Eurasian Journal of Soil Science 2022, 11(4), 316 - 321

Eurasian Journal of Soil Science

Journal homepage : http://ejss.fesss.org

Bioremediation of HCB-contaminated soil using *Comamonas testosteroni* and *Zea mays* L.

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Article Info

Received : 22.11.2021
Accepted : 13.05.2022
Available online : 01.06.2022

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Abstract

Bioremediation measures to restore soil ecosystems are environmentally safe, promising and relevant. Soil ecosystems contaminated with hexachlorobenzene require remediation measures. Studying the effectiveness of applying the microbial remediator *Comamonas testosteroni* UCM B-400, phytoremediator *Zea mays* L. cultivar Olena and microbial and phytoremediation complex to remove hexachlorobenzene contamination was carried out. The HCB content was determined by chromatographic method, the microbial groups reactions to application of various remediators in the soil were studied by classical microbiological methods. The results showed that the most effective is the complex using remediators *Comamonas testosteroni* UCM B-400 and *Zea mays* L. cultivar Olena, where HCB content was reduced to 82%.

Keywords: Bioremediation, phytoremediator, bacterial strain-destructor, hexachlorobenzene.

Introduction

Natural soil ecosystems and agrocenoses are constantly exposed to high pesticides loading, and the pesticide amount that has already accumulated requires measures to remove them from the soil. Hexachlorobenzene (HCB) is pesticide as to be removed, that is included in the list of POPs (persistent organic pollutants) prohibited for use by the Stockholm Convention (2019). The qualitative and quantitative composition of microbial representatives of ecologicy-functional groups are changes under pesticide loading conditions in soil microbiocenoses. Stressful conditions stimulate the activation of biochemical potential in microorganisms to metabolize toxic substances. Therefore, the introducing microorganisms into agrocenoses is one of the important feature of agricultural pesticides biodegradation (Jaiswal et al., 2017).

Bioremediation is a complex of processes for the recovering contaminated ecosystems, included the removal of pollutants from ecotopes, based on the physiological and biochemical activity of living organisms such as plants and microorganisms to be applicate as remediators (Jaiswal et al., 2017; Gupta et al., 2020). Bioremediation methods of soil restoration are environmentally friendly, cost-effective and efficient. Therefore, more and more research is devoted to the biological restoration of soil ecosystems. The most common bioremediation methods are microbial remediation, namely, bioaugmentation (introducing potentially capable microorganisms to destroy the target xenobiotics) (Ghosal et al., 2016), as well as phytoremediation, including rhizoremediation - the process of removing toxic substances through the microbial metabolic activity in the plant rhizosphere (Oberai and Khanna, 2018; Dubchak Bondar, 2019).

Using the potentially capable plants to remediate soils by accumulating toxic products in the phytomass is also a promising bioremediation way, but in the presence of large amounts of pesticides and other toxic substances, plant development is limited, which may reduce the phytoremediation effectiveness (Arslan et al., 2017). The same phytoremediant is noted can be absorb different amounts of toxic substances, depending on their

: https://doi.org/10.18393/ejss.1125125
: http://ejss.fesss.org/10.18393/ejss.1125125
Publisher : Federation of Eurasian Soil Science Societies
e-ISSN : 2147-4249

composition and concentration in the soil (Chigbo and Batty, 2013). It is known that plants can absorb only a certain toxicant amount (Khoudi et al., 2013). However, the plant provides the rhizosphere microbiota with useful substances, as helping to survive and increase the tolerant microorganism number that have mechanisms to reduce the content of toxic substances (Hong et al., 2015).

Given the benefits of microbial remediation and phytoremediation, researchers' attention is focused on studying the combined using plants and bacteria to improve the remediation of soils contaminated with organic pollutants, including POPs (Becerra-Castro et al., 2013).

Consequently, developing effective bioremediation methods is important to remediate HCB contaminated areas. Therefore, the aim of this work was to study the applicating effectiveness of phytoremediator (*Zea mays* L. cultivar Olena) and the HCB destructor strain *Comamonas testosteroni* UCM B-400, as well as their integrated use to recovery the soil from HCB contamination.

Material and Methods

Field experiment design

The experiment was performed in the field on gray podzolic soil contaminated with substandard pesticides residues. Characteristics of gray podzolic soil were as follows: pH = 7.3 ± 0.20, alkaline nitrogen – 206.3 ± 20.6 mg / kg , mobile phosphorus – 99.3 ± 14.9 mg / kg, exchangeable potassium – 136.3 ± 13.6 mg / kg. The HCB content in the soil was determined at the beginning and after remediation measures. The experiment (plot size 1 m^2 , three repetition) included the following variants: control without remediators (a), growing *Zea mays L.* cultivar Olena as phytoremediator (b), application of liquid culture of *Comamonas testosteroni* UCM B-400, capable to HCB-destructing (c), complex liquid culture of *Comamonas testosteroni* UCM B-400 with phytoremediator (d). We isolated the *Comamonas testosteroni* UCM B-400 strain from the organochlorine pesticides (OCP) landfill (Kalush, Ivano-Frankivsk region, Ukraine) (Dimova et al., 2022). The culture liquid of the microorganism-destructor *Comamonas testosteroni* UCM B-400 was obtained by culturing in Menkina's liquid medium for 48-72 hours before the exponential growth phase. Menkina's liquid medium consisted following composition in g per l: $NaNO_3$ – 2, KCl – 0.6, sodium succinate – 4 (instead glucose -5), $MgSO_4$ – 0.5, K_2HPO_4 – 3. Microbial biomass after cultivating was determined colorimetrically (λ = 490 nm, cuvette thickness 3 mm. The concentration corresponded to 0.6 g per l in terms of dry biomass. The culture liquid *Comamonas testosteroni* UCM B-400 was applied to the soil immediately before sowing the seeds of phytoremediators, at the rate of 1 liter per 1 m^2. Biometric parameters of remediator *Zea mays* L. were determined at the seventh leaf stage and after full ripening.

Chemical analysis

The HCB content in the soil determaned used gas chromatograph Agilent 6890 N (Agilent, USA) in combination with software HP Chemstation (Agilent, USA), two microelectron capture detectors, two injectors with distribution and without flow distribution (Split / Splitless) autosampler on 100 samples, with synchronous in enter samples without flow distribution simultaneously (EPA, 1993; Levchuk et al., 2008). HCB analysis was performed using an HP-5 column (length 30 mm, inner diameter 0.32 mm, phase thickness 0.25 μm (HP cat. № 19091J-413). To analyze the component composition of the samples, a mass-selective gas chromatograph detector was used, which makes it possible to determine the mass spectra of the components in the pollutant mixtures. For identification, the obtained spectra were compared with the position in the NIST and AMDIS data libraries [http://www.sisweb.com/software/ms/nist.htm]. The using mass spectrometry to confirm and identify substances is highly effective and is accepted in modern analytical studies as the main method (Levchuk et al., 2008).

Microbiological analysis

Rhizosphere soil samples were taken from experimental sites where different bioremediation activities were applied, as well as from the control site without remediation. The microbial quantity of the main ecologicy - functional groups was determined by sowing methods of soil suspension on agar nutrient media and expressed by the counting of colony-forming units (CFU) per 1 g of dry soil. For the cultivating amylolytic microorganisms starch-ammonium agar was used; ammonifying - meat-peptone agar (MPA); pedotrophic-soil agar (contains soil extract); oligonitrophilic and nitrogen-fixing bacteria – Ashby medium; phosphate-mobilizing – Menkina's agar medium with sodium phenolphthalein phosphate (Tepper et al., 2004). To characterize the direction of microbiological processes, the coefficients of pedotrophicity were calculated as the ratio of the pedotrophic microorganism number to ammonifying bacteria, and the coefficient of nitrogen immobilization and mineralization as the ratio of the amylolytic microorganism number to ammonificators.

Statistical analysis of the data was performed by GraphPad Prism 8.0.1 software using Student's t-test. All values showed as mean ± SD.

Results

As a result of the study, data were obtained on the effectiveness of the using remediators on pesticide-contaminated soils. The effectiveness of bioremediation measures was assessed by the following indicators: the difference in concentrations of HCB in soil samples at the beginning and end of the experiment, the number and ratio of ecological-functional groups of soil microbiocenosis, assessment of biochemical processes in soil by ecological-trophic coefficients. The HCB content in the soil after remediation measures in all variants with remediators decreased compared to the initial content. Thus, due to the using bioremediation complex based on *Zea mays* L. cultivar Olena remediator and liquid culture of *C. testosteroni* UCM B-400, the greatest reduction in HCB content was 82%. When liquid monoculture of *C. testosteroni* UCM B-400 was introduced the HCB content decreased by 70%. The smallest effect was obtained with the use of the phytoremediator, which resulted in a decrease of 27.3%. The HCB content in the soil without remediators changed at the level of statistical error (Table 1).

Table 1. The HCB contain in soil under different bioremediation methods application

Bioremediation methods	HCB contain, μg per kg of dry soil		% decomposition from the initial level
	Initial level	Final level	
Without remediators	1854.0 ± 7.66****	1804.0 ± 10.33****	3
C. testosteroni UCM B-400 and Zea mays cultivar Olena	1854.0 ± 7.66****	516.4 ± 1.03****	82
Zea mays cultivar Olena	1854.0 ± 7.66****	1237.4 ± 0,77****	27,3
C. testosteroni UCM B-400	1854.0 ± 7.66****	555.2 ± 0.65****	70

****Correlation is significant at the 0.0001 level; ± the standard deviation (SD)

HCB presence in the plant mass confirmed the effectiveness of using complex "phytoremediator – microbial culture" and phytoremediator. In the phytoremediator sample from complex remediation with *C. testosteroni* UCM B-400 HCB (0.03 μg/kg) was detected, and in the sample from phytoremediator – 0.02 μg / kg. Thus, the plant is able to fully develop and accumulate more toxicant, probably due to the phytostimulant activity of *C. testosteroni* UCM B-400. However, HCB was not detected in grain samples. Thus, our results showed that bacteria play a key role in the metabolising toxic substances, but a certain contribution to this process are made by plant-remediators. Promising for the using complex remediation are on the one hand the bacterial culture is able to use HCB as a source of energy and carbon, and on the other hand – phytoremediator provides stability of biophysical processes during the period of detoxicating target substances, as well as root secretions stimulate the development of rhizosphere microbiota. Microorganisms that perform various functions to maintain the stability of soil ecosystems is played the leading role in ensuring high soil quality. We defined the development of the main ecological-trophic and taxonomic groups of soil microbiocenoses (Table 2).

Table 2. The microbial quantity in the soil under different bioremediation methods, CFU per 1 g of dry soil

Microbial ecology-functional and taxonomic group	Bioremediation methods			
	Without remediators (control)	*C. testosteroni* UCM B-400 and *Zea mays* cultivar Olena	*Zea mays* cultivar Olena	*C. testosteroni* UCM B-400
Pedotrophics	$1.59 \pm 0.11 \times 10^8$	$2.52 \pm 0.09 \times 10^{8***}$	$2.32 \pm 0.03 \times 10^{8***}$	$2.45 \pm 0.01 \times 10^{8***}$
Nitrogen-fixing and oligonitrophilic bacteria	$2.12 \pm 0.09 \times 10^7$	$3.20 \pm 0.21 \times 10^{7**}$	$3.07 \pm 0.23 \times 10^{7**}$	$2.45 \pm 0.14 \times 10^{7*}$
Amylolytics	$1.51 \pm 0.21 \times 10^8$	$1.94 \pm 0.40 \times 10^{8***}$	$1.56 \pm 0.01 \times 10^{8*}$	$1.90 \pm 0.08 \times 10^{7**}$
Ammonifying	$8.88 \pm 0.16 \times 10^7$	$1.03 \pm 0.48 \times 10^{8**}$	$1.03 \pm 0.15 \times 10^{8***}$	$1.05 \pm 0.15 \times 10^{8***}$
Phosphate - mobilizing	$7.12 \pm 0.18 \times 10^6$	$8.56 \pm 0.54 \times 10^{6*}$	$9.12 \pm 0.30 \times 10^{6***}$	$8.85 \pm 0.42 \times 10^{6**}$
Streptomycetes	$6.08 \pm 0.30 \times 10^6$	$7.56 \pm 0.48 \times 10^{6*}$	$8.10 \pm 0.36 \times 10^{6**}$	$9.76 \pm 0.54 \times 10^{6***}$
Micromycetes	$1.52 \pm 0.18 \times 10^4$	$2.12 \pm 0.30 \times 10^{4*}$	$2.18 \pm 0.32 \times 10^{4***}$	$1.90 \pm 0.12 \times 10^{4*}$

***Correlation is significant at the 0.001 level; ** Correlation is significant at the 0.01 level; *Correlation is significant at the 0.05 level; ± the standard deviation (SD)

The number of pedotrophic bacteria, as played an important role in the formation of soil fertility, increased in all experimental variants compared to the variant without remediation. It should be noted that in variants with introduced liquid culture of strain *C. testosteroni* UCM B-400, or using it in combination with phytoremediator, the increase of the number of pedotrophic bacteria reached 30%, which indirectly suggests that the bacterial metabolic activity was associated with decreasing the concentration of toxic substances in the soil (Table 2). The quantity of amylolytic bacteria that perform the function of plant residues

transformation was higher in all remediation variants, compared to the control without remediators. Thus, in the variant with mays Olena the number of this group increased to 15%, with introducing *C. testosteroni* UCM B-400 increased to 21%. The largest increase (49.7%) in the amylolytic bacteria amount was observed under complex remediation *C. testosteroni* UCM B-400 and mays Olena. The largest increasing the number of nitrogen-fixing and oligonitrophilic bacteria was under integrated using phytoremediant mays Olena and the culture liquid of *C. testosteroni* UCM B-400, almost 50% compared to the control without remediators. By separately application mays Olena and bacterial liquid of *C. testosteroni* UCM B-400, the number increased by 20 and 16%, respectively. Number of ammonifying bacteria as transformating organic nitrogen-containing compounds under applicating remediators increased significantly. Streptomycetes, which play an important role in the formation of productive microbial-plant systems and increase soil suppression to phytopathogens, have also increased in quantity. Under the phytoremediator applicating its number increased to 62%, under augmentation of *C. testosteroni* UCM B-400 and in the case of complex "phytoremediant and microbial culture of *C. testosteroni* B-400" – to 34 and 27.5%, respectively. Micromycetes play an important role in the soil fertility formation and prohumus compounds synthesis. According to the results of the study, micromycetes also showed an increase in the number from 16 to 24%. Thus, the quantitative and qualitative composition of the soil microbiocenosis changed as a resulted bioremediation measures. In all remediated variants, the number of studied groups of microorganisms was increased. Therefore, the practical applicating bioremediators will eventually lead to the restoration of soil fertility in polluted soil agrocenoses. Coefficients of nitrogen mineralization and pedotrophicity are indicators of soil quality and fertility, which are determined by the ratio of the specific microbial group number. It reflect to some extent the microbiological processes direction. Changes in the pedotrophic and mineralization of nitrogen indices under the HCB action were revealed compared to the control (Table 3).

Table 3. Coefficients of pedotrophic and nitrogen immobilization-mineralization in different remediation variants

Variant	Coefficient of pedotrophic	Nitrogen mineralization-immobilization coefficient
Without remediators	1.80 ± 0.15	1.70 ± 0.02
C. testosteroni UCM B-400 and mays cultivar Olena	2.45 ± 0.03**	1.89 ± 0.07*
C. testosteroni UCM B-400	2.33 ± 0.02**	1.80 ± 0.05*
Zea mays cultivar Olena	2.25 ± 0.06**	1.51 ± 0.02***

***Correlation is significant at the 0.001 level; ** Correlation is significant at the 0.01 level; *Correlation is significant at the 0.05 level; ± the standard deviation (SD)

Calculated coefficients showed that the soil organic matter transformation processes after remediation were stabilized. In the control soil without remediators, the lowest pedotrophic coefficient (1.73) was noted compared to the experimental variants. It's indicated the inhibiting the processes of water-soluble humus fractions transformation. Mineralization indices after remediation measures in all variants did not exceed 2,0, as confirmed the balance of immobilization-mineralization processes. Only in the variant without remediation, this was slightly increased (2.13), that's emphasizes the imbalance in the nitrogen regime, due to the pesticides loading. Biometric parameters of plant-remediators demonstrated effectiveness of bioremediation measures. At the beginning growing season in the 6-7 leaves stage the height of the *Zea mays L.* plants growing under complex remediation with the *Comamonas testosteroni* UCM B-400 culture liquid was higher by 5% compared to the plant from phytoremediator. However, the plant mass with the root and the root system mass were greater to 46.5 and 33.6%, respectively, compared to the plants from phytoremediation variant (Table 4). The obtained results indicate the phytostimulating effect of the culture liquid *Comamonas testosteroni* UCM B-400. After full maturation, the plant-remediators were again selected, biometric indicators confirmed the presence of a stimulating effect on the growth and development of plant-remediators. The plant height under applicating *Comamonas testosteroni* UCM B-400 culture liquid was statistically significantly superior to the plant without treatment. The weight of plant cobs per 1 m^2 without treatment was lower by 9% compared to the variant applicated the *Comamonas testosteroni* UCM B-400 culture liquid. Thus, with the microbial inoculum applicating, a phytostimulating effect on plant-remediators was observed.

Table 4. Biometric indicators of *Zea mays L.* plants after full maturation

Characteristic	Phytoremediator *Zea mays L.* cultivar Olena	*C. testosteroni* UCM B-400 and *Z. mays L.* cultivar Olena
Plant heigth, cm	218.5 ± 2.65***	226.0 ± 1.58***
Mass of cobs per 1 m^2, kg	7,59 ± 0,36**	8,26 ± 0,24**

***Correlation is significant at the 0.001 level; ** Correlation is significant at the 0.01 level; ± the standard deviation (SD)

Discussion

Comamonas bacteria are known to be destructors of toxic polycyclic aromatic compounds. The effectiveness of the using *Comamonas sp.* CNB-1 isolated from activated sludge polluted with from 4-chloronitrobenzene (4-CNB) was studied. This strain was introduced into the system "plant - microbial strain-destructor 4-CNB" for bioremediating the contaminated environment. In the 8-day experiment the results of the 4-CNB degradation (100 µg per g of soil at the beginning) were compared in three variants: inoculating with *Comamonas* sp. CNB-1 strain and alfalfa, inoculation without alfalfa and alfalfa without inoculation with strain. The highest efficiency of 4-HNB degradation was in the system "alfalfa - *Comamonas* sp. CNB-1 ", where the toxicant was completely removed within 2 days. In the variant only with the strain inoculating 4-CNB degradation was lasted more than 6 days, and in the third variant (alfalfa only) removal of the pollutant after 8 days exposure was not observed. These results revealed that the *Comamonas* sp. CNB-1 played a key role in the 4-CNB degradation, and alfalfa was stimulated by bacteria (Liu et al., 2007). Similarly, our study showed that under the complex remediation the percentage of HCB degradation was the highest. It can be stated that it was due to the studied strain as the toxicant content in the soil was decreased. Since HCB and 4-CNB are derivatives of benzene compounds, the ability of our studied *C. testosteroni* UCM B-400) to destroy HCB is quite expected, as was confirmed by the our study results.

Studies concerning restoration HCB contaminated soil using a tandem of microorganisms with Reygrass, it was found which microorganisms were HCB destructors. Plant root exudates are known to affect the microbial community by providing available carbon sources. An increase in the numbers of representatives of genera *Comamonadaceae*, *Azohydromonas* and *Pseudomonas* were found, indicating as root exudates stimulated the growth of these bacteria (Yan et al., 2014; Zhang et al., 2017).

The advantage of the microbial and plant-associated bioremediation methods is that bacteria are able to metabolize HCB or other toxic substances, and plants provide bacteria with high physiological activity through the supplying nutrients contained in root exudates, as well as provide oxygen needed for bacterial metabolism, namely the biophysical processes stability depends on the plant (Singh et al., 2011). High efficiency of removing hexachlorocyclohexane isomers from the soil by means of complex bioremediation based on the association of microbial culture with leguminous *Cytisus striatus* has been reported (Becerra-Castro et al., 2013).

Plants used as phytoremediators can also grow under toxic loads, although xenobiotics can be transported to the rhizosphere through transpiration flow and inhibit root development. Therefore, a certain amount of toxicant can accumulate in the plant, which was also shown in our study. However, an important condition for plants is the colonization of the root system by bacterial cells, as a result bacteria form biofilm on the surface, which can function as a protective barrier, filtering and destroying contaminants (Liu et al., 2007). On the other hand, plant root exudates stimulate an increase in the resident microbiota and also affect their activity, which leads to a change in the ratio of functional and systematic groups in soil microbiocenoses (Jha et al., 2015). Almost 20% of all photosynthetically formed carbon as transferred to the rhizosphere through root exudates is reported to use as a carbon and energy source (Dennis et al., 2010). According to the results of our study, there was also a positive dynamics in the development of microbial functional groups in the phytoremediator rhizosphere.

Plants are able to absorb a limited amount of toxic substances. For example, this has been demonstrated in a study of the effectiveness of soil phytoremediation from heavy metals using *Arabidopsis thaliana* (Khoudi et al., 2013). The results of our studies also confirmed the above, as *Zea mays L.* plants accumulated a small HCB quantity in the variants with phytoremediator and it complex with *C. testosteroni* UCM B-400 compared to the HCB amount that was removed from the experimental plots of these variants, relative to the remediation-free. It should be noted that in the variant of complex bioremediation, the plant accumulated 50% more HCB than with phytoremediator, due to the phytostimulanting activity of *C. testosteroni* UCM B-400, as also is reflected in the biometric indicators of plant- remediators. Liquid culture of *C. testosteroni* UCM B-400 recommended to be used in soil remediation measures from hexachlorobenzene pollution both in complex with phytoremediators and in the monoculture.

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