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Large-scale remediation of oil-contaminated water using floating treatment wetlands

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The contamination of water with hydrocarbons resulting from oil exploration and production highlights the need for efficient and environmentally friendly technology to mitigate this form of water pollution. Floating treatment wetlands are a sustainable approach for remediating contaminated water. In this large-scale study, we used four different plants, *Phragmites australis*, *Typha domingensis*, *Leptochloa fusca*, and *Brachiaria mutica*, to vegetate a floating mat with an area of 3058 m² made from locally sourced materials. The floating treatment wetlands constructed in this manner were used to treat an oil-contaminated water stabilization pit resulting from oil and gas exploration activities in District Chakwal, Pakistan. The plants and the water in the pit were inoculated with a consortium of 10 different hydrocarbon-degrading bacteria. The application of floating treatment wetlands to the pit reduced chemical oxygen demand, biochemical oxygen demand, total dissolved solids, hydrocarbon content, and heavy metals by 97.4%, 98.9%, 82.4%, 99.1%, and 80%, respectively, within 18 months. All plants survived and showed growth, but maximum development and biomass production were exhibited by *P. australis*. Moreover, the bacteria used for inoculation were able to persist and show degradation activity in the water as well as in the rhizoplane, roots, and shoots of the plants. We conclude that floating treatment wetlands can be applied to oil-contaminated water stabilization pits for affordable and effective water treatment.

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INTRODUCTION

Crude oil is the world's largest non-renewable energy resource, accounting for about 33% of the total consumed energy. Drilling and extraction processes for oil and gas generate huge volumes of oil-contaminated water,^{1,2} with the global generation of oil-contaminated water estimated to be 33.6 million barrels per day.^{3,4} The worldwide demand for oil is expected to keep rising in the coming years, which will potentially increase the generation of oil-contaminated water.

Crude oil consists of various proportions of different hydrocarbons, such as alkanes, aromatics, and polycyclic aromatic hydrocarbons, as well as non-hydrocarbons, including sulfur, nitrogen, and trace metals, particularly nickel, iron, and copper.^{5,6} Human exposure to hydrocarbons occurs primarily through skin contact and consumption of contaminated food and water.⁷ Some hydrocarbons are reported to be carcinogenic, neurotoxic, and genotoxic to humans and other organisms in the environment.^{8,9} In aquatic organisms, crude oil causes DNA damage, defects in cardiac function, and oxidative stress.^{10–12} This, in turn, reduces the abundance and diversity of fish, consequently disrupting ecosystems.¹³

Worldwide, water contaminated with crude oil is usually stored in evaporation pits before eventual discharge into the environment without any treatment. The remediation of such oil-contaminated water has become a crucial problem in oil-producing countries and requires immediate attention.^{4,14–16} Most drilling and extraction sites are in remote and hilly areas. Moreover, the majority of oil and/or gas wells are not highly productive at the time of their installation or lose their efficiency

after some time. Conventional methods of oil-contaminated water treatment based on physical and chemical processes are not feasible to install in developing countries due to their high capital, operational, and maintenance costs.^{16,17} Therefore, it is difficult—both practically and economically—to develop oil-contaminated water treatment plants for oil and gas companies in these countries.

Using floating treatment wetlands (FTWs) is an innovative approach for the remediation of polluted water, which requires only vegetation attached to buoyant mats.^{18–21} They can be applied to any oil-contaminated water stabilization pit with minimal financial capital. Recent studies have revealed that the combined use of plants and bacteria in FTWs can enhance plant growth and pollutant degradation.^{18,22,23} Plants provide nutrients to rhizospheric microbes through their root exudates. These microorganisms have major roles in the degradation of organic compounds, and their efficiency in degrading hydrocarbons increases in the presence of plants.^{24–28} Once the pollutant is taken up by the plant, endophytes are actively involved in the *in planta* degradation.^{26,27} However, this combination of plants and associated microbes has not yet been evaluated at large scales for remediation of oil-contaminated water.

The aim of this study was to evaluate the remediation efficiency of oil-contaminated water generated as a waste product by an oil and gas company by applying FTWs to its oil-contaminated water (22.326 m³) stabilization pit. Four different plant species were used to develop FTWs, which were then inoculated with bacteria possessing capabilities of hydrocarbon degradation, rhamnolipid production, and plant growth promotion. Plant growth and

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reduction in pollutant content were monitored for 18 months. In addition, bacterial colonization and metabolic activities were monitored in the rhizosphere and endosphere of the plants. To our knowledge, this is the first report on the large-scale treatment of oil-contaminated water in the stabilization pit of an oil and gas company.

Table 1. Characteristics of the crude oil-contaminated water collected from a pit resulting from oil and gas exploitation in Chakwal, Pakistan

Parameter	Value	NEQS
pH	7.49 (0.1)	6–9
Chemical oxygen demand (COD) (mg l ⁻¹)	1316 (73.5)	150
Biochemical oxygen demand (BOD ₅) (mg l ⁻¹)	365 (15.4)	80
Hydrocarbons (mg l ⁻¹)	319 (9.7)	10
Dissolved oxygen (mg l ⁻¹)	0.57 (0.03)	NG
Total dissolved solids (TDS) (mg l ⁻¹)	8050 (113)	3500
Chlorides (Cl ⁻) (mg l ⁻¹)	1330 (73)	1000
Sulfates (SO ₄ ²⁻) (mg l ⁻¹)	432 (9.3)	600
Nitrogen (mg l ⁻¹)	0.8 (0.04)	NG
Phosphorous (P) (mg l ⁻¹)	0.6 (0.02)	NG
Sodium (Na ⁺) (mg l ⁻¹)	2467 (143)	NG
Potassium (K ⁺) (mg l ⁻¹)	1378 (64)	NG
Cadmium (Cd) (mg l ⁻¹)	0.98 (0.06)	0.1
Chromium (Cr) (mg l ⁻¹)	0.86 (0.03)	1.0
Copper (Cu) (mg l ⁻¹)	0.20 (0.01)	1.0
Iron (Fe) (mg l ⁻¹)	1.52 (0.08)	8
Nickel (Ni) (mg l ⁻¹)	0.53 (0.03)	1.0
Lead (Pb) (mg l ⁻¹)	0.62 (0.02)	0.5

Each value is a mean of three replicates; standard deviations are presented within parentheses
NG not given in NEQS list, NEQS National environmental quality standards

RESULTS AND DISCUSSION

Characteristics of oil-contaminated water

The physicochemical characteristics of the pre-treatment water contaminated with oil revealed values of COD (1316 mg l⁻¹), BOD₅ (365 mg l⁻¹), hydrocarbon content (319 mg l⁻¹), total dissolved solids (TDS) (8050 mg l⁻¹), chlorides (1330 mg l⁻¹), Cd (0.98 mg l⁻¹), and Pb (0.62 mg l⁻¹) that were higher than the oil-contaminated water discharge standards of Pakistan.²⁹ However, very low concentrations of total nitrogen (0.8 mg l⁻¹) and phosphorus (0.6 mg l⁻¹) were observed (Table 1).

Remediation of oil-contaminated water

The analyses of organic and inorganic contaminants throughout the experimental period of 18 months provided deep insights into the application of FTWs vegetated with the four different plant species (*Typha domingensis*, *Phragmites australis*, *Leptochloa fusca*, and *Brachiaria mutica*) at the large scale (Table 2 and Fig. 1). There was a continual decrease in contamination level over time; however, a rapid decline in pollution load was observed within the first 6 months of treatment, suggesting quick adaptability of the plants to water contaminated by crude oil. Some plant species have a natural ability to use their metabolic processes for detoxifying toxic compounds.^{30–32}

Among the different pollution parameters tested, hydrocarbons were reduced from 321 to 2.8 mg l⁻¹ (99.13%) within 18 months of applying the FTWs (Fig. 1a). In an earlier study, only 62% hydrocarbon removal was observed when applying FTWs vegetated with *Vetiveria zizanioides*.⁶ Our findings suggest that augmenting FTWs with a specific hydrocarbon-degrading bacterial consortium improves the traditional phytoremediation system by enhancing the capability of both plants and microbes to degrade recalcitrant compounds. This plant–bacterial synergism for hydrocarbon degradation in water has been reported in earlier studies.^{6,26,27,33} In our findings, a major reduction in hydrocarbon content was already observed in the first 6 months of treatment with FTWs. This might have resulted from the presence at the start of treatment of easily degradable hydrocarbons in the oil-

Table 2. Remediation of the crude oil-contaminated water by floating treatment wetlands

Month	Mar'16	May'16	Jul'16	Sep'16	Nov'16	Jan'17	Mar'17	May'17	Jul'17	Sep'17
Parameter										
pH	7.52 ^a (0.14)	7.33 ^a (0.14)	7.14 ^a (0.22)	6.93 ^a (0.27)	6.87 ^b (0.27)	6.79 ^b (0.33)	6.85 ^b (0.39)	6.99 ^a (0.33)	7.19 ^a (0.33)	7.29 ^a (0.33)
TDS	8061 ^a (453)	6252 ^b (564)	4117 ^c (734)	3652 ^d (568)	2987 ^e (342)	2883 ^e (367)	2467 ^f (235)	2193 ^f (238)	1656 ^g (198)	1417 ^h (203)
DO	0.58 ^d (0.05)	2.67 ^c (0.06)	4.88 ^b (0.09)	6.98 ^a (0.08)	6.25 (0.15)	6.81 ^a (0.25)	6.73 ^a (0.134)	6.80 ^a (0.25)	6.72 ^a (0.35)	6.75 ^a (0.55)
Na	2456 ^a (198)	1842 ^b (168)	935 ^c (12)	431 ^d (9.4)	327 ^e (9.12)	213 ^f (11.12)	194 ^f (9.13)	136 ^g (8.12)	116 ^h (6.52)	106 ^h (9.43)
K	1355 ^a (109)	1242 ^a (88)	937 ^b (92)	624 ^c (74)	404 ^d (65.7)	279 ^e (26.4)	171 ^f (16.2)	108 ^g (96.8)	61 ^h (1.93)	38 ⁱ (1.19)
Cl	1339 ^a (106)	1135 ^{a,b} (113)	926 ^b (39)	793 ^c (19.8)	638 ^d (14.7)	596 ^e (14.3)	348 ^f (9.55)	212 ^g (11.8)	171 ^h (8.4)	108 ⁱ (7.5)
SO ₄	434 ^a (7.02)	348 ^b (8.9)	298 ^c (5.94)	189 ^d (4.98)	138 ^e (7.52)	117 ^f (7.25)	101 ^g (7.50)	88 ^h (7.15)	78 ^{h,i} (3.86)	64 ⁱ (1.2)
Cd	0.91 ^a (0.07)	0.83 ^b (0.05)	0.71 ^{b,c} (0.03)	0.66 ^c (0.09)	0.58 ^{c,d} (0.02)	0.49 ^d (0.04)	0.37 ^e (0.07)	0.21 ^f (0.05)	0.16 ^f (0.02)	0.11 ^g (0.04)
Cr	0.83 ^a (0.03)	0.61 ^b (0.05)	0.57 ^b (0.02)	0.42 ^c (0.01)	0.35 ^d (0.02)	0.27 ^e (0.05)	0.18 ^f (0.02)	0.12 ^g (0.01)	0.09 ^h (0.004)	0.04 ⁱ (0.002)
Cu	0.21 ^a (0.04)	0.14 ^b (0.02)	0.09 ^c (0.01)	0.07 ^{c,d} (0.01)	0.06 ^d (0.001)	0.05 ^{d,e} (0.001)	0.04 ^e (0.001)	0.02 ^f (0.003)	0.01 ^g (0.003)	0.01 ^g (0.003)
Fe	1.52 ^a (0.05)	0.92 ^b (0.02)	0.81 ^b (0.03)	0.60 ^c (0.06)	0.43 ^d (0.05)	0.13 ^e (0.002)	0.08 ^f (0.005)	0.03 ^g (0.006)	0.02 ^g (0.003)	0.01 ^h (0.004)
Ni	0.53 ^a (0.02)	0.42 ^b (0.04)	0.31 ^c (0.05)	0.26 ^{c,d} (0.07)	0.21 ^d (0.02)	0.18 ^d (0.05)	0.13 ^e (0.02)	0.11 ^e (0.01)	0.09 ^f (0.04)	0.08 ^f (0.02)
Pb	0.62 ^a (0.16)	0.55 ^{a,b} (0.12)	0.48 ^b (0.01)	0.34 ^c (0.02)	0.22 ^d (0.03)	0.13 ^e (0.001)	0.09 ^f (0.003)	0.06 ^g (0.001)	0.05 ^g (0.002)	0.04 ^g (0.001)

All values are in mg l⁻¹ except the pH. Values are means of three samples collected from the pit every 2 h on the day of visit to the pit. Each value is a mean of three replicates, values within parentheses indicate the standard deviation and labels (a)–(i) indicate statistically significant differences between values in the row at a 5% level of significance

TDS total dissolved solids, DO dissolved oxygen, Na⁺ sodium, K⁺ potassium, Cl⁻ chloride, SO₄²⁻ sulfate, N nitrogen, P phosphorus, Cd cadmium, Cr chromium, Cu copper, Fe iron, Ni nickel, Pb lead

Fig. 1 Reduction in hydrocarbons **a**, COD (chemical oxygen demand) **b**, and BOD (biochemical oxygen demand) **c** in the oil-contaminated water in 18 months by the application of floating treatment wetlands. Each value is mean of three replicates and labels **a–f** indicate statistically significant differences between values at a 5% level of significance

contaminated water, which decreased in concentration over time.³³

Other major parameters defining the health of aquatic ecosystems are COD and BOD₅, for which low values of COD and BOD₅ indicate cleaner water. In this study, COD and BOD₅ were reduced by 97.43% and 98.93%, respectively, after 18 months of applying FTWs in the pit (Figs 1b, c). This decomposition of organic matter can be attributed to the synergism established between microbial biofilms in the rhizoplane and the plant roots that help diffuse oxygen into the plant rhizoplane.^{31,34} With respect to other physiochemical parameters, the pH of the water decreased (7.55 to 6.99) over the whole study period (Table 2). Reduction in pH can be attributed to the microbial degradation of organic matter and production of acids; plants also produce acidic root exudates, which can shift the pH of water towards acidity.^{35–37}

Fig. 2 Metal accumulation in the roots **a** and shoots **b** of different plants vegetated in floating mats for the development of floating treatment wetlands. Each value is mean of three replicates and labels **a–f** indicate statistically significant differences between values at a 5% level of significance

TDS decreased (82.42%) steadily with time following treatment by FTWs in the pit (Table 2). The concentrations of Na^+ , K^+ , SO_4^{2-} , and Cl^- were also reduced in the same proportion as the TDS. This might be due to the uptake of these elements by the plants and microbes and/or their adsorption onto the roots of the plants. Plants absorb inorganic elements from the wastewater, transport them to the aerial parts through the xylem, and finally sequester them in their roots and shoots.^{38–40} The process of bacterial-assisted phytoremediation in FTWs is analogous to rhizo/phyto degradation in the soil, where the bacterial consortia in the rhizospheres of plants work synergistically with their host to efficiently reduce the concentration of contaminants.

Heavy metal removal

In this study, the removal efficiencies of the tested heavy metals from the oil-contaminated water were in the following order: Fe (99.9%) > Cu (95.2%) > Cr (95%) > Pb (93.54%) > Cd (88.8%) and Ni (84.9%) (Table 2). A higher abundance of microbes in the environment has been proposed to reduce the toxicity of metals.^{41,42} Consistent with the decrease in metal concentrations found in oil-contaminated water over time, plant roots and shoots were observed to accumulate more metals at each harvest (Fig. 2). This can be attributed to the enhancement in translocation of metals from the roots to the leaves by bacteria in the rhizoplane. Bacteria do this by enhancing the bioavailability of metals in the rhizosphere or by promoting an increase in root length, total root surface area, or number of root hairs for entrapping these pollutants.^{39,43} Previous studies have also revealed that the bioavailability of trace elements increases with higher microbial population and activity.^{44,45} Hence, microbes and plants work best

in combination to reduce heavy metals in wastewater by improving their bioavailability and uptake by plants.⁴⁶

All the plants used in this study have previously been reported to remove trace elements from oil-contaminated water through a mechanism known as phytoextraction.^{18,22,47,48} In this study, metal accumulation in the four species used to vegetate the FTWs was in the order of *P. australis* > *T. domingensis* > *L. fusca* > and *B. mutica*. *Phragmites australis* has been previously reported as an ideal plant to accumulate moderate amounts of metal contaminants while retaining high biomass production.^{33,49,50}

Persistence of inoculation bacteria

In phytoremediation, hydrocarbons are mineralized by plant-associated microbial populations.^{27,36,51} In the present study, all of the inoculation bacteria showed a high level of persistence (colony-forming unit (CFU) and *alkB* gene abundance) and activity (*alkB* gene expression) in the oil-contaminated water, rhizoplane, as well as in the root and shoot interiors (Table 3). This might be due to the fact that the bacteria used for inoculation originated from crude oil-contaminated soil and/or plants growing in such soil; being from such sources means that they already have mechanisms/catabolic genes for growth and proliferation in the presence of hydrocarbons.

Although bacterial augmentation was applied to both the roots of the plants and the oil-contaminated water, the highest numbers of inoculate bacteria were found in the rhizoplane, followed by the root and shoot interiors and then oil-contaminated water. This may have resulted from the larger surface area provided by the roots of the plants that extend below the water surface and are thus able to support microbial proliferation by ensuring the availability of nutrients and habitat for microbes.^{52–54} Among the inoculation strains, the endophytic bacteria *Klebsiella* sp. strain LCRI-87, *Bacillus subtilis* LORI66, and *Acinetobacter* sp. BRSI56 showed higher colonization and activity inside the plant roots and shoots compared with the other inoculation strains. On the other hand, two strains, *Microbacterium oryzae* R4 and *Pseudomonas aeruginosa* R25, showed minimum persistence and activity in different tissues of the plants. Although all these strains were used to inoculate both the roots and the oil-contaminated water, these results support previous findings that, as expected, endophytes have more potential to colonize plant interiors while rhizospheric bacteria preferably reside outside the plant.^{36,55}

The bacterial population and activity decreased in the oil-contaminated water over time. However, in the rhizoplane, roots, and shoots, both abundance and expression were enhanced during the first 6 months of inoculation followed by a decrease. This may be due to the reduction in the content of bioavailable hydrocarbons over time; also, it is possible that the remaining hydrocarbons were not readily biodegradable.³⁶ Among the four plant species used to vegetate the FTWs, *P. australis* hosted the highest number of bacteria with higher abundance and expression of the hydrocarbon-degrading gene (*alkB*) as compared with the other plant species. This can be explained by the release of different nutrients from each species, which stimulates only specific microorganisms to form a synergistic relationship with the plant. Thus, colonization of inoculate bacteria in the rhizoplane and endosphere depends not only on the type and amount of contaminants but also on the plant species.^{36,51,56} Another possible reason may be that *P. australis* exhibited better growth and development than other plants, which means that it was also able to provide more nutrients and space for microbial colonization and activity.

Plant growth

The plants were harvested every 3 months; root and shoot lengths and dry biomass were determined as described earlier.²² All the

plants, *P. australis*, *T. domingensis*, *L. fusca*, and *B. mutica*, exhibited growth above the mat with the development of roots beneath the water, which provided evidence that these grasses acclimatize well to the hydrocarbon-polluted environment. This may be due to the partnership developed between the inoculate microbes and plants. These microbes have several plant growth-promoting traits that help ameliorate hydrocarbon-induced stress and thus increase plant growth and development.^{27,51,57}

Average plant dry biomass above the mat was 1.27, 0.71, 0.67, and 0.56 kg m⁻² for *P. australis*, *T. domingensis*, *B. mutica*, and *L. fusca*, respectively (Fig. 3a). The average root biomass below the mat was 0.38, 0.35, 0.24, and 0.20 kg m⁻² for *P. australis*, *T. domingensis*, *B. mutica*, and *L. fusca*, respectively (Fig. 3b). The average lengths of roots and shoots in the four tested species were 76.22 and 252.94, 64.21 and 228.62, 39.62 and 106.68, and 27.43 and 207.26 cm for *P. australis*, *T. domingensis*, *L. fusca*, and *B. mutica*, respectively (Fig. 3b). These results suggest that all four plant species had different rates of growth and biomass production, among which *P. australis* exhibited significantly higher biomass and overall development. As mentioned before, this can be due to their varying abilities to host microbes, which may ultimately enhance the development of different plant tissues (root and shoot).^{58–60}

Detoxification of oil-contaminated water

Fish survival analysis showed that after 6 and 12 months of applying FTWs in the pit, treated oil-contaminated water was only partially detoxified as indicated by the death of three and two fish, respectively, at those times. However, after 18 months, no fish expired, which indicates the complete detoxification of the oil-contaminated water from the stabilization pit (Table 4). This can be attributed to the removal of salts and hydrocarbons from the oil-contaminated water by the combined action of plants and bacteria.^{18,47} In view of these findings, FTWs are proposed as favorable and advantageous ecotechnology to restore the quality of water reservoirs.

The results obtained from using FTWs used to treat an oil-contaminated water stabilization pit generated by oil and gas exploitation provide evidence that it is a promising approach for the remediation of oil-contaminated water in this industry. *Phragmites australis* showed significantly better growth and development than the other three plant species used in this study. Inoculation bacteria showed not only persistence in the rhizosphere and endosphere but also remained metabolically active in the degradation of hydrocarbons. The successful removal of organic and inorganic pollutants from the oil-contaminated water stabilization pit gives clear evidence of the potential of FTWs to improve the quality of water.

METHODS

Site description

The FTWs were used to treat oil contamination in the water stabilization pit of an oil and gas exploration company located in the district of Chakwal in Pakistan (33°01'30.4"N 73°09'18.3"E). This closed pit (122 m × 61 m) with no inlet or outlet was built in 2014 and has been filled with crude oil-contaminated water since. Depth in the center of the pit is about 3 m from the surface of the oil-contaminated water. Chakwal is located in a semi-arid steppe climate with 519 mm of annual precipitation. The range of average temperature in summer lies between 15 °C and 40 °C, and the winter temperatures are between -4 °C and 25 °C.

Floating mat

The floating mat was made out of a Jumbolon role (cells of polyethylene resins) (<http://jumbolon.com/jumbolon-rolls/>). This material was selected to develop the floating mat due to its durability, flexibility, and high buoyancy. The mat was designed and constructed at NIBGE. Each mat (area of 7.32 m²) had dimensions of 1.83 m × 1.22 m × 0.10 m (length ×

Table 3. Persistence of the inoculate bacteria and *alkB* gene abundance and expression in water, rhizoplane (RP), root interior (RI), and shoot interior (SI) of the four plant species used to vegetate the FTWs

Months	CFU ml ⁻¹ or g ⁻¹ × 10 ⁶				<i>alkB</i> gene abundance (copy number ml ⁻¹ or g ⁻¹ × 10 ⁴)				<i>alkB</i> gene expression (copy number ml ⁻¹ or g ⁻¹ × 10 ⁴)			
	RP	RI	SI	Water	RP	RI	SI	Water	RP	RI	SI	Water
<i>Phragmites australis</i>												
3	69 ^a (0.4)	125 ^b (0.35)	5.15 ^c (0.2)	1.31 ^e (0.7)	15.6 ^a (0.45)	105 ^b (1.2)	5.3 ^b (0.02)	1.7 ^b (0.08)	7.3 ^a (0.04)	10.8 ^b (0.23)	2.4 ^b (0.08)	0.41 ^e (0.02)
6	25 ^b (0.7)	153 ^a (0.41)	28 ^a (0.11)	10.4 ^a (1.6)	8.3 ^b (0.12)	147 ^a (0.92)	15 ^a (0.14)	2.3 ^b (0.06)	4.9 ^b (0.03)	16.5 ^a (0.18)	3.7 ^a (0.06)	0.85 ^d (0.03)
12	13 ^c (0.4)	64 ^c (0.27)	12.3 ^b (0.09)	3.61 ^c (0.5)	0.38 ^c (0.04)	15 ^e (0.71)	1.6 ^e (0.02)	0.80 ^e (0.03)	1.2 ^c (0.02)	2.2 ^f (0.06)	1.0 ^d (0.02)	0.34 ^e (0.01)
18	8.6 ^d (0.08)	6.8 ^d (0.18)	2.5 ^e (0.07)	1.4 ^e (0.05)	0.15 ^d (0.02)	2.8 ^d (0.05)	0.26 ^g (0.04)	0.17 (0.01)	0.24 ^d (0.01)	0.65 ^h (0.01)	0.52 ^e (0.01)	0.08 ^h (0.02)
<i>Typha domingensis</i>												
3	69 ^a (0.4)	27 ^e (0.35)	2.83 ^e (0.20)	0.64 ^h (0.07)	15.6 ^a (0.23)	28 ^e (0.87)	2.5 ^d (0.05)	1.2 ^d (0.04)	7.3 ^a (0.17)	4.3 ^e (0.02)	1.8 ^c (0.06)	1.1 ^b (0.03)
6	25 ^b (0.7)	46 ^d (0.41)	13.7 ^b (0.41)	5.8 ^b (0.06)	8.3 ^b (0.14)	23 ^d (0.28)	3.1 ^c (0.04)	1.4 ^c (0.03)	4.9 ^b (0.21)	6.8 ^c (0.06)	2.1 ^b (0.02)	1.6 ^a (0.02)
12	13 ^c (0.4)	10.2 ^h (0.27)	4.3 ^d (0.09)	0.8 ^g (0.05)	0.38 ^c (0.09)	4.3 ^f (0.15)	1.1 ^f (0.02)	0.65 ^f (0.01)	1.2 ^c (0.06)	0.84 ^g (0.01)	0.28 ^f (0.01)	0.12 ^g (0.01)
18	8.6 ^d (0.08)	3.1 ^f (0.14)	1.6 ^h (0.04)	0.3 ⁱ (0.01)	0.05 ^d (0.01)	1.7 ^k (0.08)	0.15 ^h (0.01)	0.14 ⁱ (0.01)	0.24 ^d (0.02)	0.19 ⁱ (0.01)	0.10 ^h (0.01)	0.08 ^h (0.01)
<i>Leptochloa fusca</i>												
3	69 ^a (0.4)	13 ^g (0.35)	2.4 ^e (0.07)	0.55 ^h (0.07)	15.6 ^a (1.04)	8.45 ^f (0.67)	1.8 ^e (0.06)	0.52 ^{fg} (0.02)	7.3 ^a (0.15)	3.9 ^e (0.13)	1.7 ^c (0.02)	0.95 ^c (0.03)
6	25 ^b (0.7)	18 ^f (0.41)	3.7 ^f (0.11)	2.2 ^d (1.6)	8.3 ^b (0.45)	7.8 ^g (0.43)	2.3 ^d (0.02)	1.5 ^{bc} (0.06)	4.9 ^b (0.17)	5.8 ^d (0.16)	1.6 ^c (0.03)	1.3 ^{ab} (0.02)
12	13 ^c (0.4)	6.1 ^f (0.27)	2.1 ^g (0.09)	1.0 ^f (0.5)	0.38 ^c (0.18)	3.4 ^f (0.18)	1.4 ^e (0.03)	0.64 ^f (0.02)	1.2 ^c (0.02)	0.82 ^g (0.08)	0.22 ^f (0.01)	0.18 ^f (0.01)
18	8.6 ^d (0.08)	2.8 ^k (0.04)	1.7 ^h (0.04)	0.8 ^g (0.03)	0.05 ^d (0.01)	1.2 ⁱ (0.06)	1.1 ^f (0.02)	0.47 ^g (0.01)	0.24 ^d (0.04)	0.17 ⁱ (0.01)	0.14 ^{gh} (0.01)	0.11 ^g (0.01)
<i>Bracharia mutica</i>												
3	69 ^a (0.4)	11 ^g (0.30)	2.1 ^g (0.1)	0.54 ^h (0.05)	15.6 ^a (0.65)	14.3 ^e (0.56)	1.6 ^e (0.03)	0.43 ^g (0.02)	7.3 ^a (0.26)	2.3 ^f (0.06)	1.2 (0.02)	0.84 ^d (0.02)
6	25 ^b (0.7)	16 ^f (0.31)	3.4 ^f (0.11)	2.6 ^d (0.6)	8.3 ^b (0.15)	20 ^d (0.28)	3.9 ^c (0.48)	1.8 ^b (0.07)	4.9 ^b (0.08)	2.6 ^f (0.02)	1.5 ^c (0.03)	1.2 ^b (0.07)
12	13 ^c (0.4)	6.2 ^f (0.27)	2.3 ^g (0.03)	1.1 ^f (0.05)	0.38 ^c (0.04)	5.7 ^h (0.14)	3.4 ^c (0.19)	0.52 ^{fg} (0.02)	1.2 ^c (0.03)	0.64 ^h (0.02)	0.18 ^g (0.01)	0.16 ^f (0.01)
12	8.6 ^d (0.08)	2.1 ⁱ (0.1)	1.5 ^h (0.05)	0.7 ^g (0.03)	0.05 ^d (0.01)	1.4 ^{kl} (0.05)	1.3 ^e (0.08)	0.19 ^h (0.01)	0.24 ^d (0.01)	0.16 ⁱ (0.01)	0.11 ^h (0.01)	0.09 ^h (0.01)

Each value is mean of three replicates, values within parentheses indicate the standard deviation and labels a–l indicate statistically significant differences between values in the column at a 5% level of significance

Fig. 3 Dry biomass **a** and length **b** of root and shoot of different plants vegetated in floating mats for the development of floating treatment wetlands. Each value is mean of three replicates and labels **a–f** indicate statistically significant differences between values at a 5% level of significance

width × height) with a 2.54 cm border on all sides (Fig. 4). The borders were covered with aluminum foil to protect the mat from sunlight. Fourteen holes (12.7 cm diameter on the upper side and 10.16 cm diameter on the lower side) were drilled into each mat in a manner so that there were five holes along both sides of the mat lengthwise and four holes along the midline. Small holes (2.54 cm diameter) were also drilled at each of the four corners and polypropylene random copolymer (PPRC) pipes (length 15.24 cm and diameter 2.54) were inserted into these holes (Fig. 4). These pipes were used to connect the mats with each other with the help of plastic-coated wires. A total of 218 mats were connected together to construct a floating island with a total area of 3058 m² (Figs. 5, 6).

Plants

A nursery containing four plant species, two aquatic (*P. australis* and *T. domingensis*) and two terrestrial (*L. fusca* and *B. mutica*), was grown using plastic pots (length 10.16 cm, diameter 12.7 cm, and a hole of diameter 7.62 cm at the bottom) in the botanical garden at National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan. The seedlings of *P. australis* and *T. domingensis* had been obtained from wastewater drains and pits in the surroundings of Faisalabad. The cuttings of *L. fusca* and *B. mutica* had been obtained from the Nuclear Institute for Agriculture and Biology, Faisalabad.

Table 4. Detoxification of oil-contaminated water by bacterial-assisted floating treatment wetlands

Time (months)	Fish deaths over time				Total deaths	Detoxification status
	24 h	48 h	72 h	96 h		
0	10	0	0	0	10	No
6	0	0	2	1	3	Partially
12	0	0	1	1	2	Partially
18	0	0	0	0	0	Yes

Fig. 4 Design of a floating mat for constructing a floating treatment wetland (FTW) for application in an oil-contaminated water pit generated by oil and gas exploitation

Fig. 5 a–f Application of floating treatment wetlands in the oil-contaminated water stabilization pit of an oil and gas company. Oil-contaminated water stabilization pit **a**, vegetation of mats with plants and their inoculation with bacteria **b**, **c**, fixing of mats to each other **d**, adding the bacteria to the pit **e**, and the growth of the plants at the initial stage **f**

Bacterial strains

Ten bacterial strains that had previously been isolated and characterized by Fatima et al.⁶¹ and Tahseen et al.⁶² were used in this study. These strains were *Ochrobactrum intermedium* R2, *Microbacterium oryzae* R4, *Pseudomonas aeruginosa* R25, *P. aeruginosa* R21 (isolated from crude oil-contaminated soil), *Acinetobacter* sp. LCRH81, *Klebsiella* sp. LCRI-87 (isolated from the rhizosphere and root interior of *Lecucaena leucocephala*, respectively), *Acinetobacter* sp. BRISI56, *P. aeruginosa* BRRI54 (isolated from the shoot and root interior of *Brachiaria mutica*, respectively), *Bacillus subtilis* LORI66 (isolated from the root interior of *Lolium perenne*), and *Acinetobacter junii* TYRH47 (isolated from the rhizosphere of *T. domingensis*). These strains are able to degrade hydrocarbons, produce biosurfactants, and promote plant growth (Table 5). Each strain was grown separately in LB broth (containing 1% (w v⁻¹) diesel) overnight. Cells were recovered by centrifugation and suspended in normal saline solution (0.9% NaCl). An inoculum of each strain was prepared containing an equal number of cells by a turbidimetric method.^{62,63}

Application of FTWs in the pit

A single pot with seedlings was put in each of the 14 holes for each mat. The mats were then covered with a 2.54 cm layer of soil (70%), sand (20%), and medium-sized gravel (10%) to protect them from sunlight. A total of 418 mats were prepared: 200 mats were vegetated with *P. australis*, 118 with *T. domingensis*, and 50 each with *L. fusca* and *B. mutica*. As noted earlier, these mats were connected to one another using wires to make a single island. The total area thus covered by the floating island was 3058 m², which was equivalent to 25% of the surface area of the pit (Figs. 5, 6).

Fifty kilograms of diammonium phosphate and urea fertilizer were added to the oil-contaminated water in the pit every 3 months for 18 months. Moreover, 10 L of the inoculum for each bacterial strain (10⁹ CFU m⁻¹) was added to the oil-contaminated water and the roots of the plants during this period.

Sampling and analysis of oil-contaminated water

Samples of oil-contaminated water were collected from the pit every 2 months from March 2016 to September 2017. These samples were preserved in glass bottles at 4°C in an ice box, transported to the laboratory, and analyzed for various physicochemical parameters. The pH and electrical conductivity (EC) were measured using bench top equipment (XL 30, Fisher Scientific Pte Ltd. UE TechPark, Singapore). Chemical oxygen demand (COD), biochemical oxygen demand (BOD₅), TDS, sulfates (SO₄²⁻), and chlorides (Cl⁻) were measured as described in an earlier study.⁶⁴ Hydrocarbon content was measured using the Spectrum Two Environmental Hydrocarbons Analysis System (Perkin Elmer, USA), following extraction with hexane as the solvent.⁶⁵ Total nitrogen (N) and phosphorus (P) were estimated using a Millipore Cell Test Kit (Merck & Co). Sodium (Na) and potassium (K) were measured using a flame photometer (FP 20, SEAC, Italy). Heavy metals, including Cd, Cr, Cu, Fe, Ni, and Pb, were determined quantitatively using atomic absorption spectroscopy (Varian SpectrAA.200, Varian Australia).

Enumeration of inoculate bacteria

The persistence and survival of the bacterial strains used to inoculate the FTWs were assessed for the rhizoplane, the root and shoot interiors, and

Fig. 6 **a–f** Growth of different plants used to vegetate the floating mats for remediating the oil-contaminated water stabilization pit. Growth of different plants **a**, shoot growth of *L. fusca* **b**, shoot growth of *P. australis* **c**, roots of *P. australis* **d**, floating treatment wetlands (3058 m²) **e**, and fully grown *P. australis* **f**

Table 5. Bacterial strains used to inoculate vegetation in the floating treatment wetlands

Bacteria	Source	Activity	Gene	Reference
<i>Ochrobactrum intermedium</i> R2	COC soil	HC degradation	<i>alkB</i>	62
<i>Microbacterium oryzae</i> R4	COC soil	HC degradation	<i>alkB</i>	62
<i>Pseudomonas aeruginosa</i> R25	CO soil	BS production	<i>rhIA, rhIB, rhIC</i>	62
<i>Pseudomonas aeruginosa</i> R21	CO soil	BS production	<i>rhIA, rhIB, rhIC</i>	62
<i>Acinetobacter</i> sp. LCRH81	RH of <i>Lecucaena leucocephala</i>	HC degradation, IAA production	<i>alkB</i>	61
<i>Klebsiella</i> sp. LCRI-87	RI of <i>L. leucocephala</i>	HC degradation, phosphorous solubilization, IAA and siderophores production, ACC deaminase	<i>alkB</i>	61
<i>Acinetobacter</i> sp. BRSI56	SI of <i>Brachiaria mutica</i>	HC degradation, siderophores production	<i>alkB</i>	61
<i>Pseudomonas aeruginosa</i> BRR154	RI of <i>B. mutica</i>	HC degradation, phosphorous solubilization, IAA and siderophores production	<i>alkB</i>	61
<i>Bacillus subtilis</i> LORI66	RI of <i>Lolium perenne</i>	HC degradation, siderophores production	<i>alkB</i>	61
<i>Acinetobacter junii</i> TYRH47	RH of <i>Typha domingensis</i>	HC degradation, siderophores production	<i>alkB</i>	Unpublished data

COC soil crude oil-contaminated soil, RI root interior, SI = shoot interior, RH rhizosphere, HC hydrocarbons, BS biosurfactants, IAA indole acetic acid, ACC 1-amino-cyclopropane-1-carboxylic acid

the oil-contaminated water of the pit by the plate count method as described in an earlier study.^{18,66} Bacteria from the rhizoplane were isolated by agitating roots in a normal saline solution containing 0.1-cm (diameter) glass beads. The surfaces of roots and shoots were sterilized and homogenized in normal saline solution to isolate endophytes. Appropriate dilutions of the bacterial suspensions were plated on minimal media plates amended with 1% diesel. Similarly, different dilutions of oil-contaminated water samples were also plated on minimal medium containing 1% diesel. These plates were incubated at 37 °C. The inoculate strains were identified among the isolates by subjecting randomly selected colonies to restriction fragment length polymorphism analysis.⁶⁷

Determination of *alkB* gene abundance and expression

The abundance and expression of the *alkB* gene were estimated in the rhizoplane, root and shoot interiors, and oil-contaminated water using real-time PCR as described in previously published studies.^{27,68} Briefly, DNA and RNA were isolated from roots, shoots, and oil-contaminated water using commercially available kits. RNA was then converted to complementary DNA (cDNA) with the help of reverse transcriptase. Following their synthesis, DNA and cDNA were used as templates for the estimation of *alkB* gene abundance and expression, respectively.

Plant growth analysis

Plants were harvested every 3 months. Roots were washed carefully with tap water to remove attached sludge. Plant tissue was then separated into shoots and roots, and their lengths were determined. The shoot and root samples were oven-dried at 70 °C for 72 h, and dry biomass was determined.^{69,70}

Evaluation of toxicity of treated water

A sample of the treated oil-contaminated water was collected from the pit every 6 months for 18 months following the installation of the FTWs. All the collected samples were subject to toxicity analysis as described earlier.^{18,71} Briefly, ten healthy fish, *Labeo rohita*, of uniform weight and size were exposed to each treated sample in triplicate, and their survival rates were observed by counting the number of fish that expired every 24 h over 4 consecutive days.

Statistical analysis

Data (pollutants removal, plant biomass, and bacterial persistence) were analyzed with the software package SPSS (SPSS Inc., Chicago, IL, USA), and the standard deviation from mean values was calculated using Duncan's multiple range test.

DATA AVAILABILITY

All data reported in this study is available in this paper.

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AUTHOR CONTRIBUTIONS

M.A., K.R., and G.S. designed and conducted the experiments. K.R., G.S., R.T., and A.J.H. contributed in wastewater analysis for different physicochemical parameters and also in determination of bacterial population, gene abundance and gene expression in water, and in rhizosphere and endosphere of the plants. A.I. and H.B. were involved in data analysis and manuscript preparation.

ADDITIONAL INFORMATION

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