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Aerobic removal of methoxychlor by a native *Streptomyces* strain: Identification of intermediate metabolites

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ABSTRACT

Streptomyces strains isolated from pesticide-contaminated sediments of Santiago del Estero, Argentina were able to grow in the presence of 1.66 mg L^{-1} of methoxychlor (MTX). *Streptomyces* sp. A14 showed the best growth in the presence of MTX in culture medium at 30 °C and pH 7. When soil microcosms were contaminated with MTX and inoculated with *Streptomyces* sp. A14, a decrease in MTX was detected in both concentrations assayed (8.33and 16.60 mg kg^{-1}). This actinobacterium was able to remove the pesticide, reaching its maximum removal percentages (40% and 76%) after 28 days of incubation. Methoxychlor was gradually converted into 1,1-dichloro-2,2-bis(4-methoxyphenyl)ethane, 1,1-dichloro-2,2-bis(4-methoxyphenyl)ethylene, 1-chloro-2,2-bis(4-methoxyphenyl)ethane, and 2,2-bis(4-hidroxyphenyl)acetonitrile, indicating that MTX is dominantly degraded by dechlorination, dehydrogenation and CN-replacement, resulting in the production of several major degradation products. This is an interesting proposal as an alternative method for soil cleanup. This is the first report of methoxychlor degradation using a native *Streptomyces* strain under aerobic conditions.

Introduction

Methoxychlor [2,2-bis(*p*-methoxyphenyl)-1,1,1-trichloroethane] (MTX) is an organochlorine pesticide that was developed for use as a replacement for DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane]. DDT has been internationally prohibited since 1970s due to its high toxicity (Stuchal et al., 2006). MTX is structurally similar to DDT and it was primarily used against various species of insects that attack field crops, trees, vegetables, fruits, gardens, stored grain, livestock, and domestic pets (ATSDR, 1994). MTX is persistent in soils and its residues are found to be present even after 18 months of post-treatment with microbes that scavenge MTX (Golovleva et al., 1984). The use of MTX was banned worldwide in 2004 because of failure to register with the U.S. Environmental Protection Agency (EPA) (Stuchal et al., 2006); however several sites still exist around the world where soils and sediments are highly polluted with this compound. These places include areas of (former) pesticide manufacturing and formulation, water courses used to receive discharges from these factories, obsolete pesticide storage locations and others (Baczynski, 2012). Globally, humans and domestic animals are exposed to MTX through the extensive usage of this chemical and through consumption of agricultural products (Stuchal et al., 2006).

Despite its relatively low level of toxicity and short half-life there is a considerable amount of concern regarding MTX exposure because it is an endocrine disrupting chemical (EDC). EDCs produce their effects by mimicking, antagonizing, or altering levels of endogenous steroids (androgens or estradiol) via changes in their rates of synthesis or metabolism and/or expression or action at the receptor targets (Frye et al., 2012).

Since harmful effects of MTX on health are well known (Guo et al., 2013), it is imperative to develop methods to remove it from the environment. One of the strategies that have been adopted is bioremediation using microorganisms with the ability to degrade pesticides. MTX metabolism in higher organisms has been well investigated in mammals, birds, fish, and bivalves (Masuda

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et al., 2011), and MTX biodegradation has also been described for certain fungi and bacteria that are able to use this kind of pesticide under aerobic or anaerobic conditions (Fogel et al., 1982; Lee et al., 2006; Satsuma et al., 2012). However, little information is available on MTX biotransformation by Gram-positive microorganisms such as actinobacteria (Fuentes et al., 2010, 2013; Lal et al., 2010). These microorganisms have been shown to have great potential for biodegradation of toxic organic compounds, and several studies have demonstrated the ability of different genera of actinobacteria to degrade pesticides including lindane, chlordane, MTX, chlorpyrifos, diuron, and pentachlorophenol (Fuentes et al., 2010; Briceño et al., 2012).

It has also been determined that environmental factors such as temperature, pH, and salinity have a strong influence on microbial activity, as well as on the bioavailability of target chemicals. Therefore, the optimization of these parameters is necessary in order to achieve substantial degradation of pollutants. In this context, the aim of the present work is to determine the optimal conditions for MTX removal by indigenous *Streptomyces* strains, to characterize their potential degradation in soil microcosm, and to investigate the production of metabolites from MTX.

Materials and methods

Chemicals

Methoxychlor [2,2-bis(*p*-methoxyphenyl)-1,1,1-trichloroethane] (99.8% pure) was purchased from Sigma–Aldrich (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

Four actinobacteria strains (*Streptomyces* spp. A3, A6, A12,A14) previously isolated from a contaminated environment in Santiago del Estero, Argentina and identified as belonging to the genus *Streptomyces* were selected because of their ability to grow in the presence of MTX as a carbon source and remove MTX from the culture medium (Fuentes et al., 2010). Also, two strain were used: *Streptomyces coelicolor* A3 (2), obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), and *Streptomyces* sp. M7, previously isolated in our laboratory from sediment samples contaminated with heavy metals and pesticides (Benimeli et al., 2003).

All removal assays were carried out in Minimal Medium (MM), which contained (g L^{-1}): $(NH_4)_2SO_4$, 2.00; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.20; and $FeSO_4 \cdot 7H_2O$, 0.01 (Hopwood et al., 1985). The pH of the medium was adjusted to 7.0 prior to sterilization. All media were sterilized by autoclaving at 121 °C for 20 min.

A stock solution of MTX dissolved in acetone was poured into the sterile glass flasks and air-dried. Then autoclaved MM was added aseptically, according to the requirements of each test. The MM with pesticide was allowed to stand overnight to promote the dissolution of the MTX.

Batch cultures of Streptomyces spp. strains in minimal medium supplemented with methoxychlor

Spore suspensions of the six actinobacterium strains (150 μL) were inoculated in 125 mL flasks containing 30 mL of MM supplemented with MTX (1.66, 8.30, and 16.60 mg L^{-1}) as the carbon source. All cultures were incubated on a rotatory shaker (200 rpm) at 30 °C, for 7 days. Supernatant samples of centrifuged cultures (9900 × *g*, 30 min, 4 °C) were used to determine residual MTX by gas chromatography. Biomass was estimated after centrifugation by washing the pellets with 25 mM Tris–EDTA buffer (pH 8.0) and drying to constant weight at 105 °C.

Inoculated MM without MTX and supplemented with glucose 1 mg L^{-1} as carbon source (designed as "biotic control") and non-inoculated MM with MTX (designed as "abiotic control") samples were also included. All experiments were carried out in triplicate; results are given as the means from the three tests.

Soil microcosms: condition and inoculation

For soil microcosms, surface soil samples (5–15 cm depth) were taken from an experimental site northwest of San Miguel de Tucumán, Argentina. Samples were kept at10–15 °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.

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 8.33, and 16.60 mg kg^{-1} wet weight (w/w). Sterility was checked for each set of sterilized soil pots by the enumeration of bacteria (CFU g^{-1}) (Benimeli et al., 2008).

Before being inoculated on soil, *Streptomyces* sp. A14 was precultured in Erlenmeyer flasks containing Tryptic Soy Broth (TSB) 50 mL at 30 °C on a rotatory shaker (100 rpm) for approximately 3–4 days. Pots with MTX were inoculated with precultured strain 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}). The growth of *Streptomyces* sp. A14 was measured as CFU kg^{-1} by transferring 1 g of soil from each pot into a sterile flask, containing 9 mL of a sterile solution of sodium hexametaphosphate (1.66 g L^{-1}, pH 7) according to the method used by Benimeli et al. (2008). Soil was vortexed for 10 min. Serial 10-fold dilutions in sodium dihydrogenophosphate (0.05 M, pH 7) were made and plated in triplicate on starch casein agar. The plates were incubated at 30 °C for 72 h.

Colorimetric assay for dechlorination activity

Cell-free supernatant samples were immediately used for indirect determination of the release of chloride ions using a modification of the procedure described in Phillips et al. (2001), in which Phenol Red Sodium Salt is added to 1 mL of supernatant at a 1:10 ratio as a pH indicator. A color change from red through orange to yellow caused by the presence of chloride in the supernatant is indicative of dechlorination of MTX, and therefore represents a positive result. Culture medium with a pH indicator was used as a blank. Chloride concentrations were determined colorimetrically at 540 nm using a Beckman spectrophotometer and compared with standard HCl solutions. A decrease in optical density at 540 nm ($\Delta A_{540\ nm}$) was indicative of microbial dechlorination activity (Cuozzo et al., 2012).

Gas chromatography analysis

Residual MTX in cell-free supernatants was extracted by solid phase extraction (SPE) using a C18 column (Agilent Technologies, United States). The extraction procedure for MTX residues in soil was performed according to Quintero et al. (2005) as follows: aliquots of 5 g of homogenized soil were transferred to centrifuge tubes and mixed with 4 mL water, 1 mL methanol and 5 mL hexane.

The tubes were hermetically sealed, vortexed for 10 min in order to allow the extraction of MTX from the soil to the organic phase, and then centrifuged (9000 × g, 10 min, 4 °C) to separate the organic and aqueous phases. The organic phases were evaporated to dryness under reduced pressure; residues were then resuspended in hexane for analysis by GC.

These extracts were then quantified in a Gas Chromatograph (Agilent Technologies 7890A) equipped with an HP5 capillary column (30 m × 0.53 mm × 0.35 μm) and ^{63}Ni μECD detector, split/splitless injector (Agilent 7693B), 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 further 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, New Haven, CT, USA). The detection limit for MTX was 0.07 μg L^{-1}. The average extraction efficiencies were 86.3 ± 8.7% and 86.9 ± 6.4% for supernatants and soil, respectively.

Identification of metabolites for the methoxychlor microbial degradation by Streptomyces sp. A14

A 150 μL concentrated spore suspension (10^9 CFU mL^{-1}) of *Streptomyces* sp. A14 was inoculated in flasks with 250 mL MM containing 16.60 mg L^{-1} of MTX. These flasks were then incubated at 30 °C and 150 rpm for either 12 or 24 h; flasks without inoculum were used as control. Incubation pellets were then aseptically harvested by centrifugation (9900 × *g*, 30 min, 4 °C). The cell-free extract (obtained by French-press cell disruption) and the culture supernatant were used for extraction of MTX degradation intermediaries using solid phase extraction (SPE) with C18 columns. The extracts obtained were analysed in a Gas Chromatograph (Agilent Technologies 6890) equipped with a Network Mass Selective Detector (Agilent 5973), HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) and a split/splitless injector (Agilent 6850). The chromatographic conditions were as follows: inlet temperature 280 °C, carrier gas (helium) constant flow rate at 1.0 mL min^{-1}; oven temperature was programmed to increase from 50 to 125 °C at 3 °C min^{-1}, and then at 17.5 °C min^{-1} to 300 °C and remaining at this temperature for 10 min. The Mass Detector was used in Single Ion Monitoring.

Results and discussion

Microbial growth and degradation of methoxychlor by selected Streptomyces isolates

In previous studies Fuentes et al. (2010) detected an MTX concentration of 0.46 μg g^{-1} in soil samples from an illegal storage site with more than 30 t of obsolete pesticides. From these samples, twelve actinobacteria were isolated and four strains (*Streptomyces* spp. A3, A6, A12, A14) were selected on the basis of its ability to grow in the presence of MTX. These actinobacteria and the strains *Streptomyces* sp. M7 and *S. coelicolor* A3 (2) were used for studying the growth of these microorganisms over time in the presence of MTX.

All six strains were able to grow in MM containing 1.66 mg L^{-1} of MTX as carbon source and they showed similar growth profiles (Fig. 1). Biomass increased rapidly at 24 h of incubation without any detectable lag, the same as has been reported for other microorganisms growing in media with pesticides (De Paolis et al., 2013). The maximum growth values were obtained for *Streptomyces* sp. A14, at 7 d of incubation in the presence of the pesticide. Furthermore, no growth inhibition was observed in medium supplemented with MTX as the carbon and energy source compared to the controls without the pesticide (data not shown). These results may indicate that the xenobiotic is not toxic to the cells at the tested concentration (1.66 mg L^{-1}), and may also suggest that toxic metabolites with inhibitory effect on microbial growth are not accumulated either.

In a similar study, Benimeli et al. (2006) observed that when *Streptomyces* sp. M7 was cultured in MM supplemented with lindane, another organochlorine pesticide, it showed maximum growth at the 24 h of incubation and then the biomass remained almost constant for the next three days.

In the present study, analysis of the residual MTX concentration in the culture medium after 7 d of incubation showed that all of the studied actinobacteria presented high rates of MTX removal, with removal MTX percentages between 97% and 100% after the first 24 h of growth (Table 1).

For anaerobic MTX degradation processes, incomplete degradation of this pesticide has been observed in previous studies (Hirano et al., 2007; Baczynski and Pleissner, 2010), with these authors arguing for the need for a subsequent aerobic treatment. This type of treatment was in fact conducted by Fogel et al. (1982), who studied the aerobic biodegradation of MTX after an anaerobic incubation, achieving 90% mineralization of the pesticide after three months. It is also important to note that the removal percentages achieved by the actinobacteria assayed in the present study were similar to those reported by other authors, although the disappearance of MTX proceeded more rapidly than the removal reported for other microorganisms (Satsuma and Masuda, 2012; Satsuma et al., 2012).

In order to determine and confirm the MTX-degrading ability of the actinobacteria assayed, the release of chloride ions into the culture medium was also examined (Fuentes et al., 2010; Cuozzo

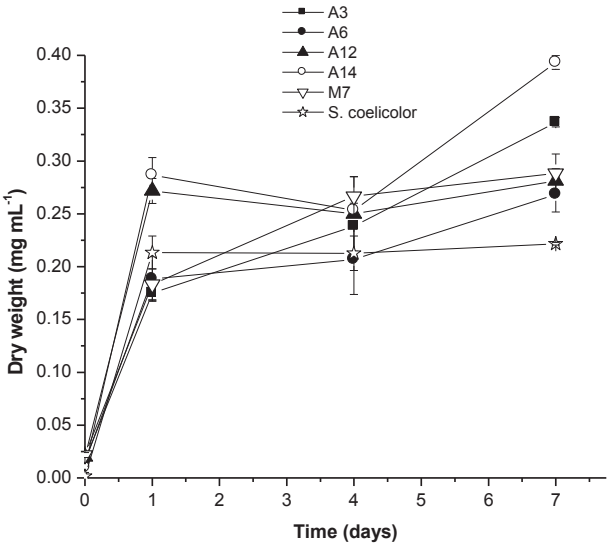

Fig. 1. Growth of *Streptomyces* spp. A3, A6, A12, A14, M7 and *Streptomyces coelicolor* A3 (2) cultured in minimal medium with MTX added at 1.66 mg L^{-1} during 7 days of incubation.

Table 1
MTX removal by *Streptomyces* spp. A3, A6, A12, A14, M7 and *Streptomyces coelicolor* A3 (2), cultured in minimal medium added with 1.66 mg L^{-1} of MTX.

Strains	% MTX removal
Streptomyces sp. A3	98.3 ± 0.1
Streptomyces sp. A6	97.0 ± 0.6
Streptomyces sp. A12	98.3 ± 0.1
Streptomyces sp. A14	100.0 ± 0.6
Streptomyces sp. M7	98.3 ± 0.1
Streptomyces coelicolor A3(2)	98.3 ± 0.6

et al., 2012). All of the cell-free supernatants revealed the presence of chloride ions, further suggesting that the microorganisms were able to degrade MTX. This chloride ion release reached its maximum values after 1–4 days of incubation depending upon the microbial growth patterns, indicating degradation of the MTX as well as its use as a carbon source, since the culture medium did not have any other carbon source (Fig. 2). No significant differences were observed among all the assayed actinobacteria strains. Elimination of halogens from halogenated xenobiotics is a key step in their degradation because the carbon–halogen bond is relatively stable (Fetzner and Lingens, 1994). Because dehalogenation plays a central role in the biodegradation of many chlorinated compounds, the current study also assayed the release of chloride ions to assess microbial degradation of MTX. In a previous work, Benimeli et al. (2006) reported on the release of chloride ions from lindane by a streptomycete strain. Cuozzo et al. (2009) demonstrated that synthesis of dechlorinase in *Streptomyces* sp. M7 was induced when the microorganism was grown in the presence of lindane as carbon source. Fuentes et al. (2010) observed chloride released into culture supernatants of actinobacteria growing on MM added with MTX, lindane, or chlordane. Following a similar methodology as the one described in the present work, Cuozzo et al. (2012) found that the strains *Streptomyces* spp. A6, M7, and *S. coelicolor* A2 (3) showed release of chloride ions into supernatants when they were cultured in MM in the presence of chlordane. The current study showed the release of chloride ions in cell-free supernatants. The six actinobacteria strains showed a positive relationship among growth, chloride release, and removal of MTX in the presence of this pesticide, and these findings were supported by the presence of intermediate metabolites.

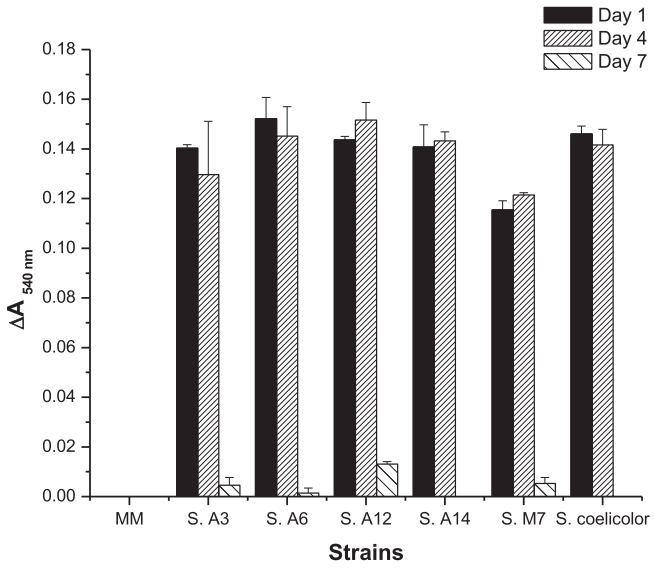

Fig. 2. Chloride ions released from MTX by *Streptomyces* spp. A3, A6, A12, A14, M7 and *Streptomyces coelicolor* A3 (2) cultured in minimal medium with MTX added at 1.66 mg L^{-1} during 7 days of incubation.

Influence of temperature, pH and pesticide concentration on methoxychlor biodegradation by Streptomyces sp. A14

Streptomyces sp. A14 was selected for further parameter optimization studies related to MTX removal because it showed the highest value for biomass after 7 days of incubation (0.39 mg mL^{-1}) compared to the rest of the studied strains, as well as the lowest time of duplication (4.96 h) and 100% MTX removal at 24 h of incubation.

MTX removal by *Streptomyces* sp. A14 at three different temperatures (25, 30, and 35 °C), was analyzed. The MTX residual concentration after the first 24 h of culture was 91.8%, 100%, and 90.5%, respectively (data not show). Although temperature did not affect the removal rate of the pesticide, 30 °C was the temperature chosen for selecting the optimal pH. Benimeli et al. (2007) observed that 30 °C was the optimal temperature for lindane removal by *Streptomyces* sp. M7 growing in a soil extract medium. On the other hand, Baczynski et al. (2010) found that incubation temperature had an influence on removal percentages for organochlorine pesticides like lindane, DDT, and MTX, although the extent of this influence varied for individual compounds and the lowest effect was seen for MTX.

The different initial pH (5.0, 7.0, and 9.0) at 30 °C did not affect the MTX removal, and no significant difference ($p < 0.05$) was observed among the removal percentages of this toxic compound (data not shown). Thus, pH 7.0 was selected for next assays of MTX degradation by *Streptomyces* sp. A14, on the bases of previous work. In fact, Benimeli et al. (2007) selected the pH 7 for lindane removal by *Streptomyces* sp. M7 because the maximum growth and lindane removal percentage were detected in this condition, in extract soil medium. Also, it is important to note that pH 7.0 is generally considered optimal for the metabolic activities of the genus *Streptomyces* (Okeke et al., 2002). Additionally, Robinson et al. (2009) observed that an acidic environment is not suitable for aerobic dehalogenation of MTX by bacteria. The fate of organic pollutants in the environment is influenced by environmental factors such as pH and temperature, which affect the activity of microorganisms. However, *Streptomyces* sp. A14 showed MTX removal percentages in the range of 90–100% over a wide range of pH values in MM.

Later, the growth of *Streptomyces* sp. A14 in MM at 30 °C, pH 7.0, with different MTX concentrations, for seven days was studied. The microbial biomass as dry weight and the MTX residual concentration were evaluated. Similar experiments were carried out using control samples without MTX. Significant differences in growth were not observed (data not shown). These results would indicate that the growth of *Streptomyces* sp. A14 in MM was not affected by the MTX concentrations assayed, suggesting that the microorganism could tolerate these concentrations or perhaps degrade the pesticide by producing the dehalogenase enzymes necessary for the mineralization process, as was demonstrated by Nagata et al. (2007) for *Sphingobium japonicum* UT26 when this strain grew in the presence of lindane.

The influence of different initial concentrations of MTX on pesticide removal was also evaluated by determining residual MTX in the supernatant samples (data not shown). At initial MTX concentrations of 1.66, 8.33, and 16.60 mg L^{-1}, 100% removal of the pesticide was obtained after 7 days of incubation. In all cases, it was demonstrated that the strain *Streptomyces* sp. A14 is capable of removing even the highest MTX concentration from the culture medium. Along these same lines, Baczynski (2012) studied the anaerobic biodegradation of DDT, a compound similar to MTX, in

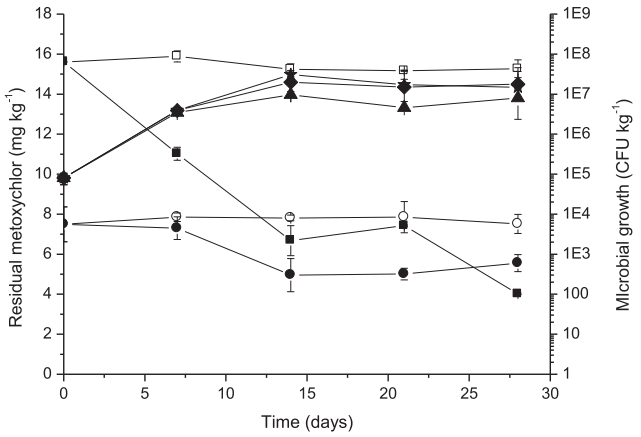

Fig. 3. The effect of MTX concentration on *Streptomyces* sp. A14 growth and MTX removal, cultured in soil microcosms with MTX added at 8.33 and 16.60 mg kg^{-1} during 4 weeks of incubation. Symbols used: (○) MTX removal, non-inoculated soil, 8.33 mg kg^{-1} MTX; (●) MTX removal, inoculated soil 8.33 mg kg^{-1} MTX; (□) MTX removal, non-inoculated soil, 16.60 mg kg^{-1} MTX; (■) MTX removal, inoculated soil, 16.60 mg kg^{-1} MTX; (▲) microbial growth, non-contaminated soil; (★) microbial growth, 8.33 mg kg^{-1} MTX; (◆) microbial growth, 16.60 mg kg^{-1} MTX.

samples from a polluted field inoculated with granular sludge, and it was observed that even at the highest DDT soil level of 102 mg kg^{-1}, no decrease of removal effectiveness was seen.

Removal of methoxychlor by Streptomyces sp. A14 in soil microcosms

Streptomyces sp. A14 was grown for 4 weeks in sterile soil microcosms containing two different MTX concentrations (8.33 and 16.60 mg kg^{-1}). In both cases, the MTX removal was determined. Similar experiments were carried out with control samples without MTX. As was observed in liquid MM, significant differences in growth were not observed at the different added MTX concentrations compared to the control without MTX (Fig. 3), reinforcing the hypothesis that actinobacteria are well adapted for proliferation in natural soils contaminated with this kind of pesticides.

The changes in MTX concentration in control samples and the biodegradation tests over time in soil are also presented in Fig. 3. Since no variations of pesticide concentrations were observed in either of the control series, there is no evidence of any noticeable contribution of abiotic processes to the MTX removal. At initial MTX concentrations of 16.60 and 8.33 mg kg^{-1}, the percentages of pesticide removal after 4 weeks of incubation were 76% and 40%, respectively. This assay demonstrated that degradation was higher in inoculated soils contaminated with the highest concentrations of the pesticide. The results obtained in this work are in accordance with those observed by Fuentes et al. (2013), who found that a *Streptomyces* spp. consortia growing in a soil microcosm spiked with MTX achieved approximately 56% MTX removal.

Similar findings in relation to lindane removal were reported by Okeke et al. (2002), who studied the ability of a *Pandoreae* sp. strain to remove lindane in liquid and soil slurry cultures. Their results indicated that the rates and extent of lindane removal increased with increasing concentrations up to 150 mg L^{-1} but declined at 200 mg L^{-1}, after 4–6 weeks of incubation.

Identification of metabolites of the methoxychlor biodegradation

Four MTX degradation metabolites were identified in both cell-free extract and culture supernatant of *Streptomyces* sp. A14 after 12 and 24 h of incubation by GC–MS analysis. On the basis of these spectra (Table 2), the peak at 34.28 min corresponded to 1,1-dichloro-2,2-bis(4-methoxyphenyl)ethane, formed by elimination of a chloride ion from the MTX molecule; the second peak, in the position 34.02 min, was identified as 1,1-dichloro-2,2-bis(4-methoxyphenyl)ethylene, a product of dehydrogenation process. The third peak in the spectrum at 34.49 min, was 1-chloro-2,2-bis(4-methoxyphenyl)ethane, formed by the second dechlorination process according to the pathway proposed for the degradation of MTX in culture medium by the white rot fungus *Stereum hirsutum* (Lee et al., 2006). Also, a fourth peak, with a retention time of 33.45 min, was identified as 2,2-bis(4-hidroxyphenyl)acetonitrile, product of the microsomal oxidative o-demethylation of the methoxy group (Fig. 4). 1,1-dichloro-2,2-bis(4-methoxyphenyl) ethane also is a detoxification reaction product present in the MTX degradation ways proposed for *Corbicula japonica* (Masuda et al., 2012) and *Bradyrzobium* sp. strain 17-4 (Satsuma et al., 2012). Also, Grifoll and Hammel (1997) reported that the white rot fungus *Phanerochaete chrysosporium* metabolized the MTX in various degradation products, one of the most prominent of these was identified as the 1-dechloro derivative 1,1-dichloro-2,2-bis(4-methoxyphenyl)ethane. Therefore, identification of the compounds as reported in the present work is clearly indicating the presence of two possible MTX degradation pathways: the one proposed by Lee et al. (2006) and the one proposed by Masuda et al. (2012), respectively.

MTX nitrile metabolites and 1,1-dichloro-2,2-bis(4-methoxyphenyl)ethane were also detected by Masuda et al. (2012) reported that the first step of MTX degradation in model systems (sediments and associated water) was both dechlorination or CN-replacement for mineralization this pesticide. These results indicate that *Streptomyces* sp. A14 could have two pathways for metabolizing MTX (Fig. 4). The presence of nitrile metabolites is not uncommon for organochlorine pesticides. For example, Albone et al. (1972) previously demonstrated the presence of a nitrile intermediate of DDT. However, with *Bradyrhizobium* sp. 17-4 it was detected that the o-demethylation of MTX yielded a monophenolic derivative [1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl) ethane] as the primary degradation product (Satsuma et al., 2012). Also, 1,1-dichloro-2,2-bis(4-methoxyphenyl)ethane was previously shown to occur as a product of MTX metabolism by a bacterium, *Klebsiella pneumoniae* (Baarschers et al., 1982), as well as with uncharacterized

Table 2
Metabolites present during the biodegradation of MTX by *Streptomyces* sp. A14 as determined by GC/MS.

Compounds[a]	Retention time (min)	Monitored ions in SIM mode
2,2-Bis(4-hidroxyphenyl)acetonitrile	33.45	225 (mp), 197, 181,132 (bp)
1,1-Dichloro-2,2-bis(4-methoxyphenyl)ethylene	34.02	310, 308 (bp, mp), 273, 238, 223, 195, 166, 152, 119
1,1-Dichloro-2,2-bis(4-methoxyphenyl)ethane	34.28	312, 310 (mp), 275, 240, 227 (bp), 212, 169, 153, 114
1-Chloro-2,2-bis(4-methoxyphenyl)ethane	34.49	276, 274 (bp, mp), 259, 239, 224, 196, 165, 152, 132
Methoxychlor	34.73	346, 344 (mp), 311, 309, 276, 274, 228, 227 (bp)

[a] Nomenclature of compounds is according to IUPAC (International Union of Pure and Applied Chemistry). bp: base peak. mp: molecular ion peak. SIM: Selected Ion Monitoring.

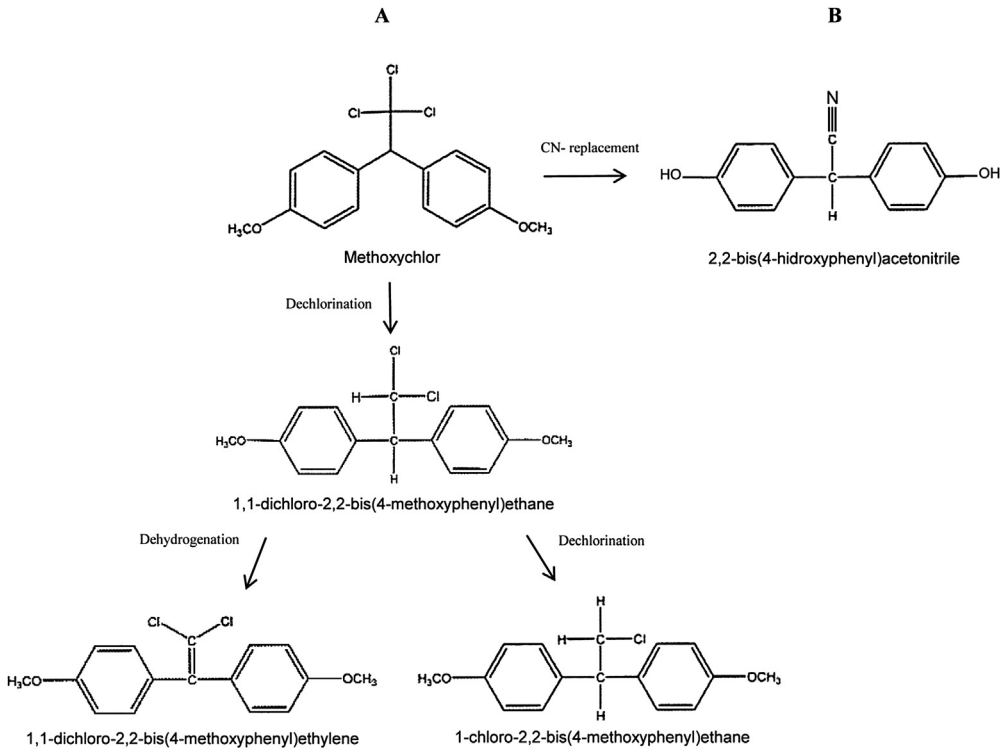

Fig. 4. Proposed major metabolic pathways of MTX removal by *Streptomyces* sp. A14, showing dechlorination (A) and CN-replacement (B) reactions, according to Lee et al. (2006) and Masuda et al. (2012).

mixed microbial cultures (Muir and Yarechewski, 1984). To our knowledge, this is the first report on the detection of the MTX metabolite (1,1-dichloro-2,2-bis(4-methoxyphel)ethane) in the MTX removal process by actinobacteria. Intermediary metabolites with fewer carbon atoms could be present, however so far, they have not been detected in this work at the assayed times. More detailed investigation is required in order to clarify the mechanisms for these reactions, as well as to determine whether the MTX degradation products reach the point of formation of carbon dioxide and water, in order to ensure a safe bioremediation process for the environment (Baczynski et al. 2010).

These results suggest that *Streptomyces* sp. A14 is capable of rapidly degrading different concentrations of MTX through monodechlorination and CN-replacement of MTX as the initial biotransformation step, as confirmed by GC/MS analysis. We believe that this is the first example of the presence of two alternative degradation pathways for MTX in an actinobacterium.

Conclusions

The conditions allowing *Streptomyces* sp. A14 to safely resist and completely remove MTX were found to be 30 °C and pH 7 in culture medium. Furthermore, no significant differences in growth were seen, at different MTX concentrations compared to the control, thereby establishing the high capacity of this strain to remove MTX at increasing concentrations. In both concentrations assayed in soil (8.33 and 16.60 mg kg^{-1}) this actinobacterium was able to remove the pesticide, reaching its maximum removal percentages (40% and 76%) after 28 days of incubation, respectively. There is also evidence that the MTX biodegradation may be taking place by dechlorination, dehydrogenation, and CN-replacement, resulting in the production of several major degradation products, which will make it an interesting proposal as an alternative method for soil cleanup. In summary, *Streptomyces* sp. A14 could have good potential for bioremediation of soils contaminated with high MTX concentrations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.ibiod.2014.09.016.

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