Ecotoxicology and Environmental Safety 116 (2015) 34–39

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Versatility of *Streptomyces* sp. M7 to bioremediate soils co-contaminated with Cr(VI) and lindane

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ARTICLE INFO

Article history:
Received 29 October 2014
Received in revised form
20 February 2015
Accepted 25 February 2015
Available online 6 March 2015

Keywords:
Bioremediation
Soil
Actinobacteria
Lindane
Chromium

ABSTRACT

The aim of this work was to study the impact of environmental factors on the bioremediation of Cr(VI) and lindane contaminated soil, by an actinobacterium, *Streptomyces* sp. M7, in order to optimize the process.

Soil samples were contaminated with 25 μg kg^{-1} of lindane and 50 mg kg^{-1} of Cr(VI) and inoculated with *Streptomyces* sp. M7. The lowest inoculum concentration which simultaneously produced highest removal of Cr(VI) and lindane was 1 g kg^{-1}. The influence of physical and chemical parameters was assessed using a full factorial design. The factors and levels tested were: Temperature: 25, 30, 35 °C; Humidity: 10%, 20%, 30%; Initial Cr(VI) concentration: 20, 50, 80 mg kg^{-1}; Initial lindane concentration: 10, 25, 40 μg kg^{-1}.

Streptomyces sp. M7 exhibited strong versatility, showing the ability to bioremediate co-contaminated soil samples at several physicochemical conditions. *Streptomyces* sp. M7 inoculum size was optimized. Also, it was fitted a model to study this process, and it was possible to predict the system performance, knowing the initial conditions. Moreover, optimum temperature and humidity conditions for the bioremediation of soil with different concentrations of Cr(VI) and lindane were determined. Lettuce seedlings were a suitable biomarker to evaluate the contaminants mixture toxicity. *Streptomyces* sp. M7 carried out a successful bioremediation, which was demonstrated through ecotoxicity test with *Lactuca sativa*.

1. Introduction

Industrialization and urbanization have led to serious problems of soil contamination, by both organic (polyphenols, pesticides, etc.) and inorganic compounds (Cd, Cu, Cr, etc.). Mixed pollution is a global problem, hence it affects more than one third of contaminated sites (Tang et al., 2010; Mansour, 2012).

Chromium contamination in soil and water has been detected in and around industrial sites (Benimeli et al., 2003; Nie et al., 2010; Srinivasa Gowd et al., 2010). Cr(VI) is a harmful pollutant, neurotoxic, dermatotoxic, genotoxic, carcinogenic and immunotoxic (Bagchi et al., 2002). On the other hand, residues of the gamma isomer of hexachlorocyclohexane (γ-HCH), commercially known as lindane, have been reported in soils, water, air, plants and animals, because of its indiscriminate use, principally in agriculture practices (Fuentes et al., 2011). Lindane is highly recalcitrant, and produces several health effects, such as neurological problems and cancer (Saez et al., 2012). Moreover, mixed pollution by chromium and lindane has been detected around the world in water, sediment and soil, at concentrations up to 140 mg kg^{-1} and 400 μg kg^{-1}, for chromium and lindane respectively (Benimeli et al., 2003; Maggi et al., 2012; Arienzo et al., 2013; Coatu et al., 2013).

The treatment of co-contaminated soils is complex, as the chemical processes and remediation technologies are different for each group of pollutants (Puzon et al., 2002; Dong et al., 2013). Bioremediation is a low cost technology, which simultaneously allows the degradation of organic compounds and the removal or stabilization of metals into non-toxic or less toxic forms (Owabor

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http://dx.doi.org/10.1016/j.ecoenv.2015.02.036
0147-6513/

et al., 2013).

The composition of the microbial population dictates the overall microbial degradation process (Owabor et al., 2013). However, the bioremediation effectiveness is subject to several factors which interact in complex ways, depending on the matrix and contaminant characteristics, among others. When a system is affected by a large number of independent factors, experimental design methods are commonly used to systematically determine the effective factors and their interactions, as well as to model and optimize the whole system. Through full factorial design maximum information regarding the factors is obtained. It is possible to identify the interactions between separate experimental factors and to predict the effect that such interactions could have on the experimental response (Antony, 2003; Mason et al., 2003). Thus, biological treatments could be improved using this approach. Actinobacteria represent an important component of the microbial population in soils. They have demonstrated bioremediation ability (Benimeli et al., 2008; Polti et al., 2009; Albarracín et al., 2010; Alvarez et al., 2012). In particular, *Streptomyces* sp. M7 was able to bioremediate simultaneously Cr(VI) and lindane from non-sterilized soils (Polti et al., 2014). However, to assess whether bioremediation processes are acceptable, it is mandatory to investigate toxic effects of microbial metabolites produced during the pollutant removal (Repetto et al., 2001). Bioindicators change their response in front of changes in environmental pollution. *Lactuca sativa* is a recommended specie for this purpose (Charles et al., 2011), since it allows evaluating lethal and sublethal effects and it can be used in samples with high turbidity, reducing pretreatment interference. Furthermore, it has high sensitivity, so it requires reduced exposure time, it has low cost and does not require sophisticated equipment (Sobrero et al., 2004).

The aims of this work were to statistically optimize environmental factors for bioremediation of lindane and Cr(VI) by *Streptomyces* sp. M7 in soil, and further, to prove the efficiency of this bioprocess by using *L. sativa* as bioindicator.

2. Materials and methods

2.1. Bacterial strain, culture medium and chemicals

Lindane (γ-HCH) (99% pure) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other chemicals used during the study were analytical grade and purchased from standard manufacturers. Cr(VI) was added as $K_2Cr_2O_7$ (Benimeli et al., 2003, 2007; Polti et al., 2007).

The actinobacterium used was *Streptomyces* sp. M7, previously isolated from sediments contaminated with organochlorine pesticides and heavy metals, and then characterized by Benimeli et al. (2003). The *Streptomyces* sp. M7 inoculum was obtained by cultivating the strain in Tryptic Soy Broth, containing (in g L^{-1}): tryptone, 15; soy peptone, 3; NaCl, 5; K_2HPO_4, 2.5; and glucose, 2.5, during 3 days at 30 °C (200 rpm) (Polti et al., 2009).

2.2. Soil samples: preparation and inoculation

Non-polluted soil samples (SS) were collected from an experimental site near the city of Tucumán, in northwest Argentina. The samples were taken from near the surface (5–15 cm deep) and stored in darkness at 10–15 °C until being used. 200 g of soil were put into each glass pot, and humidity content was fixed using distilled water.

The SS were inoculated with *Streptomyces* sp. M7 (0.5, 1, 2, or 4 g kg^{-1}) and contaminated with lindane and Cr(VI). The glass pots were then incubated during 14 days. Also, inoculated SS without toxics and non-inoculated SS with both toxics were used as controls. In all cases, samples were taken at the end of each assay to determine both lindane and bioavailable chromium residual concentrations.

2.3. Analytical determinations

The extraction procedure for γ-HCH in soil was performed as follow: 5 g of soil were transferred to centrifuge tubes and mixed with 10 mL of a 4:1:5 water-methanol-hexane solution. The tubes were hermetically sealed and shaken during 10 min in order to allow the extraction of lindane from soil to the organic phase, and then centrifuged (2500*g* during 10 min) for separation of the organic and aqueous phases. Organic phase was evaporated to dryness. The residues were suspended in hexane and analysed by Gas Chromatography. Extracts were quantified in a Gas Chromatograph Agilent 7890 A 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 Chem Station software. Quantitative sample analysis was performed using appropriate calibration standards (AccuStandard) (Fuentes et al., 2011).

Potentially bioavailable chromium in the soil was extracted by a physical method: 100 g of soil were centrifuged at 5050*g* during 60 min, in order to reproduce the maximum plant suction (soil water potential: 1500 kPa, conventional wilting point) (Csillag et al., 1999). Supernatant was recovered, filtered at 0.45 nm and analysed by Atomic Absorption Spectrometry, using a Perkin Elmer Analyst 400 (AAS) for Cr content (Polti et al., 2011).

2.4. Experimental design and statistical analysis

Experimental design and analysis were performed using MINITAB 17(PA, USA) statistical software. Statistical significance values for the means were evaluated using one-way analysis of variance. Differences were accepted as significant when $p < 0.05$. In order to identify the main effects of the selected factors and the interactions among them, a 2^3 full factorial design was applied. Three extra replicates were included as centre points. The experimental variables evaluated are presented in Table S1 (see Supplementary Table S1 in EES Online), which shows the two alternative options tested for each factor. All assays were performed in triplicate and the results are presented as the mean value ± standard deviation. Associations between variables were assessed by using Pearson's correlation coefficient.

In each sample two responses were evaluated, including residual Cr(VI) and lindane. The results of the experimental design were studied and interpreted using MINITAB 17 (PA, USA) statistical software to estimate the response of the dependent variable (Martorell et al., 2012).

2.5. Phytotoxicity test

To assess the bioremediation success, three parameters were assessed on lettuce seedlings (*Lactuca sativa*): germination, root elongation and hypocotyl elongation. Thirty seeds were placed into sterile Petri plates containing 15 g of soil sample bioremediated by *Streptomyces* sp. M7. Biotic and abiotic soil samples were used as controls. Petri plates were sealed and incubated at 22 ± 2 °C in darkness, during 5 days. At the end of the incubation period, the number of germinated seeds was registered. The length of roots and hypocotyl was measured by using a millimetre scale. Vigour index ((mean root length + mean hypocotyl length) × (percent germination/10)) was also calculated (Bidlan et al., 2004; Fuentes et al., 2013; Saez et al., 2014).

3. Results

3.1. Simultaneous removal of CR(VI) and lindane by Streptomyces SP. M7: inoculum optimization

Cr(VI) and lindane removal in SS inoculated with *Streptomyces* sp. M7 is showed in Fig. 1. In order to select the optimum inoculum concentration, a statistical analysis was carried out. The lowest Cr(VI) removal (54.6%) occurred when SS were inoculated with 0.5 g kg^{-1} of *Streptomyces* sp. M7; while the highest removal (89.5%) was achieved with 4 g kg^{-1} of *Streptomyces* sp. M7. No significant differences were observed in Cr(VI) removal achieved by the different inoculum concentrations tested; however, they were significantly higher compared to the removal observed in uninoculated SS.

The lowest lindane removal (6%) was achieved with 4 g kg^{-1} of *Streptomyces* sp. M7, showing no statistical differences ($p < 0.05$) compared to the uninoculated SS (Fig. 1). The maximum lindane removal (38%) was obtained when 2 g kg^{-1} of *Streptomyces* sp. M7 was inoculated in SS, and it was significantly greater than the obtained with 0.5 and 4 g kg^{-1}. However, no significant differences were observed with 1 g kg^{-1} of *Streptomyces* sp. M7.

Based on the statistical analysis, the inoculum of 1 g kg^{-1} of *Streptomyces* sp. M7 was selected for further assays, since it was the lowest inoculum concentration which allowed the highest simultaneous removal of Cr(VI) and lindane.

3.2. Evaluation of the influence of physical and chemical parameters on soil samples bioremediation by Streptomyces SP. M7

The effects and interactions between humidity, temperature, initial Cr(VI) and lindane concentration on SS bioremediation were evaluated by using a full factorial design, with 4 factors and 2 levels plus a centre point. The evaluated responses were residual Cr (VI) and lindane concentrations. The factorial design and experimental results are shown in Table S1.

The statistical analysis of this model employed Tukey's test. Analysis of variance (ANOVA) for residual Cr(VI) and lindane concentrations showed that residual Cr(VI) concentration was significantly lower than those found in uninoculated control, at 16 evaluated conditions (Fig. 2). On the other hand, residual lindane concentration was significantly lower than those obtained in uninoculated control, at 14 evaluated conditions (Fig. 2).

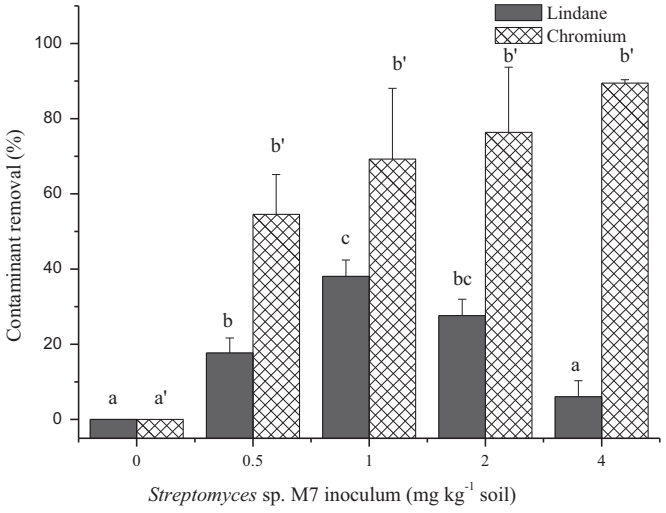

Fig. 1. Contaminants removal in Soil Samples inoculated with *Streptomyces* sp. M7, after 14 days of incubation at 30 °C. Means with different letters are significantly different ($p < 0.05$).

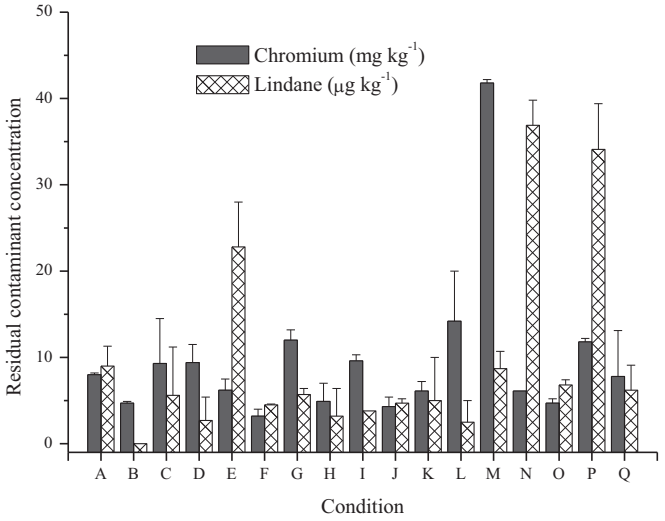

Fig. 2. Residual contaminants concentration in Soil Samples inoculated with *Streptomyces* sp. M7, after 14 days of incubation at 30 °C.

Main effects on both evaluated responses were analysed (Supplementary Fig. S1). The lowest residual Cr(VI) concentration was reached at the highest temperature and the lowest humidity and initial Cr(VI) concentration tested. Initial lindane concentration did not show statistically significant effect on Cr(VI) removal. Moreover, interactions were observed among initial Cr(VI) concentration, temperature and humidity. On the other hand, main effects analysis showed lower residual lindane concentrations by increasing the initial Cr(VI) concentration and reducing the initial lindane concentration and humidity. Temperature did not have significant effect, however, interactions were observed among temperature and both initial Cr(VI) concentration and humidity.

Significant terms were selected by backward elimination (Minitab 17) in order to assemble a first-order lineal model to explain the evaluated responses (Supplementary Tables S2 and S3). This procedure starts with all potential terms in the model and removes the least significant term for each step, obtaining the best regression equation for residual concentrations of Cr(VI) (1) and lindane (2)

$$\begin{aligned} &\textit{Residual Cr(VI) concentration} \\ &= 32.1 - 0.271Cr(VI)i - 1.119\,T - 2.05H \\ &\quad + 0.150Li + 0.0126Cr(VI)iT + 0.0679Cr(VI)iH \\ &\quad - 0.0415Cr(VI)iLi + 0.076TH \\ &\quad - 0.002008Cr(VI)iTH \end{aligned} \tag{1}$$

A high correlation was observed between residual Cr(VI) concentration, experimental values, and those predicted by the statistical model. The r^2 value was 0.8013. Moreover, predicted r^2 was 0.6094, indicating that the model could explain the 80.13% of the observed data and could predict more than 60% of new data.

$$\begin{aligned} &\textit{Residual Lindane concentration} \\ &= 43.4 - 0.292Cr(VI)i - 1.634T - 3.95H \\ &\quad +0.2153Li + 0.0122Cr(VI)iT + 0.0453Cr(VI)iH \\ &\quad + 0.1598TH - 0.001837Cr(VI)iTH \end{aligned} \tag{2}$$

The fit of this model was verified with the value of r^2 0.7343, indicating that 73.43% of the variability in the response "Residual lindane concentration" could be explained by the variation of the analysed factors and their interactions. However, predicted r^2 was 0.4910, suggesting that the model will not predict new

Table 1
Optimum bioremediation conditions obtained after run the response optimizer (Minitab 17).

Initial concentrations		Optimum conditions	
Cr(VI) (mg kg^{-1})	Lindane (μg kg^{-1})	Temperature (°C)	Humidity (%)
20	10	25	30
20	40	35	10
80	10	35	10
80	40	35	10

observations nearly as well as it fitted the existing data.

Also, a response optimizer was run. It is a method which allows compromise among various responses (Minitab 17). All possible combinations were considered, in terms of initial concentrations of Cr(VI) and lindane, which could be present in a natural co-contaminated environment (Table 1). The optimum conditions for bioremediation was 35 °C and 10% of humidity, in three of the four evaluated scenarios; while, in the last scenario (low initial concentrations of both pollutants), the optimum bioremediation conditions were 25 °C and 30% of humidity.

3.3. Assessment of the efficacy of soil bioremediation

Previous studies confirm that *Lactuca sativa* can be used as biomarker for monitoring bioremediation. For this purpose, lettuce seeds were cultured in soil, from three bioremediation conditions: D, Q and I (Table S1), which were randomly selected among the ones which showed significant differences with contaminated and non-bioremediated soil. Tested seeds were found to be adversely affected by the addition of the contaminated soil. The statistic relation between evaluated toxic effects and the initial and final factor concentrations was analysed. Only an inverse relationship between the toxic effects observed in seedlings and Cr(VI) initial concentration (r^2=0.9154) was observed.

Roots and hypocotyls lengths and the vigour index (VI) were significantly lower in contaminated SS compared to non-contaminated soils (Table 2). At D and I conditions, significant differences were not observed in roots length of seedlings grown on bioremediated and non-bioremediated SS. However, at Q condition, significant differences were observed between root lengths developed on bioremediated and non-bioremediated SS (Fig. 3A). On the other hand, at all assayed conditions, VI and hypocotyls length were significantly higher in bioremediated SS than in non-bioremediated ones. Moreover, at D and I conditions, VI and hypocotyls lengths did not show significant differences between bioremediated and non- contaminated SS (Fig. 3B).

At Q condition, lettuce seeds were not able to germinate before bioremediation. However, after this process, germination was greater than 50% (data not shown). These results are in accordance with the achieved contaminants removal.

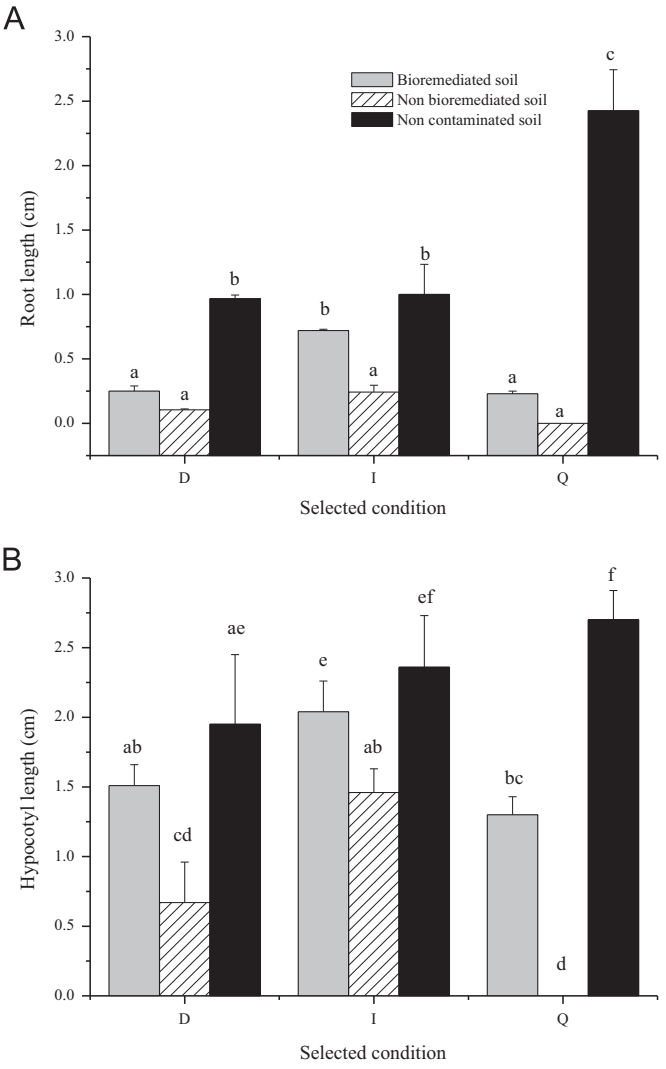

Fig. 3. Development of lettuce seedlings cultivated on soils: (A) Root length; (B) Hypocotyl length. Means with different letters are significantly different ($p < 0.05$).

4. Discussion

The optimization of a biotechnological process involves the evaluation of different parameters affecting the effectiveness and profitability of the process. It is therefore essential to optimize the quantity of microbial biomass required, since the production of the same significantly affects the costs of the process (Wolski et al., 2007). In a previous work it was observed, similarly, that the highest inoculum concentration was not the optimum for lindane removal by using *Streptomyces* sp. M7 (Benimeli et al., 2008). Previously, Polti et al. (2014) reported that chromium and lindane

Table 2
Factors and levels of selected conditions. Vigour Index (VI) of lettuce seedlings.

Selected conditions	Initial Cr (VI) (mg kg^{-1})	Initial Lindane (μg kg^{-1})	Temperature (°C)	Humidity (%)	Residual Cr (VI) (mg kg^{-1})	Residual Lindane (μg kg^{-1})	VI		
							Bioremediatedsoil	Non bioremediated control	Non contaminated control
D	20	40	35	10	2.7	9.4	$21.98 \pm 0.18^{cd*}$	9.38 ± 0.66^{be}	27.23 ± 0.92^{c}
I	50	25	30	20	7.8	9.8	11.27 ± 0.58^{ab}	2.22 ± 0.54^{ef}	18.23 ± 0.20^{ad}
Q	80	40	35	30	6.2	7.8	8.23 ± 0.98^{be}	0.00 ± 0.00^{f}	22.57 ± 3.37^{cd}

*Means with different letters are significantly different ($p < 0.05$).VI=(mean root length+mean shoot length) × percentage of germination/10.

removal did not occur simultaneously. First, metal is reduced, and then the pesticide is degraded. The Cr(VI) reduction to Cr(III) is a process which uses NADH from bacterial metabolism and, therefore, any process that affect its production affects the reduction of Cr(VI) (Polti et al., 2010). Also, electron acceptors affect significantly lindane degradation (Robles-González et al., 2012). It is a co-metabolic process, which improves with an additional energy source (Benimeli et al., 2006; Benimeli et al., 2008). Soil energy sources could be used primarily to obtain NADH for Cr(VI) reduction, and residual carbon sources could be used for lindane removal. A large inoculum size could use all the energy sources for cell reproduction and, therefore, the residual energy for lindane degradation would be lower and thus a significant removal of the pesticide would not be achieved.

Differences in biological and physicochemical properties allow establishing different accepted concentrations for Cr(III) and Cr(VI) in water and soil. Classic way to long-term isolation of chromium waste includes a permanent reducing environment and permanent immobilization of reduced chromium (Bartlett, 1991). However, it is possible to avoid Cr(VI) mobility, and hence toxicity, by regulating matrix moisture content. The moisture influences on the contaminated soil chemistry and affects the amount of dissolved minerals, the pH and the redox soil potential, which are mandatory on chromium mobility. Chromium behaviour could not be predicted based only on soil humidity content; it is necessary to evaluate simultaneously the influence of several chemical and physical parameters. According to our results, in general, low humidity levels favoured Cr(VI) removal, however, there was a particular situation, where highest Cr(VI) removal was achieved at high humidity levels. In this case, both contaminants initial concentrations were low. These observations could indicate that at high concentrations of pollutants, the major removal mechanism was physicochemical, including low mobility and high adsorption to soil particles; whereas, at low contaminant concentrations, Cr (VI) removal could result from microbial activity.

Experimental data indicated that lindane has high affinity for organic matter which is due to its hydrophobic nature (Robles-González et al., 2006). Therefore, lindane mobility decreases by increasing humidity (Willett et al., 1998). In order to extract lindane from soil, water-immiscible solvents with affinity for hydrophobic compounds are necessary. This can help in attracting the contaminants molecules adsorbed onto soil, transferring the contaminant into the solvent phase and, afterwards, facilitating the exchange of contaminant between the solvent and the aqueous phase where the microorganisms can finally degrade the pollutant (Robles-González et al., 2012). This agrees with our results, since lindane removal was increased at low humidity level.

Temperature affects several processes involved in the accumulation of organochlorine pesticides and heavy metals in the soil. This effect may be direct, by modifying the adsorption/desorption, diffusion, volatilization and degradation/chemical reduction of the compounds, or indirectly, by increasing soil microbial activity, favoring biological removal processes (Dhal et al., 2013; Navarro et al., 2013). The way these factors affect the bioremediation process is complex, and may have opposite effects and interactions. The temperature and humidity affect microbial activity, and thereby facilitate or inhibit the degradation of lindane (Ali et al., 2014). Furthermore, Cr(VI) could be toxic to the microorganisms involved in the process; however, lindane removal was higher with high Cr(VI) initial concentrations. Possible mechanisms of resistance to Cr(VI) by this actinobacterium prevent its metabolic activity is affected and therefore, its ability to remove lindane (Ali et al., 2014). We observed negative effects of temperature on Cr(VI) removal. This could be explained by the optimum growth temperature of soil actinobacteria, which is between 25 and 30 °C. However, upon optimizer response, higher temperature conditions promote contaminants removal when one of them was at high initial concentration, which could be related to non-biological activity. Several studies indicate that the presence of metals in a contaminated site affects microbial population development, causing growth rate decreasing and inhibition of degradative metabolism (Lin et al., 2006; Hong et al., 2007; Moreira et al., 2013).

Bioremediation strategies should aim at causing the least possible disturbance in the polluted area, using low-cost technologies. With this premise, it was determined the optimum temperature and humidity for the bioremediation of Cr(VI) and lindane contaminated environments. The technologies used to achieve temperature increasing involve assembling piles of soil, and subsequent coverage. In contrast, the temperature decrease is performed by removing the soil, mixing/uprising material, which besides decreasing temperature, promotes soil aeration (Kauppi et al., 2011; Kalantidou et al., 2012). The humidity can be up regulated by watering, manually or mechanically. On the other hand, humidity content can be decreased by exposing the material to the sun, evaporating water gradually. These strategies could be employed to change the temperature and humidity of the contaminated environment, during treatment with actinobacteria, for enhancing their bioremediation activity.

The observed toxic effects of these contaminants on lettuce seedlings confirm that it is an appropriate indicator for studying the process efficiency. Lindane is phytotoxic, and it disrupts vital processes in plants, including normal cell growth and photosynthesis (Calvelo Pereira et al., 2006). It can also affect germination, causing imbalance in biochemical processes in the seed (Bidlan et al., 2004; Saez et al., 2014). Moreover, high availability of metals, such as Cr(VI), can induce physiological and biochemical changes, including inhibition of root growth, and interveinal chlorosis with chlorophyll reduction (Sharma et al., 2003; Polti et al., 2011). Similar to Saez et al. (2014), lettuce roots showed less development than hypocotyls, suggesting that the capacity for exploring the substrate and obtaining resources for correct growth was reduced. It is interesting how effects were reversed after remediation. It is remarkable that at all tested conditions, properties of bioremediated soil improved significantly, even approaching its behaviour to the uncontaminated control. Although, a relationship between residual toxic concentrations was not found, indicating that factors affected the system as a whole. According to these results, it could be inferred that there was an effective reduction of lindane and Cr(VI) bioavailability in co-contaminated soil samples, with generation of less toxic or non-toxic metabolites.

These results represent a significant advance in the study of co-contaminated environment bioremediation, and the next step would be scaling the process to achieve bioremediation of bigger environments that suffer mixed contamination.

Acknowledgements

The authors gratefully acknowledge financial support of Consejo de Investigaciones de la Universidad Nacional de Tucumán (CIUNT), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). The authors would also like to thank Juliana Saez for meticulous proof-reading of the article.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2015.02.036.

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