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ORIGINAL PAPER

Methoxychlor bioremediation by defined consortium of environmental *Streptomyces* strains

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Abstract Methoxychlor is an organochlorine pesticide used worldwide against several insect pests, resulting in human exposure. This pesticide mimics endocrine hormone functions, interfering with normal endocrine activity in humans and wildlife. For this reason, it is imperative to develop methods to remove this pesticide from the environment, and though, bioremediation using microorganisms results as an excellent strategy. Five *Streptomyces* spp. strains previously isolated from organochlorine-polluted sites and capable to grow and remove methoxychlor were combined as different mixed cultures to increase methoxychlor removal. From the 39 consortia tested, one consortium (*Streptomyces* spp. A6, A12, A14, M7) was selected because of its high pesticide removal and specific dechlorinase activity to be assayed on slurry and soil systems. This consortium showed higher biomass values ($8.3 \times 10^6 \pm 5.7 \times 10^5$ CFU mL^{-1}) and methoxychlor removal (56.2 ± 2.3 %) on enriched slurry than in non-enriched slurry ($7.3 \times 10^5 \pm 1.2 \times 10^5$ CFU mL^{-1} and 45.6 ± 7.4 % of pesticide removal). In soil systems, *Streptomyces* consortium showed higher growth ($1.0 \times 10^{11} \pm 5.0 \times 10^{10}$ CFU g^{-1}) than in enriched slurry, although differences in methoxychlor removal between both culture conditions were not statistically significant. Therefore, the selected *Streptomyces* consortium may be suitable for the development of in situ (soil) and ex situ (slurry bioreactor) bioremediation methods because of their potential to remove methoxychlor from different systems.

Keywords Actinobacteria · Bioremediation · Organochlorine pesticide · Slurry bioreactor · Soil remediation

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Introduction

Methoxychlor [2,2-bis(*p*-methoxyphenyl)-1,1,1-trichloroethane] (MTX) is an organochlorine pesticide (OP) developed to be used as a replacement of DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane], which was internationally prohibited since 1970s due to its high toxicity (Stuchal et al. 2006). The physical and chemical properties of MTX are given in Table 1.

Methoxychlor [2,2-bis(*p*-methoxyphenyl)-1,1,1-trichloroethane] has been banned in most countries; however, it is still being used on agricultural products against several insect pests (Basavarajappa et al. 2011) and also in tropical environments where mosquito-borne malaria and

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Table 1 Physical and chemical properties of methoxychlor

Property	Value
Molecular formula	$C_{16}H_{15}Cl_3O_2$
Molecular weight	345.65
Color	Pale yellow
Physical state	Crystalline solid
Odor	Slightly fruity or musty
Melting point	89 °C (pure), 77 °C(technical grade)
Boiling point	Decomposes
Solubility	
Water	0.045 mg L^{-1}
Organic solvents	Soluble in aromatics, ketones, aliphatics, alcohols
Density	
Partition coefficients	1.41 g mL^{-1}
Log *K*ow	4.7–5.1
Log *K*oc	4.9
Vapor pressure (25 °C)	1.4×10^{-6} mmHg

Adapted from ATSDR (1994)

typhus are serious health problems (Llados et al. 2002). Despite its relatively low toxicity and short half-life (Metcalf 1976), there is considerable concern for MTX exposure because of its estrogenic activity (Staub et al. 2002; Fort et al. 2004). This toxic compound mimics endocrine hormone functions in the body, acting as disrupting chemicals, interfering with normal endocrine activity in humans and wildlife (Crisp et al. 1998). MTX is considered to be a proestrogen that, when converted into mono- and bis-hydroxy metabolites, exhibits greater estrogenic properties than the MTX parental compound does (Lee et al. 2006).

Since MTX health effects are well known, it is imperative to develop methods to remove it from the environment. One of the strategies adopted is bioremediation using microorganisms with pesticide degrading potential. The metabolic fate of MTX has been well investigated in eukaryotes, especially mammals (Masuda et al. 2012), although its biodegradation has also been described in fungi and bacteria able to use this kind of pesticide under aerobic or anaerobic conditions (Fogel et al. 1982; Lee et al. 2006; Yim et al. 2008; Keum et al. 2009). However, little information is available on the MTX biotransformation by Gram-positive microorganisms such as actinobacteria (De Schrijver and De Mot 1999; Fuentes et al. 2010; Lal et al. 2010). These microorganisms have a great potential for the biodegradation of organic and inorganic toxic compounds, and several studies have demonstrated oxidation and partial dechlorination and dealkylation of aldrin, DDT and herbicides like metolachlor and atrazine, particularly in *Streptomyces* (Liu et al. 1990; Radosevich et al. 1995), the main genus of actinobacteria present in soils (Xu et al. 1996). In previous studies, Benimeli et al. (2003, 2006, 2007) and Fuentes et al. (2010, 2011) isolated and selected wild-type *Streptomyces* spp. strains which were able to tolerate and remove lindane (γ-hexachlorocyclohexane), another OP, from culture media and soil, as pure and mixed cultures. The use of microbial mixed cultures has proved to be more suitable for bioremediation of recalcitrant compounds than pure cultures because their biodiversity can enhance environmental survival and increase the number of catabolic pathways available for the biodegradation of the xenobiotic (Siripattanakul et al. 2009; Yang et al. 2010). In this context, the aims of this study were the selection of the most efficient actinobacteria consortium for MTX biodegradation and their application on slurry and soil systems, as potential techniques for ex situ and in situ bioremediation, respectively. This work was performed in the Laboratory of Actinobacteria's Biotechnology, Pilot Plant of Industrial Microbiological Processes (PROIMI), CONICET, Tucumán, Argentina, from 2010 to 2012.

Materials and methods

Chemicals

Methoxychlor [2,2-bis(*p*-methoxyphenyl)-1,1,1-trichloroethane] (99.8 % pure) was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Solvents were of pesticide grade, and all other chemicals used throughout the study were of analytical grade and were purchased from standard manufacturers.

Microorganisms and culture media

Streptomyces spp. A3, A6, A12, A14, and M7 were previously isolated from sediments 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 database, accession numbers: AY45953 (M7) (Benimeli et al. 2007), GU085104 (A3), GQ867051 (A6), GQ867056 (A12), and GU085105 (A14) (Fuentes et al. 2010)]. *Streptomyces coelicolor* A3 (2) (*S. coelicolor*) obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) was also used.

Starch–casein medium (SC) used for the preparation of actinobacteria spore suspensions consisted of (g L^{-1}): starch, 10; casein, 1; K_2HPO_4, 0.5; agar, 15. The pH was adjusted to 7 prior to sterilization.

Actinobacteria were grown in liquid minimal medium (MM) containing (g L^{-1}): L-asparagine, 0.5; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; $FeSO_4.7H_2O$, 0.01 (Hopwood 1967).

Tryptone Soy Broth (TSB) contained (g L^{-1}): tryptone, 15; soy peptone, 3; NaCl, 5; K_2HPO_4, 2.5; glucose, 2.5. The pH was adjusted to 7.3 prior to sterilization. All media were sterilized by autoclaving at 121 °C for 20 min.

Selection of the most efficient mixed culture for methoxychlor biodegradation

Precultures

In order to prepare spore suspensions, *Streptomyces* strains were cultured on Petri dishes containing SC medium. Plates were incubated at 30 °C for 7 days, and then, spores were scraped and were suspended in sterile distilled water. Spore suspensions were individually cultured in Erlenmeyer flasks containing 30 mL of MM supplemented with filter sterilized (0.22 μ, Millipore) MTX (1.66 mg L^{-1}) as carbon source. Cultures were incubated at 30 °C on a rotary shaker (200 rpm) for 3 days. Afterward, they were centrifuged (9,000×*g*, 10 min, 4 °C). Supernatants were discarded and pellets were used as inocula for mixed cultures.

Mixed cultures

Microorganisms precultured (see above) were inoculated in 250 mL Erlenmeyer flasks containing 100 mL of MM with MTX (1.66 mg L^{-1}), with all possible combinations of mixed cultures of two, three, four, five, and six strains (final inoculum concentration: 2 g L^{-1}). Mixed cultures were incubated at 30 °C on a rotary shaker (200 rpm), for 4 days. Afterward, they were centrifuged (9,000×*g*, 10 min, 4 °C). Fifteen milliliter of supernatant of each culture were taken in order to determine the residual MTX concentration by Gas Chromatography (GC, see below). Pellets were washed, resuspended in 5 mL of sterile distilled water, and then were lysed in a French Press to obtain cell-free extracts (CFEs) for specific dechlorinase activity determinations.

Determination of specific dechlorinase activity in mixed cultures of *Streptomyces* strains

Specific dechlorinase activity (SDA) was indirectly determined in CFEs, by means of a colorimetric assay, in which phenol red sodium salt was added as pH indicator, at a ratio of 1/10 (Phillips et al. 2001). The change in color from red through orange to yellow in the presence of chloride ions was indicative of MTX dechlorination and therefore a positive result. Sterile distilled water with CFEs and the pH indicator, but without substrate, was used as a blank. Chloride concentrations were determined at 540 nm with reference to standard HCl solutions, using a spectrophotometer. One enzymatic unit was defined as the amount of chloride ions released (μmol) in one h (EU = μmol Cl^- h^{-1}) and the SDA was defined as EU mg^{-1} of protein.

The protein concentration was determined according to the Bradford method (Bradford 1976).

Slurry assay: conditions and inoculation

To slurry formulation, surface soil samples (5–15 cm depth) were taken from an experimental site northwest of San Miguel de Tucumán, Argentina. Samples were kept at 10–15 °C in the dark and used in the next days. Prior to use, soil was air-dried, lightly ground using mortar and pestle, and finally sieved through a 1-mm sieve. Such preparation was found necessary to improve homogeneity on the pollutant distribution. Two conditions of growth were assayed: soil slurry bioreactors (SB) formulated with distilled water (SB-water, poor culture medium) or with TSB (SB-TSB, rich culture medium) for the stimulation of microbial growth (Robles-González et al. 2008).

Experiments were conducted in Erlenmeyer flasks containing 40 g of sterile soil and 60 mL of sterile distilled water or sterile TSB, as required. Before being inoculated on SBs, selected mixed culture (see above) was precultured in Erlenmeyer flasks containing 30 mL of TSB, on a rotary shaker (200 rpm) at 30 °C, for 3 days. SBs with MTX were inoculated with precultured strains using a microbial concentration of 2 g L^{-1} (designed as "assay"). Inoculated slurries samples without MTX (designed as "biotic control") and non-inoculated slurries with MTX (designed as "abiotic control") were also used. Flasks were incubated on a rotary shaker (200 rpm), at 30 °C for 7 days. Samples (1 mL) were taken at different incubation time (at the beginning of the assay, at third and seventh day) for determining residual MTX concentration by GC. Microbial growth was also determined by the enumeration of bacteria (CFU mL^{-1}). Throughout the experiment, flasks were vigorously mixed to obtain a homogenous culture medium, as much as possible.

Soil assay: conditions and inoculation

Soil was obtained and processed as described above. Glass pots were filled with 200 g of soil at 20 % moisture (dry weight base) and kept for 36 h at room temperature so that water in the soil was balanced. Soil samples were sterilized (three successive sterilizations at 121 °C, 1 h each one, with 24 h in between) and the soil humidity was adjusted with sterile water or MTX to a final pesticide concentration of 1.66 mg kg^{-1} of soil. Sterility was checked for each set

of sterilized soil pots by the enumeration of bacteria (CFU g^{-1}). Before being inoculated on soil, selected mixed culture was precultured on TSB as described above. Pots with MTX were inoculated with precultured strains using a microbial concentration of 2 g kg^{-1} of soil (designed as "assay") (Benimeli et al. 2008). Soil, inoculum, and MTX were mixed thoroughly to ensure a uniform distribution. Inoculated soil samples without MTX (designed as "biotic control") and non-inoculated soil pots spiked with MTX (designed as "abiotic control") were also used. Soil pots were incubated at 30 °C for 4 weeks, and the soil humidity was monitored regularly twice a week. Samples were taken once a week for determining residual MTX concentration by GC and microbial growth by the enumeration of bacteria (CFU kg^{-1}).

Gas chromatography

For determining residual MTX concentration in the cell-free supernatants of mixed cultures (9,000×*g*, 30 min, 4 °C), solid phase extraction (SPE) using a C18 column (Varian, Lake Forest, USA) was carried out.

The extraction procedure for MTX residues in soil and slurry assays was performed according to Quintero et al. (2005) as follows: (1) Aliquots of 5 g of dry homogenized soil or 5 mL of slurry were transferred to centrifuge tubes and mixed with 4, 1, and 5 mL of water, methanol, and hexane, respectively. (2) The tubes were hermetically sealed and shaken for 10 min on a vortex in order to allow the extraction of MTX from soil to the organic phase and then centrifuged (9,000×*g*, 10 min, 4 °C) for separating organic and aqueous phases. (3) Organic phases were evaporated to dryness under reduced pressure; residues were resuspended in hexane and analyzed with GC.

Extracts were quantified in a Gas Chromatograph Agilent 7890A equipped with a HP5 capillary column (30 m × 0.53 mm × 0.35 m) and ^{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}, and increasing to 290 °C at 20 °C min^{-1}; detector temperature, 320 °C; and injection volume, 1 μl. Quantitative analyses were performed using appropriate calibration standards (AccuStandard).

Phytoxicity test

To test the potential toxicity of the residual MTX in SB-water assay, two endpoints were assessed: germination and root and hypocotyl elongation of lettuce seedlings (*Lactuca sativa*). Thirty seeds were placed into sterile Petri plates containing filter paper Whatman No. 1 saturated with 2 mL of slurry spiked with MTX and treated with the selected *Streptomyces* spp. consortium. Biotic and abiotic slurries were added to control plates. Petri plates were sealed with parafilm and incubated at 22 ± 2 °C in darkness, for 5 days (U.S. E.P.A. 1996). At the end of the incubation period, the number of germinated seeds was registered. The length of roots and hypocotyl was measured by means of a ruler to the closest millimeter. Distilled water control was used to monitor germination rate and the repeatability of this test. There were only small variations in average root and hypocotyls length of the lettuce seeds in the water control (data not shown). Vigor index (mean root length + mean hypocotyl length) × (percent germination/10) was also calculated (Bidlan et al. 2004).

Statistical analyses

All the results are the average of three replicates per sample. One-way analysis of variance (ANOVA) was performed to test the significant differences between treatments in slurries and *T* test was performed for soils. ANOVA was used to analyze the significant differences in the number of lettuce germinated and Nested-ANOVA to test the significant differences in the length of roots and hypocotyls. When significant differences were found, Tukey's post-test was used to separate the effects among treatments. Tests were considered significantly different at $P < 0.05$. Professional versions of Infostat and Statistica 6.0 software were used.

Results and discussion

Selection of the most efficient microbial consortium for methoxychlor biodegradation

In contrast to the great number of studies about the toxic effects of MTX, reports about its removal or biodegradation, especially by microorganisms, are scarce (Fogel et al. 1982). In view of this, five native *Streptomyces* spp. strains previously isolated from highly OPs contaminated environments and the reference strain *S. coelicolor* A3 (2) were cultured in the presence of MTX to assess their potential on pesticide removal. The results demonstrated that all the studied actinobacteria were able to grow in MM supplemented with MTX. It is important to note that neither the pesticide nor its potential toxic intermediaries (Lee et al. 2006) had an inhibiting effect on bacterial growth, since significant differences in bacterial growth with and without MTX were not observed ($P > 0.05$) (data not shown). The strains were not able to grow in MM containing L-asparagine as a sole carbon source (Benimeli et al. 2003), and there was no evidence of microbial growth in control

cultures without added MTX (data not shown). These results are very relevant, specially taking into account that there is little updated information about MTX degradation by actinobacteria.

From an applied perspective for bioremediation purposes, to introduce a microbial consortium rather than a pure culture to a polluted area is more advantageous. Considering that no growth inhibition was observed among any of the *Streptomyces* strains, all of them were cultured on MM added with MTX to evaluate their performance (SDA and pesticide removal). Mixed cultures consisting of combinations of two, three, four, five, and six strains were assayed, obtaining 39 different consortia. All of them were able to grow and to dissipate MTX from the culture medium and/or degrade it showing lower residual pesticide values than the initial concentration. These diverse catabolic activities are due to the presence of catabolic genes and enzymes (Siripattanakul et al. 2009). Indeed, it was possible to determine whether enzymatic degradation of MTX has taken place because of SDA was detected in CFEs in most the consortia (data not shown). The molecule of MTX has three chlorine atoms, and thus, the dechlorination is a very significant step in its degradation process (Fetzner and Lingens 1994). Since acidification in CFEs of each consortium was detected, we assumed that MTX is dominantly degraded by dechlorination and dehydrogenation process. In fact, previous studies support that actinobacteria were able to release chloride ions from liquid culture media contaminated with OP (Benimeli et al. 2006; Fuentes et al. 2010).

Because no linear relationship was found between the residual MTX concentration (RM) and SDA, it was decided to examine the ratio between both parameters in order to select the best mixed culture for further experiments, according to Benimeli et al. (2007). Table 2 shows the results of ratio RM/SDA for each consortium. Three of them presented the lesser values of the mentioned ratio because of the low residual MTX and high SDA, so they were found to be the most efficient consortia according to their SDA and pesticide removal ability. Considering that no statistical significant differences were found in the RM/SDA among these three consortia ($P < 0.05$), one of them consisting of *Streptomyces* spp. A6, A12, A14, and M7 was chosen for further experiments. In addition, this mixed culture showed the higher SDA.

Table 2 Ratio between residual methoxychlor concentration and specific dechlorinase activity in mixed cultures of *Streptomyces* spp. growing on minimal medium

Mixed cultures	RM/SDA × 10^{2}[a]
A6 M7	3.62
A3 M7	0.58
A12 M7	1.12
A6 *Sc*	1.31
A3 *Sc*	0.51
A3 A12	1.10
A3 A6 A12	0.31
A3 A6 A14	0.35
A3 A12 M7	1.19
A3 A12 *Sc*[b]	2.76
A3 A14 *Sc*	1.56
A6 A14 *Sc*	1.17
M7 A6 *Sc*	0.91
M7 A12 *Sc*	1.73
SC A14 M7	0.45
M7 A12 A14	1.56
A6 A12 *Sc*	2.05
A3 M7 *Sc*	0.42
A3 A6 *Sc*	2.26
A3 A14 M7	3.40
A6 A14 M7	0.14
A6 A12 M7	0.22
A3 A6 A12 A14	1.35
A3 A6 M7 *Sc*	0.13
A3 A6 SC A14	0.37
M7 A6 A14 *Sc*	0.28
A12 M7 A3 *Sc*	15.02
A12 A3 A14 *Sc*	0.44
A3 A6 A12 *Sc*	0.10
M7 A3 A14 *Sc*	0.17
A12 M7 A6 *Sc*	12.22
A3 A6 M7 A14	0.43
A3 A6 A12 M7	0.03
A12 A14 M7 A3	0
A6 A12 A14 M7[c]	0.04
A3 A12 A14 M7 *Sc*	0.21
A3 A6 A14 M7 *Sc*	0.11
A3 A6 A12 A14 M7	2.74
A3 A6 A12 A14 M7 *Sc*	1.69

[a] *RM* residual methoxychlor (mg L^{-1}), *SDA* specific dechlorinase activity (EU mg^{-1} protein)

[b] *Sc Streptomyces coelicolor* A3 (2)

[c] The most efficient mixed culture in methoxychlor biodegradation

Growth and residual methoxychlor of the *Streptomyces* spp. consortium growing on slurry bioreactor

Slurry bioreactors (SBs) are one of the most important types of ex situ techniques for the bioremediation of polluted soils (Robles-González et al. 2008). In fact, the obtained results generally reflect the actual biological depuration potential of the soil, as first step before working on soil itself (Robles-González et al. 2008). It has been shown that the degradation of xenobiotics by a given

microbial population can be favored by the addition of one or more rate-limiting nutrients to the contaminated system (biostimulation) (Delille et al. 2004). For instance, Sarkar et al. (2005) improved the biodegradation of petroleum hydrocarbons by up to 96 % after the addition of nutrient-rich organic matter resulting from the treatment of domestic sewage to diesel contaminated soils. To evaluate these issues, the selected *Streptomyces* spp. consortium was grown in SB formulated with soil-TSB (SB-TSB) or with soil-distilled water (SB-water) (Fig. 1a, b). According to the rich culture medium, maximum microbial growth was registered when strains were grown on SB-TSB spiked with MTX ($8.3 \times 10^6 \pm 5.7 \times 10^5$ CFU mL^{-1}), at the third day of the experiment (Fig. 1a). This biomass value was statistically significantly different to the biomass reached on SB-water ($P < 0.05$) at the same culture time (Fig. 1b). Furthermore, the duplication time, i.e., the period required for cells to double its biomass, was lesser and thus

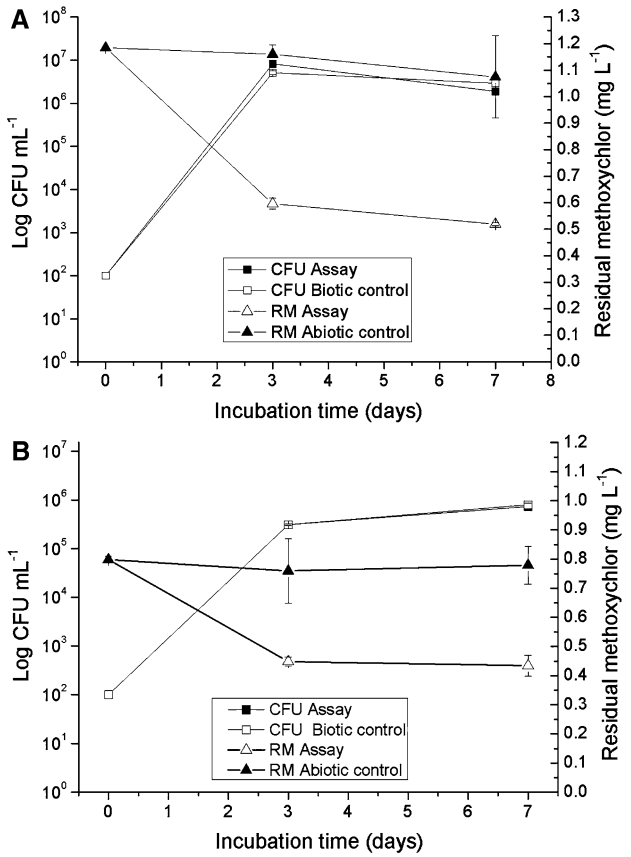

Fig. 1 Residual methoxychlor concentration and microbial enumeration of *Streptomyces* spp. consortium growing on slurry at different incubation times. *RM* residual methoxychlor, *CFU* colonial forming units, *assay* consortium cultivated with methoxychlor (MTX), *biotic control* consortium cultivated without MTX, *abiotic control* slurry spiked with MTX, without microorganisms. **a** slurry formulated with TSB and **b** slurry formulated with water

better on SB-TSB than in SB-water (Table 3) ($P < 0.005$). It is possible that the rich culture medium (TSB) together with the organic matter own of soil and the pesticide may have provided extra carbon sources to support the higher microbial growth and the faster growth rate (0.22 ± 0.01 h^{-1}) obtained in this condition. Despite these results, strains were also able to grow on SB-water. Moreover, no statistically significant differences were found between biomass reached with and without nutrient supply and with and without MTX, at the end of the experiments ($P > 0.05$) (Fig. 1a, b). This is not surprising considering that *Streptomyces* strains are already adapted to soil and hence may grow without additional carbon sources such as MTX and/or TSB, using the organic matter attached to the soil particles in suspension. This is especially true for strains growing on SB-water. The favorable performance of the consortium to grow in the presence of MTX may be due to a selective pressure of the polluted environment from which actinobacteria were isolated (Fuentes et al. 2010), which implies the adaptation of the strains with the possible acquisition of the corresponding metabolic abilities for surviving, as already noted in MM.

As it was expected, *Streptomyces* spp. selected consortium was able to remove MTX from both SB-TSB and SB-water, although nutrient supply was found to improve the pesticide removal. MTX removal, calculated as percentage of initial MTX minus percentage of residual MTX, reached 56.2 ± 2.3 % on SB-TSB and 45.6 ± 7.4 % on SB-water, at the end of the experiment (Fig. 1a, b). These findings suggest that TSB may be an appropriate carbon source as electron donor to support aerobic dehalogenation of the pesticide. In this regard, Fogel et al. (1982) studied the biodegradation of MTX in soil under different environmental conditions. The authors found evidence that a co-metabolic process may be responsible for the molecular changes which occurred with MTX because the rate of its disappearance from soil was observed to level off after exhaustion of soil organic matter. Moreover, Garcia-Rivero and Peralta-Pérez (2008) reported that co-metabolism is a very important process for the elimination of certain environmental xenobiotics. Similar results were obtained by Benimeli et al. (2007) and Alvarez et al. (2012) who found that the removal of different OPs by *Streptomyces* strains was more efficient when other carbon sources were present in the medium.

As shown in Fig. 1a, b, maximum microbial growth and MTX removal were reached at the third day of the experiment, and then it remained relatively constant until the end of the experiment, and then both parameters remained relatively constant until the end of the experiment, suggesting that these could be in direct proportion. Considering that bioremediation refers to the optimization of the environmental conditions to stimulate microbial growth

Table 3 Duplication time ($h \pm$ SD) of *Streptomyces* spp. consortium cultivated on slurry and soil

Slurry				Soil	
With TSB		With water		Assay	Biotic control
Assay	Biotic control	Assay	Biotic control		
3.13 ± 0.01a	3.22 ± 0.02a	3.94 ± 0.01b	3.94 ± 0.05b	15.21 ± 1.66c	11.77 ± 0.92c

Values that share the same letter were not significantly different ($P > 0.05$, Tukey's post-test)

and biodegradation of a given pollutant (Benimeli et al. 2007), survival and activity of the inoculum must be guaranteed. The obtained results are in accordance with this subject because an increase in the microbial population resulted in a consequent decrease in the residual MTX.

Regarding cost production, the use of water rather than TSB to cultivate the strains would be particularly appealing. Considering that no statistically significant differences were found in MTX removal between both SBs ($P > 0.05$), water may be an effective solvent to formulate SBs to potential ex situ bioremediation of MTX. Furthermore, this result confirms that *Streptomyces* strains are well adapted to a particular environment with deficient nutrients. Moreover, Robles-González et al. (2008) recently published an extensive list of several applications of SBs to remediate soils polluted with polycyclic aromatic hydrocarbons (PAHs), pesticides, polychlorinated biphenyls (PCBs), and explosives. None of the mentioned applications uses *Streptomyces* strains to remediate OPs, which suggest that biodegradation of MTX by actinobacteria has received relatively little attention. Indeed, according to the available reports, this is the first research of *Streptomyces* spp. remediating slurries polluted with MTX.

Growth and residual methoxychlor of the *Streptomyces* spp. consortium growing on soil

Actinobacteria growth capacity and MTX removal were also assessed in sterile soil samples spiked with MTX. This kind of study can provide closer insight about microorganisms and their growth requirements, before any in situ intervention for decontamination is carried out. As shown in Fig. 2, maximum microbial growth was registered when strains were grown on soil spiked with MTX ($1.0 \times 10^{11} \pm 5.0 \times 10^{10}$ CFU g^{-1}), at the end of the experiment (28 days). This biomass value was different at a statistical significant level from the biomass reached on soil without MTX ($P < 0.05$). However, no statistically significant difference was found in duplication time between both conditions ($P > 0.05$) (Table 3) reinforcing the hypothesis that actinobacteria are well adapted to proliferate in natural soils contaminated with xenobiotics. Microorganisms cultivated with MTX have not yet reached the stationary growth phase at the 28th day (Fig. 2), suggesting that the carbon source had

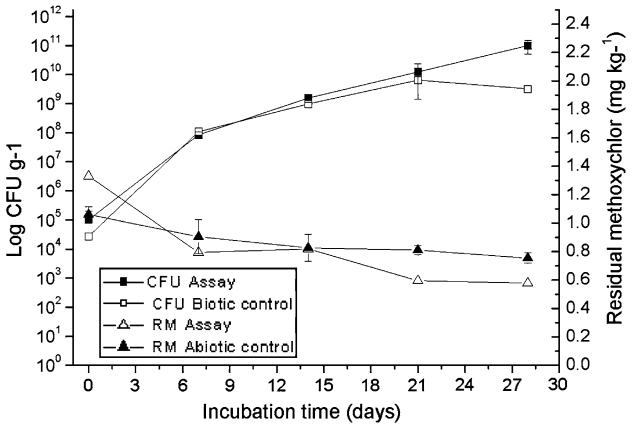

Fig. 2 Residual methoxychlor concentration and microbial enumeration of *Streptomyces* spp. consortium growing on sterile soil at different incubation times

not been exhaustive. In opposite, cells grown without the pesticide reached the plateau approximately 1 week before. It seems that MTX provides an additional source of carbon to support the cells growth, besides to own soil organic matter. Similar results were obtained by Benimeli et al. (2008), who found that *Streptomyces* spp. M7 continued growing after consuming lindane, possibly using the organic nutrients readily available in soil.

A substantial decline of the residual MTX was observed within 0–28 days of incubation with the consortium (Fig. 2). This strong biological effect, which allowed the 56.4 ± 5.5 % of pesticide removal, is very relevant taking into account the feasibility of its application: *Streptomyces* strains not only are already adapted to the soil condition but are also well adapted to the presence of this kind of pesticide. A small fraction of MTX disappears from the uninoculated control probably because of the aggregation and/or adsorption of the pesticide to soil particles (Fig. 2).

When comparing SBs with respect to the biodegradation in situ (soil), it was noted that SBs allow reducing the processing times (Robles-González et al. 2008). The results obtained in this work are in accordance with this observation, since MTX removal reached approximately 56 % on SB-TSB (Fig. 1a) and 46 % on SB-water (Fig. 1b) at the seventh day of assay, while in soil, pesticide removal reached a similar value at a time almost three times higher (Fig. 2). This is not surprising considering that SBs

Table 4 Development of lettuce seedlings cultivated on bioremediated slurry-distilled water

Treatment	Germinated seeds (No. ± SE)	Root length (cm ± SE)	Hypocotyl length (cm ± SE)	Vigor index
Bioremediated	12.00 ± 3.52a	0.26 ± 0.06bc $n = 35$	0.83 ± 0.12a	4.36
Biotic control	27.30 ± 1.71b	0.34 ± 0.03a $n = 82$	0.99 ± 0.07a	12.11
Abiotic control	21.70 ± 3.01ab	0.59 ± 0.07ac $n = 65$	1.36 ± 0.08a	14.07

Values that share the same letter were not significantly different ($P > 0.05$, Tukey's post-test)

typically increase mass transfer rates such as desorption of pollutants from soil which usually translates in faster and higher rates of contaminant biodegradation (Robles-González et al. 2008). As counterpart, SBs technology has drawbacks usually associated with the excavation, handling of the soil, and the construction and operation of the reactor itself, which results in increased costs of the process (Cookson, 1995). In opposite, in situ bioremediation may be a cheaper and feasible alternative to treat polluted sites, despite being a longer process. In support to in situ bioremediation, the results also showed that *Streptomyces* consortium grew more on soil than in SBs ($P < 0.05$, seventh day of assay) (Figs. 1a, b, 2) and that the differences in pesticide removal between SBs and soil were not statistically significantly different ($P > 0.05$, seventh day of assay). These findings indicate that the studied strains could be equally useful in the application of different ex situ and in situ remediation technologies, which in turn depend on the type and nature of the contamination.

Assessment of the efficacy of slurry bioremediation

Any planned intervention on natural environments must be preceded by ecotoxicity studies which should be used as bioindicators of the quality of the bioremediation process (Hamdi et al. 2007; Liu et al. 2010). For this purpose, lettuce seeds were cultured on bioremediated SB-water, which being an aqueous extract of soil increases the contact between the pesticide and the plant (Robles-González et al. 2008). Consequently, its use may be more appropriate for ecotoxicity assays than bioremediated soil. As shown in Table 4, tested seeds were found to be adversely affected by the addition of the bioremediated slurry, since a considerable reduction in the number of germinated seeds, root and hypocotyls length as well as in seedling vigor was observed ($P < 0.05$) (Table 4). These results may be interpreted as a confirmation that the parent compound was not completely biodegraded or it could be transformed into toxic intermediates. In effect, pollutant removal sometimes may be accompanied by the transformation of the mother contaminant into more toxic intermediates that could persist in soil and produce toxicity in certain species (Robles-González et al. 2008; Calvelo Pereira et al. 2010). The metabolic fate of MTX has been well investigated in eukaryotes, and it involves demethylation of the aromatic rings, a reaction catalyzed by cytochrome P450 monooxygenases (Masuda et al. 2012). Furthermore, dechlorination and hydroxylation have been identified as early steps in MTX metabolism in the fungus *Phanerochaete chrysosporium* (Grifoll and Hammel, 1997). Similarly, the main metabolic pathways involved in the microbial degradation of MTX in freshwater sediments are reported to be demethylation and reductive dechlorination. For example, Satsuma and Masuda (2012) observed that several environmental bacterial species convert methoxychlor to dechlorinated methoxychlor [1,1-dichloro-2,2-bis(4-methoxyphenyl)ethane] under aerobic conditions. Moreover, Masuda et al. (2012) observed that dechlorination reaction to yield 1,1-dichloro-2,2-bis(4-methoxyphenyl)-ethane [de-Cl-MTX] or CN-replacement to yield 2,2-bis(4-methoxyphenyl)acetonitrile [MTX-CN] was the first step in the degradation process for this pesticide. In this study, it appears reasonable to assume that the actinobacteria consortia are capable to carry out pathways that conduce to de-Cl-MTX because of their demonstrated SDA, but more detailed investigation is required to determine the produced metabolites during the MTX bioremediation. On the other hand, Lee et al. (2006) demonstrated that mono- and bis-hydroxy metabolites of MTX exhibit greater estrogenic properties than MTX alone does. In terms of germination, such properties are manifested as an imbalance in biochemical processes in the seed, leading to decrease in the growth (Calvelo Pereira et al. 2010). This hypothesis was reinforced when most seedlings grew in the abiotic control, where the parent compound remained almost unchanged (Table 4). On the other hand, Calvelo Pereira et al. (2010) found in *Solanum nigrum*, a relative increase in the percentage of germination at low levels of contamination with lindane, i.e., the presence of the contaminant stimulates plant responses. Such overcompensation, known as hormesis, is a relatively common phenomenon in studies of environmental contamination which involves an additional activity of the plant in an

attempt to recover from the stress to which it has been subjected (Calabrese 2005). However, results from toxicity monitoring are highly dependent on the test organisms used and it is therefore important to use several assays together (Moradas et al. 2008).

Taking all this into account, the effectiveness of the bioremediation process could be improved by removing the toxic effect of MTX intermediates, after their identification by gas chromatography-mass spectrometry (GC-MS) (Masuda et al. 2012). The proposed strategy, in this case, should involve the use of multi-component system (Ledin, 2000). For instance, an important improvement of the bioremediation process has been found using soil additives, like surfactants. Surfactants applications were observed to facilitate desorption of pollutants from soil and also decrease toxic intermediates accumulation during MTX degradation (Quintero et al. 2005; Baczynski et al. 2010). In this connection, maize plants are an excellent source of surfactants by root exudates production (unpublished results), and successful applications of maize for the remediation of xenobiotics (phytoremediation) in combination with actinobacteria had been previously reported (Gao et al. 2010; Alvarez et al. 2012).

Conclusion

Five native *Streptomyces* spp. strains previously isolated from highly OP-polluted environments were able to grow and to remove MTX when they were cultured on different systems. Pesticide removal was improved when the strains were combined as mixed cultures. In this context, because of its high pesticide removal and SDA, one consortium was selected among 39 different consortia for further experiments on slurry bioreactors and soil. Although the selected consortium showed the best performance growing on enriched slurry, differences with non-enriched slurry were not statistically significant. On the other hand, the results also showed that the consortium grew more on soil than in slurry. However, differences in pesticide removal between both culture conditions were not statistically significant. Hence, the present work has given relevant evidence that *Streptomyces* spp. may be suitable for the development of in situ and ex situ bioremediation methods. The combination of bioaugmentation, biostimulation, and biosurfactant addition might be a promising strategy to improve and speed up MTX bioremediation. In this connection, further studies evaluating the soil-microbe-additives system and its influence on MTX biodegradation are necessary so as to better explore and exploit an undoubtedly huge potential.

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