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MICROBIOLOGICAL CHARACTERISTICS OF LONG-TERM CONTAMINATED SOIL WITH ORGANOCHLORINE PESTICIDES

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Abstract

The aim of this study was to characterize the soil microbiome, involved in nitrogen transformation processes, long-term exposed to obsolete pesticides (HCH, DDT and their metabolites). The working technique was based on the usual bacteriological methods of analysis; nutrient media, suitable for the comparative study of these groups of microorganisms were used. Under the long-term impact of toxicants, the restructuring of soil microbial community in the direction of reducing microbial diversity took place. In the soil polluted with POPs, compared to the reference soil, the number of nitrogen transforming bacteria and micromycetes was diminished and the representatives of the actinomycetes group were absent. The lack or small number of Azotobacter genus was the characteristic feature for the polluted soil. The research allowed the detection of both the inhibition of soil microflora and the development of natural attenuation of pesticides in soil.

Key words*: soil microorganisms, nitrogen transformation, long-term pollution, organochlorine pesticides*

INTRODUCTION

Soil pollution with different persistent contaminants is a global problem. There are approximately 342,000 contaminated sites in Europe alone, which will increase to more than 500,000 by 2025 (Liedekerke et al., 2014; Storey, 2018).

The Republic of Moldova is no exception. Until the 1990s, significant amounts of pesticides were used in agriculture, which later contributed to the contamination of soils and crops in agricultural fields. Due to the ban on the use of persistent organic pollutants (POPs), storehouses, which contained significant amounts of pesticides, were abandoned. Currently, around 1,600 sites in the country - the former territories of pesticide deposits, reached a deplorable state (Focșa et al., 2020). These deposits, as well as the adjacent territories, are a continuous source of environmental contamination, due to the persistence of organic pollutants (Juc et al., 2006).

Synthetic organic compounds often include atoms (such as chlorine, fluorine, bromine or sulphur) inserted into their structures in positions not normally found in nature, and this complicates their natural degradation processes. As well as persisting in the environment, this also means that they can affect the structural and functional properties of soil microbial communities (Bohme et al., 2005) and at the same time create nutrient imbalances in agricultural soils. Soil microbiome including bacteria, fungi, protozoa, algae and viruses forms a vital component of agro-ecosystem and is responsible for many critical and fundamental soil functions such as nutrient-cycling, soil fertility, improving plant productivity through enhanced availability of limited nutrients and decomposition of organic as well as inorganic matter. At the same time, the soil microbial community is essential in the detoxification (bioremediation) of soils contaminated with toxins and unwanted components resulting from human activities.

In many cases, after the application of pesticides, an increase in total microbial biomass has been reported, while a corresponding reduction in functional diversity has been observed (Wang et al., 2008; Lupwayi et al., 2009). Under the long-term influence of chemical pesticides, only a few functional

groups of microorganisms tend to dominate in the soil, which affects the overall community structure and therefore different biological processes of the soil (Hussain et al., 2009; Lo, 2010; Tan et al., 2012; Ivantsova et al., 2015; Prashar & Shah, 2016).

In order to reduce the environmental impact caused by stocks of obsolete pesticides and stocks contaminated with POPs, a series of measures have been taken to implement the commitments made by the Republic of Moldova following the ratification of international environmental treaties regulating chemicals, stocks and their waste (National Implementation Plan, 2004). In the Environmental Strategy for 2014-2023 and the Action Plan for its implementation, approved by Government Decision no. 301 of 24.04.2014, activities are planned for the evacuation and destruction of unusable pesticides, including the POPs category (Focșa et al., 2020).

However, the information about the actual condition of soil after the repacking on former storages is not sufficient at present. The remains of the storages, the foundations can be a permanent source of soil contamination. Along with this, natural detoxification processes can take place in soil long-time contaminated with pesticides. In the framework of State Program Project 20.80009.7007.20 "The study and management of pollution sources for the elaboration of recommendations on the implementation of measures to reduce the negative impact on the environment and public health" (2020-2023) the investigation of pesticide pollution level and microbiological characteristics of soil around the former pesticide storage CR-Slobozia Duşca 01 was initiated.

The aim of this study was to characterize the soil microbiome, involved in nitrogen transformation processes, long-term exposed to obsolete pesticides (HCH, DDT and their metabolites) at former storage CR-Slobozia Duşca 01.

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: X = 29.087525404, Y = 47.174280 0600001; 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). Three complex soil samples were collected from different zones of the site: sample 1 - near the basement of demolished storage, up the slope; sample 2 and 3 - down the slope. The sample of the reference soil (control soil) was taken at a distance of 200 m from the deposit on the rising slope.

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).

Soil pH and soil moisture content were determined using standard methods (GOST 17.5.4.01-84; Kozlova, 2009).

The concentrations of organochlorine pesticides were determined by gas chromatography GC/MS (Agilent 6890 equipped with a μECD detector) following the USEPA Method 8081A. The calibration interval was from 0.02 to 0.5 μg/ml. The following pesticides or pesticide metabolites were analyzed in the samples: α-, β, and γ-HCH isomers, hexachlorbenzene (HCB), heptachlor, aldrin, dieldrin, endrin, chlordane, DDE, DDD, and DDT.

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 (Gerhardt, 1981; Zvyagintsev, 1991). Thus, the presence of ammonifying bacteria was determined by inoculation on Nutrient agar medium (Oxoid, England); bacteria which assimilate the mineral forms of nitrogen 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 $FeSO_4$, 30.0 Glucose, 30.0 Agar-agar, final pH 5.0 ± 0.2); and oligonitrophilic

bacteria and *Azotobacter* spp. - on Ashby's Mannitol Agar (Sigma-Aldrich).
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 AND DISCUSSIONS

The former pesticide storage CR-Slobozia Dușca 01 is located extremely close to pastures and arable land. In a radius of 300 meters 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.
The results of the laboratory analyzes regarding the soil pollution in the perimeter of the investigated land showed a high level of soil contamination with POPs (Table 1).
In the present survey, 10 POPs substances were determined in concentrations corresponding to the high level of contamination. In addition to HCH, DDT and their metabolites, traces of other pollutants have been recorded, such as Heptachlor, gamma-Chlordane, alfa-Chlordane, Endosulfan Sulfate, Toxaphene, Trifluralin and Atrazine.

Table 1. The degree of soil pollution with POPs in the territory of the pesticide storage

Chemical compound	Concentration of POPs, mg/kg soil
alfa-HCH	74.077
beta-HCH	17.922
gamma-HCH	8.983
delta-HCH	5.742
o-p-DDE	1.452
p-p-DDE	7.908
o-p-DDD	0675
p-p-DDD	6.277
o-p-DDT	17.475
p-p-DDT	74.384
Total POPs	**214.895**

Note: Maximum permissible concentration of pollutant in soil - 0.1 mg/kg soil.

The pH value of the soil in the control sample, samples 1 and 3 was neutral, while in sample 2 it had increased acidity of 3.6-3.8 (Table 2).

Table 2. pH value of the reference and contaminated soil

Soil sample	pH
Reference soil	7.6-7.7
Sample 1	7.6
Sample 2	3.6-3.8
Sample 3	7.6

The microbiological analysis of the soil samples, and in particular, of the microbiome involved in the nitrogen transformation, showed a big gap between the soil samples (Figure 1).
Thus, the most abundant population of microorganisms was found in contaminated soil samples 1 (59.21 x 10^6CFU/1 g dry soil) and 3 (54.45 x 10^6CFU/1 g dry soil), where the population density exceeded even the control sample (28.35 x 10^6CFU/1 g dry soil) by 2 times. An analysis of microbial population revealed a strong decrease in the total number of microorganisms in the soil sample 2 (1.03 x 10^6CFU/1 g dry soil), it was by 27.5 times lower than in the control sample. This indicates that high doses of pesticides and high acidity of the soil inhibit the vital functions of soil microorganisms, and lead to a reduction of their biodiversity in comparison with the biodiversity in uncontaminated soil.
Such changes in the density and diversity of the polluted soil microflora have been observed in other research (Mohn et al., 2006; Nicol et al., 2008; Manickam et al., 2010; Doolotkeldieva et al., 2018; Lu et al., 2020).
The composition of the indigenous population of microorganisms that assimilate nitrogen and survived in the hard conditions of a long-term toxic stress is presented in Table 3.
It has been observed that, under the long-term influence of the toxicants, the restructuring of the soil microbial cenosis took place in the direction of the decrease of the microbial diversity, but with the appearance of more resistant species. The soil sample 1 is the richest one, both in terms of density and diversity of microorganisms. In this sample, all groups of tested microorganisms were detected, and the density of bacteria that assimilate mineral nitrogen, ammonifiers and oligonitrophilic exceeds by 2 times the control sample. Predominant in the soil sample 1 were bacteria that assimilate mineral nitrogen.
In the soil sample, collected from location 3 of the site, although the population density is

higher than in the reference soil, actinomycetes are missing, and the number of micromycetes and representatives of g. *Azotobacter* is small. Oligonitrophilic bacteria predominated in this soil sample. The increase in number of microorganisms, the predominance of one or other functional groups in samples 1 and 3, speaks about the adaptation of the microflora to the conditions of long-term pollution with POPs and the development of natural bioremediation processes in the soil.

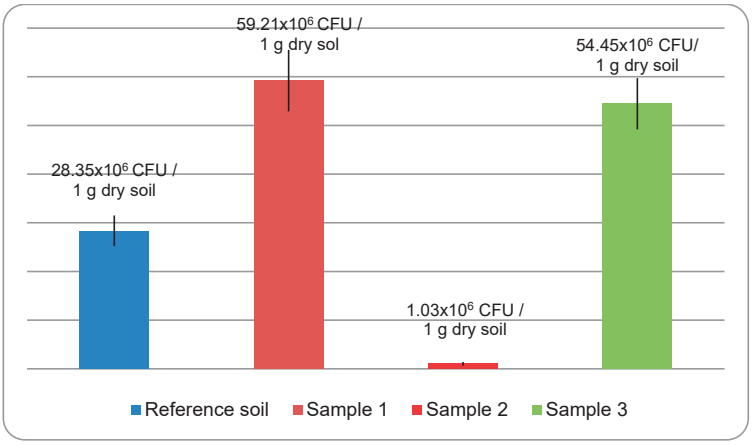

Figure 1. The total number of microorganisms in the reference and contaminated soil samples. The sample of the reference soil was taken at a distance of 200 m from the deposit on the rising slope. Soil sample 1 was collected near the basement of demolished storage, up the slope; soil sample 2 and 3 were collected down the slope

Table 3. Diversity of microorganisms involved in nitrogen transformation processes in polluted soil

Groups of microorganisms	CFU/ 1 g dry sol	Reference soil	Polluted soil		
			Sample 1	Sample 2	Sample 3
Bacteria that assimilate mineral nitrogen	$\times 10^6$	11.71 ± 1.12	26.13 ± 2.92	0.17 ± 0.18	14.20 ± 0.79
Actinomycetes	$\times 10^6$	3.99 ± 0.31	5.18 ± 0.28	0.00	0.00
Oligonitrophilic bacteria	$\times 10^6$	6.23 ± 1.55	13.14 ± 1.80	0.26 ± 0.01	27.04 ± 3.44
Ammonifying bacteria	$\times 10^6$	10.29 ± 0.47	19.89 ± 1.59	0.60 ± 0.18	13.24 ± 1.04
Micromycetes	$\times 10^3$	126.28 ± 8.80	51.58 ± 3.19	4.34 ± 0.87	5.05 ± 2.46
Azotobacter spp.	Cells	952.68 ± 39.27	61.94 ± 7.28	0.00	12.03 ± 0.10

The poor diversity of microorganisms was established for soil sample 2. In this soil no actinomycetes and *Azotobacter* spp. was detected. Representatives of the other groups were observed as single colonies.

The predominance of different groups of microorganisms in the studied soil samples can be explained by the complex pollution and the presence of a large number of POPs metabolites. There is a lot of research which shows that depending on the degradation phase and metabolic pathways involved in the decomposition of persistent pollutants, the groups of microorganisms with the best adapted set of enzymes predominate (Mohn et al., 2006; Manickam et al., 2010; Jeffries et al., 2018; Doolotkeldieva et al., 2018; Regar et al., 2019).

CONCLUSIONS

Thus, our results show that long-term contamination of soils with obsolete organochlorine pesticides affect representatives of soil microbiome differently.

In response to the conditions of toxic stress the reduction of the microbial population biodiversity, especially of fungi, actinomycetes and representatives of g. *Azotobacter* was established.

At the same time, compared to unpolluted soil, the predominance of some groups of microorganisms in polluted soil (bacteria that assimilate mineral nitrogen and oligonitrophilic bacteria) was observed. This is an indication that the microflora is adapting to long-term pollution with POPs and the natural bioremediation processes in the soil is developing.

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