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ABSTRACT: Currently, in the territory of Kyrgyzstan, 50 storage facilities of obsolete pesticides exist; they store about 5000 tons of these hazardous chemicals. The storage conditions have become unusable for a long time. They pose a serious threat to the people living there, livestock, and the environment. The main purpose of this research was the use of selected bacteria with cytochrome P450 genes for the bioremediation of polluted soils around the burial sites in model soil experiments. In the first trial of biodegradation experiments, one contaminated soil was used without any changes in chemical contents, and in the second, the physical and chemical contents of the soil were improved to maintain the bioremediation conditions. The soils in both variants were treated 3 times (ie, once a month) with suspensions of a single culture or a blend of active bacteria (1×10^8 cells/mL) selected from in vitro biodegradation experiments. Two control units without the addition of the bacteria culture were also run. The quantification of targeted persistent organic pollutants (POPs) before and after biodegradation was performed by capillary gas chromatography (GC) coupled to a mass spectrometer. In 6 months, obsolete pesticides such as dieldrin, α-endosulfan, β-endosulfan, and 4-heptachlor-epox pure were able to degrade almost completely, up to 98% to 99.0%, by the blend of bacteria and the single culture of bacteria. Endrin aldehyde showed more resistance as the blend of bacteria was able to degrade it to 59.77%. To improve the aerobic degradation for elimination of pesticides from contaminated soils, it is necessary to create optimal agrotechnical and agrochemical conditions.

KEYWORDS: Polluted soils, persistent pesticides, bioremediation, P450 genes, agrochemical soil conditions

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Introduction

The problem of environmental safety of polluted air, contaminated water, and infertile soil in particular is a relevant topic today.[1] Human activities have disturbed the balance in nature, and the waste and emissions of such activities pose a threat to human health and life as well as the environment.[2,3]

Pesticides widely used in crop production against pests are recognized as persistent organic pollutants (POPs).[4] POPs are resistant to degradation in the environment; they accumulate in the ecosystem trophic links, especially in living organisms.[5-8] They are capable of migrating as far away from their pollution sources, and their levels in the environment are regulated according to the rules of the Stockholm Convention.[9]

Of all known pesticides, only 26 are listed as POPs.[10] Although different POPs represent different degrees of hazard, these chemicals, by definition, have 4 common properties: (1) they are highly toxic; (2) they are resistance to decomposition; (3) they persist for years or even decades until they disintegrate to less dangerous forms; and (4) they have the possibility to be evaporated and transported over long distances by air and by water and to be finally accumulated in adipose tissue.[11,12]

According to reports of international organizations annually in the world, more than 2.5 tons of pesticides are used in crop production, and the number of officially registered active chemical substances exceeds 500.[13] As indicated by the World Health Organization (WHO), only 2% to 3% of used chemical pesticides can effectively reduce the harmfulness of pests, and the rest remains in the soil, water, or other environmental substrates. Thus, in the surface layers of soil and water, the residual amount of pesticides accumulates and has a toxic effect on other components of the environment.[14,15]

The Kyrgyz Republic has never produced pesticides; however, between 1960 and 1985, about 1 million hectares of crops (cotton, sugar beets, vegetables, tobacco, crops, orchards, vineyards, and pastures) were grown, consuming about 5000 tons of organochlorine and organophosphate pesticides with an average of 10 kg or more per ha.[15] There are 50 repositories of obsolete pesticides in Kyrgyzstan, which store about 5000 tons of these hazardous chemicals.[16]

In many dumping zones, the storage conditions have become unusable for a long time. They were created by destroying the integrity of the dumpsites, and people began to dig them up for reuse. They pose a serious threat to the people living there, livestock, and the environment.

The natural self-cleaning of soils from persistent pesticides and pollution by pesticides can need decades.[17] Currently, most traditional methods of restoring contaminated soil pesticides (burning, instillation, etc) are not only ineffective but also harmful. For example, during burning, pesticide undergoes pyrolysis and they form carcinogenic polycyclic aromatic

hydrocarbons. An alternative to traditional methods of cleaning contaminated soil pesticides is their recovery by bioremediation—including the microorganisms that are destructors of pesticidal hydrocarbons—and the stimulation of local soil microflora.[17,18]

Bioremediation is a special innovative process that uses the metabolism of certain groups of microorganisms to remove pollution from environmental objects.[3,19-22] In unpolluted ecological niches, each type of microorganism (bacteria, fungi, yeast, algae, protozoa, and others) performs a certain function and maintains the biosphere by catalyzing biogeochemical processes, whereas in ecosystems polluted with anthropogenic substances (pesticides, oil products, heavy metals, and others), a balance between the functions of microorganisms is disturbed. Some microorganisms can die out with a large load of pollutants; others can survive using these substances as nutrient source. When using the bioremediation approach, it comes to the aid of local microorganisms population, restores their former function, or stimulates their work—to destroy organic pollutants at a high rate.[19] For this, bioremediation develops such approaches to provide additional nutrition (nitrogen, phosphorus), electron acceptors (oxygen), carbon sources, and regulation of the pH, improving other physicochemical conditions in order to increase the metabolism of microorganisms.[3,23,24] Usually, this method includes several stages as it can apply the biostimulation process, ie, initiation of activity of viable local microbial populations. Final stages of bioremediation can be accomplished by approaches such as phytoremediation (plants) and rhizoremediation (plant and microorganism cooperation).[25,26]

Nevertheless, on the contrary, the optimization of the bioremediation process and control over its implementation are complex systems. The coordinated operation of such a system is due to factors such as the presence of an active microbial population that can decompose pollutants and of a certain load of pollutants. In addition, environmental factors such as soil type, temperature, pH, the presence of oxygen, electron acceptors, and nutrients play an important role.[27]

As can be seen from the data of many researchers, biodegradation can occur in a wide pH range, but the pH is considered optimal from 6.5 to 8.5 in most aquatic and terrestrial systems.[28-30] Other authors note the importance of environmental moisture, which directly affects the rate of biodegradation by microorganisms, and the osmotic pressure and pH of the system. The temperature factor is the most important among other factors determining the survival of degrading microorganisms and the composition of decomposing hydrocarbons[31] The rate of decomposition of pollutants often depends on the concentration of the pollutant and the amount of specific enzymes produced by active microbes, as the presence and amount of specific enzymes produced by each cell of active microbes determines the rate of decomposition of contaminants.[21,32]

The removal of heteroatoms (like halogens) or heteroatom-containing groups in organochlorine and organophosphate pesticides is frequently among the first steps in biodegradation. These steps are catalyzed by enzyme groups like monooxygenases, dioxygenases, or peroxidases,[33,34] which, in aerobic conditions, could generate large quantities of free radicals. Cytochrome P450 monooxygenases are one of the largest known superfamilies of enzyme proteins, involved in biodegradation pathways. Their gene sequences have been identified in all living organisms such as humans, bacteria, algae, fungi, and plants. These hemi-containing proteins catalyze various reactions in organisms, ranging from biosynthetic pathways of secondary metabolites in plants and hormones in animal cells to biodegradation of xenobiotics in microbial cells.[35-39]

Many active bacteria are known to harbor in their cells the P450 genes responsible for the biodegradation of organic pollutants, including organochlorine and organophosphate pesticides from the genera *Bacillus, Pseudomonas, Micrococcus, Flavobacterium, Streptomyces*, and others under aerobic and optional aerobic conditions.[40-49] The search, isolation, and identification of the microorganisms with specific genes responsible for catalyzing the pathway of organic pollutant degradation is the first step for the proper understanding of bioremediation process and selection of active microbes.[44,46,50] Thus, the primary purpose of this study was the selection of optimal soil conditions in model experiments to improve the biodegradation rate of obsolete pesticides by introducing active, in vitro–selected bacteria containing P450 genes.

Materials and Method

Soil sampling

Soil samples were collected from sites located around 2 dumping plots (Suzak A and Suzak B) that have long been exposed to pesticide contamination (N 40° 59.625, E 72° 53.796′ , 1136 m elevation above sea level). To collect soil samples from contaminated sites, standard methods of soil microbiology[51] and specific methods[52,53] were used. Specifically, the samples were collected using stainless steel handheld corer. The first 2 corers were always discarded. Three cores (0, 5 cm), taken over an area of 100 m^2, were bulked together to form one sample. The samples were wrapped in aluminum foil twice and sealed in 2 plastic bags to minimize the possibility for contamination. The samples were clearly labeled and stored in containers appropriate for the analysis being undertaken. All samples were transported to the laboratory as soon as possible (preferably within 24 hours) with appropriate chain of custody documentation.[54] Upon receipt, the samples were stored at 20°C until conduction of microbiological and chemical analyses.

The soil was air-dried, ground, and passed through a sieve with 2 mm pores before being stored in sealed containers at room temperature. The organic carbon (OC), cation exchange capacity, and other physicochemical parameters of the soil samples were analyzed (Table 1). The soil samples were suspended in distilled water (1:4 w/v), and the particles were allowed to settle. The pH of the suspension was determined using a pH meter (Thermo Scientific, Orion Laboratory Products). Electrical

Table 1. Physical and chemical characteristics of the soils around the Suzak A and Suzak B (n=3).

SOIL PARAMETERS	
Soil types	Light sierozem soils
Mechanical composition	Silt-loam, sandy loam, and loamy loess
Humus content	0.7%-1.7%
Total nitrogen	0.1%-0.14%
CO_2 in the upper layer	2%-3%
Soil pH	8.2-8.5

conductivity of the soil was determined in the filtrate of the water extract using a conductivity meter. The OC content was determined by adopting the chromic acid wet digestion method according to the standard procedure of Walkley and Black, using a diphenylamine indicator.[55] Available potassium content in the soil was determined by using turbidimetric methods; calcium was determined by titration with a standard $KMnO_4$ solution. The carbonate in the soil was determined by the rapid titration method using a bromothymol blue indicator.[56]

Analysis of contaminated soils for pesticides

Soil samples taken from the contaminated sites were analyzed for their pesticide concentration at the Chemical Laboratory of the "ILIM" Ltd, Scientific Production Association, Bishkek, Kyrgyzstan (Chromatography, Master GC). Finally, an acetone-/hexane-based extract was made, after which clean-up was performed using Florisil (Silica Company, Park Row, TX, US). The purified extracts were analyzed by capillary gas chromatography (GC) coupled to a mass spectrometer (MS; either single-quad MS or triple-quad MS-MS). Compound identifications were based on retention times and their qualifier and target ions. Quantifications were based on peak size. Chemical analysis for the quantification of targeted POPs before and after biodegradation was performed according to methods compliant with NEN-6980 of the Netherlands Standardization Institute.[57]

Extraction of total DNA from contaminated soils and DNA extraction from pure cultures of bacteria isolated from contaminated soils

DNA was extracted from the enrichment cultures during the active phase of microbial growth, using the UltraClean Soil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) and alternative protocol developed by the Mo Bio Laboratories. To process soil samples, 5 g of soil was mixed with 10 to 30 mL of phosphate-buffered saline (PBS) to create homogenized slurry. Samples were mixed for 1 hour at room temperature and then centrifuged for 5 minutes at 123×g. The supernatant was removed and centrifuged at 20 000×g for 15 minutes. The supernatant was then carefully discarded, and the pellet was resuspended in 1 mL of PBS. To extract DNA, 700 µL of the resuspended soil extraction pellet was processed. The purified bacteria were incubated in meat peptone medium (MPM) for 2 days at 25°C. Cells were harvested at the early exponential growth phase, and their DNA was then extracted by the alternative protocol of the Mo Bio Laboratories. Successful DNA extraction was determined by agarose gel electrophoresis (1.0% agarose). Amplification was performed with a Multi Gene Thermal Cycler (TC9600-G/TC, Labnet International, Edison, New Jersey, USA), using a 25 µL mixture containing 15 µL of PCR MasterMix (Taq DNA polymerase, $MgCl_2$, deoxyribonucleotide triphosphate, and reaction buffer), 2 µL of each primer,1 µL of template DNA, and 1 µL of H_2O. The amplification program was used as the follows: 94°C for 5 minutes, 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds, and 72°C for 7 minutes. PCR products were electrophoresed in a 1.0%agarose gel and visualized using the BioDoc-It Imaging Systems (Ultra-Violet Products Ltd) after ethidium bromide staining. To control contamination, we used a negative control reaction, and sterile water was added as a matrix. Almost full-length fragments of 16Sp RNA genes were amplified using the primers 16S-27F and 16S-907R. Fragments of genes encoding the subunits of alkane monooxygenases were amplified using specific sets of primers. The primer set alkB-F and alkB-R was used to amplify the alkane hydroxylase, and P450R was used to amplify the cytochrome P450 alkane hydroxylase. Sequence analysis was performed by the Macrogen Company (10F World Meridian Center, Seoul, Korea), and sequences were edited with Applied Biosystems 3730XL sequencers. Only sequences with more than 700 nucleotides were used for diversity analyses. The phylogenetic relatedness among different sites was determined using the cluster environment. The 16S ribosomal RNA (rRNA) gene sequences were deposited in the GenBank and DB of the National Center for Biotechnology Information nucleotide sequence databases.[58]

Selected local bacteria species

Bacillus polymyxa, Pseudomonas fluorescens, Flavobacterium sp, and *Micrococcus sp* bacteria were selected for in vitro biodegradation experiments with Aldrin. These bacteria were isolated from the soil according to soil microbiology methods and identified to the species using classical microbiological and PCR analysis.[58]

In situ biodegradation experiments

A surface soil treatment unit was designed and fabricated (25×12, 5×7 cm), and the contaminated soil taken from the Suzak burial sites was distributed between them. Four variants of experiments were conducted according to the soil properties of the contaminated sites. In the first, uncontaminated soil was used without any changes in chemical contents and characteristics with the following local parameters: pH of 8.2 to 8.5, temperature of 22°C to 25°C, moisture of 40% to 45%, and the soil type is light sierozem with low organic matter content (Table 1).

Table 2. In vivo biodegradation experiment design.

1. CONTAMINATE SOIL WITH LOCAL PARAMETERS		2. CONTAMINATED SOIL WITH IMPROVED PARAMETERS	
PARAMETERS	RANGE	PARAMETERS	RANGE
C: N: P	2:0.14:0.02	C: N: P	100:10:1
pH	8.2-8.5	pH	7.4-7.62
Temperature	22°C-25°C	Temperature	26°C-27°C
Moisture	40%-45%	Moisture	60%-75%
Dissolved oxygen	4.0-5.2 mg/kg	Dissolved oxygen	8.0-9.2 mg/kg
Microbial content (1×10^8 cells/mL)	Blended bacteria: *Bacillus polymyxa, Pseudomonas fluorescens, Flavobacterium sp*, and *Micrococcus sp*	Microbial content (1×10^8 cells/mL)	Blended bacteria: *Bacillus polymyxa, Pseudomonas fluorescens, Flavobacterium sp*, and *Micrococcus sp*
	Single bacteria: *Pseudomonas fluorescens*		Single bacteria: *Pseudomonas fluorescens*
Control 1, soil with local parameters, no bacterial treatment		Control 2, soil with improved parameters, no bacterial treatment	

In the second variant, to activate the soil microflora, 300 g of black soil and rotted manure in the ratio of 1:5 were added to 1000 g of polluted soil. Bioremediation conditions such as moisture (60%-75%), temperature (26°C-27°C), dissolved oxygen, and pH (7.4-7.62) were monitored and maintained in the surface soil treatment unit (Table 2). The soils in both variants were treated 3 times (ie, once a month) with suspensions of a single culture or a blend of active bacteria (1×10^8 cells/mL); they were selected for in vitro biodegradation experiments. To maintain soil moisture, the soil was humidified every 2 to 3 days and loosened for soil aeration. Two control units without the addition of the bacteria culture were also run in parallel for the sake of making comparisons: soil alone with no treatment and soil alone + improved conditions for optimization. Moreover, the bioremediation of the pesticide was carried out in triplicates; 0.05% Tween 80 was added to the soil as a surfactant to prevent the adsorption of pesticide to soil particles. The aerobic condition was maintained by supplying symmetric air with the help of an electric air pump. Bioremediation conditions such as moisture, temperature, dissolved oxygen, pH, and nutrients (C, N, and P) were monitored and maintained in the surface of the soil treatment unit by the addition of one dose of nutrient: glucose ($C_6H_{12}O_6$): 150 mg/L, potassium dihydrogen phosphate (KH_2PO_4): 80 mg/L, and ammonium sulfate (NH_4) SO_4: 80 mg/L.

Statistical analyses

Biodegradation (%) was calculated based on the difference between residual pesticides in treated samples and the uninoculated controls. The means and standard deviations (n – 1) of 3 replicates were computed using data analysis tools in the software program MS Excel (Microsoft Excel 2013). Means were compared by least significant difference (LSD) tests with statistical significance at $P \leq .05$ using the software MSTAT-C (Mstat 6.1, Michigan State University, East Lansing, MI, USA).

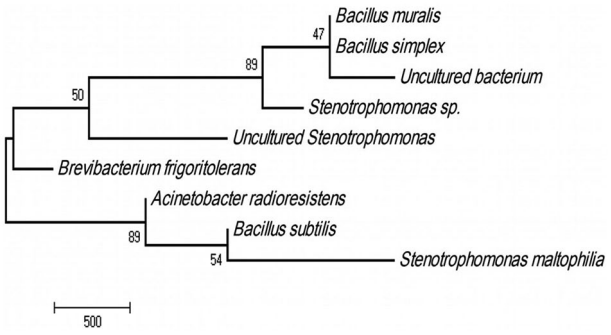

Figure 1. Phylogenetic tree of P450 alkane oxygenases containing bacteria from pesticide-contaminated soil around the Suzak A damping zone.

Results

Biodiversity of bacterial communities in contaminated soils

Using both culture-dependent and culture-independent approaches, the taxonomic composition of the bacterial communities taken from the contaminated soils was determined. The universal primer set for 16S rRNA gene and the specific primer set P450R were used to amplify bacteria with the cytochrome P450 hydroxylase gen. As Figures 1 and 2 have shown, in pesticide-contaminated soil, bacterial species with cytochrome P450 genes from *Pseudomonas, Stenotrophomonas, Acinetobacter, Bacillus*, and other genera were found. These bacteria species are well known as degraders of waste related to pesticides.[42,44-47,59]

In this context, the cytochrome P450 family is a large, well-characterized group of monooxygenase enzymes that have

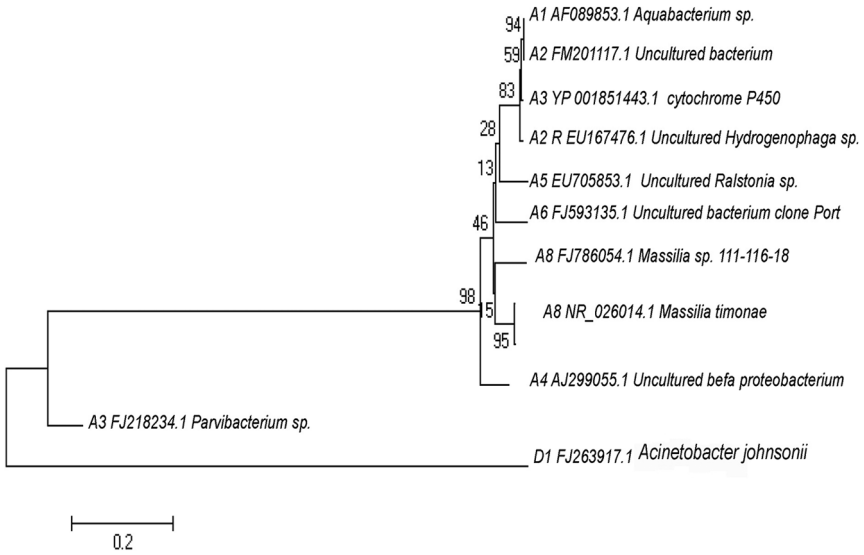

Figure 2. Phylogenetic tree of P450 alkane oxygenases containing bacteria from pesticide-contaminated soil around the Suzak B damping zone.

Table 3. Experimental variants to test biodegradation in soils contaminated with obsolete pesticides.

BURIAL SITES CONTAINING PESTICIDES IN SOUTHERN KYRGYZSTAN	SPECIES AND DOSE OF THE BACTERIA USED FOR THE BIODEGRADATION OF SOIL CONTAINING PESTICIDES
Suzak A	Blend of bacteria: *Pseudomonas fluorescens* + *Bacillus polymyxa* + *Micrococcus flavus* + *Flavobacterium sp*, 1×10^8 cells/mL of the liquid medium
Suzak A	Single culture of *Pseudomonas fluorescens*, 1×10^8 cells/ mL of the liquid medium
Suzak B	Blend of bacteria: *Pseudomonas fluorescens* + *Bacillus polymyxa* + *Micrococcus flavus* + *Flavobacterium sp*, 1×10^8 cells/mL of the liquid medium
Suzak B	Single culture of *Pseudomonas fluorescens*, 1×10^8 cells/mL of the liquid medium

long been recognized for their potential in many industrial processes.[41,44,46]

More than 200 subfamilies of P450 enzymes have been found across various prokaryotes and eukaryotes. They all contain a catalytic iron–containing porphyrin group that absorbs at 450 nm upon binding with carbon monoxide. Bacterial P450 systems employ a ferredoxin reductase and a ferredoxin to transfer electrons to P450. The Cytochrome P450–initiated pathway is a monooxygenase that catalyzes the addition of one atom of molecular oxygen into the terminal carbon, thus forming the corresponding alcohol.[60,61] In vitro biodegradation experiments were conducted using single *Bacillus polymyxa, Micrococcus sp, Flavobacterium sp, Pseudomonas fluorescens* and the blend of their cultures that was incubated for 12 days with various concentrations (0.2, 0.5, and 1.0 mg) of the pesticide Aldrin to test their degradation capability[58] Based on the results, these bacteria were selected for use in in vivo experiments.

In situ biodegradation experiments

As described in the "Materials and Method" section, the contaminated soil taken from the Suzak burial sites was treated 3 times (ie, once a month) with suspensions of active bacteria from the single culture or the blend of bacteria that were selected from in vitro biodegradation experiments. To maintain soil moisture, the soil was humidified every 2 to 3 days and loosened for soil aeration (Table 3).

The quantities (mg/kg) of obsolete pesticides before and after biodegradation were analyzed using the chromatography method. The quantities (mg/kg) of obsolete pesticides before biodegradation is shown in Table 4.

All 12 initial POPs listed under the Stockholm Convention[9] were identified through chromatography analyses of the soil samples (mg/kg dry weight). They have been recognized as causing adverse effects on humans and the ecosystem and are highly toxic to fish, birds, and other aquatic animals—particularly frogs. In addition, mothers pass POPs to their infants through the placenta and through nursing milk; they are classified as possible human carcinogens that are lethal to some animals and adversely affects their reproductive success at lower levels. Nevertheless, POP residues have been found in air, water, soil, fish, birds, and mammals, including humans.[62-64]

As the results of chromatography analyses have shown, the concentrations of identified compounds in the investigated areas

Table 4. Quantity (mg/kg) of obsolete pesticides before biodegradation.

THE NAME OF OBTAINED OBSOLETE PESTICIDES IN SUZAK A AND SUZAK B SOILS	QUANTITY (MG/KG) OF OBSOLETE PESTICIDES, N = 3
β-BHC	52.740 ± 1.07
Heptachlor-epox pure	18.000 ± 1.12
Endrin aldehyde	43.469 ± 1.09
α-Endosulfan	73.873 ± 1.05
δ-BHC	2283.103 ± 1.35
Heptachlor	1371.921 ± 1.31
Aldrin	1326.939 ± 1.36
4,4′-DDE (13C12)	462.358 ± 1.28
Dieldrin	268.031 ± 1.25
4.4 DDD	266.593 ± 1.28
β-Endosulfan	743.211 ± 1.15

Values are given as mean ± SD, n = 3, significantly different at $P \leqslant .05$.

theoretically exceed the threshold dose in terms of affecting biological organisms in the environment many times over. After 3 and 6 months, the chromatography analyses found different levels of biodegradability by destructive bacteria in obsolete pesticides from Suzak A and Suzak B in 4 variant experiments.

Soil with improved content experiment results in 3 months

After 3 months in soil with improved content by the addition of black soil and manure, the changing of pH to a neutral state, and an increase in humidity, the degradation rate of analyzed obsolete pesticides is presented in Table 5.

When analyzing the intensity of degradation of 9 pesticides from 12 in 3 months after the introduction of aerobic bacteria blend to the soil with improved agrochemical conditions, a significant difference in the degradation of pesticides was revealed. During this period, endrin aldehyde has showed a high sensitivity to blend culture bacteria and was subject to almost 72.9 ± 0.05% decomposition, α-endosulfan: 50.68 ± 0.05%; 4-heptachlor-epox pure: 39.9 ± 0.05%; β-BHC: 30.75 ± 0.05%; dieldrin: 34.44 ± 0.05% ($P \leqslant .05$), whereas pesticides such as δ-BHC, heptachlor, and aldrin were subject to low degradation (4.062 ± 0.05%, 5.683 ± 0.05%, and 7.545 ± 0.05%); accordingly, this indicates their resistance to microbial degradation.

When using a single culture of *Pseudomonas fluorescens* (1×10^8 cells/mL), the results were different from the blend bacterial effects. The degradation rate of such pesticides like β-BHC, 4-heptachlor-epox pure, and dieldrin using a single culture was lower compared with using the blend culture of bacteria, eg, the percentage of β-BHC degradation was 22.75 ± 0.05% and 4-heptachlor-epox pure was 34.5 ± 0.05%, whereas other pesticides like endrin aldehyde, β-endosulfan, and α-endosulfan have shown significant sensitivity to degradation effect of single *Pseudomonas fluorescens* culture. A comparison was made with control soil 2, where agrochemical parameters were improved, however, without the introduction of inoculum of degrading bacteria. Here, a sharp difference was observed with the soil with introduced degrading bacteria. The degradation intensity for all analyzed pesticides was almost 1.5 to 2.0 times lower. For 3 months, the degradation rate of β-BHC amounted to 8.86 ± 0.05%, 4-heptachlor-epox pure: 20.27 ± 0.05%, dieldrin: 20.0 ± 0.05%, endrin aldehyde: 3.6 ± 0.05%, and β-endosulfan: 5.9 ± 0.05 % (Figure 3).

Experiment results in 6 months, in soil with improved content

After 6 months in soil with improved content, the degradation rate of the analyzed obsolete pesticides is presented in Table 6.

When the 9 well-known obsolete pesticides of the 12 listed under the Stockholm Convention were analyzed, it was found that their degrading rates by the blend of bacteria and single culture of bacteria were different. Obsolete pesticides such as dieldrin, α-endosulfan, β-endosulfan, 4-heptachlor-epox pure, δ-BHC, and aldrin were degraded almost completely by the blend of bacteria. However, endrin aldehyde showed more resistance to degrading microorganisms as the blend of bacteria was able to degrade it to 59.77 ± 0. 05% ($P \leqslant .05$).

On the contrary, a single culture of *Pseudomonas fluorescens* was able to degrade these pesticides almost completely, ranging from 92.4% up to 99.9 ± 0. 05% ($P \leqslant .05$). Therefore, the microbial consortium degraded the β-BHC pesticide to 81.46 ± 0.05% within 6 months, whereas the single culture *Pseudomonas fluorescens* managed to reduce its content to 99.9 ± 0.05% ($P \leqslant .05$). The 4-heptachlor-epox pure pesticide was destroyed within 6 months by the blend of bacteria to 97.87 ± 0.05% and to 92.88 ± 0.05% ($P \leqslant .05$) when exposed to a single culture. Another pesticide, dieldrin, was degraded to 99.96 ± 0.05% by the blend of bacteria, whereas when exposed to a single culture, *Pseudomonas fluorescens*, it was degraded up to 99.8 ± 0.05% ($P \leqslant .05$).

When compared with control 2, where the soil conditions were improved, however, degrading bacteria as bioinoculants were not introduced into the soil. In such soils, conditions were presumably created for the activation of the local microflora of microorganisms involved in the transformation of pesticides. Analysis has shown that in 6 months, some pesticides like α-endosulfan were subjected to considerable destruction of the local microflora, and the rate of degradation reached up to 49.58 ± 0.05% ($P \leqslant .05$). Pesticides like 4-heptachlor-epox pure, dieldrin, heptachlor, and aldrin have shown moderate resistance to activate local microflora of soil, so their percentage

Table 5. The level of biodegradability of obsolete pesticides from Suzak A after 3 months from the transformation of destructive bacteria in soil with improved content.

OBSOLETE PESTICIDES	QUANTITY (MG/KG) OF OBSOLETE PESTICIDES AFTER BIODEGRADATION USING A BLEND OF BACTERIA (1×10^8 CELLS/ML)	QUANTITY (MG/KG) OF OBSOLETE PESTICIDES AFTER BIODEGRADATION USING A SINGLE CULTURE OF *PSEUDOMONAS FLUORESCENS* (1×10^8 CELLS/ML)	QUANTITY (MG/KG) OF OBSOLETE PESTICIDES IN CONTROL 2
β-BHC	16.221 ± 1.12	12.003 ± 1.02	48.125 ± 1.25
4-Heptachlor-epox pure	7.182 ± 1.27	6.225 ± 1.09	14.370 ± 1.19
Dieldrin	92.310 ± 1.23	91.720 ± 1.18	201.012 ± 1.11
Endrin aldehyde	31.340 ± 1.41	32.415 ± 1.05	41.920 ± 1.31
β-Endosulfan	335.700 ± 1.62	427.102 ± 1.3	700.09 ± 1.17
δ-BHC	92.75 ± 1.19	91.87 ± 1.07	2034.10 ± 1.14
Heptachlor	77.97 ± 1.23	69.730 ± 1.25	1287.12 ± 1.13
Aldrin	100.12 ± 1.09	121.14 ± 1.54	1185.35 ± 1.29
α-Endosulfan	37.440 ± 1.13	39.002 ± 1.25	66.312 ± 1.01
4,4′-DDE	127.24 ± 1.03	151.29 ± 1.17	400.23 ± 1.14
4.4 DDD	132.75 ± 1.35	141.83 ± 1.19	213.09 ± 1.13

Values are given as mean ± SD, n = 3, significantly different at $P \leqslant 0.05$.

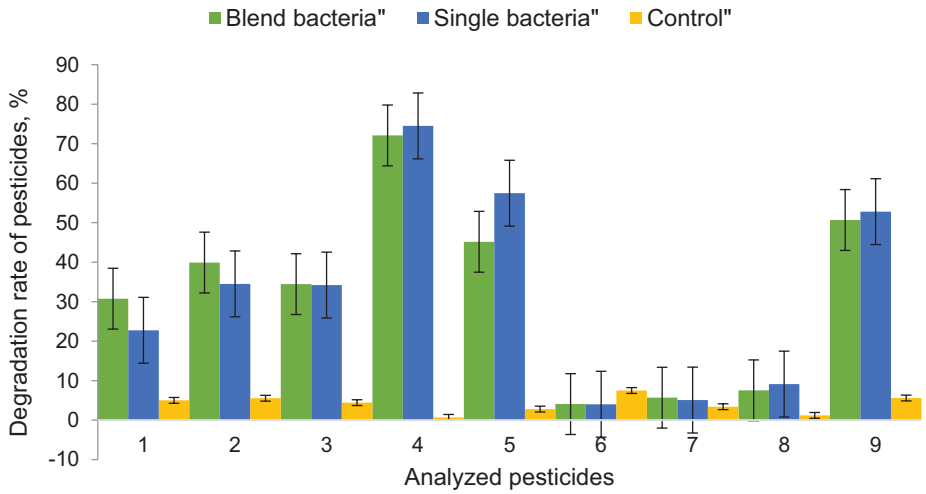

Figure 3. The degradation rate of obsolete pesticides in improved soil, during 3 months. 1: β-BHC; 2: 4-Heptachlor-epox pure; 3: Dieldrin; 4: Endrin aldehyde; 5: β-Endosulfan; 6: δ-BHC; 7: Heptachlor; 8: Aldrin; 9: α-Endosulfan; values are in mean ± SD, n = 3. Values with different letters are statistically significantly different at $P \leqslant .05$.

of transformation made was 29.62 ± 0.05%, 28.86 ± 0.05%, and 26.75 ± 0.05%, respectively. The rest-analyzed pesticides were subjected to low degradation (Figure 4).

Experiment results in 3 months, in soil with unimproved content

The degradation rate of obsolete pesticides by the blend of bacteria and single culture of bacteria in soil with local natural conditions differed from improved soil with less activity in terms of pesticides (Table 7).

After 3 months, when the blend culture of degrading bacteria was introduced into the soil with local conditions, such pesticides as β-BHC, heptachlor-epox pure-6, endrin aldehyde, and α-endosulfan were subjected to low biodegradation by the blend of bacteria. Whereas β-endosulfan has shown high sensitivity, its percentage of degradation reached 80.0 ± 0.05% ($P \leqslant .05$). Pesticides such as dieldrin, δ-BHC,

Table 6. The level of biodegradability of obsolete pesticides from Suzak A after 6 months from the transformation of destructive bacteria in soil with improved content.

OBSOLETE PESTICIDES	QUANTITY (MG/KG) OF OBSOLETE PESTICIDES AFTER BIODEGRADATION USING A BLEND OF BACTERIA (1×10^8 CELLS/ML)	QUANTITY (MG/KG) OF OBSOLETE PESTICIDES AFTER BIODEGRADATION USING A SINGLE CULTURE OF *PSEUDOMONAS FLUORESCENS* (1×10^8 CELLS/ML)	QUANTITY (MG/KG) OF OBSOLETE PESTICIDES IN CONTROL 2
β-BHC	9.778 ± 1.09	0.044 ± 0.0015	43.143 ± 1.02
4-Heptachlor-epox pure	0.382 ± 0.18	1.296 ± 0.95	12.670 ± 1.03
Dieldrin	0.096 ± 0. 23	0.32 ± 0.007	190.678 ± 1.14
Endrin aldehyde	25.982 ± 1.12	39.762 ± 1.87	4 0.457 ± 1.91
β-Endosulfan	9.778 ± 0.34	3.972 ± 1.45	49.870 ± 1.85
δ-BHC	0.048 ± 0.24	0.048 ± 0.008	1978.10 ± 1.17
Heptachlor	0.021 ± 0.11	0.024 ± 0.0002	1005.56 ± 1.27
Aldrin	0.001 ± 0.0003	0.004 ± 0.0001	980.136 ± 1.89
α-Endosulfan	0.140 ± 0.002	0.432 ± 0.003	37.245 ± 1.12
4,4′-DDE	1.123 ± 0.94	2.24 ± 1.23	378.123 ± 1.34
4.4 DDD	1.567 ± 0.87	1.928 ± 0.98	178.451 ± 1.71

Values are given as mean ± SD, n = 3, significantly different at $P \leqslant .05$.

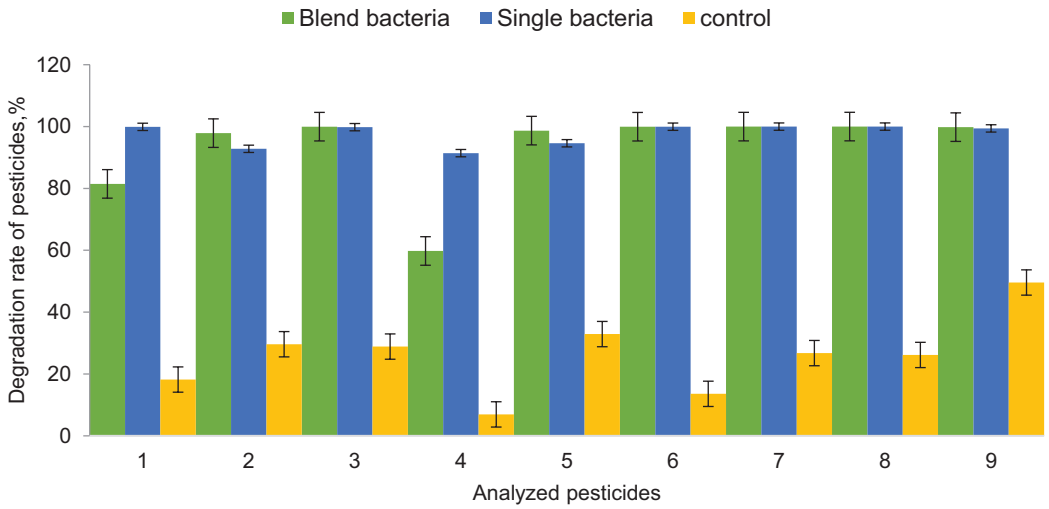

Figure 4. The degradation rate of obsolete pesticides in improved soil, during 6 months. 1: β-BHC; 2: 4-Heptachlor-epox pure; 3: Dieldrin; 4: Endrin aldehyde; 5: β-Endosulfan; 6: δ-BHC; 7: Heptachlor; 8: Aldrin; 9: α-Endosulfan; values are in mean ± SD, n = 3. Values with different letters are statistically significantly different at $P \leqslant .05$.

and aldrin in the local soil conditions were subjected to moderate degradation, and their percentage of decomposition ranged from 39.57 ± 0.05 to 57.78 ± 0.05% ($P \leqslant .05$). The degradation rate under local conditions of those resistant pesticides such as β-BHC, 4-heptachlor-epox pure, endrin aldehyde, and α-endosulfan by a single culture of *Pseudomonas fluorescens* was lower compared with the degradation by the blend of bacteria. For example, dieldrin and β-endosulfan had a very low percentage of degradation, ie, 2.57 ± 0.05% and 2.27 ± 0.05%, respectively. A higher degree of degradation was noted in 4-heptachlor-epox pure, ie, almost 83.44 ± 0.05%; moderate degradation was in α-endosulfan (61.47 ± 0.05%); and the remaining pesticides had a degradation rate of 20.8 ± 0.05% to 48.18 ± 0.05% when exposed to a single culture *Pseudomonas fluorescens*. In the control variant, without bacterial treatment, under

Table 7. The level of biodegradability of obsolete pesticides from Suzak A after 3 months by the transformation of destructive bacteria (n = 3) in soil with local nature conditions.

OBSOLETE PESTICIDES	QUANTITY (MG/KG) OF OBSOLETE PESTICIDES AFTER BIODEGRADATION USING A BLEND OF BACTERIA (1×10^8 CELLS/ML)	QUANTITY (MG/KG) OF OBSOLETE PESTICIDES AFTER BIODEGRADATION USING A SINGLE CULTURE OF *PSEUDOMONAS FLUORESCENS* (1×10^8 CELLS/ML)	QUANTITY (MG/KG) OF OBSOLETE PESTICIDES IN CONTROL 1
β-BHC	42.720 ± 0.79	40.187 ± 0.81	50.17 ± 1.05
4-Heptachlor-epox pure -6	17.131 ± 0.81	17. 535 ± 0.92	17.978 ± 1.34
Dieldrin	110.50 ± 1. 01	103.27 ± 1.23	253.49 ± 1.81
Endrin aldehyde	41.701 ± 1.15	42.05 ± 1.31	43.170 ± 1.27
β-Endosulfan	112.8 ± 1.37	123.09 ± 1.39	723.119 ± 1.43
δ-BHC	1090.01 ± 1.91	1183.21 ± 0.12	2276.205 ± 1.99
Heptachlor	931.31 ± 1.23	877.03 ± 1.07	1361.200 ± 1.13
Aldrin	802.71 ± 1.71	757.01 ± 1.31	1320.12 ± 1.02
α-Endosulfan	60.143 ± 1.51	57.133 ± 1.43	72. 421 ± 1.07
4,4′-DDE	132.71 ± 1.02	142.71 ± 1.13	457.134 ± 1.02
4.4 DDD	137.92 ± 1.17	142.04 ± 1.51	265.123 ± 1.05

Values are given as mean ± SD, n = 3, significantly different at $P \leqslant .05$.

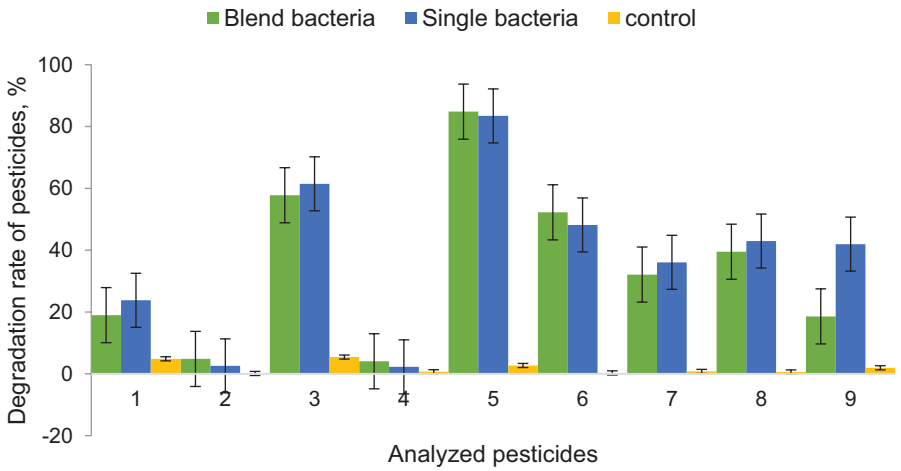

Figure 5. The degradation rate of obsolete pesticides in unimproved soil, during 3 months. 1: β-BHC; 2: 4-Heptachlor-epox pure; 3: Dieldrin; 4: Endrin aldehyde; 5: β-Endosulfan; 6: δ-BHC; 7: Heptachlor; 8: Aldrin; 9: α-Endosulfan; values are in means ± SD, n = 3. Values with different letters are statistically significantly different at $P \leqslant .05$.

unimproved soil conditions, a very low rate of degradation of pesticides was revealed. The degradation rate of many pesticides like 4-heptachlor-epox pure, endrin aldehyde, δ-BHC, heptachlor, and aldrin was so low, which was measured at 0.13 ± 0.01%, 0.69 ± 0.01%, 0.31 ± 0.01%, 0.79 ± 0.01%, and 0.6 ± 0.01% ($P \leqslant .05$) respectively. A significant rate of decomposition in comparison with others was noted in dieldrin (5.43 ± 0.05%) and β-BHC (4.87 ± 0.05%) (Figure 5).

Experiment results in 6 months, in soil with unimproved content

The degradation rate of obsolete pesticides by the blend of bacteria and single culture of bacteria in soil with local natural conditions differed from improved soil with less activity in terms of pesticides (Table 8). As opposed to improved soil with more favorable chemical compositions and pH reactions for the activation of indigenous microflora and the development of introduced blends and single bacteria, in this case,

Table 8. The level of biodegradability of obsolete pesticides from Suzak A after 6 months by the transformation of destructive bacteria (n = 3) in soil with local nature conditions.

OBSOLETE PESTICIDES	QUANTITY (MG/KG) OF OBSOLETE PESTICIDES AFTER BIODEGRADATION USING A BLEND OF BACTERIA (1×10^8 CELLS/ML)	QUANTITY (MG/KG) OF OBSOLETE PESTICIDES AFTER BIODEGRADATION USING A SINGLE CULTURE OF *PSEUDOMONAS FLUORESCENS* (1×10^8 CELLS/ML)	QUANTITY (MG/KG) OF OBSOLETE PESTICIDES IN CONTROL 1
β-BHC	19.532 ± 0.96	17.89 ± 0.01	50.800 ± 1.87
Heptachlor-epox pure-6	12. 671 ± 0.01	13. 167 ± 0.95	16.567 ± 1.34
Dieldrin	18.196 ± 0. 23	11. 34 ± 0.009	15.978 ± 1.61
Endrin aldehyde	25.782 ± 1.02	27.902 ± 1.09	43.389 ± 1.97
β-Endosulfan	60.778 ± 0.34	62. 453 ± 1.12	54.890 ± 1.87
δ-BHC	3.231 ± 0.17	4.123 ± 0.12	2112.25 ± 1.19
Heptachlor	5. 021 ± 0.11	3.145 ± 0.009	1325.317 ± 1.28
Aldrin	3.001 ± 0.001	2.112 ± 0.091	1313.123 ± 1.249
α-Endosulfan	23.786 ± 0.002	19.002 ± 0.001	437.157 ± 1.08
4,4′-DDE	5.243 ± 0.95	7.123 ± 1.09	435.267 ± 1.17
4.4 DDD	4.450 ± 0.17	5.345 ± 0.94	262.287 ± 1.19

Values are given as mean ± SD, n = 3, significantly different at $P \leqslant .05$.

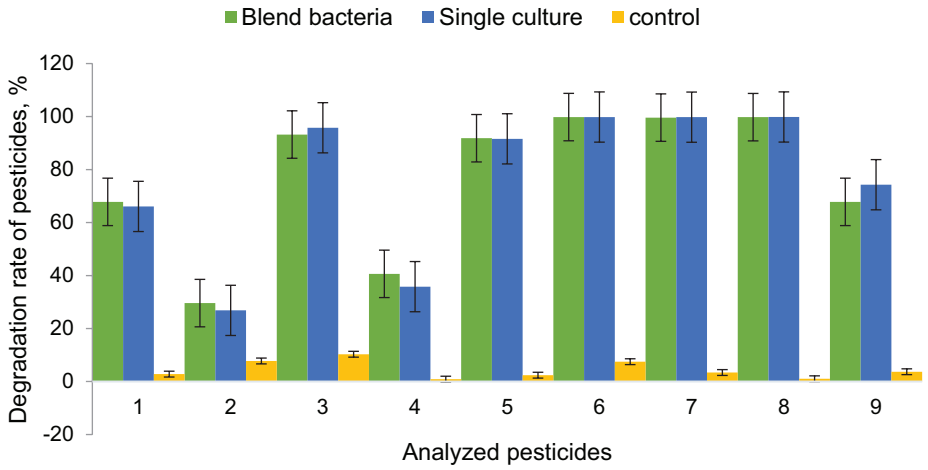

Figure 6. The degradation rate of obsolete pesticides in unimproved soil, during 6 months. 1: β-BHC; 2: 4-Heptachlor-epox pure; 3: Dieldrin; 4: Endrin aldehyde; 5: β-Endosulfan; 6: δ-BHC; 7: Heptachlor; 8: Aldrin; 9: α-Endosulfan; values are given as mean ± SD, n = 3. Values with different letters are statistically significantly different at $P \leqslant .05$.

most pesticides were degraded in no significant quantities; their content in the soil remained high. Moreover, some pesticides have shown sensitivity to degrading bacteria in such natural conditions. Thus, the decomposition rate of pesticides such as dieldrin and β-endosulfan reached 93.21 ± 0.05% and 91.82 ± 0.05% ($P \leqslant .05$), respectively, due to the blend of bacteria. These pesticides also showed significant sensitivity to a single *Pseudomonas fluorescens* culture. However, pesticides such as endrin aldehyde and 4-heptachlor-epox pure exhibited high resistance to the use of the blend of bacteria and single bacteria culture. In the control, the degrading rate of pesticides ranged from 0.9 ± 0.05% to 10.3 ± 0.05% ($P \leqslant .05$), when the soil with pesticides were cultivated without bacteria in the same temperature, humidity, and aeration conditions in the 6-month duration (Figure 6).

The degradation capacity of the local microflora present in the 2 control soils was compared.

As described in the "Materials and Method" section, in control 1, the soil agrochemical conditions remained unchanged, and in control 2, the soil conditions were improved, in both

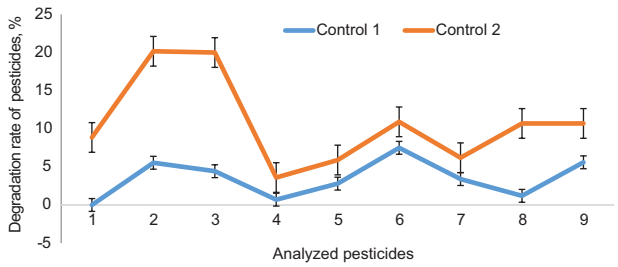

Figure 7. A comparison of the degradation rate of obsolete pesticides in control 1 (unimproved soil) and 2 (improved soil), in 3 months. 1: β-BHC; 2: Heptachlor-epox pure; 3: Dieldrin; 4: Endrin aldehyde; 5: β-Endosulfan; 6: δ-BHC; 7: Heptachlor; 8: Aldrin; 9: α-Endosulfan; values are in mean ± SD, n = 3. Values with different letters are statistically significantly different at $P \leq .05$.

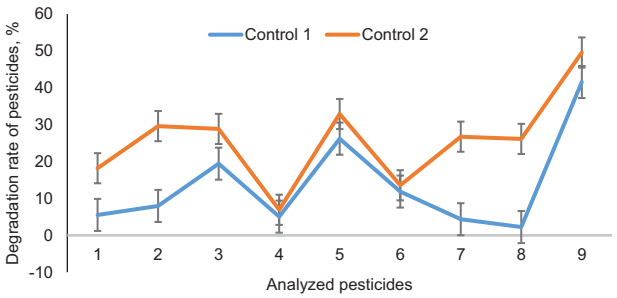

Figure 8. A comparison of the degradation rate of obsolete pesticides in control 1 (unimproved soil) and control 2 (improved soil) in 6 months. 1: β-BHC; 2: Heptachlor-epox pure; 3: Dieldrin; 4: Endrin aldehyde; 5: β-Endosulfan; 6: δ-BHC; 7: Heptachlor; 8: Aldrin; 9: α-Endosulfan; values are in mean ± SD, n = 3. Values with different letters are statistically significantly different at $P \leq .05$.

without the addition of active degrading bacteria. In 3 months, in these control soils, a marked difference was observed in the degradation rate of pollutants. Thus, in improved soil, the degrading activity of local microflora toward to some pesticides was significantly higher than in unimproved soil. For example, in control 1, the percentage of 4-heptachlor-epox pure degradation was 5.54 ± 0.05%, dieldrin: 4.43 ± 0.05%, and aldrin: 1.2 ± 0.05%, whereas in control 2, the degradation percentage of 4-heptachlor-epox pure was 20.17 ± 0.05%, dieldrin: 20.0 ± 0.05%, aldrin: 10.7 ± 0.05%, so almost 4 to 4.5 times more intensive (Figure 7).

When comparing the 2 control soils after 6 months, the difference between them in the destructive activity of soil microorganisms toward to the analyzing pesticides was even more noticeable. Of all the analyzed pesticides, α-endosulfan was degraded more intensively, and after 6 months, almost 49.5% of this pesticide was destroyed by the local microflora (Figure 8).

The results confirmed that the additional introduction of nutrient sources, neutralization of the pH of the medium, and optimization of temperature and aeration provide the activation of metabolic pathways of local microorganisms involved in the aerobic destruction of organic compounds, including organochlorine and organophosphate pesticides. However, having a low density and activity of local microorganisms and their dose necessary for the intensive destruction of high concentrations of pesticides is not enough in polluted sites. Therefore, the bioinoculation of active bacteria into the soil with natural local conditions is the obligatory step of the bioremediation process with additional agrochemical improvements in the soil content. Introduced bacteria reproducing intensively can use pesticides as a food source and turn them into simpler and more harmless compounds. At the same time, the local microflora also can contribute to the destruction of pesticides, if the soil has optimal conditions, as it was found in the control soils.

Discussion

In this study, the bioremediation process involved in degrading and removing the 12 obsolete pesticides in the Stockholm Convention list from heavily contaminated soils around burial zones in Kyrgyzstan was modulated. This artificial module experiment with very close-to-nature conditions used the action of bacteria, which were selected using in vitro experiments on their active degradation activity.

We found several types of pesticide pollutants in contaminated soils. Therefore, to eliminate every kind of pesticide, more than one species of microorganism is needed, as stated and discussed in the studies of other authors.[65] Considering this feature, we used the blend of active tested bacteria, and for comparison, we also used single cultures in parallel. Besides, different bacteria species in a consortium may have adaptability and viability to various environmental conditions. For achieving successful bioremediation, it is necessary to eliminate such environmental restrictions as water content, temperature, pH, and the presence of pollutants themselves, such as organic compounds.[28,66]

To optimize the model experiment, it was necessary to balance the lack of mineral salts in the soil and the presence of free oxygen. The most important condition for the experiment was to make the pH neutral. To this end, the addition of ammonium salt and manure provided the soil with nitrogen and other nutrient sources as the pH values in the soils around Suzak A and Suzak B were slightly alkaline at 8.05.

The research results showed that improving the chemical composition and biological activity of soils contaminated with obsolete pesticides led to the activation of local microflora and created better conditions for the intensive work of selected bacteria cultures with P450 genes introduced into the soil, which are consistent with other studies.[27,28-31]

The tested composition containing Gram-positive bacteria (GP) from the genera *Bacillus* and *Micrococcus* and Gram-negative bacteria (GN) from *Pseudomonas* and *Flavobacterium* genera in this association served as pollution-removing agents and were effective in contaminated soils to eliminate pollution by obsolete pesticides. Within 6 months, under optimal aeration conditions, humidity, pH values, and C: N: P ratio, the consortium of the above bacteria was introduced into the soil 3 times and was able to almost completely remove high concentrations

of obsolete pesticides. Pesticides such as dieldrin, α-endosulfan, β-endosulfan, and 4-heptachlor-epox pure were highly sensitive to the degrading activity of the above bacteria association.

Other studies have also confirmed in their experiments on the degradation of dieldrin and endrin, endosulfan degrades in a relatively short period when co-cultured with active bacteria.[25,44,46,67]

As reported by other studies, GP bacteria are more tolerant to stress compared with GN bacteria due to the type of cell walls and their endospore-forming abilities. On the contrary, the GN bacteria are usually more sensitive to environmental variations such as type and concentration of the carbon source and the presence of xenobiotics.[36,65] In this study, the GN bacteria, *Pseudomonas fluorescens*, showed the highest degradation ability in a single culture toward most obsolete pesticides; this is in agreement with the results of other studies, where GN bacteria were found to be more effective in terms of removal rates.[66-68] In this context, the members of genus *Micrococcus* have the highest potential in the degradation of pesticides.[69-72]

In contaminated soil, without any improvements of additional composition, a single culture of *Pseudomonas fluorescens* was introduced 3 times and showed significant activity against pesticides such as dieldrin and β-endosulfan; their transformation level approached $95.77 \pm 0.05\%$ and $91.59 \pm 0.05\%$. Under such conditions, pesticides like endrin aldehyde and 4-heptachlor-epox pure showed low sensitivity as their degradation rate ranged from $35.81 \pm 0.05\%$ to $26.85 \pm 0.05\%$, respectively.

On the contrary, in nonoptimized conditions, in unimproved soil, pesticides such as dieldrin and β-endosulfan were easily degraded with the use of bacterial associations that were applied 3 times within 6 months. This indicates that in addition to the created balanced physicochemical conditions, a sufficient dose of active bacteria also plays a role in the transformation of sensitive pesticides due to the presence of enzymes of degrading bacteria that catalyze the decomposition of carbon-containing compounds.

It is well known that through their enzymatic pathways, microorganisms act as biocatalysts and facilitate the progress of biochemical reactions that degrade the desired pollutants by using them as sources of energy and nutrients to build their own cells' components.[39-41] The expression of specific enzymes by the cells can increase or decrease the rate of contaminant degradation. The active bacterial species with P450 genes that were used are responsible for expressing specific monooxygenases and dioxygenases enzymes. In addition, their enzymatic pathways could facilitate the degradation of obsolete pesticides, as confirmed by our research results. Moreover, the results confirmed that the efficiency of bioremediation depends on many factors, including the chemical nature and concentration of pollutants, the physicochemical characteristics of the environment (type of soil, temperature, pH, the presence of oxygen or other electron acceptors, and nutrients), and their availability to microorganisms. To provide the growth and activity necessary for the bacteria used in these experiments, the mentioned environment factors were optimized according to contaminant concentration, type, solubility, chemical structure, and toxicity of pesticides. In both cases, these model experiments have shown that in the presence of active degrading bacteria, the transformation of obsolete pesticides in the soil can take place. However, when improved conditions are created to ensure the enzymatic activity of bacteria, degradation can proceed more intensively; this allowed bacteria to use pesticides as a source of nutrition and reduce their content to almost zero.

To assess the biodegradability of pesticides in the microbial consortium, many studies have been limited to laboratory scale.[21,43,45,48]

The progress of our research lies in the fact that we used contaminated soils taken from burial sites of pesticides with precisely defined concentrations and types. Using, in model experiments, the natural background of pollution and creating improved agrotechnical and agrochemical conditions, comparing the local current soil indicators, we achieved certain results. The results obtained allow us to recommend these simple, affordable bioremediation technologies for use in the field, where there is contamination with pesticides. These encouraging recommendations can work directly in pesticide-contaminated sites. This approach is cost-saving and feasible in the spring-autumn period.

Conclusions

For elimination the obsolete pesticides from the soil will be recommended the implementation of agrotechnical and agrochemical adjustments to improve aeration, the balance of chemical elements, and pH of the ground before the addition of ready-made suspensions of active degrading bacteria. The addition of the ready-made suspension of active bacteria once a month and regular aeration would be desirable in the 5 to 6 months period at the optimum ambient temperature (27°C-28°C) and pH (7.2-7.6) to ensure the complete elimination of pesticides from the soil.

So, in this study, the bioremediation of high concentrations of obsolete pesticides in the soils around the Suzak A and Suzak B burial zones was achieved by a microbial consortium (*Micrococcus flavus, Bacillus polymyxa, Pseudomonas fluorescens* and *Flavobacterium sp*) and a single culture of *Pseudomonas fluorescens*. The bioremediation of polluted soils around the Suzak A and Suzak B burial sites should be achieved based on the type of pesticide, the environmental matrix, and the organisms present in this ecosystem. Soil pH, temperature, cell count, biomass growth rate, substrate bioavailability, and moisture are important parameters to assure bioremediation. The most effective way is the use of adapted microorganisms either in a blend or as a single culture depending on the type of pesticide.

Author Contributions

This study was done in partnership with all the authors. TD designed the study, wrote the protocol, performed preliminary data analysis, and interpreted the data. MK handled the sample collection and treatment and gathered the initial data. SB worked on the literature review and produced the initial draft. All authors read and approved the final manuscript.

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