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60 Abstract

Background and aims: Plants and contaminant-degrading microbes are a suitable combination for the remediation of pesticides. The aim of this study was to evaluate the effectiveness of *Streptomyces* strains cultured with maize plants in relation to lindane removal.
Methods: Four *Streptomyces* strains were cultured and added as both single and mixed cultures, along with maize plants, to artificially polluted hydroponic systems and soils. The effectiveness of the resulting soil bioremediation was then evaluated through phytotoxicity testing using lettuce seedlings.
Results: In the hydroponic and soil experiments, similar levels of lindane removal were recorded in the inoculated and non-inoculated systems where maize plants were introduced. However, the vigor index (VI) of the maize plants was highest when grown in inoculated and artificially polluted soil. In the phytotoxicity assay, the VI of the lettuce seedlings increased with increasing bioremediation time for the soils, thus indicating the effectiveness of the process.
Conclusions: Similar levels of lindane removal were recorded in both inoculated and non-inoculated planted systems, indicating that pesticide removal was not significantly affected by the bacterial inoculant. However, inoculation an actinobacteria consortium led to an increase in the VI of the maize and protected the plants against the existing toxicity. Furthermore, maize plants may attenuate the transient toxic effects of microbial lindane degradation.

61 Keywords separated by ' - '

Lindane - *Streptomyces* strains - Maize plants - Phytoremediation - Phytotoxicity

62 Foot note information

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Electronic supplementary material

Supplementary Table 1
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Supplementary Table 2
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REGULAR ARTICLE

Lindane removal using *Streptomyces* strains and maize plants: a biological system for reducing pesticides in soils

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Introduction

Until the 1990s lindane [γ-hexachlorocyclohexane (γ-HCH)] was one of the most extensively applied organochlorine pesticides, used to control a wide range of agricultural, horticultural, and public health pests (El-Shahawi et al. 2010). Currently however its use has been restricted or even banned, since it is considered as one of the priority organic pollutants under the Stockholm Convention on persistent organic pollutants (Salam and Das 2012; Singh et al. 2011). In addition, the International Agency for Research on Cancer has classified lindane as a possible carcinogenic for animals (ATSDR 2011). Lindane therefore represents a serious risk since HCH residues are still found in the environment (Fuentes et al. 2010; Saadati et al. 2012).

Phytoremediation techniques, based on the interactions between plants and microorganisms, have been proposed as cost-effective and ecofriendly methods for cleaning up soils polluted with organochlorine pesticides (Becerra-Castro et al. 2013a; Gerhardt et al. 2009; San Miguel et al. 2013). Several studies have demonstrated enhanced dissipation of organochlorine pesticides at the root-soil interface (Becerra-Castro et al. 2013a, b; Gerhardt et al. 2009; Kidd et al. 2008, 2009). This rhizosphere effect is generally attributed to an increase in microbial activity caused by the release of plant root exudates [enzymes, amino acids, carbohydrates, low-molecular-mass carboxylic acids and phenolics; (Curl and Truelove 1986)]. Root exudates may induce the expression of genes encoding enzymes involved in the degradation process, increase contaminant bioavailability and/or promote degradation by plant enzymes (Gao et al. 2010; Gerhardt et al. 2009; Miya and Firestone 2001; Kidd et al. 2008; Schnoor et al. 1995). Although these processes occur naturally, they can also be optimized by deliberate manipulation of the soil using suitable combinations of plants and contaminant-degrading microbes (Gerhardt et al. 2009). For example, Kuiper et al. (2004) showed that grass species combined with a naphthalene-degrading microbe protected the grass seeds from the toxic effects of naphthalene, and the growing roots also allowed the naphthalene-degrading bacteria to penetrate sufficiently deeply into the soil, which would not have been possible in the absence of roots. Therefore, the development of phytoremediation systems where microorganisms interact with plants is being increasingly considered as an option for dealing with the inherent weaknesses related to application of isolated elements (microbial degraders or plant species) (Fester et al. 2014).

While complete mineralization of pesticides or their transformation into non-toxic products is desirable, consortia of microorganisms may perform this task better than single isolates, probably because of their metabolic diversity (Yang et al. 2010). In a phytoremediation context, consortia can provide multiple benefits to plants, including the synthesis of protective compounds, chelators for delivering key plant nutrients, and degradation of contaminants before they can negatively impact the plants (Gerhardt et al. 2009). In summary, the introduction of a consortium of microbial degraders into polluted and planted systems may represent an important development in relation to ecological approaches for soil remediation (Fester et al. 2014).

Many microorganisms have been isolated and studied, both as pure and mixed cultures, for their capacity to degrade HCH-isomers (Fuentes et al. 2010; Kidd et al. 2008; Lal et al. 2010). The genus *Streptomyces*, which represents the main group of actinobacteria present in soils and sediments, has shown great potential for bioremediation of toxic organic and inorganic compounds, since these bacteria are already adapted to these types of habitats (Alvarez et al. 2012; Polti et al. 2007, 2011, 2014). In addition to their potential metabolic diversity, *Streptomyces* strains may be especially well suited for soil inoculation as a consequence of their mycelial growth habit, relatively rapid growth rates, ability to colonize semi-selective substrates, and ability to be genetically manipulated (Shelton et al. 1996). Several studies have been successfully conducted on the ability of *Streptomyces* strains to degrade organochlorine pesticides (lindane, chlordane, and metoxychlor) (Benimeli et al. 2007, 2008; Cuozzo et al. 2012; Fuentes et al. 2010, 2011, 2013; Saez et al. 2012, 2014).

On the other hand, plants tolerant to HCHs have been reported in a variety of studies in which researchers have evaluated either plant species that spontaneously grow in polluted areas (Becerra-Castro et al. 2013a, b; Kidd et al. 2009) or crop plants (Calvelo Pereira et al. 2006; San Miguel et al. 2013). For example, maize plants can cope with high environmental levels of organochlorine pesticide pollution, and as a result of this, successful applications of this species for remediation of xenobiotics have been reported (Blondel et al. 2014; Gao et al. 2010; Luo et al. 2006).

In a previous study conducted by our research group, four lindane-degrading *Streptomyces* strains were

shown to be able to grow and remove lindane in the presence of maize root exudates (Alvarez et al. 2012), leading us to hypothesize that lindane removal may be improved by the combined presence of *Streptomyces* strains and maize plants. In this context, the main objective of the present work has been to evaluate the effectiveness for lindane removal of inoculation with these four *Streptomyces* strains, both as single and mixed cultures, combined with maize plants. Since sometimes certain byproducts of the biodegradation process can themselves be toxic, the success of this intervention was also evaluated by a phytotoxicity test.

Materials and methods

Chemicals

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

Microorganisms

Streptomyces strains M7, A5, A2, and A11 were previously isolated from sediment and soil samples contaminated with several organochlorine pesticides (OPs) (Benimeli et al. 2003; Fuentes et al. 2010). Taxonomic identification of these strains has been confirmed by amplification and partial sequencing of their 16S rDNA genes [GenBank IDs: AY45953 (M7) (Benimeli et al. 2007), GQ867055 (A11), GQ867050 (A5), GU085103 (A2) (Fuentes et al. 2010)].

Soils and culture media and solutions

Depending upon the objectives of the experiment, bacterial strains and/or maize plants were cultured in one of the following media, all of which were sterilized by autoclaving at 121 °C for 20 min:

Starch-Casein agar (SC agar), containing (g l^{-1}): starch, 10.0; casein, 1.0; K_2HPO_4 0.5; agar, 12.0. This medium was used to prepare spore suspensions (inoculum for the hydroponic assay) and for CFU counts (soil experiment). In the latter case, nalidixic acid and cycloheximide (10 μg l^{-1}) were added to the SC agar.

Minimum medium (MM) containing (g l^{-1}): $(NH_4)_2SO_4$ 4.0; K_2HPO_4 0.5; $MgSO_4$ $7H_2O$ 0.2; $FeSO_4$ $7H_2O$, 0.01, pH 7 (Amoroso et al. 1998). This was used in the hydroponic experiment in which maize plants and single cultures of *Streptomyces* strains were grown.

Tryptone Soy Broth (TSB) containing (g l^{-1}): tryptone, 15.0; soy peptone, 3.0; NaCl, 5.0; K_2HPO_4, 2.5; glucose, 2.5, pH 7.3. This was used for preparing microbial inocula (pre-cultures for the soil experiment).

Nutrient solution containing: KNO_3 1.5 mM; $Ca(NO_3)_2$ 1 mM; $MgSO_4$ 0.5 mM; $NH_4H_2PO_4$ 0.25 mM; EDTA-Fe 11.9 μM; H_3BO_3 11.5 μM; $MnSO_4$ 1.25 μM; $ZnSO_4$ 0.2 μM; $CuSO_4$ 0.075 μM; $(NH_4)_6Mo_7O_{24}$ 0.025 μM, pH 5.8. This was used to cultivate maize plants in order to obtain their root exudates (REs) (Luo et al. 2006).

The soil used was free of pesticide contamination, collected from an urban area in the northwestern region of Argentina. Prior to use the soil was air-dried, lightly ground using a mortar-and-pestle, and finally sieved through a 1-mm sieve. The soil samples were then sterilized (three successive sterilizations at 121 °C for 15 min each, with a 24 h pause in between) (Fuentes et al. 2013).

Experimental design with single cultures and maize plants grown hydroponically

Streptomyces strains were cultured on SC agar plates at 30 °C until sporulation (approximately 7 days). Spores were then scraped from the surface of the plates and washed twice with sterile distilled water. Spores from each strain (harvested by centrifugation at 9,000 g for 10 min) were suspended in an equal volume of sterile distilled water to prepare the spore suspensions (Amoroso et al. 1998; Hopwood et al. 1985), which were individually inoculated (150 μl) in glass tubes (40 cm deep×3 cm diameter), filled with 20 ml of MM. A stock solution of lindane dissolved in acetone (10 mg ml^{-1}) was filter-sterilized (0.22 μm Millipore) and then added to the tubes to reach a final concentration of 2 mg l^{-1}. The amount added was below lindane solubility in water (Xiao et al. 2004). The test tubes were left uncapped for 2 h to allow evaporation of the

acetone prior to the microbial inoculation described below.

Endophyte-free maize (*Zea mays*) seeds not treated with fungicide [donated by Estación Experimental Agroindustrial Obispo Colombres (EEAOC) Tucumán, Argentina] were first surface sterilized using 2 % mercuric chloride (Benimeli et al. 2008). The seeds were then placed into sterile Petri dishes with filter paper (Wattman No. 1) moistened with sterile distilled water, until germination. One seedling was then transferred to each tube and the tube was then sealed. The various treatments used were as follows: 1) *Streptomyces* strain (M7, A5, A2, or A11)-lindane-plant; 2) *Streptomyces* strain-plant; 3) *Streptomyces* strain-lindane; and 4) plant-lindane. Polluted, non-inoculated, and no-plant tubes (lindane only) were also used as controls. Each treatment was repeated five times.

In order to allow oxygenation of the culture and promote better *Streptomyces* growth, the test tubes were incubated under gentle agitation (100 rpm) at 30 °C for 10 days. Tubes were then centrifuged (9,000 g, 10 min, 4 °C) and the culture supernatants were used to determine residual lindane by gas chromatography (GC). The microbial biomass was also estimated after centrifugation by washing the pellets with sterile distilled water and drying to constant weight at 105 °C. To calculate microbial biomass, the amount of non-soluble residue (probably root debris) existing in the non-inoculated tubes (plant-lindane treatment) was subtracted from the non-soluble residue obtained in the inoculated tubes.

An attempt was also made to detect residual lindane in the plant tissues. For this purpose, at the end of the assay plants grown in polluted but non-inoculated tubes (plant-lindane treatment) were harvested and processed to extract lindane as detailed below.

Collection and analysis of root exudates

Several studies have demonstrated that plant REs may stimulate microbial growth and contribute to pesticide dissipation at the root-soil interface (Gao et al. 2010; Gerhardt et al. 2009; Kidd et al. 2008; Luo et al. 2006). In this context, maize REs were obtained and analyzed in order to detect their main components. Groups of twenty maize seedlings, germinated as described above, were aseptically transferred to flasks where they were grown in 200 ml of nutrient solution under sterile conditions, in a climate controlled room (25 °C, 16:8 light:dark, 65 % relative humidity). The nutrient solution in the culture flasks was replaced every 2 days for 2 weeks, with the exiting solution being collected and stored at 4 °C. The solution collected from each flask was used as the source of REs (adapted from Luo et al. 2006). These exudates were lyophilized, then diluted in an appropriate volume of sterile distilled water, then filter sterilized (0.22 μm Millipore).

The protein concentration was determined according to the method described by Bradford (1976). For this purpose, 1 ml of Coomassie Blue G-250 reagent was added to 100 μl of REs. Each sample was held for 10 min at room temperature and the protein concentration was then estimated at 595 nm. The standard used was Bovine Serum Albumin (BSA, Sigma-Aldrich A2153).

Carbohydrates were determined by the dinitrosalicylic acid (DNS) method described by Miller (1959) and modified as follows: 500 μl of sample and 750 μl of 1 % DNS (dissolved in 6 % NaOH) were mixed and incubated for 10 min in a boiling water bath. Absorbance was then measured at 590 nm, using D-(+)-Glucose (Sigma, G8270) as the standard.

Total phenolic compounds were determined by the Folin-Ciocalteu (FC) method, as described by Singleton et al. (1999). To 1 ml of sample, 6 ml of distilled water and 5 ml of FC reactive were added. The sample was then held for 30 s at room temperature and then 15 ml of Na_2CO_3 were added to the mixture. Distilled water was added to reach the final volume of the reaction (100 ml). The mixture was incubated for 2 h at 22 °C and absorbance was then determined at 590 nm. Gallic acid (Sigma-Aldrich, 27645) was used as the standard, so the determined values were expressed as gallic acid equivalents ml^{-1} (GAE ml^{-1}).

Specific dechlorinase activity (SDA) was indirectly determined in the REs using a colorimetric assay adapted from Phillips et al. (2001). Phenol red sodium salt (Sigma-Aldrich, P4758) was added to the sample as a pH indicator at a ratio of 1/10, with the change in color from red through orange to yellow in the presence of chlorides ions therefore being indicative of lindane dechlorination. Chloride ion concentrations were determined colorimetrically at 540 nm based on a calibration curve produced using standard HCl solutions. One enzymatic unit was defined as the amount of chloride ions released (in micromoles) in 1 h (EU = μmol $Cl^- h^{-1}$), and the SDA was defined as EU per milligram of protein.

Root exudates were also mixed with a hydrocarbon (kerosene) (1:1) by vortexing for 2 min and being left to

settle for 24 h. The emulsification index (EI_{24}) was calculated as the percentage of the height of the emulsified layer (mm) related to the total height of the liquid column (mm) (Cooper and Goldenberg 1987). Distilled water mixed with kerosene was used as a control.

Experimental design for mixed culture and maize plants grown in soil

Glass pots (10 cm deep×7.5 cm diameter) were filled with 300 g of soil at 20 % moisture and spiked with a stock solution of lindane (prepared as described above) to reach a final concentration of 2 mg kg^{-1}. Lindane was dissolved into the water added to the pots to reach 20 % soil moisture (Fuentes et al. 2011). The soils were then mixed thoroughly and the pots were left uncovered for 12 h to allow evaporation of the solvent used to produce the lindane stock solution. The *Streptomyces* strains were individually cultured in TSB as described above. Cells of each strain (harvested by centrifugation, 9,000 g, 10 min, 4 °C) were washed twice with sterile distilled water and then combined in equal proportions to produce a mixed culture for use as inoculum (final concentration: 2 g kg^{-1}). The rationale for using a consortium of 4 strains belonging to the same genus is based on the fact that this consortium has previously shown a lindane removal ability of 62 %, compared with 23–37 % lindane removal by single cultures of the 4 strains. In addition, the consortium presented specific dechlorinase activity (SDA) five times higher than the average SDA for the single cultures (Fuentes et al. 2011). Other recent studies have also demonstrated that the 4 strains present different molecular and physiologic characteristics among them (Saez 2015).

The soil, inoculum, and lindane were mixed together thoroughly to ensure uniform distribution. The previously germinated maize plants were then planted one per pot, prepared according to the various treatments: 1) lindane-plant-consortium; 2) plant-consortium (no lindane); 3) lindane-consortium (no plant); and 4) lindane-plant (no consortium). Unpolluted, non-inoculated, and planted soils (soil and plant only) as well as unpolluted, non-inoculated, and no-plant soil pots (soil only) were used as controls. For each treatment, 3 replicates were prepared and the whole experiment was repeated twice. The pots were incubated in a greenhouse at 30 °C for 21 days. Every 2 days the planted and unplanted pots were irrigated with nutrient solution. Soil samples were taken at 0, 7, 14, and 21 days for determining microbial growth (CFU g^{-1}) and residual lindane concentration and for ecotoxicity testing (detailed below). For CFU counting, plates containing SC medium supplemented with antibiotics (as described above) were incubated at 30 °C for 7 days. *Streptomyces* strains grown on SC medium were recognized based upon their color and colony morphology, the presence of diffusible pigments, and their tough appearance with leathery characteristics typical of vegetative actinobacteria mycelium (Fuentes et al. 2010; Lechevalier 1989). It is important to note that since the soil was first sterilized and since the SC-agar plates used for the CFU count were supplemented with antibiotics, only rarely did colonies grow other than the inoculated *Streptomyces* strains.

Maize plants were harvested at the end of the assay to determine the length of the shoots and roots (Calvelo Pereira et al. 2008) using a millimeter scale. The vigor index (VI) for the plants was also calculated using the following formula: VI=(SL+RL)×G/10, where SL is the average shoot length, RL is the average root length, and G is the percentage of seed germination (Bidlan and Afsar 2004).

Phytotoxicity test

In order to evaluate the effectiveness of the bioremediation process, a toxicity test was performed using lettuce seeds (*Lactuca sativa*). Lettuce was selected as a bioindicator because it has been proven to be highly sensitive to HCHs (Calvelo Pereira et al. 2008). The seeds [donated by Estación Experimental Agroindustrial Obispo Colombres (EEAOC) Tucumán, Argentina] were rinsed twice with distilled water and then grown in Petri dishes containing 2 g of bioremediated soil, for 0, 7, 14, and 21 days. Thirty seeds were placed into each plate and incubated in the dark for 5 days at controlled environmental conditions (22 °C, 70 % relative humidity) (U.S. E.P.A. 1996). At the end of the incubation period, the percentage of germinated plants and the length of each one germinated (root plus shoots) were determined, in order to calculate the vigor index (VI) of the seedlings. Sterile distilled water was used as a control to monitor the germination rate and the repeatability of this test. There were only small variations in average length of the lettuce seedlings in the water control (data not shown).

Lindane determinations

Residual lindane in the liquid phase of the hydroponic systems (9,000 g, 10 min, 4 °C) was extracted by solid-phase extraction by using a C18 column (Varian, Lake Forest, USA). The procedure for lindane residue extraction from plant tissues was performed as follows. Plants grown hydroponically in non-inoculated, lindane-polluted tubes were weighed, cut into small pieces, and frozen overnight at −20 °C. The samples were then thoroughly blended to achieve maximum homogeneity, and then processed with the Agilent Sampling QuEChERS AOAC kit to extract the pesticide.

The procedure for extracting lindane residues from soil was performed according to Quintero et al. (2005). Aliquots of 5 g of dry homogenized soil were transferred to centrifuge tubes and mixed with 10 ml of water/methanol/hexane (4/1/5). The tubes were then hermetically sealed, shaken for 10 min on a vortex, and centrifuged (9,000 g, 10 min, 4 °C). The organic phase was collected and evaporated and the residues were re-suspended in hexane for analysis by GC.

Finally, lindane concentrations in the hydroponic systems and plant and soil extracts were quantified using an Agilent 7890A Gas Chromatograph equipped with a HP5 capillary column (30 m×0.53 mm×0.35 m) and a ^{63}Ni μECD detector, 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}, then increasing to 290 °C at 20 °C min^{-1}, detector temperature: 320 °C, and injection volume: 1 μl. Quantitative sample analysis was performed using appropriate calibration standards (AccuStandard).

Statistical analysis

One-way analysis of variance (ANOVA) was used to test for significant differences among the treatments regarding microbial growth and lindane removal in the hydroponic and soil-based experiments. Nested-ANOVA was carried out to test for significant differences in germination, shoot/root length, and VIs of lettuce seedlings. When significant differences were found, Tukey's post-test was used to separate the effects among treatments. Tests were considered significantly different at $p<0.05$. These analyses were performed using professional versions of Infostat and Statistica 6.0 software.

Results

Streptomyces single cultures and maize plants grown in hydroponics

Streptomyces strains were able to grow in liquid media with maize plants as sole carbon source. Values for microbial growth (g l^{-1}) ranged between 0.55 and 0.31, depending upon the strain ($p<0.05$) (Fig. 1a–d). Maximum microbial growth values were recorded with *Streptomyces* M7 (0.55±0.06) and *Streptomyces* A11 (0.51±0.01) ($p>0.05$) (Fig. 1a and d).

In treatments with lindane as the sole carbon source, *Streptomyces* A5, A2, and M7 showed higher but generally similar growth (0.54±0.09, 0.50±0.01, and 0.48±0.01 g l^{-1}, respectively) ($p>0.05$) (Fig. 1a–c) compared to the lowest growth as exhibited by *Streptomyces* A11 (0.32±0.01 g l^{-1}) ($p<0.05$) (Fig. 1d). In contrast, when the strains were cultured on lindane-polluted media including a maize plant, *Streptomyces* A11 achieved the highest growth (0.77±0.01 g l^{-1}, Fig. 1d) ($p<0.05$) while the other strains presented lower but similar growth levels: 0.41±0.01 (*Streptomyces* M7); 0.35±0.01 (*Streptomyces* A5); and 0.34±0.01 g l^{-1} (*Streptomyces* A2) ($p>0.05$) (Fig. 1a–c).

There was evidence for lindane removal, calculated as the percentage of the initial lindane minus the percentage of residual lindane, in all of the treatments (Fig. 1a–d), although the highest values were obtained in the plant-microbe system. Lindane removal ranged between 94.1 % and 81.5 %, depending upon the strain (Fig. 1a–d) ($p>0.05$). On the other hand, lindane removal levels by *Streptomyces* strains in the absence of the maize plant were significantly lower (between 45.3±7.2 for *Streptomyces* M7 and 15.6±0.7 % for *Streptomyces* A11) than the lindane removal levels seen for the plant-microbe system reported above ($p<0.05$).

When maize plants were grown in the absence of the *Streptomyces* strains, pesticide removal levels were similar to those seen in the plant-microbe system ($p>0.05$), reaching 88.6±4.1 % (Fig. 1a–d). The concentration of lindane detected in macerated tissues of these plants was 16.65 mg kg^{-1}.

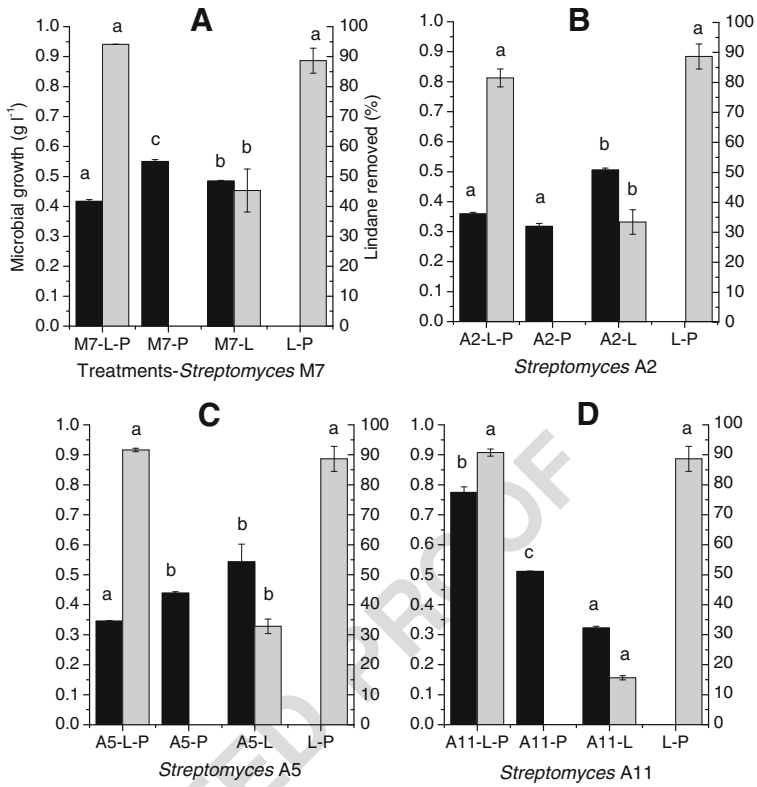

Fig. 1 Growth (■) and lindane removed (▒) by *Streptomyces* strains and maize plants cultured hydroponically. *L* lindane, *P* plant (maize). Bars showing different letters indicate they were significantly different (comparing among strains) ($p<0.05$, Tukey's post-test)

Physico-chemical characteristics of maize root exudates

The pH of the REs solution was 7.3. The content of carbohydrates, total proteins, and phenolic compounds in the REs were: 0.92±0.01 g l^{-1}, 238.00±1.83 μg ml^{-1}, and 0.32±0.02 mg EAG ml^{-1}, respectively. Specific dechlorinase activity was 12.80±0.32 μmol Cl^- h^{-1}. The emulsification index (EI_{24}) of the REs was 46.5± 9.5 %.

Streptomyces consortium and maize plants grown in soil

The bacterial consortium was able to grow in all three treatments assayed (Fig. 2), although differences in the growth values were recorded among the treatments and the incubation periods. After 7 days the consortium in the lindane-plant-consortium treatment showed higher growth than in the lindane-consortium treatment ($1.05\times10^6\pm1.55\times10^5$ versus $2.95\times10^5\pm2.04\times10^4$ CFU g^{-1}) ($p<0.05$), while microbial growth in the plant-consortium treatment was intermediate ($5.95\times10^5\pm5.30\times10^4$ CFU g^{-1}) in relation to those two treatments involving the artificially polluted soils ($p>0.05$).

On the other hand, after 14 days the microbial growth was quite similar among the treatments ($p>0.05$) (Fig. 2). At 21 days, microbial growth showed about the same value in the lindane-plant-consortium treatment ($2.00\times10^7\pm1.85\times10^6$ CFU g^{-1}) as in the lindane-consortium treatment ($1.93\times10^7\pm1.22\times10^5$ CFU g^{-1}), while microbial growth in the plant-consortium treatment was slightly lower ($1.01\times10^7\pm4.50\times10^5$ CFU g^{-1}) ($p>0.05$). No significant microbial growth was recorded in the control soils that were not inoculated, regardless of whether or not these were lindane-polluted or whether they contained plants.

Lindane removal was recorded at 0, 7, 14, and 21 days for soils assayed with the following treatments: lindane-plant-consortium; lindane-plant; and lindane-consortium. There was evidence for pesticide removal in all of these treatments (Fig. 2), although this process was variable among them over time. After 7 days significant lindane removal was recorded in the lindane-plant-consortium treatment as well as in the lindane-plant treatment, with these levels then reaching 17.6± 2.9 % (lindane-plant-consortium) and 39.6±1.3 % (lindane-plant) lindane removal after 14 days ($p<0.05$). At the end of the experiment after 21 days

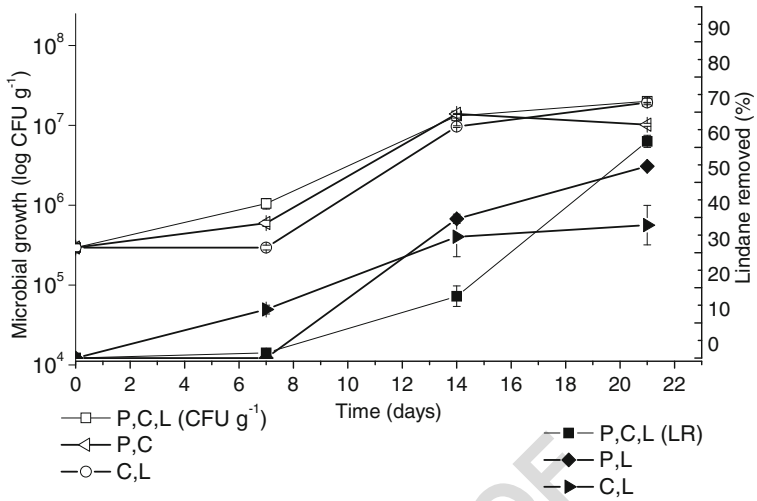

Fig. 2 Proliferation of inoculated *Streptomyces* strains (CFU g^{-1}) and lindane removed (%) in soil. *P* plant (maize), *C* consortium, *L* lindane, *LR* lindane removed. Statistically significant differences among treatments are showed in the results section ($p<0.05$, Tukey's post-test)

the highest value of pesticide removed (61.6±1.7 %) was obtained in the lindane-plant-consortium treatment, while lindane removal in the lindane-plant treatment was 54.5±1.3 % ($p>0.05$). On the other hand, the bioremediation process in the lindane-consortium treatment (with no plant) was in evidence from the beginning of the experiment, reaching 13.7±1.2 % pesticide removal after 7 days, 34.5±5.7 % at 14 days, and 37.8±5.6 % after 21 days. The lindane removal obtained in this treatment at the end of the experiment was different at the statistical level ($p<0.05$) to those registered for the same time period in the other treatments (soils with plant). Significant lindane removal was not detected in the control soil (non-inoculated and with no plant, data not shown).

The vigor index of the maize plants assessed in the various soils was as follows: lindane-plant-consortium treatment: 168.2±7.5; plant-consortium treatment: 187.1±19.9; lindane-plant treatment: 105.2±9.5; and plant-only treatment (control soil with no lindane pollution and no inoculation): 188.8±10.0. The vigor index of plants grown in the lindane-plant treatment (105.2±9.5) was different at a statistical level ($p<0.05$) from the VIs obtained in the other treatments. Shoot and root lengths are shown in Supplementary Table 1.

Phytotoxicity test

The effectiveness of the bioremediation process was evaluated using a phytotoxicity test. For this purpose lettuce seeds were cultured in soils that had been previously bioremediated. In most cases the number of germinated seeds and the length of each seedling (Supplementary Table 2), and therefore the VI (Fig. 3), were enhanced with increasing bioremediation time. An exception was recorded in lettuce grown in artificially polluted soil treated with the consortium (lindane-consortium treatment), where the VI was lower after 14 days (28.5±1.8) than after 7 days (43.0±7.5) under the same culture conditions. The tested seeds were found to be adversely affected by growing in non-remediated soil (control soil: lindane-polluted, no maize plant, and non-inoculated), since the VI of seedlings was in this case the lowest (8.5±0).

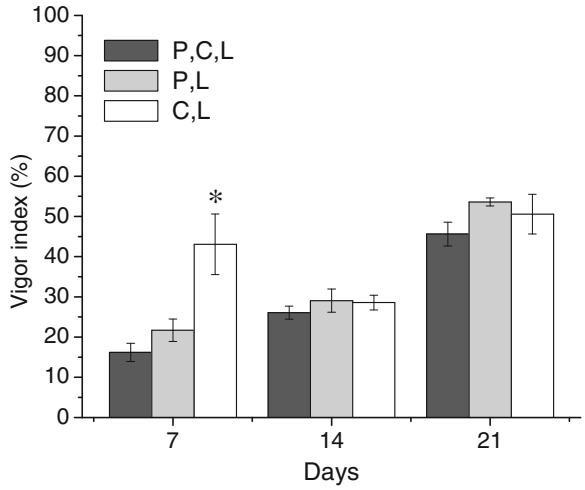

Fig. 3 Vigor index (VI) of lettuce seedlings cultivated in bioremediated soils with differing incubation times. (VI=seedling length×percentage of germination/10). Asterisk indicates significant differences among experiments ($p<0.05$, Tukey's post-test)

Discussion

During plant growth, roots release a range of organic compounds that can potentially enhance the biodegradation of xenobiotics in a variety of ways, including by stimulation of bacterial growth (provided that these bacteria have the appropriate metabolic abilities). In the present study, four native *Streptomyces* strains were found to be able to grow on liquid media with maize plants as the sole carbon source, confirming that the maize plants, and/or the REs released by them, represent a viable carbon and energy source for these strains. Also as part of the present study, carbohydrates, proteins, and phenolic compounds were detected in the collected REs of the maize plants. Moreover, in a previous study it has been shown that *Streptomyces* M7, A5, and A11 were able to grow in MM with maize REs added as the sole carbon source (Alvarez et al. 2012). In view of these results, it may be possible that the studied actinobacteria were competitive at the maize rhizosphere level. Although the complex interactions of the natural systems existing in the rhizosphere are not detectable in in vitro assays, these observable results should be interpreted as a subset of the processes that could happen in nature (Bais et al. 2006). Despite of this, hydroponics-based experiments, where the pollutant is more bioavailable (Schwitzguébel et al. 2006), seem to be favorable for initial studies related to plant-microbe behavior in polluted systems.

In experiments with lindane as the sole carbon source, *Streptomyces* M7, A5, and A2 showed significantly higher growth compared to the lowest growth level as exhibited by *Streptomyces* A11. This ability to grow in the presence of lindane may be due to selective evolutionary pressure that would have been exerted by the environment in which the microorganisms were isolated, leading to acquisition of metabolic capabilities to survive in polluted environments (Fuentes et al. 2013). It is important to note that Benimeli et al. (2003) and Fuentes et al. (2011) reported for the first time the ability of these actinobacteria to grow using lindane and other OPs as their sole carbon source.

Lindane removal was evidenced in all of the treatments studied here, but the highest value was obtained in the plant-microbe system. In agreement with other studies (Becerra-Castro et al. 2013a, b; Kidd et al. 2008; Schwitzguébel et al. 2006), these results suggest that the most viable approach for remediation of lindane will be based on rhizodegradation techniques. However, it must also be noted that the percentage of pesticide removal obtained by the plant alone (~88 %, in the absence of actinobacteria) was not significantly lower, strongly suggesting that the presence of the plant significantly influences the remediation process even without significant microbial involvement. Since hydrophobic chemicals such as lindane (log K_{ow} 3.72) (Schwitzguébel et al. 2006) tend to sorb to organic material (Rodríguez Garrido 2009), the lindane could have been retained at the root level and hence the presence of the plant significantly influenced the remediation process. In fact, 16.65 mg kg^{-1} of lindane was detected in the tissues of plants growing in non-inoculated MM. This value could be considered high, since it represents 53.2 % of the total lindane added to the medium (31.25 mg kg^{-1} of plant). Retention of lindane and other organic contaminants by plants has been also suggested by other authors. For instance, Becerra-Castro et al. (2013a) compared HCH removal in substrates planted with *Cytisus striatus* and either inoculated or not inoculated with *Rhodococcus erythropolis* ET54b and *Sphingomonas* sp. D4. They reported that planted substrates showed a higher removal of lindane compared to substrates that were both planted and inoculated.

Exudates released by plants may contain enzymes that potentially enhance biodegradation (Gao et al. 2010; Van Aken et al. 2010), and exudation of surfactant-type compounds through the roots would also be associated with this process (Becerra-Castro et al. 2013a). In view of this, it cannot be ruled out that part of the lindane removal detected in experiments with plants could be due to their own enzymatic activity. Besides having proven to be a rich carbon source, maize REs were shown to have enzymatic activity. This activity could partially explain the high lindane removal values detected in our plant-microbe and lindane-plant systems. Since each lindane molecule has six chlorine atoms, dechlorination is a very significant step in its degradation, and this is the reason for the importance of the presence of enzymes released by the roots and able to dechlorinate organohalogenated compounds. Calvelo Pereira et al. (2006) found that enzymes secreted by *Avena sativa* and *Cytisus striatus* were able to reduce HCH levels in rhizospheric soils. Other researchers have also reported dechlorination of polychlorinated biphenyls by crude nitrate reductase extracts from *Medicago sativa* and by a pure commercial nitrate reductase from maize (Magee et al. 2008).

The pesticide removal percentages achieved by *Streptomyces* strains grown with lindane (in the absence of the maize plant) were similar to those reported by Fuentes et al. (2011) for these actinobacteria (~37–23 % of pesticide removal, depending upon the strain). It is possible that a fraction of the lindane removal was due to the action of microbial dechlorinase enzymes whose activity was previously demonstrated by Cuozzo et al. (2009) and Fuentes et al. (2011). On the other hand, lindane tends to sorb to organic material because of the physico-chemical characteristics of HCH isomers, so a decrease in the pollutant by adsorption to microbial biomass also cannot be ruled out.

As was expected, in the soil experiments, the actinobacterial consortium was able to grow under the assayed conditions, with its biomass increasing along with the incubation time. Moreover, at the end of the experiment no significant differences in microbial growth were found among the treatments, confirming the ability of the actinobacteria to grow in all of the soils. Regarding the bio-phytoremediation process, the dynamics of pesticide removal differed both among the treatments and over time. In the lindane-plant-consortium and lindane-plant systems, there was evidence of pesticide removal after 7 days, increasing along with increased culture time, which was probably due to the gain of plant biomass. In relation to this, Luo et al. (2006) investigated desorption of DDT by soil washing experiments with REs of maize, rye-grass (*Lolium perenne*), and wheat (*Triticum aestivum*). The authors reported that pesticide desorption increased as the biomass of the tested plants increased (corn > wheat > rye-grass). Also, Chaudhry et al. (2005) concluded that a plant's rate of exudation changes with age, and therefore so does the effect on pesticide removal. There are several mechanisms by which the maize plants and/or the REs could have contributed to decreases in lindane such as enzymatic activities and/or adsorption phenomena, as well as the effects of surfactant-type compounds. In regard to this last mechanism, emulsifier properties were detected in maize REs, and these could be related to a slight tendency towards increased lindane removal in the lindane-plant-consortium treatment, probably due to an increase in the pollutant bioavailability. On the other hand, pesticide removal in the consortium-lindane treatment was clearly evidenced from the beginning of the experiment. Fuentes et al. (2011) found similar results, since 31.5 % lindane removal was detected after 21 days in soil treated with the same consortium of actinobacteria.

It is noteworthy that there was not a direct relationship between bacterial counts and pesticide removal over the course of the assay (i.e., more biomass did not necessarily lead to more lindane removal). This was especially true in soils with maize plants during the first and last weeks of the experiment. It may be the case that in the presence of abundant carbon sources, other than the pesticide, the strains prefer to start growing using REs and/or soil organic matter instead of lindane.

Optimal plant growth is known to be a critical factor that directly affects phytoremediation, influencing plant performance, bacterial colonization, and rhizodegradation efficiency (Afzal et al. 2011). As in this study, plant root and shoot elongation rates have frequently been used for a quick evaluation of phytotoxicity for soils and/or hydroponic systems (Calvelo Pereira et al. 2008). Plants grown in the present study in non-inoculated soils showed signs of stress (lowest growth and VI) caused by the presence of the pesticide, and the phytotoxicity of HCH has been previously observed by other authors (Abhilash and Singh 2010; Calvelo Pereira et al. 2010). However, inoculation of maize plants with the actinobacteria consortium led to an improvement in plant development, reflected through an increase in their VI. Therefore, this bacterial inoculant seems to represent a mutualistic association with high promise for developing phytoremediation strategies aimed at the clean-up of lindane-contaminated sites.

Phytotoxicity tests are versatile tools for monitoring the success of a remediation process (Chiochetta et al. 2013), and in the present study lettuce seeds were therefore cultured in soil samples previously bioremediated under different conditions and with varying incubation times. The VIs of lettuce cultured in soils that had been bioremediated both with and without maize plants increased with increasing bioremediation time, suggesting the success of the bioremediation, i.e., a decrease of soil toxicity through a biological process taking place over time. The only exception to this trend was observed in the VI of lettuce grown in soil that was inoculated but without a maize plant and with remediation for 14 days (VI=28.5), since the VI recorded for lettuce seedlings cultured in soil treated under the same condition but for 7 days was higher (VI=43.0). Considering that a pronounced increase in lindane removal was recorded on these soils with no maize plant (between 7 and 14 days; Fig. 2), the low rate of lettuce development in the 14-day soil may be interpreted as an indication that lindane might be transformed into toxic intermediates during

microbial degradation. In fact, Fuentes et al. (2013) and Saez et al. (2014) also proposed the production of toxic intermediates by these and other actinobacteria when they were cultured in soils and slurries polluted with lindane, chlordane, and metoxychlor. Several studies have described the production of chlorobenzenes (CBs) by microorganisms during anaerobic degradation of HCH and, in a few instances, during the aerobic pathway (Lal et al. 2010). In the most frequently studied pathways, a series of dechlorination steps occur and these produce trichlorobenzene (TCB) and dichlorobenzene (DCB) as stable and toxic end products that may accumulate in the environment (Fathepure et al. 1988). However, when lettuce seeds were cultured in soil that had been bioremediated for a total of 21 days, with inoculation but without a maize plant, the VI increased from 28.5 for the 14-day sample to 50.5 for the 21-day sample. Since microbial consortia can work in tandem to effectively degrade organic contaminants (Yateem et al. 2007), it could be hypothesized that one or more strains of the consortium consumed these toxic metabolites. As was mentioned before, numerous authors have reported that a decrease in a pollutant may sometimes be accompanied by transformation of the original contaminant into more toxic metabolites that could persist in soil and produce toxicity on certain species (Calvelo Pereira et al. 2010; Fuentes et al. 2013; Saez et al. 2014). The results presented here are therefore very relevant, since toxic metabolites that would have been produced during microbial degradation would have been consumed relatively quickly, and therefore soil toxicity decreased. Nevertheless, it cannot be ruled out that potentially toxic metabolites may have simply evaporated.

The VIs of seedlings cultured on bioremediated soils that included a maize plant (both inoculated and non-inoculated) increased with increasing bioremediation time. It is important to note that the VIs did not decrease at any stage of the assays, despite the fact that lindane removal in these planted soils progressively increased from the 7th to the 21st day. Taking into account our hypothesis regarding the production of toxic intermediaries by microbial degradation, these results suggest that maize plants would mitigate their negative effects on lettuce development by different mechanisms (such as transformation of lindane by extracellular plant enzymes or by root-bound enzymes). For example, Chaudhry et al. (2005) found that maize plants exposed to atrazine dissipated about 20 % of the contaminant, while simultaneously accumulating desethyl-atrazine (DEA), a toxic compound produced by phytodegradation of atrazine. The authors showed that after 15 days, 80 % of the DEA was dissipated by dechlorinase enzymes produced by the plant itself. In summary, more research is needed in order to understand metabolic transformation of lindane and also to determine whether the products produced by microbial degradation are more or less toxic in the environment than the original free chemical.

Conclusions

The levels of lindane removal recorded were similar in both inoculated and non-inoculated systems with maize plants included. However, inoculation of systems with maize plants with an actinobacteria consortium led to an increase in VI and protected the plants against the toxic. This bacterial inoculant seems to represent a mutualistic association with high promise for developing phytoremediation strategies aimed at the clean-up of lindane-contaminated sites.

The results of our phytotoxicity test were interpreted as indicating that lindane may have been transformed into toxic intermediates during microbial degradation. Nevertheless, the results presented in this study provide evidence that maize plants may attenuate or suppress the transient toxic effects of microbial lindane degradation. This use of plants is an attractive method for decontaminating soils because of the low amount of soil handling involved and the low cost of maintenance. Further research is still needed, however, in order to develop a better understanding of plant-bacteria partnerships and to thereby enhance the bio-phytoremediation potential.

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Compliance with Ethical Standards

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

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AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES.

Q1. Please check if the affiliations are presented correctly.
Q2. Bidlan et al. 2004 has been changed to Bidlan and Afsar 2004 as per the reference list. Please check if okay.