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Effect of the acclimation of a *Streptomyces* consortium on lindane biodegradation by free and immobilized cells

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ABSTRACT

Lindane is an organochlorine pesticide widely used in agriculture, human health, and veterinary. It is a persistent organic pollutant, representing a serious risk since its residues are still in the environment. The aim of this work was to evaluate the influence of the acclimation of free and immobilized *Streptomyces* consortium on lindane degradation in liquid and slurry systems. The actinobacteria consortium demonstrated stability since the survival of all members was confirmed after the acclimation period. It reached higher biomass (0.56–0.65 g L^{-1}) and lindane removal (40–97%) than the consortium without acclimation (0.37–0.44 g L^{-1}; 33–87%) when it was cultured in liquid medium with 20 and 50 mg L^{-1} of lindane. In contrast, lindane removal achieved by an acclimated and immobilized consortium in a slurry system (50%) did not present significant difference respect to the consortium without acclimation. Moreover, lindane degradation was confirmed by identification of three metabolites in liquid medium, 1,2- and 1,4-dichlorobenzene, and γ-pentachlorocyclohexene; and by determination of chloride ions released in a slurry system. Bioassays with *Lactuca sativa* demonstrated the effectiveness of the bioremediation by the acclimated consortium in both systems, since the seedlings showed an improvement in its biological parameters compared to abiotic controls, confirming a significant decrease in toxicity.

1. Introduction

The gamma isomer of hexachlorocyclohexane, γ-HCH or lindane, is a saturated chlorinated hydrocarbon which was widely used from the 1940s to the 1990s as a pesticide. It has been classified as a probable carcinogen and endocrine disrupter, with proven teratogenic, genotoxic, and mutagenic effects [1]. Due to its chemical properties (Supplementary material 1), lindane can persist in the environment, undergo volatilization in tropical conditions, migrate to long distances with air current, deposit in colder regions, and cause widespread contamination. Besides, γ-HCH-residues reach human body via food chain and get biomagnified at each trophy level [2]. For these reasons the use of lindane has been banned or severely restricted in at least 52 countries, including Argentina [3,4]. However, some developing countries still use it for economic reasons and, consequently, its residues remain in the environment and have been found in water, sediments, soil, plants and animals tissues and its derivates. It was even found in human fluids such as blood, amniotic fluid and breast milk [5].

In this context, the need to remediate different polluted sites has led to the development of technologies aimed at decontaminating the environment, such as bioremediation. There are different bioremediation techniques; among them slurry bioreactors are one of the most important types of *ex situ* techniques and it has become one of the best options for the bioremediation of soils and sediments polluted by recalcitrant compounds under controlled conditions [6]. In this connection, there are several studies describing the application of slurry bioreactors for bioremediation of soil contaminated with pesticides [7,8]. Furthermore, it is known that the use of a mixed community of microorganisms could alleviate some metabolic limitations involved by using a single population, thus enhancing the capabilities of the single cultures. In nature, microorganisms exist as part of microbial consortia, made up of multiple populations that coexist and carry out complex chemical processes and physiological functions in order to enable survival of the community [9,10].

Moreover, other biotechnological technique that has received increasing interest in the field of waste treatment is immobiliza-

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tion of microbial cells. The use of immobilized microbial cells for bioremediation purposes presents several advantages respect to free suspended cells, such as increased cell viability (weeks or months), easier solid–liquid separation, and the possibility to reuse the immobilized cells. Besides, immobilized cultures tend to have a higher level of activity and they are more tolerant to environmental perturbations such as pH, temperature or exposure to toxic compounds, than suspended biomass cultures [11,12]. In this connection, previous works have demonstrated that actinobacteria immobilized in cloth bags presented higher lindane removal efficiency than free cells [13,14].

One characteristic of biological systems that is both interesting and difficult to describe is their ability to adapt and evolve under different environmental conditions. Microorganisms might be capable of adapting to various non-native carbon compounds because they are able to adapt to environmental changes by using a number of strategies to meet their growth requirements and to achieve optimal overall performance in the new conditions [15]. However, the mechanisms used to adapt to a defined metabolic challenge are still difficult to understand. Elcey and Kunhi [16] postulated that a gradual increase in the concentration of determined substrate during acclimation may result in enhanced induction of various enzymes of the degradation pathway of such substrate. Further, in a microbial consortium the net result of this process may be a synergism through complementation of the enzymes in different strains members.

In this framework, the present study intended to evaluate the influence of the acclimation of a consortium of actinobacteria on lindane degradation in liquid and slurry systems by free and immobilized cells. Finally, the efficiency of lindane biodegradation by the actinobacteria consortium was assessed by ecotoxicity bioassays with *Lactuca sativa*.

2. Materials and methods

2.1. Chemicals

Lindane (99% pure) was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Solvents were of pesticide grade, and all other chemicals used throughout this study were of analytical grade and were purchased from standard manufacturers.

2.2. Streptomyces *consortium, culture media and conditions*

A defined consortium of actinobacteria consisting of four *Streptomyces* strains (A2, A5, A11, and M7) was used in this study. These strains were previously isolated from soils and sediments contaminated with organochlorine pesticides [17,18], and the consortium was selected based on its higher lindane removal and specific dechlorinase activity compared with the single cultures of the four strains [19].

Minimum medium (MM), containing ($g\,L^{-1}$): L-asparagine, 0.5; K_2HPO_4, 0.5; $MgSO_4{\cdot}7H_2O$, 0.2; $FeSO_4{\cdot}7H_2O$, 0.01, pH 7, was used for the acclimation of the strains and consortium, and for lindane removal assay.

Tryptic Soy Broth (TSB) was used for inoculum preparation. It consists of ($g\,L^{-1}$): tryptone, 15; soy peptone, 3; NaCl, 5; K_2HPO_4, 2.5; glucose, 2.5. Each microorganism or the consortium was cultured in TSB for 72 h at 30 °C in a rotatory shaker (200 rpm). Then, the cells were harvested by centrifugation (9000 × *g*, 10 min) and washed three times with sterile distilled water.

Starch-Casein (SC), containing in $g\,L^{-1}$: starch, 10; casein, 1; K_2HPO_4, 0.5; agar, 15 was used for determining the survival of the strains. The pH of the media was adjusted to 7 prior to sterilization by autoclaving at 121 °C for 15 min.

Soil slurry was used for lindane removal assay. It was prepared using a loam soil collected from an urban area (5 × 15 cm depth) in San Miguel de Tucumán, Argentina (26°48′35″S 65°14′26″W), free of pesticides. Organic matter and nitrogen content of the soil were 2.6% and 0.14%, respectively, and the soil pH was 7.0. It contained 47.7% sand, 40% silt, and 12.3% clay. Prior to use, soil was air-dried, lightly ground using mortar and pestle, and finally sieved through a 1-mm sieve. Subsequently, the soil samples were sterilized (three successive sterilizations at 121 °C for 15 min each, 24 h in between) [14]. The experiments were conducted in 250 mL-Erlenmeyer flasks, containing 20 g of sterile soil and 80 mL of distilled water (soil/water ratio of 1:4). The resulting mixture was autoclaved again for 15 min at 121 °C and 1 atm overpressure. The loss of liquid due to the sterilization process was calculated by difference in weight between the flasks before and after sterilization, and was then adjusted by adding sterile distilled water.

2.3. Selection of surfactant concentration for lindane removal in liquid medium

In order to evaluate the removal of high concentrations of lindane (10, 20, 50, and 100 $mg\,L^{-1}$) in MM, the influence of the addition of Tween 80 was evaluated. For this purpose, Erlenmeyer flasks containing 30 mL of MM were employed, adding the pesticide at the concentrations previously mentioned and the surfactant in the following concentrations: 0.25, 0.5, 0.75, 1.0, and 1.25 mM. Flasks were incubated for 96 h with constant stirring (150 rpm). Subsequently, samples were taken to determine lindane concentration by gas chromatography (detailed bellow, 2.6). Lindane solubilisation capacity (%) was calculated based on the data obtained from gas chromatography quantification of remaining lindane.

2.4. Microbial cells immobilization

For the *Streptomyces* consortium immobilization, cloth bags were used as support. For this purpose, bags were prepared with voile, in a size of 2.5 × 3.5 cm each one, following the methodology described by Saez et al. [13,14]. The bags, opened in one side, were individually sterilized in autoclave (121 °C, 15 min, 1 atm overpressure). Then, they were aseptically loaded with the consortium inoculum (10^7 CFU g^{-1}). Finally, they were closed with sterile thread, by using sterile tweezers.

2.5. Assessment of the consortium acclimation

2.5.1. Culture acclimation

In order to obtain acclimated cultures, a sequential transfer method was employed [16]. For this purpose, each *Streptomyces* strain or the consortium, i.e. the four strains together, were initially inoculated in MM supplemented with 10 $g\,L^{-1}$ of glucose and 1 $mg\,L^{-1}$ of lindane. After 24 h incubation at 30 °C and 150 rpm, the cultures were transferred to fresh medium, thus extending their exponential growth phase. After seven sequential transfers, lindane concentration was increased to 5 $mg\,L^{-1}$ and glucose concentration was decreased to 9 $mg\,L^{-1}$. This procedure was conducted for five weeks, gradually increasing lindane concentration to 10, 15, and 20 $mg\,L^{-1}$ in the 3rd, 4th and 5th week, respectively, and decreasing glucose concentration (8, 7 and 6 $g\,L^{-1}$ in the 3rd, 4th and 5th week, respectively). Cycloheximide and nalidixic acid (10 $mg\,L^{-1}$ each) were daily added in order to inhibit eukaryotic microorganisms and Gram-negative bacteria, respectively. Acclimated cultures were preserved by lyophilisation.

2.5.2. Antibiotic sensitivity and RAPD-PCR of Streptomyces strains

Antibiotic sensitivity test and RAPD-PCR of the four actinobacteria strains were carried out in order to find differential characteristics between the strains, with the aim of using these biochemical and molecular approaches to determine the survival of the strains after the acclimation period.

Sensitivity of the strains against 24 antibiotics was evaluated by using the agar diffusion method. For this purpose, each strain (10^9 CFU mL^{-1}) was inoculated in Mueller–Hinton agar plate; then, commercially-prepared paper antibiotic disks were aseptically placed on the inoculated agar surface. Plates were incubated for 7 days at 37 °C. Afterwards, the growth inhibition zones around each antibiotic disk were measured to the nearest millimetre and results were interpreted by using the standard criteria published by the Clinical and Laboratory Standards Institute [20].

Detection of genetic polymorphisms characteristic of each strain was carried out by RAPD-PCR (Random Amplified Polymorphic DNA-Polymerase Chain Reaction), based on the use of primers with random sequences. For this purpose the four actinobacteria and the strains isolated from the acclimated consortium were cultured in TSB for 48 h at 30 °C and 150 rpm. Total DNA extraction of each culture was performed by lysing the cells with glass beads and 10% sodium dodecyl sulfate (SDS). Proteins were extracted with phenol/chloroform/isoamyl alcohol (25:24:1); DNA was precipitated with sodium acetate 3 M/isopropyl alcohol 100%; finally the DNA pellet was washed with 70% ethanol. The primers used were DA F 5′-GAG GTC GTG CTG ACC GTG CTG CA-3' and DA R 5'-CTG GTG GTT GCC GAT GAC GTC GT-3'. Different annealing temperatures (50, 52, 55, 57, or 60 °C) were used to obtain a characteristic profile of each single actinobacterium. Finally, products were visualized in polyacrylamide gels stained with 6% $AgNO_3$.

2.5.3. Evaluation of microbial growth and lindane removal by acclimated consortia in liquid system

After the acclimation period, microbial growth and lindane removal ability were assessed in MM supplemented with Tween 80 in the concentration previously selected and lindane at the following concentrations: 10, 20, 50, and 100 mg L^{-1}. Two consortia were used: one consisting of the consortium acclimated for five weeks (Mix S5A) and the other constituted of the strains individually acclimated for five weeks [equal amount of each strain (Mix S5B)]. After 96 h incubation, microbial growth was determined by drying the biomass to constant weight at 105 °C, and lindane removal was determined by gas chromatography (detailed bellow, 2.6). Based on the results obtained, one of the consortia was selected for further studies.

2.5.4. Evaluation of the removal of lindane by the acclimated immobilized consortium in soil slurry system

The selected consortium previously acclimated, was immobilized in cloth bags and inoculated in soil slurry supplemented with 50 mg kg^{-1} of lindane with and without the addition of the commercial surfactant Tween 80. The slurry was incubated for 28 days at 30 °C with constant stirring at 150 rpm. Samples were collected at 0, 7, 14, 21, and 28 days, in order to determine residual lindane concentration, chloride ions released, and phytotoxic effects of the bioremediated slurries. Furthermore, the reuse of the immobilized acclimated consortium was tested in two additional cycles of 21 days each.

2.6. Lindane determination by gas chromatography

Residual lindane in liquid medium was extracted by solid-phase extraction by using a C18 column (Varian, Lake Forest, USA), n-hexane, and methanol.

For lindane recovery from slurries, an extraction with solvents was carried out. For this purpose, n-hexane, water, and methanol (5:4:1) were added to 5 mL of slurry. The resulting mixture was stirred for 10 min and centrifuged at 9000 × *g* for 10 min. Then, it was incubated at −4 °C for 5 min to enhance the separation of the organic phase, and subsequently 2 mL were taken. The extract was evaporated and finally suspended in an appropriate volume of n-hexane [14].

Lindane concentration in extracts was quantified in a gas chromatograph (Agilent 7890A) equipped with ^{63}Ni micro-electron capture detector (GC-μECD), HP5 capillary column (30 m × 0.53 mm × 0.35 m), a split/splitless Agilent 7693B injector and Agilent ChemStation software. The chromatographic conditions were as follows: carrier gas (nitrogen) flow rate: 25 cm s^{-1}, initial oven temperature: 90 °C increasing to 180 °C at 30 °C min^{-1}, and increasing to 290 °C at 20 °C min^{-1}, detector temperature: 320 °C and injection volume: 1 mL. Quantitative sample analysis was performed using a calibration curve with appropriated dilutions (1–30 mg L^{-1}) of lindane calibration standards (AccuStandard, New Haven, USA).

2.7. Identification of metabolites of lindane degradation

In order to determine the presence of intermediate metabolites of lindane degradation in the culture supernatant of acclimated consortium, a separation and identification of the components was performed, by using a gas chromatograph (Agilent Technologies 6890) with mass spectrometry detector (GC–MS) (Agilent Technologies 5973 Network).

For this purpose, the sample extract (obtained as described in 2.6) was injected into an Agilent VF-5ms column (phenyl methyl siloxane; 30 m, 0.25 mm, 0.25 μm). The injection (1 μL) was performed in splitless mode at 280 °C. Chromatographic run started at 50 °C, then the temperature increased to 125 °C at a rate of 3 °C min^{-1} and finally to 300 °C at a rate 17 °C min^{-1}. Helium was used as carrier gas (1 mL min^{-1}). The GC–MS was operated at scan mode from 40 to 500 amu and detector temperature was 280 °C. Compounds were identified by comparing with standards registered on Wiley7NIST05 Library.

2.8. Chloride ions determination

The chloride ions concentration in solution was estimated by a modified method of Bidlan and Manonmani [21]. One mL of the culture was centrifuged and 100 μL of the supernatant were taken. Then, 50 μL of each 0.15 N HNO_3 and $AgNO_3$ 0.1 N were added to the supernatant with mixing at each step. Turbidity was measured at 600 nm after standing for 20 min at room temperature. The amount of chloride was computed from a standard curve prepared for NaCl in a similar way.

2.9. Phytotoxicity test

A phytotoxicity test using *Lactuca sativa* (lettuce) was performed since it is a versatile tool to assess the potential environmental impact, allowing the identification of the effect of pollutants present in the matrix, as well as to monitor the success of a remediation process [22]. For this purpose, 30 seeds were grown in Petri dishes containing sterile filter paper (Wattman No. 1), moistened with 2 mL of the sample (liquid medium or slurry). They were incubated in darkness for 5 days under controlled environmental conditions (22 °C and 70% of relative humidity). At the end of the incubation period, the number of germinated plants was determined, and the length of the root and hypocotyl of each one was measured by using a millimetre scale. The vigour index of the

seedlings (VI) was also calculated, by using the following formula [23]:

$$VI = (LR + LT) \times \frac{G}{10}$$

LR is the average roots length, LT is the average hypocotyls length and G is the germination percentage. G was calculated through the following formula [23]:

$$G(\%) = \frac{N_g}{N_s} \times 100$$

N_g: number of germinated seeds at the end of the experiment.
N_s: number of seeds used in the experiment.

2.10. Statistical analyses

All assays were conducted in triplicate and the results are the average of them. One-way analysis of variance (ANOVA) was used to test the significant differences among treatments or among incubation times (for each treatment) and its abiotic control, as appropriate. When significant differences were found, Tukey post-test was used to separate the effects. Tests were considered significantly different at $p < 0.05$. Statistical analyses were performed using a professional version of Infostat software.

3. Results and discussion

3.1. Selection of surfactant concentration for lindane removal in liquid medium

The strong solubilisation power of Tween 80 and its reported lack of toxicity to several microorganisms suggest that this surfactant may be an attractive tool for solubilising high concentrations of hydrophobic compounds [24]. In this context and considering the low solubility of lindane in aqueous phase, the effect of Tween 80 on solubilisation of lindane in liquid culture medium was evaluated.

Results showed that the addition of 0.25 mM of Tween 80 to the culture medium supplemented with 10 mg L^{-1} of lindane enhanced significantly the pesticide solubility respect to control (without Tween 80), obtaining 91% of γ-HCH solubilised (Supplementary material 2). In contrast, when 20 mg L^{-1} of lindane were spiked in MM, the addition of 0.25–0.75 mM of surfactant did not increase significantly the recovery of the pesticide; while 1 mM of Tween 80 led to 100% of lindane solubilised (Supplementary material 2). This could be explained considering that the solubilisation depends on the concentration of the surfactant.

At higher lindane concentrations (50 and 100 mg L^{-1}), its solubility significantly increased in the presence of surfactant ($p < 0.05$), achieving the highest solubilisation degree when 1 and 1.25 mM of Tween 80 were added to the culture medium, although no significant differences were observed between these two concentrations (Supplementary material 2). Based on these results, the concentration of 1 mM of Tween 80 was selected for further studies.

Several authors found similar results. Manickam et al. [25] showed that the solubilisation of the four major isomers of HCH was 3–9 folds higher by the addition of biosurfactants, being more effective between 40 and 60 μg mL^{-1}, while at higher concentrations the solubility of the isomers decreased. Moreover, Kang et al. [26] evaluated the effect of several commercial and microbial surfactants on the solubilisation of hydrocarbons. They demonstrated that the solubility of 2-methylnaphthalene in aqueous phase was dependent on the concentration of surfactant used, being Tween 80 the agent which provided the highest hydrocarbon solubilisation from six other non-ionic surfactants tested.

3.2. Assessment of consortium acclimation in liquid medium

After the acclimation period, both consortia, Mix S5A (consisting of the strains acclimated all together) and Mix S5B (consisting of the strains individually acclimated) obtained biomass values significantly higher than consortium without acclimation (Mix S0) when they were cultured at high concentrations of lindane (20, 50, and 100 mg L^{-1}). However the biomass of acclimated consortia showed no significant difference with Mix S0 when grown in the presence of 10 mg L^{-1} of the pesticide or in its absence (Fig. 1A). This could be explained considering that the acclimation process could have contributed to increase the tolerance of the actinobacteria to high concentrations of the pesticide, which is essential for degradation studies [27].

Regarding lindane removal, it was significantly enhanced in both acclimated consortium, Mix S5A and Mix S5B, compared to the consortium without previous acclimation (Mix S0) when the culture medium was supplemented with 20 and 50 mg L^{-1} of the pesticide. However, when defined consortia were grown in the presence of 100 mg L^{-1} of lindane, no significant differences were detected in lindane removal (Fig. 1B).

Other authors reported similar results. Manonmani et al. [28], for instance, found that after the acclimation of a consortium to increasing concentrations of α-HCH isomer, it was able to degrade 50 μg mL^{-1} of the xenobiotic in 72 h, while the consortium without acclimation was unable to completely mineralize the same amount of substrate even after 15 days. Conversely, individual strains and different combinations of them did not show any improvement in the ability to degrade α-HCH, even after acclimation. Moreover, Elcey and Kunhi [16] isolated a consortium able to mineralize 25 μg mL^{-1} of γ-HCH over a period of 10 days, reaching a maximum growth of 37.5 g of protein per gram of cells, after 5 days of incubation. When the consortium was acclimated, it reached twice the biomass in 24 h, using the same concentration of pesticide as substrate. Besides, the acclimated consortium could mineralize 12-fold the concentration of γ-HCH in less than half the time.

Interestingly, in the present work, both acclimated and unacclimated consortia reached lindane removal percentages greater than 85% and 95% in liquid systems spiked with 50 and 100 mg L^{-1} of lindane, respectively. This is not surprising considering that it has been already reported that the presence of surfactants not only facilitate the bioavailability of the pesticide but may also stimulate the metabolic machinery whereby both the γ-HCH and the surfactant could have been consumed by bacteria as substrates [25]. It is further known that several species of *Streptomyces* genus are able to degrade this surfactant, as well as Tween 60 and Tween 40, among others [29].

Based on the results, and considering that in most cases no significant difference was found in both biomass and lindane removal achieved by Mix S5A and Mix S5B, the first one was selected for further studies in slurry system. This selection is based on the easier and economical process of acclimation of the consortium, compared to four strains separately.

3.3. Survival test of Streptomyces strains

Results of the sensitivity of the actinobacteria strains against antibiotics showed that all four strains were resistant to ampicillin/sulbactam, cefotaxime, ceftazidime, among others; while all of them were sensitive to ciprofloxacin, levofloxacin, minocycline, and streptomycin at the studied conditions. Also, differences in antibiotic sensitivity were found among the four actinobacteria studied. For instance, *Streptomyces* sp. A2, A5, and A11 were inhibited by teicoplanin, while *Streptomyces* sp. M7 was resistant. *Streptomyces* sp. A5 was the only strain resistant to imipenem; and *Streptomyces* sp. A2 and A11 were resistant to vancomycin, while

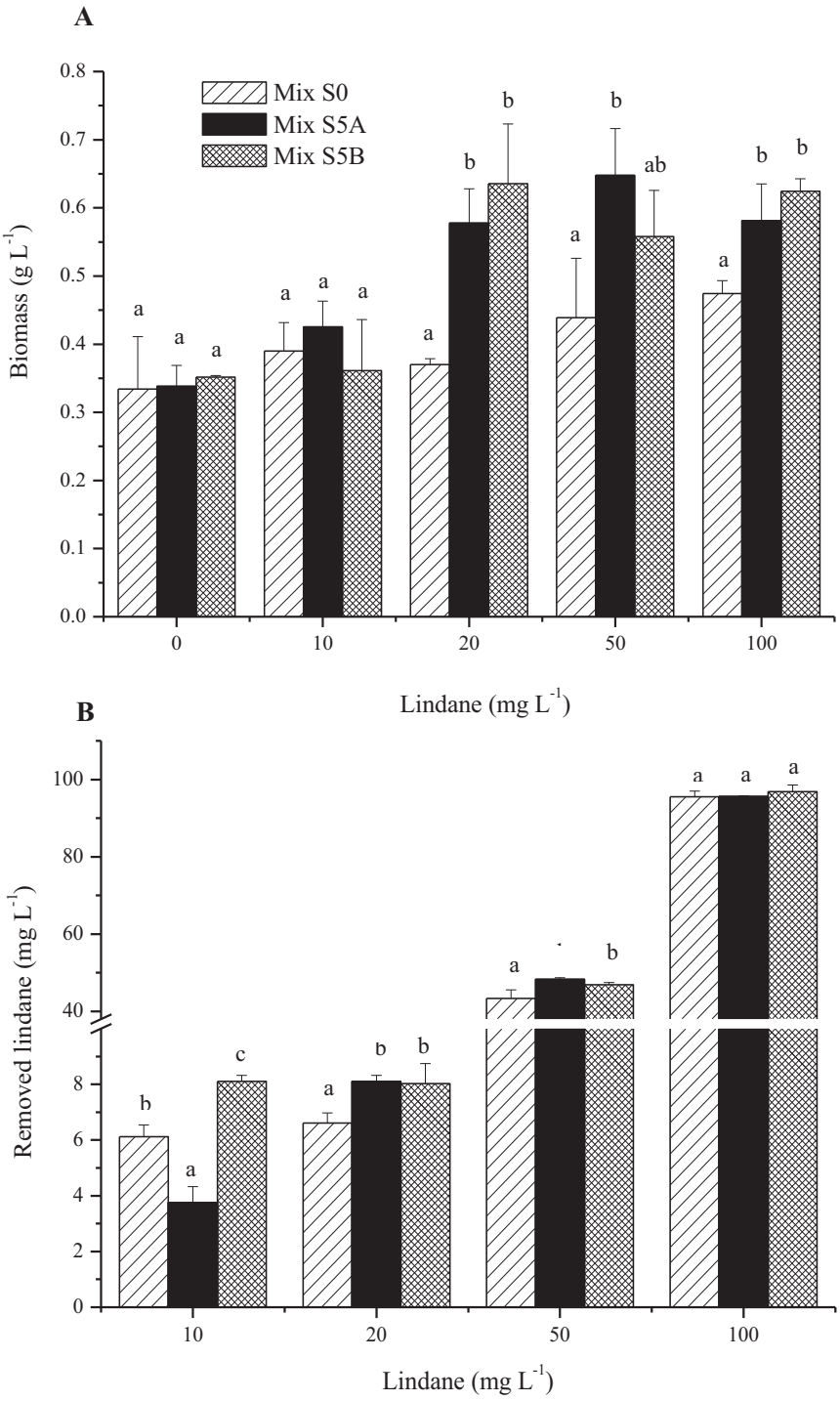

Fig. 1. (A) Biomass ($g\,L^{-1}$), and (B) removed lindane ($mg\,L^{-1}$) of the actinobacteria consortia grown in minimal medium with different lindane concentrations. Mix S5A: acclimated consortium, Mix S5B: consortium consisting of strains individually acclimated, Mix S0: consortium without acclimation. Different letters indicate significant differences among treatments ($p < 0.05$, Tukey test).

Streptomyces A5 and M7 were sensitive to it (Supplementary material 3).

In nature, the main function of antibiotics is to inhibit competitors, which in turn are induced to inactivate these compounds by chemical modification (hydrolysis), or changes in the action site and the membrane permeability [30]. Thus, several authors found similar antibiotics sensitivity to those produced by the actinobacteria studied in the present work. A study performed with *Streptomyces* isolated from urban soil showed that most of the strains were resistant to multiple antibiotics, suggesting that these genes are common in this environment [31].

On the other hand, RAPD-PCR method detects differences throughout the genome bacterial and not just in particular sequences, so it is useful for the characterization of bacteria belonging to the same genus [32]. The results obtained by using this technique revealed that at an annealing temperature of 55 °C, characteristic profiles were obtained for each strain (Supplementary material 4).

Table 1
Development of *Lactuca sativa* seedlings in minimal medium supplemented with 50 mg L^{-1} of lindane and bioremediated by the acclimated consortium (Mix S5A) and respective controls. Each treatment was conducted in triplicate and the results are the average of them. Different letters indicate significant differences among treatments ($p < 0.05$, Tukey test).

Treatment	G* (%)	Root length (cm)	Hypocotyl length (cm)	VI**
Abiotic control	92.2 ± 1.9[a]	0.74 ± 0.01[a]	1.11 ± 0.01[a]	16.94 ± 0.46[a]
Biotic control	97.8 ± 1.9[a]	1.46 ± 0.08[b]	1.95 ± 0.11[c]	33.34 ± 0.91[c]
Bioremediated	93.3 ± 3.3[a]	1.30 ± 0.18[b]	1.38 ± 0.11[b]	25.00 ± 0.19[b]

* Germination percentage of the seeds.
** Vigour index of the seedlings.

Based on these results, Mix S5A was plated on SC supplemented with either teicoplanin (15 μg mL^{-1}), imipenem (15 μg mL^{-1}), or vancomycin (30 μg mL^{-1}). After one week incubation, the separated colonies obtained in each case were used to perform RAPD-PCR. Notably, in the presence of vancomycin two different colony morphologies and colours were obtained, designated as colonies A and B.

The profiles obtained in polyacrylamide gels confirmed the identity, and hence survival, of the four *Streptomyces* sp. strains constituent of the defined consortium (Supplementary material 4), thus demonstrating its stability over the acclimation period and its resistance to the high lindane concentrations employed during this process. This result is very important since the stability during long-term storage and operation is an essential factor for practical application of a microbial consortium [33]. However, this finding is not always observed. For example, Bidlan and Manonmani [21] reported that during the acclimation of a consortium, a succession of microbial members had taken place resulting ultimately in the survival of four members at the end of acclimation period.

3.4. Assessment of the efficiency of lindane bioremediation by acclimated consortium in liquid medium

Lettuce seeds were negatively affected by the presence of the pesticide. This was reflected in the lower values of VI, radicle and hypocotyl lengths of seedlings when grown in the abiotic control (MM spiked with 50 mg L^{-1} of lindane) in contrast with the parameters registered in seedlings grown in biotic control (uncontaminated medium) (Table 1). The toxic effects observed on lettuce seedlings confirm that it is an appropriate indicator for studying the efficiency of the process under the studied conditions. In contrast, the germination percentages (G) were not significantly different in plants grown in the presence and absence of lindane. This may be probably because lindane concentration used could have not been toxic enough to inhibit the germination of lettuce seeds; however, it may retard or completely inhibit radicle and/or hypocotyl elongation processes. Thus, the inhibition in those processes are very sensitive sub-lethal indicators for assessing biological effects in plants, providing complementary information to that provided by studying the effect on germination [34].

In MM bioremediated by the *Streptomyces* acclimated consortium, the negative effects of lindane on seeds were reversed, since the hypocotyls and radicles lengths, as well as the VI of *Lactuca sativa* seedlings were significantly higher compared to those obtained in seedlings grown in the abiotic control (Table 1), confirming a significant decrease in the toxicity of the bioremediated medium. In fact, the root length of bioremediated medium was not significantly different to the biotic control.

Several studies have described the phytotoxic effects of pesticides in general, and lindane in particular, which are expressed as an imbalance in biochemical processes in the seed [8,23]. It is well known that during the first days of seedling development, numerous physiological processes occur, so in the presence of a toxic compound, it may disturb the survival and normal development of the plant, and hence it is a time of great sensitivity to adverse external factors. Therefore, the evaluation of the development of radicle and hypocotyl is a representative indicator to determine the ability of establishment and development of the plant [34]. In this context, these results demonstrate that the bioremediated system was less toxic than the abiotic control, i.e. with no treatment, hence confirming that during the bioremediation process it would not have been released to the medium metabolites more toxic than lindane.

Furthermore, three metabolic intermediates of lindane degradation were identified by GC–MS in the liquid system bioremediated by the consortium previously acclimated, thus demonstrating that lindane was not only removed but also degraded by the consortium. Based on the GC–MS spectrum and the fragmentation spectra of the detected compounds (Fig. 2, Supplementary material 5), the peak at 9.899 min corresponded to 1,4-dichlorobenzene (1,4-DCB); the second peak, at 10.227 min, was identified as 1,2- dichlorobenzene (1,2-DCB), and the third peak in the spectrum at 27.497 min, was the γ-1,2,3,4,5-pentachlorocyclohexene (γ-PCCH).

Several microorganisms have been described to degrade lindane, with the consequent release of intermediate metabolic compounds [35]. The γ-PCCH is the first compound in lindane degradation pathway reported for *Sphingobium japonicum* UT26 [36]. It is one of the most commonly found metabolites in lindane aerobic degradation, in addition to 2,5-diclorobenzoquinone, chlorohydroquinone (CHQ), chlorophenol, and phenol [35]; although, other metabolites were also reported. Geueke et al. [37] detected the presence of γ-PCCH and 1,2,4-TCB, among other metabolites, during aerobic degradation of different HCH isomers by *Sphingobium* strains, previously isolated from contaminated sites. Other authors also reported 1,4-DCB as lindane degradation product, such as Camacho-Pérez et al. [35] who found this metabolite in a slurry bioreactor contaminated with 100 mg L^{-1} of lindane and inoculated with a native consortium previously acclimated.

Few studies have reported the toxic effects of lindane metabolites. It is known, for instance, that 1,2-DCB presents acute and chronic toxicity in fish, amphibious, bacteria, algae and aquatic invertebrates; although its half-life is approximately 3 weeks, i.e. much shorter than lindane ($t_{1/2}$ = 2.6 years) [7,38]. In this context, it is noteworthy that the metabolites detected in the bioremediated liquid system, besides being less recalcitrant than the parent compound, they were also less toxic to *Lactuca sativa* seeds, compared to lindane initially added to the culture medium. This result is critical considering that for an effective bioremediation process, it is necessary to ensure that metabolites produced through biotransformation are non-toxic or reactive, and its release does not entail further risk for the environment or human health [39]. In this sense, Yáñez-Ocampo et al. [40] confirmed a decrease in the toxicity of an effluent contaminated with pesticides after bioremediation with a bacterial consortium, through the application of acute toxicity test on *Eisenia fetida*. In opposite, Fuentes et al. [8], observed a significant reduction in the number of germinated lettuce seeds as well as in the roots and hypocotyls lengths, and consequently the vigour index of seedlings, after a bioremediation of slurry contaminated

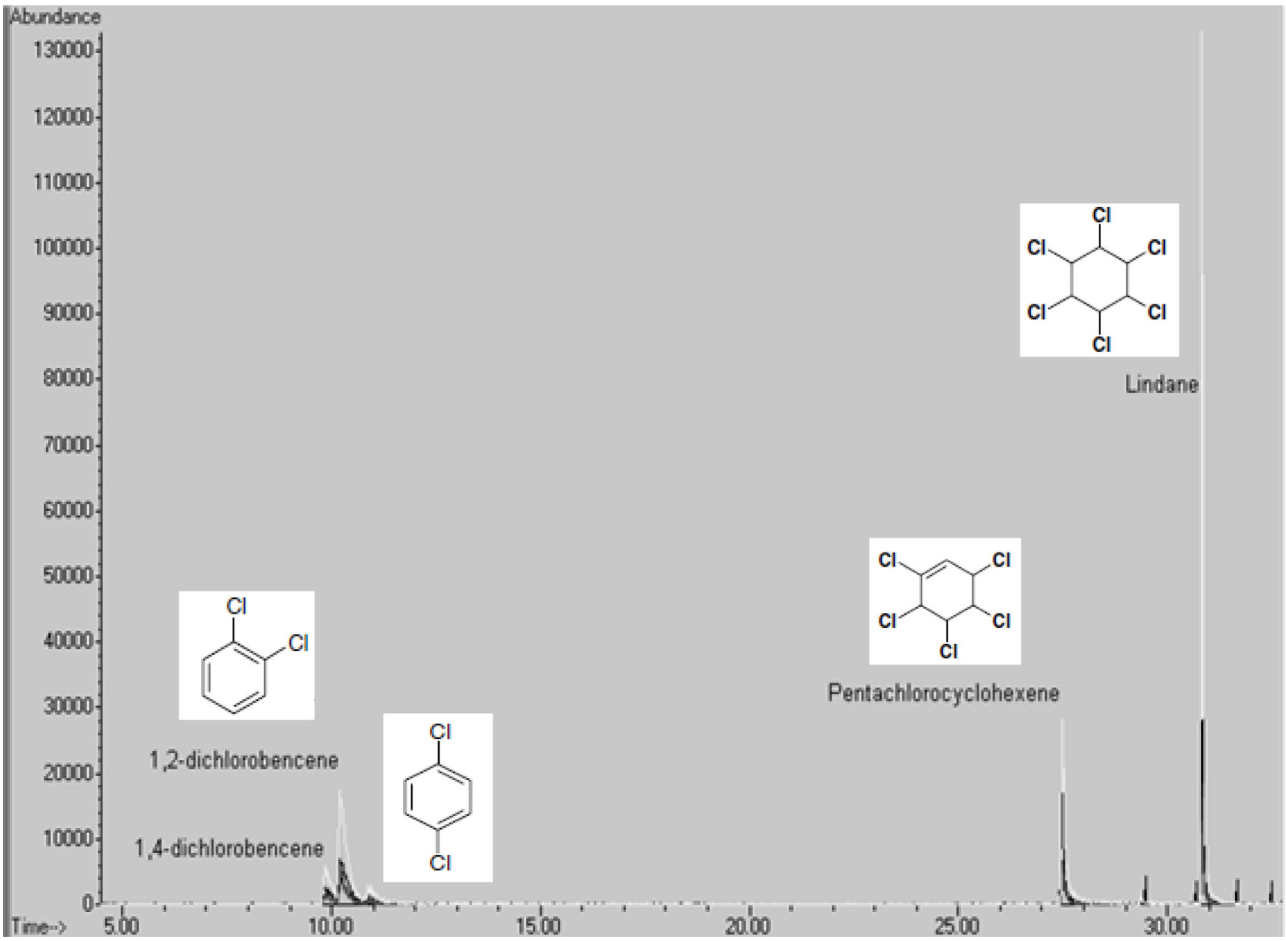

Fig. 2. GC–MS analysis of the extracts corresponding to minimal medium contaminated with 50 mg L^{-1} of lindane and bioremediated by the acclimated consortium of *Streptomyces*.

with methoxychlor. These results could be interpreted as a possible transformation of the pesticide into more toxic intermediates.

3.5. *Assessment of lindane biodegradation by acclimated and immobilized consortium of* Streptomyces *in slurry system*

The acclimated and immobilized consortium removed 20 mg kg^{-1} of lindane at seven days of incubation, representing the 40% of lindane initially added to the slurry. A significant increase in pesticide removal was observed after 21 days-incubation, reaching approximately 50% of pesticide removal; however extending the incubation period up to 28 days, did not improve the pesticide removal (Fig. 3A). Furthermore, the addition of Tween 80 did not represent a significant increase in lindane removal by the acclimated and immobilized *Streptomyces* consortium (Fig. 3B). It is also important to note that the removal obtained in abiotic control was less than half the obtained in the biologic treatments, and remained constant throughout the process.

Subsequently, the reuse of the acclimated immobilized consortium was assessed in two additional cycles of 21 days each. Results revealed that Mix S5A removed 27.6 and 24.4 mg–kg^{-1} of lindane (i.e. approximately 55 and 48% of initial lindane added) in the second and third cycle, respectively, in the slurry without Tween 80 (Fig. 4A). In slurry supplemented with Tween 80, pesticide removed in the second and third cycle were 26.8 and 24.5 mg kg^{-1} of lindane respectively, (i.e. 54 and 49%) (Fig. 4B). Thus, the acclimated immobilized consortium retained its removal ability, i.e. no significant difference was registered in the second and third use respect to the first use of immobilized cells. This is an valuable result since one of most interesting advantages of using immobilized cells for the treatment of pollutants compared to the use of free cells is the real possibility of recycling them for their use in subsequent cycles [41,42]. Nigam et al. [12] showed that *Streptomyces* sp. immobilized in agar was used for bioconversion of acrylonitrile for twenty cycles, without much loss of activity. Other authors also obtained similar results using a defined consortium immobilized in PVA cubes [33]. In contrast, Ellaiah et al. [43] were able to use *Aspergillus niger* entrapped in sodium alginate, carrageenan, and polyacrylamide, for lipase production during three cycles. However, thereafter blocks started disintegrating, thus resulting in the release of biomass to the culture medium. It is noteworthy that the stability of the support used in this study was already demonstrated in a previous work [13]. In that study, the cells released from the bag at the end of the assay represented only 0.12% of the cells grown inside the support and 1.2% of the initial inoculum, thus revealing that the immobilization technique was efficient and the support did not suffer any disintegration. Moreover, the cloth bags could be reused for two additional culture cycles in liquid minimal medium with a removal efficiency of 59% in each case.

Regarding the addition of Tween 80, the results obtained in the present work are in agreement with some reports, although different responses in pesticides removal were observed with the addition of surfactants. Quintero et al. [44], for instance, studied the effect of the addition of synthetic surfactants on the anaerobic biodegradation of HCH isomers in slurry cultures. These authors demonstrated that the degradation of α- and γ-HCH isomers pre-

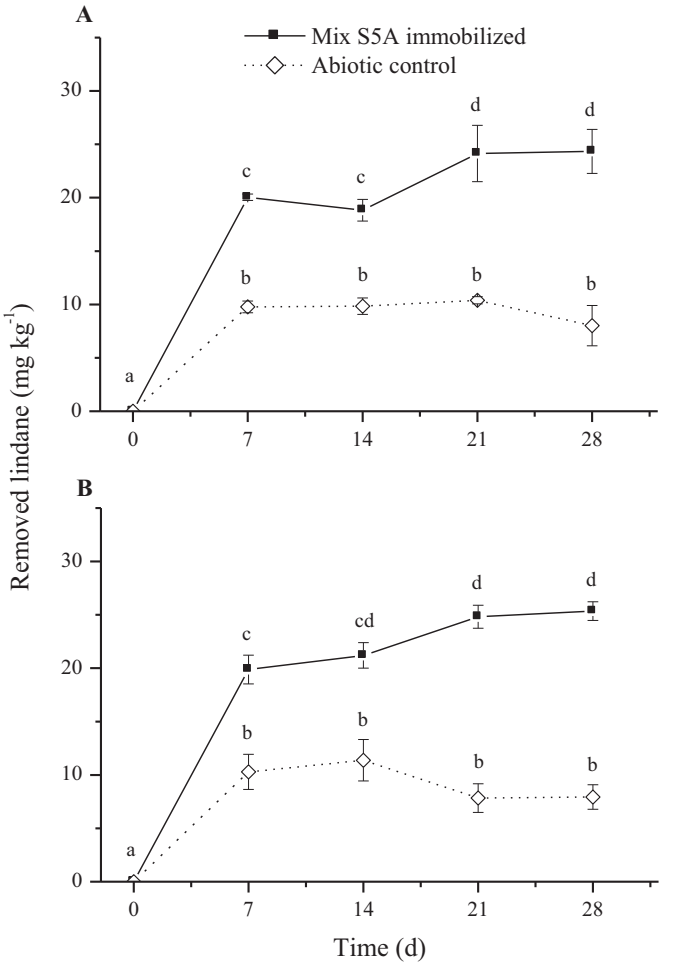

Fig. 3. Removed lindane (mg kg^{-1}) by the acclimated and immobilized consortium (Mix S5A) on slurry system. (A) Slurry without Tween 80, (B) Slurry supplemented with Tween 80. Different letters indicate significant differences among incubation times (for each treatment) and its abiotic control ($p < 0.05$, Tukey test).

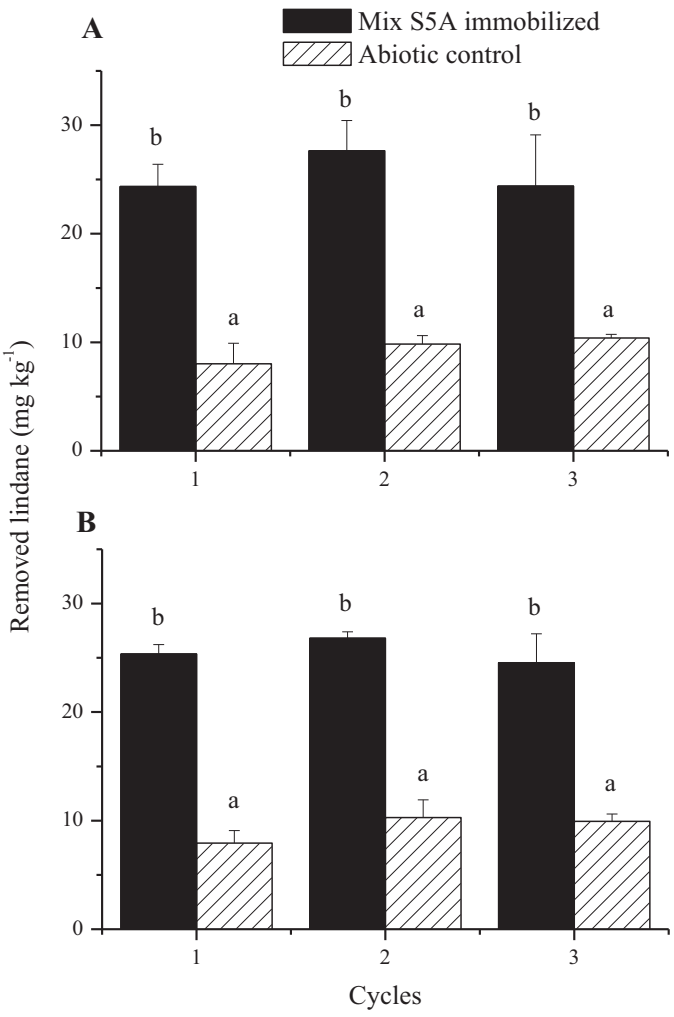

Fig. 4. Removed lindane (mg kg^{-1}) by the acclimated consortium of *Streptomyces* (Mix S5A) immobilized in cloth bags, in cycles of 21 days each. (A) Slurry without Tween 80, (B) Slurry supplemented with Tween 80. Different letters indicate significant differences among cycles (for each treatment) and its abiotic control ($p < 0.05$, Tukey test).

sented the highest rates in the absence of surfactant. The negligible effect of Tween 80 on the biodegradation of these isomers may rely not on desorption rates but on the intrinsic capability of degradation of the microorganisms. In contrast, the low biodegradation rates for β- and δ-HCH are attributable not only to the spatial distribution of the chlorine atoms in the molecule, which makes them more stable and recalcitrant to degradation, but also to their poor bioavailability with high mass transfer limitations, which can be improved by the addition of Tween 80.

In opposite, other authors postulated that surfactants would be a good alternative to improve the remediation of hydrophobic compounds in soils or slurries. In this sense, Manickam et al. [25] revealed an increase of about 30–50% in HCH degradation by *Sphingomonas* sp. NM05 in soil slurry by adding surfactants.

On the other hand, when comparing lindane removal achieved by the acclimated and immobilized consortium (Fig. 3A), respect to the consortium without prior acclimation in the same slurry system [14], it could be concluded that the acclimation period is not necessary, since no significant differences were observed in lindane removal obtained in both cases. This represents a great advantage from a biotechnological point of view, considering that the acclimation period besides being long and intensive; it also increases the costs of bioremediation. Similarly, De Paolis et al. [45] studied the ability of *Arthrobacter* strains to degrade HCH, and they also concluded that a pre-conditioning period in contact with the pesticide might be not necessary. Bidlan and Manonmani [21] also found no improvement in the degradation rate of DDT after a continuous exposure of a consortium to increasing concentrations of the xenobiotic. Other authors also reported that the strategy of biomass adaptation by long exposure to an anionic surfactant did not lead to an increase of the surfactant removal in real wastewater. They attributed this effect to the high microbial richness of the inoculum, which results in a metabolic response to different influent conditions [46].

It is known that the degrading ability of a culture strongly depends on its origin and adaptation degree [47]. In fact, microbial communities living in contaminated ecosystems tend to be dominated by organisms capable of using and/or tolerating toxic compounds. It is possible that nutritional conditions of the environment result in an immediate reaction at metabolic level and secondly, at genetic level [48]. The actinobacteria employed in the present work were isolated from soil and sediments contaminated with mixtures of organochlorine pesticides and other toxic compounds, thus the microorganisms were already exposed to extreme conditions. Therefore, they may have acquired new metabolic capabilities which allow them to tolerate and degrade high concentrations of toxic substances [47,49]. In ecosystems containing toxic anthropogenic substances, multidirectional and long-term effects

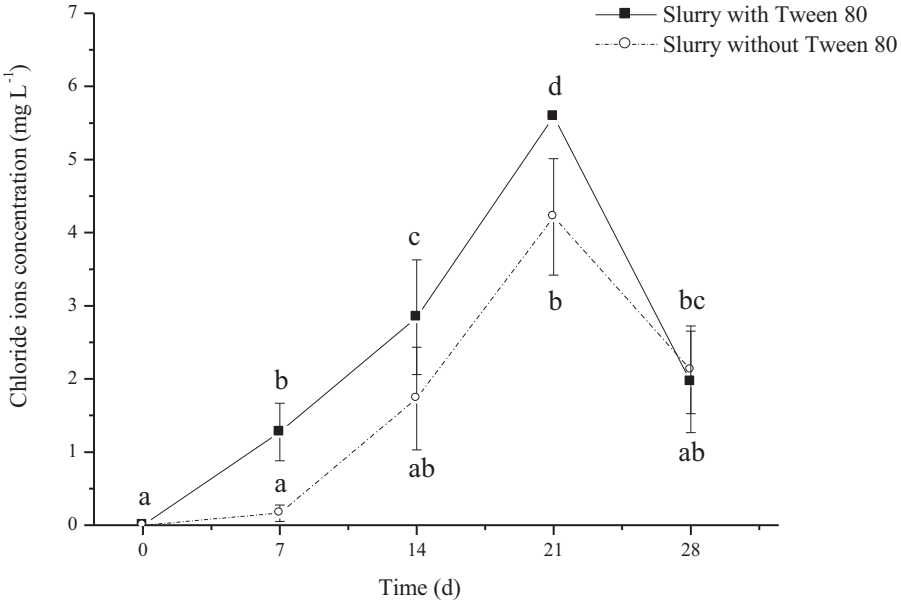

Fig. 5. Chloride ions concentration (mg L^{-1}) determined in slurries bioremediated by acclimated consortium (Mix S5A) immobilized in cloth bags. Different letters indicate significant differences among incubation times (for each treatment) ($p < 0.05$, Tukey test).

of pesticides were observed, hence affecting the organisms living there. Thus, natural populations exhibit a great range of responses to these pollutants, and faster reactions were observed in bacteria and fungi, which can use diverse substances present in the environment as carbon and energy sources [50].

The key reaction during microbial degradation of halogenated compounds is the removal of the halogen atom, i.e. dehalogenation of the organic halogen. During this step, the halogen atom(s), which is (are) usually responsible for the toxic and xenobiotic character of the compound is most commonly replaced by hydrogen or a hydroxyl group. Halogen removal reduces both recalcitrance to biodegradation and the risk of forming toxic intermediates during subsequent metabolic steps [35]. Therefore, in order to confirm lindane degradation in slurries bioremediated by the defined consortium of acclimated and immobilized actinobacteria, the presence of chloride ions was determined. Chloride ions released showed a maximum value at 21 days in both slurries, in the presence and absence of Tween 80 (Fig. 5). Notably, in the same incubation period the maximum lindane removal values were also detected in both slurries, with and without Tween 80. In this connection, Salam et al. [51] found that the release of chloride ions from lindane by *Rhodotorula* sp. VITJzN03 was slow at the beginning and then reached 100% after five days of incubation, obtaining a stoichiometric ratio between the disappearance of lindane, the increase in biomass and the release of chloride ions. In previous studies, Benimeli et al. [52] confirmed that chloride ions release occurred when *Streptomyces* sp. M7 was grown in the presence of lindane as sole carbon source. Also, the presence of dechlorinase activity and the ability to release chloride ions have been previously demonstrated in liquid medium by the microorganisms employed in the present work, both as pure as well as mixed cultures, but with a 30-fold lower concentration of lindane [18]. Moreover, Cuozzo et al. [49] found that some members of the consortium used in the present study, released chloride ions when cultured in the presence of chlordane as carbon source, demonstrating that they are capable of degrading different organochlorine pesticides. There is a large variety of dehalogenating enzymes and mechanisms involved in the degradation of halogenated compounds. The microorganisms used in this study were isolated and selected based on their microbial growth and tolerance and/or removal ability of specific organochlorine pesticides. Thus, it is not surprising they have different dehalogenase enzymes with varying substrate specificities [53].

Table 2
Development of *Lactuca sativa* seedlings in soil slurries supplemented with 50 mg kg^{-1} of lindane and bioremediated by the acclimated and immobilized consortium (Mix S5A) and respective controls. Each treatment was conducted in triplicate and the results are the average of them. Different letters indicate significant differences among treatments ($p < 0.05$, Tukey test).

Treatment	G (%)	VI
Slurry		
Abiotic control	86.7 ± 0.1^{a}	14.31 ± 0.77^{a}
Day 7	94.4 ± 6.9^{ab}	21.56 ± 2.40^{b}
Day 14	92.2 ± 1.9^{ab}	21.17 ± 3.95^{b}
Day 21	92.2 ± 3.8^{ab}	22.74 ± 0.39^{b}
Day 28	97.8 ± 1.9^{b}	26.18 ± 2.57^{b}
Slurry with Tween 80		
Abiotic control	92.2 ± 3.8^{a}	19.15 ± 0.78^{a}
Day 7	87.8 ± 5.1^{a}	23.50 ± 0.28^{b}
Day 14	90.0 ± 5.8^{a}	25.93 ± 2.03^{c}
Day 21	90.0 ± 5.8^{a}	23.82 ± 0.87^{bc}
Day 28	93.3 ± 3.3^{a}	27.42 ± 0.16^{c}

Phytotoxic effects of the slurry bioremediated by the consortium previously acclimated and immobilized were evaluated on *Lactuca sativa* seeds. Results showed that the vigour index of seedlings significantly increased on the 7th day in both slurries, with and without the surfactant (Table 2), hence confirming that the toxicity of the slurries on lettuce plants decreased significantly. Germination percentage (G), however, showed no significant difference in bioremediated slurry with Tween 80, compared to untreated slurry (abiotic control); while in the absence of surfactant an increase in the G was detected at 28 days of treatment, respect to abiotic control (Table 2). Other authors demonstrated that total germination is not a sensitive variable for the detection of phytotoxic effects of HCH in the early stages of plant growth. In fact, most of the plants investigated by these authors did not suffer direct negative effects during germination. This may be due to the adsorption of HCH onto the organic matter in soils, thereby producing a protective effect. On the opposite, in general, HCH isomers limit the development of lettuce seedlings, which is reflected in the VI [23].

4. Conclusions

The survival of the four microorganisms of the consortium after the acclimation period was confirmed, thus demonstrating its stability during long period. The ability of the acclimated *Streptomyces* defined consortium to degrade lindane was demonstrated by the identification of three intermediate metabolites of lindane degradation in liquid medium, 1,2-dichlorobenzene, 1,4-dichlorobenzene, and γ-pentachlorocyclohexene, and by the determination of chloride ions released in the slurry system. The acclimation of actinobacteria defined consortium stimulated growth and increased lindane removal in liquid systems. Although in slurry system the acclimation is not necessary, since it did not improve significantly the pesticide removal.

The bioassay with *Lactuca sativa* was appropriate to evaluate the effectiveness of bioremediation. The defined acclimated actinobacteria consortium carried out a successful bioremediation process in liquid and slurry systems, which was demonstrated through ecotoxicity test with *Lactuca sativa*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.procbio.2015.08.014.

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