DYNAMICS OF MICROBIAL POPULATION IN THE SOIL DURING BIOREMEDIATION

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Abstract. In the presence of pesticides in the soil, there is a tendency for the predominance of only a few functional groups of microorganisms, thereby affecting not only the general structure of the community, but also different biological processes in the soil. The research presented in this article aims to monitor groups of microorganisms during the bioremediation of soil long-term and complex contaminated with persistent organic pollutants (POPs). The experimental scheme for bioremediation of soil polluted with POPs involves the alternation of anoxic-oxic conditions and the addition of a fertilizer in different concentrations. The microbiological analysis of the polluted soil before bioremediation revealed the predominance of three functional groups of microorganisms: bacteria that assimilate mineral nitrogen, oligonitrophilic bacteria and ammonifying bacteria. Small amounts of micromycetes and *Azotobacter* and total lack of actinomycetes in the polluted soil were determined. In the experimental variants with added fertilizer, after five cycles of bioremediation, the microbial biomass increased significantly (by 15 and 155 times), and the degradation of pesticides was more intense.

Key words: soil pollution; bioremediation; microbial population.

INTRODUCTION

Soil pollution is a global problem, which during the years of industrialization and modernization has turned into a threat, both for the health of the population and biodiversity, and for the food security of the world. The sources of pollution are diverse, starting with domestic activities and ending with industrial ones. In modern agriculture the use of fertilizers and pesticides is a common practice, but over the years it has led to the accumulation of toxic substances in the soil.

Although the use of a number of persistent organic pollutants (POPs) was banned by the Stockholm Convention in 2001, the presence of pollutants and their metabolites in soil and groundwater is still recorded in different countries, which have previously used these pesticides extensively.

In the Republic of Moldova, the main sources of environmental contamination with persistent organic pollutants are intensive agriculture, energy production facilities, transport units, waste incineration, and pesticide industry. The issue of former pesticide depots, which are currently being demolished or partially demolished, and adjacent land contaminated with POPs, which continue to be a source of environmental pollution, remains a priority [3, 8].

Soil pollution with POPs residues has negative effects on the soil microbiome, which in turn affects the fundamental functions of the soil, such as nutrient cycle, soil fertility, availability of nutrients for plants [1, 22].

Currently, bioremediation is considered the most eco-friendly and cost-effective method of remediation of contaminated sites. This technology is used all over the world, including in Europe, and is very promising, especially when it comes to certain contaminated sites [16, 18].

Bioremediation involves the use of the metabolic abilities of microorganisms to reduce soil contamination with pesticides. Microorganisms use pollutants, along with other nutrients, as a substrate in metabolic reactions, reducing pesticides to simpler substances such as CO_2, water, oxides, or mineral salts, which can be used as carbon, mineral, and energy sources [16, 18].

The efficiency of pesticide degradation processes depends on both the characteristics of the pesticides and the properties of the soil and the environmental conditions. In the case of complex and long-term contaminations, only a few groups of microorganisms survive and dominate in the soil, which affects the general structure of the soil microbiome, therefore, changes occur in the biological processes of the soil [6, 7, 11, 14, 21].

The research presented in this article aims to monitor groups of microorganisms during the bioremediation process of soil complex contaminated with POPs in the presence of the fertilizer.

MATERIALS AND METHODS

The object of study was the polluted soil collected from the territory of the former pesticide storage, located near the village Slobozia-Duşca, Criuleni district, Republic of Moldova. Geographical coordinates of the warehouse: 47.1742800600001 N, 29.087525404 E; the approximate area of the site: 7600 m².

Primary sampling of soil samples from the pesticide-contaminated site was performed according to the protocol (GOST 17.4.4.02-2017). After air-drying at 22-23°C and the removal of vegetal parts and other impurities, the samples were ground and sieved (mesh No. 2), homogenized, and sampled by the incremental sampling approach.

Soil pH and soil moisture content were determined using standard methods [5, 9].

Analysis of toxic substances in soil samples was made by gas chromatography coupled with mass spectrometry (GS/MS) technology using Agilent 6890/5973 system. The following methods were used in this work: EPA method 3546 Microwave-assisted extraction; EPA method 8081B Organochlorine Pesticides by gas chromatography; ISO/CD 23646 Soil quality – Determination of organochlorine pesticides by gas chromatography with mass selective detection (GC/MS) and gas chromatography with electron-capture detection (GC/ECD). Soil samples were analyzed by GC/MS with electron capture detector ^{63}Ni μECD, split-splitless injector and HP-5 column: 30 m length, 320 μm internal diameter, 0.25 μm film thickness, maximum temperature of 325°C. Carrier gas was Helium, 1.2 $mL \cdot min^{-1}$, average velocity of 28 cm/s, constant flow. For data collection was used ChemStation software.

The following pesticides or pesticide metabolites were analyzed in the samples: α-, β-, and γ-HCH isomers, hexachlorbenzene (HCB), heptachlor, aldrin, dieldrin, endrin, chlordane, DDT, DDE, and DDD.

Based on the nature of the detected pollutants and the age of the pollution, the bioremediation experiment was designed to focus on stimulating the indigenous soil microflora. The bioremediation was established in plastic jars, each containing 300 g of contaminated soil. The jars with soil were placed outdoors in real environmental conditions. During the experiment the temperature variation was from 18°C at night to 40°C at day in the sun.

The experimental scheme included the following technological compartments:

- **Compartment 1** – the experiment took place under oxic conditions with a constant soil humidity of 60% of the water holding capacity (WHC).
- **Compartment 2** – consisted of cyclic alternation of anoxic and oxic conditions. Anaerobic conditions were created by saturating the contaminated soil with water (80-90% of WHC) in jars sealed with black polyethylene. The duration of the anaerobic phase was 14 days. At the beginning of the aerobic phase, the soil samples were open and mixed. Soil moisture was adjusted to approximately 60% WHC. The aerobic phase took 7 days.

At the same time, at the beginning of the experiment in the Compartment 2, a mixture of mineral and organic compounds in the amount of 3.0% and 6.0% were added as a bioremediation factors.

The fertilizer has the following composition (in report to the mass): wood sawdust – 50.0%; iron filings – 40.0%; organic fertilizer – 10.0%.

Thus, for the bioremediation of the polluted soil, 4 experimental variants were established, as follows in the treatment protocol presented in Table 1.

In this study, we did not pretend to provide a comprehensive microbiological analysis of the studied soil samples, but focused on the main groups of microorganisms that participate in nitrogen transformation in soil. Isolation of soil microorganisms was performed by spread plate method, on nutrient media considered the most informative for the comparative study of microorganisms involved in nitrogen transformation [4, 25]. Thus, the presence of ammonifying bacteria was determined by inoculation on Nutrient agar medium (Oxoid, England); bacteria which assimilate the mineral forms of nitrogen (BAMN) and actinomycetes – on Inorganic Salt Starch agar (ISP No.4) (in grams per liter: 10.0 Starch soluble, 1.0 K_2HPO_4, 1.0 $MgSO_4 \cdot 7H_2O$, 1.0 NaCl, 2.0 $(NH_4)_2SO_4$, 2.0 $CaCO_3$, 0.001 $FeSO_4 \cdot 7H_2O$, 0.001 $MnCl_2 \cdot 7H_2O$, 0.001 $ZnSO_4 \cdot 7H_2O$, 20.0 Agar-agar, final pH 7.2±0.2); micromycetes – on Czapek-Dox agar (in grams per liter: 2.0 $NaNO_3$, 1.0 K_2HPO_4, 0.5 $MgSO_4 \cdot 7H_2O$, 0.5 KCl, 0.01 $FeSO_4$, 20.0 Glucose, 20.0 Agar-agar, final pH 5.0±0.2); oligonitrophilic bacteria and *Azotobacter* spp. – on Ashby's Mannitol Agar (Sigma-Aldrich).

The results were evaluated visual. For each experimental variant, both the groups of microorganisms were counted and their percentage content of the total number was calculated [12]. The dynamics of the microbial population in the soil subjected to bioremediation was evaluated after the first and fifth cycles.

Statistical analysis was performed using MS Excel. All results were expressed as mean of three individual replicates ± CI (confidence intervals). All differences were considered significant at $P<0.05$.

RESULTS

The former pesticide storage CR-Slobozia Duşca 01 is located extremely close to pastures and arable land. In a radius of 300 m from the investigated land, the following risk receptors were identified: arable land/annual crops – distance 5 m, pastures – distance 5 m. In the sector up to 1000 m down on the relief from the investigated land, the following risk receptors were identified: river – distance 450 m, water basin – distance 690 m.

Table 1. Treatment protocol for the bioremediation of the polluted soil

Experimental variants	Soil moistening, % of WHC	Periodic tilling	Amendments, %
Compartment 1			
1 (Control)	60	-	-
Compartment 2			
2	80 / 60	- / +	-
3	80 / 60	- / +	3.0
4	80 / 60	- / +	6.0

In the previous survey, 10 POPs substances were determined in the polluted soil, collected from territory of CR-Slobozia Dușca 01, in concentrations corresponding to the high level of contamination. The organochlorine pesticides, such as HCH, DDT and their metabolites are prevailed, and also the traces of other pollutants: Heptachlor, gamma-Chlordane, alfa-Chlordane, Endosulfan Sulfate, Toxaphene, Trifluralin, and Atrazine were recorded [17].

In our case, the analysis of POPs substances in soil prepared for bioremediation study showed that the main groups of contaminants are HCH isomers (α–, β–, γ–, δ–) and DDT isomers, accompanied with their transformation products (o-p-DDE, p-p-DDE, o-p-DDD, p-p-DDD, o-p-DDT, p-p-DDT). The total concentration of substances from the POPs group was around 600 mg/kg. The determination of the total concentration of POPs in the soil from the Control variant, after five cycles of bioremediation, showed an insignificant decrease in concentration – up to 3%. The change in the concentration of POPs in the other experimental variants is presented in Figure 1.

The obtained results demonstrate the reduction of concentrations of POP substances in each experimental variant. The experimental variant without amendments, maintained in alternating anoxic/oxic conditions (Variant 2) showed a decrease in POP concentration for all bioremediation cycles. This decrease is 12.4% of the initial concentration value: approximately 4.5% for the first two cycles of the experiment, and 2.0% for the last ones. The POPs degradation rate in the soil was in the same level for variant 3. The dynamics of POP concentration reduction in experimental variant 4 was similar for five cycles of changing the redox conditions (6.5-7.0%, for each cycle).

Applying double quantity of the fertilizer to the soil (from 3.0% to 6.0%) facilitated the degradation of harmful substances – from 12 to 24%. This process is accompanied by an increase in the activity of microorganisms in the soil, which are the main agent of degradation of POPs substances.

The initial pH value of the soil before the start of the bioremediation experiment was 7.0. During five remediation cycles in all experimental variants, to a greater or lesser extent, the pH of the soil has increased (Table 2). In the Control and Variant 2, where no fertilizers were added, the soil pH increased by 0.4 units. In Variants 3 and 4, which contained respectively 3.0% and 6.0% of fertilizers, after Cycle 1 the pH decreased; but with the development of redox reactions towards the end of the Cycle 5 the pH increased by 0.9-1.0 units.

The count of microorganisms in the polluted soil revealed that under the influence of toxicants the population is low in numbers, and probably the most resistant microorganisms have survived (Table 3). The number of micromycetes was very low, bacteria of the genus *Azotobacter* spp. were found as single cells, and actinomycetes were not detected.

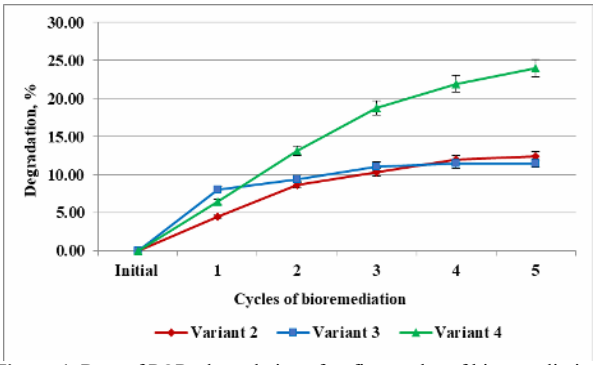


Figure 1. Rate of POPs degradation after five cycles of bioremediation

Table 2. Soil pH value during bioremediation cycles

Experimental variants	Soil pH value				
	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5
1	7.1	7.1	7.2	7.3	7.4
2	7.1	6.9	7.0	7.3	7.4
3	6.9	6.9	7.3	7.5	7.9
4	6.8	7.0	7.3	7.5	8.0

Table 3. Number of microorganisms from different functional groups in the polluted soil, until the initiation of remediation procedures

Groups of microorganisms	CFU / 1 g dry sol	Polluted soil
Bacteria that assimilate mineral nitrogen (BAMN)	$\times 10^6$	1.47 ± 0.26
Actinobacteria	$\times 10^6$	0.00
Oligonitrophilic bacteria	$\times 10^6$	1.75 ± 0.40
Ammonifying bacteria	$\times 10^6$	2.10 ± 0.26
Micromycetes	$\times 10^3$	3.77 ± 1.17
Azotobacter spp.	cells	10.48 ± 0.10

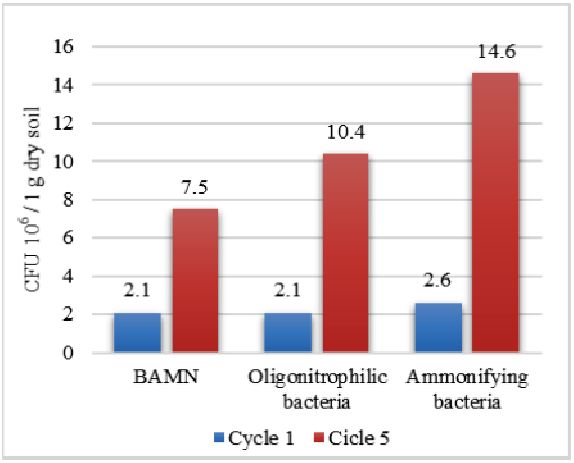
According to some research, micromycetes and actinobacteria can be used as indicators of the degree of soil pollution [12]. Physiological stress of microorganisms, induced by pesticide contamination, leads to changes in the structural and functional diversity of microbial populations. These changes initially lead to the predominance of opportunistic, fast-growing bacteria able to take advantage of transient conditions, followed by slower-growing, resource-efficient microorganisms [14]. The lack of actinobacteria, the low number of fungi and azotobacter (Table 3) in our research, attest to the high degree of soil pollution.

During the bioremediation process in the 4 experimental variants, at the end of each cycle, functional groups of microorganisms, which are involved in the processes of nitrogen transformation, were determined. It was observed that depending on the created conditions there was a change in the population of microorganisms, both in the direction of increasing the number of some groups of microorganisms, and the disappearance of other groups.

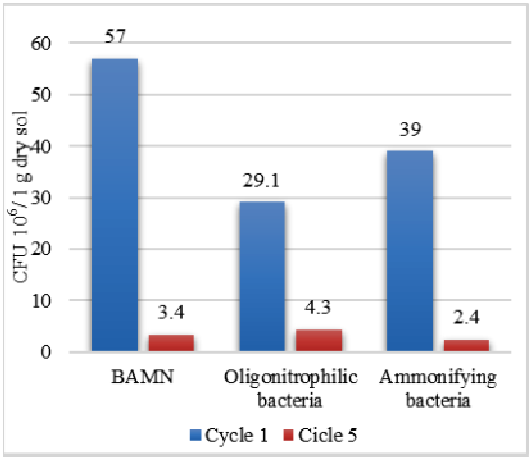
Thus, even after the first cycle and then throughout the bioremediation cycles, microorganisms of the actinomycetes group and bacteria of genus *Azotobacter* spp. have not been registered. After the first cycles, micromycetes were present in very small numbers, and during next cycles they completely disappeared in all bioremediation variants. Predominant were 3 groups of microorganisms: bacteria that assimilate mineral nitrogen, oligonitrophilic bacteria and ammonifying bacteria.

In experimental Variant 1, which was also control, where bioremediation took place under oxic conditions, it was established the gradual increase in the number of microorganisms and towards the end of Cycle 5 the population of bacteria assimilating mineral nitrogen increased by 3.5 times, of oligonitrophiles about 5 times, and that of ammonifying bacteria by 5.6 times.

In Variant 2 where the oxic-anoxic conditions were alternated, after Cycle 1 of bioremediation there was a significant increase of the population of microorganisms involved in the nitrogen transformation processes. But starting with Cycle 2, the number of microorganisms began to decrease, so that towards the end of Cycle 5 there was a drastic decrease: the number of bacteria that assimilate mineral nitrogen decreased by 16.7 times, the population of oligonitrophils by 6.7 times, and the of ammonifying bacteria by 16.3 times.



Variant 1



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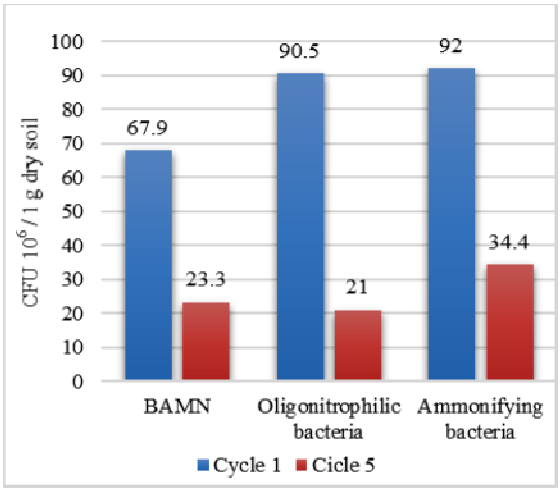
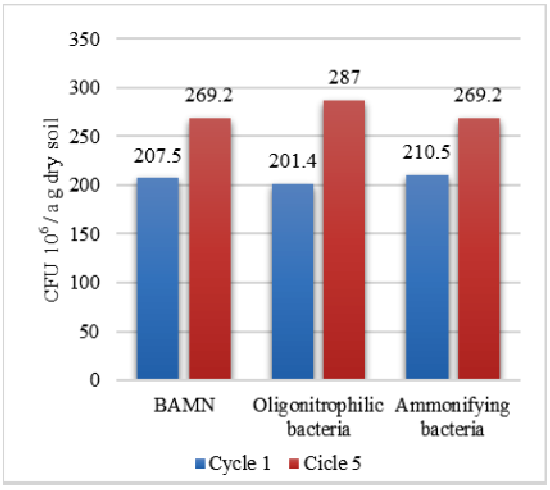


Variant 3



Variant 4

Figure 2. Change in the number of microorganisms after five cycles of bioremediation

The same picture was observed in Variant 3, where alternating oxic-anoxic conditions were maintained and a mixture of fertilizer in the amount of 3.0% was introduced, the significant increase in the population of microorganisms after Cycle 1 and decrease in their number in the next cycles. Thus, at the end of Cycle 5, the number of bacteria that assimilate mineral nitrogen decreased approximately 3 times, the population of oligonitrophiles by 4.3 times, and that of ammonifying bacteria by 2.7 times. Compared to Variant 2, the addition of the fertilizer to the soil favored the viability of the microorganisms.

Unlike Variants 2 and 3, where at the end of the remediation period there was a decrease in the number of microorganisms, in Variant 4 the population of microorganisms increased. In this variant the fertilizer was added in an amount of 6.0%. Thus, after Cycle 5, the number of bacteria that assimilate mineral nitrogen increased by 30%, the population of oligonitrophiles by 42.5%, and of ammonifying bacteria by 28%.

If we compare the total number of microorganisms in the soil samples after 5 cycles of bioremediation (Fig. 3), then it is obvious that Variant 4 of bioremediation was the most favorable for the growth of microorganisms. Compared to the polluted soil, the population of microorganisms in Variant 4 increased by 155 times, and compared to variant 1 (Control) by 25.4 times. Remediation by alternating oxic-anoxic conditions (Variant 2) did not have such major effects on the population of microorganisms, while the addition of the fertilizer (Variants 3 and 4), under the same conditions, significantly stimulated their viability. The optimum concentration for the growth of microorganisms was 6.0%.

Although, during the remediation cycles, groups of microorganisms such as micromycetes and bacteria of the genus *Azotobacter* disappeared, at the end of Cycle 5 in the soil samples 3 groups of microorganisms predominated, among those studied by us: bacteria that assimilate mineral nitrogen, oligonitrophilic bacteria and ammonifying bacteria (Fig. 4). The analysis of the ratio of these groups of microorganisms in the polluted soil, showed that the ammonifying bacteria predominate, followed by the oligonitrophilic bacteria, then by the bacteria that assimilate the mineral nitrogen.

After five cycles of remediation under aerobic conditions (Variant 1) the same tendency of predominance of ammonifying bacteria was maintained, the number of which increased, followed by oligonitrophilic bacteria, then by bacteria that assimilate mineral nitrogen, whose population decreased. Bioremediation in alternating of oxic-anoxic conditions (Variant 2) led to the predominance in the soil of oligonitrophilic bacteria, followed by the population of bacteria that assimilate mineral nitrogen, then by ammonifying bacteria. The addition of the fertilizer in different concentrations also influenced the ratio between the populations of microorganisms at the end of the bioremediation cycles. Thus in Variant 3, where the fertilizer concentration was 3.0%, the population of ammonifying bacteria predominated, followed by bacteria that assimilate mineral nitrogen and oligonitrophilic bacteria. In Variant 4, where the fertilizer concentration was 6.0%, the ratio between the 3 groups of microorganisms was 1: 1: 1.

DISCUSSION

The community of microorganisms in the soil inevitably reacts to the presence of pesticides in the soil by changing both the total number of microorganisms and the ratio between the functional groups. In the presence of pesticides in the soil, there is a tendency for the predominance of only a few functional groups, thereby affecting not only the general structure of the community, but also different biological processes in the soil [2, 14]. The same results were also established in our research: the predominance of three functional groups of microorganisms, small amounts of micromycetes and *Azotobacter* and the total lack of actinomycetes in the polluted soil.

With the initiation of bioremediation, micromycetes and *Azotobacter* were no longer detected in the soil. This can be explained by the fact that the decomposition of pesticides generates new chemical substances, which are often more toxic than the original pesticide [13]. Some groups of microorganisms, such as fungi and actinomycetes are discussed to be sensitive to the presence of even traces of xenobiotics in the soil [2, 12]. Another factor would

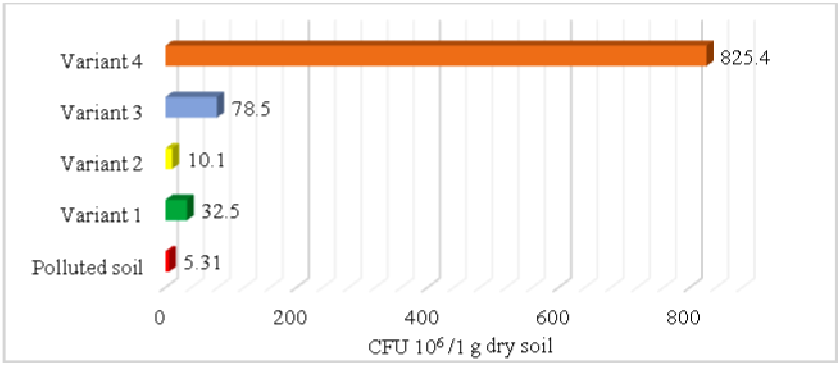


Figure 3. Total number of microorganisms in soil samples after five cycles of bioremediation

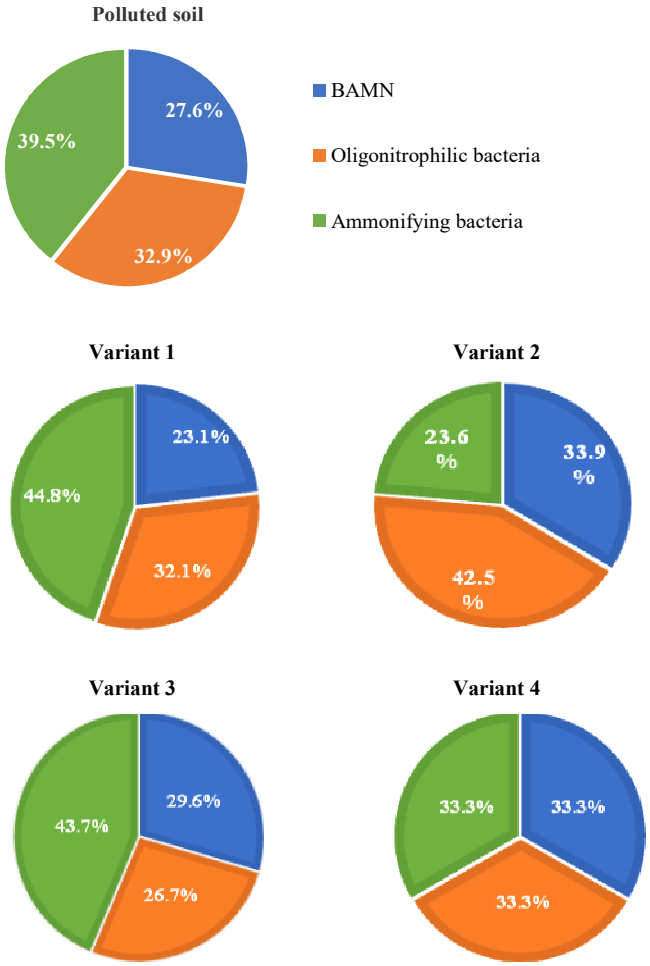


Figure 4. The ratio of the groups of microorganisms in the soil samples

be the change in soil pH towards basic, less preferable for the growth of micromycetes.

It is well known that biodegradation under natural conditions is running slowly mainly due to the poor potential of microorganisms in polluted environments to degrade pollutants. That is why a widespread practice is biostimulation by addition of fertilizers of organic and mineral origin, which play the role of a trigger in switching the metabolic processes of microorganisms and, respectively, stimulating their growth.

Mineral and organic fertilizers, separately, as well as in combination, lead to an increase in the abundance of microorganisms due to an increase in the amount of total nitrogen in the soil. Mineral fertilizers stimulate the growth of ammonifying and nitrifying microorganisms and bacteria that assimilate mineral nitrogen and fungi. Organic fertilizers stimulate organotrophic microorganisms and non-symbiotic diazotrophic bacteria [19, 23, 24]. Thus, the improvement of polluted soils with fertilizers of different origin gave good results in the case of bioremediation of sites polluted with HCH, heavy metals and petroleum products [10, 15, 20].

The results obtained in our research correspond with the data in the literature regarding the addition of fertilizers in purpose to facilitate the degradation of xenobiotic compounds of various chemical natures. With increasing concentration of the fertilizer to 6.0% the degree of degradation reaches the level of 24%. Applying of the fertilizer leads to significant increase in the abundance of soil microorganisms that participate in nitrogen metabolism and also influenced the ratio between the populations of microorganisms at the end of the bioremediation cycles.

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