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Bioaugmentation-Enhanced Remediation of Crude Oil Polluted Water in Pilot-Scale Floating Treatment Wetlands

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Abstract: Floating treatment wetlands (FTWs) are cost-effective systems for the remediation of polluted water. In FTWs, the metabolic activity of microorganisms associated with plants is fundamental to treatment efficiency. Bioaugmentation, the addition of microorganisms with pollutant-degrading capabilities, appears to be a promising means to enhance the treatment efficiency of FTWs. Here, we quantified the effect of bioaugmentation with a four-membered bacterial consortium on the remediation of water contaminated with crude oil in pilot-scale FTWs planted with *Phragmites australis* or *Typha domingensis*. The bacteria had been isolated from the endosphere and rhizosphere of various plants and carry the alkane hydroxylase gene, *alkB*, involved in aerobic hydrocarbon degradation. During a treatment period of 36 days, FTWs planted with *P. australis* achieved a reduction in hydrocarbon concentration from 300 mg/L to 16 mg/L with and 56 mg/L without bioaugmentation. In the FTWs planted with *T. domingensis*, respective hydrocarbon concentrations were 46 mg/L and 84 mg/L. The inoculated bacteria proliferated in the rhizoplane and in the plant interior. Copy numbers of the *alkB* gene and its mRNA increased over time in plant-associated samples, suggesting increased bacterial hydrocarbon degradation. The results show that bioaugmentation improved the treatment of oil-contaminated water in FTWs by at least a factor of two, indicating that the performance of full-scale systems can be improved at only small costs.

Keywords: nature-based solutions; water pollution and treatment; plant-bacteria interaction; *Phragmites australis*; *Typha domingensis*

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1. Introduction

The burning of petroleum products still accounts for about one-third of the global society's energy budget [1]. Next to being unsustainable, onshore oil field exploitation alone generates over 5 million m^3 per day of polluted water globally [2–4]. The polluted water contains a slew of organic and inorganic contaminants such as hydrocarbons, phenols, and heavy metals with high human and ecotoxicological significance [4–6]. In particular in Low-Middle-Income Countries (LMIC) with oil production, treatment of the water is limited to storage in so-called evaporation pits as the cheapest approach. The water discharged or leaking from the pits is of poor quality [6]. Physical and chemical treatment technologies are available but have significant demands on energy input, capital investment, and operation and maintenance cost requirements, and are therefore rarely used in LMIC [7,8].

Floating treatment wetlands (FTW) are nature-based solutions for the cost-effective treatment of various contaminated waters, including those polluted with oil [4,6,8–19].

These systems consist of aquatic plants, i.e., emergent macrophytes, whose roots can form filter mats submerged in the water. They are buoyant due to the aerenchyma of the roots and gas trapped in the root mats. The buoyancy can be further enhanced by simple rafts. Thus, quite effective water treatment systems can be implemented at minimal financial cost [8]. While the plants themselves can attenuate the concentration of pollutants in the water via uptake, sequestration, and in planta transformations, the major share of pollutants is degraded by members of the microbial communities in the various compartments of FTWs [4,20]. The relevant compartments are the areas of free water, the rhizosphere and rhizoplane, and the endosphere. Particularly in the latter two compartments, mutually beneficial interactions between the plants and their microbiomes take place [6,10,21–23]. Thus, a high reactive surface area of the root system is key to the success of FTWs. The roots' exterior and interior are residence spaces for the microorganisms where they can capture plant-derived organic substrates as well as oxygen provided by the aerenchyma for their enhanced survival and metabolic activity. In turn, the plant can benefit from the microbial transformation of toxic compounds into harmless products. In addition, it has been shown that several bacterial strains isolated from the rhizosphere or endosphere have properties that could promote plant growth, such as stress alleviation, inorganic phosphorous solubilization, and siderophores formation [24–30].

Several studies using laboratory-scale systems have indicated that bioaugmentation with microbes capable of hydrocarbon transformation can enhance the treatment performance of FTWs [4,31–33]. In essence, such investigations are of empirical nature in which bacterial strains or consortia with the desired metabolic properties are tested in combination with particular plant species. Typically, the microorganisms had been isolated from the root system of aquatic plants, phenotypically or genotypically tested for relevant properties, and if suitable used in bioaugmentation. Previously, we demonstrated that hydrocarbon degradation in laboratory-scale FTWs planted with various macrophytes improved after bioaugmentation with a four-membered bacterial consortium [4,34]. A limitation of the previous studies, for which 20 L-sized containers were used, was that the rhizosphere took up a greater relative portion of the total volume than can be expected for full-scale systems. Considering that the rhizosphere is the hot spot for microbial activity, it was not clear how well the performance of the laboratory systems could be translated to large-scale FTWs.

Here, we quantified the effect of bioaugmentation on hydrocarbon degradation in pilot-scale FTWs (1000 L) planted with either *Phragmites australis* (common reed) or *Typha domingensis* (southern cattail), in which the ratio of rhizosphere to free water volume was similar to that in full-scale systems. The added bacteria proliferated in the FTWs, suggesting that full-scale systems can be effectively bioaugmented at minimal costs.

2. Materials and Methods

2.1. Establishment and Operation of Pilot-Scale FTWs

FTWs systems in plastic tanks having 1 m^3 of water holding capacity [dimensions: 1.2 m (length) × 1.0 m (width), × 1.0 m (height)] were designed and constructed at National Institute for Biotechnology and Genetic Engineering (NIBGE) (Faisalabad, Pakistan) (Figure 1). Floating rafts of fitting dimensions were prepared using Jumbolon Roll (Diamond Foam, Lahore, Pakistan). The raft's surface was covered with aluminum foil and a soil-sand mixture to reduce weathering effects such as damage by ultraviolet radiation. The two emergent aquatic plant species, *P. australis* and *T. domingensis*, were selected, as in previous laboratory-scale studies [4,33–36]. They are available locally, develop aerenchyma in flooded environments, are capable of forming a strong root network, and are able to thrive in harsh environmental conditions. The nursery of these plants was previously established at NIBGE in plastic pots. For plantation purposes, eight holes of equal diameter (7.5 cm) were drilled into the rafts and twenty-four seedlings of either *P. australis* or *T. domingensis* (each ~60 cm high and 45–65 g in weight) were inserted into the holes (three per hole). In the case of rain, the tanks were covered with plastic sheets to avoid addition of rainwater to the tanks. Initially, the plant seedlings were grown in the presence of Hoagland solution

(5%) for a period of 2 months to establish a strong root network. Then, the following FTW set-ups were established in triplicates:

(1) oil-contaminated water without vegetation and bacterial inoculation (1st control, C1);
(2) oil-contaminated water with bacterial inoculation only (Treatment 1, T1);
(3) oil-contaminated water with vegetation only, (Treatment 2, T2);
(4) oil-contaminated water with vegetation and bacterial inoculation (Treatment 3, T3);
(5) Vegetation in tap water (2nd control, C2).

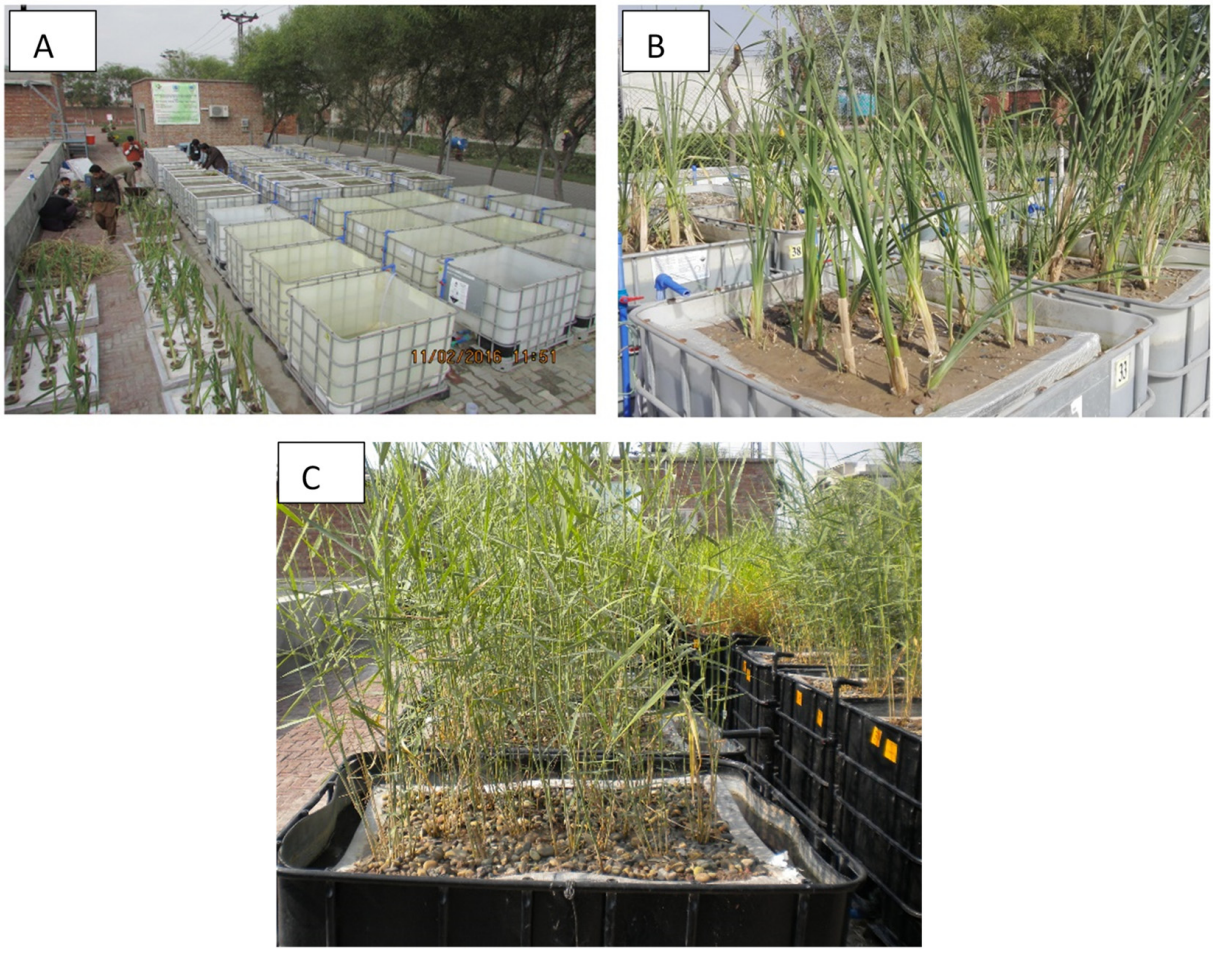

Figure 1. The development and installation of FTW macrocosms with *Typha domingensis* (**A**,**B**), and *Phragmites australis* (**C**).

The contaminated water was from an oil production site near Fimkassar (32.55_N 72.51_E), Chakwal, Pakistan. The physicochemical characteristics have been described previously [4,6], and are also presented in Table S1. Several of the quality parameters such as hydrocarbon content exceed the National Environmental Quality Standards (NEQS) of Pakistan. The experiment was set up at local environmental conditions of Faisalabad. No rain was recorded during the experimental period.

2.2. Chemical Analyses and Determination of Plant Growth

For chemical analyses, water samples were collected from the macrocosms every 12 days over a period of 36 days. Hydrocarbon concentrations (mainly C10–C30 alkanes) were determined as described previously via a Spectrum Two Environmental Hydrocarbon Analysis System [37,38]. The chemical oxygen demand (COD) and the biochemical oxygen demand (BOD) were measured with the standard method 5210B and 5200D, respectively [39].

To record plant growth and biomass, the roots of harvested plants were carefully washed with tap water to remove loosely attached particles. The plants were then separated into shoots and roots, and their respective lengths measured. The shoot and root samples were oven-dried at 70 °C for 72 h, and dry biomass was gravimetrically determined.

2.3. Bioaugmentation with a Four-Membered Bacterial Consortium

A synthetic consortium of four bacterial strains previously isolated in our laboratory was used to enhance oil degradation in the pilot-systems. These strains were *Acinetobacter junii* TYRH47 (NCBI GenBank accession: KJ620859), *Acinetobacter* sp. LCRH81 (accession: KJ620868), *Bacillus subtilis* LORI66 (accession: KF478216), and *Klebsiella* sp. LCRI87 (accession: KF478220). The strains TYRH47 and LCRH81 were isolated from the rhizospheres of *T. domingensis* and *Lecucaena leucocephala*, respectively, whereas the strains LORI66 and LCRI87 were isolated from the root interior of *Lolium perenne* and *L. leucocephala* [40].

In vitro, these strains are highly effective in crude oil degradation and have plant-growth promoting capabilities [indole-3-acetic acid (IAA) and siderophore production, P-solubilization, and 1-aminocyclopropane-1-carboxylate (ACC)-deamination]. Further, these strains carry the *alkB* gene, which is involved in catabolic hydrocarbon degradation [34,40–43]. To prepare the consortium, the four strains were individually cultured in Lysogeny-Broth (LB) at 37 °C for 24 h and harvested via centrifugation at 10,000 rpm (Eppendorf Centrifuge 5810R, Eppendorf, Hamburg, Germany), followed by re-suspension in 0.9% normal saline solution. The suspensions were diluted to a density of 10^7 cells mL^{-1} and mixed with an equal ratio (1:1:1:1). One litre of the consortium was inoculated to the FTWs subsets T1 and T3.

Water samples were collected from the bioaugmented FTWs every 12 days over a period of 36 days and investigated as follows. The cultivable bacterial communities from the rhizoplane, and roots and shoots' interior were counted as colony forming units (CFUs) coupled with restriction fragment length polymorphism (RFLP) analysis. Bacteria from the rhizoplane were isolated by agitating root cuts at 180 rpm with glass beads in normal saline solution for 1 h at room temperature. For in planta bacterial quantification, root and shoot samples were surface-sterilized with 70% ethanol for 2 min followed by treatment with 1% sodium hypochlorite solution for 5 min, and washed thrice with sterile distilled water for 2 min. After the sterility test of cultivable community on LB agar plates [38], 2 g of surface-sterilized roots and shoot samples were ground using a mortar and pestle in the presence of 10 mL of 0.9% sodium chloride solution. The ground material was then shaken for 1 h at 37 °C. The solid material was allowed to settle for some time, and then 100 µL of the suspension and treated water were plated onto minimal media (M9) containing 1% (v/v) diesel as a sole source of carbon and energy. The plates were incubated at 37 °C followed by counting of CFUs. To check if the inoculated strains survived and proliferated in the pilot-systems, the identity of isolates was confirmed through RFLP analysis of PCR amplified 16S-23S rDNA intergenic spacer (IGS) region product as reported earlier [36].

2.4. Abundance and Expression of the alkB Gene in the FTWs

Furthermore, the copy numbers of the *alkB* gene and its transcript were recorded in the various compartments of the bioaugmented FTWs over 36 days at intervals of 12 days. Total DNA and RNA were extracted from the treated water, rhizoplane, roots, and shoots by using the FastDNA™ and FastRNA™ spin kits (MP Biomedicals, Irvine, CA, USA). The quality and quantity of DNA and RNA were assessed through agarose gel electrophoresis and by NanoDrop spectrometry (Thermo Scientific, Waltham, MA, USA). RNA was reverse transcribed to cDNA by using Superscript II reverse transcriptase (Invitrogen, Waltham, MA, USA). Real-time PCR (qPCR) was used to determine the copy numbers of gene and transcript using the specific primer-set and conditions reported earlier [41,44].

2.5. Transmission Electron Microscopy

Transmission electron microscopy was used to visually observe the localization of bacteria in the rhizoplane and *in planta* [45]. Briefly, surface sterilized roots were cut into small agar cubes (2–3 mm^3) and fixed in 2% glutaraldehyde for 16–18 h at room temperature. After washing thrice with 0.2 M PIPES buffer (pH 6.8) for 1 h and rinsing with distilled water, samples were treated with 0.2% osmium tetraoxide (prepared in 0.2 M PIPES buffer) for 16–18 h and again washed for 30 min. The samples were then treated with 5% uranyl acetate for 16–18 h, washed, dehydrated with 100% ethanol for 1 h, and then kept in acetone for 30 min. Pure Spurr resin equal in amount of acetone (1:1) was added gently and left for 16–18 h. Afterwards acetone/Spurr mixture was replaced with pure Spurr resin and left for 16–18 h on a specimen rotator. The specimens were embedded in rubber moulds and allowed to polymerize at 65 °C for 72 h. Ultra-thin plastic sections of processed roots were cut with ultra-microtome (RML-70, RMC, Ontario, NY, USA), picked on 400 mesh copper grids and double stained with uranyl acetate and lead citrate. Finally, the sections were observed under transmission electron microscope (JEOL, JEM-1010, Akishima, Tokyo, Japan) at 80 KV.

2.6. Toxicity Bioassay

The toxicity of the reclaimed water of all the treatments was assessed using a fish survival assay [45]. Ten individuals each with approximately equal weight and size of a locally available fish (*Labeo rohita*) were exposed to treated water samples. The mortality rate was determined by counting the number of live and dead fish every 24 h of exposure for a period of 4 days.

2.7. Statistical Analysis

All parameters were subjected to one-way analysis of variance (ANOVA) using Statistix 9, and *p*-values were considered to be significant at $p < 0.05$.

3. Results

3.1. Attenuation of Hydrocarbon Concentration, COD and BOD in Pilot-Scale FTW

Pilot-scale FTW performance was evaluated by examining water quality parameters at 0, 12, 24, and 36 day time points (Figure 2). The reduction in hydrocarbon concentration, COD, and BOD was significantly lower in the unplanted macrocosms C1 (without bioaugmentation) and T1 (with bioaugmentation) than in the macrocosms planted with either *P. australis* or *T. domingensis*. Total hydrocarbons were reduced from 300 to 276 mg/L (8%) in C1, and to 115 mg/L (61%) in T1 over a 36 day period. In the FTW macrocosms without bioaugmentation (T2), hydrocarbon reduction improved, reaching a concentration of 56 mg/L with *P. australis* and 84 mg/L with *T. domingensis* (81% and 72% removal, respectively) at the 36-day time point. The better performance in the experiments with *P. australis* was not statistically significant. When plant and bacterial augmentation were applied together, the maximum reduction in hydrocarbon content was achieved. Bacterial augmentation reduced hydrocarbons to 16 mg/L (95% reduction) with *P. australis* and to 46 mg/L (85% reduction) with *T. domingensis* (setup T3).

The removal of COD and BOD mirrored the hydrocarbon removal. It was important to analyze those parameters as well to estimate how much the hydrocarbon fraction contributed to the overall contaminant load. The COD was reduced from 1440 mg/L to 1116 mg/L (23% reduction) in the unplanted and uninoculated setup C1, whereas the bacterial inoculation in setup T1 lowered COD to 418 mg/L (71% reduction). In the FTW setup without bioaugmentation (T2), COD was reduced to 163 mg/L (89%) with *P. australis*, and to 263 mg/L (82%) with *T. domingensis*. The COD removal efficiency of both plants was further improved when bacteria was inoculated in the system (T3). Bioaugmentation resulted in a reduction of COD to 87 mg/L (94%) in the FTW with *P. australis*, and to 107 mg/L (93%) in the FTW with *T. domingensis* (Figure 2). The reduction in BOD, with an initial concentration of 645 mg/L, showed the same pattern.

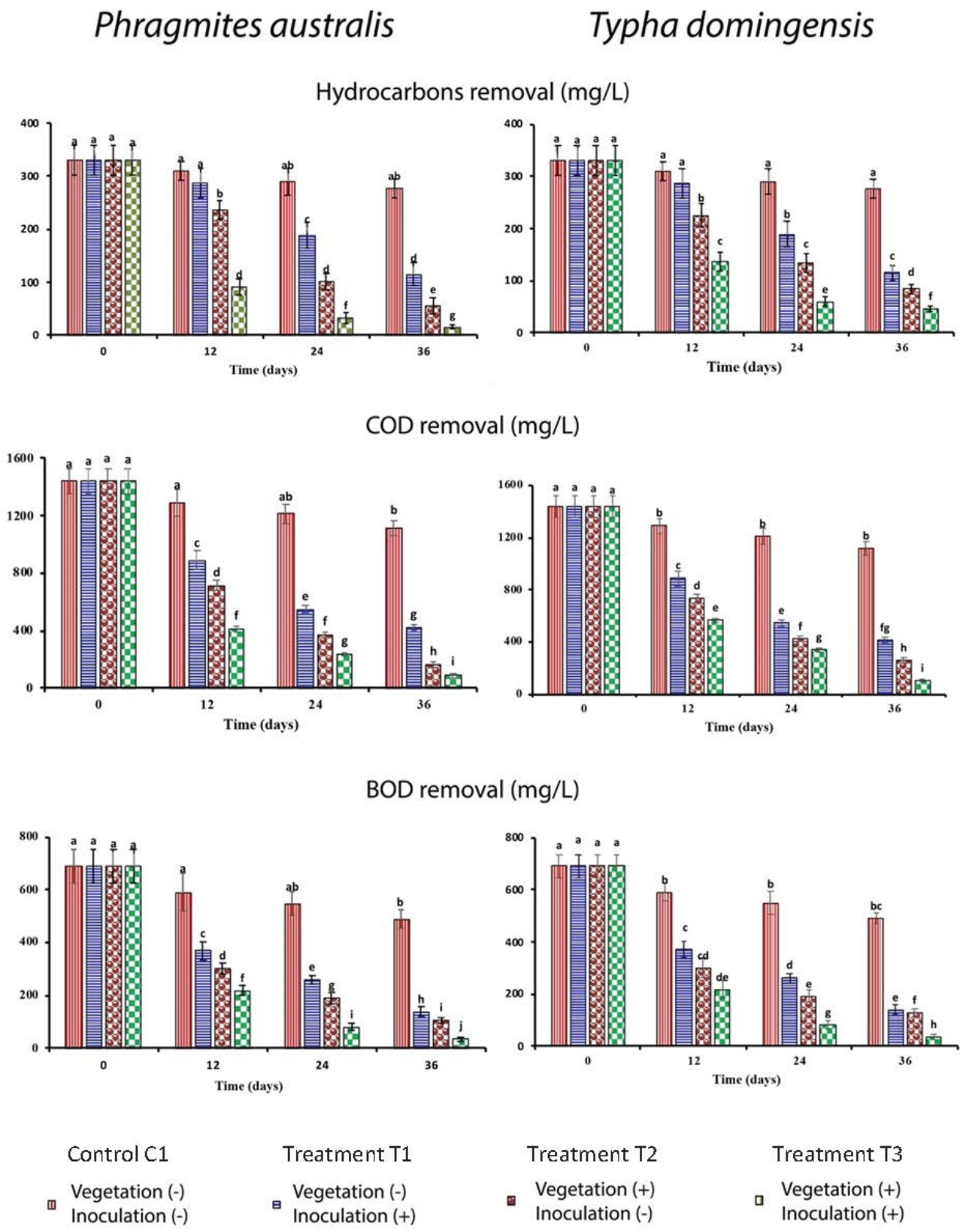

Figure 2. Removal of hydrocarbons, chemical oxygen demand (COD) and biochemical oxygen demand (BOD) in FTWs. The mean and standard error of three replicates is provided. The superscript letters behind the mean represent statistical comparison (ANOVA) per column. Same letters represent no differences and different letters represent statistical differences at 5% level of significance.

3.2. Plant Growth in FTWs

Plant growth parameters were determined by harvesting plants at the end of the experiment (Table 1). The oil contamination reduced the plant growth and biomass in terms of root length, shoot length, and fresh and dry biomass for both *P. australis* and *T. domingensis*, with the former being more affected.

Table 1. Effect of bacterial inoculation on mean length of roots and shoots of *Phragmites australis* and *Typha domingensis*, and plant biomass per m^3 of water.

	Length (cm)		Fresh Biomass (g m^{-3})		Dry Biomass (g m^{-3})	
	Roots	**Shoots**	**Roots**	**Shoots**	**Roots**	**Shoots**
			P. australis			
Tap water	58.3 ^{a} ± 3.4	218 ^{a} ± 20	654 ^{a} ± 17	187 ^{a} ± 9	183 ^{a} ±13	112 ^{a} ± 7
Inoculation (−)	34.7 ^{c} ± 3.8	135 ^{c} ± 15	448 ^{c} ± 31	130 ^{c} ± 7	119 ^{c} ± 8	78 ^{c} ± 6
Inoculation (+)	45.1 ^{b} ± 3.1	172 ^{b} ± 16	511 ^{b} ± 24	151 ^{b} ± 6	145 ^{b} ± 7	94 ^{b} ± 6
			T. domingensis			
Tap water	52.1 ^{a} ± 4.2	178 ^{a} ± 12	576 ^{a} ± 16	164 ^{a} ± 7	116 ^{a}± 7	107 ^{a} ± 9
Inoculation (−)	33.4 ^{c} ± 3.7	124 ^{c} ± 8.2	384 ^{c} ± 21	119 ^{c} ± 6	81 ^{c} ± 5	68 ^{c} ± 4
Inoculation (+)	42.2 ^{b} ± 3.8	150 ^{b} ± 9.5	482 ^{b} ± 26	142 ^{b} ± 7	96 ^{b} ± 6	87 ^{b} ± 5

The mean and standard error of three replicates is provided. The superscript letters behind the mean represent statistical comparison (ANOVA) per column. Same letters represent no differences and different letters represent statistical differences at 5% level of significance.

Compared to the control with tap water (control C2), the final length of roots and shoots were shorter by 40% (34.7/58.3 cm) and 38% (135/218 cm) for *P. australis*, respectively, and 36% (33.4/52.1 cm) and 30% (124/178 cm) for *T. domingensis*, respectively, in the FTW setup with oil-contaminated water (T2). Bioaugmentation (T3) lessened the negative effect of the oil-contaminated water on growth of both plants. Root and shoot lengths were shorter by only 23% (45.1/58.3 cm) and 21% (172/218 cm) for *P. australis*, respectively, and 19% (42.2/52.1) and 16% (150/178) for *T. domingensis*, respectively. The results of fresh and dry biomass of both types of FTWs matched the pattern recorded for the length of the roots and shoots.

The effective surface area of the roots is thought to have a governing influence on treatment performance. Since the surface area cannot be precisely determined in the wetlands, we computed the ratio of dry root biomass per m^3 of water as surrogate to allow for comparison with a bioaugmented full-scale FTW treating water from the same source as in the present study [6]. In the full-scale system, the mean dry root biomass of *P. australis* and *T. domingensis* were approximately 127 g m^{-3} and 117 g m^{-3}, respectively, which are similar to 145 g m^{-3} and 96 g m^{-3} calculated for the bioaugmented FTWs in this study.

3.3. Abundance of Inoculated Bacteria, and of the alkB Gene and Transcripts

The inoculated bacteria successfully persisted in the hydrocarbon-contaminated water, the rhizoplane, as well as in the root and shoot interiors as enumerated via CFU counts coupled with RFLP. The total numbers of bacteria in the various plant compartments were in the following order: rhizoplane > water > root interior > shoot interior (Table 2). This result was expected based on knowledge about typical bacterial abundance in the various compartments [4,46].

In the free water zone, the CFU counts decreased over time, but increased in the rhizoplane as well as the root and shoot interior. The composition of the communities present in the compartments on day zero was not determined. However, the temporal dynamics of the total abundance matched the plant-associated isolation origin of the bioaugmented strains [31,40,47]. At the end of the observational period after 36 days, the abundance of each of the four strains was at least 18-fold higher than provided with the inoculum (Table 3). There were small but statistically significant ($p < 0.05$) differences in the abundance of the strains in the FTW compartments. With both plants, *Klebsiella* sp. strain LCRI87 and *B. subtilis* LORI66 colonized the plant interior parts better than *Acinetobacter*

sp. LCRH81 and *A. junii* TYRH47, while the latter two showed better persistence in the rhizoplane and water than the former. Furthermore, each of the four strains was more abundant in the rhizoplane of *P. australis* than in *T. domingensis*. Other compartments were not statistically different between the plant species ($p > 0.05$).

Table 2. Total colony forming units (CFU) in the water, rhizoplane (RP), root interior (RI), and shoot interior (SI) of *Phragmites australis* and *Typha domingensis* in bioaugmented FTWs.

Time Days	CFU mL^{-1} or $g^{-1} \times 10^6$			
	Water	RP	RI	SI
		P. australis		
0	$59^{a} \pm 4$	$55^{c} \pm 9$	$5.6^{c} \pm 0.2$	$1.3^{d} \pm 0.1$
12	$55^{ab} \pm 7$	$153^{b} \pm 7$	$7.8^{b} \pm 0.5$	$1.4^{c} \pm 0.1$
24	$53^{ab} \pm 4$	$156^{ab} \pm 9$	$12.3^{a} \pm 0.9$	$2.6^{b} \pm 0.1$
36	$46^{b} \pm 8$	$168^{a} \pm 2$	$12.5^{a} \pm 0.7$	$2.8^{a} \pm 0.1$
		T. domingensis		
0	$64^{a} \pm 8$	$27^{b} \pm 1$	$2.8^{e} \pm 0.2$	$0.7^{b} \pm 0.1$
12	$58^{ab} \pm 4$	$46^{a} \pm 4$	$3.7^{b} \pm 0.4$	$0.8^{a} \pm 0.1$
24	$51^{b} \pm 5$	$52^{a} \pm 2$	$4.3^{d} \pm 0.5$	$0.8^{a} \pm 0.1$
36	$43^{c} \pm 5$	$49^{a} \pm 1$	$4.6^{h} \pm 0.5$	$0.8^{a} \pm 0.1$

The mean and standard error of three replicates is provided. The superscript letters behind the mean represent statistical comparisons (ANOVA) per column. Same letters represent no differences and different letters represent statistical differences at 5% level of significance.

Table 3. Total colony forming units (CFU) of the 4 added bacterial strains after 36 days in the water, rhizoplane (RP), root interior (RI), and shoot interior (SI) of *Phragmites australis* and *Typha domingensis* in the FTWs. At day zero the water had been inoculated with 0.25×10^6 CFU mL^{-1} of each strain.

Strain	CFU mL^{-1} or $g^{-1} \times 10^6$			
	Water	RP	RI	SI
		Phragmites australis		
Acinetobacter junii TYRH47	6.9 ± 1.2	58.8 ± 4.2	1.3 ± 0.1	0.2 ± 0.004
Acinetobacter sp. LCRH81	9.2 ± 1.6	81.2 ± 5.8	1.5 ± 0.1	0.2 ± 0.004
Bacillus subtilis LORI66	6.9 ± 4.2	61.6 ± 4.4	3.8 ± 0.21	0.4 ± 0.007
Klebsiella sp. LCRI87	4.6 ± 0.8	39.2 ± 2.8	1.7 ± 0.1	0.3 ± 0.005
		Typha domingensis		
Acinetobacter junii TYRH47	10 ± 1.1	13.9 ± 0.4	0.8 ± 0.08	0.2 ± 0.002
Acinetobacter sp. LCRH81	9.3 ± 1.1	12.3 ± 0.4	0.4 ± 0.04	0.1 ± 0.002
Bacillus subtilis LORI66	5.7 ± 0.7	21.2 ± 0.6	0.8 ± 0.08	0.2 ± 0.002
Klebsiella sp. LCRI87	4.3 ± 0.5	21.2 ± 0.6	1.0 ± 0.1	0.1 ± 0.002

The mean and standard error of three replicates is provided.

Imaging with transmission electron microscopy of the rhizoplane and the root endosphere of *P. australis* confirmed the higher abundance of bacteria in samples from the bioaugmented FTWs as compared to the non-bioaugmented FTWs (Figure 3). No high-quality EM images could be obtained with samples from *T. domingensis*.

To provide evidence that the inoculated bacteria were indeed involved in hydrocarbon degradation in situ and in planta, we monitored the abundance and expression of the *alkB* gene in the water, rhizoplane, and root and shoot interiors for both plants (Table 4). In samples from the free water phase, the copy numbers of gene and transcript decreased over time, concomitant with the decrease in CFU counts. In contrast, the copy numbers increased in the rhizoplane and the root and shoot interiors. At the end of the experiment, the highest abundance and expression of the gene was seen in the root interior, i.e., 1×10^4 and 2×10^3 copies g^{-1}, respectively. The increase in copy numbers of gene and transcript positively

correlated (R > 0.7) with hydrocarbon removal, supporting that the bioaugmented bacteria were involved in hydrocarbon degradation.

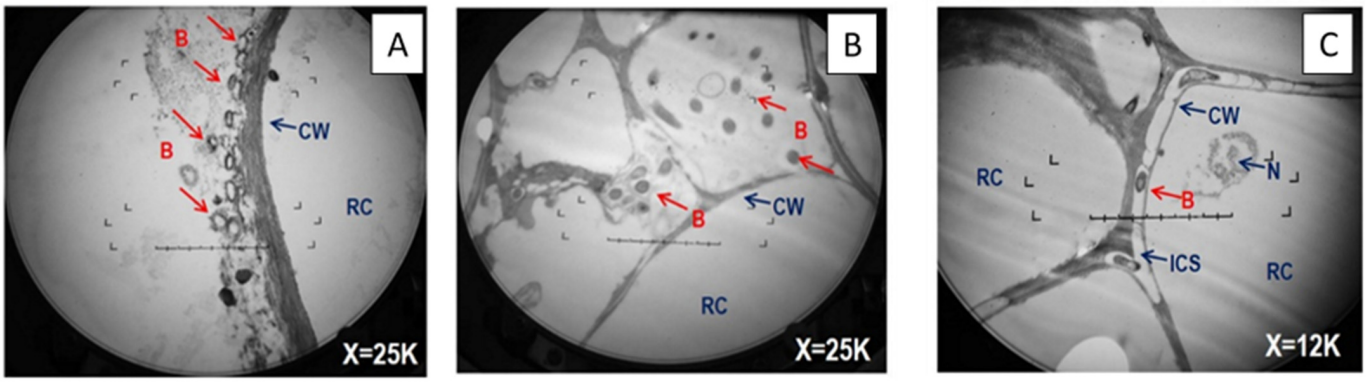

Figure 3. Transmission electron micrographs of bacterial communities associated with *Phragmites australis*: (**A**) in the rhizoplane; (**B**) inside root cells; (**C**) in intracellular space of the root. B = bacteria; CW = plant cell wall; ICS = intercellular space; N = nucleus.

Table 4. Abundance and expression of *alkB* in the water, rhizoplane (RP), root interior (RI), and shoot interior (SI) of *Phragmites australis* and *Typha domingensis* in FTWs.

Time Days	***alkB* Abundance**				***alkB* Expression**			
	Copy Number mL^{-1} or g^{-1} × 10^4				**Copy Number mL^{-1} or g^{-1} × 10^4**			
	Water	RP	RI	SI	Water	RP	RI	SI
				P. australis				
0	18.6 ^{a} ± 0.5	1.3 ^{c} ± 1.2	0.3 ^{d} ± 0.02	0.2 ^{d} ± 0.1	7.3 ^{a} ± 0.04	0.7 ^{c} ±0.2	0.04 ^{c} ± 0.1	0.01 ^{d} ± 0.02
12	17.3 ^{b} ± 0.7	14.7 ^{b} ± 0.9	4.8 ^{c} ± 0.1	2.3 ^{c} ± 0.1	6.6 ^{b} ± 0.03	10.5 ^{b} ± 0.2	3.7 ^{a} ±0.1	0.4 ^{c} ±0.03
24	13.8 ^{c} ± 0.04	15.2 ^{b} ± 0.7	6.6 ^{b} ± 0.1	2.8 ^{a} ± 0.03	3.2 ^{c} ± 0.02	11.2 ^{b} ± 0.1	3.0 ^{b} ±0.02	0.6 ^{b} ±0.01
36	11.5 ^{d} ± 0.02	15.8 ^{a} ± 0.1	7.2 ^{a} ± 0.04	2.7 ^{b} ± 0.01	2.2 ^{d} ± 0.01	12.5 ^{a} ± 0.1	3.5 ^{a} ±0.01	0.8 ^{a} ± 0.02
				T. domingensis				
0	13.3 ^{a} ± 0.2	1.1 ^{d} ± 0.9	0.05 ^{d} ± 0.1	0.02 ^{c} ± 0.04	6.1 ^{a} ± 0.2	0.3 ^{d} ± 0.02	0.8 ^{d}± 0.1	0.03 ^{b} ± 0.03
12	12.3 ^{b} ± 0.1	3.3 ^{c} ± 0.3	1.1 ^{c} ± 0.04	0.4 ^{b} ± 0.03	5.3 ^{b} ± 0.3	2.8 ^{c}± 0.2	1.1 ^{c}± 0.1	0.1 ^{a} ±0.02
24	13.8 ^{c} ± 0.1	4.3 ^{b} ± 0.2	2.1 ^{b} ± 0.02	0.7 ^{a} ± 0.1	2.1 ^{c} ± 0.7	3.8 ^{b}± 0.3	1.3 ^{a} ± 0.01	0.1 ^{a} ±0.01
36	12.5 ^{d} ± 0.01	4.7 ^{a} ± 0.1	2.5 ^{a} ± 0.01	0.64 ^{a} ± 0.1	1.4 ^{d} ± 0.02	4.2 ^{a}± 0.2	1.1 ^{b} ± 0.01	0.2 ^{b} ±0.02

The mean and standard error of three replicates is provided. The superscript letters behind the mean represent statistical comparisons (ANOVA) per column. Same letters represent no differences and different letters represent statistical differences at 5% level of significance.

3.4. Fish Toxicity

Fish toxicity analysis showed that the water contaminated with hydrocarbons was very toxic (Table 5). Fish mortality was 100% after 96 h of exposure in the control without FTWs or added bacteria. The water treated with bioaugmentation or vegetation alone reduced fish mortality to 70% and 30%, respectively. In contrast, no fish died in the water treated by the combined use of bioaugmentation and *P. australis* or *T. domingensis*.

Table 5. Fish toxicity assay with initially 10 individuals of *Labeo rohita* in oil-contaminated wastewater.

Treatment	Fish Mortality up to Time Point			
	24 h	**48 h**	**72 h**	**96 h**
Wastewater only	10	-	-	-
with bioaugmentation	0	3	4	5
Phragmites australis without bioaugmentation	0	0	2	3

Table 5. *Cont.*

Treatment	Fish Mortality up to Time Point			
	24 h	**48 h**	**72 h**	**96 h**
with bioaugmentation	0	0	0	0
Typha domingensis				
without bioaugmentation	0	0	2	4
with bioaugmentation	0	0	0	0

4. Discussion

The success of treatment wetlands relies on effective plant-bacteria interactions [47,48]. This includes oil-contaminated wastewater, where hydrocarbons are mineralized by plant-associated microbial communities [46,49,50]. The plants support the microbial activity via the provision of oxygen and root exudates, the so called rhizospheric effect, but are not extensively involved in hydrocarbon degradation themselves. It has been argued that various plant species release different nutrients that recruit and stimulate specific microbial communities to establish a synergistic relationship with the plant [25,47,50]. In exploratory investigation at the laboratory scale, it was shown that the treatment efficiency of FTWs can be improved through bioaugmentation with certain bacteria [34,51]. Here, we quantified the added removal efficiency of bioaugmentation with a four-membered bacterial consortium at pilot-scale for the first time. The approximate volumes of root zone and free water were at a similar ratio to fully established full-scale systems [9].

FTWs with *P. australis* or *T. domingensis* but without bioaugmentation achieved already a sizable removal of the hydrocarbon fraction (83%) as well as COD (88.7%) and BOD (84.5%) from the water. The plants were selected based on their comparably high ability to withstand harsh environmental conditions, including the presence of oil constituents at concentration typically found in oil-processing water evaporation pits [36,52]. The survival and growth of plants in the presence of the contaminants is obviously highly important as it affects the efficiency of floating and substratum-based treatment wetland. Here, plant growth parameters were about 40% lower in the oil-contaminated water as compared to the plant grown in tap water, showing that the toxicity level of crude oil contaminated water can be substantial [53,54].

The bacterial strains selected for bioaugmentation were rather suitable for hydrocarbon removal in oil-contaminated water. Bacterial inoculation alone resulted in 65% removal, which at this level is not often reported [34,40]. In particular the two *Acinetobacter* strains, *A. junii* TYRH47 and strain LCRH81, could proliferate well in the rhizoplane of *P. australis* even after 36 days after their inoculation. Members of this genus are frequently isolated or detected via molecular means in soils, water, and wastewater, including habitat contaminated with petroleum [55]. They are heterotrophs capable of growing on a variety of organic compounds such as aliphatic hydrocarbons and phenols. Furthermore, they are rather stress-tolerant, as exemplified by their survival of disinfectant exposure.

When FTWs and bacteria were deployed together, the system's performance improved remarkably with removal efficiencies for hydrocarbons of 95% and 85% with *P. australis* or *T. domingensis*, respectively. The toxicity of the water was reduced significantly, as shown by much better plant growth in the systems as well as survival of fish in the treated water. These observations are in line with previous studies on the positive effect of the combined application of plants and bacterial inoculation on water treatment [4,21,34,51,56,57]. This effect was likely a result of the removal of toxic compounds via specific catabolic activities of the inoculated bacterial strains [29,58,59], and the improvement of plant fitness by the bacteria due to multiple plant growth promoting activities such as production of siderophores, IAA, and ACC deaminase [46]. The four bacterial strains used for bioaugmentation exhibited successful colonization of the rhizoplane and plant tissues (Figure 3). This was likely due to the fact that these bacteria were originally isolated from the plants growing in crude oil-contaminated soil, and already possessed the mechanisms and catabolic genes for

growth and proliferation in the presence of hydrocarbons [40]. The increase of *alkB* gene abundance and expression in plant-associated samples of time strongly suggested that the added bacteria were indeed involved in hydrocarbon degradation (Table 2). Higher bacterial colonization on the roots of *P. australis* compared to *T. domingensis* might be due to its larger root surface area and extended network below the water surface that allow successful proliferation of the microbial communities [60–62].

5. Conclusions

Treatment of oil-contaminated water in FTW pilot-scale systems planted with *P. australis* or *T. domingensis* was substantially improved through bioaugmentation with a four-membered bacterial consortium, consisting of *Klebsiella* sp. strain LCRI87, *B. subtilis* LORI66 *Acinetobacter* sp. LCRH8, and *A. junii* TYRH47. The bacteria proliferated also in plant tissue. Overall, FTWs planted with *P. australis* performed better than those with *T. domingensi.* Although the observational period was relatively short, we think the results are of relevance for full-scale systems. It is reasonable to assume that bioaugmentation of full-scale FTW systems can be highly effective with only small financial demands, rendering this enhanced nature-based solution an attractive treatment option in particular to LMIC.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/w13202882/s1, Table S1: Characteristics of the crude oil contaminated water collected from a pit resulting from oil and gas exploitation in Chakwal, Pakistan.

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