***Stenotrophomonas maltophilia* IMV B-7288, *Pseudomonas putida* IMV B-7289 and *Bacillus megaterium* IMV B-7287 – new selected destructors of organochlorine pesticide hexachlorocyclohexane.**

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Running headline: microbial destruction of organochlorine pollutants

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Abstract: Microbial destruction of organochlorine pollutants is the one of the most effective approaches, the safest and promising methods for remediation the environment from pollution. This study presents strains of microorganisms that destroy hexachlorocyclohexane: *Stenotrophomonas maltophilia* IMV B-7288, *Pseudomonas putida* IMV B-7289 and *Bacillus megaterium* IMV B-7287 - new selected destructors of

organochlorine pesticide hexachlorocyclohexane. Their advantages and features are considered, namely - exclusively of natural origin - microorganisms are isolated from places of total pollution. The studied strains are characterized by high resistance to the HCH contaminant (in the range of 100-1000 mg / l) and the ability to decompose in soil and liquid medium. We have found that strains *B. megaterium* IMV B-7287 and *P. putida* IMV B-7289 showed a high efficiency of destruction of HCH in laboratory studies when cultivated on a chlorine-free MM medium at 70.4-89.3% from initial content. For *S. maltophilia* IMV B-7288 has been found that the ability to degrade HCH-isomers depends on the season a little and it was at maximum in the summer for every studied HCH-isomer: 61.6–82.1% from initial amount. The investigated strains are promising for further work to create microbial compositions with the aim to provide an effictive destruction of HCH-isomers complex.

Key words: microbial destruction, hexaclorocyclohaxane, chloroorganic degradation, resistance.

Abbreviations:

HCH, hexachlorocyclohexane; IMV, D.K. Zabolotniy Institute of Microbiology and Virology of National Academy of Science of Ukraine; MPC, maximum permissible concentrations; CFU, colony forming units; MM medium, Menkina's mineral medium.

1. Introduction

Halogenated compounds are often considered to be relatively recalcitrant in various surface environments, such as soils, sediments, and groundwaters, due in part to their chemical stability and in other part to the lack of appropriate microbial activity for their degradation (Nayyar at al., 2014). Moreover, gamma-hexachlorocyclohexane (γ-HCH or Lindane) can negatively affects to the activities of microbial communities in impacted habitats (Timmis at al, 2009, Bhaganna at al., 2010).

The development of effective strategies for the decontamination of chloroorganic contaminated ecotopes is thus needed. Physical and chemical strategies, such as chemical inactivation, combusting, photolysis, soil vapor extraction and soil washing, have been used as decontamination strategies for polluted soils (Xu et al., 2013; Varjani at al., 2017). However, these methods are not economically viable and can involve significant polluted areas disturbances (Shahi et al. 2016). As an alternative, bioremediation taking advantage of the capacity of a wide range of microorganisms, has shown to be a cost-effective and ecologically acceptable clean-up biotechnology for treating chlororganic contaminated soils (Varjani at al., 2017). Bioremediation treatments can extensively apply for the decontamination of polluted soils using laboratory or in situ approaches (Margesin at al., 2007; Azubuike et al. 2016).

Therefore, there is necessity to find indigenous soil microorgamisms resistant to chlorinated pesticides at different concentrations: from low in agricultural applications, to medium and high at wood treatment or spill sites. As a rule, recalcitrance of organic pollutants increases with increasing halogenation. Substitution of halogen as well as nitro and sulfo groups at the aromatic ring is accomplished by an increasing electrophilicity of

the molecule. These compounds resist the electrophilic attack by oxygenases of aerobic bacteria. To overcome the relatively high persistence of halogenated xenobiotics, reductive attack of anaerobic bacteria is of significance (Fritsche at al., 2000). There are a sufficient number of reports about the destruction of a chlorinated cycloaliphatic compound γ-HCH under anaerobic conditions (Quintero at al., 2005).

But there are a lot of areas with varying levels of pollution where it is not possible to create anaerobic conditions for microbial destruction of HCH-isomers (Report, 2010/ UNEP/OCHA). In consequence of above it is necessary to research the indigenous aerobic soil microorganisms having natural resistance to HCH-isomers complex in heavily polluted areas, in order to obtain highly efficient destructors capable to decompose HCH-isomers.

Previously we had selected the bacterial strains from pesticides polluted soils and predicted possible their biordegradating properties. The microbial strains have been identified by us as *Pseudomonas putida* IMV B-7289 and *Stenotrophomonas maltophilia* IMV B-7288 (Yamborko at al. 2010). They had appeared sustainable property to decompose HCH-isomers complex (Yamborko at al., 2018). The representatives of *S. maltophilia* are ubiquitously distributed in the environments with regard to habitat and geography, also often associated with roots of various plant species as typical rhizospheric microorganisms (Ryan et al., 2009). It is known that *S. maltophilia* is an facultative aerobic, non-fermentative, gram negative bacterium; motile due to polar flagella, catalase-positive, oxidase-negative slightly smaller (0.7–1.8×0.4–0.7 mm; which distinguishes them from most other members of the genus). While *S. maltophilia* is an facultative aerobe, it can still grow using nitrate as a terminal electron acceptor in the absence of oxygen (Crossman et al., 2008). *S. maltophilia* is widespread, plant-associated important soil microorganisms. Pathogenic and conditionally (opportunistic) pathogenic strains are also widespread and capable to forme stable biofilms. However, the ability to decompose HCH has not been enough previously described.

The choice of microorganisms was connected with their degradation ability, with growth characteristics and other useful biological properties. So far, several strains of soil microorganisms have known their useful property to synthesize some complex of plant growth regulators and our strain *Bacillus megaterium* IMV B-7287 also has the property (Yamborko at al., 2016), in contrast to the majority representatives of *Bacillus* genus (Kornelia at al., 2013). Resulting in laboratory selection of these microorganisms, consistently high degradation properties were formed to decompose the HCH isomers by the 70-95 % of initial content (Yamborko et al., 2012). *Pseudomonas* and *Bacillus* genera are the predominant bacteria in polluted soils. The best-suited microbes for bioremediation are often those isolated from sites contaminated with a particular target compound (Guinn at al., 1996).

P. putida is a Gram-negative, rod-shaped, saprotrophic soil bacterium. Based on 16S rRNA analysis, *P. putida* was taxonomically confirmed to be a Pseudomonas species and placed, along with several other species, in the *P. putida* group, to which it lends its name. *P. putida* has one of the highest degradative potential (Atterby at al., 2002).

B. megaterium is a Gram-positive, mainly aerobic spore-forming bacterium found in widely diverse habitats from soil to seawater, sediment, rice paddies, honey, fish, and dried food. It can grow in simple media on over 62 carbon sources out of 95 tested including all tricarboxylic acid cycle 10 intermediates, formiate, and acetate. This

has made it an ideal industrial organism for over 50 years (Vary at al., 2007). Species *B. megaterium* is often found in unusual and sometimes toxic environments and may have potential as a detoxifying agent. The ability of *B. megaterium* to degrade persistent chloroorganic insecticides such as metachlor, Baytext and Paris green, and utilize them as carbon sources has also been documented (Vary at al., 1994).

The aim of the study is to research growth characteristics of new perspective strain-destructors at presence of toxicant and to study efficiency of HCH-degradation and stability the properties to degrade the last.

2. Material and Methods

2.1. The objects of the study.

At our previous studies we have isolated and identified strains *S. maltophilia* IMV B-7288 and *P. putida* IMV B-7289 from high polluted soil area where γ-HCH has been applied and stored more over 40 years for industrial purposes. This site has very high organochlorines contamination level (Report, 2010/ UNEP/OCHA). Also, the ability to decompose HCH-isomers complex was established in the deposed strain *B. megaterium* IMV B-7287. All strains are stored in the depository of the institute.

2.2 *Cultivation of microorganisms.*

The range of stability investigated microorganisms to HCH-isomers complex (α-HCH, β-HCH, γ-HCH, δ-HCH) were determined on agar plates M17 medium in concentration range from 100 to 1000 $mg \cdot L^{-1}$. The Petri plates were incubated at 28 ± 0.1°C for 48 h, when the colonies were counted manually and calculated in CFU (colony forming units). For monitoring the growth properties the strains were cultivated on liquid Menkina's mineral (MM) medium containing per liter: 4,0 g of glucose, 2,0 g of $NaNO_3$, 0,5 g of KCl, 200 mg of K_2HPO_4, 100 mg of $MgSO_4 \times 7H_2O$ (pH 7.2) for 11 days at rotating condition with 240 rpm at 28 ± 0.1°C.

Microbial biomass was determined in the initial inoculum using spectrophotometry. Optical density (OD) is directly proportional to the biomass in the cell suspension in a given range that is specific to the cell type and the one of every microbial suspension have been measured (at λ=590 nm, №3) and the cell biomass was adjusted to the same level for all strains. As inoculum were used microbial cultures at the middle of the exponential growth phase (72 h) that corresponds 0.6 $mg \cdot L^{-1}$ of biomass concentration, using inoculating volume 5% of all variants (on MM nutrition medium).

The microbial growth has been occurred in liquid nutrition MM medium without and with HCH-isomers complex (20 $mg \cdot L^{-1}$ for γ-HCH, calculating active insecticide substance). The number of microbial cells also has been determined during cultivation on 4-th, 7 and 11-th days by multiple dilution method using Petri plates with M17 agar medium. As a control has been used sterile mineral nutrition medium with toxicant HCH without microorganisms.

To determine the efficiency of HCH-isomers complex degradation in a liquid medium and evaluate of microbial growth the acetone concentrate of commercial preparation hexachlorane (Syngenta) was eluted calculating the final concentration of active substance γ-isomer of HCH 20 $mg \cdot L^{-1}$, which corresponds to 200 MPC (maximum permissible concentrations). The total content of α-, β-, γ- and δ-HCH isomers was determined 95% in the preparation; the others were present at the mixture in trace amounts.

The seasonal destruction of HCH-isomers by the studied strains of microorganisms have been studied in a liquid nutrient medium as described above.

Also we have been studied properties the bacteriall strains to destruct HCH-isomers complex during growth on Menkina's mineral (MM, nutrient composition see above) chlorine-free nutrient medium (KCl was excluded from the nutrient medium). In this case, the organochlorine pollutant HCH is the only source of chlorine, which is a necessary chemical element for the vital activity of cells of microorganisms. The toxicant concentration was as well 20 $mg \cdot L^{-1}$.

2.3. HCH-isomers determination

Microbial cells have been separated from cultural liquid after cultivation, by centrifugation (5600 g, 30 min). The determination of HCH-isomers amounts were carried out in the obtained microbial supernatants samples according to the recommendations of the US Environmental Protection Agency (Chemical methods, EPA, 1990). To 30 ml of supernatant was added 30 ml of saturated shaken NaCl solution (in ratio 6:5) and was shaken for 5 min at 280 rpm. After shaking with NaCl solution, 10 ml of hexane was added to mixture and was shaken again for 5 minutes. All manipulations were performed in 100-ml flasks. The resulting slurry was putted to a separating funnel and settle for phase separation, after which the lower aqueous fraction was separated for re-shaking with 10 ml hexane, and the upper hexane fraction was collected in a 50 ml rotary flask. Extraction with hexane was performed three times; all portions of the hexane fraction were collected together and dried on a rotary evaporator (HEIDOLPH Laboporta 4000 efficient, Germany) on a water bath temperature of 40 ° C and a residual vacuum of 20-30 mmhg. The obtained dry residue in a rotary flask was washed three times with 2 ml of hexane and collected in a 10 ml rotary flask, concentrated under vacuum and washed with 0.5 ml of hexane again, then all portions of hexane concentrate with HCH-isomers were transferred into vials, the volume of hexane in vials adjusted to 1.5 ml. Hexachlorobenzene was added (0.56 ng/sample, dissolved in hexane) to the combined 1 ml of hexane extract, to serve as an internal standard.

2.3 Gas chromatographic analyses.

For chemical analysis of α-, β-, γ-, δ- HCH, the confirmation of pesticides and their metabolites was performed on an Agilent Technologies 7890A gas chromatograph with an Agilent Technologies 5975C mass spectrometric detector using Agilent Technologies DRS A.04.00 software, which includes NIST and AMDIS databases. Gas chromatograph was applied in combination with HP Chemstation software and two microelectroncapture detectors, two injectors with and without distribution of the flow (Split/Splitless) by autosampler per 100 samples, with simultaneous introduction of samples. The analysis of HCH-isomers was performed applying an HP-5 column (30 m x 0.32 mm x 0.25 μm), HP cat. No.19091J-413.

2.4. Statistical analysis.

Using statistical programs of Micrisoft Exel have been performed all experimental data and the basic statistical parameters (M – mean, m – standard error, M±m) have been calculated. The difference was considered statistically significant at $P < 0.05$.

3. Results and Discussion

There is known the fact that species *B. megaterium* is frequently found with *Pseudomonas* species in contaminated environments, their capability of metabolizing unusual substrates have been suspected (Eppinger at al., 2011). For example there is known a new halogenated hydrocarbon-degrading bacterium CTBmeg1 identified also as *B. megaterium* (Kazim at al., 2019). One more example, has been isolated and identified an

endophytic bacterium strain *B. megaterium* Q3 that can effectively degrade chloroorganic herbicide quinclorac (Liu at al., 2014).

The versatility of *B. megaterium* spans its habitats, its unusual enzymes, its industrial record, its secretion capacity, its cloning host capabilities, and its many plasmids, altogether, all the features make *B. megaterium* an ideal organism for industrial, environmental, and experimental applications (Vary at al., 2007).

Pseudomonads are also known for their wide range of enzymatic activity concerning to exotic substrates and are ubiquitous microorganisms in the environment, including contaminated areas as well.

All three selected strains were studied for their ability to grow on solid nutrition medium at the presence of high HCH-isomers complex concentrations. So, *S. maltophilia* IMV B-7288 and *P. putida* IMV B-7289 actively grew on agar medium M17 at 100, 200, 400 and 1000 $mg \cdot L^{-1}$ of HCH, at the same time *B. megaterium* IMV B-7287 showed growth at a concentration of 100, 200 $mg \cdot L^{-1}$ but at concentration 400 $mg \cdot L^{-1}$ weaker growth was observed, and at 1000 $mg \cdot L^{-1}$ growth was absent (Fig.1).

Therefore, all these microorganisms can be further considered as promising for further studies of their biological and detruction properties for use in the practice of bioremediation.

The one of them – *S. maltophilia* IMV B-7288 demonstrates the strong ability to grow at the presence the mixture of four HCH-isomers on agar plates contrast to other microbial strains. The ability to grow was revealed at the highest tested concentration – 1000 $mg \cdot L^{-1}$ or 10^6 ppm (Fig.1*)*.

The result is very exciting for natural selected strain isolated from polluted area without any genomic manipulations. This was to be expected, because such a huge potential for biodegradation is consistent with the well-known literature on the high functional flexibility and ubiquity of *Stenotrophomonas*. The great genetic and metabolic diversity within *S. maltophilia* makes it omnipresent in environment (Fig.2).

But the concentration (1000 $mg \cdot L^{-1}$) have not been taken for studying microbial growth features because it was very high for other researched microbial strains. According to the literature data, concentration range for effective microbial destruction of HCH-isomers is 5-30 $mg \cdot L^{-1}$ and concentration maximum is approximately 100 $mg \cdot L^{-1}$ in a liquid medium (Mannonmani at al., 2000). The chosen concentration of toxicant at the experiment is 20 $mg \cdot L^{-1}$ – there is average range for cultivation in liquid nutrition medium.

S. maltophilia IMV B-7288 demonstrated rapid growth and short lag-phase both – in the control without HCH and in presence of toxicant. The maximal quantity of living cells 112 $^{x}10^6$ CFU and meaning of μ_{HCH} was 0,030 but meaning $\mu_{control}$ was approximately the same – 0.028, there was observed on the 4-th day of cultivation (Fig.3A). But after the 7 day of cultivation the viable count decreased till 6.5 $^{x}10^6$ (ln 15,83) in the control, and till $7.5^{x}10^6$ CFU (ln 15,68) – in the presence of HCH (Fig.3A).

At the initial stages of degrading process the short lag-phase and active growth and of *S. maltophilia* IMV B-7288 lead to more rapid HCH-destruction – on the 4-th day we have observed stationary phase, while other studied strain-destructors – *B. megaterium* IMV B-7287 and *P. putida* IMV B-7289 only start its active metabolism (Fig.3). The growth rate of *B. megaterium* IMV B-7287 was maximal on 7-th day of cultivation: μ_{HCH} was 0.028 and $\mu_{control}$ was 0.029 $hour^{-1}$. They rate practically didn't differ from growing activity of

Stenotrophomonas. The same growing activity had *P. putida* IMV B-7289 on 7-th days of cultivation: μ_{HCH} was 0,029 and $\mu_{control}$ was 0.029 $hour^{-1}$.

Thus, the total duration of active metabolism phases of studied microorganisms will be much longer and as a result HCH-degradation will be much more effective at the simultaneous using the three strains.

It could be note the presence of HCH-isomers mixture in cultural liquid at given concentration didn't cause the growth inhibitory effect to *S. maltophilia* IMV B-7288, *B. megaterium* IMV B-7287 and *P. putida* IMV B-7289 (Fig.3). As a consequence, we have established that tested concentration (20 $mg \cdot L^{-1}$) was non-toxic and didn't affect to growth dynamic to the investigated microorganisms. The scientists explain such effect by antimutagene process, this is a genetic homeostasis that provides the ability of cell to maintain stable state of its genome. Antimutagenesis is inherent in all the living cells (Słoczyńska at al. 2014).

We can state that studied concentration of HCH-isomers were noncritical because significant difference between the experimental growth parametrs under HCH-isomers complex action and the control one was not observed. The genetic homeostasis of cells system had worked effectively and antimutagenic protection was realised by several mecanisms simultaneously using compounds with antimutagenic properties (adenosine, guanosine, acridine, Mg^{2+} , Co^{2+}, Se^{2+}-ions, ascorbic and uric acids, humic acids, steroid hormones, retinol, tocopherols, chlorophylls) (Kada et al, 1987). It is known that microbial cells activated and concentrat all the resources on DNA replication and cell division (DNA repairation system, glutathione (GSH)-transferase activity, etc.) (Namiki at al., 1990).

In the strains *B. megaterium* IMV B-7287 and *P. putida* IMV B-7289 selected for the study, we also assumed the presence of extraordinary properties with respect to the toxicant HCH.

Microbial destruction of HCH-isomers by *B. megaterium* IMV B-7287 and *P. putida* IMV B-7289 have been studied at deficiency conditions of chlorine ions in the nutrient medium. There is well known, Cl^- ions are necessary component of physiological solution of any cells. Therefore, at chlorine-free conditions in nutrition medium would promote more active elimination of chlorine ions from HCH-molecules. Thus, the first step of HCH-destruction - dehalogenation - will be occure more intense (Fig.4).

We have found that strains *B. megaterium* IMV B-7287 and *P. putida* IMV B-7289 showed a high efficiency of destruction of HCH in laboratory studies when cultivated on a chlorine-free MM medium. The residual amounts of HCH isomers after destruction on a chlorine-free nutrient medium were for the *B. megaterium* IMV B-7287 strain - 29.6% - α-HCH, 17.3% - β-HCH, 9.4% - γ-HCH, 10,7- δ-HCH. Destruction activity of *P. putida* IMV B-7289 strain was also at high level on a chlorine-free nutrient medium – the residiual amounts were 17,8% for α-HCH, 16,7% - β-HCH, 14,7% - γ-HCH, 18,9 - δ-HCH (Fig.4).

We have studied the possible changes in HCH-isomers degrading activity of selected microbial strains seasonally for year under laboratory conditions in liquid nutrient medium (Fig.5). For *S. maltophilia* IMV B-7288 has been found that the ability to degrade HCH-isomers depends on the season a little and it was at maximum in the summer for every studied HCH-isomer (Fig.5). Destruction of α-HCH was 73,4 % from initial amount, β-HCH – 61,6%, γ-HCH – 82,1% and δ-HCH – 74,5% was degraded.

The significant differences between degradation certain isomers were revealed to *S. maltophilia* IMV B-7288 and *B. megaterium* IMV B-7287 in the autumn: the lowest degrading level have been observed for β-HCH –

42,1% and 57,4% respectively.

As a result, *S. maltophilia* IMV B-7288 had the sustainable ability to decompose HCH-isomers complex among the studied bacterial strains. The property was stable during long time regardless of season. The researchers noted about the possibility to change the intensity of microbial growth in laboratory conditions, depending on the season of year and solar activity (Guicharnaud at al., 2010). *S. maltophilia* has also been found to play important role in the bioremediation of chlorinated pesticides like Chloropyrifos (Dubey, at al., 2012) and endosulfan (Barragaán-Huerta at al., 2007). Soil isolates of *Stenotrophomonas spp*. degraded dichloro diphenyl-trichloroethane (DDT) two l,l-dichloro-2,2-bis(p-chlorophenyl) ethane (DDD) (Mwangi et al., 2010). The three main enzyme families implicated in pesticide degradation are esterases, glutathione S-transferases (GSTs) and cytochrome P450 (Ortiz-Hernández et al., 2013).

In our case fluctuation of destruction activity for *B. megaterium* IMV B-7287 also was observed. The strain had not demonstrated the stability of HCH-isomers decomposition for year (Fig.5). In the summer we observed the minimal destruction activity – 57,4-75,1% were destroyed in the liquid nutrient medium only from initial content, comparing with 95,5-97,7% in the winter-spring period (Fig.5).

The biggest difference between destructive activity HCH-isomers complex at different seasons was observed for *Pseudomonas putida* IMV B-7289. It is known that a lot of microorganisms have stages with active growth, which are replaced by slow growth stages and stability of season growth properties is strain feature for microorganisms (Minkevich, 2011).

In this regard, the ability to decompose some kind of pollutants by *S. maltophilia* may be interesting relating to creation microbial combinations with other effective strains for destruction of chloroorganic pollutants and promising for development of soil remediation technology.

Conclusions

The study has found the strong resistance of all tested strains on Petri plates (M17 nutrition medium) to concentration 100, 200, 400 and 1000 $mg \cdot L^{-1}$of HCH. The gained results emphasized that strains *B. megaterium* IMV B-7287 and *P. putida* IMV B-7289 showed a high efficiency of destruction of HCH in laboratory studies when cultivated on a chlorine-free MM medium at 70.4-89.3% from initial content. For *S. maltophilia* IMV B-7288 has been found that the ability to degrade HCH-isomers depends on the season a little and it was at maximum in the summer for every studied HCH-isomer: 61.6–82.1% from initial amount. The investigated strains are promising for further work to create microbial compositions with the aim to provide an effictive destruction of HCH-isomers complex.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Author Contribution to Study

Nadia Yamborko, Galyna Iutynska, Irina Levchuk wrote the article. All authors contributed to the conception, design and critically revised the manuscript

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Figures legend and captions

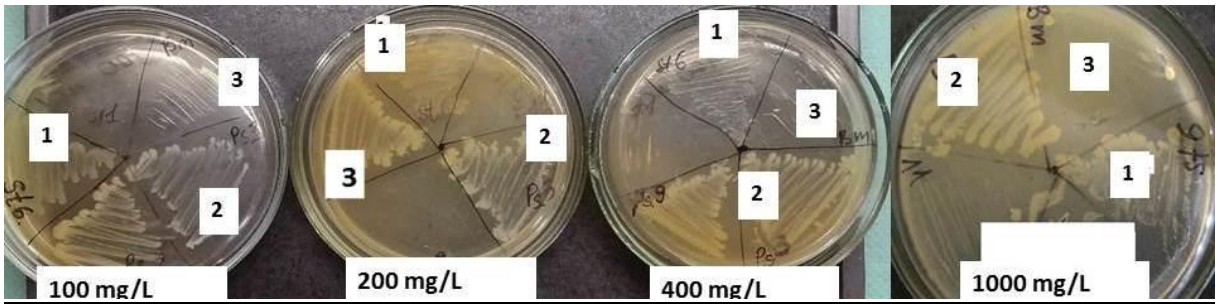


Fig. 1 Resistance and growth of tested strains on Petri plates (M17 nutrition medium) with concentration 100, 200, 400 and 1000 $mg \cdot L^{-1}$ of HCH-isomers complex: 1- *S. maltophilia* IMV B-7288; 2- *P. putida* IMV B-7289; 3- *B. megaterium* IMV B-7287.

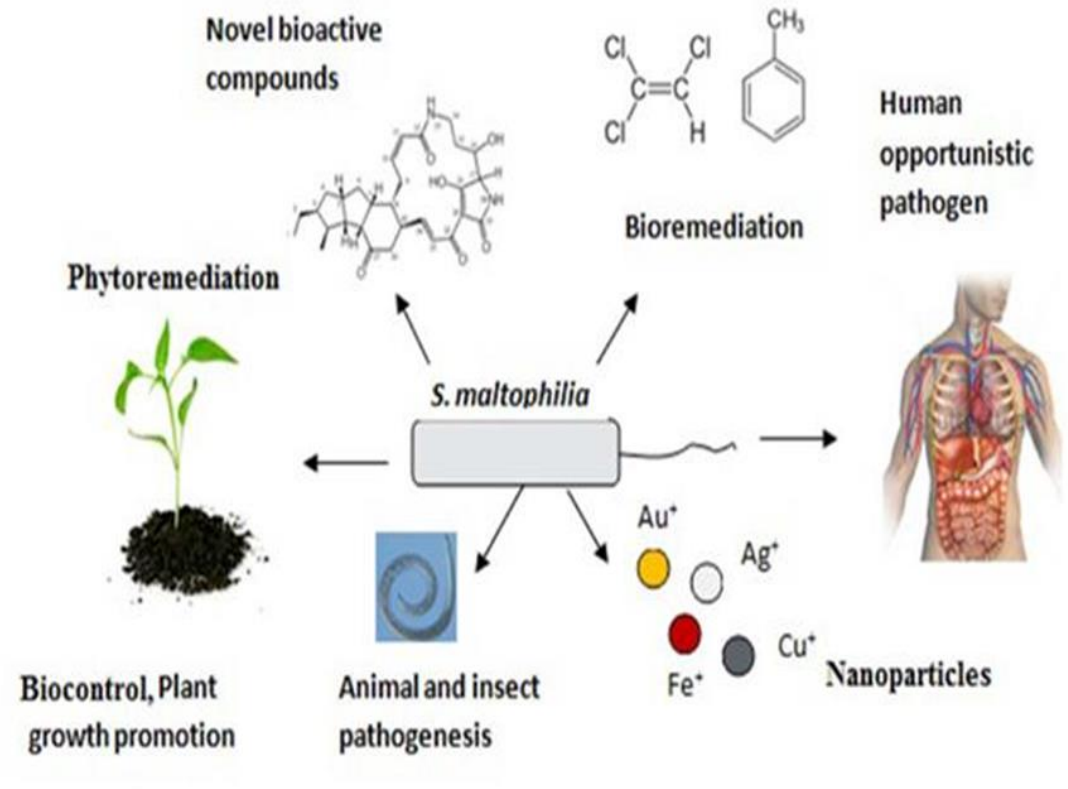


Fig. 2. The multifaceted role of *S. maltophilia* in environment signifies its substantial genetic, metabolic, and residential diversity (Mukherjee at al., 2016).

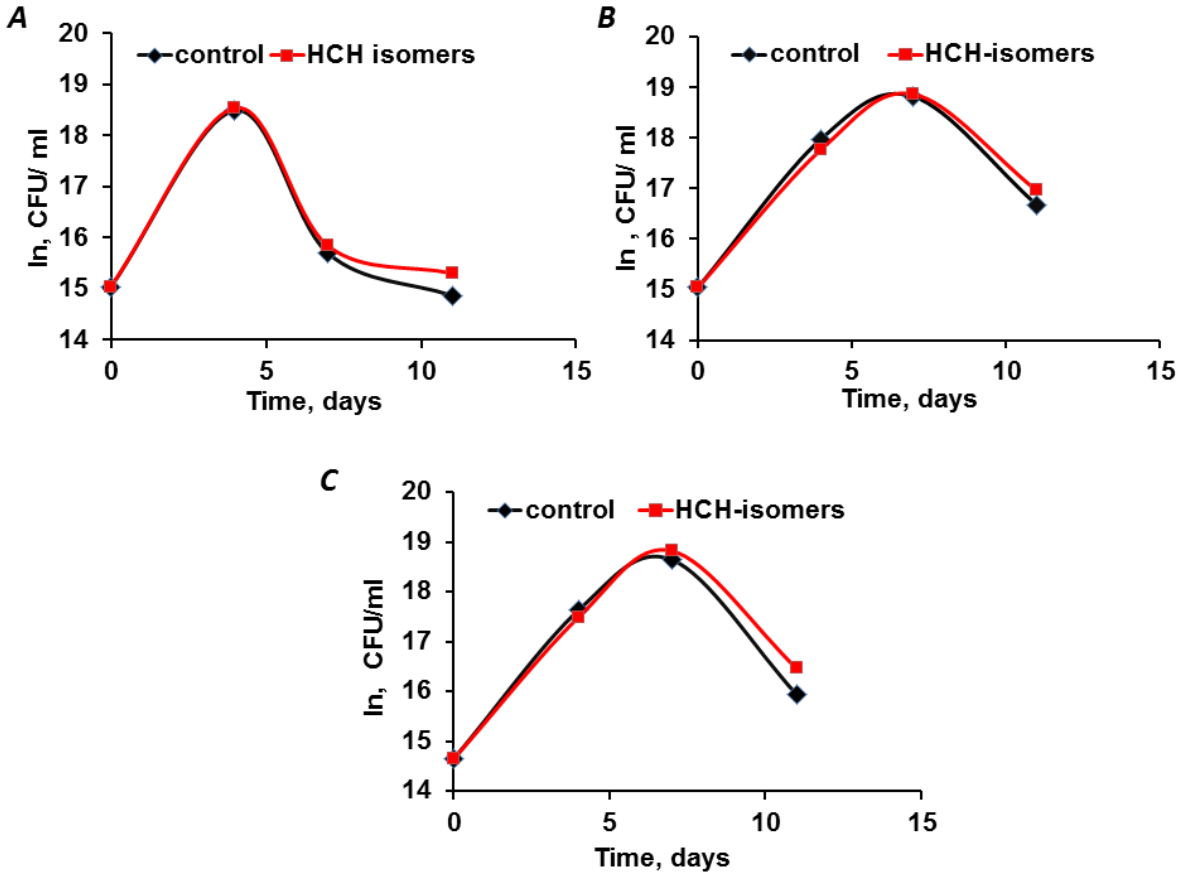


Fig. 3. The quantity of microbial cells during destruction HCH-isomers complex by *Stenotrophomonas maltophilia* IMV B-7288 (A), *Bacillus megaterium* IMV B-7287 (B) and *Pseudomonas putida* IMV B-7289 (C).

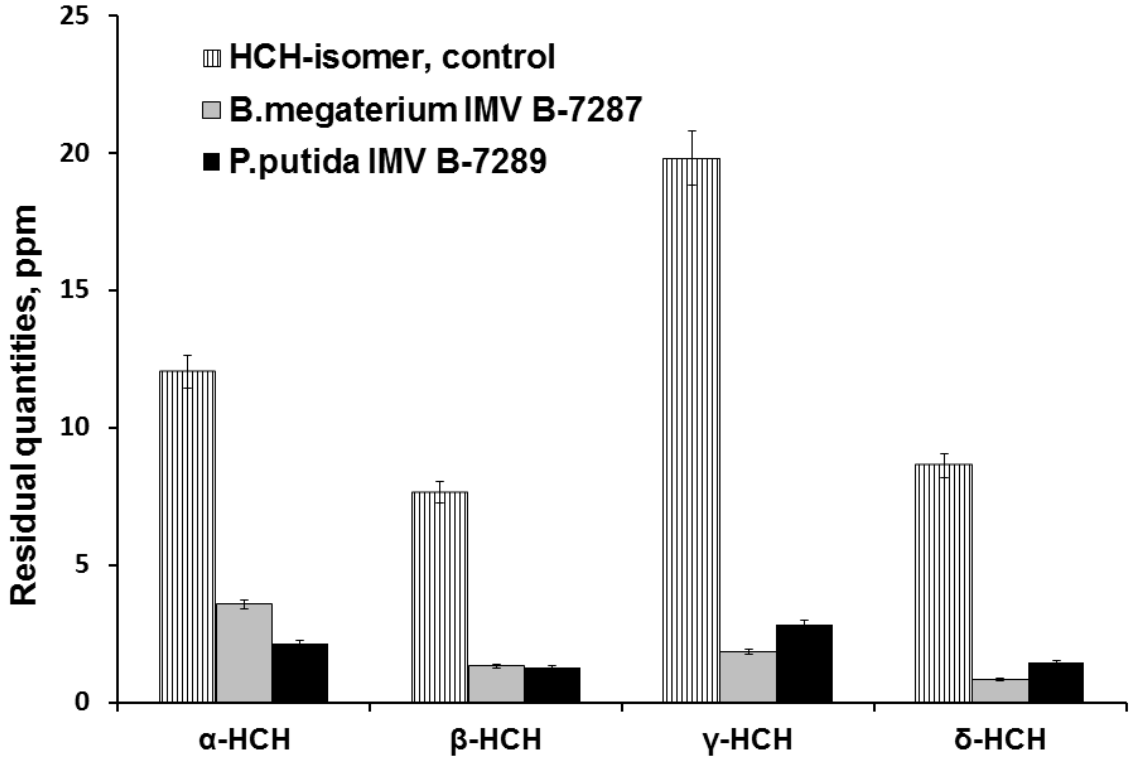
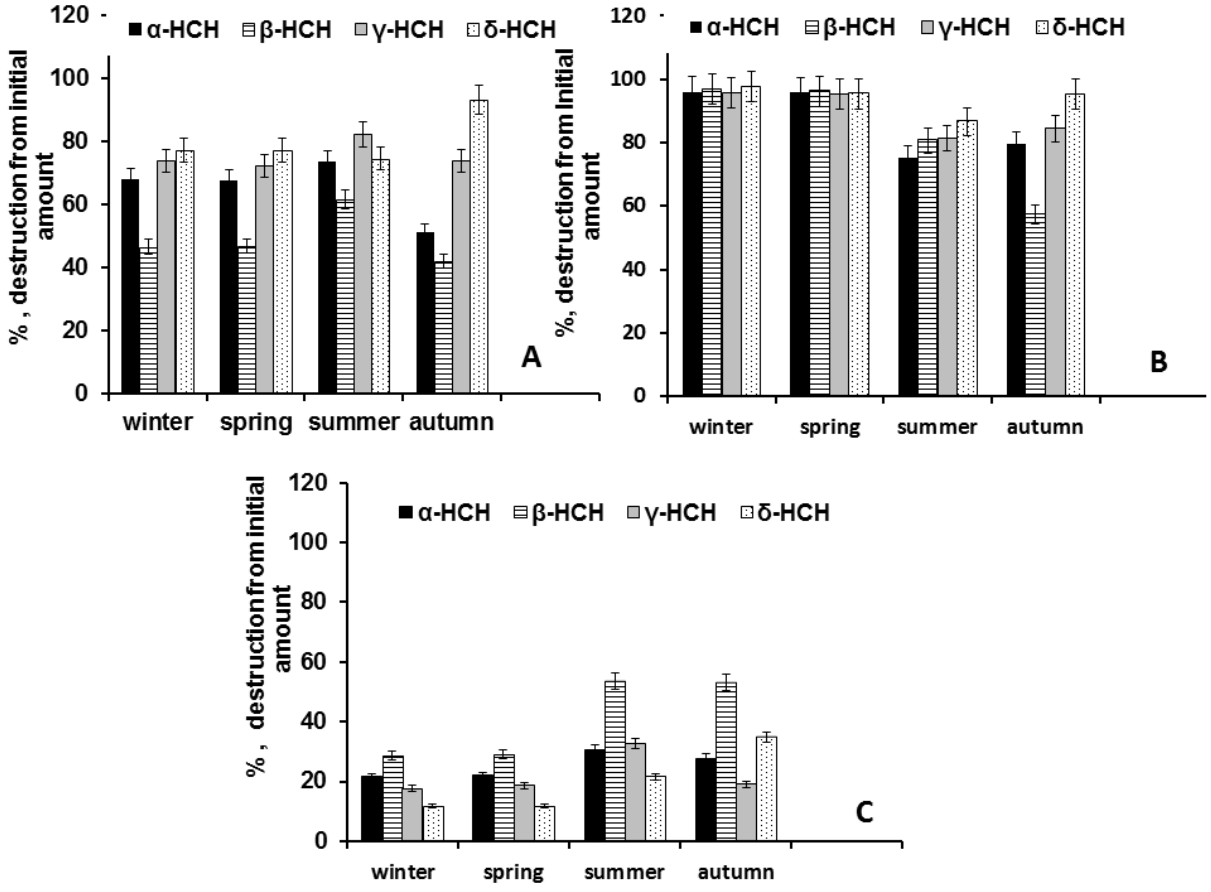


Fig. 4. Destruction of HCH-isomers by microorganisms on a chlorine-free synthetic nutrient medium.



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Figures legend and captions

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