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# Enhanced remediation of $Cr^{6+}$ in bacterial-assisted floating wetlands

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**Keywords**
*Brachiaria mutica*; chromium; hydroponic root mats; phytoremediation; plant-bacteria partnership.

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## Abstract

Hexavalent chromium ($Cr^{6+}$) is reported to have negative effects on fauna and flora. The present study establishes plant-bacterial synergism in floating wetlands (FWs) for the maximum removal of $Cr^{6+}$ from the contaminated water. A common wetland plant, *Brachiaria mutica* (para grass), was planted in combination with three Cr-resistant rhizo- and endophytic bacteria. Results indicated that FWs vegetated with *B. mutica* showed the potential to remove Cr (53%) from water and their efficacy was significantly enhanced by bacterial inoculation (88%). The inoculated bacteria were able to colonize the plant interior successfully, that is, roots and shoots. The un-vegetated control tanks, however, showed the least bacterial persistence in the water. The perceptible phytotoxicity symptoms on *B. mutica* were only observed for the treatments without bacterial inoculation. The study suggests that *B. mutica* could be an effective choice as a wetland macrophyte to establish a partnership with the Cr-resistant bacteria for improved remediation of $Cr^{6+}$ contamination.

## Introduction

Water pollution by chromium (Cr) is of serious concern due to increasing discharges to the water environment. It is used in many industries such as textile, tanneries, chromium plating, steel production, wood preservation and refractories (Afzal *et al*., 2014b, Homa *et al*., 2016). The associated hazards in the environment nevertheless depend on its solubility and oxidation state (Mishra and Bharagava, 2016). The trivalent form ($Cr^{3+}$) serves as an essential trace element in metabolism, whereas the hexavalent form ($Cr^{6+}$) is toxic, acidic, carcinogenic and mutagenic (Garg *et al*., 2007). This is because of the high mobility/bioavailability of $Cr^{6+}$ in aquatic ecosystems (Singh and Singh, 2002). Many of the industrial effluents are expected to contain mainly $Cr^{3+}$; nevertheless, a series of redox reactions occurring in the sludge/water interface can lead to an increased proportion of $Cr^{6+}$ (Stêpniewska and Wolinska, 2005). The tanning industry is one of the main contributors to Cr contamination of the water environment releasing ~40% of the applied Cr directly into the effluent. This can cause increased levels up to 1,500–3,000 mg/L which is more than 300-fold the permissible limits in the wastewater, that is, 5 mg/L for $Cr^{3+}$ and 0.05 mg/L for $Cr^{6+}$ (Sharma and Goyal, 2009).

In recent years, bacterial-assisted phytoremediation has been widely adopted for the remediation of a variety of heavy metals (HMs) (Ijaz *et al*., 2015; Khan *et al*., 2015). The technique is economical and environment-friendly but the efficiency depends on the environmental setting as well as on the choice of plant. For wastewater reclamation, several variations in wetland engineering have been proposed (Ijaz *et al*., 2015; Rehman *et al*., 2018). Floating wetlands (FWs) allow macrophytes to grow on self-buoyant mats, while extending their roots deep down into the contaminated waters (Yeh *et al*., 2015). This allows the: (a) development of a hydraulic gradient between plant roots and the bottom of the treatment system for water filtration (Rehman *et al*., 2018); (b) phytostabilization of the soluble/bioavailable HMs

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to immobilize them and make them less available for uptake or translocation (Dary *et al.*, 2010; Karczewska *et al.*, 2013); and (c) establishment of biofilms for physical entrapment, metal speciation and sequestration (Rehman *et al.*, 2019).

Chromium has been recognized as a phytotoxic element that can affect plant growth by interfering with photosynthetic and respiration processes, inducing structural alterations, producing reactive species, causing oxidative damage and eventually leading to the death of plant (Rai *et al.*, 2004; Singh *et al.*, 2013; Gill *et al.*, 2015). To reverse these effects, plant-bacteria partnerships are emerging as an effective choice that alleviates stress due to aminocyclopropane-1-carboxylate (ACC) deaminase potential of the inoculated bacteria, as well as helping the plants gain more biomass by producing phytohormones such as siderophores, cytokinin, indole acetic acid (IAA), etc. (Afzal *et al*., 2014a). Additionally, these bacteria minimize the mobility of HMs by chelation, acidification, redox changes, phosphate solubilization, etc. (Abou-Shanab *et al.*, 2008; Ahemad, 2015; Fatima *et al.*, 2015). Harnessing these partners for the sake of water treatment can be an appealing choice for hydroponic as well as soil-based phytoremediation systems (Arslan *et al.*, 2014; Saleem *et al.*, 2018).

*Brachiaria mutica*, also known as a para grass, is reported to withstand harsh environmental conditions including waterlogging and Cr stress (Mohanty and Patra, 2012; Mohanty *et al.*, 2012; Liu *et al.*, 2013). This is because of the: (a) presence of hollow stem with large aerenchyma in its roots that helps it thrive in long-term flooding under low oxygen levels and (b) hyperaccumulator nature that promotes luxuriant root growth with massive fibrous roots even at Cr concentration up to 11 170 mg/kg dry soil (Mohanty and Patra, 2012). In this study, FW microcosms were established by planting *B. mutica* in combination with one rhizospheric and two endophytic Cr-resistant bacterial strains. This study particularly aims to elucidate: (1) the suitability of using *B. mutica* in a bacterial assisted FWs for the efficient remediation of Cr from contaminated waters, as well as (2) the response of inoculated bacterial species in the rhizosphere and endosphere of the plant towards enhanced remediation. A formal hypothesis was established as follows:

**Hypothesis 1** *Chromium can be removed effectively from wastewater by the combined action of plants and bacteria. Effectively is defined as >80%.*

## Materials and methods

### Establishing FW microcosms

Fifteen FW microcosms were established using polyethylene tanks of 20 L capacity. The floating mats were developed using the Diamond Jumbolon sheet (Diamond Foam Company, Pvt. Ltd. Pakistan) after cutting the sheet into 50 (length) × 40 (width) × 7.5 (thickness) cm portions (Fig. 1). Each mat was drilled to create 5 holes at an equal distance to insert five healthy seedlings of *B. mutica* in each hole. The plants were then fixed with the help of coconut shavings and gravel, while keeping their roots free. The seedling containing mats were placed over the tanks and 15 L of tap water was added accordingly. The microcosms were allowed to establish root network for a period of 1 month, while 7.5 mL of the diammonium phosphate (DAP) solution (1%, w/v) was applied to augment the root-establishment process. After the acclimatization, plant roots were surface-sterilized with a 5% sodium hypochlorite solution. The water (15 L) was spiked with 2.125 g of potassium dichromate ($K_2Cr_2O_7$) to get Cr 50 mg/L. This concentration was selected based on previous studies reporting the harmful effects on plant metabolism for several species (Singh *et al.*, 2013).

### Preparation of bacterial strains for the experimental setup

Three Cr-resistant bacterial strains namely *Pseudomonas aeruginosa* PJRS20, *Ochrobactrum* sp. ASI14 and *Enterobacter* sp. HU38 were used in the present study. The strain *P. aeruginosa* PJRS20 was previously isolated from the rhizosphere whereas *Ochrobactrum* sp. ASI14 and *Enterobacter* sp. HU38 were isolated from the shoot interior of the *Prosopis juliflora*. The bacteria were selected based on their Cr-resistance and plant growth-promoting activities (Khan *et al.*, 2015). All of the strains were found to have ACC-deaminase potential, a stress alleviation trait, as tested on 0.7 g of ACC/L (Khan *et al.*, 2015). The bacterial strains were then cultivated in 10% Luria Bertani (LB) broth for 24 h at 37°C and agitated at 150 rpm. Subsequently, bacterial cells were harvested by centrifugation followed by washing and resuspension in 0.9% NaCl solution. Finally, for each bacterial culture, the optical density was adjusted to obtain $10^7$ cells/mL and 150 mL of the consortium was inoculated in the microcosms as per the experimental design.

### Experimental setup

The experiment was conducted at NIBGE, Faisalabad, Pakistan for a period of 2 months. A total of five treatments (in triplicates) were established which were: (T-1) microcosm containing tap water with *B. mutica,* (T-2) microcosm containing Cr-contamination without vegetation, (T-3) microcosm containing Cr-contamination and bacterial consortium, (T-4) microcosm containing Cr-contamination with *B. mutica*, (T-5) microcosm containing Cr-contamination with *B. mutica* and bacterial consortium. The pots were placed randomly in the natural climate of NIBGE, Faisalabad, Pakistan. The average day/night temperatures were 28/15°C and humidity was 57%. The

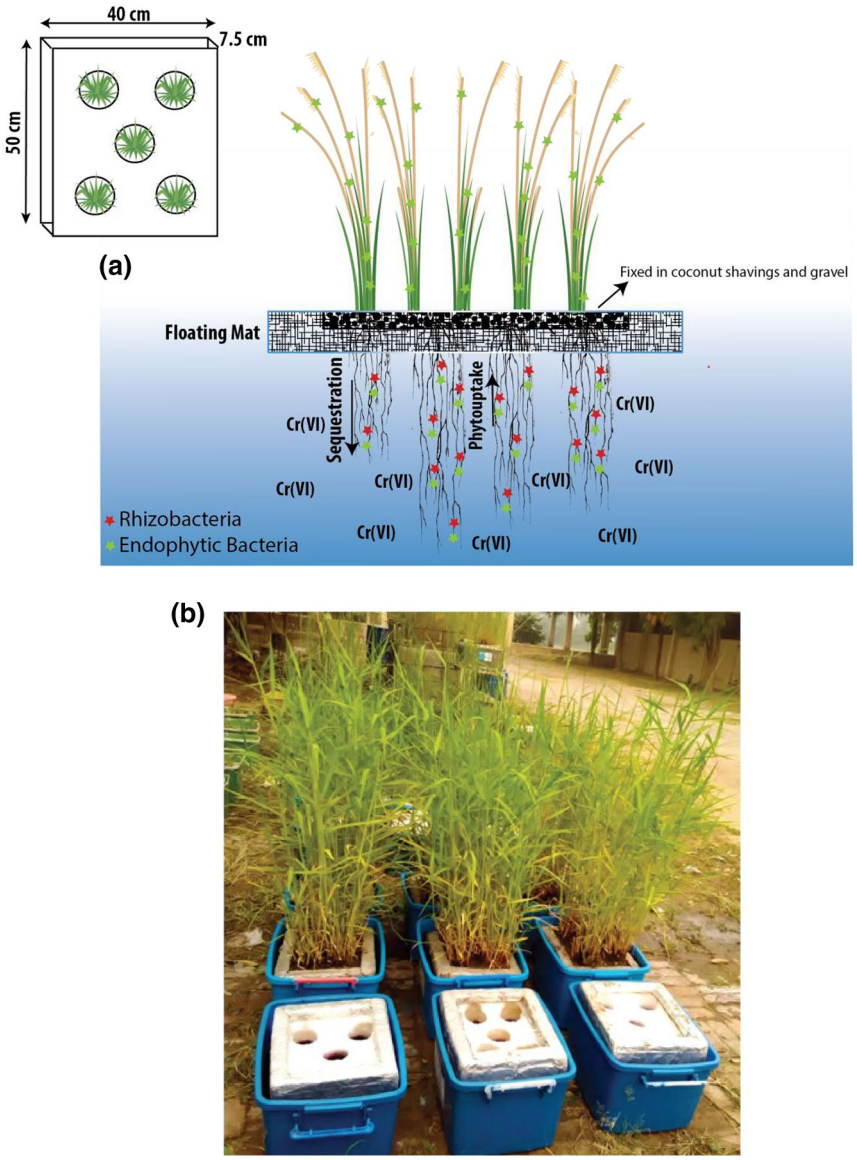


**Fig. 1.** (a) Schematic representation of the experiment and (b) experimental setup at the end of the experiment.

experiments were carried out in March-April 2017; each treatment was replicated thrice. No rain was observed in the study period. The level of water in each tank was maintained at each sampling time by the addition of sterilized distilled water. The initial biomass (fresh weight) of each seedling was 10 ± 1.5 g. The pH of the water was 6.0 and Cr concentration was 50 mg/L. The macronutrients in water were calcium (59 mg/L), magnesium (21 mg/L), chlorides (100 mg/L), nitrogen (0.1 mg/L), phosphorus (0.16 mg/L) and sulphates (42 mg/L).

## Plant and water analyses

Plants were harvested at the end of the experiment in order to compare the fresh and dry biomass of both roots and shoots. The dry biomass was determined after 48 h of incubation in an oven at 70°C (Singh *et al.*, 2013). Furthermore, samples were ground in a mortar and pestle to obtain ground mixture for subsequent Cr concentration analysis (Khan *et al.*, 2015). The comminuted mixture was sieved (0.5 mm) and then digested in a microwave digestion system (Multiwave3000, Anton Paar GmbH Graz, Austria). Lastly, Cr concentration in the shoot and root tissues was quantified by the atomic absorption spectrometer (FAAS, Perkin-Elmer Analyst 300).

The water quality parameters namely pH and electrical conductivity (EC) were measured at 5-day interval. The pH measurements used a bench-top digital pH meter (781 pH/

ion meter, Metrohm Herisau, Switzerland), whereas EC was determined using a conductivity meter (XL 30, Fisher Scientific Pte Ltd. Singapore).

### Enumeration of inoculated bacteria

The bacterial populations were determined in the water, rhizoplane and in the root and shoot through colony forming units (CFU) method (Kuffner *et al.*, 2008; Saleem *et al.*, 2018). For the determination of bacterial population in the rhizoplane, roots were agitated in 0.9% (w/v) NaCl solution and serial solutions (up to $10^{-6}$) were made of the water. Before analyzing the endophytic population, plant tissues (roots and shoots) were surface sterilized using 70% (v/v) ethanol and 2% (v/v) sodium hypochlorite solution. The roots and shoots were first treated with ethanol for 5 and 2 min, and then with sodium hypochlorite solution for 2 and 1 min, respectively. Afterward, the surface-sterilized plant tissues were washed several times in distilled water. The sterilized roots and shoots were put on LB plates, with no bacterial growth indicating the sterilization of the roots and shoots. Subsequently, 10 g of roots and shoots was used to prepare plant material suspension by grinding in a mortar and pestle followed by serial dilutions up to $10^{-6}$, after the settlement of the plant tissues. Approximately, 100 μL aliquots of each dilution was then spread over LB agar medium containing 50 mg/L of Cr for water, rhizoplane and plant samples. The dilutions containing 30-50 CFUs were considered for the quantification of the total bacterial counts $\left(\frac{\text{number of CFU}}{100\ \mu L \times \text{dilution used}}\right)$. The plates were incubated for 48 h at 37°C for CFU count and at least 10 bacterial colonies were picked randomly from each plate which had a total of 30-50 colonies. The identity of isolates was confirmed through the restriction fragment length polymorphism (RFLP) analysis of PCR amplified 16S-23S rDNA intergenic spacer (IGS) region product as reported earlier (Saleem *et al.*, 2018).

### Statistical analysis

The phytoaccumulation ability of *B. mutica* was estimated by measuring the bioconcentration factor (BCF) using the following expression,

$$\text{BCF} = \frac{\text{Cr concentration in plant tissues (roots/shoots)}}{\text{Cr concentration in water}}$$

Additionally, plant biomass, water quality parameters, Cr removal and CFU estimation were statistically analyzed using the R statistical language. One-way ANOVA was performed to determine the significant differences among treatments, while considering post hoc Tukey's Honestly Significant Difference (HSD) test function for multiple comparisons (HSD.test). Differences were considered significant when $P < 0.05$ (Afzal *et al.*, 2019).

## Results

### Plant response in the presence of Cr contamination and bacterial inoculation

The fresh and dry biomass was measured to determine the effect of Cr contamination and bacterial inoculation on plant growth and development. Chromium contamination (T-4) significantly decreased (32%) the plant biomass when compared with the plants grown without contamination (T-1). However, inoculation of bacterial consortium (T-5) enhanced the plant biomass. The increase in biomass was 34% for roots and to 27% for shoots ($P < 0.05$). The highest biomass was observed for the plants grown in tap water without Cr contamination (T-1) (Fig. 2).

Chromium concentrations within plant tissues (roots and shoots) were also determined to elucidate the effect of plant growth-promoting rhizo- and endophytic bacteria on Cr uptake and bioaccumulation (Fig. 3a). The bacterial inoculation displayed a pronounced effect on Cr accumulation within plant tissues, that is, Cr concentration increased by 35% in the roots and 15% in the shoots. ANOVA testing confirmed that these results were significantly different at $P < 0.05$. Additionally, the values of BCF were consistent with the findings as high BCF was recorded for the inoculated plants along with the more accumulation in roots (Fig. 3b).

### Water quality parameters and Cr removal

Established FWs were able to improve the quality of water by optimizing pH, maintaining EC values and removing Cr from the wastewater. In the presence of vegetation only (T-4), the pH of the treated water remained alkaline (7.68), whereas inoculum resulted in a pH of 7.17 (T-5) (Fig. 4a). At the end of the experiment, a change in pH was similar to the control (T-1) where no Cr was spiked ($P < 0.05$). Hydroponic mats with bacterial consortium alone also helped in improving the water quality by neutralizing the pH to a certain extent (7.92); nevertheless, results remained less significant (T-3) ($P < 0.05$). Similar observations were made for EC whose values decreased from 7.30 to 4.44 mS/cm in the presence of both vegetation and inoculum, whereas 5.90 and 6.49 mS/cm of EC was recorded for vegetation and inoculum separately at the end of the experiment, respectively, (Fig. 4b). These values were less significant than the combined application plant and bacteria ($P < 0.05$). Chromium removal was also highly efficient for the inoculated and vegetated FWs (88%) whereas less removal was observed for the FWs with bacterial

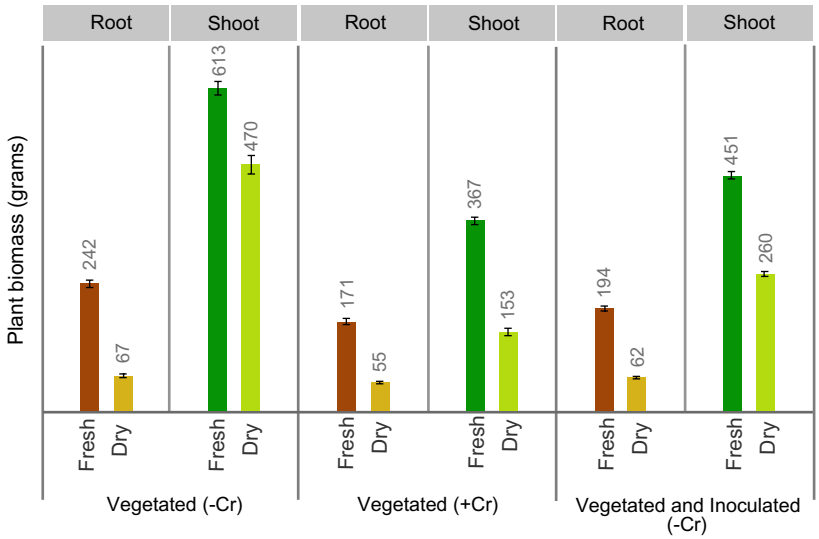


**Fig. 2.** Effect of Cr contamination and bacterial inoculation on plant biomass.

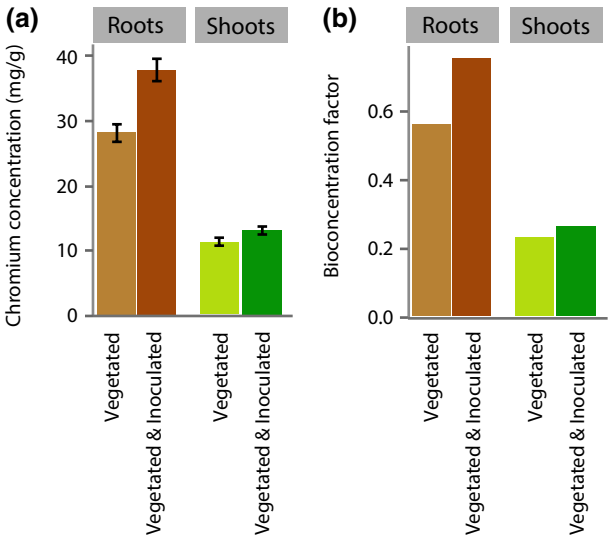


**Fig. 3.** Cr uptake and bioaccumulation within plant roots and shoots.

inoculation and vegetation individually, that is, 20 and 53%, respectively, (T-3 and T-4) (Fig. 4c).

### Bacterial persistence in the plant rhizo- and endosphere

Figure 5A shows the results of colony PCR performed on some of the isolates morphologically identical to the inoculated ones. The inoculated bacteria's presence and persistence in roots are shown by RFLP analysis (Fig. 5b-d). For the presentation purpose, restriction patterns of PCR products of a subset of the samples are shown in the form of a figure. However, complete results on the quantification of persisted bacteria in the rhizoplane and endosphere, that is, root interior and shoot interior of *B. mutica*, are shown in Table 1. The inoculated bacteria displayed high persistence in the plant roots followed by shoots and rhizoplane. Moreover, a decrease in CFU counts was observed for the water sample from the un-vegetated reactor (T-3). The decrease was statistically insignificant during the first 10 days of the experiment whereas a sharp decrease was seen in the last 5 days ($P < 0.05$). Similar behavior of bacterial counts was recorded for the inoculants isolated from the rhizoplane. In the root interior and shoot interior, however, total CFUs increased with the passage of time and values recorded after every 5 days of the experiment were statistically different from the initial values ($P < 0.05$). RFLP analysis further confirmed that 59–72% of the isolates were the inoculated strains in the treated water, 66–74% in the rhizoplane, 76–84% in the root interior and 59–74% in the shoot interior. The statistical relevance of results obtained in RFLP was in accordance with the results obtained for the CFUs.

## Discussion

The observations made in this study support the established hypothesis that FWs were able to effectively remove Cr from wastewater by the combined action of plants and bacteria. A bacterial consortium of one rhizospheric and two endophytic bacteria, previously isolated from the rhizo- and endosphere of *P. juliflora*, was found to establish a successful partnership with *B. mutica* and allowed the enhanced removal of Cr in the established FW.

At first, we found that Cr contamination inhibited plant growth (T-4), whereas inoculation of rhizospheric and endophytic bacteria helped to prevent this inhibition (T-5).

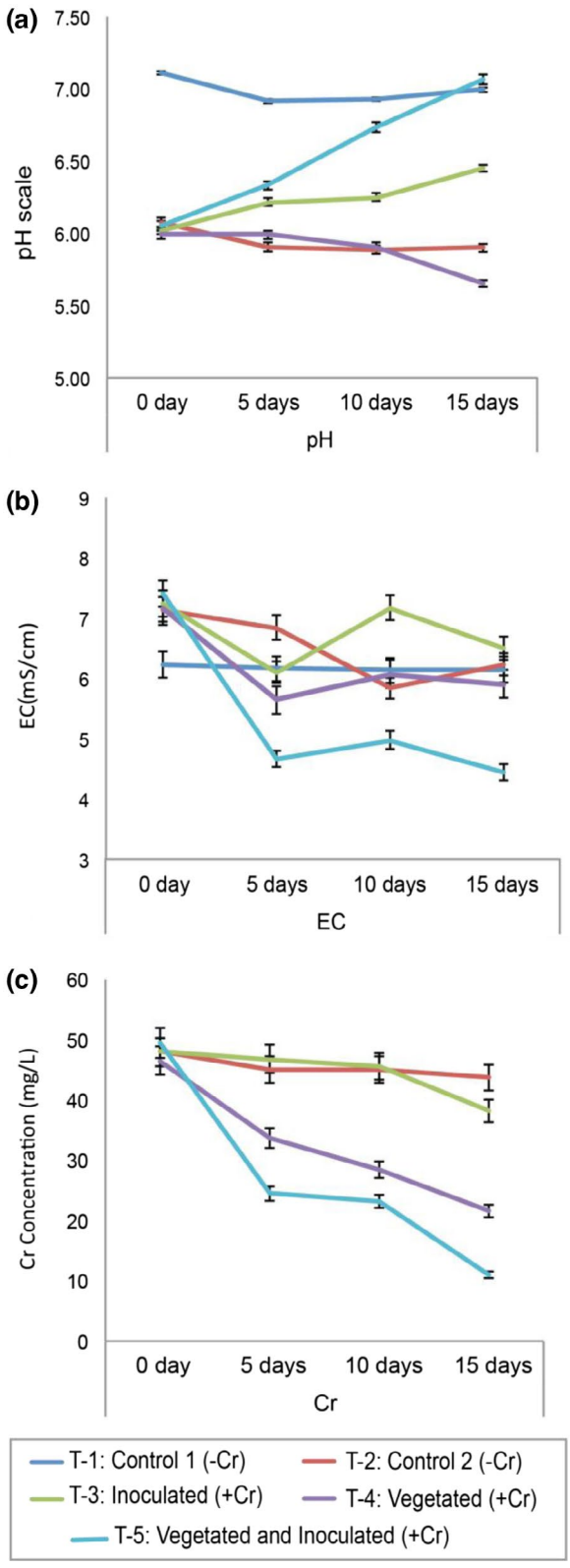


**Fig. 4.** Water quality parameters and Cr removal during the experiment.

This can be attributed to the ACC deaminase enzyme activity of the inoculated bacteria, which help alleviate abiotic stress by lowering the ethylene concentration and preventing inhibition of root elongation (Glick, 2014; Ahemad, 2015). Additionally, an increase in plant biomass in the presence of bacterial inoculation can also be linked to the plant growth-promoting activities of the bacteria including phosphorous solubilization, nitrogen fixation, siderophores production (metal chelating agents) and release of necessary phytohormones such as IAA (Souza *et al.*, 2015). IAA helps plants gain more biomass by favoring root growth especially root hair elongation along with the absorption of plant essential nutrients (Taghavi *et al.*, 2009; Ahemad, 2015). Similarly, siderophores increase the synthesis of chlorophyll by making iron available to the HM-stressed plants (Ahemad, 2015) and by enhancing metals availability in the rhizosphere through complexation. Accordingly, high BCF values for roots confirm the plant phytostabilization potential and Cr immobilization in the vacuoles of root cells (Ullah *et al.*, 2015). Similar observations are reported in other studies where plant-bacteria partnership is employed for the removal of Cr from contaminated soils (Khan *et al.*, 2015; Ahsan *et al.*, 2018).

The bacterial inoculation also helped to improve the water quality by removing Cr and by lowering pH and EC. The highest removal by vegetated and inoculated FW (T-5) can be associated with high plant biomass as well as the bacterial potential to reduce metal toxicity (Jiang *et al.*, 2008). Endophytic bacteria are key candidates that help reduce metal toxicity in plants grown in the HM-polluted environment, along with the reduction, sequestration and chelation services offered by rhizospheric bacteria (Chatterjee *et al.*, 2009). Moreover, inefficient Cr removal by microcosms with bacterial inoculation only (T-3) can be attributed to the poor survival/colonization of rhizospheric and endophytic bacteria in the absence of vegetation (Arslan *et al.*, 2014; Saleem *et al.*, 2018). Similarly, partial removal of Cr by vegetated microcosms (T-4) can be associated with the innate phytoremediation potential of *B. mutica*; which came with the symptoms of toxicity such as root tissues necrosis and reduction in biomass (visual observations). In parallel to the Cr removal, the pH of the contaminated water was neutralized and EC decreased significantly for the vegetated and inoculated microcosms (T-5). This could be due to the successful uptake/translocation of Cr by plant tissues (Cervantes *et al.*, 2001). Initial alkalinity could be associated with the formation of chromate ($CrO_4^-$) and/or dichromate ions ($Cr_2O_7^{2-}$), but sequential uptake of Cr by plants favored by bacteria might have served the purpose accurately. Nevertheless, direct evidence of Cr efflux *in planta*

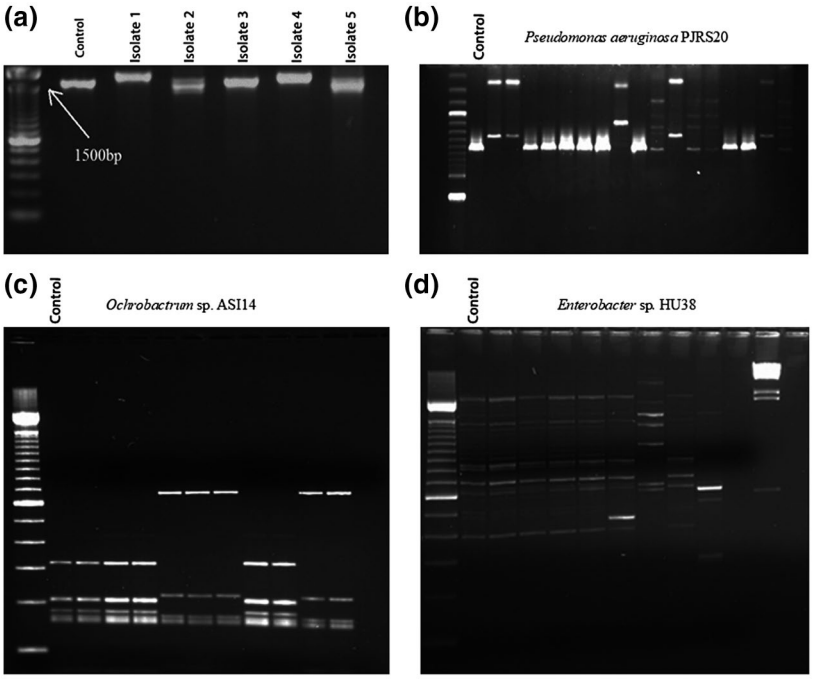


**Fig. 5.** (a) PCR products of some of the isolates from rhizoplane, root interior and shoot interior of *Brachiaria mutica* and (b-d) restriction patterns of the PCR products for the inoculated strains.

**Table 1** Bacterial persistence in the rhizoplane, root and shoot interior, and water of hydroponic system with and without vegetation of *Brachiaria mutica*

| | | | CFU × $10^4$ | | |
|---|---|---|---|---|---|
| | Treatment | | 5 days | 10 days | 15 days |
| CFU | Inoculated & unvegetated | Water ($mL^{-1}$) | $13.2^{a}$ (1.54) | $11.8^{ab}$ (0.91) | $9.47^{b}$ (1.14) |
| | Inoculated & vegetated | Rhizoplane ($g^{-1}$) | $15.4^{a}$ (2.14) | $15.6^{a}$ (1.75) | $14.2^{b}$ (1.12) |
| | | Root interior ($g^{-1}$) | $2.63^{c}$ (0.08) | $5.38^{b}$ (0.15) | $9.17^{a}$ (0.04) |
| | | Shoot interior ($g^{-1}$) | $0.21^{c}$ (0.05) | $0.45^{b}$ (0.03) | $0.61^{a}$ (0.08) |
| RFLP | Inoculated & unvegetated | Water (%) | $71.6^{a}$ (2.8) | $68.1^{ab}$ (4.1) | $59.8^{b}$ (6.2) |
| | Inoculated & vegetated | Rhizoplane (%) | $71.3^{a}$ (7.2) | $73.7^{a}$ (5.7) | $65.8^{b}$ (5.2) |
| | | Root interior (%) | $76.1^{a}$ (8.1) | $83.5^{b}$ (5.1) | $82.8^{c}$ (3.7) |
| | | Shoot interior (%) | $58.7^{a}$ (4.5) | $65.1^{b}$ (6.2) | $73.5^{c}$ (5.8) |

The values presented are the means and the standard deviation is given in parenthesis (n = 3). Significant differences ($P < 0.05$) were determined using a two-way ANOVA followed by a Bonferroni post hoc test. Letters (a–c) indicate statistically significant differences between treatments at a 5% level of significance.

Abbreviations: CFU, colony forming unit; RFLP, restriction fragment length polymorphism (RFLP) analysis.

was not investigated in the study. Likewise, biosorption, diminished accumulation and precipitation might have contributed to the decrease as well (Alvarez *et al.*, 1999; Cervantes *et al.*, 2001). This is even clearer when compared with the un-vegetated and un-inoculated microcosms (T1) where respective values of pH and EC remained the same throughout the experimental period. Both pH and EC are important water quality parameters whose recovery suggests the cleaning of water resources (Ijaz *et al.*, 2015).

The persistence of inoculated bacteria in phytoremediation is fundamental to the system's performance (Saleem *et al.*, 2018). The present study establishes another example of the successful partnership of inoculated bacteria with the host (i.e. *B. mutica*) rhizosphere and endosphere. The successful colonization by rhizosphere and endophytic bacteria during the phytoremediation of various HMs has been reported in multiple studies (Burd *et al.*, 2000; Rajkumar *et al.*, 2012; Ullah *et al.*, 2015). Moreover, inoculated bacteria were previously isolated from the plant

rhizo- and endosphere (already grown in Cr contaminated soil); therefore, they would be pre-adapted to Cr contaminated environment and successful colonization of the host (Khan *et al.*, 2015). This colonization actually aids the endophytic community to phytostabilize the HMs along with the rhizospheric sequestration as described previously (Ahsan *et al.*, 2019). The lower persistence in the unvegetated (T-3) hydroponic mats strengthens the fact that the inoculated bacteria were restricted to their performance only in the presence of host (Arslan *et al.*, 2014). Nevertheless, Cr accumulation in the roots can be correlated with the combined application of plant and bacteria that offers a series of ecological services, that is, microbial reduction, sequestration, chelation, phytostabilization, biotransformation, bioaccumulation, etc. (Ahsan *et al*., 2018).

## Conclusions

1). It is concluded that $Cr^{6+}$, which is highly toxic to plants and least likely to be translocated, can be removed successfully from the wastewaters using bacterial-assisted FWs. Inoculated bacteria were able to persist in different components of the FWs leading to effective remediation. 2). This study also recommends the applications of similar wetland systems for the viable and economic restoration of Cr-contaminated wastewaters. Nevertheless, this is a preliminary study that sets the basis for deeper studies in the future regarding the genes involved in the underlying mechanisms of Cr-uptake and translocation effects. 3). Lastly, metabolic activities and turn overpotential of the inoculated bacteria and their capability to be employed at field-scale should be investigated.

## Acknowledgments

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## Conflict of interest

Authors declare that no conflict of interest exists for this study.

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