Phragmites australis — a helophytic grass — can establish successful partnership with phenol-degrading bacteria in a floating treatment wetland

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A B S T R A C T

Helophytic plants contribute significantly in phytoremediation of a variety of pollutants due to their physiological or biochemical mechanisms. Phenol, which is reported to have negative/deleterious effects on plant metabolism at concentrations higher than 500 mg/L, remains hard to be removed from the environmental compartments using conventional phytoremediation procedures. The present study aims to investigate the feasibility of using *P. australis* (a helophytic grass) in combination with three bacterial strains namely *Acinetobacter lwofii* ACRH76, *Bacillus cereus* LORH97, and *Pseudomonas* sp. LCRH90, in a floating treatment wetland (FTW) for the removal of phenol from contaminated water. The strains were screened based on their phenol degrading and plant growth promoting activities. We found that inoculated bacteria were able to colonize in the roots and shoots of *P. australis,* suggesting their potential role in the successful removal of phenol from the contaminated water. *Pseudomonas* sp. LCRH90 dominated the bacterial community structure followed by *A. lowfii* ACRH76 and *B. cereus* LORH97. The removal rate was significantly high when compared with the individual partners, i.e., plants and bacteria separately. The plant biomass, which was drastically reduced in the presence of phenol, recovered significantly with the inoculation of bacterial consortia. Likewise, highest reduction in chemical oxygen demand (COD), biochemical oxygen demand (BOD), and total organic carbon (TOC) is achieved when both plants and bacteria were employed. The study, therefore, suggests that *P. australis* in combination with efficient bacteria can be a suitable choice to FTWs for phenol-degradation in water.

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1. Introduction

Phenol (or hydroxybenzene) is a commonly found pollutant in industrial wastewaters. The compound is produced both synthetically and naturally; nevertheless, synthetic production has been increased multi-fold recently. It has been reported that in 2015, world's annual production of the phenolic compounds exceeded 10 million tons, which is expected to increase up to 11.6 million tons in 2020 (Plotkin, 2017). Almost every industry *viz a viz* petrochemical, pharmaceutical, agriculture, tanning, paper and pulp, iron smelting, food, resin, etc. depends heavily on phenolic compounds for its manufacturing processes (Herouvim et al., 2011; Tatoulis et al., 2015). Many of these phenol-containing wastes are discharged directly into the wastewaters causing further contaminations of surface and groundwater (Huang et al., 2012; Stefanakis and Thullner, 2016). Some of the phenolic compounds have been detected up to 10,000 mg/L of concentration (van Schie and Young, 2000); whose genotoxic, immunotoxic, carcinogenic, mutagenic and teratogenic effects are also reported accordingly, Cf., list of priority pollutants (USEPA, 1979; Wu et al., 2012). The persistence nature also allows their bioaccumulation in the biotic elements, rendering them as a pollutant of concerns (Liu et al., 2011; Liu et al., 2012).

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Recent advances in ecological engineering have helped establish innovative phytoremediation systems for the treatment of wastewaters (Chen et al., 2016; Ijaz et al., 2016; Wang et al., 2015). These include floating treatment wetlands (FTW) that harbor rooted and/or emergent plants of aquatic or terrestrial origin, grown on self-buoyant mats being fixed on wastewater reservoirs (Keizer-Vlek et al., 2014; Lynch et al., 2015; Yeh et al., 2015). These mats allow plants to extend their roots deep into the contaminated waters; allowing development of a large biologically active area in the form of biofilms for the physical entrapment, biochemical transformation, and degradation of the organic pollutants (Kyambadde et al., 2005; Weragoda et al., 2012). The success of such a system purely relies on microbial community in the biofilm as well as on the plant species (Arslan et al., 2017b; Glick, 2010; Weyens et al., 2009). Some of the pollutants are, however, toxic enough to downgrade the overall cleaning process by reducing the plant growth and inhibiting bacterial degradation (Alkio et al., 2005). Phenol is among these pollutants, and can affect both of the processes due to its bactericidal properties and plant metabolic malfunctioning potential (Adeboye et al., 2014; Kottuparambil et al., 2014; Phenrat et al., 2017; Ucisik and Trapp, 2006). To overcome such a situation, artificial augmentation of rhizobacteria and/or endophytic bacteria, having antagonistic activities for the specific pollution type, have been proposed in a variety of phytoremediation experiments (Afzal et al., 2014; Khan et al., 2013; Weyens et al., 2009). The successful partnership allows plants to provide nutrients and residency to the inoculated bacteria while bacteria in return improve the plant health by reducing stress, producing phytohormones and nutrients, and by providing pollutant degradation services (Glick, 2010; Glick, 2014; Weyens et al., 2009).

The common reed *Phragmites australis* (Cav.), a helophytic grass, is reported to withstand harsh environmental conditions including contaminant stress (Davies et al., 2005; Hechmi et al., 2014; Schröder et al., 2008). The species grows in marshes, swamps, and wet waste areas and therefore can be exploited in FTWs for the remediation of wastewaters. In principle, the species allows adsorption of the organic contaminants to the roots, without being translocated to aboveground plant parts, rendering it suitable for the removal of organic volatile compounds (Van der Werff, 1991). Moreover, members of the helophytic grasses are able to transport atmospheric oxygen to the rhizosphere hence helping them survive in waterlogged conditions. This oxygen flow from leaves to roots further helps the microbial respiration by creating gradients of redox conditions ultimately supporting the biofilm functioning (Syranidou et al., 2016).

Keeping in mind these facts, we established FTW microcosms by employing *P. australis* as a model plant, in partnership with the two rhizospheric bacterial strains (*Bacillus cereus* LORH97 and *Pseudomonas* sp. LCRH90) and one endophytic bacterial strain (*Acinetobacter lwofii* ACRH76). This study aims to elucidate (1) the potential of employing *P. australis* in a bacterial assisted FTW, and (2) the success of established FTWs for remediation of phenol-contaminated water. Moreover, bacterial persistence in the rhizosphere and endosphere of the plant is evaluated.

2. Materials and methods

2.1. Bacterial strains

Three bacterial strains namely *Acinetobacter lwofii* ACRH76 (NCBI accession number KF478224), *Bacillus cereus* LORH97 (NCBI accession number KF478239), and *Pseudomonas* sp. LCRH90 (NCBI accession number KF478222) were used in the current study (Fatima et al., 2015). The strains *A. lwofii* ACRH76 and *B. cereus*

LORH97 were isolated from the rhizosphere of *Acacia ampliceps* and *Lolium perenne*, respectively, whereas *Pseudomonas* sp. LCRH90 was isolated from the shoot interior of *Lecucaena leucocephala*. The strains were selected based on their ability to utilize phenol as a sole carbon source, i.e., carrying clusters of *alk* and *cyp* genes; metabolic functioning was tested on minimal salt medium containing 100 mg l^{-1} of phenol. Moreover, *B. cereus* LORH97 and *Pseudomonas* sp. LCRH90 possessed 1-aminocyclopropane-1-carboxylate (ACC) deaminase activities, a stress alleviation trait of plant growth promoting bacteria, tested on 0.7 g of 1-aminocyclopropane-1-carboxylate (ACC) l^{-1} (Kuffner et al., 2008). The strains were then cultivated in 10% Luria–Bertani (LB) broth at 37 °C, previously amended with 50 mg l^{-1} of phenol. Cells were harvested by centrifugation followed by re-suspension in 0.9% (w/v) of NaCl solution. Finally, the optical density of each bacterial strain was adjusted to get 10^7 cells ml^{-1}, the suspension was mixed in 1:1:1 ratio and 150 ml of the consortium was inoculated in each reactor, where required.

2.2. Construction of FTW microcosms

The experiment was conducted in March 2017 at National Institute for Biotechnology and Genetic Engineering, Faisalabad, Pakistan for two months. Fifteen FTW phytoreactors were established using Diamond Jumbolon sheet (Diamond Foam Company, Pvt. Ltd. Pakistan) and polyethylene tanks (20-liter capacity). The sheet was used to make floating mat after cutting it into 20 (length) × 15 (width) × 3 (thickness) inches; and five holes were drilled at equal distance to insert healthy seedlings of *P. australis* in the mat, i.e., 40–50 seedlings for each reactor (Fig. 1) (Patil et al., 1994; Mehmood et al., 2013). The seedlings were then allowed to develop roots for a month in the tap water while Hoagland solution was applied fortnightly to augment the process of root-establishment. After 30 days of acclimatization, tanks and plant roots were surface-sterilized with 5% NaOCl solution and the water of the tank was spiked with phenol (500 mg l^{-1}). The concentration was selected based on earlier observations that of phenol (500 mg l^{-1}) in water can decrease 50% of evapotranspiration in willow trees (Ucisik and Trapp, 2006). The observations were made in triplicates and different treatments were:

- T1: vegetated reactor containing tap water – control
- T2: un-vegetated reactor containing phenol-contaminated water – control
- T3: un-vegetated reactor containing phenol-contaminated water and bacterial consortium
- T4: vegetated reactor containing phenol-contaminated water
- T5: vegetated reactor containing phenol-contaminated water and bacterial consortium

2.3. Plant biomass

Plant tissues were cut 2 cm above the mat surface after 15 days experimental period. The fresh and dry biomass of both roots and shoots was then measured to reveal the effect of bacterial inoculation and phenol contamination on plant growth and development. Dry biomass was determined after three days in an oven at 50 °C (Arslan et al., 2014).

2.4. Phenol estimation

APHA standard methods for the examination of water and wastewater were used to estimate the residual phenol concentration in water samples (APHA, 2005).
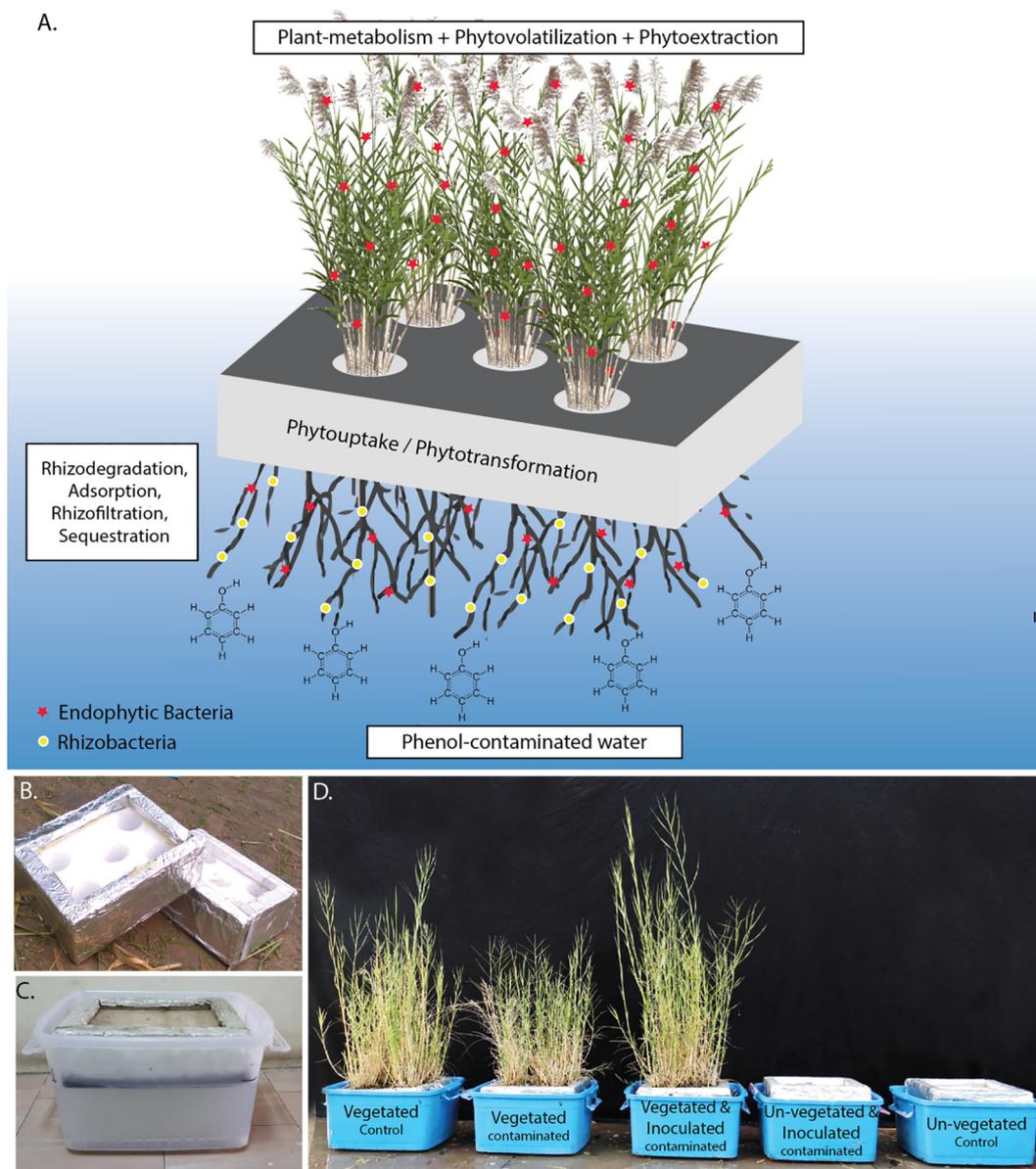
Fig. 1. Schematic representation of floating mat with holes for plantation of healthy seedlings of *P. australis* (A); construction of phytoreactors with container and floating mat (B-C); experimental setup showing plant biomass (C). The tanks were covered to avoid sunlight penetration.

2.5. Water quality parameters analyses

The water quality parameters were evaluated using established protocols (APHA, 2005). These included BOD, COD, TOC, and pH as they define the quality of water (Eaton et al., 2005). Additionally, dissolved oxygen (DO) was measured temporally to evaluate wetland performance over time.

2.6. Survival/colonization of inoculated bacteria

The survival of the inoculated bacteria was determined in the treated water, rhizospheric water, and plant endosphere at the end of the experiment. The endophytic bacteria were isolated from the shoots and roots interior was performed after surface sterilization as per established protocols (Yusuf et al. 2010). Briefly, roots and shoots were washed for 2 min in sterile distilled water followed by treatment with 70% ethanol for 10 min (for roots) and 5 min (for shoots). Subsequently, the tissues were rinsed for 1 min in 1% NaOCl amended with 0.01% Tween 20 solution. Finally, the surface sterilized roots and

shoot were washed thrice with sterile distilled water and the surface sterility of the last rinse was checked by spreading 100 μl aliquot on nutrient enriched LB medium. Afterwards, 5 g of the surface sterilized roots and shoots were grounded with a pestle and mortar in 10 ml NaCl solution (0.9%, w/v) to make the suspension. Thereon, 100 μl of the suspension and rhizospheric water were plated on M9 media (up to 10^{-6}) containing phenol as a sole energy source (50 mg l^{-1}). The plates were then incubated at 37 °C for 48 h, followed by counting of colony forming units (CFU) using OpenCFU software (Geissmann, 2013). A statistically significant number of colonies were picked and subjected to IGS-PCR and RFLP analyses (Ijaz et al., 2015). Each RFLP reaction constituted 7 μL PCR product, 1 μL HindIII enzyme, 1.5 μL R-buffer, and 5.5 μL deionized water, to make a total of 15 μL reaction.

2.7. Statistical analysis

The plant biomass, residual phenol, pollution parameters, CFU counts, and mutagenicity levels were subjected to statistical

analysis in R statistical language. One-way ANOVA was used to test the significant differences among different treatments, considering Tukey's test, after testing the homogeneity of variance. We also performed nMDS analysis (Bray-Curtis similarity) to compare the distribution of inoculated bacteria based on their RFLP profile.

3. Results and discussion

3.1. Plant biomass and growth

The fresh and dry biomass of plant tissues (root and shoot) was measured to elucidate the influence of phenol toxicity and respective bacterial inoculation on growth of *P. australis* (Table 1). It can be seen that the phenol contamination (T4) inhibited plant biomass development, whereas bacterial inoculation (T5) improved the biomass production. The reduction in biomass can be attributed to the presence of phenol in water since its toxic nature can drastically affect the plant health parameters such evapotranspiration, photosynthesis rate, etc. (Kottuparambil et al., 2014; Ucisik and Trapp, 2006). It has been reported that, in willow plants, 50% of the evapotranspiration was dropped after its exposure to water containing phenol 500 mg l^{-1} whereas complete plant death occurred at 1000 mg l^{-1} of phenol exposure (Ucisik and Trapp, 2006). The molecular mechanisms behind this drop in evapotranspiration have remained least investigated; however, in our other studies of antibiotics exposure (100 μg l^{-1}), we have seen direct damages to plant vascular bundles, increased production of reactive oxygen species (ROS), necrosis of plant roots, and dysbiosis in indigenous endophytic bacteria, ultimately leading to weakening of plant basal defense mechanisms (Arslan et al., 2017a). In the present study, we also observed that the root network of *P. australis* turned necrotic in the presence of phenol (T4) without bacterial inoculation. This investigation is in agreement with Phenrat et al., (2017), which also reported severe degradation of plant roots at 500 mg l^{-1} concentration of phenol in water in the first few days of exposure. Another reason for decreased biomass is the overloading of the plant detoxification system after continuous uptake/accumulation of phenol in plant tissues. This is because of the optimal octanol/water partition coefficient (log Kow = 1.46) of phenol. Nevertheless, indigenous bacteria and plants cannot increase the enzymatic efficiency significantly to cope with the external pressures and therefore can lead to severe toxic effects, i.e., the concept of internal exposure concentration (Escher et al., 2004).

On the other hand, bacterial inoculation increased the biomass of *P. australis* in the presence of phenol in water (T5); this could be linked to the ACC deaminase activities of the two inoculated strains, i.e., *B. cereus* LORH97 and *Pseudomonas* sp. LCRH90. It is an established fact that the bacteria possessing ACC deaminase capabilities are able to reduce the contaminant induced stress for the developing plant, ultimately improving the plant health even in the presence of contamination (Compant et al., 2010; Glick,

2010; Saleem, 2016; Saravanakumar et al., 2011). This improved biomass is further confirmed by ANOVA since less significant differences (p < .05) were observed between control (T1) and inoculated plants (T5).

3.2. Phenol removal

The phenol removal by different FTWs is presented in Table 2. Lowest phenol removal was observed in the reactors with bacterial consortium only (T3), whereas vegetated reactors were able to remove a higher amount of phenol (T4). This could be due to the fact that the phenolic compounds, when present in the environment at high concentrations, are toxic for bacterial communities and can inhibit/reduce the overall metabolic efficiency (Adeboye et al., 2014). However, higher phenol removal by plants, as compared to bacteria, can be associated with the optimal octanol–water partition coefficient leading to successful uptake/removal of phenol by plants. This is more evident in the initial 5-day exposure phase since most of the phenol was removed in this period and, thereafter, only a smaller fraction was removed suggesting the effect of physiological damages as explained earlier. (Riaz et al., 2017) reported that the accumulation of organic pollutants within plant tissues could trigger high ROS production, ending up into plant anatomical damages. Nevertheless, vegetated reactors in combination with bacterial consortia (T5) were able to remove the highest concentration of phenol; which is verified at statistical inference of p < .01. This is in agreement with the earlier studies describing the usefulness of bacterial augmentation in classical phytoremediation systems (Afzal et al., 2013). Such partnership systems have been widely adopted around the world with engineered modifications, aiming to accelerate the natural phytoremediation process with designed objectives (Chen et al., 2016; Ijaz et al., 2016; Newman and Reynolds, 2005; White and Cousins, 2013).

3.3. Water quality parameters

Established FTWs were able to improve the quality of water by lowering their respective COD, BOD, and TOC values (Table 3). The pollution load was highest in the un-vegetated reactors (T2); while a slight reduction was observed in un-vegetated reactors containing only bacterial consortium (T3) followed by vegetated contaminated reactors (T4). Anyhow, significantly highest reduction of COD, BOD, and TOC was observed in the bacterially assisted vegetated reactors (T5), i.e., COD reduced from 1057 to 122 mg l^{-1}, BOD from 423 to 78 mg l^{-1}, and TOC from 359 to 53 mg l^{-1}, after 15 days of the treatment. The high oxygen concentration in water is one of the fundamental parameters of water quality and high concentration of organic matter in water can decrease oxygen concentration, and affect metabolic functioning of the indigenous microbial community (Trivedy and Goel, 1984). The inoculation of phenol-degrading bacteria (T3), however, would have decreased the contaminant level but at the expense of oxygen (Chen et al., 2010; Demoling and Bååth, 2008). While in vegetated reactors

Table 1
Reed root and shoot biomass measured after 15 days of the experiment period.

Treatments	Root biomass (g)		Shoot biomass (g)	
	Fresh	Dry	Fresh	Dry
Vegetated (-phenol)	181.1[a] (21.9)	57.4[a] (4.1)	963[a] (98)	268[a] (34)
Vegetated and un-inoculated (+phenol)	76.5[b] (7.2)	22.9[b] (2.4)	593[b] (53)	124[b] (26)
Vegetated and inoculated (+phenol)	159.7[a] (27.8)	51.1[ab] (9.9)	815[ab] (46)	256[a] (39)

Each value is a mean of three replicates; means in the same column followed by different letter are statistically different at 5% level of significance; standard deviations are presented in parentheses.

Table 2
Effect of inoculum and vegetation on phenol removal.

Treatments	Initial	5 days	10 days	15 days
Control	500	389.4[a] (26.6)	347.7[a] (34.4)	333.5[a] (23.8)
Inoculated	500	327.7[ab] (24.2)	293.3[ab] (23.1)	194.6[b] (19.3)
Vegetated	500	202.3[b] (29.3)	188.1[b] (17.4)	167[bc] (21.3)
Vegetated and Inoculated	500	149.4[bc] (15.6)	45.3[c] (11.3)	19.3[c] (3.1)

Each value is a mean of three replicates; means in the same column followed by different letter are statistically different at 5% level of significance; standard deviations are presented in parentheses.

Table 3
Water quality parameters for the treated water at the end of experiment.

Treatments	COD	BOD	pH	TOC
Control	1057[a] (45)	423[a] (34)	5.8[b] (0.0)	359[a] (34)
Inoculated	514[b] (25)	281[b] (14)	6.6[ab] (0.01)	165[b] (15)
Vegetated	476[b] (29)	223[b] (22)	6.7[ab] (0.01)	172[b] (11)
Vegetated & Inoculated	122[c] (7)	78[c] (6)	7.2[a] (0.01)	53[c] (5)

Each value is a mean of three replicates; means in the same column followed by different letter are statistically different at 5% level of significance; standard deviations are presented in parentheses.

(T4), comparatively high removal can be attributed to the continuous uptake of the pollutant as well the potential of transporting atmospheric oxygen to the rhizosphere of *P. australis* (Van der Werff, 1991). In the bacterial assisted vegetated reactors (T5), successful interactions between plant-roots and rhizospheric bacteria are obvious to be established causing diffusion of surrounding oxygen into the plant rhizosphere as well as removal of contaminant load with the passage of time (Morris and Monier, 2003; Vymazal, 2010; Vymazal, 2013). This was revealed during temporal measurement of dissolved oxygen, where bacterially assisted reactors were able to recover while all other reactors suffered from oxygen depletion (Fig. 2). The pH of the treated water was also improved (acidic to neutral conditions), which is also attributed to the combined action of phenol transformations by bacteria as well as the use of organic acids by the plants (Ijaz et al., 2015).

3.4. Survival/colonization of inoculated bacteria

The survival of inoculated bacteria was determined in the rhizospheric water, roots interior, and shoots interior of the plant (Fig. 3). Comparatively, bacterial persistence was less commonly observed in the un-vegetated reactors (T3) as compared to the vegetated reactors (T5). This could have been caused by the absence of plant roots (symbiotic partner) resulting in reduced survival of inoculated bacteria (Arslan et al., 2014). Moreover, as all of the inoculated bacteria were previously isolated from the plant rhizosphere and/or endosphere; they may have developed necessary mechanisms of proliferation in the

presence of host only (Afzal et al., 2014). This is the reason that persistence of inoculated bacteria was more evident in the vegetated reactors (T5). In the vegetated reactors (T5), 38% of the total population was appeared to be *A. lwofii* ACRH76, followed by 24% of *B. cereus* LORH97. The endophytic species *Pseudomonas* sp. LCRH90 dominated the plant endosphere as it was recorded to be 68% of the total bacterial endophytes. Many of the earlier studies have reported the similar findings that the rhizospheric bacteria acclimatize and proliferate the rhizosphere whereas, endophytic bacteria colonize plant interior (Ho et al., 2013; Ho et al., 2012; Ijaz et al., 2016; Ijaz et al., 2015; Li et al., 2011; Li et al., 2010; Vacca et al., 2005). The colonization potential of endophytic bacteria appeared to be highly significant for *P. australis,* which support its suitability as wetland plant in addition to its helophytic properties. The parallel experiments on phenol degradation using *Typha domingensis* also achieved successful removal; nevertheless, bacterial persistence was limited to only *Pseudomonas* sp. LCRH90 (Saleem et al., In Press). The present study therefore presents not only an effective solution to overall phytoremediation success but also elucidate the nature *P. australis* as potential niche provider to endophytic and rhizospheric bacterial communities. This finding is further confirmed by nMDS analysis that distinctly separates the communities of endophytic bacteria from rhizospheric bacteria (Fig. 4); which was previously observed to be valid for the medium only (Cf. Saleem et al., In Press).

Based on these facts, it can be argued that efficient bacterial colonization can help develop an efficient system for the cleanup phenol especially at higher concentration, which remained difficult in classical phytoremediation experiments. A number of studies have reported potential key species of rhizospheric and endophytic bacteria which can be employed in combination with plants for efficient removal of phenol (Krastanov et al., 2013; Liu et al., 2016; Gu et al., 2016). Iqbal et al., (2017) reported such a partnership in which phenol and benzene were removed by endophytic bacterial strains namely *Achromobacter* sp. (AIEB-7), *Pseudomonas* sp. (AIEB-4), and *Alcaligenes* sp. (AIEB-6) when inoculated with *Cannabis sativa*. Nevertheless, studies employing plant-bacteria partnerships are relatively few and most of them rely only to the bacterial potential of phenol degradation in classical lab experiments. Therefore, there is a need to validate if these key species can be used for bacterial assisted phytoremediation studies. Moreover, to find plant species that can serve as appropriate host for these bacteria is equally crucial.
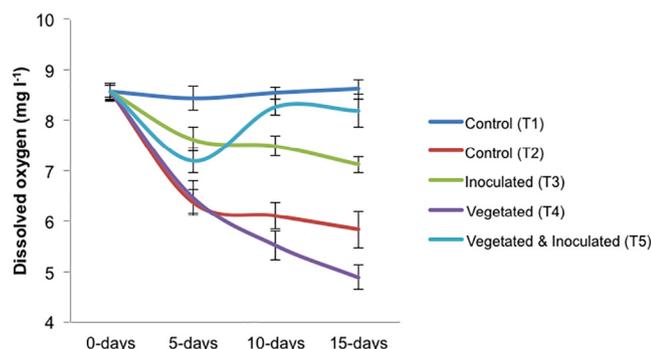
Fig. 2. Temporal assessment of dissolved oxygen for evaluating system's performance.
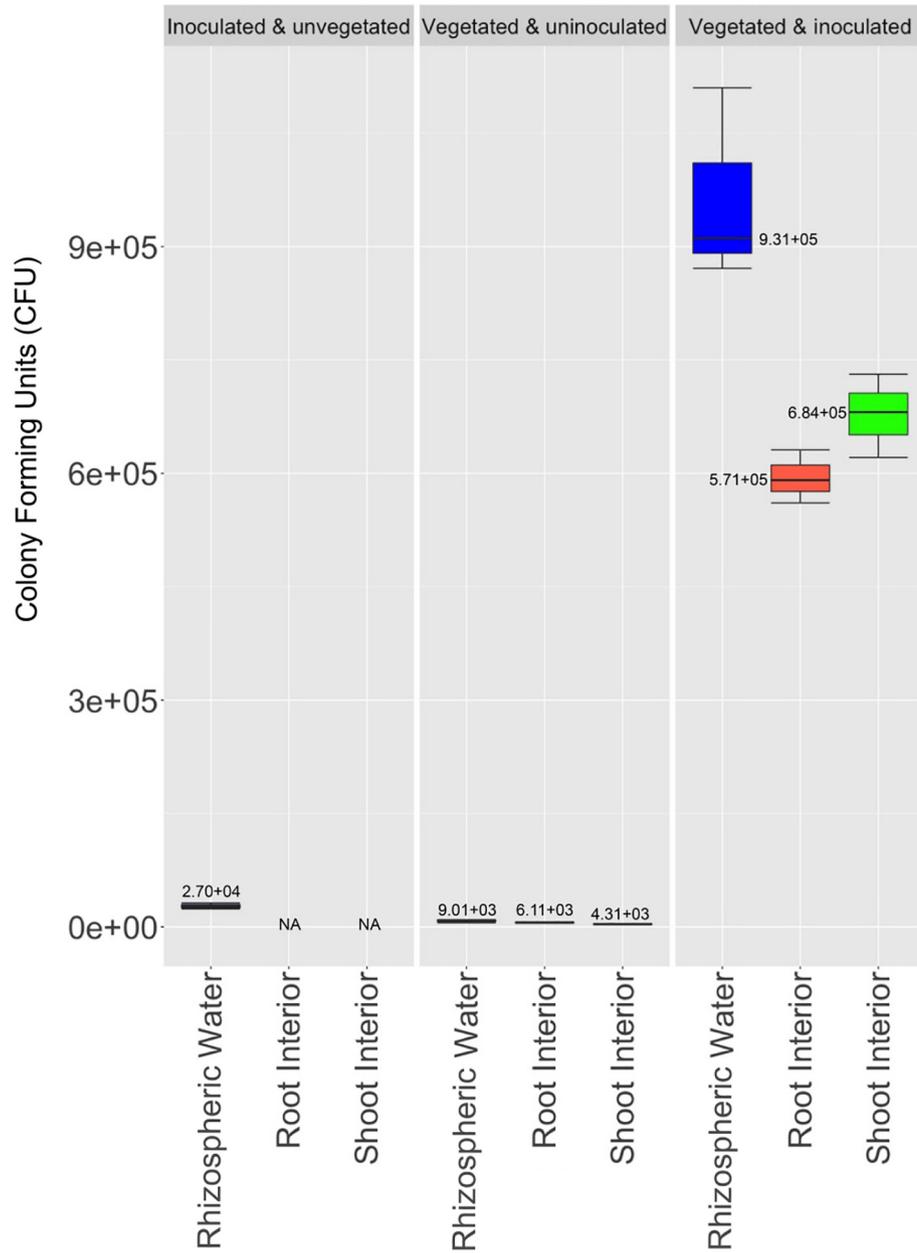
Fig. 3. Persistence of inoculated bacteria in the treated water, root interior, and shoot interior of *T. domingensis.*
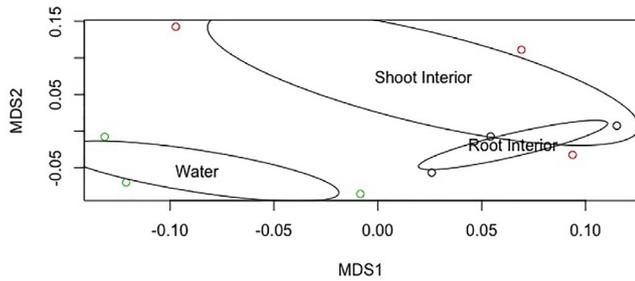
Fig. 4. The nMDS ordination of inoculated bacterial consortium based on RFLP profile.

4. Conclusions

Helophytic plants can be a suitable choice in FTW for phytoremediation of phenolic compounds. The technology is an innovative product of ecological engineering and can be engineered to harness phytological and microbiological remediation at all levels. The current study is an example of such a system that was engineered in a way to restore contaminated water with phenolic compounds. The system performance was up to the mark as successful removal of phenol was observed in the established FTWs with reduced COD, BOD, and TOC levels. Although the study was conducted in microcosm, we observed successful interaction of inoculated bacteria with *P. australis* suggesting the potential role of helophytic grasses in plant-bacteria partnership. Therefore, engineering the potential of helophytic plants with pollutant-degrading bacteria could be a promising area in the wastewater treatment. Last but not least, the technology relies on near-natural means of remediation and comparable little energy is needed for its operation, this makes it particularly attractive for the countries with more economic constraints such as Pakistan.

Acknowledgements

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