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Comparative study of single and mixed cultures of actinobacteria for the bioremediation of co-contaminated matrices

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ABSTRACT

With the aim of comparing the behavior of single and mixed cultures of actinobacteria for the removal of chromium and lindane from different co-contaminated systems, the actinobacteria *Streptomyces* sp. M7, MC1, A5 and *Amycolatopsis tucumanensis* DSM 45259 were assayed as simple, double, triple, and quadruple cultures in minimal medium and soil artificially polluted with Cr(VI) and lindane. In liquid medium, *Streptomyces* sp. MC1 and the quadruple consortium reached the highest Cr(VI) and lindane removal (94% and 52%, respectively, in both cultures), although they did not present statistically significant differences in Cr(VI) removal respect to six other consortia. The best removal of bioavailable chromium and lindane from artificially contaminated soil were achieved by *Streptomyces* sp. M7 (50% and 60%, respectively) and the quadruple consortium (60% and 55%, respectively). The bioassay with *Lactuca sativa* demonstrated the success of the bioremediation in soil by *Streptomyces* sp. M7 and the quadruple consortium through an increase in the vigor index measured in lettuce seedlings compared to non-treated soil. The quadruple consortium demonstrated to be appropriate to bioremediate Cr(VI) and lindane co-contaminated both liquid and soil systems. The viability of the four strains after the bioremediation process was confirmed. These results represent an approximation for conducting a field-scale bioremediation strategy.

1. Introduction

Increasing water and soil pollution problems resulting from industrial and agricultural activities have caused worldwide concerns [1]. Large numbers of contaminants entering the environmental matrices pose a huge threat to human health and natural ecosystem [2]. This problem is extremely acute in areas where the presence of different families of organic pollutants is accompanied by heavy metals, at concentrations exceeding permissible levels [3].

Chromium is a heavy metal widely used in a variety of industrial processes (leather tanning, steel production, metal corrosion inhibition), mainly as Cr(VI) [4]. Industrial effluents containing Cr(VI) are released into water courses, mostly without proper treatment, resulting in anthropogenic contamination. Due to its strong oxidizing potential, Cr(VI) induces acute and chronic toxicity, neurotoxicity, dermatotoxicity, genotoxicity, carcinogenicity, immunotoxicity, and general environmental toxicity [5–8].

Lindane, the gamma isomer of hexachlorocyclohexane, has been widely used for both agriculture and medical purposes before its use has been restricted in most countries [9]. This organochlorine pesticide is highly recalcitrant and produces several health effects [10] and it was recently classified as carcinogenic to humans (Group 1) by the International Agency for Research on Cancer [11]. Moreover, the presence of lindane residues has been reported in soils, water, air, plants, agricultural products, and animals, as well as in human body [12,13].

Mixed pollution by chromium and lindane has been detected in sediment and soil samples, at concentrations up to 140 mg kg^{-1} and 400 mg kg^{-1}, respectively [14–16]. Argentina is no stranger to this problem. Recently, Aparicio et al. [17] detected lindane and total Cr in soils from Lerma Valley (Salta, Argentina) at concentrations over the permissible levels established in the Federal Hazardous Waste Law N° 24051 (10 µg kg^{-1} and 250 mg kg^{-1}, respectively).

The restoration of environments co-contaminated with organic compounds and heavy metals is a big challenge because of the different

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nature of these pollutants and the interaction between them [18]. Remediation technologies involve any operation that alters the characteristics of polluting wastes in order to reduce their toxicity, volume or mobility [19]. Some of the physical and chemical technologies studied are oxidation of fuels, solvents, and pesticides, photocatalysis of pesticides [20], chemical reduction of heavy metals, supercritical fluid extraction, adsorption, filtration, and precipitation [21]. These technologies can be effective in reducing the levels of pollutants, but they also have several disadvantages, including high specificity, complexity, high costs, and lack of acceptance by the population [22]. In contrast, biological treatments have received considerable attention as an effective biotechnological tool to degrade, remove, and/or transform hazardous wastes through the use of different organisms or their derivatives [23]. This eco-friendly remediation, called bioremediation, allows the degradation of organic compounds and the removal or stabilization of metals into non-toxic or less toxic forms, either simultaneously or sequentially [24]. In this sense, studies are focusing on microorganisms with both abilities. Among such microorganisms, actinobacteria stand out, presenting a cosmopolitan distribution in aquatic and terrestrial ecosystems. They are a group of bacteria with an important role in the environment and great ability to remove several pollutants such as oil, hydrocarbons, pesticides, and heavy metals, among others [25–31]. In this context, the treatment of co-polluted matrices with actinobacteria with the ability to degrade pesticides and detoxify heavy metals represents a promising bioremediation approach [32,33].

In natural environments, communities dominate the microbial world and the existing diversity allows an increase of metabolic capabilities, division of labor, and survival to perturbations [34]. In particular, in bioremediation, the division of labor is crucial, and for this purpose, the formulation of defined consortia would facilitate the examination of the characteristics of each one of its members, and the monitoring of their dynamics together [35]. In this sense, an actinobacteria consortium composed of the strains *Streptomyces* sp. A5, MC1, M7, and *Amycolatopsis tucumanensis* DSM 45259 was formulated for the bioremediation of real co-contaminated soil samples from the Northwest of Argentina [17]. However, it is relevant to consider that although simple culture has metabolic limitations, the use of mixed culture has technological limitations [36]. For the development of a bioprocess, thus, it should be pursued the best cost-benefit relationship. The novelty of the present work lies in evaluating, for each particular scenario, the convenience of using single or mixed culture as bioremediation strategy. In this sense, the performances of pure and mixed cultures of actinobacteria to remove Cr(VI) and lindane from artificially co-contaminated liquid and soil systems were comparatively evaluated. Also, the effectiveness of soil bioremediation and the viability of the inoculated actinobacteria were assessed.

2. Materials and methods

2.1. Chemicals

Lindane (γ-HCH, 99% pure) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All chemicals used in this study were purchased from standard manufacturers. Solvents were of pesticide grade and all other chemicals were of analytical grade. Lindane was dissolved in acetone in order to obtain a stock solution (50 mg mL^{-1}). Cr(VI) was added as $K_2Cr_2O_7$.

2.2. Bacterial strains and culture media

Four previously isolated actinobacteria were used in this study: *Streptomyces* sp. M7 [27] and *Amycolatopsis tucumanensis* DSM 45259 [26], isolated from wastewater of a copper filter plant, *Streptomyces* sp. MC1, isolated from sugar cane plant contaminated with pesticides and heavy metals [37], and *Streptomyces* sp. A5, isolated from soil samples contaminated with several organochlorine pesticides [38]. These actinobacteria were selected based on their ability to remove chromium and lindane [26,28,31,38].

The strains were cultured in Petri dishes with Starch Casein medium (SC), containing in g L^{-1}: starch, 10.0; casein, 1.0; K_2HPO_4, 0.5; agar, 15.0. The pH was adjusted to 7.0 ± 0.2 prior to sterilization. Petri dishes were incubated at 30 °C for 7 d.

Removal assays in liquid culture were carried out in Minimal Medium (MM), which contained in g L^{-1}: glucose, 5; $(NH_4)_2SO_4$, 2; K_2HPO_4, 0.5; $MgSO_4{\cdot}7H_20$, 0.2; $FeSO_4{\cdot}7H_20$, 0.01. The pH was adjusted to 7.0 ± 0.2.

Tryptic Soy Broth (TSB), containing in g L^{-1}: tryptone, 15; soy peptone, 3; NaCl, 5; K_2HPO_4, 2.5; and glucose, 2.5, was used for the preparation of inocula for removal assays in soil. The pH was adjusted to 7.3 ± 0.2.

Mueller–Hinton medium (MH) was used to evaluate the sensitivity to antibiotics. It contained in g L^{-1}: acid hydrolysate of casein, 17.5; beef extract, 3; starch, 1.5; agar, 15. The pH was adjusted to 7.3 ± 0.1.

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

2.3. Bioremediation assays

2.3.1. Removal assays in liquid system

Flasks with 30 mL of MM were supplemented with 250 μg L^{-1} of lindane and Cr(VI) at a final concentration of 25 mg L^{-1}. Standardized spore suspensions of the four strains (10^9 CFU mL^{-1}) harvested from SC were inoculated, either individually or by making all possible combinations of two, three or four strains to obtain the consortia. The cultures were incubated on an orbital shaker (0.85 × *g*) at 30 °C. Non-contaminated MM and non-inoculated MM were used as biotic and abiotic controls, respectively. After 120 h of incubation, microbial biomass was harvested by centrifugation at 8385 × *g* for 15 min at 4 °C; cells were washed twice with sterile distilled water and dried at 105 °C to constant weight. The supernatants of the cultures were used to determine residual lindane and Cr(VI) concentrations.

2.3.2. Removal assays in soil

2.3.2.1. Preparation of the inocula.. Spore suspensions of the four actinobacteria strains harvested from SC were individually cultured in TSB and incubated at 30 °C for 72 h on an orbital shaker (0.85 × *g*). Then, the pellets were harvested by centrifugation at 8385 × *g* and washed twice with sterile distilled water. The biomass was resuspended in 0.9% NaCl at 0.1 g L^{-1}.

2.3.2.2. Preparation and inoculation of soil.. Non-contaminated soil (NCS) was collected from an urban area in the city of Tucumán, in northwestern Argentina (26°48′36.6″S 65°14′28.0″W). It was taken from near the surface (5–15 cm deep) and stored in the dark at 10–15 °C until being used. The physicochemical characteristics of the soil are listed in Table 1. Glass pots were filled with 200 g of soil and 20% humidity was fixed using distilled water. The pots were kept for 36 h at room temperature so that water in the soil was balanced.

The soil pots were then contaminated with 25 μg kg^{-1} of lindane and 50 mg kg^{-1} of Cr(VI). After a stabilization period of 14 days, the contaminated soils were inoculated with either each actinobacterium strain individually or with all possible combination of double, triple, or quadruple mixed culture to reach a final inoculum concentration of 2 g kg^{-1}. Soil, inoculum and both contaminants were mixed thoroughly to ensure a uniform distribution. Also, non-inoculated co-contaminated soils were used as controls. The pots were incubated at 30 °C for 14 days. Soil humidity was monitored twice a week. Samples were taken at the end of the assay to determine both residual lindane and bioavailable chromium concentrations and to perform the phytotoxicity bioassay.

Table 1
Physicochemical characteristics of the soil used in this study.

Parameters	
pH[1]	6.75
Calcareous[2], %	20–40
Organic matter[3], %	0.92
Organic carbon[3], %	0.25
Total Nitrogen[4], %	0.214
Phosphorus[5], ppm	14.5
Clay[6], %	42.9
Silt[6], %	40.8
Sand[6], %	16.2
Texture[6]	Loamy

[1] Soil to distilled water ratio of 1:2.5.
[2] Calcimetry.
[3] Walkley-Black method.
[4] Kjeldahl method.
[5] Bray-Kurtz method.
[6] Soil texture analysis by hydrometer: modification of the Bouyoucos method.

2.3.3. Comparative evaluation of pure and mixed cultures performances

In order to compare the pollutants removal obtained by the defined consortia versus the obtained by the pure cultures in both liquid and soil systems, the ratio R was calculated as follows: R = O/E, where O is the removal obtained by the mixed culture and E is the expected removal, calculated as the average of the removal obtained by the pure cultures of each strain forming the mixed culture values [12]. When $R > 1$ it indicates possible synergism, $R < 1$ indicates possible antagonism and $R = 1$ indicates that there is no detectable interaction among the actinobacteria forming the mixed culture.

2.4. Lindane determination

The extraction procedure of lindane residues from soil was performed according to Fuentes et al. [12]. Briefly, 10 mL of a water–-methanol–hexane solution (4:1:5) were added to 5 g of soil and stirred for 10 min. After a centrifugation, an adequate volume of the organic phase was collected and evaporated to dryness. Finally, lindane residues were resuspended in hexane to obtain the extract.

In liquid cultures, residual lindane was extracted from the supernatants (8385 g, 30 min, 4 °C) by solid phase extraction using a C18 column (Agilent, Lake Forest, USA).

Extracts obtained from liquid and soil systems were quantified in a Gas Chromatograph Agilent 7890A equipped with an HP5 capillary column (30 m × 0.53 mm × 0.35 m) and ^{63}Ni micro-electron capture detector, a split/splitless Agilent 7693 B injector, and Agilent Chemstation software. Quantitative analyses were performed using appropriate calibration standards (AccuStandard, New Haven, CT, USA).

The recovery of the method was 86 ± 9% for the liquid system and 103 ± 5% for soil samples.

2.5. Determination of Cr(VI) and bioavailable chromium

Cr(VI) concentration was determined in aliquots of supernatants from liquid cultures, using the Cr(VI) specific colorimetric reagent 1,5-diphenylcarbazide, dissolved in acetone to a final concentration of 5 mg mL^{-1}, as described in EPA method 7196A [7].

Potentially bioavailable chromium in soil was determined by a physical method: 100 g of soil were centrifuged at 5050 × *g* for 60 min, to reproduce the maximal plant suction (soil water potential: 1500 kPa, conventional wilting point) [33,39]. After centrifugation, the supernatant was recovered and filtered at 0.45 mm. Soil Extracts were analyzed by atomic absorption spectrometry using a Perkin Elmer Analyst 400 for Cr content [40].

2.6. Bioassay

To assess the success of the soils bioremediation, a bioassay with lettuce (*Lactuca sativa*) was performed. Thirty seeds were placed into sterile Petri dishes containing 15 g of a soil sample from each condition. Artificially contaminated soil (CS) and non-contaminated soil (NCS) samples were used as controls. Petri dishes were sealed and incubated at 22 ± 2 °C in darkness for 120 h. Then, the number of germinated seeds was registered, and the length of roots and hypocotyls of the seedlings was measured by using a millimeter scale. Vigor index was calculated as (mean root length + mean hypocotyl length) × germination percentage/10 [32].

2.7. Strains survival evaluation

Antibiotic sensitivity test and Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) of the four actinobacteria strains were performed in order to find differential characteristics between the strains, with the aim of using these biochemical and molecular approaches to determine their survival in the soil after the bioremediation assay.

The sensitivity of the strains against 25 antibiotics was evaluated by using the agar diffusion method [41]. Each strain was inoculated in plates containing MH; then, commercially-prepared antibiotic disks were aseptically placed on the inoculated agar surface. Plates were incubated for 7 days at 37 °C. Afterward, the growth inhibition zones around each antibiotic disk were measured by using a millimeter scale and results were interpreted by using the standard criteria [42]. Different concentrations of the selected antibiotics were later assayed to determine the lowest effective concentration necessary to reisolate each strain.

Detection of genetic polymorphisms characteristic of each strain was performed by RAPD-PCR. The four actinobacteria were individually cultured in TSB. Total DNA extraction of each culture was performed as described by Polti et al. [37]. The primers used were DA F 5′ −GAG GTC GTG CTG ACC GTG CTG CA-3′ and DA R 5′-CTG GTGGTT GCC GAT GAC GTC GT-3′ [41]. Different annealing temperatures (50, 52, 55, 57, or 60 °C) were used to obtain a characteristic profile of every single actinobacterium. Finally, products were visualized in polyacrylamide gels stained with 6% $AgNO_3$.

2.8. Statistical analyses

Three replicates were conducted in all assays and the results are the average of them. One-way analysis of variance (ANOVA) was used to test the significant differences. When significant differences were found, Tukey test was used to separate the effects. Tests were considered significantly different at $p < 0.05$. Statistical analyses were performed using a professional version of Minitab®17 statistical software (PA, USA).

3. Results and discussion

3.1. Removal of Cr(VI) and lindane by single and mixed cultures of actinobacteria in liquid systems

The microbial growth of the single and mixed cultures in the presence of the contaminants did not show statistically significant differences with their respective controls without pollutants (Fig. S1), thus evidencing that the concentrations used did not result toxic for the actinobacteria. This is not surprising considering that these strains were isolated from contaminated sites, where they have been exposed to extreme conditions; hence they may have developed adaptive mechanisms to tolerate and grow under those adverse conditions [43]. In fact, previous studies have already demonstrated that these strains were tolerant to higher concentrations of both pollutants in solid medium

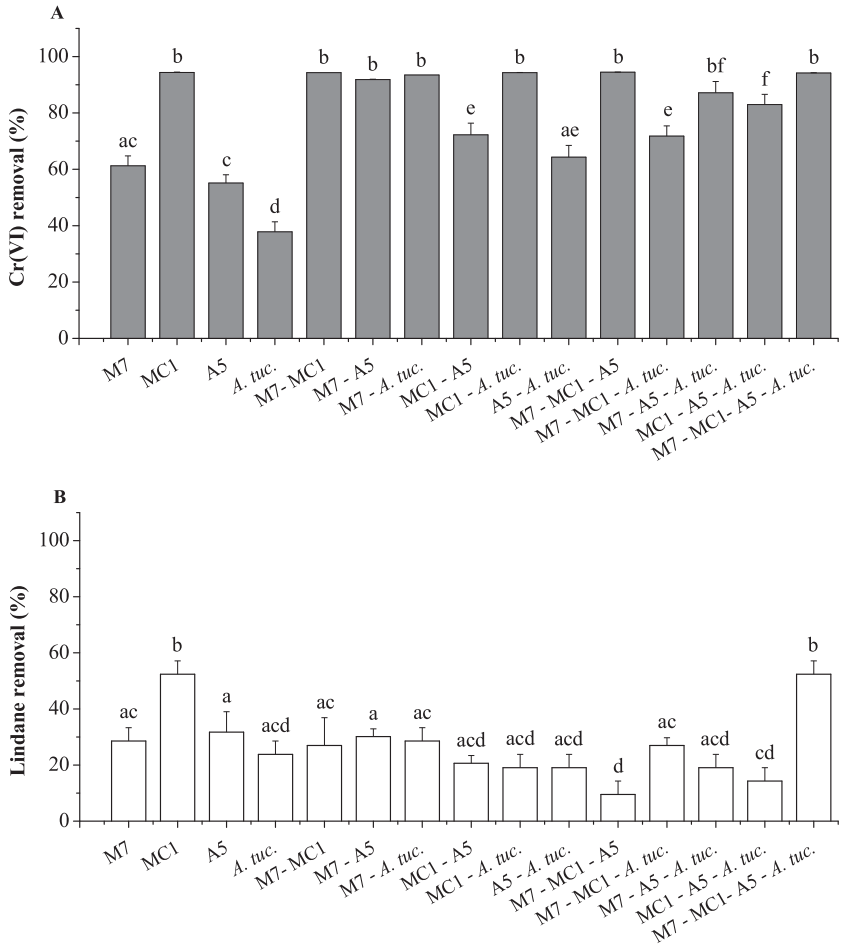

Fig. 1. Removal (%) of (A) Cr(VI) and (B) lindane by pure and mixed cultures of actinobacteria in liquid medium. M7: *Streptomyces* sp. M7, MC1: *Streptomyces* sp. MC1, A5: *Streptomyces* sp. A5, *A. tuc.*: *Amycolatopsis tucumanensis* DSM 45259. Different letters indicate significant differences between conditions ($p < 0.05$).

and in soil [32,33].

Cr(VI) and lindane removal (%) in MM inoculated with single or mixed cultures are shown in Fig. 1. In non-inoculated MM contaminated with both pollutants, abiotic removal was not observed (data not shown).

Nowadays, the most accepted mechanisms of bacterial Cr(VI) reduction comprise two ways. One of them is the extracellular Cr(VI) reduction to Cr(III) by enzymes deliberately produced and exported to the medium. On the other hand, the intracellular Cr(VI) reduction occurs in four major steps: (1) Cr(VI) biosorption, (2) Cr(VI) transport into cells, (3) cytosolic Cr(VI) reduction, and (4) Cr(III) accumulation [44]. However, the fraction immobilized inside the cell is usually minimal (around 10% of the total Cr) [31]. Even so, when cells die, they lyse and release the cell content to the medium, including the Cr(III) previously immobilized. For this reason, in the present study, only Cr(VI) was determined in the culture supernatants, which is the real measure of the metal removal in liquid systems.

Cr(VI) removal by single cultures were 61%, 55%, and 38% for *Streptomyces* sp. M7, *Streptomyces* sp. A5, and *Amycolatopsis tucumanensis*, respectively, while *Streptomyces* sp. MC1 reached the maximal removal from single cultures (94%) (Fig. 1).

In order to compare the pollutants removal performance of the defined consortia versus pure cultures, the ratio R was determined for both pollutants and the results are shown in Table 2. The R revealed that the removal of chromium improved using almost all the consortia ($R > 1$) due to a possible synergism among the strains, except for the double mixed culture of *Streptomyces* sp. MC1 and A5, which was almost the same than the corresponding pure cultures ($R \approx 1$), i.e. these strains did not reveal detectable interactions. Similarly, Kiliç et al. [45] have demonstrated that the purification of the yeast strains present in a mixed culture decreased the capacities of the mixed culture to remove Cu(II) and Cr(VI) in activated sludge medium. A similar effect was observed by Akpomie and Ejechi [46], by using *Pseudomonas aeruginosa*, *Escherichia coli*, *Aspergilllus niger*, and *Penicillium chrysogenum* to remove chromium in tannery wastes. In this sense, they postulated that the combination of microorganisms approximates to the natural environment where different organisms co-exist and combine their metabolic activities in order to bring about efficient organic and inorganic transformations.

Table 2
Pollutants removal values expected and ratio (R) between the removal values obtained (O) and expected (E) in the mixed cultures of actinobacteria in liquid medium. R = O/E.

Mixed cultures	E	R for Cr(VI)	E	R for lindane
M7-MC1	77.8 ± 16.6	1.21	40.5 ± 11.9	0.67
M7-A5	58.2 ± 3.1	1.58	30.2 ± 1.6	1.00
M7-AB0	49.5 ± 11.7	1.89	26.2 ± 2.4	1.09
MC1-A5	74.7 ± 19.6	0.97	42.1 ± 10.3	0.49
MC1-AB0	66.1 ± 28.3	1.43	38.1 ± 14.3	0.50
A5-AB0	46.5 ± 8.7	1.38	27.8 ± 4.0	0.69
M7-MC1-A5	70.2 ± 17.2	1.35	37.6 ± 10.6	0.25
M7-MC1-AB0	64.5 ± 23.2	1.11	34.9 ± 12.5	0.77
M7-A5-AB0	51.4 ± 9.9	1.70	28.0 ± 3.3	0.68
MC1-A5-AB0	62.4 ± 23.7	1.33	36.0 ± 12.0	0.40
M7-MC1-A5-AB0	62.1 ± 20.5	1.52	34.1 ± 10.9	1.53

Lindane removal obtained by the single cultures of *Streptomyces* sp. M7, *Streptomyces* sp. A5 and *Amycolatopsis tucumanensis* ranged between

24 and 32%, approximately; while *Streptomyces* sp. MC1 achieved 52% of lindane removal, being statistically higher than the other single cultures. In contrast with the results obtained for Cr(VI), the R for lindane was in most cases ≤ 1, i.e. the use of multiple microorganisms together did not produce an enhancement in the pesticide removal. Only the quadruple consortium improved lindane removal by more than 50% (R = 1.53) respect to its corresponding pure cultures (Fig. 1, Table 2). In a previous study, Fuentes et al. [12] reported that the removal of lindane did not improve when five or six *Streptomyces* strains were combined to form defined mixed cultures, respect to the obtained by the pure strains. Besides, in such case lindane was the only pollutant present in the medium. In the present study, the removal or degradation of lindane may have been inhibited by the presence of chromium. It is known that the degradation of organic contaminants by microorganisms may be related to an inducible system. However, in co-contaminated environments, the presence of heavy metals can inhibit the degrading metabolism of the organic pollutants [33]. Pornwongthong et al. (2014) have reported a dose-dependent inhibition of 1,4-dioxane biodegradation by Cd(II), Cu(II), and Ni(II) in the bacterium *Pseudonocardia dioxanivorans*. This toxic effect may be due to interactions of metals with enzymes involved in the biodegradation (e.g., pollutant-specific oxygenases) or with enzymes involved in general metabolism, thus inhibiting the pollutant biodegradation [48].

3.2. Removal of bioavailable chromium and lindane by single and mixed cultures of actinobacteria in soil systems

In control flasks, bioavailable chromium was reduced from 50 to 26 mg kg^{-1} (data not shown). Cr(VI) is highly reactive; when it spills on the ground, it immediately reacts with the organic matter and clay minerals present in the soil. After exceeding the saturation level of the metal in the soil, the solution phase starts to enrich itself in Cr(VI) [49]. The physical method developed by Csillag et al. [39] allows measuring the bioavailable chromium, defined as the fraction of dissolved metal species in the pore water which can be taken up by plants roots or other soil organisms [49]. Polti et al. [50] have demonstrated that this fraction of dissolved metal corresponds specifically to Cr(VI). Bioavailable chromium concentration detected in control flasks was considered as the 100% for further removal calculations. In contrast, no variations on lindane concentrations in control were observed, so there was no evidence of a noticeable contribution of autochthonous microbiota or abiotic removal of the pesticide (data not shown).

Bioavailable chromium and lindane removal (%) in soil inoculated with single and mixed cultures are shown in Fig. 2. Bioavailable chromium removal by actinobacteria from soil was notably lower than the obtained in MM. The same trend was reported by Polti et al. [31,50]. They demonstrated that *Streptomyces* sp. MC1 was able to remove 50% of Cr(VI) in a liquid minimal medium [31], whereas the same microorganism achieved just 10% of bioavailable chromium removed after 14 days in non-sterilized soil samples contaminated only with the metal [50]. The removal ability of this strain could have been reduced by the microorganisms present in the non-sterilized soil sample. The negative interaction observed between the indigenous microbial community and the inoculum may be explained by a competition for nutrients resources [51]. Nevertheless, in the present work, all the single cultures exceeded 30% of removal of bioavailable chromium and the single culture of *Streptomyces* sp. M7 removed was able to remove 50% of the bioavailable fraction of the metal (Fig. 2). Among the double and triple mixed cultures, the maximal chromium removal efficiencies were obtained by *Streptomyces* sp. M7-A5, *Streptomyces* sp. MC1-*A. tucumanensis*, and *Streptomyces* sp. MC1-A5-*A. tucumanensis*, which were able to remove 53.5%, 58.7%, and 62.2% of bioavailable chromium, respectively. The greatest bioavailable chromium removal (64.4%) was achieved by the mixed culture consisting of the four strains, possibly due to positive interactions among the members of the consortium [52]. Community life can generate stability over time in the face of environmental fluctuations [53]. In addition, mixed cultures have a greater capacity to resist the invasion of other species [54].

The removal values of both pollutants obtained by the mixed cultures were also compared with the expected values (Table 3). In all the mixed cultures where the removal of bioavailable chromium exceeded 50% (Fig. 2), a synergy among the microorganisms involved was observed (R > 1) (Table 2). For instance, in the quadruple consortium, the ability to remove the bioavailable fraction of the metal increased by 61%.

On the other hand, *Streptomyces* sp. M7, *Streptomyces* sp. MC1 and *A. tucumanensis* were able to individually remove above 60% of lindane, while *Streptomyces* sp. A5 showed around 50% of lindane removal (Fig. 2). Polti et al. [33] evaluated the same single cultures in sterilized soil co-contaminated with 25 μg kg^{-1} of lindane and 50 mg kg^{-1} of Cr (VI) observing lower removal values than in non-sterilized soil in all cases (fewer than 40%). This greater lindane removal observed in non-sterilized soil could be due to the presence of the native population of the soil, which, although it could be sensitive to high concentrations of bioavailable chromium, when this concentration was reduced enough by the added actinobacteria strains it could have started expressing lindane degrading activity [48].

Lindane removal obtained by all mixed cultures was lower than the expected removal (R < 1), thus indicating possible negative interactions among the actinobacteria (Table 3). In the mixed cultures of two and three strains, lindane removal percentages obtained were generally under 35%. Okerentugba and Ezeronye [55] observed a similar behavior with bacteria and fungi for the degradation of crude oil; single cultures were better degraders than the mixed cultures. In the present work, only in the presence of the four strains, the lindane removal achieved was similar to the expected removal. It is important to highlight that each of the strains forming the consortium has different properties. *Streptomyces* sp. MC1 and *A. tucumanensis* have demonstrated to be excellent bioremediation agents with a high adaptation towards heavy metals [30,31,56]. This adaptation allows the strain to remove these compounds and survive the stress. On the other hand, *Streptomyces* sp. M7 and *Streptomyces* sp. A5 have shown a high capacity to grow, remove and degrade different organochlorine pesticides [28,38,57]. Therefore, in real environments, where Cr(VI) and lindane coexist with other heavy metals and pesticides, the use of a mixed culture of multiresistant actinobacteria is potentially promising. In contrast to simple cultures, a mixed culture has a high capacity to survive stress due to the complex interactions that occur among the members of the consortium and the native microbiota, maintaining or even improving its removal abilities. These interactions include communication through signals, horizontal gene transfer, competitive or cooperative relationships, and changes in the environment to influence the growth of neighbors [34]. In this sense, an extreme negative interaction was observed among the strains *Streptomyces* sp. M7, MC1, and *A. tucumanensis*, where there was a complete inhibition of the degradation of lindane. The simple cultures of these three strains generated similar lindane removal; it could be possible that they use similar pathways and there was a competitive inhibition among them. Surprisingly, the incorporation of *Streptomyces* sp. A5 into the mixed culture reversed this inhibition, revealing the complexity of the interactions.

On the other hand, besides possible interactions among the actinobacteria, the lowest lindane removal obtained by most of the tested consortia could also be related to the presence of Cr(VI) in the soil. Polti et al. [33] showed that the removal of Cr(VI) and lindane did not occur simultaneously. In a first stage the metal is reduced and then the pesticide is degraded. The reduction of Cr(VI) to Cr(III) is a process which involves the use of NADH for microbial metabolism and, therefore, any process that affects its production would consequently affect the reduction of Cr(VI). Soil energy sources can be used in a first instance to obtain NADH for the subsequent reduction of Cr(VI), and the residual energy could be then used for the removal of lindane. It is likely that the

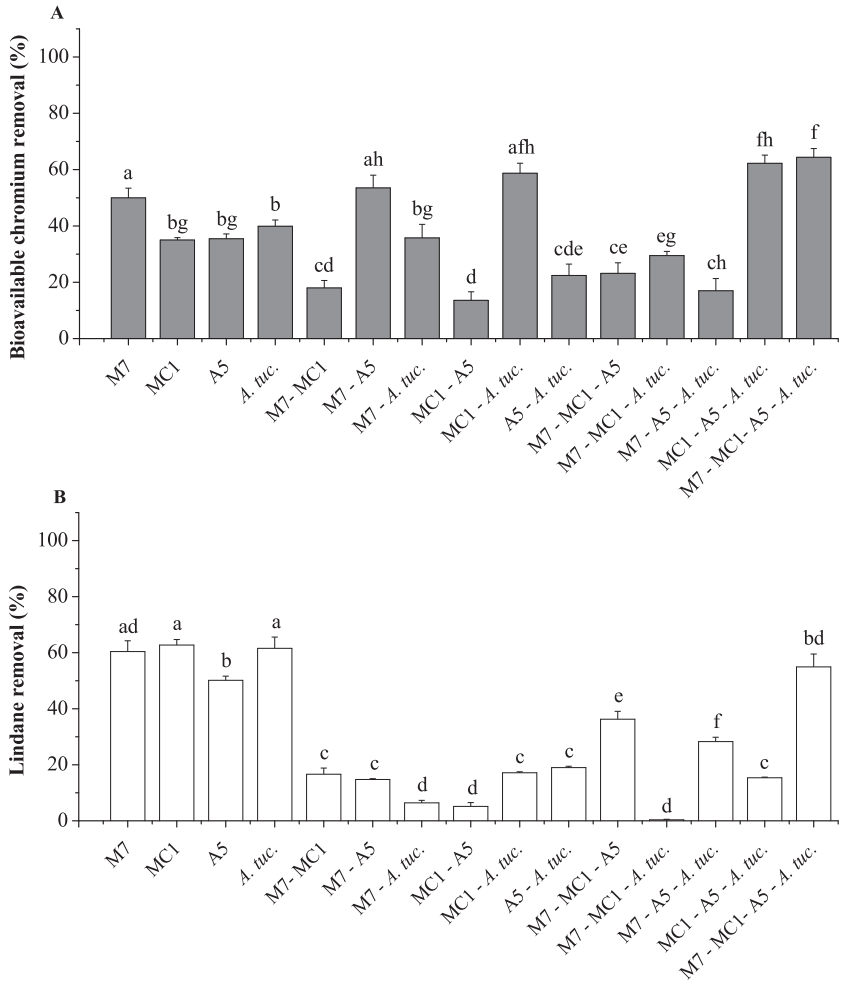

Fig. 2. Removal (%) of (A) bioavailable chromium and (B) lindane by pure and mixed cultures of actinobacteria in artificially co-contaminated soil. M7: *Streptomyces* sp. M7, MC1: *Streptomyces* sp. MC1, A5: *Streptomyces* sp. A5, *A. tuc.*: *Amycolatopsis tucumanensis* DSM 45259. Different letters indicate significant differences between conditions ($p < 0.05$).

Table 3
Expected removal values for bioavailable chromium and lindane, and ratio (R) between the removal values obtained (O) and expected (E) in the mixed cultures of actinobacteria in soil system. R = O/E.

Mixed cultures	E	R for Cr(VI)[a]	E	R for lindane
M7-MC1	42.5 ± 7.5	0.42	61.6 ± 1.2	0.27
M7-A5	42.7 ± 7.3	1.25	55.3 ± 5.1	0.27
M7-AB0	44.9 ± 5.0	0.80	61.0 ± 0.6	0.10
MC1-A5	35.2 ± 0.2	0.39	56.4 ± 6.3	0.09
MC1-AB0	37.4 ± 2.4	1.57	62.2 ± 0.6	0.28
A5-AB0	37.7 ± 2.2	0.59	55.9 ± 5.7	0.34
M7-MC1-A5	40.2 ± 6.9	0.58	57.8 ± 5.5	0.63
M7-MC1-AB0	41.6 ± 6.2	0.71	61.6 ± 1.0	0.01
M7-A5-AB0	41.8 ± 6.1	0.41	57.4 ± 5.1	0.49
MC1-A5-AB0	36.8 ± 2.2	1.69	58.1 ± 5.7	0.26
M7-MC1-A5-AB0	40.1 ± 6.0	1.61	58.7 ± 5.0	0.94

[a] Bioavailable fraction.

consortium had used a greater proportion of energy sources to improve the reduction of Cr(VI). Therefore, the residual energy for the degradation of lindane would be lower, which could be reflected in a lower elimination of this contaminant [32]. In this sense, longer time trials could give more information about the events that occur. It is known that once the metal is inactivated, the degradation processes are stimulated.

Furthermore, no relationship was observed between the removal of both pollutants in liquid medium and soil for simple, double and triple cultures; however, in both matrices, the quadruple culture showed the maximum removal values.

3.3. Assessment of the efficacy of soil bioremediation

An important aspect of any process of restoration of a contaminated environment is the verification of the effectiveness of the method applied by using a biological approach.

The analysis of soils and related matrices is complex mainly due to the intrinsic problems of the matrix, which can result in non-quantitative analyte recoveries or in the lack of accuracy and reproducibility of the results, as a consequence of interferences in the stage of detection and quantification [58]. Currently, evaluations of quality, health, the impact of disturbances and the effectiveness of the remediation processes of contaminated soils focus only on physicochemical parameters. However, these assessments cannot rely solely on analytical determinations of environmental samples, since this approach offers no indication of the actual effects that pollutants cause on organisms and biodiversity. Thus, biological indicators have an integrating nature and, due to their greater sensitivity and speed of response to fluctuations in the system, they can react in advance to irreversible changes and disturbances, and allow a correct and integrated assessment of soil health [59].

Therefore, lettuce seeds were used as bioindicators for monitoring the success of the bioremediation process in the soil system. *Lactuca sativa* is a recommended species for this purpose since this toxicological test represents a useful tool to compare the toxicity of treated and non-

Table 4
Vigor index (VI) of lettuce seedlings grown on soils bioremediated by pure and mixed cultures of actinobacteria. CS: contaminated soil, NCS: non-contaminated soil. Vigor indexes with different letters in superscript indicate statistically significant differences ($p < 0.05$). VI = (mean root length + mean hypocotyl length) × germination percentage/10.

Condition	VI
NCS	49.33 ± 2.91^{a}
CS	8.27 ± 0.76^{b}
Streptomyces sp. M7	27.75 ± 0.25^{c}
Streptomyces sp. MC1	17.17 ± 1.04^{d}
Streptomyces sp. A5	14.95 ± 0.38^{def}
A.tucumanensis	14.50 ± 0.70^{defg}
Streptomyces sp. M7-MC1	9.55 ± 0.05^{bfg}
Streptomyces sp. M7-A5	9.65 ± 0.15^{bfg}
*Streptomyces*sp. M7-*A.tucumanensis*	13.43 ± 3.24^{defg}
Streptomyces sp. MC1-A5	9.03 ± 0.03^{bg}
Streptomyces sp. M7-*A.tucumanensis*	15.73 ± 0.27^{de}
Streptomyces sp. A5-*A.tucumanensis*	10.37 ± 1.04^{befg}
Streptomyces sp. M7-MC1-A5	7.03 ± 0.97^{b}
Streptomyces sp. M7-MC1-*A.tucumanensis*	9.35 ± 0.28^{bfg}
Streptomyces sp. M7-A5-*A.tucumanensis*	10.50 ± 0.90^{befg}
Streptomyces sp. MC1-A5-*A.tucumanensis*	18.48 ± 0.12^{d}
Streptomyces sp. M7-MC1-A5-*A.tucumanensis*	24.72 ± 1.91^{c}

treated soil samples, and thus to assess the effectiveness of the actinobacteria cultures in reducing the co-contamination [60].

The vigor index combines data of germination and seedling length for a single value and thus simplifying the comparison between conditions. The vigor indexes of seedlings grown on CS were significantly lower compared to those grown on NCS (Table 4), thus confirming that *Lactuca sativa* serves as an appropriate indicator for evaluating the efficiency of the bioremediation process under the studied conditions.

Vigor indexes were significantly greater in eight conditions compared to the obtained in CS (Table 4). This could indicate that toxic effects of Cr(VI) and lindane on lettuce seeds decreased after those bioremediation processes. Under those conditions, Cr(VI) removal was higher than 38%; however, lindane removal ranged from 5 to 60%. Moreover, only one condition (the double culture of *Streptomyces* sp. M7-*Streptomyces* sp. A5) showed Cr(VI) removal higher than 38% but did not show significant differences in the vigor index with the CS. Although a considerable percentage of both pollutants have been removed by this culture, an accumulation of toxic metabolites could be possible.

In the case of organic compounds bioremediation, their mineralization is desired, i.e. complete degradation to carbon dioxide and water. In contrast, in the case of metals, such as Cr(VI), the bioremediation process is based on their conversion to a less toxic form and/or immobilization in order to reduce their bioavailability [53]. However, in some cases, the pollutants removal may be accompanied by their transformation into more toxic intermediates that could persist in the environment and produce toxicity to certain species [61,62].

The absence of a noticeable relationship between lindane removal and the vigor index values could indicate that the use of another bioindicator could be necessary to assess the bioremediation in soil contaminated with lindane at this concentration.

Notably, the maximal vigor index values were reached in soils bioremediated by the single culture of *Streptomyces* sp. M7 and the quadruple consortium (Table 4), both conditions corresponding to the greater percentages of removal for bioavailable chromium and lindane (Fig. 2). Those vigor index values represented around 3-fold the vigor index of the CS, hence confirming that the bioremediation process was effective since in addition to an effective reduction of both lindane and Cr(VI) bioavailable concentrations in the soil samples, less toxic or non-toxic metabolites would have been produced by the actinobacteria. This is a critical result in order to ensure the effectiveness of the bioremediation process since the release of the metabolites produced does not entail further risk for the environment or human health [41].

3.4. Survival of actinobacteria strains after soil bioremediation process

The interactions between inoculum and indigenous microbes are complex, therefore monitoring the survival and activity of the inoculum is essential [51]. In this context, the survival of the strains composing the consortium at the end of the soil bioremediation process was evaluated. For this purpose, two approaches were studied; one of them was antibiotic sensitivity (biochemical approach). Results revealed that the four actinobacteria showed different antibiotic sensitivity profiles (Table 5). *Streptomyces* sp. A5 was the only strain resistant to imipenem, while *Streptomyces* sp. MC1 was resistant to minocycline and *Amycolatopsis tucumanensis* was resistant to gentamicin. However, none of the tested antibiotics individually allowed the development of *Streptomyces* sp. M7 alone. For this reason, a combination of lincomycin and erythromycin was successfully used to reisolate *Streptomyces* sp. M7 from the soil. In addition, the RAPD-PCR methodology showed a characteristic profile for each of the four actinobacteria at an annealing temperature of 55 °C. Based on the above, dilutions of the soil samples obtained after the bioremediation assay were plated on MH supplemented with 10 μg mL^{-1} of nalidixic acid and 10 μg mL^{-1} of cycloheximide (to inhibit gram-negative bacteria and fungi, respectively) plus either imipenem (10 μg mL^{-1}), gentamicin (25 μg mL^{-1}), minocycline (15 μg mL^{-1}), or lincomycin + erythromycin (70 μg $mL^{-1}$and 20 μg mL^{-1}, respectively). After one week of incubation, separated colonies were obtained (Fig. 3A), which were selected according to their morphology and then used to perform RAPD-PCR. The profiles obtained in polyacrylamide gels confirmed the identity, and hence

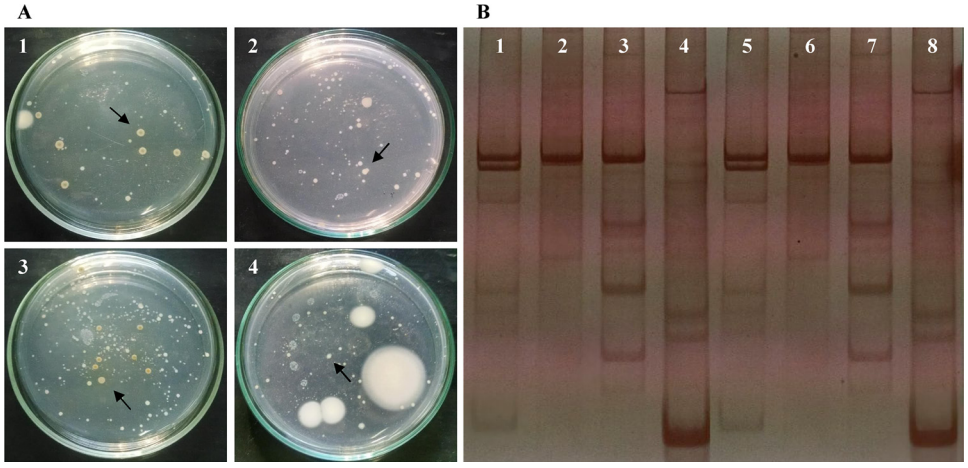

Fig. 3. (A) Microbial development of soil samples extraction on SC plates, and (B) polyacrylamide gel electrophoresis of the fragments amplified. (1) Colony isolated from SC plate added with erythromycin 25 μg mL^{-1} plus lincomycin 70 μg mL^{-1}, (2) Colony isolated from SC plate added with minocycline 15 μg mL^{-1}, (3) Colony isolated from SC plate added with imipenem 10 μg mL^{-1}, and (4) Colony isolated from SC plate added with gentamicin 25 μg mL^{-1}. Pure cultures of (5) *Streptomyces* sp. M7, (6) *Streptomyces* sp. MC1, (7) *Streptomyces* sp. A5, and (8) *Amycolatopsis tucumanensis* were used as reference control.

survival, of the four actinobacteria strains at the end of the bioremediation process (Fig. 3B). This is a very important result since long-term stability is an essential factor for practical application of a microbial culture, even more in bioremediation processes where they are exposed to high concentrations of environmental contaminants. These actinobacteria strains may have acquired mechanisms to tolerate and grow under adverse conditions achieving a strong adaptation to the adverse environment due to the time they were exposed to such conditions [53]. Fan et al. [63] also found that the yeast SK21 inoculated in soil microcosms remained viable at the end of the process, which showed the applicability of this specific yeast for bioremediation of oil-contaminated soil. Saez et al. [41] also confirmed the survival of four *Streptomyces* sp. strains constituent of a defined consortium after a five-week acclimation period employing similar methodologies to those used in the present study, although they had been applied to axenic cultures. This is the first study using this methodology in non-sterilized matrices.

Robustness is an emergent property of microbial communities and is necessary for survival in the wildly changing world [34]. It is known that microorganisms may be inhibited when introduced to field scale because of predation, competition by autochthonous microorganisms and neighboring roots that release organic compounds [64]. In the present study, it was demonstrated that the four actinobacteria strains introduced in co-contaminated non-sterilized soil microcosms were able to survive not only to the adverse conditions created by the presence of Cr(VI) and lindane but also to the presence of the native microbiota present in the soil.

The success of a bioaugmentation process depends on the selection of a culture able not only to remove the contaminants presents in the soil, but also able to grow in a hostile environment, tolerating others contaminants and not be inhibited by autochthonous microbiota [48]. For this reason, bioremediation must be tailored to specific conditions of the environment or sample to be treated and therefore requires a treatability study prior to the actual cleanup by bioremediation processes [65,66].

4. Conclusions

Actinobacteria single and mixed cultures were able to remove Cr (VI) and lindane from artificially co-contaminated liquid and soil systems. The single cultures of *Streptomyces* sp. MC1 and *Streptomyces* sp. M7 reached the highest removal percentages of both pollutants in the liquid medium and soil, respectively, whereas the quadruple consortium consisting of *Streptomyces* sp. M7, *Streptomyces* sp. A5, *Streptomyces* sp. MC1 and *Amycolatopsis tucumanensis*, achieved the best removal profiles of Cr(VI) and lindane from both matrices, liquid medium, and soil. The success of the bioremediation process in soil by those cultures was demonstrated through the ecotoxicity test with *Lactuca sativa*, due to an increase in the vigor index measured in lettuce seedlings compared to non-treated co-contaminated soil. This may be indicating that more toxic or reactive metabolites had not been produced. The four actinobacteria strains inoculated in co-contaminated soil microcosms were able to survive to high concentrations of Cr(VI) and lindane and also to the presence of autochthonous microbiota of the soil. The results of the present work indicate that the defined consortium formed by the four actinobacteria would represent a promising tool for the bioremediation of soils co-contaminated with inorganic and organic compounds, such as chromium and lindane, while single actinobacteria cultures have different performance according to the evaluated matrix.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jece.2018.03.030.

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Figure S1. Growth of pure and mixed cultures of actinobacteria in liquid medium supplemented with Cr(VI) and lindane (▬) and their respective controls without pollutants (▭). M7: *Streptomyces* sp. M7, MC1: *Streptomyces* sp. MC1, A5: *Streptomyces* sp. A5, *A. tuc.*: *Amycolatopsis tucumanensis* DSM 45259.