EFFECT OF IRON NANOPARTICLES AND HERBICIDE TRIFLURALIN ON THE FORMATION OF MICROBIAL CONSORTIA

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Abstract. The aim of our study was to assess the effect of iron NPs (magnetite (Fe_3O_4) and zero-valent iron Fe(0)) on the formation of microbial consortia, in the presence of the herbicide trifluralin. In our researches the increase in the trifluralin concentration in the growth medium led to the decrease of both the density and the number of species of the microbial population. Addition of iron NPs to the culture medium clearly diminished the cytotoxic action of trifluralin on the microbial communities from the enrichment cultures. The effect of NPs on the formation of consortia of microorganisms depended on their chemical structure. Magnetite NPs showed a cytotoxic effect on microbial consortium, especially against bacteria. The population of bacteria in this consortium was well below the control level. Fe(0) NPs had the most favorable effect on the formation of microbial consortium; the resulting trifluralin-resistant consortium consisted of 4 strains of bacteria and 1 fungal strain.

Keywords: microbial consortia; trifluralin; iron nanoparticles.

INTRODUCTION

The production, use and disposal of numerous chemicals that offer improvements in agriculture, industry, medical treatment, and even household chemicals generate increasing concerns about their potential negative effects on human and ecological health, over the last decades. Protecting the integrity of soil and water resources is one of the most essential environmental issues of the 21st century [2, 6].

In the Republic of Moldova huge amounts of pesticides were imported during the intensive agriculture practice in the 70-80s of the 20th century. Many of these substances have not been used for the reason of their ban or obsolescence. During 2006-2010, under the project "Management and Destruction of Persistent Organic Pollutants Stocks" financed by the Global Environment Fund through the World Bank and the Government of the Republic of Moldova and implemented by the Ministry of Ecology and Natural Resources, obsolete and unusable pesticides were repacked and stored in district warehouses, and some were transported abroad for destruction. However, in the Republic of Moldova currently over a thousand of former pesticide storehouses are in the deplorable state and represent a continuous danger for the environment and public health [31, 40].

Among halogenated pesticides trifluralin was also widely used in Moldova. Trifluralin, a synthetic fluorinated dinitroaniline herbicide, was first registered in 1963 and was marketed in a number of names, such as Treflan, Triflurex, Triflusan and others. Trifluralin was considered persistent in soil and the water/sediment systems due to the low degree of mineralization and the formation of high amounts of bound residues, and was shown to be “not readily biodegradable” [7]. During 2010-2011, on the decision of Interdepartmental Council for registration of Plant Protection Products (PPPs) and fertilizers, with acceptance of Ministry of Health and Ministry of Environment, about 30 PPPs containing active substances that are not approved in EU (trifluralin, diazinon, benomyl, carbosulfan etc.) were excluded from the State Register of Phyto-Sanitary Means and Fertilizers allowed for use in the Republic of Moldova. However, residues of this pesticide are registered in soils of Moldova up to the present day.

There are many methods for the removal of pollutants from soils. They involve both physico-chemical and biological approaches. Although the first ones are more effective than biological methods they are expensive and require high energy demand and consumption of many chemical reagents. This is a reason why the use of microorganisms capable of degrading toxic compounds, known as bioremediation, has become an attractive, environmentally friendly technology [6, 10, 28, 36].

One of the difficulties faced by researchers in developing of bioremediation technologies for pesticides is their toxicity to soil microorganisms. Interaction between pesticides and microorganisms can alter the physiological and biochemical behavior of soil microbes, which can lead to the inhibition or kill of certain groups of microorganisms. Some microbial groups are capable of using pesticide as a source of energy and nutrients to multiply. Thus, the application of pesticides reduces microbial diversity but increases functional diversity of microbial communities [18]. Due to the complex interactions that occur between consortium members – signal communication, horizontal gene transfer, competitive relationships or cooperation, microbial communities have a higher capacity to survive stress and broader degradation capacity, compared to single cultures [13, 28, 36].

The ability to adapt to hostile environmental conditions, due to the genetic heterogeneity of microbial communities inhabiting pesticide-contaminated soil, is used by researchers to create consortia able to survive and degrade xenobiotic substances. There is a series of researches that demonstrate the effectiveness of bacterial consortia to

degrade various pollutants [6, 10, 11, 13, 19, 24, 26, 36, 37].

Among metal-based engineered nanomaterials, iron nanoparticles (NPs) are, probably, the most used for bioremediation of a broad spectrum of pollutants, including halogenated organic chemicals, polycyclic aromatic hydrocarbons, pesticides and heavy metals [8, 29, 35, 38]. Remediation of polluted soil and groundwater with iron-based NPs represents a faster, cheaper and a potentially more effective treatment option than current *ex situ* and *in situ* methods. But the direct application to soils of large amounts of iron-based NPs for remediation purposes raises specific concerns about potential consequences on soil microbial communities and their key functions for soil fertility and biodegradation of pollutants [16, 39].

There are data that metal NPs can influence the bacterial growth kinetics both in the direction of stimulation, or inhibition. Regarding iron NPs, they demonstrated low negative effects, and, in most of cases, stimulated the growth of microbial consortia [5, 9, 15, 20].

Previously, two consortia of microorganisms were isolated by us from the polluted soil, using the method of enrichment cultures, and adapted to high concentrations of toxicants. Throughout the process of adaptation to high concentrations of DDX / HCH, the environmental toxicity and the systematic groups of microorganisms (fungi, bacteria, actinomycetes) were monitored. Research has shown that in the presence of the toxicants the microbial cenosis of the soil had restructured, and the microbial diversity decreased, but more resistant species survived [34].

Taking into consideration both the growing popularity of NPs in technologies for detoxification of contaminated environments, as well as the proven efficacy of iron NPs in persistent organic pollutants (POPs) remediation, the aim of our study was to assess the effect of iron NPs on the formation of microbial consortia, in the presence of the herbicide trifluralin.

MATERIAL AND METHODS

Materials. In our experiment we used magnetite (Fe_3O_4) NPs and zero-valent iron (Fe(0)) NPs in the form of a colloidal aqueous solution.

Encapsulated NPs Fe_3O_4-PVP and Fe(0)-PVP were synthesized by chemical co-precipitation method, in the presence of poly-N-vinylpyrrolidone (PVP) used as a stabilizer. Fe_3O_4 NPs were prepared using iron(II) sulfate and iron(III) chloride. Fe(0) NPs were prepared by chemical reduction from ferric chloride solution [14, 25].

Iron(II) sulfate (≥ 99.7%), a saturated iron(III) chloride solution (≥ 99.0%), poly-*N*-vinylpyrrolidone (PVP, MW: 8000), and ammonium hydroxide (≥ 99.9%) were purchased from Sigma-Aldrich.

The resulting Fe_3O_4 NPs and Fe(0) NPs were characterized by X-ray powder diffraction (XRD) analysis, X-ray fluorescence analysis (XRF), scanning electron microscopy (SEM), and FTIR-spectroscopy.

Trifluralin (α, α, α-trifluoro-2-6-dinitro-N-N-dipropyl-p-toluidine), a pre-emergent herbicide belonging to the dinitroaniline chemical family, was used as a solution in acetone at stock concentration of 100 mg/mL.

Soil sampling. Soil was collected near the former storage of persistent organic pesticides located in the central part of Republic of Moldova, Chişinau municipality, Sîngera village. Soil sample was cleaned of roots and other impurities, homogenized, sieved (mesh No. 2) and air-dried at 22-23°C.

Media. A mineral salt medium PAS was used for the isolation of microbial consortia. The composition of the media is (per liter): 4.35 g K_2HPO_4, 1.7 g KH_2PO_4, 2.1 g NH_4Cl, 0.2 g $MgSO_4$, 0.05 g $MnSO_4$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.03 g $CaCl_2 \cdot 2H_2O$ [42]. The pH of the media was 6.5. The density and diversity of the microorganism's population was determined on the Nutrient Agar medium (Oxoid, England) and Czapek-Dox agar (per liter: 2.0 g $NaNO_3$, 1.0 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4$, 30.0 g Glucose, 30.0 g Agar-agar, pH = 4.5-5.0). For identification of bacteria were used King Agar B (Sigma-Aldrich, USA) and Nutrient Agar media; for identification of fungi was used Czapek-Dox agar.

Research methods. The consortia of microorganisms were obtained by the enrichment cultures method. In the conical flask with 100 mL of PAS medium 10 g of contaminated soil were added and incubated at 24-28°C on a shaker (180-200 rpm). After 7 days, 10 ml inoculum was transferred into 90 ml of PAS medium, previously enriched with trifluralin in concentration of 100 mg/L and colloidal solutions of NPs (Fe_3O_4 or Fe(0)) in concentration of 25 mg/L and incubated under similar conditions for the next 7 days. Thus, three experimental variants were formed:

1) PAS + trifluralin (100-400 mg/L);

2) PAS + trifluralin (100-400 mg/L) + nano Fe_3O_4 (25-100 mg/L);

3) PAS + trifluralin (100-400 mg/L) + nano Fe(0) (25-100 mg/L).

The procedure for transferring the enrichment cultures was repeated exactly at the following passages. Totally 12 passages were performed. Over each 3 passages (1 enrichment cycle) the concentration of trifluralin in the PAS medium increased on 100

mg/L and concentrations of Fe_3O_4 and Fe(0) NPs were doubled. Each cycle was repeated 4 times so that the final concentration of trifluralin in the PAS medium was 400 mg/L and concentrations of Fe_3O_4 and Fe(0) NPs were 100 mg/L (Fig.1).

After passage 1 (without trifluralin) and at the end of each cycle, 1 mL of culture media was used for serial dilution followed by spreading of 50 µL on plates with Nutrient Agar and Czapek-Dox agar with trifluralin, in concentrations corresponding to each cycle, and incubated at 28°C for 3 days. The last passage was spread on the PAS medium plate

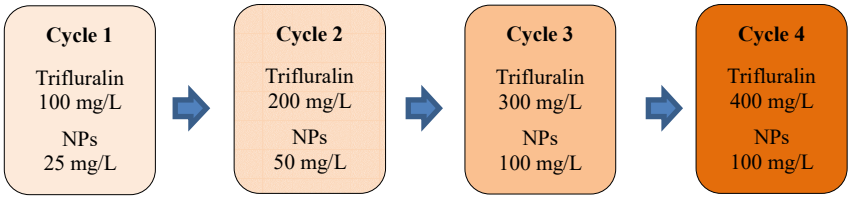

Figure 1. Experimental design for the isolation of microbial consortia

containing trifluralin as a sole source of carbon and energy, and the density (colony forming units – CFU/mL) and the number of species of the microorganisms' population were estimated.

The resulting colonies were isolated and spread onto plates containing fresh King Agar B (selective medium for *Pseudomonas*) and Nutrient Agar media for bacteria and Czapek-Dox agar for fungi to purify trifluralin-tolerant isolates. All the isolates were studied for their colony morphology, cell morphology (Gram reaction), pigmentation and spore production as per Bergey's Manual of Determinative Bacteriology [17]. Plates with King B medium were examined under UV light for detection the production of fluorescent pigments by *Pseudomonas* species [22]. The identification of fungi isolates was performed according to the colonial aspects and microscopic morphology [12]. The morphological characters observation of isolates was performed with Leica 2500DM optical microscope.

RESULTS

The shape of Fe_3O_4 NPs was spherical, the size was of 20-25 nm, then NPs agglomerated in solution into larger entities with a size of 25-39 nm; Fe(0) NPs size was computed to 4-4.7 nm. IR spectra of Fe_3O_4-PVP NPs, Fe(0)-PVP NPs, and PVP proved the formation PVP-encapsulated nanoparticles. X-ray diffraction confirmed the crystallinity of the resulting magnetite NPs. Size d of the Fe_3O_4 crystallites is d = (25 ± 1) nm; this value corresponds to microscopic data. The presence of Fe(0) was confirmed by the diffractogram of the Fe(0)/PVP NPs, with the maximum diffraction at 2θ = 44.8° . Particles size was computed according to Debye-Scherrer formula, which corresponds to 4 nm. The X-Ray Fluorescence Spectroscopy (XRF) of encapsulated nano-sized iron (~ 4.7 nm) showed that the transition energy corresponding to the gravity centre of the FeK-α_1 line shifts by 1.33 eV as a result of a transition from a flat polycrystalline sample to nanoscale iron.

Considering physical and chemical properties of the collected soil, the soil type was classified as carbonated chernozem. Analytical data indicated that the studied site was long-term and complex polluted by pesticides, the major component of contamination was trifluralin (19.67 mg/kg dry soil) and the minor component was organochlorine insecticide DDT (0.37 mg/kg dry soil) and its metabolites DDE and DDD (DDTs); the total content of pesticides was 21.5 mg/kg dry soil [33].

To assess the density and number of species of microorganisms in the polluted soil, inoculation on Petri dishes was performed at the beginning of the experiment, after the first passage. The results showed that, although numericaly the bacterian population exceeded the population of fungi, as a diversity, the fungi in the polluted soil were represented by more species (Table 1).

Table 1. Density and number of species of microorganisms after the first passage

Density / Diversity	Microorganisms	
	Bacteria, × 10^6	Fungi, × 10^3
CFU / mL	46.04 ± 4.53	20.20 ± 2.69
Number of species	6	7

After the addition of trifluralin and nanoparticles to the PAS medium, have occurred numerical changes in the population of microorganisms. The population of bacteria and fungi from the enrichment culture, grown in the presence of trifluralin, increased exponentially, and exceeded the initial values (passage 1) (Fig. 2). For bacteria, the CFU index reached maximum values at the Cycle 3 (by 12 times), and for fungi at the Cycle 2 (by 23 times). At the end of Cycle 4 the density of

microorganism population decreased significantly, however, remained higher than in the first passage by 2.4 times for bacteria and 4 times for fungi. As for species diversity, the addition of trifluralin to the culture medium has led to a decrease in the number of species after the Cycle 1, from 6 to 4 for bacteria and from 7 to 4 for fungi. After the increase in trifluralin concentration, the diversity of microorganisms had reduced, so at the end of the Cycle 4 the consortium consisted of 3 strains of bacteria and 1 fungus strain.

Trifluralin and NPs of magnetite when added to the culture medium resulted in a 4.8 times increase of the bacteria number after the Cycle 1 (Fig. 3). But, with the increase of trifluralin and magnetite concentrations, the index of CFU of bacteria decreased dramatically, having much lower values than at the beginning of the experiment, and by the end of Cycle 4 the number of bacteria was with 41% lower than after the first passage. Unlike bacteria, the population of fungi throughout the cultivation period had significantly higher values than in the first passage, reaching maximum density (by 31 times) after the Cycles 2 (Fig. 3). By the end of the Cycle 4, as a result of the increase of trifluralin and magnetite concentrations, the numerical diminution of fungi occurred, but their number still remained higher on 5 times, compared to the passage 1. The diversity of species of microorganisms in the enrichment culture also reduced, especially in the case of fungi. Following each cycle, the number of species of bacteria and fungi decreased so at the end of the Cycle 4, the consortium consisted of 3 bacterial and 1 fungus strain was obtained.

Compared with NPs of magnetite, Fe(0) NPs had more beneficial effects on the formation of the consortium of microorganisms, both in terms of density as well as number of species (Fig. 4). The bacterial CFU index increased considerably compared to 1 passage, reaching maximum values after the Cycles 3 (by 150 times), and the CFU of fungi after the Cycles 2 (by 68 times). Increased concentration of trifluralin resulted in a decrease in the number of microorganisms at the end of the Cycle 4, but, compared to passage 1, the CFU values remained high, especially at bacteria (by 36 times). The diversity of the microorganisms in the enrichment culture reduced, especially that of the fungi, so at the end of the Cycle 4 the consortium was made up of 4 strains of bacteria and 1 fungal strain.

The analysis of cultural and morphological characters of the isolated bacterial strains showed, that the 3 consortia consists of bacteria belonging to the genus *Bacillus* (strains No. 2, 5, and 8, and No. 3, 6,

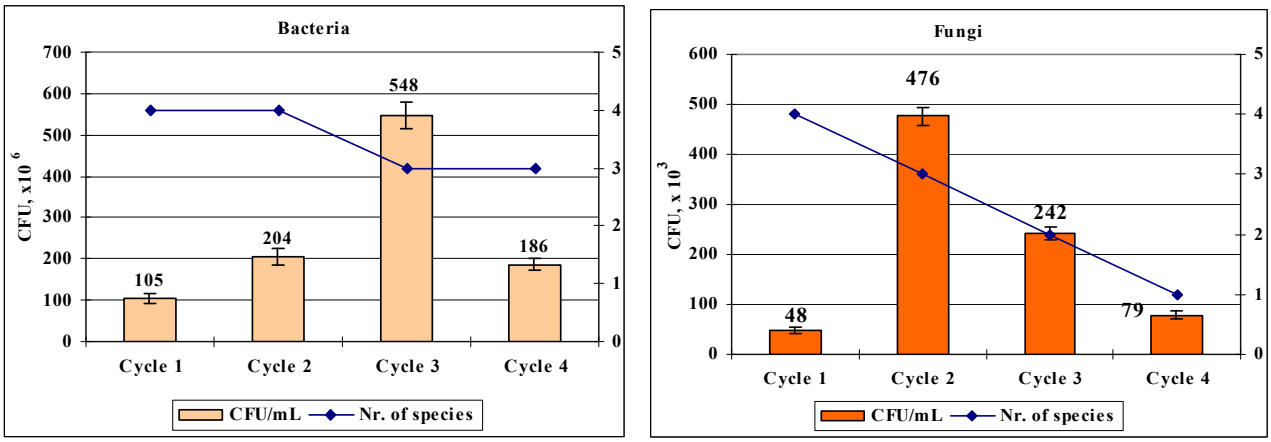

Figure 2. Modification of the density and number of species of bacteria and fungi from enrichment culture, cultivated in the presence of trifluralin

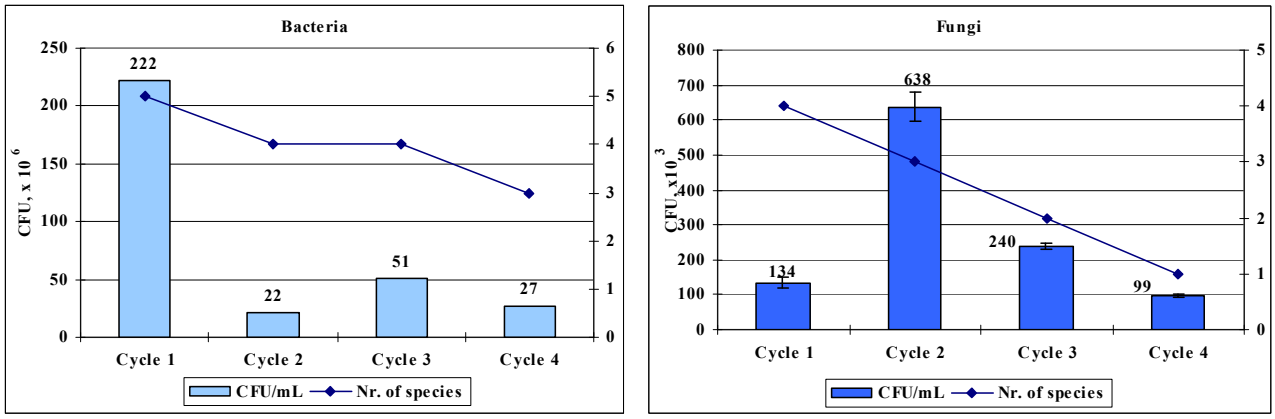

Figure 3. Modification of the density and number of species of bacteria and fungi from enrichment culture, cultivated in the presence of trifluralin and NPs of magnetite

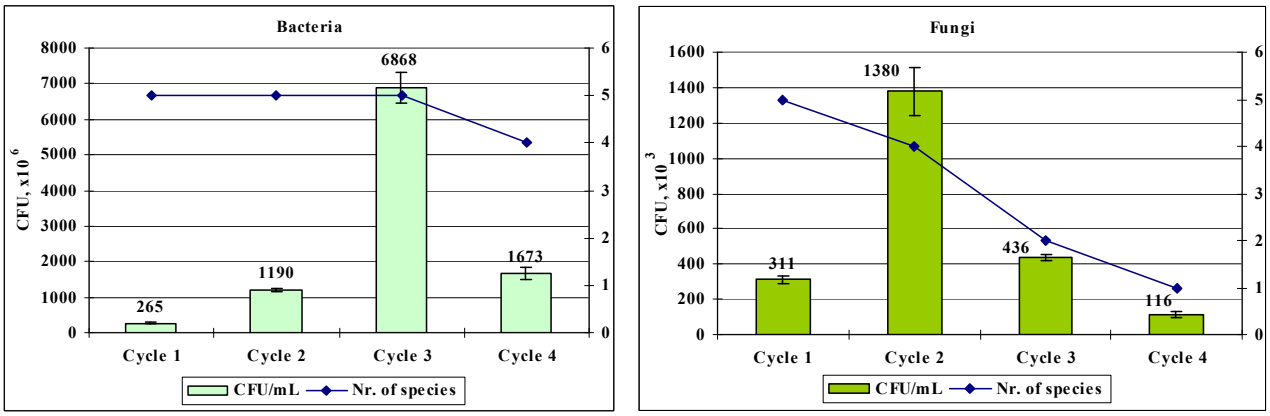

Figure 4. Modification of the density and number of species of bacteria and fungi from enrichment culture, cultivated in the presence of trifluralin and NPs of Fe(0)

Table 2. Cultural and morphological characteristics of bacterial isolates

Isolate	Nutritive medium	Colony characteristics				Microscopic observations		
		Shape	Color	Margin	Surface	Shape	Sporulation	Gram reaction
No. 1, 4, 7	King's B	Round	Yellowish green	Round	Smooth shiny	Shot rods	Negative	Negative
No. 2, 5, 8	Nutrient agar	Irregular	Yellow	Irregular	Rough	Shot rods	Positive	Positive
No. 3, 6, 9	Nutrient agar	Irregular	Pale	Irregular	Rough	Shot rods	Positive	Positive
No. 10	King's B	Round	Green	Round	Smooth shiny	Long rods	Negative	Negative

and 9) and to the genus *Pseudomonas* (strains No. 1, 4, and 7, and No. 10) (Table 2). Isolates No. 1, 4, and 7, and No. 10 were found to produce fluorescent yellow-green or bluish green diffusible pigment of variable intensities on King's B medium under UV light.

On the Czapek Dox agar the colonies of isolated fungi have floccose texture, with white aerial mycelium and colorless reverse. Mycelium consists of branched hyphae. Macroconidia are three to five-septate (frequently three-septate), fusiform, cylindrical, often moderately curved. Microconidia are abundant, cylindrical to oval, one to two-celled and formed from lateral phialides. According to the microscopic data, the strain was determined to belong to the genus *Fusarium*.

Thus, the consortium obtained by enrichment culture on the PAS medium with trifluralin was composed by the strains *Pseudomonas sp. 1, Bacillus spp. 2 and 3, and Fusarium sp.* All three species of bacteria were found in the consortium in equal proportions.

The consotium isolated on medium PAS+trifluralin + Fe_3O_4 NPs was composed by the strains *Pseudomonas sp. 4, Bacillus spp. 5 and 6, and*

Fusarium sp. Isolate *Bacillus sp.* 5 was predominant in the consortium.

The consotium obtained on medium PAS+trifluralina + Fe(0) NPs was composed by the strains *Pseudomonas spp. 7 and 10, Bacillus spp. 8 and 9, and Fusarium sp*. Isolate *Pseudomonas sp. 10* was discovered only in this consortium.

DISCUSSION

In long-term pollution environmental sites the natural self-cleaning mechanisms contribute to the development of specific microorganisms. However, at high concentrations of pollutants in soil and water, natural bioremediation processes occur slowly. In soil, pesticides suffer a variety of degradative, transport, and adsorption / desorption processes, depending on the chemical nature of the pesticide and soil properties. The soil organisms also interact with pesticides, which inevitably can lead to changes in their physiological and biochemical behavior [18, 21]. The literature describes the facts of changes in the diversity of soil microorganism population under the influence of various substances that pollute the environment [27, 39].

In natural environments, the microbial diversity, that underlying microorganism's communities, allows an increase of metabolic capacity, division of labor, and survival in unfavorable conditions [36]. The indigenous communities exposed for a long time to the action of xenobiotic substances become acquainted, exhibiting selective enrichment and genetic modifications. The adapted microbial communities can respond to the presence of pollutants within a relatively short span of time and exhibit higher biodegradation rates than communities with no history of exposure in such conditions [30].

The presence of a microbial community, formed under mixed and lasting pollution, was also determined by us at the beginning of the experiment (Table 1). With the increase in the concentration of trifluralin in the growth medium, both the density and the number of species of the population decreased, but, finally, consortia resistant to high pesticide concentration were formed.

Microorganisms communities, formed at the end of the Cycle 4, were predominantly consisted of 3-4 species of bacteria and 1 fungal species. The fact that, with the increase of the concentration of toxic substances in the soil, the decrease in average values of the number of microorganisms in the soil, especially fungi, actinomycetes and cellulolytic microorganisms, was observed by other scientists [27]. In our research, the sudden decrease in the number of fungi in all experimental variants took place during the Cycle 3 that could be explained by the fact that trifluralin concentrations higher than 200 mg/L are toxic to most fungi in the population. Another factor that influenced the viability of the fungal population was the pH of the medium (6.5), which is more favorable for bacteria. The bacteria population has proved to be more resistant to the pesticide. The decrease in number of CFU occurred during the Cycle 4, i.e. at trifluralin concentrations higher than 300 mg/L.

Addition of iron NPs to the culture medium clearly diminished the cytotoxic action of trifluralin on the microorganism communities from the enrichment cultures. This was best observed in the Cycle 4, where, compared to the Cycle 3, microorganisms were cultivated in a higher concentration of trifluralin (400 mg/L), whereas concentration of iron NPs remained unchanged (100 mg/L).

Currently iron NPs are used not only to remediate contaminated environments, but also to stimulate the activity of microorganisms [1, 5, 8, 20, 23, 41]. However, there are researches that demonstrated that iron NPs added to soil perhaps have an impact on both the compositional structure and functional capacity of the soil microbial community [39]. The iron NPs action appeared to be selective, inhibiting some microbial groups but promoting the dominance of others [15, 41].

In our case the effect of iron NPs on the formation of consortia of microorganisms depended on the chemical composition of NPs. Fe(0) NPs had the most favorable effect on the formation of the consortium of microorganisms. By the end of the Cycle 4, the consortium had the highest density and diversity of the population of microorganisms (4 strains of bacteria and 1 fungal strain).

The microorganisms, especially bacteria, have been shown to be more sensitive to magnetite NPs. The concentrations of magnetite NPs higher than 25 mg/L led to a sharp decrease in the number of bacteria, but not in the number of fungi in the same consortium. The decrease in the number of fungi occurred most likely due to the increase of trifluralin concentrations. The negative effect of magnetite NPs could have several explanations. In the literature there are data that in metal oxide NPs the surface characteristics such as charge and reactivity are intensified, which therefore make them potentially more dangerous to organisms [9]. In addition, all iron NPs could act cytotoxically on living organisms, which is due to several aspects: 1) nanoparticles exhibited magnetic properties that might interact with the electric polarity of the bacteria and influence their growth; 2) in biological systems the iron NPs could induce oxidative stress, resulting in lipid peroxidation and DNA damage; 3) cell wall structure – Gram-positive bacteria have been shown to be more resistant due to the presence of the thick peptidoglycan layer [3, 4, 41].

In conclusion, the obtained results allow us to say that the use of iron NPs permits to create more numerous and diversified microbial consortia, which are considered to lead to an increase in the spectrum of their metabolic activity, and, subsequently, to the capacity of the consortia to survive under unfavorable conditions. In our case, Fe(0) NPs were found to be most effective in creation of a microbial consortium

capable to resist, and perhaps to degrade, high concentrations of trifluralin.

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