

Biodegradation of Persistent Chlorinated Hydrocarbons Using Selected Freshwater Bacteria

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Abstract

Isolation and selection of potent degradative microorganisms against naturally persistent compounds considered as a powerful means for environmental detoxification. In the present study, the natural biotic capacity of water to degrade a mixture of four chlorinated hydrocarbons; DDT (1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane); DDE (1,1-dichloro-2,2-bis (p-chlorophenyl) ethane); Lindane (1,2,3,4,5,6-hexachlorocyclohexane (γ -HCH); Endrin (1,2,3,4,10,10,hexachloro-6,7-epoxy-1, 4,4a,5,6,7,8a-octahydro-1,4-endo-5,8-dimethanophthalene) at two elevated levels (0.05 and 50 ppm) was investigated. Five bacterial species (*Pseudomonas cepacia*, *Enterobacter agglomerans*, *Arthrobacter sp.*, *Eschericia coli* and *Staphylococcus aureus*) isolated and identified from a heavily polluted lake (L. Mariut) were investigated as biodegraders during the present work. Also, the ability of these pesticides to challenge the growth of the selected species has been examined. The tested bacterial species found to efficiently promote the degradation of the tested compounds. Degradation of DDT, DDE, Lindane and Endrin were mediated by all the species. At the low concentration (0.05 ppm), the pesticide mixture was diminished by *P. cepacia* after 24 h exposure. On the other hand, *Arthrobacter sp.*, *E. coli*, *S. aureus* and *E. agglomerans* were able to degrade the pesticide mixture totally after 48, 72, 72 and 96 h, respectively. At the high concentration (50.0 ppm), the tested bacteria were able to degrade >87.0% of the pesticide mixture. The inhibitory effect ($p < 0.001$) of the pesticides mixture (DDT, DDE, Lindane and Endrin) on the bacterial growth at 0.05 and 50.0 ppm was noticed. The magnitude of growth inhibition was found to be a function of the pesticide mixture concentration, bacterial species and the exposure time.

Keywords: Biodegradation; Chlorinated hydrocarbons; Growth; Inhibition; Pesticides; Water pollution

Introduction

Halogenated aliphatic compounds are often considered to be recalcitrant in many subsurface environments, such as soils, sediments, and ground waters, due to their chemical stability and in part to the lack of appropriate microbial activity for their degradation [1]. For example, DDT and its degradation products continue to be detected in the aquatic and terrestrial species including human decades after their application to soil [2,3]. Such bioaccumulation represents a great threat since DDT metabolites are both toxic and estrogenic and can alter reproductive development in reptiles and mammals [4-8]. Therefore, remediation of the environment using biodegradation considered as an intensive requirement to minimize the negative consequences of pesticide pollution.

Biological agents mediate the most significant degradation of organic compounds in the natural environment. Very often organic compounds such as pesticides are degraded sequentially through the activity of a series of different organisms. The main degraders in nature are microorganisms, mostly bacteria and some fungi [9]. The rate at which pesticides are degraded varies widely from days to years in some cases. DDT, DDE and lindane characterized by low

degradability (3-10 years), thus pose a serious ecological long-term problem. However, DDE and its unchlorinated analog, 1,1-diphenylene (DPE) was found to be degraded by *Pseudomonas acidovorans* [10]. Moreover, *Pseudomonas sp.* is known as a powerful degrader to γ -Hexachlorocyclohexane (γ -HCH) [11] as well as soil [12] and marine [13] microorganisms.

Lake Mariut, south of Alexandria is a hypertrophic lake heavily polluted with agricultural, industrial and domestic untreated discharges. Lake Mariut suffers from severe organic, heavy metals and pesticide pollution particularly chlorinated hydrocarbons. In the present study, the interaction between five bacterial species isolated from Lake Mariut's water and a mixture of organochlorine pesticides was investigated concerning their ability to degrade such pesticides as well as the toxic effects of the selected pesticides on bacterial growth.

Materials and methods

Pesticides

DDT, 1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane 99%; DDE, 1,1-dichloro-2,2-bis(p-chlorophenyl) ethane 99%; Lindane, 1,2,3,4,5,6-hexachlorocyclohexane (α -HCH) 97%; and Endrin, 1,2,3,4,10, hexachloro-6,7 epoxy-1,4,4a, 5,6,7,8a-octahydro-1,4-endo-5,8-dimethanophthalene 95% (Aldrich, USA) were used during the

present study. They were selected based on their availability in L. Mariut, south of Alexandria which exceeds their levels in the most polluted lakes worldwide. Stock and standard solutions of DDT, DDE, Lindane and Endrin were prepared for Gas Chromatography (GC) analysis in n-hexane Analar (BDH grade, England). They were examined as mixtures at two elevated levels, 0.05 and 50 mg/l, based on their levels in L. Mariut.

Microorganisms

As a result of the intensive pollution, L. Mariut considered as an extreme environment where its biotic elements (micro and macro) have been shifted to highly resistant species. During the present study, five different bacterial species were selected from the Institute of Graduate Studies and Research (IGSR) collection to investigate their biodegradation capabilities for the selected pesticides. They were originally isolated from L. Mariut and identified as *Pseudomonas cepacia*, *Enterobacter agglomerans*, *Staphylococcus aureus*, *Arthrobacter sp.*, and *Eschericia coli* [14]. Their occurrence in L. Mariut's environment which characterized by very high levels of pesticides reflects their resistance to such type of pollution.

Preparation and culturing conditions

For each species, three flasks containing nutrient broth (100 ml each) (Oxoid, England) were prepared, sterilized and heavily inoculated. After inoculation, the three cultures of the five species were incubated in a shaker incubator at 100 rpm and 37°C for 24 h after which, pesticides were added. One of the three replicates was used as a control culture where no pesticides were added. The other two were injected with the low and high concentrations of the selected pesticides as mixtures respectively. All the treated and the untreated cultures were incubated for another 24 h under the same conditions. After incubation, a specific fraction from each culture including the control was aseptically drawn and used for analyzing residual concentration of the pesticides as well as the inhibition in the growth of the selected bacteria. Then samples of the treated and untreated cultures were drawn every 24 h for 4 successive days and subjected to the same analysis. Survival of the selected microbes for the experiment duration (5 days) is attributed to the use of the nutrients in the rich medium for the first 24 h and the use of pesticides as sole carbon and energy sources after supplement to the treated cultures. This was confirmed later by the great ability of such bacteria to degrade the highly recalcitrant pesticides selected. Beside, these bacteria were adapted to extreme pollution and starvation conditions where they can stand and survive. This can explain the viability of our selection (treated and control) for such short period of time (5 days) is highly expected.

Inhibitory effect of chlorinated pesticides on bacterial growth

Inhibitory effect of the tested pesticides mixture on the growth of the selected bacteria at the two elevated levels was determined by comparing total viable count of bacteria (TVC) of the control culture with those of the treated ones using the Standard Plate Count (SPC) technique [15] after appropriate decimal dilutions (up to 10⁻⁸). At each exposure time, 0.1 ml of the final dilution from each culture was inoculated on nutrient agar in triplicates. Plates were incubated at 37°C for 24 h. After incubation, bacterial colonies were counted and numbers of the colony forming units (CFU) were calculated using dilution factors. This procedure was repeated every 24 h for four successive days. Then, the inhibitory effect of pesticide mixture on the

tested species at both concentrations and each exposure (contact) time on bacterial growth was determined. Inhibition% of bacterial growth was calculated according to the following equation:

$$\text{Inhibition \% of Bacterial Growth} = \frac{\text{BTVC of the Control} - \text{BTVC of the Sample}}{\text{BTVC of the Control}} \times 100$$

Where BTVC= Bacterial total viable Count

Biodegradation assays

Bacterial cultures with the two pesticides concentrations were left in contact with pesticides mixture for 24 h. To harvest bacterial cells, cultures were aseptically drawn, centrifuged (Heraeus-labfuge centrifuge, USA) at 6000 rpm for 5 min. Bacterial pellets were discarded and the supernatants were filtered through 0.22 µm Millipore membrane filter to retain the rest of bacteria. Then pesticides residues or their metabolites were extracted in the clear supernatants, after biodegradation, cleaned up and determined using GC. Biodegradation assay was run for four days at the lowest pesticides concentration and was extended to 6 days for the highest pesticides concentration to investigate the effect of the contact time on pesticides biodegradation and to trace the decline of the tested compounds Samples were withdrawn at 24 h interval.

Pesticide residual analysis

Extraction and clean up technique

Extraction and cleanup of the tested pesticides (p, p'-DDT, DDE, Lindane and Endrin) from water samples were carried out. Clear supernatant of the 5 ml sample with the remaining residues of the tested pesticides was extracted three times with 50 ml methylene chloride (1:10 v/v) using shaking in separatory funnel or mechanical tumbling in bottle. The lower phase was then collected, filtered through standard Watman filter paper (47 mm) on which 5 g of 25% anhydrous sodium sulphate was placed to retain all water, air-dried and dissolved in 2 ml n-hexane. The extracts were evaporated to dryness using a rotary vacuum evaporator at 30°C and adjusted volume to 15 ml with methylene chloride. Cleanup of the extract was performed using 25×2 cm chromatography column packed with florisil (PR grade, 6-100 mesh) which was preheated at 130°C for 24 h to remove moisture. The column was first washed with 50 ml 1 M methylene chloride, and then the residue was eluted with another 50 ml methylene chloride 1 M. The cleaned extract was collected in 250 ml flask. The solvent was evaporated at a rotary vacuum evaporator at 30°C. The residue was quantitatively transferred to standard glass stoppered test tube with n-hexane. The solvent was evaporated and concentrated using a micro-Snyder column fitted on a concentrator tube and the volume was adjusted to exactly 2.0 ml with chromatographic n-hexane [16].

GC determination

An appropriate aliquot of each sample plus the standards of the investigated pesticides were injected into a Hewlett Packard GC model. GC 5890 series II capillary USA, equipped with electron captures detector (GC-ECD). Column, HP5 (cross-linked 5%, PH ME Silicon), 30 m×0.32 mm×0.25 mm film thickness, made in USA, was used. The injection took place at 200°C oven temperatures, 250°C injection temperatures and 300°C ECD temperatures with flow rate 3 ml/min. Identification of tested pesticides was accomplished by their retention time, which compared with known standards at the same

conditions. The concentration of the tested compounds or metabolite (DDE) was then calculated by comparing its total area with the total area of the standard detected insecticides.

Statistical analysis

Bacterial counts were converted to \log_{10} CFU/ml and subjected to ANOVA using Statistic Version 5 (Stat-Soft In., Tusla OK, USA). Means, standard error and the least significance between the means were determined ($p < 0.05$).

Results and discussion

Inhibitory effect of chlorinated pesticides on bacterial growth

Measurements of growth inhibition, the viability of bacterial cells, and the effects on respiration and other bacterial metabolic parameters have been used as short-term biological screening tests of toxicity [17-19]. Under the controlled experimental conditions, the combined effect of the selected pesticides on the individual isolated species was remarkable. Densities (CFU/ml) of the selected bacteria during the biodegradation assays are presented on their figures 1 and 2. These densities of the treated and untreated cultures were used to calculate the inhibition% of their growth due to the pesticide toxicity. During the experiment duration (4 days), almost 99% inhibition was induced ($P < 0.001$) in the growth of all of the selected species at both tested concentrations (Figures 1 and 2) after 24 h exposure. Slight differences were noticed related to bacterial species, exposure time and the concentration of the pesticides mixture. In some cases, recovery in the growth occurred such as for *P. cepacia* and *E. agglomerans* which could be attributed to acclimatization and acquired resistance against the inhibitors. This sharp toxicity towards the selected species arose mainly from the synergism among the selected pesticides as well as the controlled conditions during the experiment where no interferences occurred. Such interferences and interactions either with chemical or biological components reduce to great extent the toxicity of these compounds. This is confirmed by the prevalence of these species in L. Mariut ecosystem that is heavily polluted with pesticides and other toxic compounds. This is also clearly shown by the great biodegradation efficiencies achieved by the very few numbers of the resistant and adapted cells left in the culturing medium after growth suppression as also found by Fuentes et al. [20] and Gao et al. [21]. The present results are in accordance with the high inhibitory effects of DDT, 2, 4-D and the related compounds on bacterial growth obtained by Donato et al. [22] for the effect of DDT on the growth of *B. stearothersophilus* upon the addition of 1 μM DDT to basal and Ca^{2+} growth media. Similar concentration-dependent effect was reported on the growth of microorganisms in batch cultures for several polycyclic aromatic hydrocarbons [23]. These and other studies provide experimental data useful for developing structure-activity relationships. In addition, these studies have important ecological impacts contributing to the design of selective analogs and defining upper-limit criteria for concentration of pollutants in the environment [19].

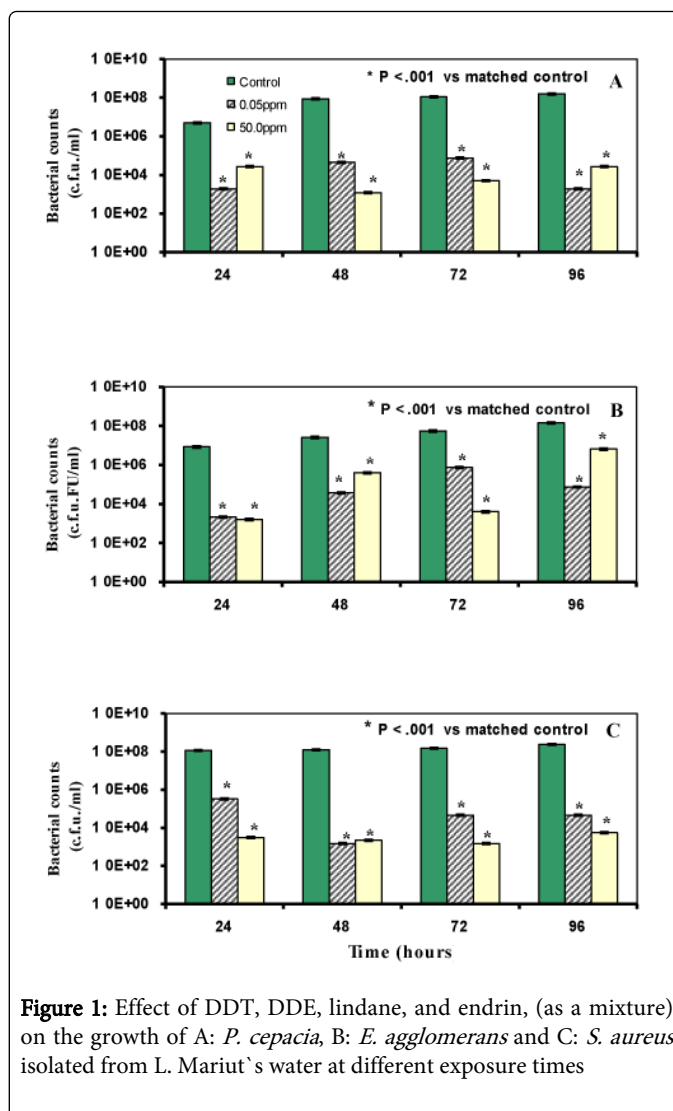
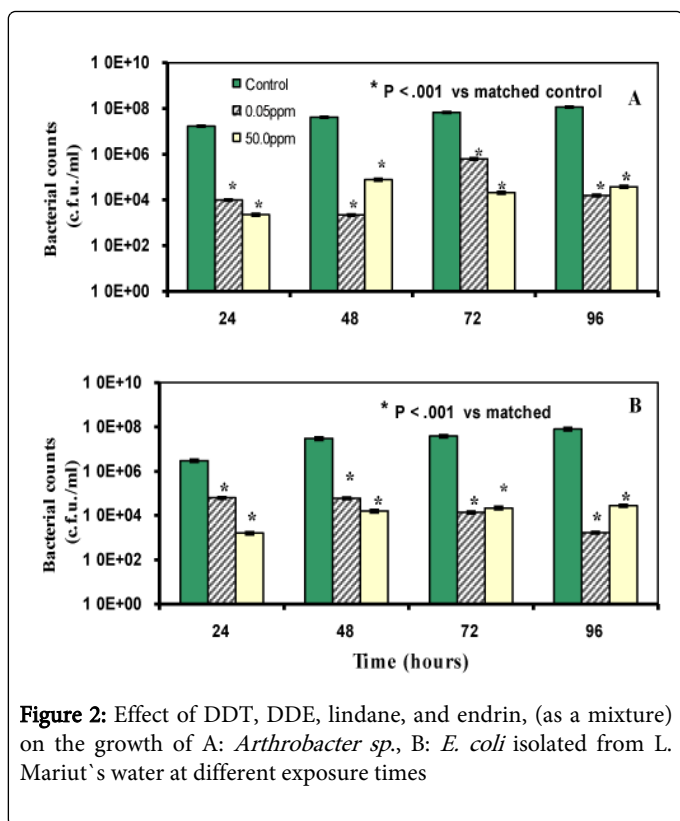


Figure 1: Effect of DDT, DDE, lindane, and endrin, (as a mixture) on the growth of A: *P. cepacia*, B: *E. agglomerans* and C: *S. aureus* isolated from L. Mariut's water at different exposure times

Biodegradation of pesticides

Biodegradation of pesticides is dependent on the presence, numbers and activity level of microorganisms, which possess the appropriate enzymatic capability. In addition, environmental conditions must be suitable such that degradation can proceed. Degradation rates are strongly influenced by environmental factors all of which affect microbial activity [24,25]. Microorganisms or their enzymes must be in contact with the pesticide, and the pesticide or its metabolites must be transported into the cell. The five bacterial species dominating the heavily pesticides polluted system (L. Mariut) were investigated for their biodegradation capabilities against a mixture of four chlorinated hydrocarbons polluting that system.



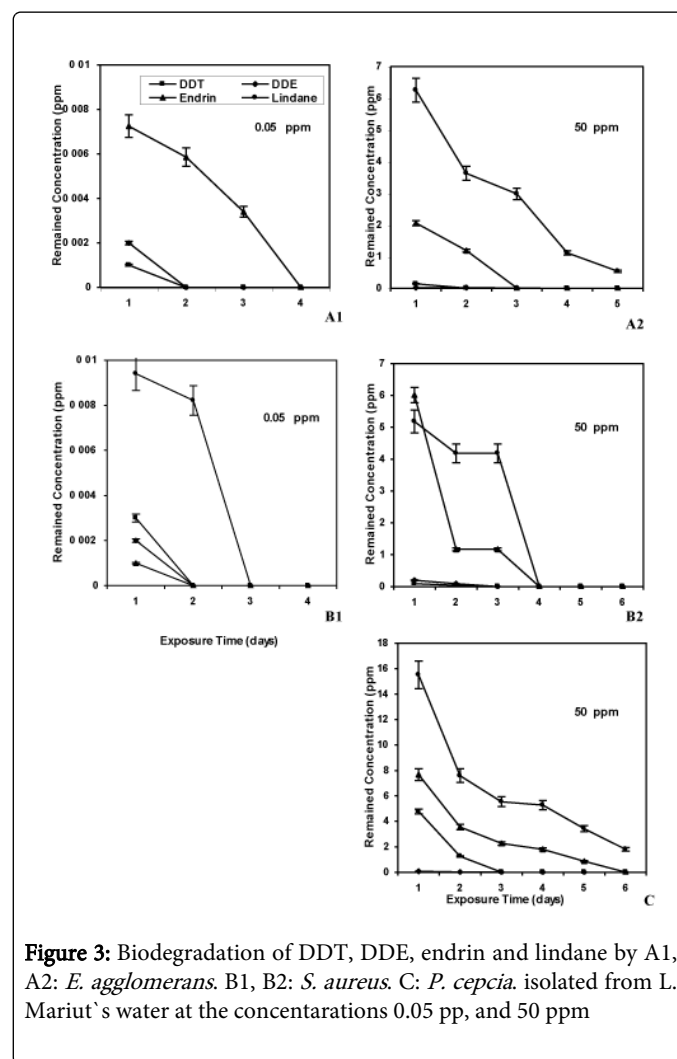
At the lowest concentration (0.05 ppm)

Generally, all of the selected species exhibited superior capabilities to degrade all of the examined compounds at that concentration with a degradation efficiency ranged between 81.2 and 100% (Figures 3 & 4). They also exhibited selective behavior for degradation depends on bacterial species, chemical structure of the compounds as well as exposure time. *P. cepacia* came on the top with a superior ability reached 100% degradation of all the tested compounds at a very fast rate where none of them were detected in the growth medium after the first 24 h and throughout the experiment. This was followed by *S. aureus* which had 100% degradation for all the compounds after 48 h except for lindane although achieving high degradation efficiency (81.2, 83.6 and 100% after one, two and three exposure days respectively). *Arthrobacter sp.* showed similar results with higher efficiencies for lindane (84, 93.2 and 100%) after the same contact time respectively. *E. coli* showed great degradation efficiency towards the most recalcitrant compounds selected (DDT, DDE, endrin and lindane) reached 100% after 48 h. *E. agglomerans* achieved the same results for DDT, DDE and lindane but needed longer contact time (4 days) to achieve 100% degradation for endrin where it degraded 85.5, 88.3 and 93.2% of the compound after one, two and three days respectively.

At the highest concentration (50.0 ppm)

It was expected that one thousand fold increases in the pesticides concentration will remarkably reduce the degradation efficiency by the selected organisms. However, only slight decrease was induced indicating high efficiencies for such selection as pesticides biodegraders. At 50.0 ppm, DDT had the highest removal rate by all the selected species ranged between 90.4 and 100% after two to three

exposure days. This was followed by DDE which achieved a range of 99.6 and 100% degradation after an exposure time ranged from one day with *P. cepacia* to four days with *E. coli*. Endrin was third in the order with a degradation rate ranged between 67.4 and 99.9% of the parent compound after three to six days while lindane was the most recalcitrant although achieving high degradation (68.9-94%) by the selected species at the same exposure time (Figures 3 & 4). Figure 5 illustrates GC chromatograms of biodegradation the tested compounds at their highest concentration (50 ppm) using the selected microorganisms which clearly confirmed high biodegradation efficiency as well as differences among the tested species.



At this concentration, the effect of the exposure time was clearly manifested where for each species, degradation capability for each compound increased regularly with increasing the intact time between the microorganism and the compound. Also, type of the pesticides had a clear effect on their degradation where all of the tested compounds were selectively biodegraded. The same finding was also found by Aislabie and Lloyd-Jones [24] and Pino et al. [25] who reported that the effectiveness of biodegradation in the natural environment was largely determined by availability, but the degree of recalcitrance was also influenced by the chemical structure of the organic compound.

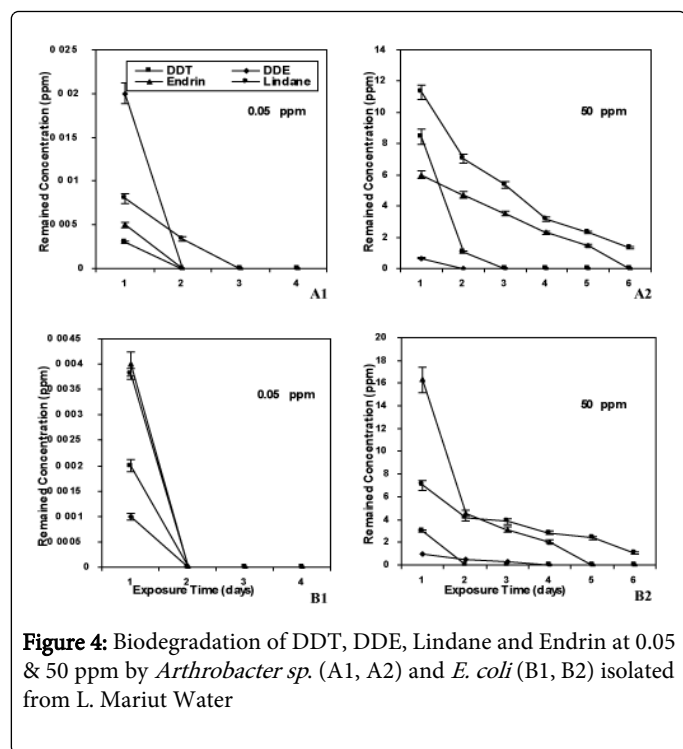


Figure 4: Biodegradation of DDT, DDE, Lindane and Endrin at 0.05 & 50 ppm by *Arthrobacter sp.* (A1, A2) and *E. coli* (B1, B2) isolated from L. Mariut Water

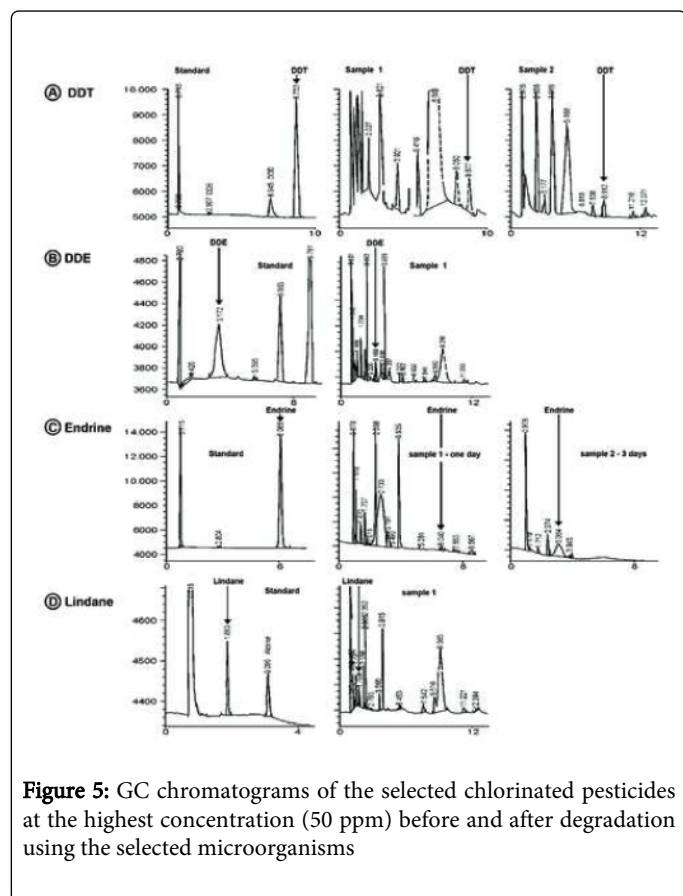


Figure 5: GC chromatograms of the selected chlorinated pesticides at the highest concentration (50 ppm) before and after degradation using the selected microorganisms

Many authors supported the present results and reported biodegradation capabilities of numerous bacteria towards recalcitrant compounds such as DDT by dechlorination to DDD under anaerobic

conditions [26,27] and Lindane [28,29]. Availability of essential nutrients for microbial growth such as carbon, nitrogen, phosphorus and oxygen may also limit the rate at which microorganisms degrade pesticides. The concentration of the pesticide must not be so high as to be toxic, nor so low such that biodegradation does not proceed due to a lack of induction of appropriate degradative enzymes and uptake mechanisms, failure to induce sufficient enzyme activities, or problems in providing sufficient energy even for cell maintenance [30].

Conclusion

The present results confirmed and supported the fact that *P. cepacia*, *E. agglomerans*, *S. aureus*, *Arthrobacter sp.*, and *E. coli* proved a great deal of DDT, DDE, Lindane and Endrin degradation and can be efficiently used in bioremediation process for that purpose.

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