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# Lindane dissipation in a biomixture: Effect of soil properties and bioaugmentation

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ABSTRACT

The biomixture is the major constituent of a biopurification system and one of the most important factors in its efficiency; hence the selection of the components is crucial to ensure the efficient pesticides removal. Besides, bioaugmentation is an interesting approach for the optimization of these systems.

A mixed culture of the fungus *Trametes versicolor* SGNG1 and the actinobacteria *Streptomyces* sp. A2, A5, A11, and M7, was designed to inoculate the biomixtures, based on previously demonstrated ligninolytic and pesticide-degrading activities and the absence of antagonism among the strains. The presence of lindane and/or the inoculum in the biomixtures had no significant effect on the development of culturable microorganisms regardless the soil type. The consortium improved lindane dissipation achieving 81–87% of removal at 66 d of incubation in the different biomixtures, decreasing lindane half-life to an average of 24 d, i.e. 6-fold less than $t_{1/2}$ of lindane in soils. However, after recontamination, only the bioaugmented biomixture of silty loam soil enhanced lindane dissipation and decreased the $t_{1/2}$ compared to non-bioaugmented. The biomixture formulated with silty loam soil, sugarcane bagasse, and peat, inoculated with a fungal-actinobacterial consortium, could be appropriate for the treatment of agroindustrial effluents contaminated with organochlorine pesticides in biopurification systems.

## 1. Introduction

Pesticides are among the most employed organic compounds worldwide and play an important role in modern agriculture and food production. However, their inadequate management can lead to contamination of soil, surface, and groundwater (Castillo and Torstensson, 2007; Chin-Pampillo et al., 2015).

Environmental pesticide contamination can occur through diffuse sources or point sources. Point source contamination by pesticides derives from improper handling or leaks of the spraying liquid, remnants, and washes of the spraying equipment, and is considered as one of the main causes of pesticide contamination in water and soils (Campos et al., 2017).

In the last decades, point source pesticides pollution has been rigorously addressed, through the evaluation and implementation bioprophylaxis protocols, whose objectives are to reduce or avoid point source pollution. For this purpose, biopurification systems or biobeds are among the most promising technologies. These systems initially developed in Sweden in the '90s, consist of a simple, ecological and cost-effective technology construction designed to retain and degrade pesticides. It consists of three main components: a clay layer at the bottom; a biomixture; and a grass layer that covers the surface, all arranged in an excavation in the soil at 60 cm-depth (Castillo et al., 2008). The biomixture represents the biologically active part of a biopurification system, where the processes of adsorption and degradation of pesticides take place. It is composed of a lignocellulosic substrate (originally straw), a humic rich component (peat or compost) and soil (Castro-Gutiérrez et al., 2017). Each component plays an important role in the dissipation of pesticides. The lignocellulosic substrate promotes the growth of ligninolytic microorganisms and the production of extracellular ligninolytic enzymes as peroxidases and phenol-oxidases; peat or compost provides high water retention and sorption capacity of

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pesticides, while the soil is an important source of bacteria and fungi which can degrade the pesticides (Castro-Gutiérrez et al., 2017; Chin-Pampillo et al., 2015).

Agricultural and forestry residues generated as lignocellulosic wastes increase every year the environmental pollution. However, these natural compounds can be converted to several value-added products (Elgueta et al., 2016). In this sense, the design of a biopurification system should be adapted to every region; hence the composition of the biomixtures will depend on the availability of agricultural by-products of low commercial value, and the physicochemical parameters of local soils, such as pH, organic matter content, moisture, temperature, among others (Góngora-Echeverría et al., 2017; Ruiz-Hidalgo et al., 2015). Intensive researches have been carried out in order to adapt the systems to the local conditions, availabilities, and needs, obtaining good performances even when the pesticides were added in successive applications and high concentrations (Chin-Pampillo et al., 2015; Góngora-Echeverría et al., 2017; Ruiz-Hidalgo et al., 2015).

The bioaugmentation of biopurification systems with pesticide-degrading microorganisms represents a very interesting approach in the design and optimization of these systems (Ruiz-Hidalgo et al., 2016). Among a variety of microorganisms known to degrade or mineralize pesticides, actinobacteria, especially those belonging to the *Streptomyces* genus, stand out as they have a great capacity to degrade various pesticides such as lindane, chlordane, methoxychlor, chlorpyrifos, diuron, diazinon and pentachlorophenol (Alvarez et al., 2017; Benimeli et al., 2008; Briceño et al., 2016, 2012; Fuentes et al., 2016, 2014). On the other hand, given that a half of the biomixture consists of lignocellulosic substrates, the bioaugmentation with ligninolytic fungi is of particular interest to potentially increase the degrading capacity of the biomixture. Also, ligninolytic fungi such, as white rot fungi, are recognized for their ability to transform a wide range of organic pollutants, including pesticides (Camacho-Morales et al., 2017; Purnomo et al., 2017; Ruiz-Hidalgo et al., 2016; Tortella et al., 2015).

In this regard, several studies have evaluated the bioaugmentation of biopurification systems with ligninolytic fungi obtaining satisfactory results (Elgueta et al., 2016; Madrigal-Zúñiga et al., 2016; Ruiz-Hidalgo et al., 2016). Moreover, Verhagen et al. (2013) demonstrated that bioaugmentation of a biopurification system with a pesticide-degrading enriched mixed culture showed an improvement in the removal of chloropropham compared to the systems inoculated with a single pesticide-degrading strain. Only a few studies have evaluated the bioaugmentation of biomixtures with either single or mixed bacterial cultures (Briceño et al., 2017; Campos et al., 2017; Karas et al., 2016), but none, to our knowledge, have explored the use of a fungal-actinobacterial mixed culture for the bioaugmentation of biopurification biomixtures.

Lindane is an organochlorine pesticide, primarily used as an insecticide and fumigant against a wide range of soil-dwelling and phytophagous insects. Although the use of lindane has been banned or severely restricted in at least 52 countries (Madaj et al., 2017; Ministerio de Salud, 2016), some developing countries are still using it for economic reasons. Therefore, it was chosen as model pesticide because due to its widely utilization in the past and its persistence in the environment, it is still being found in different environmental compartments such as water courses, sediments, soils, animal and plant tissues (Li et al., 2015; Villaamil Lepori et al., 2013; Yadav et al., 2015); thus it is imperative to develop methods to minimize, or if possible avoid, its release to the environment.

In view of the above, the aim of this study was to evaluate the performance of biomixtures formulated with an agro-industrial by-product derived from a local industry and different soil textures, and the effect of the bioaugmentation with a consortium of actinobacteria and fungi, on their lindane removal capacity.

## 2. Materials and methods

### 2.1. Microorganisms

The actinobacteria *Streptomyces* sp. A2, A5, A11, and M7 were used in this study. These strains were isolated from soils and sediments contaminated with organochlorine pesticides (Benimeli et al., 2003; Fuentes et al., 2010), and selected based on its lindane removal capacity (Fuentes et al., 2011).

Filamentous fungi *Trametes versicolor* S5NG1, *Fusarium solani* S2EG3, and *Trichoderma atroviride* S1EG1, isolated from leaf litter collected from a mountain forest of Northwestern Argentina (Fernandez et al., 2017), were used due to its lignocellulolytic activities.

### 2.2. Culture media and chemicals

Tryptic Soy Broth (TSB) was used for the actinobacteria inoculum preparation. It consists of ($g\,L^{-1}$): tryptone, 15; soy peptone, 3; NaCl, 5; $K_2HPO_4$, 2.5; glucose, 2.5 (pH 7).

Starch Casein (SC) medium, containing in $g\,L^{-1}$: starch, 10; casein, 1; $K_2HPO_4$, 0.5; agar, 15 (pH 7), was used for the antagonism assay among the strains (Hopwood, 1985).

Yeast extract-Malt extract (YM) broth, containing in $g\,L^{-1}$: glucose, 10; peptone, 5; malt extract, 3; yeast extract, 3 (pH 6.4) (Pajot et al., 2011), was used for the fungus inoculum preparation.

Plate Count Agar (PCA) medium was used for counting the total heterotrophic microorganisms. It contains ($g\,L^{-1}$): tryptone, 5; glucose, 1; yeast extract, 2.5; agar, 15 (pH 7).

All the culture media were sterilized by autoclaving at 121 °C for 15 min.

Lindane (γ-HCH, 99% pure) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and all other chemicals used in this study were purchased from certified manufacturers. A stock solution of γ-HCH dissolved in acetone ($50\,mg\,mL^{-1}$) was employed to contaminate the biomixtures. Solvents were of pesticide grade and all other chemicals were of analytical grade.

### 2.3. Antagonism assay

Each of the filamentous fungi was seeded in the center of a Petri dish containing SC medium and faced transversally with the actinobacteria, making all possible combinations. Petri dishes were incubated at 28 °C for 7 days. At the end of the assay, the development of the microorganisms was macroscopically evaluated and the presence of antagonism between the four actinobacteria and the three fungi under study, evidenced as an inhibition halo, was determined. The strains presenting no antagonism among them were selected for the bioaugmentation assays.

### 2.4. Soils sampling and conditioning

Three types of soils with different textures were collected from various regions of Tucumán province, free of contamination with OPs. Soil samples were taken from the upper layer (5–15 cm deep) and were stored in dark at 15–20 °C. The main physicochemical properties of the soils are detailed in Table 1.

Soil samples were conditioned; the undesirable macroscopic particles were separated and soils were dried at 30 °C for three days for decreasing the humidity rate. Table 2

### 2.5. Preparation of the biomixtures

According to the methodology described by Castillo et al. (2008), the biomixtures were prepared using a lignocellulosic material, peat, and soil in a proportion of 50:25:25 vol%. Sugarcane bagasse, provided by the Sugar mill Cruz Alta (Tucuman, Argentina), was used for the

**Table 1**
Physicochemical characteristics of the soils used for the formulation of the biomixtures.

| Parameters | Sample A | Sample B | Sample C |
|---|---|---|---|
| pH[a] | 7.56 | 8.95 | 6.80 |
| Oxidable organic matter[b], % | 1.0 | 1.0 | 1.3 |
| Organic carbon[b], % | 0.61 | 0.58 | 0.80 |
| Total nitrogen[c], % | 0.07 | 0.04 | 0.10 |
| Available phosphorus[d], ppm | 37.4 | 19.3 | 21.6 |
| Clay[e], % | 62.5 | 2.5 | 14.3 |
| Silt[e], % | 13.8 | 4.0 | 59.8 |
| Sand[e], % | 23.7 | 93.5 | 25.9 |
| Texture[e] | Clayey | Sandy | Silty loam |

[a] Soil to distilled water ratio of 1:2.5.
[b] Walkley-Black method.
[c] Kjeldahl method.
[d] Bray-Kurtz method.
[e] Analysis by hydrometer: modification of the Bouyoucos method.

**Table 2**
Chemical characteristics of the sugarcane bagasse used to formulate the biomixtures.

| Parameters | % |
|---|---|
| Total nitrogen[a] | 0.22 |
| Total phosphorus[b] | 0.03 |
| Total organic carbon[c] | 52.7 |
| Total organic matter[c] | 90.8 |

[a] Kjeldahl method.
[b] Spectrophotometry.
[c] Calcination.

preparation of the biomixtures in replacement of wheat straw which is the traditional material used for this purpose. This organic material was selected based on its low cost and wide availability in the in the province of Tucumán, Northwest Argentina, where the sugar industry is the main activity in the region.

The sugarcane bagasse was crushed and sieved using a 5 mm-mesh and then combined with each kind of soil and the commercial peat (50:25:25 vol%) in order to formulate the biomixtures. The three components were thoroughly homogenized and distributed into aluminum pots of 30 cm long, 20 cm wide and 10 cm high. The moisture content of the biomixtures was adjusted with sterile distilled water to approximately 60% of its water holding capacity. In order to stabilize the systems, the biomixtures were incubated at 30 °C for 30 days (Tortella et al., 2012). The moisture lost by evapotranspiration was monitored weekly by weight difference and was adjusted by adding the required amount of sterile distilled water.

### 2.6. Inoculum preparation

The actinobacteria were individually cultured in TSB for 72 h at 30 °C and 200 rpm. The cultures were centrifuged (9000 × *g*, 10 min, 4 °C) and the microbial biomass was washed twice with sterile distilled water and weighed.

The selected filamentous fungus was cultured in YM medium for 72 h at 25 °C and 250 rpm. After mechanically rupturing the mycelium by using a mixer, the biomass of the fungus was combined with the actinobacteria in order to obtain the final inoculum consisting of equal proportions (w/w, wet weight) of each strain. This mixed culture was used to inoculate the biomixtures in a final concentration of 2 g $kg^{-1}$ of biomixture (w/w, wet weight).

### 2.7. Microbial count and lindane removal capacity in the biomixtures

The biomixtures were inoculated with the selected mixed culture in a concentration of 2 g $kg^{-1}$ and stirred vigorously to ensure the correct homogenization of the system. Subsequently, they were artificially contaminated by adding an appropriate amount of lindane stock solution, in order to obtain a final lindane concentration of 100 mg $kg^{-1}$. The biomixtures were kept at room temperature for 2 h in laminar flow in order to allow evaporation of the acetone present in the stock solution. Then they were incubated in darkness at 30 °C and samples were taken at 10, 20, 30 and 60 d to determine the residual lindane concentration and the total heterotrophic microorganisms.

After 66 d of incubation, a new contamination of the system was performed by adding lindane at a final concentration of 100 mg $kg^{-1}$. Samples were taken at 66, 76 and 86 d of incubation.

In addition, controls of inoculated uncontaminated biomixture, and contaminated not inoculated biomixture were performed.

In order to determine the development of the inoculated microorganisms and the native populations of the biomixtures, the enumeration of the total heterotrophs (CFU per gram of biomixture) was performed according to the method described by Jézéquel et al. (2005). Briefly, 1 g of biomixture was transferred to a tube containing 9 mL of sterile sodium hexametaphosphate (1.66 g $L^{-1}$, pH 7) in order to facilitate the dispersion of the humic complexes, and stirred in a vortex. Serial decimal dilutions were performed with sodium dihydrogenphosphate (0.05 M, pH 7) and plated in triplicate in Petri dishes with PCA medium. Plates were incubated at 30 °C for 96 h, and the CFU $g^{-1}$ were determined.

### 2.8. Lindane analysis

The recovery of lindane from the biomixture samples was performed through an extraction with solvents. For this purpose, 5 g of each biomixture were placed in a 50 mL-capacity centrifuge tube. 4 mL of distilled water of chromatographic quality, 1 mL of methanol and 5 mL of n-hexane were added. The mixture was vigorously shaken and then centrifuged at 8500 rpm for 10 min. Subsequently, it was incubated at − 4 °C for 5 min in order to improve the separation of the organic phase. An appropriate volume of the organic phase was transferred to a hemolysis tube, evaporated to dryness and resuspended in 1 mL of n-hexane (Quintero et al., 2005).

Lindane concentration in the extracts obtained 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 automatic 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 then increasing to 280 °C at 10 °C $min^{-1}$. The detector temperature was 300 °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 (AccuStandard, New Haven, USA). The detection limit of the method was 0.01 µg $L^{-1}$.

### 2.9. Kinetic parameters of lindane removal

The removal of lindane in the biomixtures was fitted to a first-order kinetic model, which responds to the following equation:

$$\frac{C_t}{C_0} = e^{-kt}$$

$C_0$ is the concentration of lindane in the biomixtures at the initial time of the experiment, $C_t$ is the lindane concentration at time t, k is the degradation constant ($d^{-1}$) and t is the degradation time (d). The half-life of lindane ($t_{1/2}$), i.e. the time in which the concentration of the pesticide is reduced by 50%, was calculated by the following equation:

$$t_{1/2} = \ln(2)/k$$

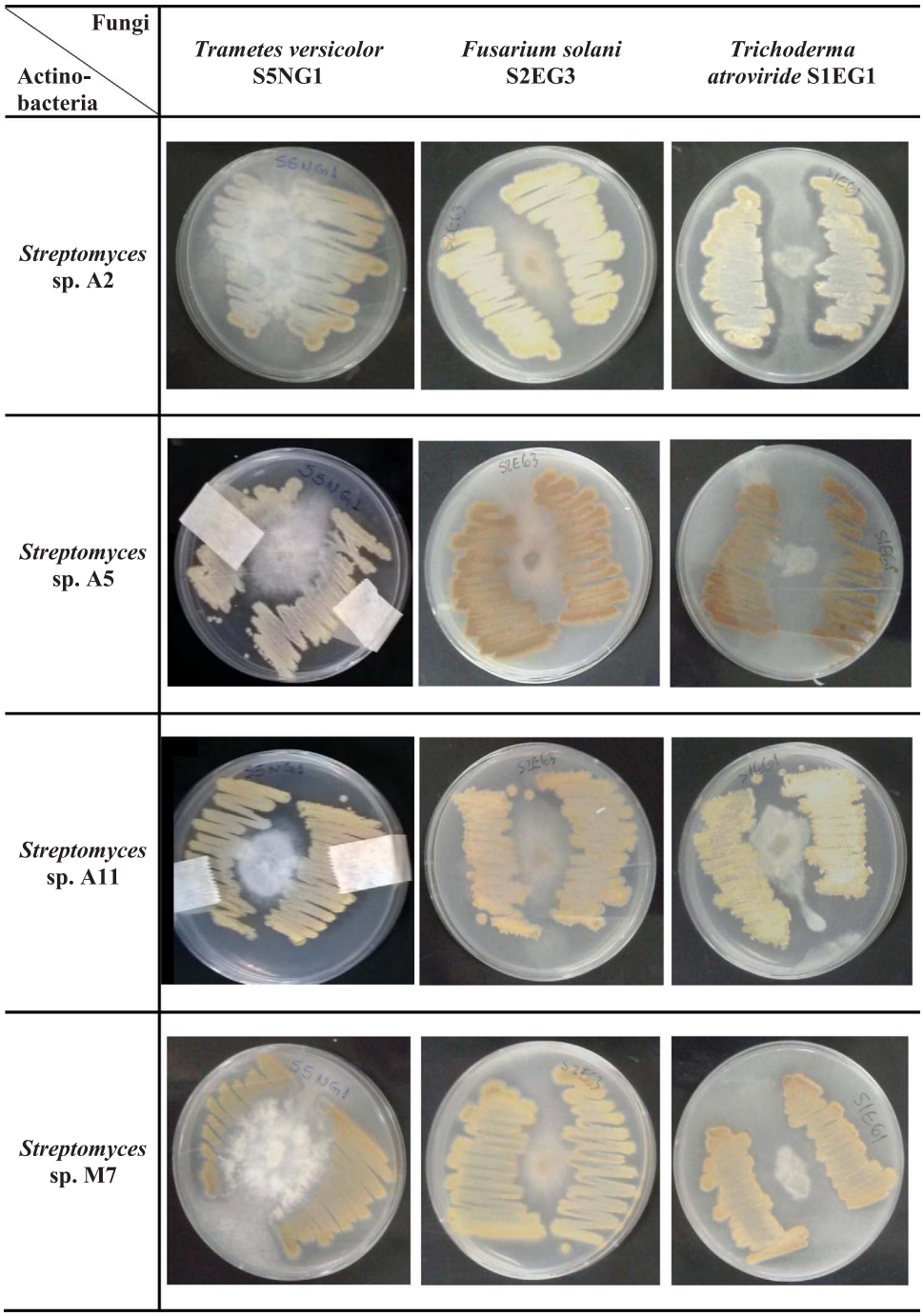


**Fig. 1.** Antagonism assay among fungi and actinobacteria strains in Starch Casein Agar medium.

### 2.10. Statistical analysis

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 the treatments. When significant differences were found, Tukey 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

### 3.1. Antagonism assay among actinobacteria and fungi

The presence of antagonistic effects among the studied strains in SC medium is shown in Fig. 1. After 7 days of incubation, the four actinobacteria showed no antagonism effects on the growth of *Trametes versicolor* S5NG1. In contrast, the radial growth of *Trichoderma atroviride* S1EG1 was inhibited by all the *Streptomyces* sp. strains. Similarly, *Streptomyces* sp. A2, A5, and M7 inhibited the growth of *Fusarium solani* S2EG3. Based on these results, the filamentous fungus *Trametes versicolor* S5NG1 was selected to formulate a defined mixed culture with the previously selected *Streptomyces* strains sp. A2, A5, A11, and M7.

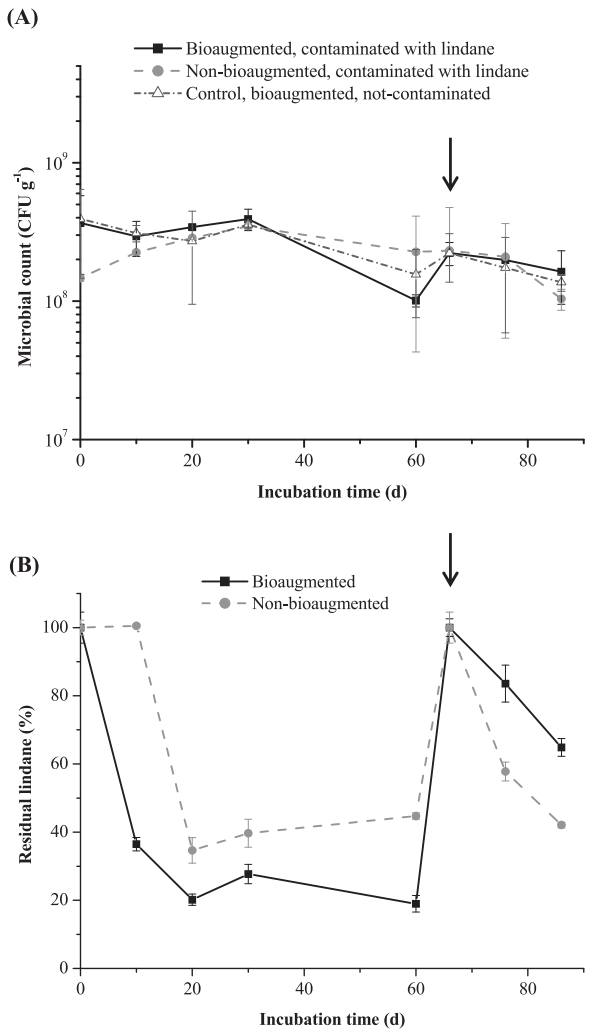


**Fig. 2. (A)** Cell enumeration of total heterotrophic microorganisms (CFU $g^{-1}$) and **(B)** residual lindane (%) in biomixtures formulated with clayey soil. The arrow indicates the second lindane contamination. Error bars represent standard deviation. Treatments presenting significant differences are mentioned in the text ($p < 0.05$).

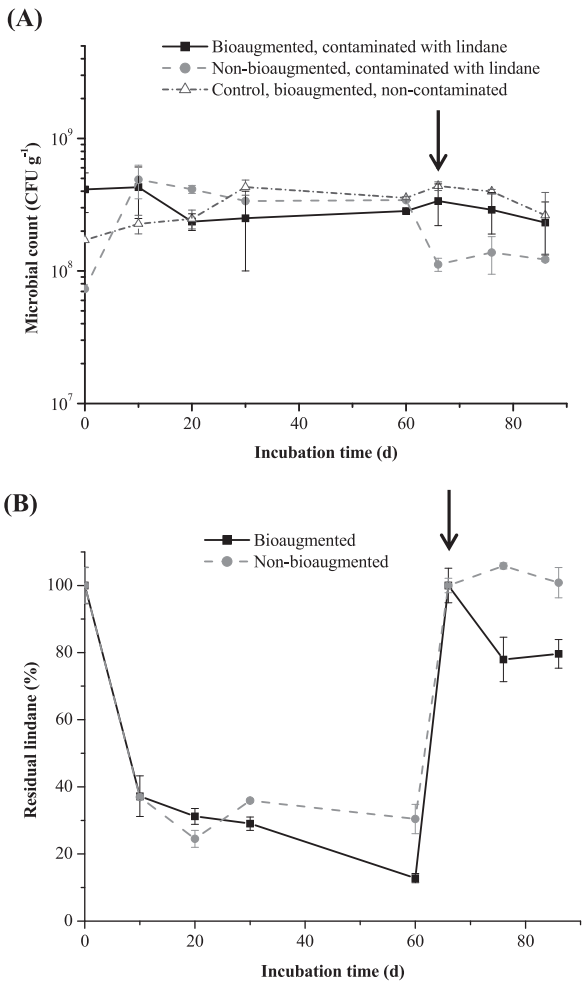


**Fig. 3. (A)** Cell enumeration of total heterotrophic microorganisms (CFU $g^{-1}$) and **(B)** residual lindane (%) in biomixtures formulated with sandy soil. The arrow indicates the second lindane contamination. Error bars represent standard deviation. Treatments presenting significant differences are mentioned in the text ($p < 0.05$).

### 3.2. Microbial count and lindane removal in biomixtures

Figs. 2–4 show the enumeration of total heterotrophic microorganisms and the removal of lindane along the incubation time in biomixtures (bioaugmented and non-bioaugmented) prepared with clayey soil, sandy soil, and silty loam soil, respectively.

In the biomixture formulated with clayey soil, the count of heterotrophic microorganisms showed a constant profile up to 30 days of incubation, regardless of the type of treatment, and no significant differences were observed in the microbial count of the bioaugmented and non-bioaugmented biomixtures respect to the non-contaminated control ($p > 0.05$) (Fig. 2A). Between 30 and 60 days of incubation, a decreasing profile was observed in the enumeration of cultivable microorganisms; however, from that moment it remained practically constant, even after the recontamination with the pesticide. Statistical analysis showed no significant differences ($p > 0.05$) in the total heterotrophic microorganisms count during the 86 days of incubation under any of the conditions tested. In biomixture inoculated with the microbial consortium, the pesticide removal was greater than 60% at 10 days of incubation; whereas lindane concentration remained constant in the uninoculated biomixture until the 10 days. At 60 days of incubation, the removal achieved by the bioaugmented biomixture was 81%, while the non-inoculated reached only 55% of removal. In contrast, after recontamination, the inoculated biomixture was able to remove 35% of lindane while the uninoculated biomixture achieved 58% of pesticide removal (Fig. 2B).

In the sandy soil based biomixture, a variable profile was observed in the total microbial count until the 30 days of incubation, depending on the type of treatment. Between 30 and 60 days of incubation, the total heterotrophic microorganisms remained constant in the three biomixtures analyzed. After the recontamination with lindane, a significant decrease in the microbial count was observed in the non-bioaugmented biomixture; while the enumeration of the total microorganism population remained practically constant in the bioaugmented ones (Fig. 3A). Regarding lindane dissipation, 63% of the pesticide was removed at 10 days of incubation in both bioaugmented and non-bioaugmented biomixtures ($p > 0.05$). At 60 days of incubation, the removal in the inoculated biomixture was 87%, whereas in the uninoculated control it reached almost 70%. Moreover, after the second lindane application, the inoculated biomixture achieved 20% of lindane removal, whereas, in the absence of the inoculum, no significant removal was observed (Fig. 3B).

A growing profile on the microbial development was observed in the biomixture prepared with silty loam soil (Fig. 4A). In all the evaluated conditions a decrease in the total heterotrophic microorganisms count was observed after recontamination with lindane (day 66); however, at 86 days of incubation, the total microbial count was significantly higher than the obtained at the initial time (day 0) in all the treatments performed ($p < 0.05$). Lindane removal was 50% at 10 days of incubation,

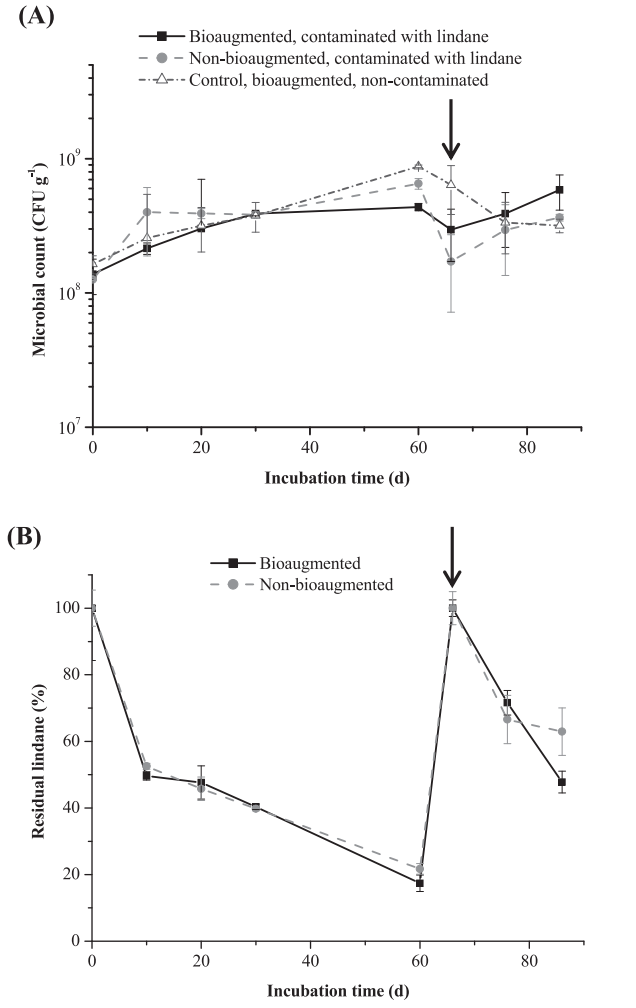


**Fig. 4. (A)** Cell enumeration of total heterotrophic microorganisms (CFU $g^{-1}$) and **(B)** residual lindane (%) in biomixtures formulated with silty loam soil. The arrow indicates the second lindane contamination. Error bars represent standard deviation. Treatments presenting significant differences are mentioned in the text ($p < 0.05$).

and reached 80% after 60 days of incubation in the biomixture inoculated with the consortium, although no statistically significant differences were registered in the pesticide removal in the presence and absence of the consortium ($p > 0.05$). However, after recontamination, the inoculated biomixture achieved a 52% removal of lindane, while the uninoculated system reached only 37% of lindane removed (Fig. 4B).

The statistical analysis of the enumeration of the total heterotrophic microorganisms at the end of the assay showed no significant differences between the biomixtures contaminated with lindane but non-inoculated respect to the biomixtures contaminated twice with 100 mg $kg^{-1}$ of lindane and inoculated with the consortium, regardless the soil texture used ($p > 0.05$).

In order to compare the lindane dissipation rate in the different biomixtures, the kinetic parameters were determined (Table 3). The biomixtures formulated with three different textured soils and bioaugmented with the microbial consortium showed significantly lower $t_{1/2}$ values than the biomixtures without bioaugmentation during the first lindane contamination ($p < 0.05$). After the second application of the pesticide, the bioaugmented biomixtures formulated with clayey and sandy soils showed lower lindane removal rates than the observed during the first contamination. In contrast, the silty loam soil biomixture inoculated with the microbial consortium maintained a high lindane dissipation capacity (approximately 50%) after the recontamination, with a significant decrease in the pesticide $t_{1/2}$ compared to the first contamination ($p < 0.05$).

## 4. Discussion

The microorganisms inoculated in a biomixture play a key role in the degradation of the pesticides. Microbial communities possess increased metabolic capabilities allowing members of communities the division of labor and survival to perturbations (Hays et al., 2015). However, for the construction of a defined mixed culture, the compatibility among the strains must be evaluated. The phenomenon of antagonism is a common event that has been observed in mixed microbial populations (Odjadjare et al., 2008). In particular, bacteria of the *Streptomyces* genus are recognized as important producers of secondary metabolites with antibacterial, immunosuppressive, antitumor and antifungal effects, among others (Alvarez et al., 2017). Previous studies have demonstrated that the quadruple consortium constituted by the *Streptomyces* strains A2, A5, A11, and M7 presented efficient lindane degradation, achieving good removal of the pesticide and also showing a high specific dechlorinase activity. In addition, the absence of antagonism among these actinobacteria strains has been already probed (Fuentes et al., 2013, 2011). On the other hand, the biotransformation of lignocellulosic wastes can be attributed to microorganisms, especially white-rot fungi, due to their extracellular ligninolytic enzymes (laccases, lignin peroxidases, and manganese peroxidases) able to attack and transform not only lignin but also organic complex molecules as pollutants (Rao et al., 2014). For this reason, *Trametes versicolor* S5NG1, based on its laccase activity over different substrates (Fernandez et al., 2017), and the absence of antagonistic effects with the *Streptomyces* strains, was selected to formulate a defined mixed culture with the pesticide-degrading actinobacteria for the bioaugmentation of the biomixtures. Thus, the fungus could be not only actively involved in the degradation of the bagasse added to the biomixture, thus providing additional sources of nutrients and energy, but also could have participated in the degradation of lindane. In fact, *Trametes versicolor* belongs to the group of white-rot fungi, and it is known for its capacity to degrade a wide range of organic pollutants (Fang et al., 2014; Yang et al., 2013), and specifically lindane degradation has been already demonstrated in other *T. versicolor* strains (Ulčnik et al., 2013).

In the present study, the microbial population was not significantly affected by the presence of lindane since no significant differences in microbial enumeration were detected in the presence and absence of the pesticide. Moreover, in the biomixture composed of silty loam soil, the final count of total heterotrophs was higher than the initial count, thus revealing the non-existence of inhibitory effects due to the presence of the pesticide. These results are indicating that the pesticide in the high concentration applied would not have a toxic effect on the microbial population present in the different systems evaluated. Fuentes et al. (2017) also observed that a defined consortium of actinobacteria composed of *Streptomyces* sp. A2, A5, A11, and M7 was able to colonize three microcosms of soils of different textures (clay silty loam, sandy, and loam) contaminated with a mixture of organochlorine pesticides without presenting an inhibiting effect on their growth in the presence of the xenobiotics, although the pesticides concentration was 20-fold lower than the tested in the present study.

Notably, in the non-bioaugmented sandy soil based biomixture a decrease in the microbial count was detected after the recontamination with lindane. This may be an evidence of an inhibitory effect of lindane on the development of the native microbiota, whereas the inoculated consortium may be more resistant to the pesticide showing similar growth than the obtained in the uncontaminated system. The four actinobacteria composing the consortium could have adequate enzymes to carry out the degradation of various organochlorine pesticides since they were isolated from an environment contaminated with these compounds (Benimeli et al., 2003; Fuentes et al., 2010). In ecosystems containing toxic substances of anthropogenic origin, multidirectional

**Table 3**
First-order kinetic parameters for lindane removal in biomixtures formulated with different soil types bioaugmented and non-bioaugmented, with two successive pesticide additions (100 mg $kg^{-1}$, each one). Different letters indicate significant differences between bioaugmented and non-bioaugmented systems ($p < 0.05$, Tukey test).

| Biomixtures | Parameters | | | |
|---|---|---|---|---|
| | $k$ ($d^{-1}$) Fist lindane contamination | $t_{1/2}$ (d) | $k$ ($d^{-1}$) Second lindane contamination | $t_{1/2}$ (d) |
| CS-bioaugmented | 0.028 ± 0.002[b] | 25.0 ± 2.0[a] | 0.022 ± 0.002[a] | 32.2 ± 3.0[b] |
| CS-non-bioaugmented | 0.013 ± 0.001[a] | 51.7 ± 1.4[b] | 0.043 ± 0.001[b] | 16.0 ± 0.4[a] |
| SS-bioaugmented | 0.034 ± 0.002[b] | 20.2 ± 1.0[a] | 0.011 ± 0.003 | 63.1 ± 16.1 |
| SS-non bioaugmented | 0.021 ± 0.001[a] | 32.9 ± 2.0[b] | ND | ND |
| SLS-bioaugmented | 0.029 ± 0.002[a] | 23.8 ± 1.9[a] | 0.037 ± 0.003[b] | 18.8 ± 1.7[a] |
| SLS-non bioaugmented | 0.026 ± 0.001[a] | 27.2 ± 1.3[a] | 0.007 ± 0.001[a] | 99.8 ± 1.6[b] |

CS: clayey soil; SS: Sandy soil; SLS: silty loam soil; $k$: degradation constant; $t_{1/2}$: half life time; ND: not determined.

and long-term effects have been observed, affecting all the organisms living in those environments (Skibniewska, 2010). Thus, natural populations exhibit a number of responses to these pollutants, and the fastest reactions have been observed in bacteria and fungi, which can use diverse substances present in the environment as carbon and energy sources (Lew et al., 2011).

Few studies have evaluated the impact of pesticides on microorganisms in the biomixtures of a biopurification system. In this sense, Tortella et al., (2014, 2013a, 2013b) observed that after three successive applications of the pesticides atrazine, carbendazim, and diazinon in a biomixture, culturable fungi were reduced but rapidly recovered, without significant changes in culturable bacteria and actinobacteria populations compared to the uncontaminated control.

In the biomixtures formulated with the three types of soils, both bioaugmented and non-bioaugmented, there was a significant removal of lindane in the first 10 days of incubation. This may be attributable to the combined effect of microbial degradation and adsorption of the pesticide to soil particles and organic material. Adsorption is the first process that takes place when pesticides are in contact with a soil, affecting processes such as leaching, bioavailability or toxicity (Morillo and Villaverde, 2017). The most important soil parameters controlling pesticide adsorption, and hence bioavailability, involve organic matter, clay contents, pH, and water content (Cycoń et al., 2013). In this case, the oxidable organic matter was not so different among the three soils types, ranging from 1.0% to 1.3%, whereas the clay content was highly variable, between 2.5% and 62.5%, presenting completely different textures. There is evidence that soil texture influences the removal of a pesticide; water movement is controlled to a large extent by soil texture and susceptibility to leaching is typically associated with organic matter content, low water retention capacity and sandy texture (Fuentes et al., 2017). However, the contrasting characteristics of the soils may not be as relevant in a biopurification system as in the *in situ* soils systems due to the destructive mixing process and the inclusion of the other components during the formulation of the biomixture. In this regard, Fogg et al. (2004) demonstrated that the use of soils with different textures (sandy loam, clayey and clay silty) in the formulation of biomixtures did not affect the yield of the biobed for the removal of different pesticides, so it would be possible to use local soils in the construction process. Independently of the physicochemical characteristics of the soil, it represents 25% of the total mixture and is the main source of microorganisms. In this sense, the composition of the biomixture will determine the predominant microbial activity, that is, the amount, the activity and the genotypic and phenotypic versatility of the microorganisms responsible for the degradation of pesticides and their metabolites (Castillo et al., 2008). However, the participation of the soil native microbiota in the processes of removal and degradation of pesticides may be variable. For instance, Benimeli et al. (2008) did not find evident changes in lindane concentration in non-sterile soil samples, suggesting that the soil microorganisms were not involved in the pesticide removal. In contrast, Cycoń et al. (2013) evaluated the biodegradation of three organophosphorus pesticides in soils with different textures and observed that the autochthonous microbiota of each soil was capable of degrading the pesticides.

On the other hand, at 60 days of incubation, in the three types of soils, the highest percentages of lindane removal were observed in the bioaugmented systems. Other authors also found good performances of bioaugmentation in biomixtures. For instance, a biomixture inoculated with the iprodione-degrading *Arthrobacter* C1 strain showed faster dissipation of the pesticide compared to the non-bioaugmented biomixture (Campos et al., 2017). Also, Elgueta et al. (2016) reported higher atrazine degradation in a biomixture inoculated with the white-rot fungus *Anthracophyllum discolor* Sp4 CCCT 16.5 than in the non-inoculated one. However, the bioaugmentation not always favors the pesticide removal. This was observed in biomixtures formulated with soil, coconut fiber and compost, bioaugmented with *Trametes versicolor* ATCC 42.530. Despite the fungus was able to remove tebuconazole in a liquid system, the bioaugmentation failed to enhance the removal capacity of the fungicide of the biomixture, which could be ascribed to competition-related factors due to interactions with the matrix microbiota, which were absent in the liquid-phase systems, resulting in poor colonization of the matrix (Murillo-Zamora et al., 2017). Similarly, Rodríguez-Rodríguez et al. (2017) reported that the bioaugmentation of a biomixture with the ligninolytic fungus *Trametes versicolor* did not improve the pesticides dissipation; nonetheless it reduced the accumulation of transformation products, and hence, decreased the toxicity of the matrix along the process. Therefore, the obtained results at the present work are promising since they highlight the importance of using appropriate mixed microbial cultures for the bioaugmentation of biomixtures for the removal of recalcitrant pesticides.

In the present study, at the end of the incubation period, only biomixtures formulated with sandy and silty loam soils bioaugmented with the consortium presented higher pesticide removal percentages than non-bioaugmented biomixtures. Moreover, after the second application of lindane, the biomixtures formulated with clayey and sandy soils bioaugmented with the microbial consortium presented lower removal rates than in the first contamination. This is in agreement with the observations of Tortella et al. (2013a), who found that after the second application of atrazine in a biomixture formulated with soil, wheat straw, and peat, the degradation efficiency of the system decreased almost 20%. In this context, a possible strategy to increase the pesticides removal after successive applications could be a periodic re-inoculation of the biomixtures with degrading microorganisms or consortia, as demonstrated by Rodríguez-Rodríguez et al. (2014). In fact, one of the main drawbacks associated with bioaugmentation is the failure of the strains introduced to survive, since competition with the native microbiota could hinder the colonization of the contaminated matrix, thus affecting their metabolism, which may be finally reflected as a decrease in the number of exogenous microorganisms after the inoculation (Tortella et al., 2015).

Regarding the kinetic parameters of lindane removal, in all the

evaluated biomixtures, both inoculated and non-inoculated with the microbial mixed culture, the half-life of lindane ($t_{1/2}$) was much lower than 148 d, the $t_{1/2}$ of lindane in soils, as reported in the International Pesticide Properties Database FOOTPRINT (PPDB, 2017). This suggests that the biomixtures formulated with soils of diverse textures and agricultural economic byproducts, such as sugarcane bagasse, may be efficient on dissipating lindane from effluents contaminated with this kind of pesticide. Even more, after the first contamination of the biomixtures, in all cases, the $t_{1/2}$ of lindane was lower in the presence of the inoculum. This finding reinforces the hypothesis that the inoculated consortium would be actively involved in the removal or degradation of lindane, so this strategy would be beneficial when designing a biopurification system. According to these results, we suggest that the mixed culture composed of *Streptomyces* sp. A2, A5, A11 and M7 and the filamentous fungus *Trametes versicolor* S5NG1 has the ability to act in a complex biomixture of a biopurification system, decreasing the lindane concentration by 50% in an average of only 23 d, i.e. 6-fold less than the half-life reported for lindane in soils (PPDB, 2017). Similarly, other authors reported a considerable reduction in the $t_{1/2}$ of two organophosphorus pesticides in a biomixture inoculated with a *Streptomyces* mixed culture, compared to bioaugmented soils and to results obtained with the single pesticides (Briceño et al., 2017, 2016).

Nevertheless, after the second lindane application, only the biomixture formulated with silty loam soil bioaugmented with the mixed culture presented a significant reduction in the $t_{1/2}$ of lindane respect to the non-inoculated ones. It is possible that sandy or clayey textures may not have favored the microbial growth or metabolism of the inoculated consortium. In fact, soil does not always favor the optimal growth of fungi and bacteria or the efficient enzymatic activity that is required for the transformation of a contaminant. Sandy soils are usually permeable to air and water due to the large size of its pores, but the water holding and nutrient storage capacity is very low. Clay soils, meanwhile, have high water holding capacity, but aeration is usually poor. The larger surface area of clayey soils may favor the contact between microorganism and contaminant, which may improve contaminant biodegradation. However, organic pollutants such as organochlorine pesticides tend to adsorb onto clay particles, thus limiting their bioavailability and their biodegradation. On the opposite, loam and silty loam soils retain adequate amounts of water and they have no aeration difficulties (Fuentes et al., 2017). Besides, as mentioned before, adsorption greatly influences the percentages of lindane removal. In this case, it is also possible that sorption sites of the sandy or clayey soil may have been saturated after the first contamination with the pesticide. Considering that an ideal sorption process occurs when only one molecule of solute can occupy one sorption site forming a mono layer on the sorbent, once all available sorption sites have been occupied, sorption stops and no other layers are formed (León-Santiestebán et al., 2011).

The results obtained in the present work demonstrate that the biomixture formulated with silty loam soil, sugarcane bagasse, and peat, inoculated with an autochthonous microbial consortium could be used in biopurification systems for the treatment of agroindustrial effluents contaminated with organochlorine pesticides. However, the fact that the strategy of bioaugmentation of the biomixture for the enhancement of pesticides removal efficiency was more effective in one type of soil than in the others, or in the first contamination than in the second one, highlights the importance of a careful design and evaluation of biomixtures before their large-scale use. Besides, this is preliminary work and the information available is related to studies under laboratory conditions. Therefore, further studies are required to evaluate the use of the selected biomixture in field-scale treatments, assessing other operational parameters such as the concentration of inoculum used, reinoculation at different times and their effect on the populations, indigenous microorganisms, among others.

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