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ORIGINAL PAPER

The impact of mineral and organic supplements on the abundance of selected groups of culturable microorganisms in soil contaminated with heavy metals*

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Abstract

The impact of lignite and leonardite combined with a NaX-C composite on the abundance of selected groups of culturable microorganisms in soil contaminated with Pb, Cd and Zn was tested. For this purpose, a 7-month incubation experiment and two-year pot experiments were conducted. The pattern of both experiments was analogous and included: soil without fertilization (C); soil fertilized with NPK mineral fertilizers (MF); soil with NPK + 3 or 6% lignite (MF+CW3%, MF+CW6%) and 3% zeolite-carbon composite (NaX-C); soil with NPK + 3 or 6% leonardite (MF+CL3%, MF+CL6%) and 3% zeolite-carbon composite (NaX-C). The test plant in the pot experiment was the Kosynier variety of maize. The soil material was used to estimate the abundance of general bacteria, mold fungi, actinomycetes (actinomycetales), ammonification bacteria and bacteria from the *Azotobacter* genus. The study found that the application of lignite and leonardite combined with a NaXC composite had generally contributed to a significant increase in the total nitrogen content and a decrease in the total carbon content in soil in the pot experiment. In general, the mineral-organic mixtures had a stimulating effect on the analyzed groups of soil microorganisms in both experiments, but the dominant groups in the pot experiment were total bacteria and ammonifying bacteria, while in the incubation experiment, fungi and actinomycetes were the prevalent groups. An RDA analysis indicated that mold fungi, bacteria, actinomycetes and amminofers were positively correlated with the EC, total nitrogen and carbon.

Keywords: bacteria, mold fungi, soil, maize, fertilization, heavy metals

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INTRODUCTION

Microorganisms are an essential component of any ecosystem, as they have a role in many of natural processes (Banerjee, van der Heijden 2023). The ecological diversity of microorganisms and the variety of biochemical processes they participate in are influenced by their broad adaptability to changing conditions, such as temperature fluctuations, nutrient availability, and pH changes (Wani et al. 2022). Soil is composed of the gas phase, mineral parts, organic parts and living organisms (Hartmann, Six 2023).

Soil microorganisms play an extremely important role in the metabolism of soil nutrients of nutritional importance to crop plants (Tataranni et al. 2012). Hence, it is essential to know their abundance, species diversity and activities in this environment to ensure optimal conditions for healthy plant growth, development and yield. The interaction of microorganisms and higher plants leads to a kind of equilibrium in biocenotic systems of soil environments. However, this equilibrium can be disrupted by any chemical inflow or by a sudden change in the physical and chemical properties of the soil caused for example by fertilization. This can cause a decrease in soil fertility, which adversely affects the growth and development of crop plants, and results in a decrease in the size and quality of the crop yield (Mierzwa-Hersztek et al. 2020). The development of microorganisms in the soil hinges upon its physical and chemical properties, fertilization, atmospheric conditions and agrochemical factors, especially the abundance of organic matter, being a source of energy and nutrients for microorganisms. Microorganisms, together with the plant cover, determine both the direction and nature of biochemical processes, whose course is influenced by tillage, fertilization and chemical protection. Among the many anthropogenic factors that have a substantial impact on soil microorganisms, fertilization ranks very high (Lupwayi et al. 2001). Substituting mineral fertilizers with organic inputs and supplementing the soil with specific root-associated microbes that mineralize organic-bound nutrients can lead to more sustainable agricultural practices. Organic additives can be obtained in a more environmentally friendly manner compared to mineral fertilizers. Various agricultural, industrial, and municipal processes generate significant volumes of nutrient-rich by-products, which are presently discarded but have the potential to be transformed and utilized as fertilizers (Paungfoo-Lonhienne et al. 2012, Jacoby et al. 2017).

Soil health plays a crucial role in achieving the goals of the European Green Deal, i.e. restoring biodiversity, healthy and sustainable food systems, climate neutrality and resilient environment. However, soils are very often under threat from pollution caused by anthropogenic activities, which, according to the Intergovernmental Technical Panel on Soils (ITPS), is the third most significant threat to soil function in Europe (FAO, ITPS 2015). The negative impact of soil degradation on human health can be attributed

to the deterioration of a number of ecosystem services provided by the soil, such as food supply, carbon sequestration, water availability and nutrient circulation, resulting in the worsening of people's quality of life. Soil contamination is one of the main factors contributing to the reduction and/or loss of soil biodiversity. Contaminants (heavy metals, PAHs, pesticides, antibiotics, microplastics, etc.) can have toxic effects on soil organisms, causing changes in their biomass, abundance, enzymatic and metabolic activity, and community structure (Muhlbachova et al. 2015). Changes in soil biological activity lead to a decrease in the decomposition rate of mulch and fresh organic matter, affecting biogeochemical cycles in contaminated areas, which ultimately results in a decrease in organic carbon content and nutrient availability (Krull et al. 2003).

Soil contamination reduces yields and deteriorates the quality of food produced, thereby affecting agricultural economics and farmers' incomes along with food security. It is estimated that soil contamination accounts for 15-25% of the loss of agricultural productivity. Severe soil contamination leads to land degradation and the inability to use it for production, housing and recreation, which ultimately contributes to land abandonment and a decline in the value of adjacent areas (FAO, ITPS 2015). For this reason, it is necessary to take measures to support soil regeneration processes. One such measure may be the use of mineral-organic mixtures containing the addition of zeolite-carbon composites (NaX-C) – Bandura et al. (2021). These materials are not only sources of carbon, but because of their mesoporous structure and large specific surface area, they can act as a sorbent for organic and inorganic contaminants (Jarosz et al. 2022).

The aim of this study was to assess the impact of soil application of mineral-organic mixtures (containing a composite of zeolite-carbon synthesized from fly ash and either leonardite or lignite) on: (i) the abundance of selected groups of cultured microorganisms, (ii) changes in soil pH and selected chemical parameters, and (iii) to compare the effects of the mineral-organic mixtures on the above parameters (i, ii) under conditions of a pot experiment with maize cultivation and under conditions of an incubation experiment conducted in strictly controlled conditions. This approach allowed the assessment of changes in the chemical and microbiological properties of the soil after the application of mineral-organic mixtures in two independent experiments with a common denominator, which was the type of fertilization (design of the experiment) and the soil used for the research.

MATERIALS AND METHODS

Amendments and soil material properties

The pot and incubation experiments used zeolite-carbon composites (NaX-C), which were obtained from fly ashes via hydrothermal synthesis (Panek et al. 2021). The synthesis of NaX–C was conducted using a hydrothermal method and high carbon fly ash (HCFA) from hard coal combustion (class F according to ASTM C 618-08) collected from a thermal power plant (Janikowo, Poland), after the electromagnetic separation of fly ash, and NaOH solution (P.P.H. "Stanlab", Lublin, Poland) as reagents. A technological line for zeolite synthesis was loaded with 20 kg of HCFA and 90 dm^3 of 3 M NaOH. The reaction temp. of 70°C was maintained for 48 h using three heaters of 3 kW. The mixing procedure was as follows: 5 min of mixing in a 1 h interval coupled with rotating for 10 min every hour. After the synthesis, the solid product was rinsed with distilled water and dried at 105°C (Mokrzycki et al. 2023, Szerement et al. 2023). Leonardite came from the Energy Investment Company Ltd. The country of origin was Ukraine. The moisture content of leonardite: 25-45%, humic acid content: 75-86.6%, fulvic acid content: 19.8-22.8%, acidity: 4.20-7.50, organic matter content: 65-83.40%, fraction: 0.01-8.0 mm.

Characteristics of soil used to conduct the pot and incubation experiments

Agricultural soil was sampled from an agricultural field located near a coniferous forest in South Malopolska (50°05′35.9″N 19°39′52.9″E), and was extracted from the upper soil layer of 0-30 cm. The soil was air-dried, then sieved through a 2 mm mesh and manually homogenized for the following pot and incubation experiments. The soil used for the experiment was slightly loamy sand (WRB 2015). The soil had an acidic pH and elevated concentrations of cadmium, lead, and zinc (Table 1). Taking into account the guidelines of the Institute of Soil Science and Plant Cultivation (IUNG-PIB, Pulawy) used to evaluate the degree of contamination of soils with heavy metals in the soil, the content of Cd can be defined as the first degree of soil contamination, and on the basis of Zn and Pb can be defined as the second degree of soil contamination (Kabata-Pendias, Pendias 2001).

The pot and incubation experiments included 6 treatments carried out in 4 replications (Table 2). The reference was the control treatment without fertilization and with mineral fertilization (chemically pure salts, N – NH_4NO_3; P – $Ca(H_2PO_4)_2 \cdot H_2O$; K – KCl).

Incubation experiment

Incubation tests were carried out in containers filled with 300 g of dry soil. The moisture content in each sample was maintained at 60% of soil

Table 1

Selected soil properties before setting up the pot experiment

Determinant	Value
Fraction 2-0.5 mm	85%
Fraction 0.05-0.002 mm	12%
Fraction <0.002	3%
pH H_2O	5.24
pH KCl	5.03
EC	273 μS cm^{-1}
N total	0.40 g kg^{-1} D.M.
C total	5.74 g kg^{-1} D.M.
S total	0.118 g kg^{-1} D.M.
Pb total	188±15 mg kg^{-1} D.M.
Cd total	1.15±0.08 mg kg^{-1} D.M.
Zn total	267±18 mg kg^{-1} D.M.
Cr total	5.32±0.73 mg kg^{-1} D.M.
Cu total	5.14±1.33 mg kg^{-1} D.M.
Ni total	2.22±0.18 mg kg^{-1} D.M.

Table 2

Description of experimental treatments

Symbol	Mineral salt	Zeolite-carbon composite	Lignite	Leonardite
C	-	-	-	-
MF	NPK	-	-	-
MF+ CW3%	NPK	3%	3%	-
MF+ CW6%	NPK	9%	6%	-
MF+ CL3%	NPK	3%	-	3%
MF+ CL6%	NPK	9%	-	6%

C – soil without fertilization; MF – soil fertilized with NPK mineral fertilizers; MF+CW3%, MF+CW6% – soil with NPK + 3 or 6% lignite and 3% zeolite-carbon composite (NaX-C); MF+CL3%, MF+CL6% – soil with NPK + 3 or 6% leonardite and 3% zeolite-carbon composite (NaX-C)

water capacity. The control sample in both experiments was stored with no mineral-organic mixtures added. The soil samples were incubated for 224 days at 25°C±1°C. The experiment was carried out in a thermostatic cabinet, in conditions of controlled temperature, humidity and without access to light. After incubation, soil samples were collected from each container for chemical and biochemical analyses. The soil for the biological analysis was sieved through a sieve with a mesh diameter of 2 mm and stored at 4°C.

The soil for the physicochemical analysis was air-dried, sieved through a sieve with a mesh diameter of 1 mm and stored at 25°C.

Pot experiment

The pot experiment was conducted in a greenhouse of the University of Agriculture in Krakow, in the years 2020-2021. The experiment was set up in PVC pots holding 9 kg of air-dry soil mass. The doses of nutrients (in the incubation and pot experiments) introduced into the treatment soils were: nitrogen 0.20 g kg^{-1} D.M. soil, phosphorus 0.10 g kg^{-1} D.M. soil, and potassium 0.25 g kg^{-1} D.M. soil. After applying mineral salts and mineral-organic mixtures and mixing them with the soil, maize grains of the Kosynier variety (15 seeds in each pot) were sown and then left to 5 seedlings after germination. Soil moisture during plant growth was maintained at 40% to 60% of the maximum water capacity of soil (depending on the plant's development stage). The experiment was continued until the fully ripe stage of the maize plants. In the pot experiment during the growing season, in the presence of nitrogen deficiency symptoms, additional doses of N in the form of NH_4NO_3 solution were applied (top dressing). The supplemental nitrogen dose was 0.02 g N kg^{-1} D.M. soil.

After the plant growing period in the pot experiment finishd (126 days in 1st year and 118 days in 2nd year), the plants were harvested. Collected straws (stalks + leaves) and cobs were dried at 65°C. The dry mass was calculated, and dried samples were ground in a laboratory mill for further analysis. Soil samples were collected from each pot for chemical and biochemical analyses. The soil for the biological analysis was sieved through a sieve with a mesh diameter of 2 mm and stored at 4°C. The soil for the physicochemical analysis was air-dried, sieved through a sieve with a mesh diameter of 1 mm and stored at 25°C.

Chemical analyses in soil material

The following parameters were determined in the soil: pH in a soil and water suspension – potentiometrically, electrical conductivity (EC) conductometrically, total nitrogen was determined in a Flash SMART (Thermo Fisher Scientific, Bremen, Germany) apparatus and calculated as the mean value from three consecutive analyses of prepared samples (50 mg). Soil organic carbon content was determined by the Turin's oxidation and titration method. The total trace element content of the soil was determined by the ICP-OES method on a PerkinElmer Optima 7300DV apparatus (Oleszczuk et al. 2007).

Microbiological analysis of soil

In order to assess the abundance of selected soil microorganisms, 10 g were weighed from each soil sample with the natural moisture content and submitted to the serial dilution method according to Koch (Kopeć et al.

2020, Mierzwa-Hersztek et al. 2020), using a range of microbiological substrates, which enabled the identification of selected culturable microorganisms (Table 3). Changes in the abundance of specific microbial groups are important for assessing fertilizer efficiency, and they indirectly convey information about soil fertility and quality from an agricultural standpoint (Wolny-Koładka, Żukowski 2019).

Table 3

Culture conditions of the labelled microorganisms

Microorganism	Substratum	Incubation temperature	Culture duration
General bacteria	Trypticasein Soy Lab Agar (BTL, Poland)	37°C	24 h
Mold fungi	Malt Extract Agar (BTL, Poland)	28°C	5 days
Actinomycetes	Actinomycete Isolation Lab Agar (Biocorp, Poland)	28°C	7 days
Ammonification bacteria	medium according to Rougieux (Malinowski, Wolny-Koładka 2017)	28°C	7 days
Azotobacter spp.	Ashby's Mannitol Agar (Atlas 2010)	28°C	7 days

The number of colony-forming units (CFU) of microorganisms was determined using the dilution culture method, converting the result of the measurement to 1 g D.M. of soil.

Statistical analysis

Differences between each treatment and control as well as between individual treatments were evaluated using a two-way analysis of variance (ANOVA, Duncan's test, $p \leq 0.05$). Variation within treatments was determined by calculating standard deviation values (±SD). All statistical analyses were performed using Statistica PL 13 software (StatSoft Inc., Tulsa, OK, USA). The data graphs were made by using Origin Pro.8.5 version, and the RDA analysis between selected microbes and environmental factors was made in CANOCO 5.

RESULTS

Changes in soil physicochemical properties

The determined pH and EC in soil with mineral-organic mixtures additions after both the first and the second years of the study were lower than the values determined in the control soil (Table 4). The EC values in the soil with the application of mineral-organic mixtures, after the second year

Table 4

Value of pH in soil after 1st and 2nd year of the pot experiment and soil of the incubation experiment

Treatment	Pot experiment		Incubation experiment
	1st year	2nd year	
C	$5.91^{c} \pm 0.10$	$5.97^{c} \pm 0.12$	$5.92^{c} \pm 0.04$
MF	$5.28^{ab} \pm 0.06$	$5.34^{b} \pm 0.16$	$4.90^{b} \pm 0.06$
MF+ CW3%	$5.12^{a} \pm 0.12$	$5.15^{ab} \pm 0.13$	$4.77^{c} \pm 0.05$
MF+ CW6%	$5.24^{ab} \pm 0.06$	$5.27^{ab} \pm 0.18$	$4.99^{b} \pm 0\ 04$
MF+ CL3%	$5.25^{ab} \pm 0.16$	$5.10^{a} \pm 0.05$	$4.91^{b} \pm 0.02$
MF+ CL6%	$5.27^{ab} \pm 0.05$	$5.15^{ab} \pm 0.13$	$5.00^{b} \pm 0.11$

Means marked with the same letters do not differ significantly according to the Duncan's test at $p \leq 0.05$; ±SD; factors: treatment, year x fertilization; $n=4$.
C – soil without fertilization; MF – soil fertilized with NPK mineral fertilizers; MF+CW3%, MF+CW6% – soil with NPK + 3 or 6% lignite and 3% zeolite-carbon composite (NaX-C); MF+CL3%, MF+CL6% – soil with NPK + 3 or 6% leonardite and 3% zeolite-carbon composite (NaX-C)

of testing, showed greater variation than after the first year of experiment (Table 4). A similar trend was found in the incubation experiment (Table 5). Among treatments with mineral-organic mixtures, the highest pH value after the second year of testing was determined in the soil of the MF+CW6% treatment (5.27), and the lowest in the soil of the MF+CL3% treatment (5.10).

Electrolytic conductivity (EC) values after the application of mineral--organic mixtures increased both after the first and second year of the study, compared to the values obtained for the soil of the control site (Table 5). Undoubtedly, the increase in EC values in both years of the study stems from the release of elements or easily soluble compounds from mineral--organic mixtures. Due to the lack of uptake of elements by the plants, the EC values in the soil from the incubation experiment were much higher than in the soil of the treatments from the pot experiment.

Total carbon content ranged from 6.46 to 8.20 g kg^{-1} D.M. soil (1st year), 6.09 to 7.70 g kg^{-1} D.M. soil (2nd year) and 7.50 to 8.38 g kg^{-1} D.M. soil (incubation) – Table 6. After the second year of testing, with the exception of the MF+CW6% treatment, a decrease in total carbon content was observed.

The content of total nitrogen (TN) in the soil after the 1st and 2nd year of the study and at the end of the incubation experiment is shown in Table 7. The content of TN in all tested soils was higher after second year of experiment in compared to first year. The largest increase in TN content (by 50%) was found in the MF+CL3% object. For the incubation experiment, a significant increase in TN content relative to the soil of the MF treatment was found only in the soil of the MF+CL6% and MF+CW6% treatments. On the

Table 5

Value of EC (μS cm^{-1}) in soil after 1st and 2nd year of the pot experiment and soil of the incubation experiment

Treatment	Pot experiment		Incubation experiment
	1st year	2nd year	
C	$366^{a} \pm 59$	$341^{a} \pm 9$	$703^{a} \pm 63$
MF	$427^{b} \pm 47$	$763^{f} \pm 21$	$1210^{b} \pm 31$
MF+ CW3%	$544^{d} \pm 12$	$708^{e} \pm 24$	$1348^{c} \pm 36$
MF+ CW6%	$449^{bc} \pm 4$	$698^{e} \pm 44$	$1494^{c} \pm 61$
MF+ CL3%	$441^{bc} \pm 9$	$1263^{h} \pm 63$	$1478^{c} \pm 93$
MF+ CL6%	$484^{c} \pm 18$	$825^{g} \pm 20$	$1520^{c} \pm 66$

Means marked with the same letters do not differ significantly according to the Duncan's test at p ≤ 0.05; ±SD; factors: treatment, year x fertilization; n=4.
C – soil without fertilization; MF – soil fertilized with NPK mineral fertilizers; MF+CW3%, MF+CW6% - soil with NPK + 3 or 6% lignite and 3% zeolite-carbon composite (NaX-C); MF+CL3%, MF+CL6% – soil with NPK + 3 or 6% leonardite and 3% zeolite-carbon composite (NaX-C)

Table 6

Total carbon content (g kg^{-1} D.M.) in soil after 1st and 2nd year of the pot experiment and soil of the incubation experiment

Treatment	Pot experiment		Incubation experiment
	1st year	2nd year	
C	$8.20^{h} \pm 0.17$	$7.28^{fg} \pm 0.16$	$7.50^{a} \pm 0.35$
MF	$6.58^{bcde} \pm 0.43$	$6.09^{ab} \pm 0.09$	$7.79^{ab} \pm 0.32$
MF+ CW3%	$6.46^{abcd} \pm 0.61$	$6.36^{abcd} \pm 0.28$	$7.63^{ab} \pm 0.37$
MF+ CW6%	$6.69^{cde} \pm 0.46$	$7.70^{gh} \pm 0.25$	$8.38^{c} \pm 1.31$
MF+ CL3%	$7.93^{h} \pm 0.61$	$6.61^{bcde} \pm 0.29$	$8.15^{bc} \pm 0.63$
MF+ CL6%	$7.36^{fg} \pm 0.05$	$5.94^{a} \pm 0.20$	$8.35^{c} \pm 1.23$

Means marked with the same letters do not differ significantly according to the Duncan's test at $p \leq 0.05$; ±SD; factors: treatment, year x fertilization; n=4.
C – soil without fertilization; MF – soil fertilized with NPK mineral fertilizers; MF+CW3%, MF+CW6% – soil with NPK + 3 or 6% lignite and 3% zeolite-carbon composite (NaX-C); MF+CL3%, MF+CL6% – soil with NPK + 3 or 6% leonardite and 3% zeolite-carbon composite (NaX-C)

other hand, compared to the soil of treatment C, a significant ($p \leq 0.05$) increase in Ntot content in the soil was shown in each of the sites where mineral-organic mixtures were applied.

Abundance of culturable microorganisms in soil

After the second year of the pot experiment, the abundance of bacteria in all soil samples increased relative to the first year, or remained at a similar level (MF+CW3%). This trend is particularly evident in soil with lignite

Table 7

Total nitrogen content (g kg^{-1} D.M.) in soil after the 1st and 2nd year of the experiment and soil of the incubation experiment

Treatment	Pot experiment		Incubation experiment
	1st year	2nd year	
C	$0.477^{bc} \pm 0.05$	$0.512^{cd} \pm 0.04$	$0.438^{a} \pm 0.004$
MF	$0.435^{abc} \pm 0.05$	$0.449^{abc} \pm 0.09$	$0.382^{b} \pm 0.011$
MF+ CW3%	$0.377^{a} \pm 0.10$	0.525^{cd}e ± 0.05	$0.401^{b} \pm 0.011$
MF+ CW6%	$0.452^{abc} \pm 0.06$	0.595^{d}e ± 0.03	$0.388^{b} \pm 0.003$
MF+ CL3%	$0.401^{ab} \pm 0.00$	0.595^{d}e ± 0.07	$0.383^{b} \pm 0.007$
MF+ CL6%	$0.402^{ab} \pm 0.00$	$0.515^{cd} \pm 0.03$	$0.494^{c} \pm 0.006$

Means marked with the same letters do not differ significantly according to the Duncan's test at $p \leq 0.05$; ±SD; factors: treatment, year x fertilization; $n=4$.
C – soil without fertilization; MF – soil fertilized with NPK mineral fertilizers; MF+CW3%, MF+CW6% – soil with NPK + 3 or 6% lignite and 3% zeolite-carbon composite (NaX-C); MF+CL3%, MF+CL6% – soil with NPK + 3 or 6% leonardite and 3% zeolite-carbon composite (NaX-C)

(MF+CW6%) and leonardite (MF+CL6%) in combination with zeolite-carbon composite (NaX-C), where the difference in bacterial counts between 1st and 2nd year was significant. The proposed fertilization significantly increased bacteria counts (soil without fertilization, C) from 96 500 CFU g^{-1} D.M. (1st year) and 328 000 CFU g^{-1} D.M. (2nd year) to as high as 280 000 CFU g^{-1} D.M. (1st year) and 801 200 CFU g^{-1} D.M. (2nd year) for soil with leonardite addition in combination with zeolite-carbon composite (MF+CL6%). In the analysis of the averages of the two years of the experiment, it was found that the 6% addition of lignite and leonardite was the most effective in increasing bacteria abundance (MF+CW6% and MF+CL6%) relative to the control sample (C). After the second year of the experiment, a trend was observed in that as the percentage of lignite and leonardite doubled (from 3 to 6%), there was a proportional increase in the abundance of soil bacteria. By analyzing soil samples from the incubation experiment, we found that despite the absence of plants, our proposed additions of lignite and leonardite contributed to an increase in the abundance of analyzed group of microorganisms. The best effects were observed with lignite (MF+CW6%) combined with NaX-C, where bacterial abundance increased from 48 667 (C) to 186 000 CFU g^{-1} D.M. (MF+CW6%) – Figure 1.

In the case of mold fungi, an opposite trend to that of bacteria was observed, related to the fact that after the second year of the experiment, their abundance decreased significantly in all soil samples. This trend is particularly evident in soil with leonardite (MF+CL6%) in combination with zeolite-carbon composite (NaX-C), where the difference in fungal abundance was significant and amounted to, respectively: 200 000 CFU g^{-1} D.M. (1st year) and 47 500 CFU g^{-1} D.M. (2nd year). Based on the analysis of the

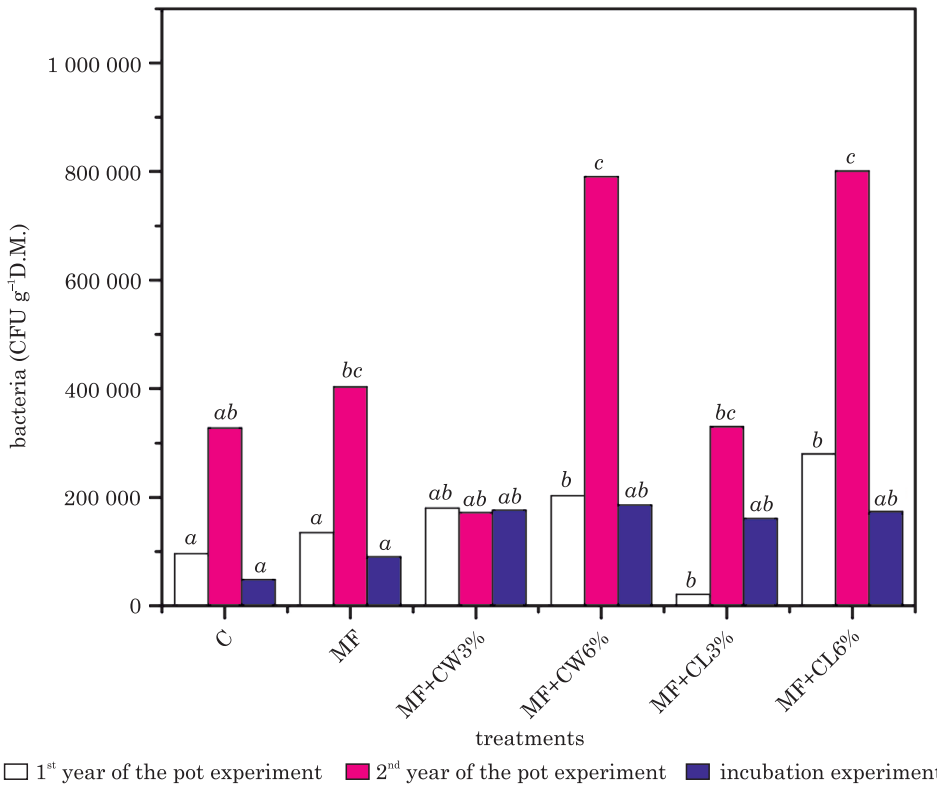

Fig. 1. Average abundance of bacteria in soil (after the 1st and 2nd year of the pot experiment and the incubation experiment)
Each value represents the mean of four replicates. Different letters indicate a significant difference at $p \leq 0.05$ according to the Duncan's multiple range tests, factors: year×treatment
C – soil without fertilization; MF – soil fertilized with NPK mineral fertilizers; MF+CW3%, MF+CW6% – soil with NPK + 3 or 6% lignite and 3% zeolite-carbon composite (NaX-C); MF+CL3%, MF+CL6% – soil with NPK + 3 or 6% leonardite and 3% zeolite-carbon composite (NaX-C)

results of two years, it was concluded that the MF+CW6% treatment created the most favorable conditions for the development of mold fungi, their average abundance was 155 500 CFU g^{-1} D.M. An increase in the abundance of mold fungi was observed from 108 000 CFU g^{-1} D.M. (1st year) and 44 000 CFU g^{-1} D.M. (2nd year) in soil without fertilization (C) to a maximum of 200 000 CFU g^{-1} D.M. (1st year) for MF+CW3% and MF+CL6% (1st year) and 136 000 CFU g^{-1} D.M. (2nd year) for MF+CW6%. As a result, the analysis of the results of the incubation experiment showed that the abundance of mold fungi increased from 15 500 CFU g^{-1} D.M. (C) to 252 667 CFU g^{-1} D.M. for MF+CL6%. The applied fertilizer supplements in the form of lignite and leonardite significantly increased the abundance of mold fungi, which exceeded abundance that was found in the pot experiment (Figure 2).

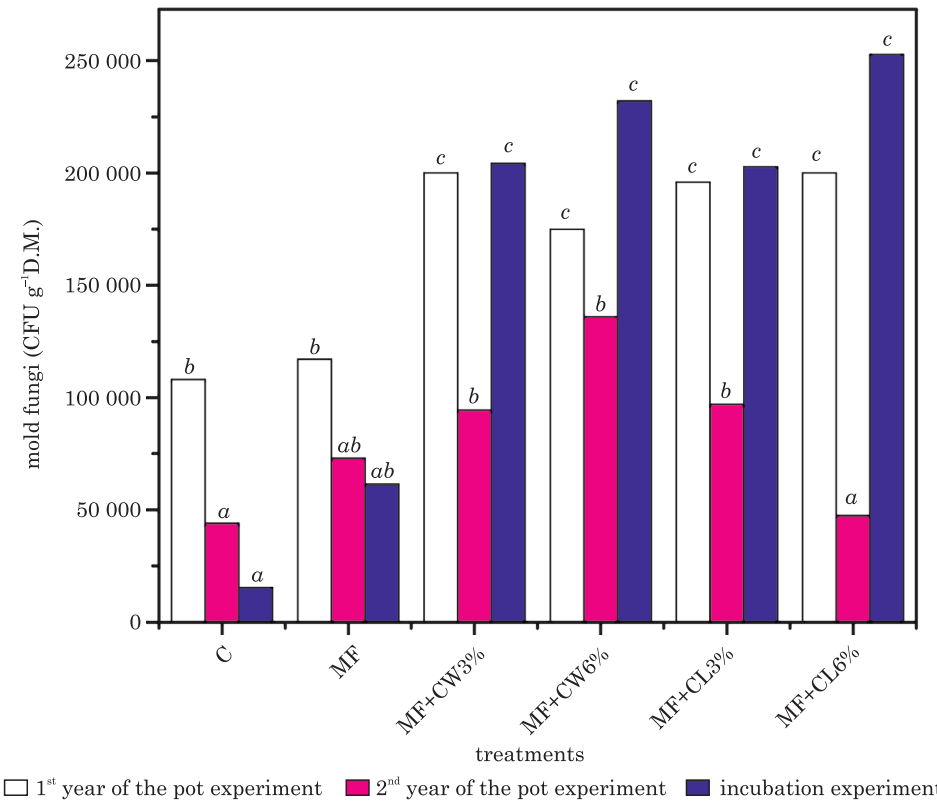

Fig. 2. Average abundance of mold fungi (after the 1st and 2nd year of pot experiment and incubation experiment) in soil samples
Each value represents the mean of four replicates. Different letters indicate a significant difference at $p \leq 0.05$ according to the Duncan's multiple range tests, factors: year×treatment
C – soil without fertilization; MF – soil fertilized with NPK mineral fertilizers; MF+CW3%, MF+CW6% – soil with NPK + 3 or 6% lignite and 3% zeolite-carbon composite (NaX-C); MF+CL3%, MF+CL6% – soil with NPK + 3 or 6% leonardite and 3% zeolite-carbon composite (NaX-C)

NPK fertilization (MF) had the greatest effect on the increase in the abundance of actinomycetes compared to the control (C), and it had the highest average value over the two years of the experiment (3175 CFU g^{-1} D.M.). The abundance of actinomycetes increased from 203 CFU g^{-1} D.M. (1st year) and 1100 CFU g^{-1} D.M. (2nd year) in soil without fertilization (C) to 4000 CFU g^{-1} D.M. (1st year) for MF and 3650 CFU g^{-1} D.M. (2nd year) for MF+CL3%. Actinomycetes were the microorganisms which proved to be difficult in terms of the interpretation of the results obtained. After the first year of the study, they were not detected in three samples of soils containing NaX-C composite: MF+CW3%, MF+CL3% and MF+CL6%, while after the second year of the study the microbial development changed, actinomycetes were detected and

their abundance reached 3650 CFU g^{-1} D.M. for MF+CL3%. The results obtained in the incubation experiment indicate a very strong impact of lignite and leonardite on the population of actinomycetes in the analyzed soil samples. Most notably, in the incubation experiment, much higher abundances of actinomycetes were found than in the pot experiment. The best effects were obtained for leonardite, where the abundance of actinomycetes increased from 5533 CFU g^{-1} D.M. (C) to 65 050 CFU g^{-1} D.M. (MF+CL6%) – Figure 3.

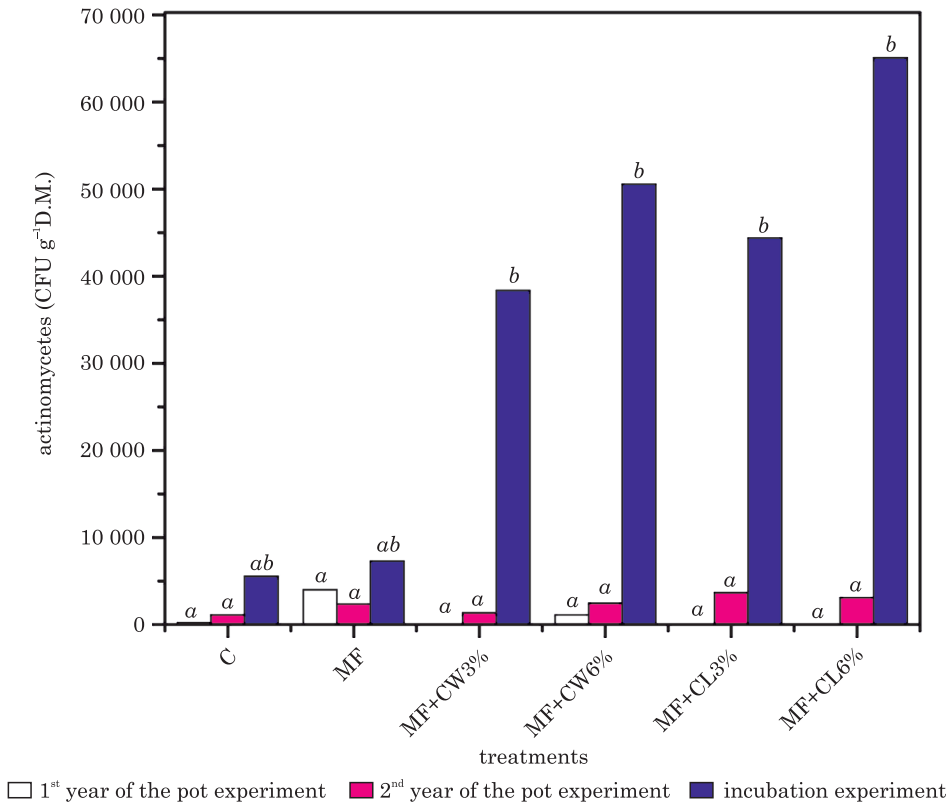

Fig. 3. Average abundance of actinomycetes (after the 1st and 2nd year of the pot experiment and the incubation experiment) in soil samples
Each value represents the mean of four replicates. Different letters indicate a significant difference at $p \leq 0.05$ according to the Duncan's multiple range tests, factors: year×treatment
C – soil without fertilization; MF - soil fertilized with NPK mineral fertilizers; MF+CW3%, MF+CW6% – soil with NPK + 3 or 6% lignite and 3% zeolite-carbon composite (NaX-C); MF+CL3%, MF+CL6% – soil with NPK + 3 or 6% leonardite and 3% zeolite-carbon composite (NaX-C)

The presence of actinomycetes in the soil without fertilizer (C) and in the soil with NPK (MF) was determined, while their absence was revealed in the samples from treatments: MF+CW3%, MF+CL3% and MF+CL6% after the first year of the experiment. In turn, the high increase in the abundance

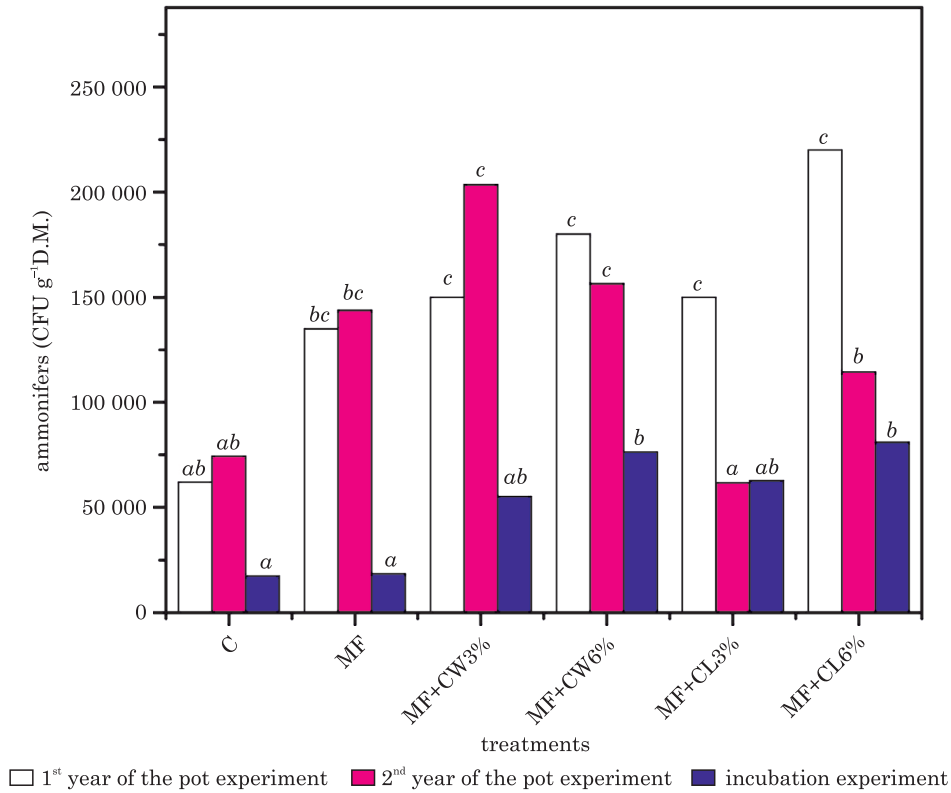

Fig. 4. Average abundance (CFU g^{-1} D.M.) of ammonifiying bacteria (after the 1st and 2nd year of experiment and incubation experiment) in soil samples
Each value represents the mean of four replicates. Different letters indicate a significant difference at $p \leq 0.05$ according to the Duncan's multiple range tests, factors: year×treatment
C – soil without fertilization; MF - soil fertilized with NPK mineral fertilizers; MF+CW3%, MF+CW6% – soil with NPK + 3 or 6% lignite and 3% zeolite-carbon composite (NaX-C); MF+CL3%, MF+CL6% – soil with NPK + 3 or 6% leonardite and 3% zeolite-carbon composite (NaX-C)

of actinomycetes in the incubation experiment indicates the positive effect of fertilization on these microorganisms.

The proposed fertilization measurably increased the abundance of ammonification bacteria (soil without fertilization - C) from 62 000 CFU g^{-1} D.M. (1st year) and 74 300 CFU g^{-1} D.M. (2 nd year) to a maximum of 220 000 CFU g^{-1} D.M. (1st year) for MF+CL6% and 203 600 CFU g^{-1} D.M. (2nd year) for MF+CW3%. Mineral fertilization with NPK also increased the abundance of ammonification bacteria, and it was comparable to the abundance determined in soil samples with the addition of lignite and leonardite combined with zeolite-carbon composite (NaX-C). The analysis of data from the two years of the pot experiment revealed that the highest average abundance (176 800 CFUg^{-1} D.M.) of ammonifiers was achieved in the soil with the

addition of MF+CW3%. Interestingly, after the second year of the experiment a decrease in the abundance of ammonifiers was observed in samples: MF+CW6%, MF+CL3% and MF+CL6%, as was the case with mold fungi. As shown by the results obtained in the incubation experiment, the increase in the abundance of ammonifiers was noticeable, while it was not as spectacular in the pot experiment. The lack of plants in the soil had an effect on the abundance of ammonifiers. The abundance of ammonification bacteria increased from 17 417 CFU g^{-1} of D.M. (C) to a maximum of 81.000 CFU g^{-1} of D.M. (MF+CL6%) – Figure 4.

Redundancy analysis of microbes and environmental factors

Redundancy analysis (RDA) was conducted to assess the relationships between EC, pH, TN, TC, mold fungi, bacteria, actinomycetes, ammonifers, azotobacter spp in soil after 1st and 2nd year of pot experument and incubation experiment (Figure 5). RDA exposed that the studied parameters can

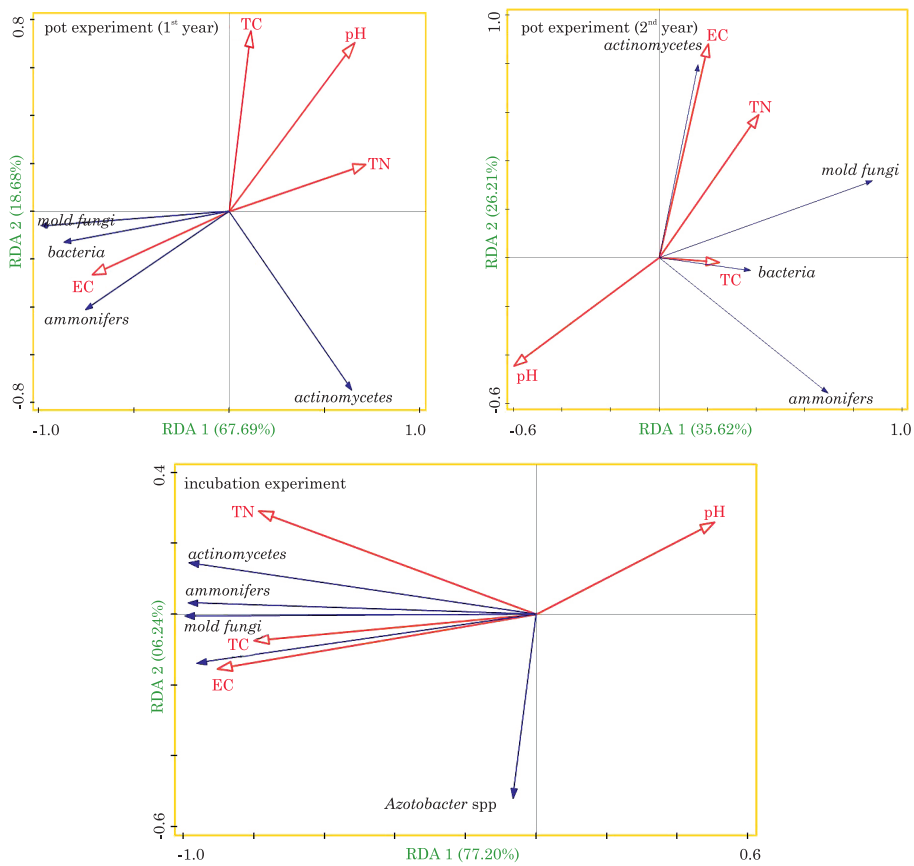

Fig. 5. Redundancy analysis (RDA) indicating the relations between tested microorganisms and selected soil properties from the pot experiment (after the 1st and 2nd year) and properties of soil from the incubation experiment

explain (86.37%) of the total variance after 1st year of pot experiment in soil. The mold fulgi, bacteria, ammonifers, *azotobacter spp* were clusterd to gether with EC and negatively correlated with pH, TC and TN. However, actinomycetes was positively correlated with TN in soil. RDA showed that the studied parameters can explain (61.83%) of the total variance after 2nd year of pot experiment in soil. The actinomycetes proportion was positively correlated with EC and TN, whereas negative correlation was observed with soil pH. Furthermore, mold fungi, bacteria and ammonifers population were positively correlated with EC, but negatively correlated was observed with soil pH. RDA indicated that the studied parameters can explain (83.46%) of the incubation experiment in soil. The actinomycetes, ammonifers were positively associated with TN, whereas negatively correlated with soil pH. Mold fungi, bacteria and *azotobacter spp* were positively correlated with EC and TC, but negative correlation was observed with soil pH.

DISCUSSION

The changes in soil pH values observed after the 2nd year of the study may be due to chemical processes occurring in the rhizosphere Hinsinger et al. (2003). The main process that contributes to pH changes in the rhizosphere caused by roots is the release of charges carried by H^+ or OH^- to balance the uptake of cations and anions at the soil-root interface. Also contributing to the drop in pH may be changes in the rhizosphere caused by the release of organic acids, sugars, phenols, amino acids by plant roots, leading to the accumulation of CO_2 and the formation of complexes of these compounds with toxic metals such as aluminum (Maurer et.al. 2021). Plant roots in an acidic environment produce more organic acids than in a neutral environment, causing detoxification of Al^{3+} ions. As a result of this reaction, the pH of the rhizosphere may increase. The greatest differences in rhizosphere pH values are observed in the uptake of NO_3^- or NH_4^+ (Custos et al. 2020). Finally, plant roots and associated microorganisms can also alter the pH of the rhizosphere through redox coupled reactions or acid phosphatase activity. These various processes involved in rhizosphere pH changes mediated by roots also depend on environmental constraints, particularly nutritional ones, to which plants can be responsive. Custos et al. (2020) reported that when more cations than anions are absorbed by the root cells, protons are released into the apoplast to compensate for the excess positive charges in the cell, thereby resulting in a decrease in the rhizosphere pH. However, when more anions are absorbed, either H^+ is taken up, or OH^- or HCO_3^- (resulting from the reaction of OH^- with CO_2) is excreted, thence leading to the alkalinisation of the rhizosphere. A study by Moeen et al. (2020) showed an increase in soil pH of up to 5%, depending on zeolite dose and incubation duration. In contrast, Doni et al. (2020) demonstrated that soil application

of zeolite in grapevine cultivation had no significant effect on soil pH values. In our study, the addition of NaX-C was relatively small (3% or 6% in the mixture composition) and no alkalizing effect of the mixtures was observed.

In the case of electrolytic conductivity, due to the lack of uptake of elements by the plants, the values of this parameter in the soil from the incubation experiment were much higher than the soil of the treatments from the pot experiment. Other researchers have also reported an increase in the EC of soil with the addition of zeolite (Mahboub 2011, Karami et al. 2020). The increase in soil EC after zeolite application is attributed to the presence of mineral ions in the zeolite, as well as its sorption capacity related to salt retention (Al-Busaidi et al. 2008). Also, according to Nursanti, Supriyanto (2022), there is an increase in pH and EC values in zeolite-treated soil to the high content of Ca^{2+} and Mg^{2+} ions.

Ours research shows taht, the highest decrease in carbon content was observed in the treatments with the leonardite-containing mixture (MF+CL3% and MF+CL6%). This may have been a result of soil microorganisms exploiting a readily available carbon source derived from leonardite. Leonardite is a fossil (an intermediate form between peat and lignite) that is the product of a humification process of organic matter lasting tens of millions of years. Leonardite is rich in humic acids (the leonardite used in the study contained between 75-86.6%), which provide a source of readily available carbon for microorganisms (Nardi et al. 2017).

Supplementation of external organic matter to the soil in the form of fertilizers affects changes in the abundance and biodiversity of the microbial populations inhabiting it, and the increase or decrease in their activity (Tian et al. 2016). Soil microorganisms, including the bacteria, mold fungi, actinomycetes and ammonification bacteria identified in this study, are actively involved in shaping soil fertility and providing nutrients to plants, as well as decomposing harmful substances (Mierzwa-Hersztek et al. 2020).

The increasing of total abundance of bacteria in fertilized soil indicates favorable soil conditions for these microorganisms, the presence of nutrients and an optimal pH for them (Malinowski et al. 2019). By contrast, in the case of mold fungi, it is clear that both the applied fertilization and its absence (C) induced a decline in the abundance of these microorganisms after the second year of the pot experiment. This may be due to the insufficient abundance of soil nutrients for the microorganisms. Even the addition of external mineral and organic matter in the form of the proposed fertilization did not balance these deficiencies. When analyzing the pH of the soil should be considered fully favorable in the context of mold fungi, and close to optimal for bacteria (Rousk et al. 2009, Malinowski et al. 2019).

Actinomycetes are microorganisms that are isolated in large numbers from soils, where, as saprophytes, they decompose dead organic matter contributing to the formation of humus. Additionally, they have an aptitude for breaking down substances that are difficult to degrade, including lignin,

chitin and cellulose (Bereza-Boruta 2002). For actinomycetes, as for mold fungi, higher abundances of these microorganisms were found in the incubation experiment (lacking the test plant – maize) than in the pot experiment. Our results show that there are no simple correlations involving transparent soil-plant-microbe interactions. The presence or absence of plants in the experiment affected the abundance of the analyzed microorganisms, while no clear hypotesis of these complex interactions can be built based on this (Mierzwa-Hersztek et al. 2020). Microorganisms respond to their surrounding environment in a variety of ways. As can be seen, the addition of exogenous organic matter in the form of lignite and leonardite combined with zeolite-carbon composite (NaX-C) compensated for their lack of plants in the soil environment. Our functionalized material (NaX-C zeolite composite), due to its unique properties and porous structure, retains water, binds nutrients, and provides an ideal substrate for microorganisms to grow and develop (Jarosz et al. 2022, Mokrzycki et al. 2023, Szerement et al. 2023). Given the above, this leads us to conclude that the proposed fertilization based on lignite and leonardite in conjunction with the NaX-C composite is not unequivocally optimal for actinomycetes. They can survive unfavorable conditions in a dormant state or produce various types of endospores and remain in this form for a long time and, as the results indicate after the second year of the experiment, return to a vegetative form and be detected in the analysis (Wolny-Koładka, Żukowski 2019).

Numerous bacteria are involved in the ammonification process, i.e., *Bacillus* sp., *Clostridium* sp., *Proteus* sp., *Pseudomonas* sp., *Serratia* sp. and *Escherichia coli*, which convert amino acids to ammonia. Biochemical transformations occur mainly due to the presence of deaminases, enzymes secreted by microorganisms. Ammonification is a desirable process, since compounds that are hard for plants to access are converted into easily assimilable ones (NH_4^+, NO_3^-) – Malinowski, Wolny-Koładka (2017), Jones et al. (2018). A variety of bacteria, with different aerobic, temperature, moisture or pH preferences, are involved in the ammonification process. Because of this, the process of ammonification in the soil can take place regardless of conditions (Jones et al. 2018). As shown by the results obtained in our study, the proposed fertilization measurably increased the abundance of ammonification bacteria (soil without fertilization – C) from 62 000 CFU g^{-1} D.M. (1st year) and 74 300 CFU g^{-1} D.M. (2nd year) to a maximum of 220 000 CFU g^{-1} D.M. (1st year) for MF+CL6% and 203 600 CFU g^{-1} D.M. (2nd year) for MF+CW3%. The enormous biodiversity of this group of microorganisms obtained in ours research and the coexistence of different species of ammonification bacteria means that they can fight each other or interact with each other on a synergistic basis. Given the vastness and complexity of the soil environment, many of the processes that take place there are not recognized at all, while others are not fully explained or understood. Therefore, there is a need to identify more soil microorganisms and learn more about the interactions in which they are

involved, as well as to make a thorough analysis of the relationships so far unexplained (Schmidt et al. 2019).

Such efforts in the context of proposing innovative fertilizer formulas are particularly valuable and contribute to the development of sustainable agriculture. An additional aspect necessary to consider when suggesting new fertilizer formulas is the soil-plant-soil microbiome interaction. In the incubation experiment, a positive effect of fertilization on the increase of bacterial and ammonifer abundance was observed, while it was not as spectacular as in the pot experiment. Considerably better results in the incubation experiment were achieved for mold fungi and actinomycetes, whose abundance even exceeded the one detected in the vase experiment. As can be observed, it is not possible to determine one simple relationship regarding the effect of the proposed fertilization in the context of the absence/presence of the test plant on the microorganisms studied. One reason being that these relationships are remarkably complex and multi-level. The strongest interactions between microorganisms and plants take place in the root zone, known as the rhizosphere (Moore-Kucera, Dick 2008, Zhou et al. 2016, Rao et al. 2021).

As the study shows the type of crop grown has a huge impact on the abundance and species composition of soil microbes due to the variability of root exudates and the presence of crop residues and symbiotic associations. In turn, soil microbial communities make nutrients available to plants by decomposing plant residues and producing siderophores (Chamberlain et al. 2020). Newton et al. (2021) claim that the most intense changes in the population of soil microorganisms due to interactions with plants occur with crop rotation, due to the diversity of crops grown. Meanwhile, with continuous multi-year cultivation of one type of crop, such as maize or wheat (monoculture), the diversity of soil bacteria is lower than with crop rotation and decreases over time. The interaction of soil microorganisms with plants and their soil secretions leads to changes in the structure of the microbial community, which has not yet been thoroughly characterized due to the extraordinary complexity of the processes underway (Navarro-Noya et al. 2013). Nonetheless, most authors agree that the cultivation of plants has a stimulating effect on soil microbial abundance, which was also confirmed in the current study (Rao et al. 2021).

RDA showed that the studied parameters can explain (61.83%) of the total variance after 2nd year of pot experiment in soil and that the studied parameters can explain (83.46%) of the incubation experiment in soil. The obtained in ours research data, clearly indicated that the TN, EC and TN were positive correlated with soil microbes, but negative correlated was observed with soil pH after 1st year pot experiment in soil. In addition, soil pH was dominantly negative correlated with microbes after 2nd year pot experiment and incubation experiment in soil with application of additives. Su et al. (2022) observed that the positive correlation between soil fungul

structure and environmental factors. Bai et al. (2022) used RDA analysis and noted that TP, SOC and EC were the main factors affecting the diversity of composition of bacterial communities. Consequently, Zhao et al. (2023) stated that the bacterial community structure has negative correlation with N, whereas soil pH shows least effect of bacterial community.

CONCLUSIONS

In general, the addition of exogenous mineral and organic matter to the soil caused an increase in the abundance of the analyzed microbial groups in relation to non-fertilized soil. The application of lignite and leonardite (6%) combined with zeolite-carbon composite (NaX-C) had the greatest stimulating effect on bacteria, whereas for fungi it was also the addition of 6% lignite, while for actinomycetes only the addition of NPK was sufficient to obtain the greatest increase in their counts. Ammonification bacteria had a very good response to both NPK and carbon and leonardite soil additives combined with zeolite-carbon composite (NaX-C), as their abundance increased significantly with respect to the control. Doubling the addition of organic materials (from 3 to 6%) resulted in a proportional increase in the abundance of only bacteria after the second year of the experiment, so for the soil microbial life, the lower percentage of the supplement (3%) should be considered as fully sufficient. Summarizing the results of the incubation experiment, it is particularly noteworthy that the additives used in the form of lignite and leonardite with zeolite-carbon composite (NaX-C), due to the content of the easily accessible carbon source for microorganisms contained in leonardite and lignite, have a stimulating effect on microorganisms and boost their abundance in the soil. In the incubation experiment, the soil showed higher levels of acidification following the use of mineral-organic mixtures compared to the soil in the pot experiment. In contrast, there was a reverse relationship regarding the carbon content, with the soil after incubation exhibiting much higher carbon content than the one determined in the soil during both years of the pot experiment. Smaller differences were observed in the case of total nitrogen content. The dominant groups in the pot experiment were total bacteria and ammonifying bacteria, while in the incubation experiment, fungi and actinomycetes were the dominant groups.

Competing interests

The authors declare no competing interests.

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