp. (*B. cereus*), and five are from *Pseudomonas* sp. – two *P. putida* and three *P. fluorescens*. Thus, according to evolutionary history (Fig. 1), two groups are formed from the isolates. One is represented by *B. cereus*, while the second belongs to the species from genera *Pseudomonas*. The last groups possessed subgroups originating from corresponding *P. putida* and *P. fluorescens* isolates.

Contaminated soil and compost content

The contaminated soil used in the present study is characterized by a relatively low content of nutrients and total organic carbon. In contrast, the concentration of presented heavy metals is high according to the Bulgarian Regulation on standards for maximum permissible content of harmful substances in arable soil medium (Council of Ministers, 2008). Cadmium concentration is determined as 21.31 mg.kg⁻¹, but the maximum permissible

concentration (MPC) is 3.0 mg.kg⁻¹, while the intervention concentration is 12.0 mg.kg⁻¹. In the case of Pb, the concentrations are as follows: 519.12 mg.kg⁻¹, 120.0 mg.kg⁻¹, 500 mg.kg⁻¹. This means that the regulatory framework requires intervention for both elements to reduce their concentration in the soil. In addition, the concentration of Zn was above the MPC of 400 mg.kg⁻¹ but below the IC of 900 mg.kg⁻¹. The above shows the urgent need for intervention activities to reduce the concentration of heavy metals in the soil. On the other hand, the compost was very rich in nutrients, while the organic carbon accounts for 32.27%. It showed a very low presence of Pb and Zn (Table 1).

Plant biomass and heavy metal accumulation

The amendments (compost and isolates) affected the biomass of spinach plants grown in contaminated soil (Fig. 2A). The introduction of compost did not lead to changes in the formed biomass of spinach, while the use of the bacterial isolates had significant consequences.

The greatest biomass was formed when applying the consortia of T-3 and T-4, which was 103.6% and 111.1%, respectively, greater than the control. A similar difference was observed with T-10, where the compost was applied without bene-

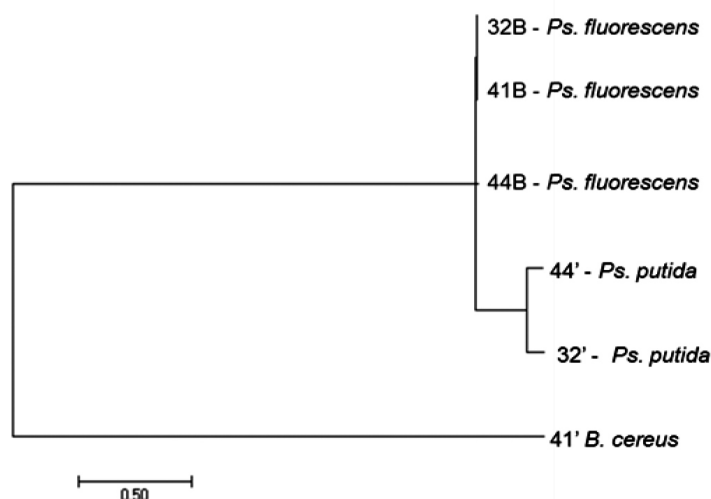


Fig. 1. Molecular phylogenetic analysis by the maximum likelihood method

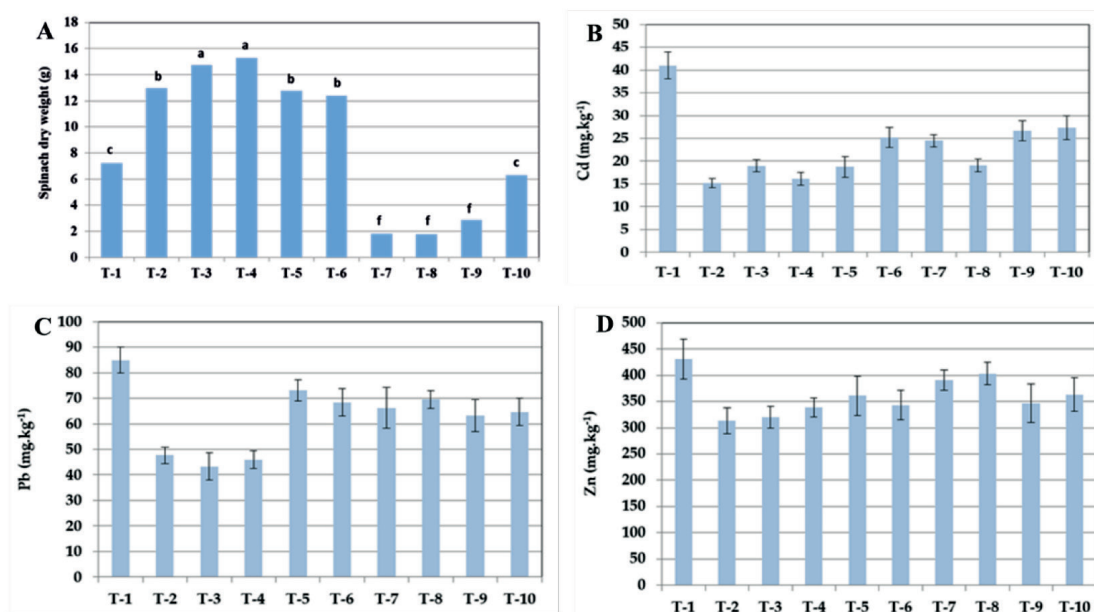


Fig. 2. Shoots dry weight of spinach plants (A); accumulation of heavy metals in plant shoots: Cd (B); Pb (C); Zn (D). The results showed the meaning of three replicates and standard deviation. Different letters of plant biomass results indicate statistically significant differences between treatments according to the LSD test ($p \leq 0.05$).

ficial bacterial populations. The next group of treatments in terms of biomass formed was T-2, T-5, and T-6, where the values were lower than T-2 and T-3, but significantly higher than the control. At the other extreme are treatments T-7, T-8, and T-9 with significantly reduced dry biomass compared to the control, by 74.97%, 75.4%, and 60.64%, respectively.

High concentrations of Cd were found in the shoot biomass of spinach in the control treatment (Fig. 2B), 40.95 mg.kg^{-1} . The remaining treatments showed significantly lower values. In that sense, Xiong and collaborators (2023) reported increasing heavy metals immobilization with the increment of compost dose in soil. They found lower availability in the rhizosphere than in bulk soil. We also suggested that this is one reason for decreasing metal accumulation when adding compost. Other researchers suggested that immobilization is due to the formation of complexes, absorption, and so on (Ye *et al.*, 2017). The accumulation in treatments T-6, T-7, T-9, and T-10 plants was similar within concentrations of $24.50 - 27.30 \text{ mg.kg}^{-1}$. The lowest accumulation of Cd was found in treatment T-2 with a concentration of 15.19 mg.kg^{-1} . In addition, accumulated Pb concentrations followed the same trend, with the highest value in the control of 59.43 mg.kg^{-1} and the lowest in treatments T-2, T-3, and T-4 (Fig. 2C). Zafar-ul-Hye *et al.* (2020) found a significant decrease in Pb accumulation in spinach plants applying compost and biochar with different bacterial populations of *B. amyloliquefaciens*. In contrast, researchers reported higher bioaccumu-

lation of metals such as Zn, Pb, and Cr, after soil amendment with municipal green-waste compost (Medynska-Juraszek *et al.*, 2020) recommending avoiding the consumption of such leafy vegetables. The inoculation of *A. faecalis* and *B. amyloliquefaciens* significantly increased spinach biomass formation. Treatments T-5 to T-10 showed similar values of accumulated concentrations. The highest concentrations of the studied elements were found for Zn (Fig. 2D), but there was not such a strong stratification by treatments as for the other two elements. In control, the concentration was $301.49 \text{ mg.kg}^{-1}$, while the lowest was found in T-2, $219.24 \text{ mg.kg}^{-1}$.

Soil enzymes and microbial activity

The study of β -glucosidase activity showed higher activity in rhizosphere soil than in non-rhizosphere soil (Figs. 3A and 3B). However, the difference between the two soil types was smaller than in the case of dehydrogenase activity, 70-75%. Incorporating the consortia into T-2, T-3, and T-4 increased activity compared to the remaining treatments with bacterial populations that were more pronounced in the presence of plants. As with dehydrogenase activity, a reduction in activity was found at T-5, T-6, and T-9 in the non-rhizosphere soil.

The dehydrogenase activity in the non-rhizosphere and rhizosphere of spinach plants was studied in all treatments three times during the vegetation (Fig. 3C and 3D). In both areas, the activity of these enzymes increased throughout the experi-

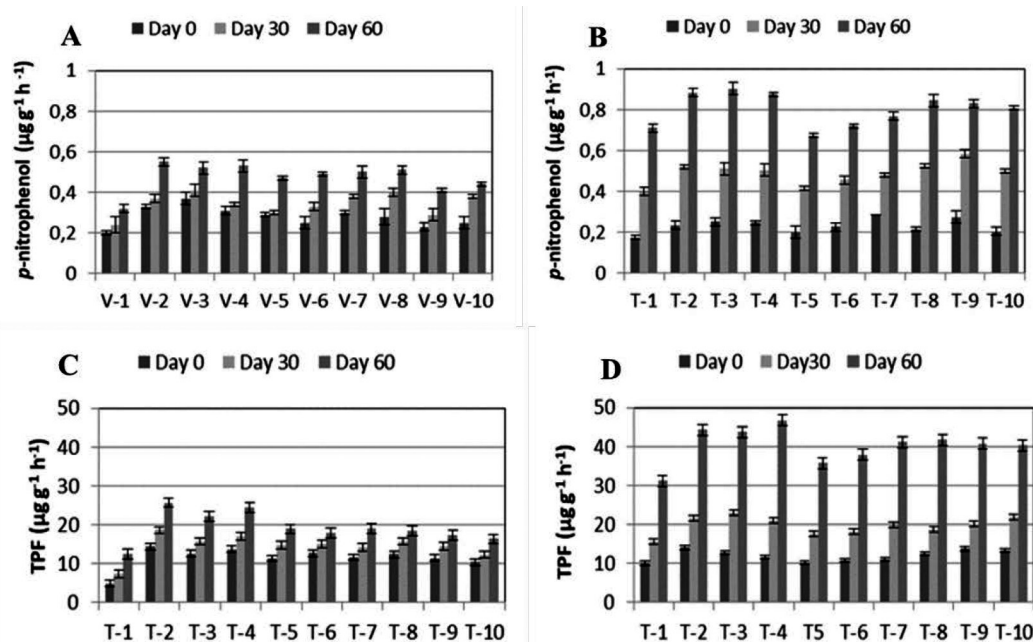


Fig. 3. Enzyme activity in non-rhizosphere and rhizosphere soil of spinach expressed as $\mu\text{g g}^{-1} \text{soil h}^{-1}$. (A) dehydrogenase in the non-rhizosphere; (B) dehydrogenase in the rhizosphere; (C) β -glucosidase in the non-rhizosphere; (D) β -glucosidase in the rhizosphere.

The data show the average of three repetitions and the standard deviation.

ment, with the highest being at the end of day 60. In the non-rhizosphere, the values were lower than in the rhizosphere, and as a rule, in control (T-1), the enzyme activity was lower than in the treatments with compost. Similarly, Wasilkowski *et al.* (2019) established a positive effect of compost on the enzymes dehydrogenases, urease, phosphatases, total enzyme activity, and microbial functional capacity in heavy metal-contaminated soil. Composts considerably affected soil enzyme activities, such as hydrolase, phosphomonoesterase, β -glucosidase, and urease, while the AWCD and Shannon index didn't change significantly (Scotti *et al.*, 2016). In the treatments with plants, the dehydrogenase activity increased since the beginning of the study, with the most significant increase observed after day 30, when the aboveground biomass was forming. The presence of the plants significantly affected the activity of this enzyme, which was particularly pronounced in control, where the increase in the rhizosphere was 1.5 fold (Fig. 3B). The most significant increase in dehydrogenase activity was observed in the second, third, and fourth treatments, both in the rhizosphere and the non-rhizosphere. In contrast, the activity was slightly reduced in the rhizosphere in T-5 and T-6. Lacalle and collaborators examined only microbial parameters of the rhizosphere in agricultural soil but in mine soil compared to non-rhizosphere. Highly increased microbial biomass and activity (dehydrogenase and urease) were found in the rhizosphere of compost-amended contaminat-

ed soil, which indicated the promotional effects on microbial communities. They observed the highest rise of pyruvic acid methyl ester, L- α -glycerol phosphate, glucose-1-phosphate, and D, L- α -glycerol phosphate utilization (Lacalle *et al.*, 2023).

The differences in the microbial activity in the rhizosphere and non-rhizosphere were confirmed (Fig. 4). Thus, spinach roots affected the soil microbial community's activity. This was evident in the control (without compost), which increased by 161.54%. The trend was similar in the treatments with amendments. In T-10, the compost studied alone contributed by 135% to the increase in released dioxide, while the presence of consortium in T-2 in the rhizosphere increased microbial activity. Higher concentration of released CO₂ was found in T-2, T-3, T-4, and T-9, while lower concentration we noted in T-5, T-6, and T-7. This was similar to the abovementioned enzyme activities.

Thus, the total rate of microbial activity was due to, on one hand, the effect of the root, while on the other hand, the soil amendments (compost and bacterial populations). Similar results were observed by other investigators, reporting increased basal respiration in most of the amended soils (Galende *et al.*, 2014). They reported a lack of substantial modification of microbial functional diversity reflected by Biolog EcoPlates™ data. In addition, Lacalle and coauthors emphasized the beneficial effect of plant roots on microbial communities in metal-contaminated soils (Lacalle *et al.*, 2023).

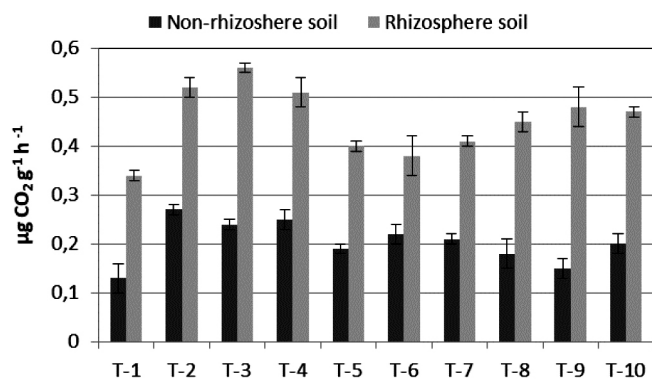


Fig. 4. Basal soil respiration in non-rhizosphere and rhizosphere soil, expressed as mg CO₂ per kg⁻¹ h⁻¹ soil. The data show the average of three repetitions and the standard deviation.

Correlation between plant biomass, heavy metal accumulation, and soil microbial activities

In the present study, the dry weight of spinach plants, β-glucosidase activity, dehydrogenase activity, and basal soil respiration in the rhizosphere were affected by the presented soil heavy metals (Pb, Cd, and Zn). At the same time, the correlation dependencies found were determined as medium to strong and statistically proven (Table 4).

Table 4. Correlations between the accumulation of heavy metals (Pb, Cd, and Zn), dry weight of spinach plants, β-glucosidase activity, dehydrogenase activity, and soil respiration in rhizosphere soil at different experimental treatments

Number		1	2	3	4	5	6	7
1	Cd	1	0.771**	0.687*	-0.205	-0.596	-0.786**	-0.762*
2	Pb		1	0.917**	-0.498*	-0.733*	-0.878**	-0.877**
3	Zn			1	-0.659*	-0.513	-0.687*	-0.731*
4	dry weight of spinach plants				1	0.057	0.151	0.266
5	β-glucosidase activity					1	0.871**	0.910**
6	dehydrogenase activity						1	0.864**
7	soil respiration							1

* Sufficient evidence for reliability $\alpha=0.05$; ** sufficient evidence for reliability $\alpha=0.01$

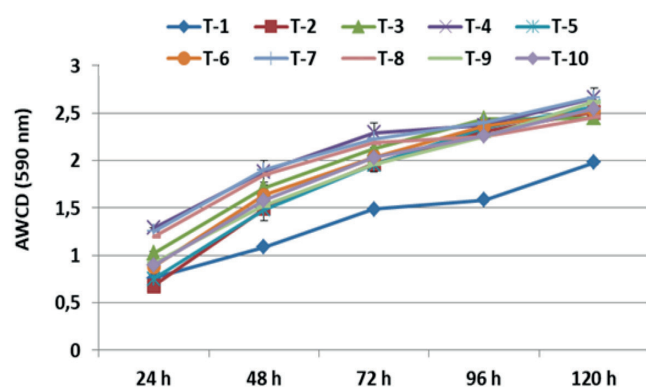


Fig. 5. Effect of treatments on carbon substrate utilization of ecoplates (AWCD at 590 nm). The results show the means of three replicates $\pm$ standard deviation.

There was a strong negative, very well-proven ($r = 0.786^{**}$; $r = 0.878^{**}$) and strong, well-proven ($r = 0.687^{*}$) correlation between dehydrogenase activity in the soil and Cd, Pb and Zn accumulation in plants, respectively. Data showed a strong negative, very well-proven correlation ($r = 0.877^{**}$) between soil respiration and absorption of Pb in spinach plants and a strong negative correlation ($r = 0.762^{*}$; $r = 0.731^{*}$) between Cd and Zn. A strong negative correlation ($r = 0.733^{*}$) between β-glucosidase activity in the rhizosphere and Pb accumulation in plant tissue was observed. In addition, the accumulation of Pb and Zn correlated negatively with the dry weight of spinach plants. On the other hand, there was a strong positive, very well-proven correlation ($r = 0.871^{**}$) between β-glucosidase activity and dehydrogenase activity in rhizosphere soil, but also between soil respiration and β-glucosidase ($r = 0.910^{**}$) and dehydrogenase activity ($r = 0.864^{**}$). The study data also showed a positive correlation between the accumulation of Pb in spinach and the accumulation of Cd and Zn ($r = 0.771^{**}$; $r = 0.917^{**}$), respectively, and between Cd and Zn. In similar studies, the dehydrogenase activity was

negatively correlated with Cd and Zn, while a positive relationship was found of β-glucosidase with Cu and As (Tang *et al.*, 2020; Hu *et al.*, 2014).

Community-level physiological profiling

To reveal the changes produced by the compost and different microbial populations added to the contaminated soil, a community-level physiological profiling method was applied. The changes over time in carbon source utilization are presented in Fig. 5.

The substrate utilization curve throughout the 120-h experiment was standard, and the increase in utilization rate was significantly reduced. Control had a lower carbon source utilization capacity than

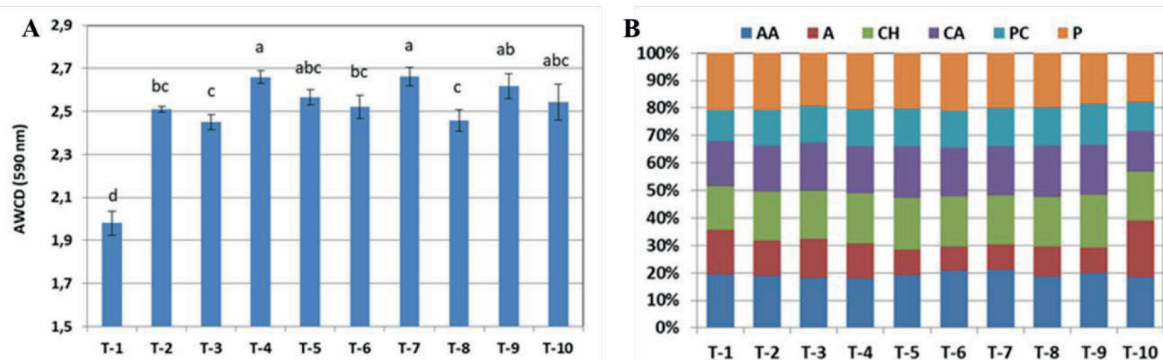


Fig. 6. Carbon sources utilization: (A) at 120 h (AWCD at 590 nm); (B) distribution by group of substrates as a portion of the total capacity.

The results show the meaning of three replicates $\pm$ standard deviation. Different letters indicate statistically significant differences between treatments according to LSD test ($p \leq 0.05$).

the rest of the treatments. Although a clear separation in source utilization was observed at the start of the trial at 24 h, this difference became significantly smaller at the end at 96 h and 120 h. Reduced utilization in T-1 is clearly seen, while communities in all compost treatments showed a significant response (Fig. 6A). We got the strongest response in T-4 and T-7, and the weakest of the options with compost was in T-3 and T-8.

Figure 6B and Figure 7 show the utilization of six groups of organic substrates presented in the ecoplates. Polymers and amino acids were the most dominant groups within the microbial communities, with a slight advantage over the polymers. The next group in terms of capacity for utilization is carbohydrates, followed by carboxylic acids. The weakest response was found for phenolic compounds, amines, and amides. The highest capacity to utilize polymers was found in T-4 and the lowest in control (T-1). The rest of the treatments are grouped. The amino acids' strongest absorption was again observed in treatments T-4 and T-7, with the weakest absorption in control. The highest consumption of amines and amides was found again in treatment T-4 and with compost only (T-10). The microbial community in the rest of the treatments showed much lower utilization capacity. Little difference was observed between the compost treatments in terms of carbohydrate utilization. The highest mean value was found at T-9 but was not significant at $p=0.05$ with T-2, T-5, T-7, and T-10. It was lowest in control. In addition, all treatments with beneficial populations were grouped concerning carboxylic acid consumption. Lower utilization was found in the compost-only treatment and lowest in the control. The relative uptake of phenolic compounds between treatments was similar to that of carboxylic acids, but the overall uptake rate was 23.23% lower.

Figure 6B shows the distribution of the microbial community's capacity to utilize the substrate groups as a fraction of the total utilization capacity. It can be seen that the structure of the microbial community is changed at T-1 and T-10 compared to the other treatments in relation. In the communities in these treatments, the populations consuming phenolic compounds are less represented, but those absorbing amines and amides are more strongly expressed. The last carbon sources are less represented in the treatments from T-5 to T-9.

The heat map shows clearly the microbial community's capacity to utilize the relevant carbon source from the eco-plates (Fig. 8). In this regard, polymers and amino acids are the preferred grouping substrates. In polymers, α -cyclodextrin predominates, while in amino acids, it is L-asparagine. The poor absorption of amines and amides, 2-hydroxybenzoic acids, and D,L- α -glycerol phosphate is striking. These results are confirmed by the abovementioned data.

Table 5 presents the soil microbial community's main indices of metabolic activity. In general, the values of the Shannon Diversity Index range from 2.49 to 3.29, which can be defined as a stable and balanced structure of habitat communities. However, in the control treatment, there is a noticeable decrease in this index and the Pielou index. Higher Shannon diversity index and substantially improved carbon source utilization are reported in organically amended soil compared to the control soil (Zhang *et al.*, 2020; Li *et al.*, 2016).

Principle component analysis

The Principal Component Analysis revealed that two principal components having greater than 1 eigenvalue contributed 97.089% of the total variation. It was found that Principal Component 1 (PC1) contributed 80.087% and PC2 contributed 17.002%, respectively. All traits contributed

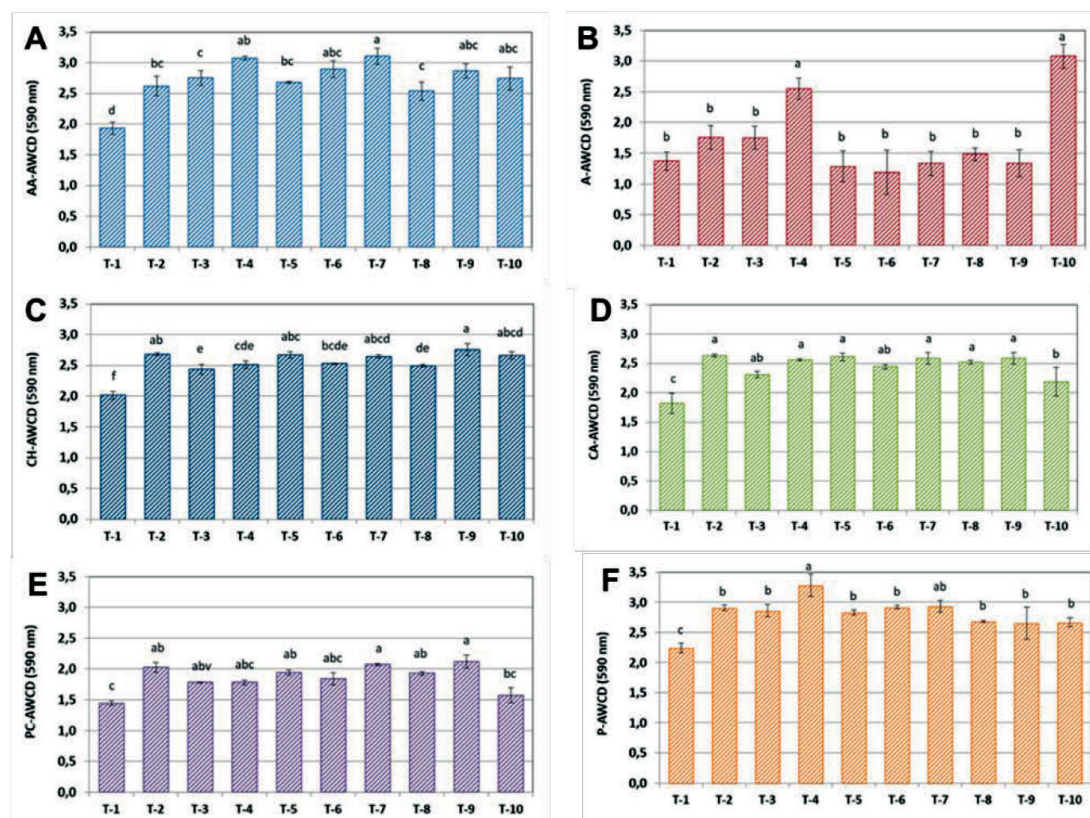


Fig. 7. Utilization of specific groups of carbon sources in the ecoplates at 120 h (AWCD at 590 nm). The results show the meaning of three replicates $\pm$ standard deviation. Different letters indicate statistically proven differences between treatments according to LSD test ($p \leq 0.05$). Weight of different groups of carbon-sources utilization patterns in the individual treatments.

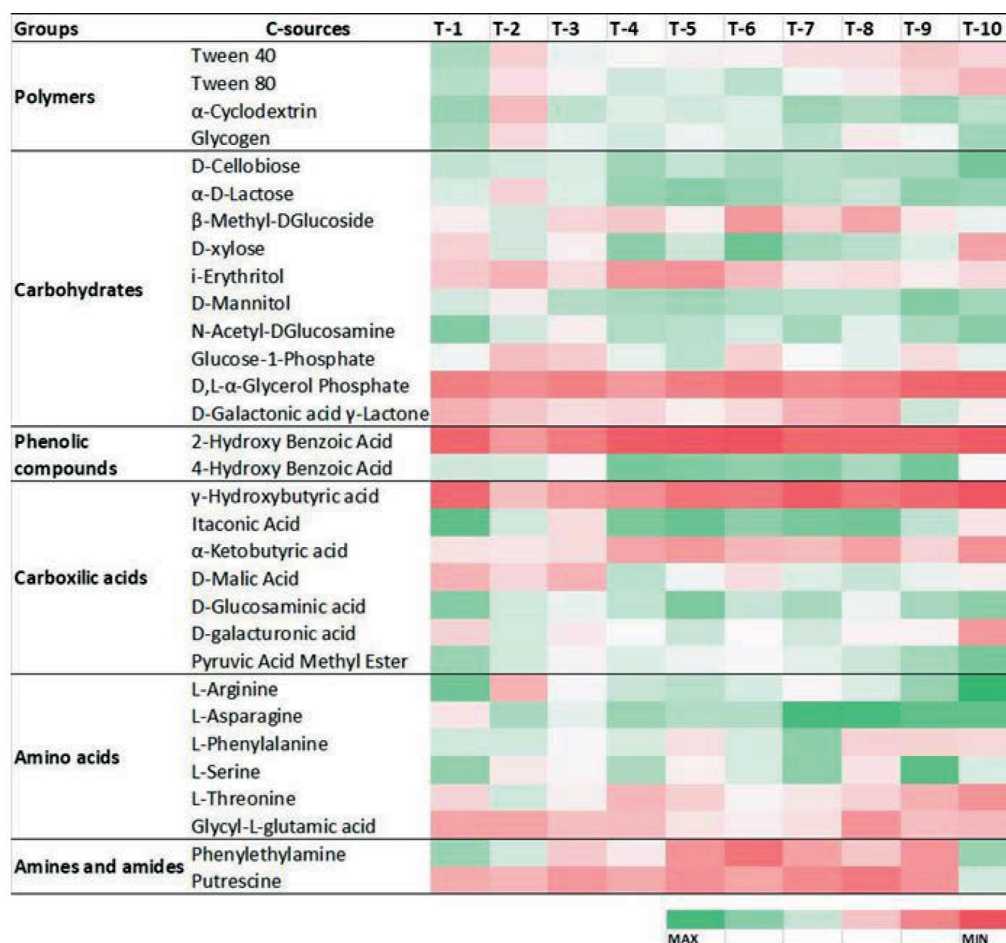


Fig. 8. A heat map based on the utilization of 31 carbon sources of Ecoplates at 120 h. The intense green color shows very good utilization, while the intense red color demonstrates a lack of capacity for it.

Table 5. Main indices of metabolic activity of soil microbial communities in different treatments of the experiment

Treatments	Shannon Diversity Index (H')	Pielou uniformity index (E)	Functional wealth (R)
1	2.49±0.19	0.76±0.06	23±0.4
2	3.15±0.12	0.97±0.01	26±1.1
3	2.92±0.17	0.97±0.00	29±0.2
4	2.94±0.05	0.98±0.01	27±1.8
1	2.98±0.07	0.98±0.01	28±0.6
6	3.25±0.11	0.99±0.00	26±0.4
7	3.28±0.12	0.98±0.01	28±0.5
8	3.26±0.15	0.98±0.00	28±0.3
9	3.22±0.13	0.99±0.00	26±1.1
10	3.14±0.16	0.98±0.00	27±0.5

The results show the mean of three replicates ± standard deviation

positively to PC1. Figure 9 represents a biplot in the dimensions of the first and second PCs. Amino acids and polymers had positive values for the first and second PC.

These two carbon groups were distinguished by the highest utilization rate in all experimental treatments. The lowest consumption rate was the phenolic compounds and amines and amides, where the A had negative values for PC1, while the phenolic compounds had negative values for PC1 and PC2. The highest correlation of T-10 (contaminated soil + compost) was found with T-1 (contaminated soil). At the same time, T-2 (a bacterial consortium of *P. fluorescence*, *P. putida*, and *B. cereus*), T-3 (a bacterial consortium of *P. putida*, *P. fluorescence*)

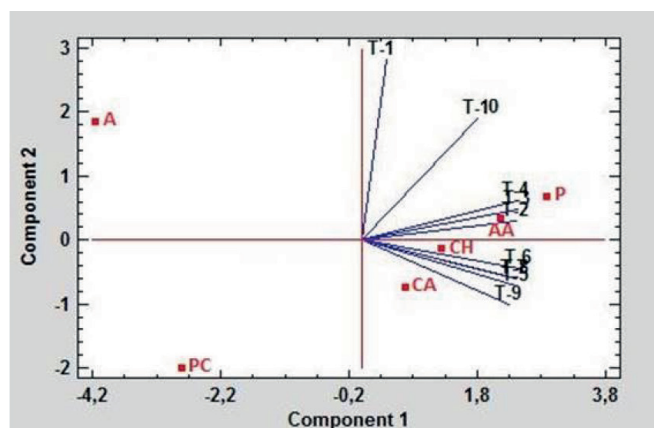


Fig. 9. Principal component analysis among experimental treatments ordinations for different carbon sources among treatments (AA: amino acids; A: amines and amides; CH: carbohydrates; CA: carboxylic acids; PC: phenolic compounds; P: polymers).

and T-4 (a bacterial consortium of *P. putida*, *P. fluorescence*), presented highest correlation with each other because they form an acute angle between among themselves.

Conclusions

1. The lowest heavy metal accumulation was found in the treatments amended with compost and consortium of beneficial bacteria, while higher was in control.

2. The incorporation of a consortium of beneficial bacteria resulted in an increase in the biomass of spinach plants by over 100% compared to the control and compost application alone.

3. CLPPs were affected by compost and beneficial bacterial populations dominating amino acids and polymer-utilizing communities in treatments with bacterial consortia. In contrast, treatments amended with compost and single-strain populations were dominated by carbohydrates and carboxylic acids utilizers. Lower consumption was found for phenolic compounds, amines, and amides.

4. The use of quality organic amendments and beneficial soil bacterial populations is a promising approach for the phyto-stabilization of soils contaminated with heavy metals.

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References

- Abou Jaoude, L., P. Castaldi, N. Nassif, M. V. Pinna, G. Garau (2020). Biochar and compost as gentle remediation options for the recovery of trace elements-contaminated soils. *Sci. Total Environ.* **711**: 134511. <https://doi.org/10.1016/j.scitotenv.2019.134511>
- Alef, K. (1995a). Dehydrogenase activity. In: Alef, K., P. Nannipieri (Eds.), *Methods in Applied Soil Microbiology and Biochemistry*, Academic Press: Cambridge, MA, USA, pp. 228-231. <https://doi.org/10.1016/B978-012513840-6/50022-7>.
- Alef, K. (1995b). Soil respiration. In: Alef, K., P. Nannipieri (Eds.), *Methods in Applied Soil Microbiology and Biochemistry*, Academic Press: Cambridge, MA, USA, pp. 214-219. <https://doi.org/10.1016/B978-012513840-6/50022-7>.
- Alef, K., P. Nannipieri (1995). β -glucosidase activity. In: Alef, K., P. Nannipieri (Eds.), *Methods in Applied Soil Microbiology and Biochemistry*, Academic Press: Cambridge, MA, USA, pp. 350-352. <https://doi.org/10.1016/B978-012513840-6/50022-7>.
- Anwar, Z., M. Irshad, Q. Mahmood, F. Hafeez, M. Bilal (2017). Nutrient uptake and growth of spinach as affected by cow manure co-composted with poplar leaf litter. *Int. J. Recycl. Org. Waste Agric.* **6**: 79–88. <https://doi.org/10.1007/s40093-017-0154-x>.
- Caldwell, B. A. (2005). Enzyme activities as a component of soil biodiversity: a review. *Pedobiologia* **49**: 637e644.

- <http://dx.doi.org/10.1016/j.pedobi.2005.06.003>.
- Council of Ministers of Bulgaria (2008). Regulation on standards for permissible content of harmful substances in soils, <https://lex.bg/laws/ldoc/2135595432> (Accessed on 21.05.2023).
- Falkowski, P. G., T. Fenchel, E. F. DeLong (2008). The microbial engines that drive Earth's biogeochemical cycles. *Science* **320**: 1034–1039. <https://doi.org/10.1126/science.1153213>.
- Galende, M. A., J. M. Becerril, M. T. Gómez-Sagasti, O. Barutia, L. Epelde, C. Garbisu, A. Hernández (2014). Chemical stabilization of metal-contaminated mine soil: early short-term soil-amendment interactions and their effects on biological and chemical parameters. *Water Air Soil Pollut.* **225**: 1863. <https://doi.org/10.1007/s11270-013-1863-z>.
- Garland, J. L., A. L. Mills (1991). Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Appl. Environ. Microbiol.* **57**: 2351–2359. <https://doi.org/10.1128/aem.57.8.2351-2359.1991>.
- Hinojosa, M. B., J. A. Carreira, R. García-Ruiz, R. P. Dick (2004). Soil moisture pretreatment effects on enzyme activities as indicators of heavy metal contaminated and reclaimed soils. *Soil Biol. Biochem.* **36**: 1559e1568. <http://dx.doi.org/10.1016/j.soilbio.2004.07.003>.
- Hu, X. F., Y. Jiang, Y. Shu, X. Hu, L. Liu, F. Luo (2014). Effects of mining wastewater discharges on heavy metal pollution and soil enzyme activity of the paddy fields. *J. Geochem. Explor.* **147**: 139–150. <https://doi.org/10.1016/j.gexplo.2014.08.001>.
- Insam, H., M. Goberna (2008). Use of Biolog for the community level physiological profiling (CLPP) of environmental samples. *Mol. Microb. Ecol. Manag.* **452**: 853–860. http://dx.doi.org/10.1007/978-1-4020-2177-0_401.
- Kaurin, A., Z. Cernilogar, D. Lestan (2018). Revitalisation of metal-contaminated, EDTA-washed soil by addition of unpolluted soil, compost and biochar: Effects on soil enzyme activity, microbial community composition and abundance. *Chemosphere* **193**: 726e736. <https://doi.org/10.1016/j.chemosphere.2017.11.082>.
- Kidd, P., J. Barceló, M. P. Bernal, F. Navari-Izzo, Ch. Poschenrieder, S. Shilev, R. Clemente, C. Monteroso (2009). Trace element behavior at the root-soil interface: Implications in phytoremediation. *J. Environ. Exp. Bot.* **67**: 243–259. <http://dx.doi.org/10.1016/j.envexpbot.2009.06.013>.
- Koner, S., J.-S. Chen, B.-M. Hsu, J. Rathod, S.-W. Huang, H.-Y. Chien, B. Hussain, M. W. Y. Chan (2022). Depth-resolved microbial diversity and functional profiles of trichloroethylene-contaminated soils for Biolog Eco-Plate-based biostimulation strategy. *J. Hazard. Mater.* **424**: 127266. <https://doi.org/10.1016/j.jhazmat.2021.127266>.
- Kumar, S., G. Stecher, K. Tamura (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **33**: 1870–1874. <https://doi.org/10.1093/molbev/msw054>.
- Lacalle, R., M. P. Bernal, M. J. Alvarez-Robles, R. Clemente (2023). Phytostabilization of soils contaminated with As, Cd, Cu, Pb and Zn: Physicochemical, toxicological and biological evaluations, *Soil Environ. Health* **1**: 100014. <https://doi.org/10.1016/j.seh.2023.100014>.
- Li, F. X., S. R. Wang, X. Wu, C. J. Wang (2016). Effect of long-term fertilisation on soil microbial diversity in irrigation silting soil of Ningxia. *Chin. J. Soil Sci.* **47**: 1134–1141. (in Chinese).
- Lin, H., C. Liu, B. Li, Y. Dong (2021). *Trifolium repens* L. regulated phytoremediation of heavy metal contaminated soil by promoting soil enzyme activities and beneficial rhizosphere associated microorganisms. *J. Hazard. Mater.* **402**: 123829. <https://doi.org/10.1016/j.jhazmat.2020.123829>.
- Lozano-Rodríguez, E., M. Luguera, J. J. Lucena, R. O. Carpena-Ruiz (1995). Evaluation of two different acid digestion methods in closed systems for trace element determinations in plants. *Quim. Anal.* **14**: 27–30.
- Medynska-Juraszek, A., M. Bednik, P. Chohura (2020). Assessing the influence of compost and biochar amendments on the mobility and uptake of heavy metals by green leafy vegetables. *Int. J. Environ. Res. Public Health* **17**: 7861. <https://doi.org/10.3390/ijerph17217861>.
- Neip, M., S. Kumar (2000). Molecular Evolution and Phylogenetics. Oxford University Press, New York, ISBN 0-19-513584-9.
- Renella, G., A. L. Reyes Ortigoza, L. Landi, P. Nannipieri (2003). Additive effects of copper and zinc on cadmium toxicity on phosphatase activities and ATP content of soil as estimated by the ecological dose (ED50). *Soil Biol. Biochem.* **35**: 1203e1210. [https://doi.org/10.1016/S0038-0717\(03\)00181-0](https://doi.org/10.1016/S0038-0717(03)00181-0).
- Scotti, R., C. Pane, R. Spaccini, A. M. Palese, A. Piccolo, G. Celano, M. Zaccardelli (2016). On-farm compost: a useful tool to improve soil quality under intensive farming systems. *Appl. Soil Ecol.* **107**: 13–23. <https://doi.org/10.1016/j.apsoil.2016.05.004>.
- Shilev, S. (2013). Soil rhizobacteria regulating the uptake of nutrients and undesirable elements by plants. In: N. K. Arora (Ed.) Plant microbe symbiosis – fundamentals and advances, Springer, India, pp. 147–167. https://doi.org/10.1007/978-81-322-1287-4_5.
- Shilev, S., H. Azaizeh, D. Angelova (2019). Biological treatment: a response to the accumulation of biosolids. In: Singh, D.P., V.K. Gupta, R. Prabha (Eds.), Microbial Interventions in Agriculture and Environment, Vol. 2: Rhizosphere, Microbiome and Agroecology. Springer, Singapore, pp. 149–178. https://doi.org/10.1007/978-981-13-8383-0_5.
- Shilev, S., M. Naydenov, V. Vancheva, A. Aladjadjian (2007). Composting of food and agricultural wastes, In: Oreopoulou, V., W. Russ (Eds.), Utilisation of By-Products and Treatment of Waste in the Food Industry, Series: Integrating Safety and Environmental Knowledge Into Food Studies towards European Sustainable Development, Springer, Vol. 3, pp. 283–302. https://doi.org/10.1007/978-0-387-35766-9_15.
- Singh, B. R., S. Gupta, H. Azaizeh, S. Shilev, D. Sudre, W. Song, E. Martinoia, M. Mench (2011). Safety of food crops on land contaminated with trace elements. *J. Sci. Food Agric.* **91**: 1349–1366. <https://doi.org/10.1002/jsfa.4355>.
- Sun, Q., Y. Zhang, C. Ming, J. Wang, Y. Zhang (2023). Amended compost alleviated the stress of heavy metals to pakchoi plants and affected the distribution of heavy metals in soil-plant system. *J. Environ. Manag.* **336**: 117674. <https://doi.org/10.1016/j.jenvman.2023.117674>.
- Tabatabai, M. A. (1982). Soil enzymes. In: Page, A.L., E.M. Miller, D.R. Keeney (Eds.), Methods of soil analysis, part

- 2, Chemical and microbiological properties, *Am. Soc. Agron.* Madison, WI, pp. 903-947.
- Tang, J., L. Zhang, J. Zhang, L. Ren, Y. Zhou, Y. Zheng, L. Luo, Y. Yang, H. Huang, A. Chen (2020). Physicochemical features, metal availability and enzyme activity in heavy metal-polluted soil remediated by biochar and compost. *Sci. Total Environ.* **701**: 134751. <https://doi.org/10.1016/j.scitotenv.2019.134751>.
- Wasilkowski, D., A. Nowak, J. Michalska, A. Mroziak (2019). Ecological restoration of heavy metal-contaminated soil using Na-bentonite and green compost coupled with the cultivation of the grass *Festuca arundinacea*. *Ecol. Eng.* **138**: 420-433. <https://doi.org/10.1016/j.ecoeng.2019.08.004>.
- Xiong, T., J. Cui, L. Jiang, H. Wang, Y. Li, C., Su, Y. Chen, X. Cao, X., Yuan, G. Yi (2023). Dose-dependent impact of compost on rhizosphere bacterial community in heavy metal-contaminated paddy soil. *Trans. Non-ferrous Met. Soc. China* **33**: 642-652. [https://doi.org/10.1016/S1003-6326\(22\)66135-1](https://doi.org/10.1016/S1003-6326(22)66135-1).
- Ye, S.-J., G.-M. Zeng, H.-P. Wu, C. Zhang, J. Dai, J., Liang, J.-F. Yu (2017). Biological technologies for the remediation of co-contaminated soil. *Crit. Rev. Biotech.* **37**: 1062-1076. <https://doi.org/10.1080/07388551.2017.1304357>.
- Yuichi, H., M. Ohkuma, T. Kudo, H. Yuzawa (2003). Evaluation of primers and PCR conditions for the analysis of 16S rRNA genes from a natural environment. *FEMS Microbiol. Lett.* **221**: 299-304. [https://doi.org/10.1016/S0378-1097\(03\)00218-0](https://doi.org/10.1016/S0378-1097(03)00218-0).
- Zafar-ul-Hye, M., M. Tahzeeb-ul-Hassan, M. Abid, S. Fahad, M. Brtnicky, T. Dokulilova, R. Datta, S. Danish (2020). Potential role of compost mixed biochar with rhizobacteria in mitigating lead toxicity in spinach. *Sci. Rep.* **10**: 12159. <https://doi.org/10.1038/s41598-020-69183-9>.
- Zhang, B., J. Hong, Q. Zhang, D. Jin, C. Gao (2020). Contrast in soil microbial metabolic functional diversity to fertilisation and crop rotation under rhizosphere and non-rhizosphere in the coal gangue landfill reclamation area of Loess Hills. *PLoS One* **15**: e0229341. <https://doi.org/10.1371/journal.pone.0229341>.