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# Improvement of lindane removal by *Streptomyces* sp. M7 by using stable microemulsions

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ABSTRACT

Lindane is an organochlorine pesticide which persists in the environment and can cause serious health problems due to its chlorinated and hydrophobic nature. Microemulsions are isotropic and macroscopically homogeneous systems with high solubilization capacity of hydrophilic and hydrophobic compounds. The aim of this study was to evaluate the removal of high concentrations of lindane by the actinobacterium *Streptomyces* sp. M7 in aqueous and soil systems in the presence of stable microemulsions. Three stable microemulsions were successfully formed with Tween 80, 1-pentanol and three vegetable oils. In most cases, an increase in the cosurfactant/surfactant ratio in the microemulsions favored the solubilization of lindane, while an increase in the oil/surfactant ratio negatively affected the stability of the system. The microemulsion prepared with soybean oil allowed the solubilization of 66% of lindane added to the aqueous medium and 4.5 times more than the surfactant solution at the same concentration. This microemulsion increased the bioavailability of lindane in the aqueous medium and hence enhanced its removal by *Streptomyces* sp. M7 almost two times respect to the achieved with the surfactant solution. In loam soil system, the addition of the microemulsion allowed an 87% of lindane removal by *Streptomyces* sp. M7, increasing almost 50% the removal respect to the obtained without the addition of surfactant agents, although it did not present significant difference respect to the obtained with the surfactant solution. This is the first report on enhanced lindane removal by actinobacteria by using direct microemulsions as bioremediation tools.

## 1. Introduction

Pesticides take an important place within the total number of substances that man is exposed to. Although they have played an important role in the success of modern food production, the extensive and sometimes inadequate use or final disposal of these chemicals can lead to risks to ecosystems and adverse effects on biota including humans (Lake et al., 2012; Villaamil Lepori et al., 2013). Organochlorine pesticides (OPs) constitute a serious environmental problem due to their toxicity, persistence, and bioaccumulation in trophic chains (Kumari et al., 2008). The gamma isomer of hexachlorocyclohexane (γ-HCH), also known as lindane, is a cyclic and saturated OP, which due to its low solubility in water and its chlorinated nature, persists in the environment and presents both high resistance to microbial degradation and toxicity to non-target organisms (Phillips et al., 2005; Manickam et al., 2008). Currently, this pesticide is considered as an endocrine disruptor, potential carcinogen, immunosuppressive and it is known to exert detrimental effects on the reproductive system and the nervous system in mammals (Salam and Das, 2012). It has also been reported as potential teratogenic, genotoxic and mutagenic (ATSDR, 2011). This pesticide is a widespread contaminant; therefore, the development of potent cleanup methodologies from different environmental compartments is necessary.

Bioremediation has received attention as an effective biotechnological tool to remediate polluted environments, and it is defined as a process based on the use of biological mechanisms to reduce (degrade, detoxify, mineralize or transform) the concentration of pollutants to an innocuous state (Azubuike et al., 2016). This technology is considered to be cost-effective and environmentally friendly compared to other methods such as chemical decomposition, incineration, and photodegradation (Zheng et al., 2012a). Among the organisms used for bioremediation, actinobacteria have demonstrated extraordinary degradative abilities, which added to their metabolic diversity and their association with the environment, make these microorganisms as great candidates for bioremediation purposes (Alvarez et al., 2017). Actinobacteria belonging to the genus *Streptomyces* have received considerable attention as effective agents to clean up pesticide-polluted environments. In this context, Benimeli et al. (2003) isolated *Streptomyces* sp.

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**Table 1**
Combinations and proportions of the components constituting the microemulsions.

| μE[a] type[b] | Surfactant (S) | Co-surfactant (C) | Oil (O) | μE subtype[c] | C/S ratio | O/S ratio |
|---|---|---|---|---|---|---|
| **1** | Tween 80 | 1-pentanol | Linseed | A | 1:3 | 1:10 |
| | | | | B | 1:2 | 1:10 |
| | | | | C | 1:1 | 1:10 |
| | | | | D | 1:3 | 2:10 |
| | | | | E | 1:3 | 3:10 |
| **2** | Tween 80 | 1-pentanol | Soybean | A | 1:3 | 1:10 |
| | | | | B | 1:2 | 1:10 |
| | | | | C | 1:1 | 1:10 |
| | | | | D | 1:3 | 2:10 |
| | | | | E | 1:3 | 3:10 |
| **3** | Tween 80 | 1-pentanol | Sunflower[d] | A | 1:3 | 1:10 |
| | | | | B | 1:2 | 1:10 |
| | | | | C | 1:1 | 1:10 |
| | | | | D | 1:3 | 2:10 |
| | | | | E | 1:3 | 3:10 |
| **4** | Triton X-100 | 1-pentanol | Linseed | – | – | – |
| **5** | | | Soybean | – | – | – |
| **6** | | | Sunflower | – | – | – |
| **7** | Brij L-23 | 1-pentanol | Linseed | – | – | – |
| **8** | | | Soybean | – | – | – |
| **9** | | | Sunflower | – | – | – |

- not evaluated.
[a] Microemulsion.
[b] For stability evaluation.
[c] For evaluation of lindane solubilizing capacity.
[d] Previously used for frying.

M7 strain, which had the ability to use γ-HCH as the only carbon source in minimal medium (Benimeli et al., 2006). This strain was also evaluated in soil microcosms assays, showing lindane removal percentages ranging from 14% to 78%, depending on the concentration of pesticide used (Benimeli et al., 2008).

In the case of hydrophobic contaminants, such as OPs and particularly lindane, due to their poor solubility in water, they tend to adhere strongly to soil particles by adsorption, electrostatic interaction and covalent bonding (Zheng and Wong, 2010). Thus, the bioremediation of matrices contaminated with these compounds can result often slow and unsatisfactory. In this sense, surfactants can improve the solubility and bioavailability of hydrophobic organic contaminants, so they are extensively utilized as a complementary strategy in remediation or bioremediation techniques of polluted water or soil (Mulligan, 2005; Bustamante et al., 2012). However, in the last years, considerable attention has been paid to the study of microemulsions for bioremediation processes. Microemulsions are macroscopically homogeneous dispersions of two immiscible fluids, generally, oil and water, stabilized by the presence of a surfactant, either alone or in combination with a cosurfactant (Zhang et al., 2011; Sanchez-Dominguez et al., 2012). These are isotropic and transparent mixtures with moderate viscosity, low interfacial tension and high solubilization capacity of hydrophilic and hydrophobic compounds (Zheng et al., 2011). Microemulsions have attracted attention in various fields of application, such as cosmetics manufacturing, biodiesel production, food and pharmaceutical industry (Worakitkanchanakul et al., 2008; Fanun, 2012). A special emphasis in the last decades has been taken in the use of microemulsions for remediation and bioremediation of organic and inorganic compounds (Bragato and El Seoud, 2003; Castro Dantas et al., 2009; Vargas-Ruiz et al., 2016). Microemulsions can improve degradation of OPs in two ways. First, they increase the bioavailability of the hydrophobic contaminant for microorganisms, and second, they interact with the cell surface and allow the hydrophobic substances to be more easily bound to the microbial cells for further degradation (Salam and Das, 2013).

In this framework, the objective of the present study was to evaluate the removal of high concentrations of lindane by the actinobacterium *Streptomyces* sp. M7 in aqueous and soil systems in the presence of stable microemulsions.

## 2. Materials and methods

### 2.1. Chemicals

Three non-ionic surfactants, Triton X-100, Tween 80, and Brij L23, were tested in the present study, based on their prevailing use in soil washing (Zheng and Wong, 2010; Zheng et al., 2011). Surfactants were purchased from Sigma–Aldrich Co. The formula and properties of the surfactants are listed in Supplementary Table. Three vegetable oils were tested to check their suitability as the oil phase, namely soybean oil, the most commonly produced vegetable oil worldwide; linseed oil, the most widely used vegetable oil in industry, and sunflower frying oil, an abundant gastronomic waste with a difficult final disposal. 1-pentanol (purity > 98%, Sigma–Aldrich Co.) was used as cosurfactant.

Lindane [γ-HCH (99% pure)] was purchased from Sigma–Aldrich Co. A stock solution of γ-HCH dissolved in acetone (50 mg $mL^{-1}$) was prepared and employed in all the assays.

Solvents were of pesticide grade, and double distilled water was used for all tests. All other chemicals used throughout this study were of analytical grade and were purchased from standard manufacturers.

### 2.2. Microorganism and culture media

The actinobacterium *Streptomyces* sp. M7 was used in this study. This strain was isolated from wastewater sediments of a copper filter plant located in an agricultural area of Tucumán, Argentina, containing heavy metals and OPs (Benimeli et al., 2003). *Streptomyces* sp. M7 has demonstrated its ability to biodegrade γ-HCH in liquid and soil systems (Benimeli et al., 2006, 2007a, b; 2008).

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. The microorganism was cultured in TSB for 72 h at 30 °C in a rotatory shaker (150 rpm). Then, the cells were harvested by centrifugation (9000 × *g*, 10 min) and washed three times with sterile distilled water.

Minimal 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 lindane removal assay.

The pH of both media was adjusted to 7 prior to sterilization by autoclaving at 121 °C for 15 min.

### 2.3. Selection of microemulsions and solubilization of lindane

Precursors of microemulsions were obtained by mixing the surfactant (S) (Tween 80, Triton X-100 or Brij L23), the cosurfactant (C) (1-pentanol) and the oil (O) (soybean oil, linseed oil or sunflower oil previously used for frying), in order to obtain a C/S ratio of 1:3 (w/w) and O/S ratio of 1:10 (w/w). Combinations of the components are listed in Table 1. Subsequently, the precursors were diluted with double distilled water to obtain different concentrations of the microemulsions. The stability of the microemulsions was evaluated by two criteria: first by macroscopic observation of their transparency after being kept at room temperature for 12–15 h, and then by centrifugation at 100 × *g* for 5 min (Zheng and Wong, 2010). Those transparent and showing no phase separation after centrifugation were considered stable and were selected for the following tests. These microemulsions were subsequently prepared by using different proportions of their components, as described in Table 1, for their evaluation of lindane solubilizing ability.

In order to determine the apparent solubility of γ-HCH in the presence of these microemulsions, a solution of γ-HCH dissolved in acetone was placed in glass tubes to obtain a final concentration of 250 mg $L^{-1}$. After complete evaporation of acetone, 10 mL of the microemulsions were added to the tubes and they were incubated for 96 h in a rotary shaker at 30 °C and 150 rpm to equilibrate the mixtures. After equilibration, 5 mL of each sample were taken to determine γ-HCH concentration (Zheng et al., 2012b). Controls were carried out by replacing the microemulsions by double distilled water or surfactant solution. The microemulsion with higher lindane solubilizing capacity was selected for subsequent assays.

### 2.4. Lindane removal by Streptomyces sp. M7 in liquid medium with the selected microemulsion

Erlenmeyer flasks containing 30 mL of MM were added with a solution of γ-HCH dissolved in acetone to reach pesticide concentrations of 50, 100, 250 and 500 mg $L^{-1}$, and left in sterility until acetone was completely evaporated. Then, the selected microemulsion was added and *Streptomyces* sp. M7 was inoculated (2*g* $L^{-1}$). Flasks were incubated for 96 h at 30 °C and 150 rpm. Samples were taken every 24 h to determine microbial growth.

For subsequent assays, the highest concentration of γ-HCH which did not inhibit the growth of *Streptomyces* sp. M7 in the presence of the microemulsion was selected to evaluate γ-HCH removal at 30 °C and 150 rpm for 7 days. Samples were taken periodically, sacrificing the flasks, to determine the residual γ-HCH concentration and microbial growth by dry weight (Saez et al., 2015). The assay was also performed replacing the microemulsion by double distilled water or the surfactant solution. Also, non-inoculated flasks containing γ-HCH without any surfactant, and γ-HCH with the microemulsion or surfactant solution were included as abiotic controls.

### 2.5. Assessment of lindane desorption in soil

A loam soil, free of pesticides, was collected (5 × 15 cm depth) from an urban area in San Miguel de Tucumán, Argentina (26°48´35´´S 65°14´26´´W). The soil pH was 7.0 and it contained 2.6% of organic matter, 0.14% of nitrogen, 47.7% sand, 40% silt and 12.3% clay. The soil was air-dried, sieved through a 1-mm sieve and sterilized (three successive cycles at 121 °C for 15 min each, 24 h in between) in glass pots containing 100 g of soil at 20% moisture. The soil humidity was adjusted with sterile water after sterilization (Saez et al., 2014). Subsequently, γ-HCH dissolved in acetone was spiked into the soil to reach the desired concentration and stirred vigorously to promote homogeneous distribution of the pesticide. Acetone was allowed to evaporate under sterility and then the contaminated soil was kept at room temperature for two weeks before desorption and removal experiments were performed. The concentration of γ-HCH in the spiked soil was determined after two weeks to reveal the real concentration (Adapted from Zheng et al., 2012b).

Desorption of γ-HCH in soil − water systems was performed in 50-mL glass flasks containing 5*g* of contaminated soil and 5 or 10 mL of the microemulsion. Controls with double distilled water or surfactant solution were performed. Flasks were shaken at 150 rpm under dark at 30 °C for 96 h and then samples were centrifuged for 5 min at 3000 × *g* and filtered through 0.22 μm filter paper to separate the aqueous phase from the soil particles. The pesticide in the aqueous phase was extracted and quantified as described in 2.7.

### 2.6. Lindane removal by Streptomyces sp. M7 in soil with the selected microemulsion

Soil previously contaminated with lindane was fractionated in glass flasks (5 g in each one) and an appropriate volume of the microemulsion was added. Subsequently, each flask was inoculated with *Streptomyces* sp. M7 (2 g $kg^{-1}$). The same methodology was also carried out by replacing the microemulsion by a surfactant solution or double distilled water. Samples were taken at 0, 1, 3 and 7 days, to determine microbial growth, by enumeration of bacteria as CFU $g^{-1}$ soil (Polti et al., 2014), and residual lindane (detailed below).

### 2.7. Lindane analysis

Lindane extraction from aqueous systems was carried out by ultrasonication. For this purpose, 3 mL of n-hexane were added to 1 mL of sample and sonicated for 30 min. Then, an appropriate volume of the organic phase was taken and dehydrated with $Na_2SO_4$. Finally, 1 mL of the extract obtained was put in a sealable vial for subsequent injection into the gas chromatograph with microelectron capture detector (GC/μECD).

For γ-HCH recovery from soil samples, an extraction with solvents was carried out (Saez et al., 2014). Briefly, n-hexane, water, and methanol (5:4:1) were added to 2.5 g of the soil sample. 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 improve phase separation, and subsequently, 1 mL of the organic phase was taken. The extract was evaporated and finally suspended in an appropriate volume of n-hexane, to be injected into the GC/μECD.

Lindane concentration in extracts was quantified in a gas chromatograph (Agilent 7890 A) equipped with $^{63}Ni$ microelectron capture detector, 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: inlet temperature: 250 °C, carrier gas (nitrogen) flow rate: 25 cm $s^{-1}$, initial oven temperature: 180 °C increasing to 250 °C at 40 °C $min^{-1}$ and increasing to 280 °C at 10 °C $min^{-1}$. The detector temperature was 320 °C and the injection volume was 1 μL. In these conditions, the retention time of lindane was 2.809 min. Calibration was performed using appropriated dilutions of γ-HCH calibration standards, with a linear curve ranging from 1 to 25 mg $L^{-1}$ (AccuStandard, New Haven, USA). In order to determine recoveries of lindane in aqueous and soil systems, a mix of decachlorobiphenyl and tetrachloro-m-xylene (AccuStandard, New Haven, USA) was used as the surrogate with three fortification levels. When the statistical evaluation was carried out between recoveries of the surrogate from fortification level 1, 2 and 3, no significant differences ($p > 0.05$) were observed. In these conditions, the recovery of the method was 86 ± 9% for the liquid system and 103 ± 5% for soil samples.

**Table 2**
Evaluation of the stability of microemulsions.

| μE | 1st stability criterion | 2nd stability criterion |
|---|---|---|
| **1** | + | + |
| **2** | + | + |
| **3** | + | + |
| **4** | + | - |
| **5** | + | - |
| **6** | + | - |
| **7** | + | - |
| **8** | - | ND |
| **9** | - | ND |

μE: microemulsion.
+: meets the stability criterion.
-: does not meet the stability criterion.
ND: not determined.

### 2.8. 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. 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. Evaluation of the stability of microemulsions

With the aim of selecting stable microemulsions, they were evaluated by two excluding criteria. Results revealed that microemulsions prepared with Brij L-23, 1-pentanol and both soybean oil and sunflower oil previously used for frying, presented a milky or opaque appearance; hence they were considered unstable and were discarded (Table 2). After centrifugation, the combinations of Triton X-100 with the three types of oils and the mixture prepared with Brij L23, 1-pentanol and linseed oil presented two phases separation, i.e. they did not meet the second stability criterion, so they were also discarded. On the opposite, microemulsions consisting of Tween 80 as the surfactant and the three types of oils as the oil phase were stable since they presented the two criteria mentioned above (Table 2), and hence were selected to continue the subsequent studies. Similarly, Zheng et al. (2011) reported that at the same surfactant and C/S ratio, the oil type did not significantly influence the formation of microemulsions, probably due to similar compositions of the evaluated vegetable oils. Zheng and Wong (2010) also determined that soybean oil and linseed oil could be used as the oil phase to obtain stable microemulsions with Tween 80, Triton X-100 and Brij 35, while no stable microemulsions were formed with bile salts as the surfactant. However, this is the first work in which stable microemulsions were obtained using sunflower oil previously used for frying. Zhao et al. (2006) suggested that the tail length of the surfactant should be close to the carbon chain length of the oil fatty acids, and the size of the surfactant head group should not be too small (less than 5) or too large (greater than 40) to obtain stable microemulsions. Zheng and Wong (2010) achieved a larger number of stable microemulsions using Tween 80 as surfactant than with Triton X-100 or Brij 35. They attributed this result to the chemical structure of Tween 80, containing 18 $CH_2$ groups, which is close to the length of the carbon chain of the fatty acids of both soybean oil and linseed oil having 23 ethoxylate groups.

On the other hand, the hydrophilic-lipophilic balance value of Tween 80, as well as the content of oil used, indicates that the microemulsions formed are oil-in-water, also called direct, in which vegetable oils are emulsified by the surfactant and cosurfactant (Flanagan and Singh, 2006; Zheng and Wong, 2010).

### 3.2. Lindane solubilization by the selected microemulsions

In microemulsions, oil and water are separated by the surfactant/cosurfactant interface. When a hydrophobic solute is solubilized by a microemulsion, it may exist either in the oil fraction or in the volumetric fraction corresponding to the interfacial layer. Thus, in addition to the surfactant content, both cosurfactant and oil fractions also influence the solubilizing ability of a microemulsion system (Zheng and Wong, 2010). In this context, in order to elucidate the effect of the cosurfactant and oil content on their lindane solubilizing capacity, microemulsions previously selected were prepared using different proportions of the components (Table 1).

Microemulsions consisting of Tween 80, 1-pentanol and soybean oil, linseed oil or sunflower oil previously used for frying, were called microemulsions 1, 2 and 3, respectively. When they were prepared with O/S ratio of 2:10 and 3:10, they presented a milky and opaque appearance immediately. This could indicate that they would have lost stability by increasing the O/S ratio and therefore were discarded for further studies. In contrast, Zheng et al. (2011) reported that an increase in the O/S ratio in microemulsions from 1:20 to 1:10 resulted in an improvement in the solubilization of DDT and lindane; however they did not evaluate the use of greater amounts of oil, as in the present study.

Subsequently, the solubility of lindane was examined in microemulsions 1, 2 and 3 prepared with increasing C/S ratios: (A) 1:3, (B) 1:2 and (C) 1:1, and constant O/S ratio (1:10). The percentages of lindane solubilized as a function of the concentration of surfactant in the microemulsions are shown in Fig. 1. When the microemulsions were replaced by double distilled water, only 2.7% of lindane was solubilized, equivalent to 6.75 mg $L^{-1}$. This is in accordance to the reported aqueous solubility of lindane, which is around 7 and 10 mg $L^{-1}$ (Phillips et al., 2005). On the opposite, when lindane was solubilized by all the microemulsions or the solution of Tween 80, higher percentages of pesticide were obtained in all cases (Fig. 1). Also, it can be noted that the aqueous solubility of lindane improved when the cosurfactant content in the microemulsion was increased, obtaining in most cases the maximal solubilization of the pesticide in those systems with a C/S ratio of 1:1 (Fig. 1). For instance, in the microemulsion 1, containing linseed oil, a maximal of 34% of lindane was solubilized by the microemulsion with the C/S ratio of 1/1 (microemulsion 1 C) at 1000 mg $L^{-1}$ of surfactant, while with the solution of Tween 80 at the same concentration, only 14.7% of the pesticide could be solubilized (Fig. 1I). The microemulsion 2 C, containing soybean oil and the maximal content of cosurfactant evaluated, allowed the solubilization of 66% of the lindane added to the aqueous medium, at a concentration of 1000 mg $L^{-1}$ of surfactant in the microemulsion (Fig. 1II). This represented 4.5 times the obtained with the surfactant solution at the same concentration and more than twice the obtained by the microemulsion of soybean oil with a C/S ratio of 1:3 (microemulsion 2 A). In the case of the microemulsions prepared with sunflower oil previously used for frying, the lowest percentages of solubilized lindane were obtained, ranging between 5% and 22%, approximately. Besides, most of them did not present a statistically significant difference respect to those obtained by the solution of Tween 80 at the same concentration (Fig. 1III). Based on these results, microemulsion 2 C, allowing the highest lindane solubilization capacity, which was 16-fold the aqueous solubility reported for lindane, was selected for lindane removal assays.

Several studies revealed that the C/S ratio is a very important factor in the microemulsion existence domain (Castro Dantas et al., 2003). Cosurfactant is a non-ionic molecule which associates with the surfactant in order to provide a suitable balance between lipophilic and hydrophilic properties for oil and water phases (Rosen, 2004). Zheng et al. (2011) revealed that the C/S ratio had a significant influence on the area of microemulsions, by incorporating higher oil content in microemulsion systems when C/S ratio increased from 1:6 to 1:1. Thus, the presence of a higher content of cosurfactant in microemulsions

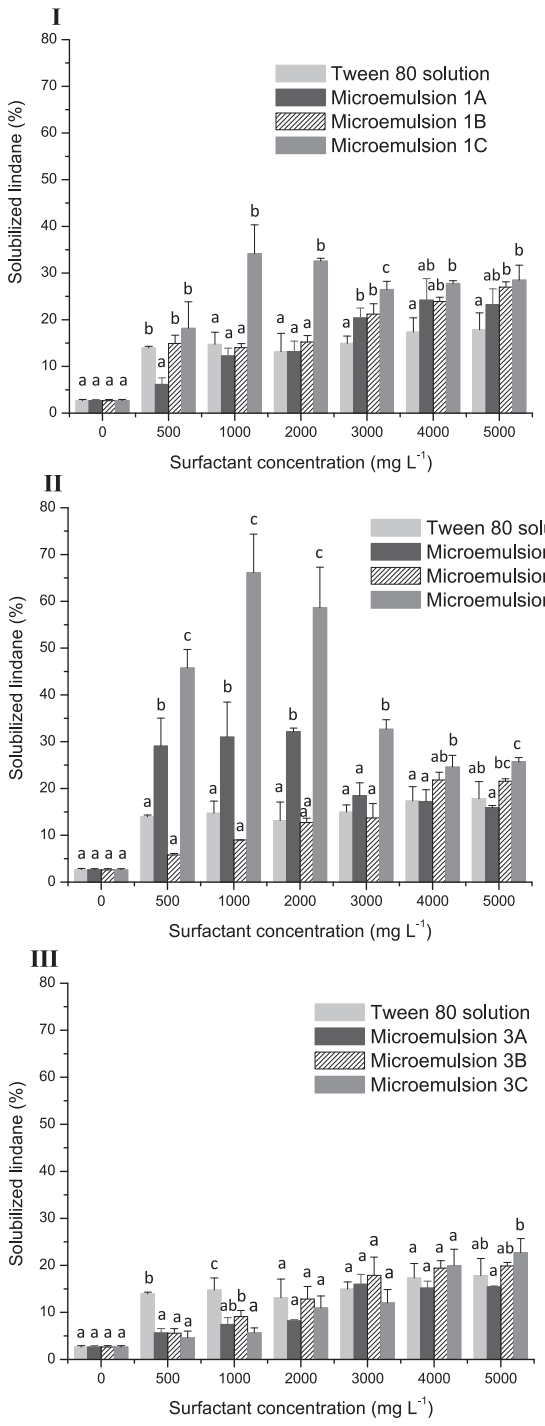


**Fig. 1.** Lindane solubilized (%) by microemulsion systems consisting of Tween 80, 1-pentanol and (I) linseed oil, (II) soybean oil, or (III) sunflower oil (previously used for frying). A, B, and C correspond to C/S ratios of 1:3, 1:2, and 1:1, respectively. Bars showing different letters indicate significant differences among treatments at a given surfactant concentration ($p < 0.05$, Tukey test).

contributed to an increased solubility of hydrophobic pesticides by those microemulsions.

### 3.3. Microbial growth and lindane removal by Streptomyces sp. M7 in liquid medium with the selected microemulsion

The microbial growth of *Streptomyces* sp. M7 in MM in the presence of different concentrations of lindane and the previously selected microemulsion was evaluated. In the presence of the microemulsion without the addition of lindane, the actinobacterium achieved a biomass of 0.2 g $L^{-1}$ after 24 h of incubation, which remained practically constant until the end of the assay. In the presence of Tween 80 solution, *Streptomyces* sp. M7 reached the stationary phase after 48 h of incubation, with a biomass value statistically higher at the end of the assay, respect to the obtained in the presence of the microemulsion ($p < 0.05$) (Fig. 2).

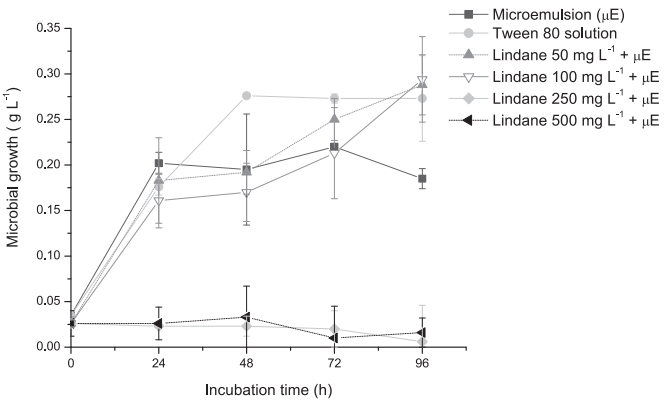


**Fig. 2.** Microbial growth (g $L^{-1}$) of *Streptomyces* sp. M7 in minimal medium supplemented with different concentrations of lindane and the selected microemulsion. Biotic controls (without lindane) of emulsion and microemulsion are also included. Treatments presenting significant differences are mentioned in the text (Section 3.3) ($p < 0.05$).

When 50 or 100 mg $L^{-1}$ of lindane were added to the medium with the microemulsion, the microbial growth of *Streptomyces* sp. M7 was similar in both concentrations of the pesticide ($p > 0.05$) and did not present statistically significant difference respect to the growth obtained in absence of the pesticide until 72 h of incubation ($p > 0.05$). However, at 96 h significantly higher biomass values were recorded in the presence of both lindane concentrations respect to the obtained with the microemulsion alone ($p < 0.05$) (Fig. 2). These results may indicate that these concentrations of lindane would not have been toxic or inhibitory for the growth of the actinobacterium under the evaluated conditions. In addition, it can be observed that in the presence of 50 and 100 mg $L^{-1}$ of lindane and the microemulsion, *Streptomyces* sp. M7 presented a typical diauxic curve. This would indicate that the actinobacterium obtained energy preferably from the surfactant and/or the oil present in the microemulsion up to 24 h, and then used the pesticide as a carbon source after 48 h incubation. Benimeli et al. (2006) also reported a diauxic growth in *Streptomyces* sp. M7 cultured in the presence of glucose (0.6 g $L^{-1}$) and lindane (100 μg $L^{-1}$); the microorganism used the glucose as a substrate until 24 h of incubation and then from 48 h, when the carbohydrate was exhausted, consumed the pesticide as a carbon source.

When *Streptomyces* sp. M7 was cultivated in the presence of the microemulsion and higher concentrations of lindane (250 and 500 mg $L^{-1}$), the biomass remained constant over time, i.e. there was no statistically significant growth ($p > 0.05$) (Fig. 2). This may be attributed to a possible inhibitory effect of these concentrations of lindane on the growth of the bacteria. In a previous study, it was already demonstrated that *Streptomyces* sp. M7 was able to grow in the presence of 50 and 100 mg $L^{-1}$ of lindane when it was integrating a consortium of four actinobacteria; however, in that case, higher concentrations had not been evaluated (Saez et al., 2015).

Based on the above, lindane concentration of 100 mg $L^{-1}$ was selected to perform the pesticide removal studies by *Streptomyces* sp. M7 since it was the highest concentration which did not inhibit the microbial growth of the actinobacterium in the presence of the microemulsion. Results of microbial growth are shown in Fig. 3. *Streptomyces* sp. M7 could grow in MM supplemented with 100 mg $L^{-1}$ of lindane without the addition of any surfactant, thus indicating that the actinobacteria could have used the pesticide as the carbon source. However, the microbial growth of *Streptomyces* sp. under this condition was

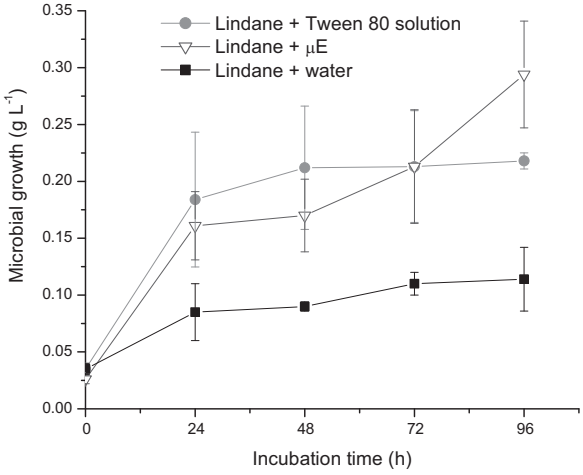


**Fig. 3.** Microbial growth (g $L^{-1}$) of *Streptomyces* sp. M7 in minimal medium supplemented with 100 mg $L^{-1}$ of lindane. Treatments presenting significant differences are mentioned in the text (Section 3.3) ($p < 0.05$).

significantly lower (0.11 g $L^{-1}$) respect to the obtained in the presence of the pesticide and the solution of Tween 80 or the microemulsion, as solubilizing agents (0.22 g $L^{-1}$ and 0.29 g $L^{-1}$, respectively) ($p < 0.05$) (Fig. 3). This may be explained, on one hand, by the fact that lindane is a hydrophobic compound with low aqueous solubility. Therefore, although the pesticide was added to the culture medium at a concentration between 10 and 15 times above its aqueous solubility limit, it may have been a limitation in the accessibility of the microorganism to this hydrophobic compound in the absence of surfactant agents (Manickam et al., 2012; Salam and Das, 2013). On the other hand, the non-ionic surfactant Tween 80, the cosurfactant 1-pentanol and the soybean oil present in the microemulsion, could have also exerted a stimulation effect on the microbial growth of the actinobacterium. Other authors reported that the presence of surfactants may facilitate the bioavailability of a hydrophobic compound and/or stimulate the metabolic machinery by which both the hydrophobic compound and the surfactant may serve as substrates for the bacteria (Manickam et al., 2012). It is also known that several species of the *Streptomyces* genus are able to degrade this surfactant, as well as Tween 60 and Tween 40, among others (Kumar and Goodfellow, 2010; Smaoui et al., 2011). Zheng et al. (2012a) reported a similar behavior in the growth of *Phanerochaete chrysosporium* in the presence of the organochlorine pesticide DDT and a microemulsion prepared with Tween 80, linseed oil and 1-pentanol.

Lindane removal by *Streptomyces* sp. M7 was estimated by quantifying the residual lindane and the results obtained are shown in Fig. 4. When no surfactant or microemulsion was added to the culture medium, an initial concentration of 10.8 mg $L^{-1}$ of soluble lindane was determined. This is consistent with data reported in the literature concerning to the aqueous solubility of lindane (Phillips et al., 2005). When the actinobacterium was inoculated in this condition, lindane removal was only 3.8 mg $L^{-1}$ after 96 h incubation, although it did not present statistically significant difference respect to that obtained in the respective abiotic control ($p > 0.05$). This poor pesticide removal ability without any solubilizing agent would indicate that lindane may have crystallized, thus being hardly available for the actinobacterium; therefore a suitable solubilizing agent could play a key role in the pesticide removal and degradation when high concentrations of hydrophobic compounds are employed in an aqueous medium (Zheng et al., 2012a). In fact, when a solution of Tween 80 was added to the culture medium, the initial concentration of lindane solubilized increased to 31.4 mg $L^{-1}$, and after a 96 h-incubation in the presence of *Streptomyces* sp. M7, a removal of 9.5 mg $L^{-1}$ of the pesticide was detected, representing 30% of the initial soluble lindane. Moreover, the addition of the microemulsion to the culture medium increased the aqueous solubility of lindane approximately seven-fold respect to the obtained in the absence of surfactant or microemulsion, detecting an initial concentration of soluble lindane of 72.3 mg $L^{-1}$. After four days of incubation, 40.3 mg $L^{-1}$ of lindane were removed by *Streptomyces* sp. M7 in the presence of the microemulsion, representing a removal efficiency of 56% of the initial soluble lindane, and thus, bioavailable for the actinobacterium. Also, it should be highlighted that a significant decrease was not detected in the concentration of residual lindane detected in the abiotic controls, either with the Tween 80 solution or the microemulsion ($p > 0.05$). These results demonstrate that biological action is the responsible for the removal of lindane in the systems inoculated with *Streptomyces* sp. M7. Similarly, Zheng et al. (2012a) demonstrated that in the absence of surfactant agents, *Phanerochaete chrysosporium* could only degrade 10% of the DDT added to an aqueous culture medium, while the removal of the pesticide increased to 35.4% and 74.2% in the presence of a solution of Tween 80 or a microemulsion prepared with the same surfactant. Salam and Das (2013) also reported a 40-fold increase in lindane solubility respect to its aqueous solubility in the presence of a bio-microemulsion, allowing the complete removal of the pesticide by the yeast *Pseudozyma* VITJzN01 in half the time than in the absence of solubilizing agents.

### 3.4. Lindane desorption from loam soil by the selected microemulsion

Due to their hydrophobicity, OPs tend to accumulate in the organic matter of the soil. Therefore, the use of surface active agents may increase the diffusive mass transfer rate of these hydrophobic organic compounds from soil particles to the aqueous phase, as well as increase the desorption of these pollutants from soils (Zhang et al., 2011). Lindane desorption process is an important phenomenon to be considered

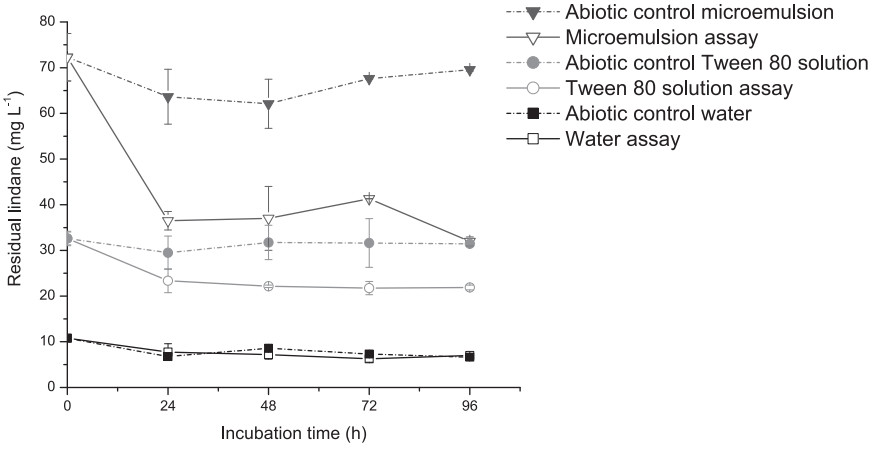


**Fig. 4.** Residual lindane (mg $L^{-1}$) determined in minimal medium supplemented with 100 mg $L^{-1}$ of lindane. Treatments presenting significant differences are mentioned in the text (section 3.3) ($p < 0.05$).

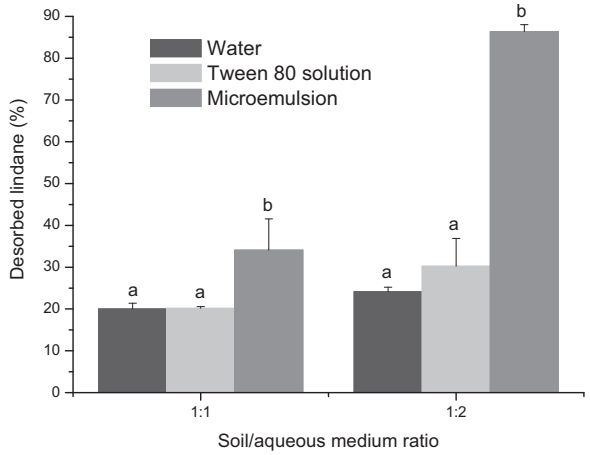


**Fig. 5.** Lindane desorbed (%) from loam soil contaminated with 100 mg $kg^{-1}$ of lindane. Bars showing different letters indicate significant differences among treatments at a given soil/aqueous medium ratio ($p < 0.05$, Tukey test).

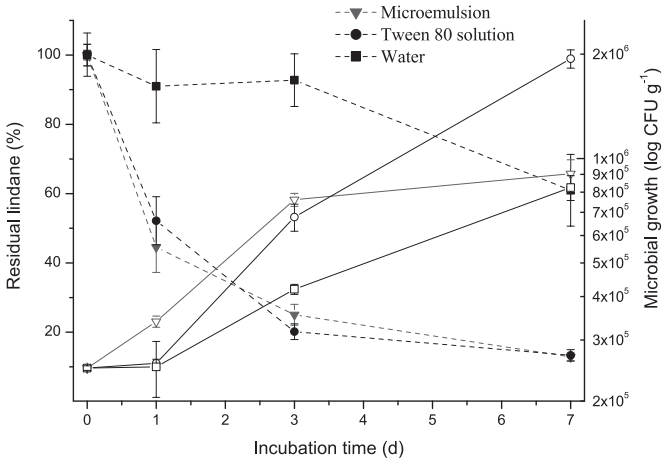


**Fig. 6.** Microbial growth (log CFU $g^{-1}$, empty symbols and solid line) and residual lindane (%, black symbols and dashed line) in soil contaminated with 100 mg $kg^{-1}$ of lindane and inoculated with *Streptomyces* sp. M7 in the presence of the microemulsion, surfactant solution, or water with no surfactant agents. Treatments presenting significant differences are mentioned in the text (Section 3.5) ($p < 0.05$).

since it determines the release rate and the potential mobility of the pesticide in soil (Rama Krishna and Philip, 2008).

In order to evaluate the capacity of the microemulsion to desorb lindane from a loam soil, a desorption assay was performed. Results revealed that at both soil/aqueous medium ratios evaluated, the microemulsion was more efficient at desorbing the pesticide from the soil than water or the surfactant solution ($p < 0.05$). Also, at both ratios evaluated, lindane desorbed from the soil by water and the surfactant alone did not present statistically significant difference between them ($p > 0.05$). At the lowest content of the aqueous medium evaluated (soil/aqueous medium 1:1), water and the surfactant solution showed a desorption capacity of 20%, while the microemulsion reached almost 35% of pesticide desorbed. When the twice of aqueous medium was added to the soil (soil/aqueous medium 1:2), lindane desorption was significantly greater for all three treatments ($p < 0.05$) than the obtained in the 1:1 ratio. In fact, the highest lindane desorption percentage was achieved by the microemulsion (soil/microemulsion 1:2), reaching more than 85% of lindane desorbed from soil, which represents around 3 and 3.5 times the obtained by the solution of surfactant and water, respectively (Fig. 5).

Surfactant-enhanced solubilization results from contaminant partitioning into the hydrophobic core of surfactant micelles (Zhao et al., 2005). In the microemulsions, molecules of 1-pentanol and oil can penetrate into the surfactant micelles. Thus, both compounds play a very important role since they enlarge the effective hydrophobic micelle core size compared to empty micelles of the surfactant (Zheng et al., 2012b).

In the same way, Zheng and Wong (2010) observed that microemulsions prepared with Triton X-100 reached higher desorption of DDT from a sandy loam soil than the surfactant solution, using a soil to the aqueous medium ratio of 1:20. In the present study, a concentration of 1000 mg $L^{-1}$ of Tween 80 in the microemulsion allowed the desorption of 56% more than the surfactant solution employing a soil/microemulsion ratio 10 times lower than the reported by Zheng and Wong (2010). This result represents a great advantage from an environmental and biotechnological point of view since the volumes handled for a microemulsion-aided soil washing or a bioremediation process mediated by this microemulsion would be much smaller.

### 3.5. Microbial growth and lindane removal by Streptomyces sp. M7 in soil with the selected microemulsion

*Streptomyces* sp. M7 showed a similar microbial growth in soil system contaminated with lindane, in the presence of the solution of Tween 80 and the microemulsion until the third day of incubation ($p > 0.05$). However, the biomass of *Streptomyces* sp. M7 at the end of the assay was significantly greater in the soil system added with the solution of Tween 80 than the soil-microemulsion system ($p < 0.05$) (Fig. 6). The microbial growth of the actinobacterium with no surfactant agents was slower until the third day, respect to the other two conditions, but at the end of the assay, it achieved a similar microbial growth to the obtained in the presence of the microemulsion ($p > 0.05$). These results indicate that *Streptomyces* sp. M7 has the ability to grow and survive in loam soil contaminated with 100 mg $kg^{-1}$ of lindane. Also, it could be inferred that the presence of the microemulsion or the surfactant solution could have enhanced the growth rate of the bacterium respect to its growth without the addition of solubilizing agents (Fig. 6). This could be due to an increased solubilization of lindane in the presence of the surfactant or the microemulsion, and therefore more lindane was bioavailable to be used as a carbon source by *Streptomyces* sp. M7 (Manickam et al., 2012). Also, the surfactant could have been used as a carbon source more easily available than the organic matter present in the soil. Although soil is the natural habitat of most streptomycetes, the availability of soil substrates depends on the structure and composition of the soil, among other factors, and therefore influences the metabolic activity of the microbiota present (Katsifas et al., 2000).

Regarding lindane removal, in the soil-water system (with no surfactant agents), a slow lindane removal was observed until the third day (only 8%), achieving at the end of the assay 39% of lindane removed. In contrast, in the presence of the microemulsion or the solution of surfactant, *Streptomyces* sp. M7 removed the pesticide much faster allowing between 44–52% of lindane removed at the first day of incubation (Fig. 6). These results indicate that the addition of the surfactant or the microemulsion could have favored the desorption of the pesticide from soil and thus increase the substrate bioavailability for the actinobacterium. For both treatments, 87% of lindane removal was registered at seven days of incubation, presenting no statistically significant difference between both treatments ($p > 0.05$). Similarly, Salam and Das (2013) also found that 40% of lindane was removed from a soil slurry system by *Pseudozyma* VITJzN01 when no surfactants or microemulsions were added. When a biosurfactant produced by the yeast or a biomicroemulsion was added to the soil suspension as solubilizing agent, lindane removal increased to 50% and 80%, respectively.

Although microemulsions possess a much higher solubilizing capacity for OPs over its counterpart empty micelles of surfactant, their advantages in solubilization could not guarantee consequential advantages in desorption of OPs in a soil-water system (Zhang et al., 2011;

Zheng et al., 2012b). Therefore the application of microemulsions in soil-washing technologies or bioremediation processes may be advantageous over surfactants depending on the type of soil and the microorganism involved in the case of bioremediation, among other factors influencing the efficiency of the process. For instance, Zheng et al. (2012b) demonstrated that microemulsions may be advantageous for soil-washing or bioremediation in loam or sandy soil environments, which have low sorption capacity for oil molecules, but not for clay soil possessing much higher sorbing capacity for oil molecules. Although the present study used a loam soil, soil properties such as pH, the content of organic matter, nitrogen, and clay could have also affected lindane removal by *Streptomyces* sp. M7.

## 4. Conclusions

Stable microemulsions were obtained with the non-ionic surfactant Tween 80, 1-pentanol and three different vegetable oils. In most cases, an increase in the cosurfactant content in the microemulsions favored the solubilization of lindane, whereas an increase in the oil phase negatively affected the stability of microemulsions. The microemulsion prepared with soybean oil showed the highest lindane solubilization capacity in the aqueous medium, allowing the solubilization of more than 16 times the aqueous solubility reported for lindane and more than 4.5-fold the obtained with the surfactant alone at the same concentration. This microemulsion effectively increased the bioavailability of lindane in the aqueous medium and hence enhanced its removal by *Streptomyces* sp. M7 almost two times respect to the obtained with the surfactant Tween 80 alone. In a loam soil system, the microemulsion allowed an 87% of lindane removal by *Streptomyces* sp. M7, although it did not present statistically significant difference respect to the obtained with the surfactant solution. Therefore, microemulsions formed with Tween 80, 1-pentanol and soybean oil could be could be used as potential tools in soil washing technologies or *ex situ* bioremediation processes of wastewaters containing not only lindane but also others hydrophobic organic compounds. However, this is a preliminary study and the information available is related to laboratory scale. For this reason, further and deeper research is needed to expand the usage of microemulsions for bioremediation purposes in field scale, assessing its effect on indigenous microorganisms.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2017.06.026.

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