Chapter 11
Co-contaminated Soils Bioremediation by Actinobacteria

Juan D. Aparicio, María Z. Simón Solá, Mariana C. Atjián, Claudia S. Benimeli, and María Julia Amoroso

Abstract More than one-third of contaminated areas are found to have more than one type of pollutant. Co-contaminated environments with metals and organic compounds are difficult to remediate because of the mixed nature of the pollulants. Actinobacteria is an important group of microorganisms found in soils, with high metabolic versatility and abilities to bioremediation. Actinobacteria are currently studied for bioremediation of soils contaminated by pesticides and heavy metals. In this chapter we review the potential of actinobacteria isolated from contaminated environments for simultaneous soil bioremediation of Cr(VI) and the organochlorine pesticide lindane. Four actinobacteria, tolerant to Cr(VI) and lindane mixture were used: *Streptomyces* spp. A5, M7, MC1 and *Amycolatopsis tucumanensis* DSM 45259. Sterilized soil samples were inoculated with actinobacteria strains, either individually or as a consortium (formed by all selected actinobacteria) then contaminated with Cr(VI) and lindane, and incubated at 30 °C for 14 days. All actinobacteria

J.D. Aparicio (✉) • M.Z. Simón Solá
Planta Piloto de Procesos Industriales Microbiológicos (PROIMI), CONICET, Av. Belgrano y Pasaje Caseros, 4000 Tucumán, Argentina

Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, 4000 Tucumán, Argentina
e-mail: dani_aparicio@hotmail.com.ar

M.C. Atjián
Planta Piloto de Procesos Industriales Microbiológicos (PROIMI), CONICET, Av. Belgrano y Pasaje Caseros, 4000 Tucumán, Argentina

C.S. Benimeli
Planta Piloto de Procesos Industriales Microbiológicos (PROIMI), CONICET, Av. Belgrano y Pasaje Caseros, 4000 Tucumán, Argentina

Universidad del Norte Santo Tomás de Aquino (UNSTA), 4000 Tucumán, Argentina

M.J. Amoroso
Planta Piloto de Procesos Industriales Microbiológicos (PROIMI), CONICET, Av. Belgrano y Pasaje Caseros, 4000 Tucumán, Argentina

Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, 4000 Tucumán, Argentina

Universidad del Norte Santo Tomás de Aquino (UNSTA), 4000 Tucumán, Argentina

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were able to grow and remove both contaminants, the consortium formed by *Streptomyces* spp. A5, M7, MC1 and *A. tucumanensis* showed the highest Cr(VI) removal, while *Streptomyces* sp. M7 produced the maximum lindane removal. In non-sterile soil samples, *Streptomyces* sp. M7 and the consortium removed more than 40 % of the lindane, while *Streptomyces* sp. M7 demonstrated the greatest Cr(VI) removal. According to these results, it could be concluded that the use of *Streptomyces* sp. M7 is the strategy more appropriate for the bioremediation of soils contaminated with Cr(VI) and lindane.

11.1 Introduction

The great expansion of industrial activity has resulted in an increase in scenarios of serious and complex environmental contamination by both organic compounds (herbicides, plastics, tannins, polyphenols, pesticides, etc.) and inorganic compounds (As, Cd, Cu, Pb, Cr, Hg, etc.) (Volke Sepúlveda and Velasco Trejo 2002). Co-pollution is a very important issue because more than one third of contaminated areas are found to have more than one type of pollutant (Mansour 2012; Tang et al. 2010). Moreover, environments co-contaminated with metals and organic compounds are difficult to remediate because of the mixed nature of these pollutants.

11.1.1 Chromium (VI)

Chromium (VI) (Cr(VI)) compounds have several uses in industry (Bhadra and Mahananda 2013; Polti et al. 2007) and contamination by these compounds has been detected in soil and water and around a wide variety of industrial sites (Benimeli et al. 2003; Nie et al. 2010; Srinivasa Gowd et al. 2010). Cr(VI) is a harmful pollutant characterized by its chronic toxicity, neurotoxicity, dermatotoxicity, genotoxicity, carcinogenicity, and immunotoxicity (Bagchi et al. 2002), and it is approximately 1,000 times more toxic and mutagenic than Cr(III) (Dana Devi et al. 2001; USEPA 1998).

11.1.2 Lindane

The systematic use of pesticides has led to great improvements in terms of agricultural production levels. However, massive and indiscriminate application of pesticide products has also led to adverse effects on human health, the environment, and even the effectiveness of the products themselves (Johri et al. 2000; Phillips et al. 2005). The gamma isomer of hexachlorocyclohexane (γ-HCH), commercially

known as lindane, is a highly chlorinated, recalcitrant organochlorine pesticide (OP). Lindane residues persist in the environment and have been reported in soils, water, air, plants, agricultural products, animals, foods, and microbial environments, as well as in the human body. Since the toxicity of γ-HCH has been established, it is now imperative to develop methods to remove lindane from the contaminated environments (Fuentes et al. 2011).

11.2 Bioremediation

The intense search for a solution to co-contamination has led to the development of remediation technologies that can simultaneously deal with multiple contaminants (Ma et al. 2010; Srivastava et al. 2007; Wasi et al. 2011).

In the last 10 years a stronger emphasis has come to be placed on the study of the physiological, biochemical, and molecular approaches to microbial bioremediation of environments co-contaminated with heavy metals and pesticides. Soils with long-term exposure to mixed contamination with organic compounds and heavy metals have been shown to have structural and functional microbial communities with the ability to adapt and grow under these conditions. This suggests that bioremediation based on microorganisms is feasible for recovery of such sites by microbial transformation of both organic compounds and heavy metals into nontoxic products. These strategies depend mainly upon the catabolic biological activities of the microorganisms, and therefore their ability to utilize the contaminants as nutrients and energy sources (Atlas and Unterman 1999; Boopathy 2000).

The impacts that metals have on biodegradation are complex and are influenced by the matrix structure, which determines the bioavailable metal concentrations. Metals inhibit biodegradation using different mechanisms and patterns, which depend upon the biological and physicochemical characteristics of each system. A variety of approaches to bioremediation of co-contaminated sites are under development, and they include addition of metal-resistant microorganisms as well as additives that reduce metal bioavailability (Sandrin and Hoffman 2007).

Several authors have evaluated bioremediation in media co-contaminated with metals and persistent organic compounds. Olaniran et al. (2009) investigated the impact of lead and mercury on biodegradation of 1,2-dichloroethane in soils, and they concluded that heavy metals have a negative impact on this bioprocess. These authors also found that biostimulation can have a positive influence on 1,2-dichloroethane degradation.

Another emerging approach is bioaugmentation. Sprocati et al. (2012) used this strategy to remediate soils co-contaminated with diesel oil and heavy metals. The bioaugmentation was performed by introducing a consortium composed of 12 allochthonous bacterial strains, previously isolated from a site with long-term pollution. This strategy showed high efficiency in the bioremediation process.

11.3 Actinobacteria

It is important to consider that when allochthonous microorganisms are incorporated into a soil, they usually cannot fully participate in the community activity in a meaningful way. This is why the use of indigenous microorganisms in bioremediation processes is so important. The actinobacteria are a group of bacteria that is found in high concentrations in soils. They play an important ecological role in recycling substances in the natural world, using humic acids for their growth as well as organic matter, which is difficult to degrade (Kieser et al. 2000). The physiological diversity of actinobacteria allows the production of a large number of metabolites with biotechnological importance included antibiotics, which are synthesized and excreted into a medium (Ensign 1990; Genilloud et al. 2011; Goodfellow et al. 1988). The important role played by actinobacteria in the environment is also demonstrated by their ability to remove oil, rubber, plastics, pesticides, and heavy metals, among other substances (Albarracín et al. 2005, 2010b; Benimeli et al. 2003, 2006, 2007; Goodfellow et al. 1988; Polti et al. 2009, 2011; Vobis 1997).

There have been previous studies focused on biotransformation of OPs by actinobacteria, particularly in relation to lindane degradation (Benimeli et al. 2006, 2007; Fuentes et al. 2011; Saez et al. 2012). *Streptomyces* spp. M7, A2, A5, and A11, isolated from sediments and soils contaminated with OPs, were found to be able to degrade lindane, as revealed by the release of chloride ions when the microorganisms were grown on media containing this pesticide as a sole carbon source (Benimeli et al. 2003, 2006; Cuozzo et al. 2009; Fuentes et al. 2010). Biotransformation of heavy metals Cu(II), Cd(II) and Cr(VI) by actinobacteria, particularly in terms of uptake and/or reduction to less toxic forms, has also been studied (Albarracín et al. 2008a; Polti et al. 2007; Siñeriz et al. 2009). *Streptomyces* sp. MC1, isolated from contaminated sugar cane, has shown the ability to reduce Cr(VI) to Cr(III) in both liquid and solid culture media (Polti et al. 2009, 2010). *Amycolatopsis tucumanensis* DSM 45259, isolated from sediments contaminated with heavy metals has also shown resistance to copper and chromium under a variety of culture conditions (Albarracín et al. 2005, 2008b, 2010a).

In this chapter we could see how actinobacteria, as pure or mixed culture, could be able to remediate soil co-contaminated with lindane and Cr(VI).

11.4 Cr(VI)-Lindane Tolerant Actinobacteria

Studies of tolerance to Cr(VI) and lindane were performed using Minimal Medium (containing in g L^{-1}: glucose, 10.0; L-asparagine, 0.5; K_2HPO_4, 0.5; $MgSO_4 \times 7H_2O$, 0.20; $FeSO_4 \times 7H_2O$, 0.01) agar plates because the toxic elements do not interact with the medium components and they therefore remain bioavailable to the actinobacteria (Amoroso et al. 2001, 2002; Rathnayake et al. 2013). Rectangular troughs were cut in the center of the plate and then filled with 500 mg L^{-1} of Cr(VI) and/or 250 μg L^{-1} of lindane.

Six previously isolated actinobacteria were assayed: three isolated from environments contaminated with pesticides and heavy metals (*Streptomyces* sp. M7, *Streptomyces* sp. MC1, and *Amycolatopsis tucumanensis* DSM 45259) (Albarracín et al. 2005; Benimeli et al. 2003; Polti et al. 2007), and three isolated from a lindane-contaminated environment in Santiago del Estero, Argentina, where in 1994 about 30 tons of organochlorine pesticides were spilled: *Streptomyces* sp. A2, *Streptomyces* sp. A5, and *Streptomyces* sp. A11 (M. S. Fuentes et al. 2010). The strains were inoculated by streaking perpendicular to the troughs, and the Petri dishes were incubated at 30 °C for 7 days. Microbial growth was used as a qualitative parameter of toxicity tolerance. Control samples for growth were also created using a medium without the addition of toxics (Fuentes et al. 2013). When the individual toxic elements were assayed, *Streptomyces* spp. A5, A11, M7, MC1, and *Amycolatopsis tucumanensis* all showed similar growth to that observed in the uncontaminated control, while *Streptomyces* sp. A2 showed little growth and was thus considered to have low tolerance to Cr(VI) and lindane. Previously, Polti et al. (2007) used Cr(VI) 260 mg L^{-1}, and Benimeli et al. (2003) used 10 μg L^{-1} of lindane to select Cr(VI) or lindane tolerant actinobacteria, respectively. Therefore, used toxic concentrations ensure selection of bacteria with high tolerance to such compounds.

Degradation of organic contaminants by microorganisms generally corresponds to an inducible system. However, in co-contaminated environments the presence of heavy metals inhibits the degrading metabolism, so it is necessary to evaluate the toxicity of both pollutants in combination in order to select the most suitable microorganisms for bioremediation processes (Alisi et al. 2009; Moreira et al. 2013; Thavamani et al. 2012). Therefore, as described above the two types of contaminants were mixed to evaluate their combined effect on the six evaluated actinobacteria.

No inhibition of growth was seen in *Streptomyces* spp. A5, A11, M7, MC1, or *Amycolatopsis tucumanensis*. However, little growth was seen in *Streptomyces* sp. A2 and it was thus considered to be a strain that with low tolerance to this mixture of contaminants, probably because the combination of Cr(VI) and lindane enhanced their inhibitory activity against this strain. This effect has in fact already been observed by other authors studying co-contaminated systems (Alisi et al. 2009; Sandrin and Hoffman 2007; Sandrin and Maier 2003). However, these results indicate that the contaminant concentrations used were not inhibitory for the growth of five of the six actinobacteria under the experimental testing conditions. The contaminant concentrations tested were selected based upon previous studies, while also taking international standards for permissible levels in soils into consideration (9 mg kg^{-1} for Cr(VI) and 10 μg kg^{-1} for lindane), in order to ensure that microorganisms with high toxicity resistance could be obtained (Benimeli et al. 2006). The concentrations used are also consistent with those observed by a variety of authors in co-contaminated environments (El Deeb and Altalhi 2009; Olaniran et al. 2009, 2013; Roane et al. 2001; Shi et al. 2013), who have reported lindane and Cr(VI) concentrations in the order of μg L^{-1} and mg L^{-1}, respectively, in different environmental compartments such as soil, groundwater, rainwater, etc. Such contamination levels produce acute toxicity in animals (Harris et al. 2011; Srivastava et al. 2007). Based upon their tolerance to the individual toxic elements and the mixture, the

strains *Streptomyces* spp. A5, A11, M7, MC1, and *Amycolatopsis tucumanensis* were initially selected for use in the further studies discussed below.

The use of a single population involves many metabolic limitations, which could be avoided by using a mixed community of microorganisms. In nature, microorganisms exist as elements of microbial consortia, made up of multiple populations that coexist and carry out complex chemical processes and physiological functions in order to enable survival of the community. Microbial consortia can combine the catalytic specialties of different species to metabolize new substrates, including pesticides (Dejonghe et al. 2003; Fuentes et al. 2011; Shong et al. 2012; Smith et al. 2005; Yang et al. 2010).

A microbial consortium formed by resistant actinobacteria could thus enhance the potential to simultaneously remove Cr(VI) and lindane, however, the absence of antagonism between the consortium members is a major issue. The presence of potential antagonistic effects among the isolated strains was evaluated (Fuentes et al. 2011), Petri dishes with solid MM were sown as follows: one of the strains was spread in the center of the plate and faced transversely with the other microorganisms to be assayed. It was considered a strain to be antagonistic to the other evaluated strains if a growth inhibition was observed. In this way, the presence of antagonism among the strains studied was assessed by considering all possible combinations. However, when these individual strains confronted each other on solid MM, it was observed that *Streptomyces* sp. A11 had an inhibitory effect on the growth of *Streptomyces* sp. MC1 and *Streptomyces* sp. M7. These results suggested that it would be best to develop a consortium with *Streptomyces* spp. A5, MC1, M7, and *Amycolatopsis tucumanensis* for removal of lindane and Cr(VI).

11.5 Soil Bioremediation Performance

11.5.1 Sterilized Soil Samples Co-contaminated with Cr(VI) and Lindane

This study was conducted in order to determine the ability of the selected actinobacteria to grow and to remove Cr(VI) and lindane in sterilized SS.

Non-polluted soil samples (SS) were collected from near the surface (5–15 cm deep) and stored in the dark at 10–15 °C until being utilized. Glass pots were filled with 200 g of soil and kept at 20 % humidity using distilled water. The SS were steam-sterilized (three successive sterilizations at 24 h intervals, at 100 °C for 1 h each) (Polti et al. 2009). The sterilized soil samples (SSS) were each inoculated with either an individually selected actinobacterium or with the mixed culture (the four actinobacteria selected after the resistance assay) to a final inoculum concentration of 2 g kg^{-1} of soil (wet weight).

The inoculated SSS were then contaminated with 25 μg $kg^{-1}$of lindane and 50 mg kg^{-1} of Cr(VI). Also, inoculated SSS without toxics and non-inoculated SSS with both toxics were used as controls.

Table 11.1 Microbial growth in SSS after 14 days at 30 °C

Strain	Non contaminated SS	Contaminated SS
A. tucumanensis	2×10^8 (a)[a,b]	4×10^7 (ab)
Streptomyces sp. MC1	2×10^8 (a)	5×10^7 (a)
Streptomyces sp. M7	2×10^8 (a)	4×10^8 (a)
Streptomyces sp. A5	2×10^8 (a)	2×10^8 (a)
Consortium	1×10^9 (c)	2×10^8 (c)

[a]Different letters indicate significant differences ($p<0.05$)
[b]CFU L^{-1}

After 14 days at 30 °C, samples were taken at the end of each assay to determine the lindane and chromium concentrations, also microbial growth was evaluated.

Microbial growth was determined as CFU g^{-1} by transferring 1 g of soil from each pot into a sterile flask, containing 9 ml of a sterile sodium hexametaphosphate solution (1.66 g L^{-1}, pH 7), Samples were then vortexed during 10 min and tenfold serial dilutions were made in NaH_2PO_4 (0.05 M, pH 7) and plated out onto solid MM in triplicate. Plates were incubated at 30 °C for 72 h (Polti et al. 2009).

After 14 days, the growth of the individual strains *Streptomyces* spp. A5, M7, and *Amycolatopsis tucumanensis* as well as the growth of the consortium was significantly inhibited by the contaminants ($p<0.05$). However, *Streptomyces* sp. MC1 showed similar growth levels in the presence or absence of both contaminants (Table 11.1). This result agrees with those of previous studies carried out in sterilized SS contaminated with 50 mg $kg^{-1}$of Cr(VI) and inoculated with *Streptomyces* sp. MC1 (Polti et al. 2009). On the other hand, for *Streptomyces* sp. M7, Benimeli et al. (2008) found no growth inhibition in SS contaminated with 100 µg $kg^{-1}$of lindane, and it would thus appear that the combined presence of the two types of contaminants probably caused the observed growth inhibition in this strain.

Potentially bioavailable chromium in the soil was measured by a physical method: 100 g of soil were centrifuged at 5,050 × *g* during 60 min, to reproduce the maximal plant suction (soil water potential: −1,500 kPa, conventional wilting point) (Csillag et al. 1999). After centrifugation, the supernatant was recovered, filtered at 0.45 µm and analyzed by AAS for Cr content (APHA 1989). After 14 days of incubation, bioavailable chromium levels were determined. In control flasks, bioavailable chromium was reduced from 50 to 12 mg kg^{-1}. This result agree with previously reported by other authors (Kotas and Stasicka 2000; Mandiwana et al. 2007; Polti et al. 2011; Stewart et al. 2003), where a fraction of chromium was adsorbed by soil compounds. Over time, this concentration was kept constant. Bioavailable chromium concentration detected in control flasks (12 mg kg^{-1}) was considered as 100 % to further calculations. *Streptomyces* spp. MC1, M7, A5, and the consortium were able to completely remove the bioavailable chromium, while *Amycolatopsis*

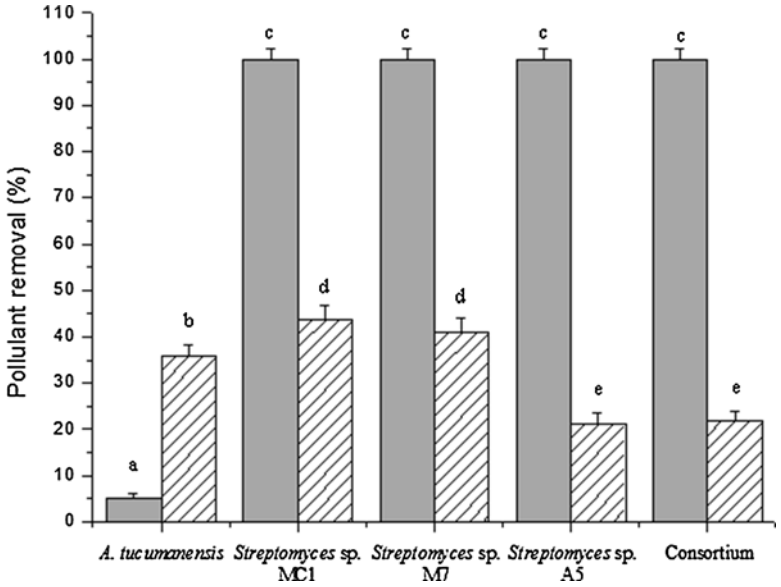

Fig. 11.1 Bioavailable chromium (*grey shaded box*) and Lindane (*stripped box*) removal in sterilized SS, after 14 days at 30 °C. Means with *different letters* are significantly different ($p < 0.05$)

tucumanensis removed only 5 % (Fig. 11.1). Previous studies have demonstrated that the bioavailable chromium fraction is exclusively formed by Cr(VI) (Polti et al. 2011), and it can therefore be inferred that the reduction of bioavailable chromium is due to either Cr(VI) reduction to Cr(III) or to bioaccumulation of chromium by *Streptomyces* spp. MC1, M7, and A5. However, since *Amycolatopsis tucumanensis* showed little ability to reduce the bioavailable chromium, its Cr(VI) tolerance must reflect a different mechanism, such as metal exclusion by a permeability barrier or active transport of the metal away from the cell (Bruins et al. 2000). In this case, the metal resistance mechanism does not have relevance in terms of bioremediation processes. It can also be mentioned that Albarracín et al. (2008b) demonstrated copper accumulation by *Amycolatopsis tucumanensis*, with electron microscopy studies demonstrating the presence of copper binding-proteins inside the cell. This mechanism would thus seem to be specific to copper, or at least it is not utilized with chromium.

Polti et al. (2009) previously reported that *Streptomyces* sp. MC1 removed more than 90 % of bioavailable chromium after 14 days of incubation in SSS contaminated with 50 mg kg^{-1} of Cr(VI). In the present work, this strain maintained this ability despite the presence of a second pollutant.

The extraction and determination procedure for γ-HCH residues in soil was performed according to Fuentes et al. (2011). The changes in lindane concentration in controls were also evaluated. No variations of lindane concentrations in both control

series were observed (data not shown), so, there was no evidence of noticeable contribution of abiotic processes to pesticide removal. *Streptomyces* spp. MC1, M7, A5, *Amycolatopsis tucumanensis*, and the consortium were all able to remove significant amounts of lindane ($p < 0.05$). *Streptomyces* sp. MC1 and *Streptomyces* sp. M7 showed the highest removal levels (44 and 41 % respectively), while *Streptomyces* sp. A5 and the consortium removed 22 and 21 %, respectively. *Amycolatopsis tucumanensis* removed 36 % (Fig. 11.1).

The actinobacteria showing better performance in the sterilized SS were selected to carry out studies in non-sterilized SS (NSSS), in order to evaluate the influence of the native microbial flora on their ability to remove Cr(VI) and lindane.

11.5.2 Nonsterilized Soil Samples Co-contaminated with Cr(VI) and Lindane

Non-sterilized soil samples (NSSS) were inoculated with the selected actinobacteria, then contaminated with 25 μg $kg^{-1}$of lindane and 50 mg kg^{-1} of Cr(VI). Flasks were incubated at 30 °C during 14 days. Similarly, inoculated NSSS without toxics and non-inoculated NSSS with both toxics were used as controls.

In control flasks, bioavailable chromium was reduced from 50 to 18 mg kg^{-1}. This result agree with previously found in non-sterilized soils (Polti et al. 2011). The bioavailable chromium fraction was lower in SSS than in NSSS, sterilization process modifies adsorption properties of soil, probably exposing or activating reactive groups of soil, and also the adsorbing surfaces that control the heavy metals solubility (Egli et al. 2006). Similarly to that observed in SSS, over time, this concentration was kept constant. Bioavailable chromium concentration detected in control flasks (18 mg kg^{-1}) was considered as 100 % to further calculations.

Bioavailable chromium removal of by *Streptomyces* sp. M7 (28 %) was significantly higher ($p < 0.05$) than by the consortium (14 %) (Fig. 11.2). It is noticeable that the bioavailable chromium removal produced by the consortium decreased significantly in the NSSS in comparison with the SSS. The main barrier to the use of communities in bioprocesses is the need for simultaneous control of both individual organisms as the ecosystem as a whole. It is possible that the different consortium members had different behaviors in relation to the native flora, resulting in a decrease in the overall performance of the consortium (Shong et al. 2012).

The changes in lindane concentration in controls were also evaluated. No variations of lindane concentrations in both control series were observed, so, there was no evidence of noticeable contribution of autochthonous microflora on the pesticide removal.

Lindane removal by the consortium and Streptomyces sp. M7 was higher than 50 % (Fig. 11.2).

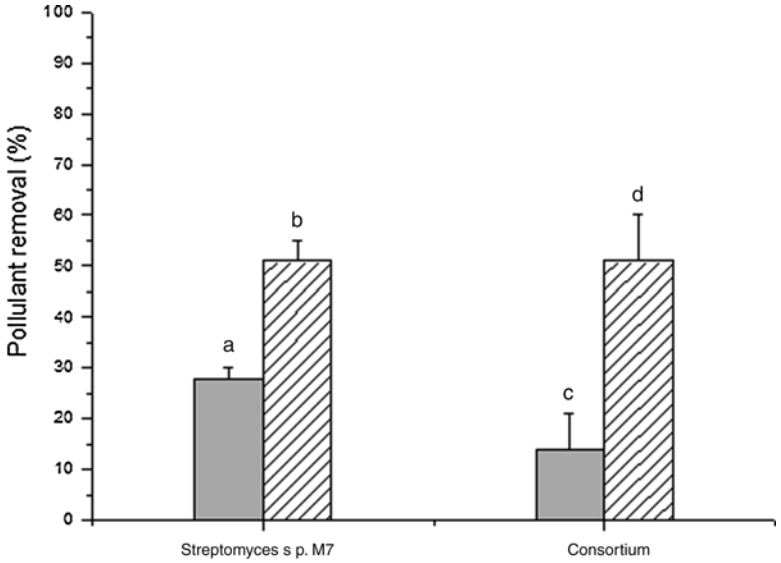

Fig. 11.2 Bioavailable chromium (*grey shaded box*) and Lindane (*stripped box*) removal in non sterilized SS, after 14 days at 30 ºC. Means with *different letters* are significantly different ($p < 0.05$)

11.6 Concluding Remarks

Based upon these results, it appears that *Streptomyces* sp. M7 and the consortium makeup of the four actinobacterial strains tested could be useful for bioremediation of soils co-contaminated with Cr(VI) and lindane. However, taking into account the importance of cost–benefit ratios in biotechnological processes and the fact that the use of a consortium is more complex and time consuming and also carries higher risks of contamination, the use of *Streptomyces* sp. M7 alone would seem to be most suitable for these types of processes.

In a next step, laboratory testing must be scaled up to field. It is mandatory to transfer the acquired knowledge to benefit the affected population.

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