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Applied of actinobacteria consortia-based bioremediation to restore co-contaminated systems

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Abstract

Global industrialization and natural resources extraction have left cocktails of environmental pollutants. Thus, this work focuses on developing a defined actinobacteria consortium able to restore systems co-contaminated with pollutants occurring in Argentinian environments. In this context, five actinobacteria were tested in solid medium to evaluate antagonistic interactions and tolerance against lindane (LIN), Reactive Black B-V (RBV), phenanthrene (Ph) and Cr(VI). The strains showed absence of antagonism, and most of them tolerated the presence of individual pollutants and their mixtures, except *Micromonospora* sp. A10. Thus, a quadruple consortium constituted by *Streptomyces* sp. A5, M7, MC1, and *Amycolatopsis tucumanensis* DSM 45259^{T}, was tested in liquid systems with individual contaminants. The best microbial growth was observed in the presence of RBV and the lowest on Cr(VI). Removals detected were 83.3%, 65.0% and 52.4% for Ph, RBV and LIN, respectively, with absence of Cr(VI) dissipation. Consequently, the consortium performance was tested against the organic mixture, and a microbial growth similar to the biotic control and a LIN removal increase (61.2%) were observed. Moreover, the four actinobacteria of the consortium survived the mixture bioremediation process. These results demonstrate the potential of the defined actinobacteria consortium as a tool to restore environments co-contaminated with organic pollutants.

Keywords: Defined consortium; Actinobacteria; Co-contamination; Bioremediation; Microbial survival.

Abbreviations: lindane (LIN), Reactive Black B-V (RBV), phenanthrene (Ph), minimal medium (MM), starch-casein agar medium (SC), tryptic soy broth (TSB), water-agar medium (WAM), biotic control (BC), abiotic control (AC), individually contaminated systems (ICS), simultaneously contaminated system (SCS).

1. Introduction

The tremendous increases in industrialization and natural resource extraction have created extreme environmental contamination. A vast evidence shows higher risks to human health by the presence of cocktails of pollutants in nature that are causing a global epidemic of cancer and other degenerative diseases [1]. These cocktails can include different pollutants such as pesticides, hydrocarbons, metals, or dyes, which are able to generate dangerous co-contaminated systems. Argentina is no stranger to this problem, whereby, some co-contaminated sites of this country can be mentioned. For example, Aparicio et al. (2018a) [2] reported the simultaneous presence of chromium and lindane in soil samples from Chicoana and Lerma Valley, in Salta province, in concentrations ranged between 26 and 1296 mg Kg^{-1} and 111 and 586 μg Kg^{-1}, respectively. Some of the detected concentrations exceed the maximum values established in the Argentine Hazardous Waste Law Nº 24051 (lindane: 10 μg kg^{-1}, total Cr: 250 mg kg^{-1}). Also, contaminated sites with pesticide mixtures, such as glyphosate and atrazine in the Pampa region [3], and organochlorine pesticides in the Southwest of Buenos Aires province [4] were detected. Other organic pollutants, such as polycyclic aromatic hydrocarbons, were found in contaminated areas of Buenos Aires province. Among them, phenanthrene was one of the most abundant compounds detected in all the soil analyzed samples, with concentration values ranging between 30.69 and 135.36 ng g^{-1} [5]. In addition, dyes from textile effluents, were detected in the Medrano Creek waters, one of the fresh watercourses that goes through the Metropolitan Area of Buenos Aires [6]. The clean-up of these co-contaminated environments is more complex and challenging than those sites with single contamination due to the diverse remediation pathways required for the different types of pollutants [7]. Bioremediation is a suitable strategy to solve this environmental problem, especially using microbial consortia. It is clear the complex nature of the environmental bioremediation and the advantages of using microbial consortia robust, stable, and with

synergistic activity to remove toxic compounds [8]. Inspired by the properties of microbial communities naturally present in the environment, the consortia-based bioremediation concept has become promising. In these systems, synthetic microbial consortia with two or more key species carry out different functions in the bioremediation processes. These functions include cooperative work based on microbial interactions and labor division [9]. These functions are crucial when looking to restore co-contaminated areas, which can be achieved by employing microbial consortia to increase the number of catabolic pathways available to biodegrade contaminants, improving the overall resource utilization efficiency and reducing the formation of byproducts [10]. In this sense, there is previous information about the advantage of using actinobacteria consortia for bioremediation processes [11, 12, 13, 14]. These bacteria with cosmopolitan distribution play relevant ecological roles since they are involved in the recycling of substances, degradation of complex polymers, and production of bioactive molecules. These versatile microorganisms with biotechnological potential also show abilities to remove organic and inorganic pollutants, which are the reasons why actinobacteria have received special attention as candidates for bioremediation processes [8]. The physiological properties of these bacteria and the advantages to use them as microbial consortia, give the possibility to design an actinobacteria defined consortium with potential to bioremediate co-contaminated systems. The importance of the defined consortia lies in the fact that they provide a technology capable of being reproduced, as needed, and exhaustively studied to elucidate the role of each constituent member. In addition, with the knowledge of the genomic information of consortium members, the current technology can generate metatranscriptomics and metaproteomics data to compare the temporal gene expression (mRNA and protein) between pure cultures and consortia, and provide information which allows a comprehensive approach to decipher the interactions among the microorganisms of the consortium [9].

Herein, the present work was focuses on designing an actinobacteria defined consortium constituted by strains with individual abilities to remove different toxic compounds as a versatile bioremediation tool able to be reproduced as needed and applied to restore systems co-contaminated with pollutants occurring in Argentinian environments.

2. Materials and methods

2.1 Microorganisms

Five actinobacteria from the culture collection of PROIMI-CONICET (Pilot Plant of Microbiological Industrial Processes, Tucumán, Argentina) with capabilities to remove different toxic compounds, were selected to conduct this study: *Streptomyces* sp. A5, M7 and *Micromonospora* sp. A10, able to remove organochlorine pesticides [15, 16], *Streptomyces* sp. MC1, resistant to Cr(VI) [17], and *Amycolatopsis tucumanensis* DSM 45259^T (AB0^T strain) resistant to Cu(II) [18], which also presents capability to degrade phenanthrene [19].

2.2 Culture media and solutions

The following culture media were used: Minimal medium (MM) containing in g L^{-1}: L-asparagine, 0.5; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; $FeSO_4.7H_2O$, 0.01; glucose, 10.0, was used for removal assays. To formulate solid MM, agar 10 g L^{-1} was added. Starch-Casein Agar medium (SC) was used to obtain actinobacteria spores and for antagonism assays among the strains. It contains (g L^{-1}): starch, 10.0; casein, 1.0; K_2HPO_4, 0.5; agar, 15.0. Tryptic Soy Broth (TSB) was used for the preparation of actinobacteria inoculum in survival evaluation tests. It contains (g L^{-1}): tryptone, 15.0; soy peptone, 3.0; NaCl, 5.0; K_2HPO_4, 2.5; glucose, 2.5. Water-agar medium (WAM) containing in g L^{-1}: Agar 15.0, was used for sealing channels in tolerance tests. All culture media were adjusted to pH 7.0 ± 0.2, and sterilized by autoclaving at 121 °C, for 15 min.

The following stock solutions were prepared: 1 mg mL^{-1} of lindane (LIN) and 25 mmol L^{-1} of phenanthrene (Ph), both dissolved in acetone as solvent [19, 20]; 2 g L^{-1} of the azo dye Reactive Black B-V (RBV) was dissolved in distilled water [21], and 1 mM of Cr(VI), as

$K_2Cr_2O_7$ were dissolved in water [22]. The solutions were sterilized by filtration, using Millipore filters of 0.22 µm pore size (Millipore Corp., Bedford, USA).

2.3 Antagonism and tolerance assays

Antagonism test: in order to determine the presence of antagonistic effects among the studied actinobacteria, each strain was spread in the center of a Petri dish containing SC medium and faced transversely with the other strains, making all possible combinations. Petri dishes were incubated 7 days at 30 °C. The positive antagonistic effect was considered when growth inhibition among the evaluated strains was detected [13].

Tolerance test: actinobacteria tolerance against LIN, Ph, RBV, Cr(VI) and their mixtures was assayed. First, Petri dishes filled with solid MM were inoculated with each actinobacteria on study, as spore lawn. Then, rectangular troughs were cut in the center of the plate, sealed with WAM, filled with the contaminants (LIN: 2 mg L^{-1}, Ph: 17.8 mg L^{-1}, RBV: 200 mg L^{-1}, Cr(VI): 52 mg L^{-1}) pure or in all double, triple, and quadruple mixtures, and incubated at 30 °C for 7 days. The pollutants concentrations assayed were selected according to previous bioremediation studies [17, 19, 21, 23]. Tolerance was evaluated by taking into account the microbial growth and RBV discoloration of mixtures containing the dye, considering the optimal growth and any grade of color removal as a positive result. The negative result was considered if any grades of growth inhibition or absence of discoloration were detected [13]. The results were expressed in percentages and calculated based on the total number of double, triple, or quadruple mixtures assayed, as corresponding.

2.4 Actinobacteria consortium performance in contaminated liquid systems

Four actinobacteria (*Streptomyces* sp. A5, M7, MC1 and *A. tucumanensis* AB0^T) were selected, based on antagonism and tolerance tests, to formulate a microbial consortium. The capability of the designed consortium to grow and remove the contaminants in individual conformations or mixtures was evaluated. A spore suspension of the consortium was inoculated at a final concentration of 5×10^6 CFU mL^{-1} (each strain was added equally) in flasks with 30 mL of MM, individually or simultaneously contaminated with LIN (2 mg L^{-1}), Ph (17.8 mg L^{-1}), RBV (200 mg L^{-1}), and Cr(VI) (52 mg L^{-1}), as appropriate. Each mixture component was supplemented with the same concentration used to test individual contaminants. The flasks were incubated at 30 °C for 7 days on a rotary shaker (200 rpm). The cultures were then centrifuged (8,500 × *g*, 10 min, 4 °C) in order to determine the residual concentration of toxic compounds in cell-free supernatants and the microbial growth. The procedure was performed in triplicate, including inoculated flasks without contaminants and non-inoculated flasks with contaminants, which were used as biotic (BC) and abiotic (AC) controls, respectively.

2.5 Analytical procedures

2.5.1 Biomass determination

The microbial growth was estimated by biomass determination, washing the pellets with 25 mM Tris-EDTA buffer (pH 8.0) and then drying at 105 °C until constant weight [16]. Results was expressed as grams of dry weight per liter of culture.

2.5.2 Residual contaminants determination

For the determination of residual LIN concentration, first, a solid phase extraction using a C18 column (Agilent Technologies Inc., USA) was carried out. The extracts obtained were analyzed by GC-μECD in a Gas Chromatograph Agilent 7890A equipped with a HP5 capillary

column (30 m × 0.320 mm × 0.25µm), a ^{63}Ni-µECD detector, a split/splitless Agilent 7693B injector and Agilent Chem-Station software, following the chromatographic conditions described previously [24]. Quantitative sample analysis was performed using appropriate dilutions of calibration standards (AccuStandard, New Haven, CT, USA).

Residual Ph was quantified according to Bourguignon et al. (2014) [19]. Briefly, Ph was extracted from the supernatants by adding acetone (30 mL) and filtering with a 0.22 µm–nylon membrane (Microclar, Argentina). The Ph determination was carried out by RP-HPLC using an HPLC equipment coupled to a PDA 2998-detector (Alliance e2695, Waters Co., MA, USA), operating at a fixed wavelength (λ=276 nm). Samples were automatically injected into C18 µBondapak HPLC column (4.6×250 mm, 50 Å pore size, 5 µm particle size). Ph concentrations were calculated applying the external standard method.

Cr(VI) concentration was determined in aliquots of supernatants, using the Cr(VI) specific colorimetric reagent 1,5 diphenylcarbazide, dissolved in acetone to a final concentration of 5 mg mL^{-1}, as described Aparicio et al. (2018b) [11].

RBV discoloration was monitored by using a microplate reader, from culture supernatants, at 595 nm. Color removal was reported as percentage decolorization: $(A_0-A_T)/ A_0*100$, where A_0 and A_T were the absorbance of dye-amended medium at the start point (0) and at a cultivation time (T), respectively [21].

2.6 Consortium survival evaluation

The survival of the four strains of the microbial consortium at the end of bioremediation assays was determined by an antibiotic sensitivity test, and a Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR), according to the methodology of Raimondo et al. (2020a) [25]. Considering the sensitivity of the strains belonging at the designed

consortium, different antibiotics were evaluated in SC medium, to allow the differential growth of each strain. The antibiotic sensibility profiles were used for the re-isolation of each actinobacteria from the MM at the end of the bioremediation assay. For that, appropriate dilutions of samples obtained from bioremediated systems were seeded on SC plates supplemented with Imipenem (10 μg mL^{-1}), Lincomycin (20 μg mL^{-1}), Erytromycin (70 μg mL^{-1}), Gentamicin (25 μg mL^{-1}), Minocycline (15 μg mL^{-1}), according to the re-isolated strain. The four actinobacteria of the consortium (control) and the actinobacteria reisolated from the bioremediated systems were individually cultured in TSB. Then, total DNA from these cultures was extracted and purified. Detection of characteristic genetic polymorphisms of each strain was carried out by DNA amplification through RAPD-PCR, based on the use of two primers with random sequences: DA F 5′-GAG GTC GTG CTG ACC GTG CTGCA-3′ and DA R 5′-GTT GAT GTG CTG GCC GTC GACGT-3′, at 50 °C as the annealing temperature [26]. The obtained products of each individual strain profile were visualized by using polyacrylamide gels stained with 6% $AgNO_3$.

2.7 Statistical analysis

All the assays were carried out in triplicate and the results are presented as means ± standard deviation. Statistically significant differences in means were tested using one-way analysis of variance (ANOVA). Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1 Antagonism and tolerance assays

Under the assayed conditions, all studied actinobacteria showed an absence of antagonistic effects among them (Fig. 1). This result suggests that these strains could be cultured together as a defined microbial consortium.

The antagonism phenomenon is a common event showed in a mixed microbial population [27]. In order to use a defined actinobacteria consortium, it is important to study this phenomenon due to the capacity of these bacteria to produce different secondary metabolites [8]. These metabolites could affect the growth of the microbial consortium strains and their different metabolic abilities. In the present work, the absence of antagonistic effects observed between the strains would allow us to use them simultaneously as a defined microbial consortium for a particular purpose. These results agree with those reported by Fuentes et al. (2013, 2016) [13, 12]; they noticed that different *Streptomyces* strains did not show growth inhibition when they were tested in antagonism assays for their afterwards use, to remove chlordane or a mixture of chlorpyriphos and pentachlorophenol. Saez et al. (2018) [28] also studied the presence of antagonism among different actinobacteria and fungi, observing the absence of this phenomenon between *Trametes versicolor* S5NG1 and *Streptomyces* sp. A2, A5, A11, M7; therefore, the authors used these five microorganisms as a defined mixed culture. In contrast, in the compatibility study of five actinobacteria for bioremediation soils contaminated with Cr(VI) and LIN, one of the strains demonstrated a negative effect on the growth of the other tested bacteria [22].

The absence of antagonism is not the only phenomenon that must be studied when a consortium with the ability to remediate contaminated systems is designed. The microbial tolerance to toxic compounds is another crucial factor that must be evaluated. Therefore, in the

248 present work, the tolerance of the actinobacteria against LIN, Ph, RBV, Cr(VI) individually and
249 their mixtures were explored, considering that optimal microbial growth indicates the absence
250 of toxicity of contaminants on the microorganisms. In addition, any extent of discoloration was
251 considered a reflection of the ability of the assayed strain to metabolize RBV.

252 All the strains showed optimal growth in the presence of the contaminants except
253 *Micromonospora* sp. A10, which was sensible against Cr(VI), RBV, the double mixture
254 LIN/Ph, and the triple mixtures Ph/Cr(VI)/RBV and Cr(VI)/RBV/LIN (Table 1). This strain
255 didn't show growth in the simultaneous presence of the four contaminants. The color removal
256 was detected for most of the evaluated systems added with RBV, except for those inoculated
257 with *Micromonospora* sp. A10 in the presence of the triple mixture Ph/LIN/RBV, and the
258 quadruple mixture. Also, *Streptomyces* sp. A5 could not eliminate the dye color from the triple
259 mixture Ph/LIN/RBV.

260 Actinobacteria have received special attention as candidates for bioremediation because of
261 their ability to remove organic and inorganic pollutants [8]. Thus, in the present study, the
262 tolerance of each strain against the different toxic compounds, either individual or in mixtures
263 form was explored. Four of the five assayed actinobacteria showed optimal growth in the tested
264 conditions, except *Micromonospora* sp. A10. Despite the previous information about the ability
265 to grow and remove hydrocarbons, organochlorine pesticides, azo dye, and Cr(VI), by
266 microorganisms belonging to *Streptomyces, Amycolatopsis* and *Micromonospora* genera [29,
267 16, 22, 30], *Micromonospora* sp. A10 showed a variable behavior with respect to their growth
268 in the presence of Cr(VI), RBV, and the different mixtures of compounds. However, the growth
269 of the strains belonging to the *Streptomyces* and *Amycolatopsis* genera seemed unaffected by
270 the simultaneous or individual presence of the tested pollutants. In this sense, previous studies
271 demonstrated the ability of *Streptomyces* and *Amycolatopsis* strains to tolerate LIN, Ph, and
272 Cr(VI). For example, Polti et al. (2014) [22] evaluated the growth of different actinobacteria in

the presence of 500 mg L^{-1} of Cr(VI) and/or 250 μg L^{-1} of LIN, observing that *Streptomyces* sp. A5, A11, M7, MC1, and *A. tucumanensis* DSD 45259^T showed similar growth to that detected in the uncontaminated control. In addition, Bourguignon et al. (2014) [19] evaluated the use of 15 actinobacteria for the removal of polycyclic aromatic hydrocarbons, including Ph, naphthalene, and pyrene. Interestingly, the maximum Ph degradation was observed in systems inoculated with *A. tucumanensis* DSM 45259^T and *Streptomyces* sp. A12.

It is important to highlight that the environment often receives a cocktail of pollutants rather than a single compound, so these co-contaminated systems can be more toxic than those impacted with only one contaminant. In this sense, Khudur et al. (2018) [31] studied the effects of the co-contamination with heavy metals and total petroleum hydrocarbons and observed that the associated toxicity was significantly increased. In the present study, the complex mixture of the four pollutants seems to exert a negative effect on *Micromonospora* sp. A10 which was especially affected in terms of growth and RBV discoloration abilities. Based on these results, *Micromonospora* sp. A10 was discarded for the following experiments. The remaining actinobacteria (*Streptomyces* sp. A5, M7, MC1 and *A. tucumanensis* AB0^T) were selected to formulate a microbial consortium and evaluate its ability to bioremediate contaminated liquid systems.

3.2 Evaluation of the actinobacteria consortium performance in contaminated liquid systems

3.2.1 Microbial growth evaluation

The influence of the individually studied contaminants on the actinobacteria consortium growth was analyzed over time. The consortium biomass showed an increase until 48 h of incubation, in the presence of Ph and Cr(VI), as well as in the biotic control. In RBV presence,

an increase in the microbial biomass was detected until 96 h. The only case in which the actinobacteria consortium grew until the end of the assay (144 h) was in LIN presence (Fig. 2).

The best growth value (1.38 ± 0.01 g L^{-1}) in individually contaminated systems was detected in RBV presence. This finding could be due to the ability of the studied consortium to degrade this dye, and probably use it as carbon source. In this regard, it is noteworthy that the microbial growth in the system contaminated with RBV was higher than that detected in the biotic control (Fig. 2). This result is not strange since existing previous reports about the actinobacteria potential, especially of the *Streptomyces* genus, to degrade azo dyes [32]. In contrast, the system contaminated with Cr(VI) showed the lowest microbial growth values (0.14 ± 0.02). This scarce microbial growth could be due to the toxicity of this metal with high solubility and mobility, able to penetrate the microbial membrane and damage the cell [33].

Taking into account the lowest growth of the actinobacteria consortium in the system contaminated with Cr(VI), its behavior was subsequently evaluated in presence of a metal-free mixture that included Ph, LIN, and RBV (Fig. 2). In simultaneous occurrence of these organic pollutants, the biomass of the consortium increased until 96 h, reached at this incubation time a biomass value similar to observed in the biotic control. From this time, biomass values detected were similar both, in presence or absence of the organic mixture, without statistically significant differences ($p > 0.05$) between them. The positive response of the consortium against this toxic organic mixture could be due to the effect of the cooperative work among the four actinobacteria strains, which were individually selected for their ability to growth and remove the individual pollutants [19, 16, 17]. This result agrees with the study of Fuentes et al. (2013) [13], who observed that actinobacteria growth on a chlorpyrifos and pentachlorophenol mixture was higher in mixed cultures than in axenic cultures. This behavior was attributed to a possible metabolic complementary action among the actinobacteria constituents of consortia, which allowed a most efficient use of these pesticides as carbon source.

Considering all the assayed conditions, the microbial growth observed at 144 h of incubation showed the following order: RBV > LIN > MIX > CB > Ph > Cr(VI), and the corresponding biomass values were 1.38 ± 0.01 g L^{-1}; 1.21 ± 0.04 g L^{-1}; 1.19 ± 0.16 g L^{-1}; 1.15 ± 0.25 g L^{-1}; 0.57 ± 0.06 g L^{-1}, and 0.14 ± 0.02, respectively.

3.2.2 Contaminants removal evaluation

Bioremediation involves using microorganisms capable of removing pollutants individually or in mixtures [34]. Therefore, the ability of the designed actinobacteria consortium to remove Ph, Cr(VI), LIN, RBV, and the triple mixture Ph/LIN/RBV from liquid systems through time, was analyzed (Fig. 3 and 4).

As observed in Fig. 3, the removal of individual toxicants by the microbial consortium action showed a positive dissipation of organic compounds and the absence of metal removal. The studied consortium showed the ability to remove Ph through time, reaching the highest removal percentage at the end of the assay (144 h), with a value of 83.3 ± 7.8%, against the 13.5 ± 0.0% reached by the abiotic control (AC), which allows inferring that the 69.8% of the detected removal corresponds to the consortium action. This 13.5% of Ph removal detected in the AC could be associated with volatilization processes, as was postulated by Isaac et al. (2015) [35]. Notably, the Ph removal obtained in this work practically doubles the percentage obtained in a previous study (36.2%), where a pure culture of *A. tucumanensis* DSM 45259^{T} was assayed to remove this hydrocarbon from liquid systems [19]. This result highlights the advantages to using microbial consortia since they allow an increase in the metabolic pathways available to degrade pollutants; especially if each strain plays a fundamental role in the transformation of a compound, providing to other consortia members, intermediate products less toxic or compounds able to be metabolized [10]. In this sense, it is known that during the degradation

of hydrocarbons, the intermediary compounds generated can be subsequently used by microorganisms that benefit others, for which they could be toxic. These synergistic relationships among the constituents of a consortium promote a more significant degradation process [36], as was observed in the present work.

In LIN presence, the inoculated systems and the AC registered 52.4 ± 0.4% and 12.4 ± 0.0% of pesticide removal, respectively, at the end of the assay (Fig. 3). The removal detected in the inoculated systems at the 96 and 144 h of incubation, not shown statistically significant differences ($p > 0.05$). Taking into account the removal percentage values detected in the AC and the inoculated system, 40.0% of LIN removal can be attributed to the actinobacteria consortium activity. The removal level reached by the consortium was higher than the one reported by Fuentes et al. (2011) [14], when they studied two of the strains belonging to the microbial consortium formulated in the present work (*Streptomyces* sp. A5 and M7), in pure cultures.

Regarding RBV, its discoloration was evident after 48 h of incubation, showing a color removal of 65.0 ± 0.0% at the end of the assay. In contrast, the AC showed a practically null color decrease (1.1%) (Fig. 3). In this case, it is important to highlight that the glucose in the media supported the microbial consortium growth until 48 h of incubation in the BC (Fig. 2), and in the systems added with the azo dye the microbial growth continued until 96 h, reaching a final biomass value higher and statistically difference ($p < 0.05$) than the BC one (Fig. 2), showing a detectable discoloration from 48 h of incubation. This is due to the ability of the microbial consortium to metabolize the azo dye, which was exhibited in 65% of the color removal detected (Fig. 3). These results demonstrate the microbial consortium's ability to tolerate and remove RBV, and allow inferring on its capability to use the dye as a carbon source. Chemical dye structures are complex; however, there are a great number of microorganisms capable to eliminate their color through mechanisms such as biosorption, anaerobic or aerobic

biodegradation, and the production of enzymes able to catalyze their discoloration process [37]. Among them, actinobacteria, especially those belonging to the *Streptomyces* genus, demonstrated their ability to decolorize and mineralize different textile dyes [37]. In this sense, it was reported that oxidative and reductive enzymes in actinobacteria make them excellent biological systems for the degradation of textile dyes [38]. Moreover, it was found that microbial consortia prove a significant efficiency in dyes decolorization, showing advantages compared to pure cultures due to the concerted metabolic activities of the microbial community [39].

In opposition to the removal of the organic pollutants observed, the microbial consortium was unable to remove Cr(VI) under the current assay conditions (Fig. 3). Some heavy metals serve, in trace amounts, as essential nutrients for many organisms; however, they are toxic in higher quantities [40]. This is probably the reason for the low growth and null removal of this pollutant by the assayed consortium at a high concentration of the metal (52 mg L^{-1}). In this respect, Mansilla (2016) [41] evaluated Cr(VI) removal by *Streptomyces* sp. M7 in liquid systems added with three different concentrations of the metal and observed that at higher concentrations, the removal and the microbial growth detected were less. Based on the obtained results, it was decided to continue with the study using systems contaminated with a mixture of organic compounds.

The residual concentration of each pollutant from the organic mixture significantly decreased along the assay in inoculated culture media (Fig. 4). This result highlights the ability of the designed consortium to remove them when the contaminants are simultaneously present in a complex mixture. In contrast to these findings, Krishna and Philip (2011) [42] observed lower degradation efficiencies of methyl parathion, LIN, and carbofuran in soils contaminated with their mixture regarding the detected in individually contaminated soils. In the present work, in the presence of the mixture, the percentages of Ph removal were 48.5 ± 8.3% and 10.7

398 ± 0.3% in inoculated systems and in the AC, respectively. Therefore, 37.8% of removal was
399 attributed to the microbial consortium action. Regarding pesticide dissipation, it was detected
400 a removal percentage of 61.2 ± 3.9% for LIN in inoculated systems at the end of assay (144 h).
401 In absence of the consortium, only 2.1 ± 0.0% of LIN removal was observed; thereby, the 59.1%
402 to the LIN removal resulted as a consequence of the consortium effect. For RBV, 19.6 ± 0.3%
403 of color removal was observed in inoculated systems and 2.0 ± 0.1% in the AC. Thus, the 17.6%
404 of RBV color decrease was attributed to the action of the microbial consortium.

405 The removal percentages of Ph, LIN and RBV, obtained at the end of the bioremediation
406 assays, were compared among the individually or simultaneously contaminated systems. For
407 LIN, a removal increase (8.8%) significantly higher in co-contaminated than in individually
408 contaminated systems ($p < 0.05$) was detected. However, the removal of Ph and RBV in the
409 mixture was significantly lesser than the detected in individually contaminated systems ($p <$
410 0.05), with 34.8 and 45.4% of removal decrease, respectively (Table 2). Fuentes et al. (2018)
411 [23] observed a similar phenomenon when they studied the ability of a native and non-GMO
412 *Streptomyces* strain to remove three organochlorine pesticides (LIN, chlordane and
413 methoxychlor), in individual and simultaneously contaminated systems. The authors detected
414 an increase in LIN removal (from 57.4% to 62.2%) in the presence of a mixture of the three
415 pesticides. In contrast, when Aparicio et al. (2021) [43] studied the coupling of bacterial and
416 physicochemical treatments to remediate wastewater containing Cr(VI) and organic pollutants,
417 they detected less LIN removal from the mixture. This phenomenon was attributed to the Cr(VI)
418 presence, which is able to inhibit the pesticide degradation from the mixture. These findings
419 support the results obtained in the present work, which demonstrate the toxicity exerted by the
420 metal on the actinobacteria consortium, reflected in a drastic reduction of the microbial growth
421 in its presence.

The present study highlights the ability of the designed actinobacteria consortium to remove the constituents of the tested mixture. The removal percentages detected in inoculated and simultaneously contaminated systems at the end of the assay were: 61.2% (LIN) > 48.5% (Ph) > 19.6% (RBV).

3.3 Consortium survival evaluation

Phenotypic approaches by studies of sensitivity to antibiotics, and genotypic approaches by studies of restriction fragment length and detection of genetic polymorphisms, were conducted to evaluate the survival of the four actinobacteria belonging to the designed consortium at the end of the mixture remediation assay. For these purposes, and based on the antibiotic sensitivity profiles of each actinobacteria strain, Imipenem, Minocycline, Gentamicin, Lincomycin and Erythromycin were used to re-isolate each one, as appropriate, from samples of the bioremediated systems [11]. The re-isolated colonies of the actinobacteria were used to perform RAPD-PCR. After an electrophoretic run of the amplification products, the profiles obtained in polyacrylamide gels confirmed the identity and survival of the four members of the designed microbial consortium, at the end of the test, as can be seen in Fig. 5.

The obtained results confirm the ability of actinobacteria strains to survive the remediation process of the mixture of pollutants as a microbial consortium. This finding, combined with the pollutants removal percentages detected in the inoculated systems (Table 2), demonstrates the potential of the designed consortium to restore wastewater contaminated with multiple organic compounds and survive the process. This is not the first time that the ability of actinobacteria consortia to survive bioremediation processes was shown. In this sense, previous works demonstrate the survival ability of actinobacteria consortia after bioremediation processes in slurry, liquid, and soil systems, polluted [44, 11, 26]. These findings confirm the robustness and

versatility of the designed actinobacteria consortium and allow proposing its use as a possible clean-up tool for co-contaminated environments.

4. Conclusions

This study demonstrates the absence of antagonistic effects among the evaluated actinobacteria and their tolerance against LIN, Ph, RBV and Cr(VI) in individual forms or mixtures. All actinobacteria strains presented optimal growth in tolerance tests, except *Micromonospora* sp. A10. RBV discoloration was observed in most of the tolerance assays carried out with the azo dye. Based on these findings, a quadruple actinobacteria consortium was formulated constituted by *Streptomyces* sp. A5, M7, MC1 and *Amycolatopsis tucumanensis* ABO^{T}. In liquid systems with single contamination, the best and lowest growth values of the consortium were detected in presence of RBV and Cr(VI), respectively. The concomitant depletion of all the organic compounds and the absence of Cr(VI) removal was observed. In liquid systems contaminated with the triple mixture Ph/LIN/RBV, the microbial growth was similar to the observed in the absence of the pollutants, and an increase in the LIN removal was detected. Moreover, the four actinobacteria constituents of the consortium survived the bioremediation process of the systems contaminated with the mixture. These findings demonstrate the feasibility to design a defined actinobacteria consortium to be used as a promising bioremediation tool to restore environments co-contaminated with organic pollutants.

Conflict of interest

The authors declare no conflict of interest.

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625

626 **Legends to figures**

627

628 **Fig. 1.** Antagonism assay among *Streptomyces* sp. A5, M7, MC1, *Micromonospora* sp. A10
629 and *Amycolatopsis tucumanensis* DSM 45259^T, in Starch Casein medium.

630 **Fig. 2.** Growth of the actinobacteria consortium in liquid media added with single or mixed
631 pollutants. BC: biotic control; Ph: phenanthrene; Cr(VI): chromium hexavalent; LIN: lindane;
632 RBV: Reactive Black B-V; MIX: Ph/LIN/RBV.

633 **Fig. 3.** Removal percentages of the pollutants from systems with single contamination,
634 inoculated with the actinobacteria consortium. Ph: phenanthrene; LIN: lindane; Cr(VI):
635 hexavalent chromium; RBV: Reactive Black B-V; AC: abiotic control. The percentage
636 informed for RBV corresponds to the percentage of color removal.

637 **Fig. 4.** Removal percentages of the pollutants from the organic mixture, inoculated with the
638 actinobacteria consortium. Ph: phenanthrene; LIN: lindane; RBV: Reactive Black B-V; AC:
639 abiotic control. The percentage informed for RBV corresponds to the percentage of color
640 removal.

641 **Fig. 5.** Polyacrylamide gel electrophoresis of the fragments amplified. (1) Colony isolated from
642 SC plate added with Erythromycin plus Lincomycin, (2) Colony isolated from SC plate added
643 with Minocycline, (3) Colony isolated from SC plate added with Imipenem, and (4) Colony
644 isolated from SC plate added with Gentamicin. Pure cultures of (5) *Streptomyces* sp. M7, (6)
645 *Streptomyces* sp. MC1, (7) *Streptomyces* sp. A5, and (8) *Amycolatopsis tucumanensis* DSM
646 45259^T, used as reference control.

647

Table 1. Percentages of growth and RBV discoloration by actinobacteria, in systems individually contaminated with LIN, Ph, RBV, Cr(VI) or with their mixtures.

Pollutants / Actinobacteria	Growth (%)*				RBV Discoloration (%)**			
	IP	**DM**	**TP**	**QM**	**IP**	**DM**	**TP**	**QM**
Streptomyces sp. A5	100	100	100	100	100	100	67	100
Streptomyces sp. M7	100	100	100	100	100	100	100	100
A. tucumanensis DSM 45259^{T}	100	100	100	100	100	100	100	100
Streptomyces sp. MC1	100	100	100	100	100	100	100	100
Micromonospora sp. A10	50	83	50	0	100	100	67	0

IP: individual pollutants [LIN, Fn, RBV, Cr(VI)]; **DM**: double mixes [Cr(VI)/RBV, Cr(VI)/LIN, LIN/Fn, LIN/RBV, RBV/Fn, Cr(VI)/Fn]; **TM**: triple mixes [Fn/Cr(VI)/RBV, Cr(VI)/LIN/RBV, Fn/Cr(VI)/LIN, Fn/LIN/RNV]; **QM**: quad mix [Fn/Cr(VI)/LIN/RNV]. **RBV**: Reactive Black B-V, **LIN**: Lindane, **Ph**: phenantrene.

*Percentage of IP and mixes in which microbial growth was optimal.

**Percentage of IP and mixes containing RBV in which discoloration was positive.

Table 2. Comparison of the removal percentages of organic pollutants among inoculated systems, either, individual or simultaneously contaminated.

Pollutant	Removal percentages (%)		
	ICS	SCS	Δ (SCS-ICS)
Ph	**83.3**	48.5	-34.8
LIN	52.4	**61.2**	8.8
RBV	**65.0**	19.6	-45.4

ICS: individually contaminated systems, **SCS:** simultaneously contaminated system

Figure 1

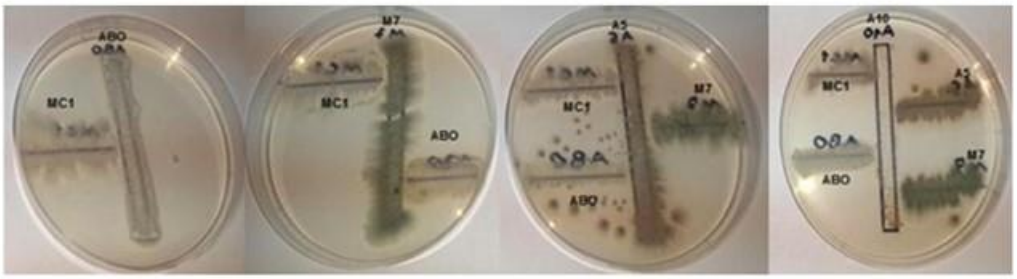


Figure 2

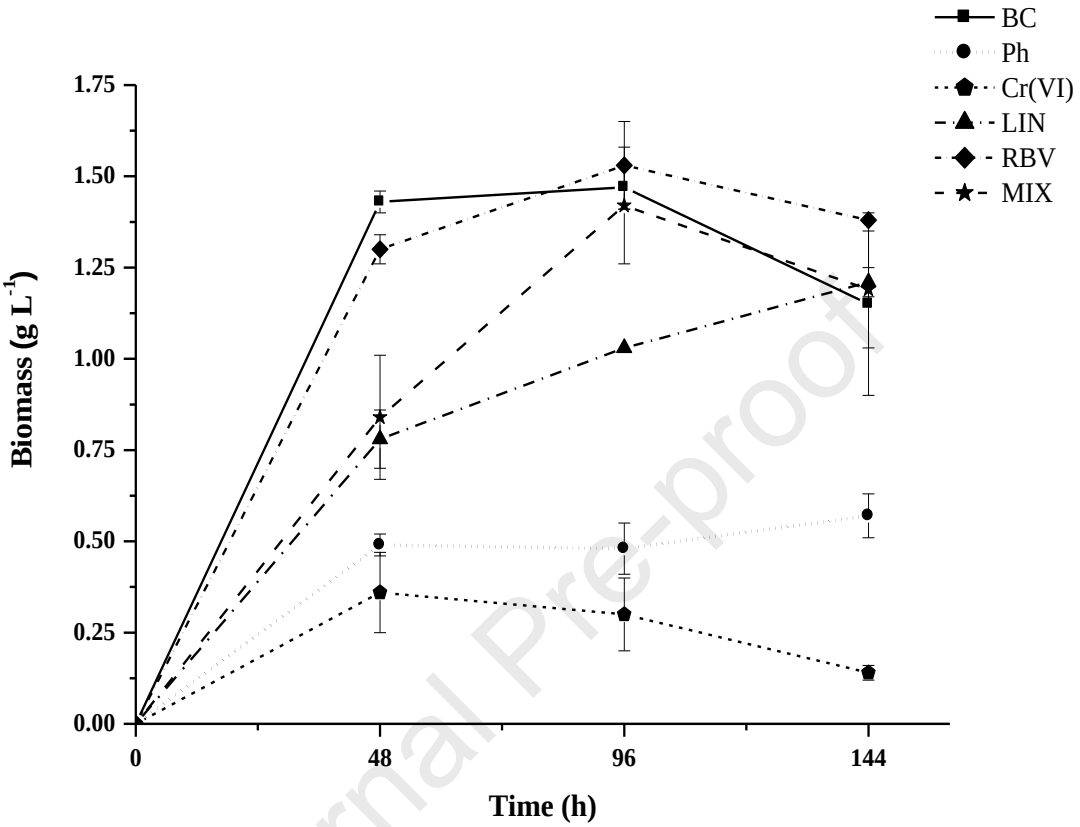


Figure 3

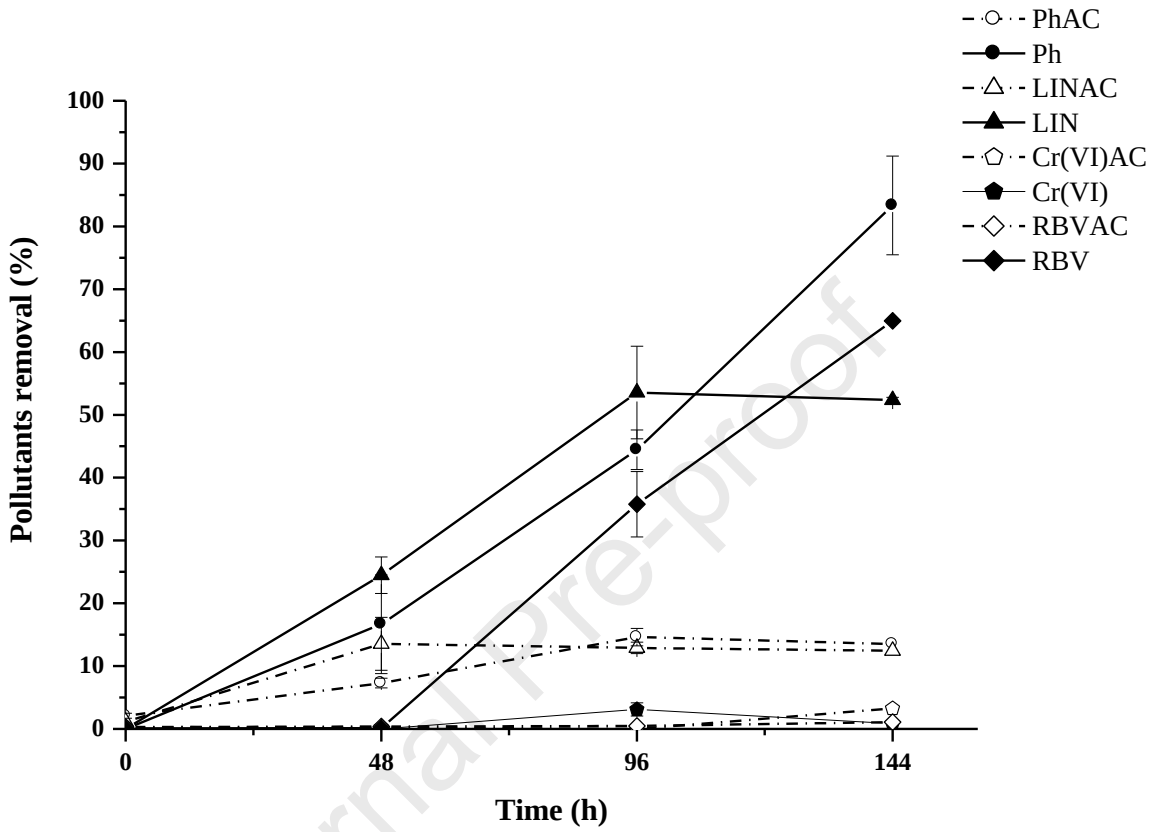
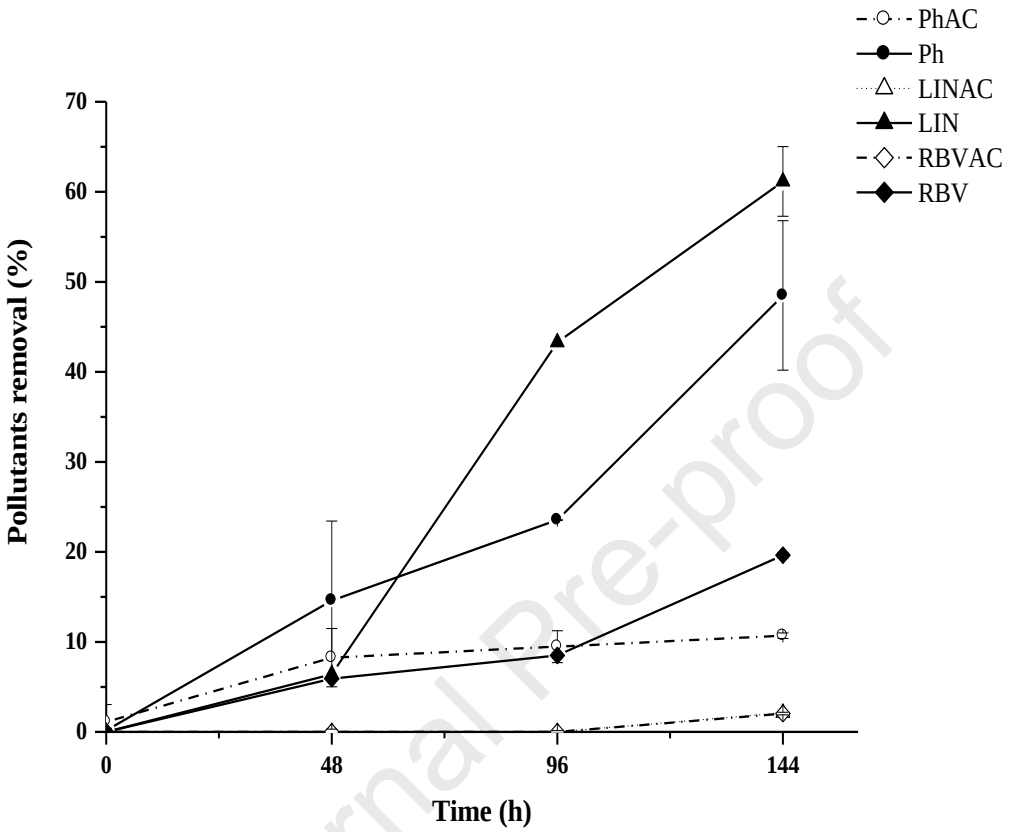


Figure 4



1 **Figure 5**

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