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Research article

Remediation of saline soils contaminated with crude oil using the halophyte *Salicornia persica* in conjunction with hydrocarbon-degrading bacteria

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ABSTRACT

The negative impact of salinity on plant growth and the survival of rhizosphere biota complicates the application of bioremediation to crude oil-contaminated saline soils. Here, a comparison was made between the remedial effect of treating the soil with *Pseudomonas aeruginosa*, a salinity tolerant hydrocarbon-degrading consortium in conjunction with either the halophyte *Salicornia persica* or the non-halophyte *Festuca arundinacea*. The effect of the various treatments on salinized soils was measured by assessing the extent of total petroleum hydrocarbon (TPH) degradation, the soil's dehydrogenase activity, the abundance of the bacteria and the level of phytotoxicity as measured by a bioassay. When a non-salinized soil was assessed after a treatment period of 120 days, the ranking for effectiveness with respect to TPH removal was *F. arundinacea* > *P. aeruginosa* > *S. persica* > no treatment control, while in the presence of salinity, the ranking changed to *S. persica* > *P. aeruginosa* > *F. arundinacea* > no treatment control. Combining the planting of *S. persica* or *F. arundinacea* with *P. aeruginosa* inoculation ("bio-augmentation") boosted the degradation of TPH up to 5–17%. Analyses of the residual oil contamination revealed that long chain alkanes (above C20) were particularly strongly degraded following the bio-augmentation treatments. The induced increase in dehydrogenase activity and the abundance of the bacteria (3.5 and 10 fold respectively) achieved in the bioaugmentation/*S. persica* treatment resulted in 46–76% reduction in soil phytotoxicity in a saline soil. The indication was that bioaugmentation of halophyte can help to mitigate the adverse effects on the effectiveness of bioremediation in a crude oil-contaminated saline soil.

1. Introduction

One of the negative consequences of the large-scale consumption of petroleum products driven by economic growth and the rise in the world's population is the pollution of soils and water resulting from the accidental spillage of crude oil or its derivatives (Pokethitiyook, 2017). Sites, which have been contaminated in this way, are in urgent need of effective remediation. Certain plants and/or micro-organisms have been shown to be able sequester and/or neutralize petroleum-based hydrocarbons in a low cost, environmentally friendly, sustainable way (Cai et al., 2016; Leewis et al., 2013; Panchenko et al., 2017). According to Agnello et al. (2016) and Nanekar et al. (2015), the effectiveness of plants as a remediant can be enhanced by supplementation with specific plant-associated microbes. To date, the focus of this sort of research has been centered on the remediation of non-saline soils, but the soil in many of the regions where crude oil is extracted suffers from a high level of salinity (Gao et al., 2015).

The growth of most plant species is strongly suppressed by salinity, as is the activity of many soil microorganisms, with the result that the effectiveness of bioremediation can be compromised (Cai et al., 2016; Hua et al., 2010). In such environments, the agents chosen for bioremediation must be able to tolerate soil salinity. The plant kingdom includes a group of highly salinity tolerant species, referred to as halophytes. Earlier, several halophyte species have

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been used for desalinization of saline/saline-sodic soils (Hasanuzzaman et al., 2014; Rabhi et al., 2009; Ravindran et al., 2007), as well as removal of heavy metals from saline soils (Liang et al., 2017; Manousaki and Kalogerakis, 2011). Some of these, namely *Scirpus triqueter* (Zhang et al., 2014), *Halimione portulacoides* (Couto et al., 2011), *Suaeda salsa* (Gao et al., 2014), *Juncus maritimus* and *Phragmites australis* (Ribeiro et al., 2014), have been shown to be capable of sequestering petroleum-based hydrocarbons present in a saline soil. Halophytes vary markedly with respect to their root morphology and the nature of their root exudates, thereby influencing the identity of the rhizosphere microbial community which they support, and consequently the efficiency with which petroleum hydrocarbons can be degraded in the rhizosphere (Ribeiro et al., 2014). According to Oliveira et al. (2015), supporting plant remediants with hydrocarbon-degrading bacteria can enhance the removal of crude oil from a contaminated soil. In recent studies, the use of some microorganisms such as mycorrhiza fungi (Gao et al., 2014), growth promoting bacteria (Xun et al., 2015) or microbial suspension (Ribeiro et al., 2014) in combination with halophyte plants have been investigated to improve the remediation of hydrocarbon compounds from saline soils. However, the presence of salt-tolerant oil-degrading bacteria in the halophyte rhizosphere seems can be more effective in improving the remediation efficiency of such soils.

Salicornia halophyte grows naturally in different regions of Iran, which the ability of this halophyte to absorb or degrade hydrocarbon compounds from saline sediments or soils have been investigated in previous studies (Ghazisaeedi et al., 2014; Meudec et al., 2006). Most of the oil-field and contaminated regions of Iran are located in arid and semi-arid areas, which are mainly affected by high salinity (Ebadi et al., 2017b; Soleimani et al., 2013, 2010). These conditions may further increase the challenge of bioremediation attempts. Therefore, studying the potential of halophyte plants and oil-degrading bacterial consortium simultaneously can be useful to improve the remediation efficiency of contaminated saline soils and increase our understanding of the conditions of such soils. Here, the effectiveness of a variety of remediation treatments were tested on such a soil: the remediants involved were the bacterium *Pseudomonas aeruginosa*, the halophyte *Salicornia persica* and the established non-halophytic hydrocarbon sequestrator tall fescue (*festuca arundinacea*).

2. Material and methods

2.1. Sample soil

Soil was collected on the spot from the top layer (0–30 cm) of a non-contaminated, non-saline site in Karaj (35° 45′ 16″ N, 50° 57′ 56″E). After air drying, the soil was passed through a 2 mm sieve, and a number of standard soil characteristics were measured (pH, electrical conductivity, cation exchange capacity and organic carbon content) (Table 1). The soil was then mixed with a combination of either 10 or 30 g/kg light crude oil and 0, 150 or 300 mM NaCl. Levels chosen to reflect the range of contamination documented across Iran (Ebadi et al., 2017b; Soleimani et al., 2013, 2010).

Table 1
Physical and chemical characteristics of the experimental soil.

Soil Property	Value
Sand	53%
Silt	25%
Clay	22%
pH	7.33
EC (Electrical Conductivity)	1.48 dS m^{-1}
CEC (Cation Exchangeable Capacity)	14.3 meq 100g^{-1} dry soil
Organic Carbon	0.55%
Organic Matter	0.95%
Sodium	71 mg kg^{-1} dry soil
Potassium	204 mg kg^{-1} dry soil

2.2. P. aeruginosa consortium

The *P. aeruginosa* consortium was a mixture of four strains, previously isolated from two aged oil-contaminated saline soils with 8–12 dS/m electrical conductivity, which located in Tehran and Isfahan oil refinery. An evaluation was conducted to measure the capacity of each strain to produce biosurfactant and degrade crude oil in a saline medium (Table 2). Details on isolation and identifying of isolates essentially described in our previous study (Ebadi et al., 2017b). The strains were then cultured individually in aerobic tryptic soy broth at 30 °C for 24 h, the cells were harvested by centrifugation (10,000 g, 10 min) and then resuspended in sterile 0.9% NaCl. The concentration of the subsequent suspensions was obtained by turbidimetry measured at 630 nm. The consortium represented a 1:1:1:1 ratio mixture of the four isolates (Tahseen et al., 2016).

2.3. Plant materials

Seeds of salicornia (*S. persica*) and tall fescue (*F. arundinacea*) were obtained from natural stands in Iran. As their germination, which preliminary examined in both salinized (0, 150 & 300 mM NaCl) and contaminated (0, 10 & 300 g/kg) soil, proved poor, established plants were used as phytoremediant agents. For this purpose, seeds were germinated on water-agar, and the resulting seedlings grown hydroponically under stress-free conditions for 20 days before being transplanted into the treated soil.

2.4. Biologically based remediation of contaminated soil

A 120 day pot experiment was conducted in a greenhouse in which the air temperature varied from 25 to 30 °C. Each pot was filled with 3 kg of sieved (4 mm), salinized (0, 150 & 300 mM NaCl) and light (33.6° API) crude oil-contaminated (10 & 30 g/kg) soil. Soil moisture was maintained at about 70% of the soil's water holding capacity. The experiment was fully randomized, and each treatment was triplicated. The six treatments (T1 through T6) were as follows: T1 - no additives; T2 - 10^6 CFU/g soil of *P. aeruginosa* along with adjusting C:N:P ratio; T3, T4 - three seedlings of, respectively, salicornia and tall fescue; T5, T6 – a combination of respectively, T3+T2 and T4+T2 (Fig. 1). The soils were sampled from each pot after 30, 60 and 120 days, and the samples stored at 4 °C prior to their analysis. At the end of the experiment, the plants were harvested, rinsed in distilled water and oven-dried (65 °C) for 48 h, after which the weight of shoot and root material was determined.

2.5. Quantification of hydrocarbon-degrading bacteria

The abundance of hydrocarbon-degrading bacteria in the soil was estimated using the “most probable number” (MPN) protocol, based on bacterial growth in MSM medium in the presence of various amounts of crude oil. Tenfold serial dilutions were made from a suspension of 1 g of soil in 10 mL MSM, and each well in a 96 well microtiter plate was inoculated with 10^{-4}–10^{-8} serial dilutions. A 5 μL volume of filtered 50 mg/L resazurin was added to each well, the plate was sealed with Parafilm and then held at 30 °C for one week. Wells in which the medium had changed color from blue to pink were deemed to be positive and the MPN of hydrocarbon-degrading microbes per g of soil was calculated following Guerin et al. (2001).

Table 2
Characterization of the individual components of the *P. aeruginosa* consortium. Values expressed in the form of mean/median±S.E/range ($n = 3$).

Isolates	Oil spreading (mm)	Emulsification index (%)	Glycolipid production (g l-1)	Oil degradation (%)	16S rDNA identification
T4	3 ± 0.28	22.2 ± 2.1	2.08 ± 0.09	39.2 ± 2.8	*P. aeruginosa* (MF 289,987)
T27	2.85 ± 0.15	33.5 ± 5.5	3.72 ± 0.11	33.3 ± 1.2	*P. aeruginosa* (MF 289,986)
T30	2.4 ± 0.51	38 ± 2.9	2.12 ± 0.28	38.4 ± 1.5	*P. aeruginosa* (MF 289,985)
E1	1.85 ± 0.2	24.5 ± 3	2.2 ± 0.28	33 ± 3.9	*P. aeruginosa* (MF 289,988)

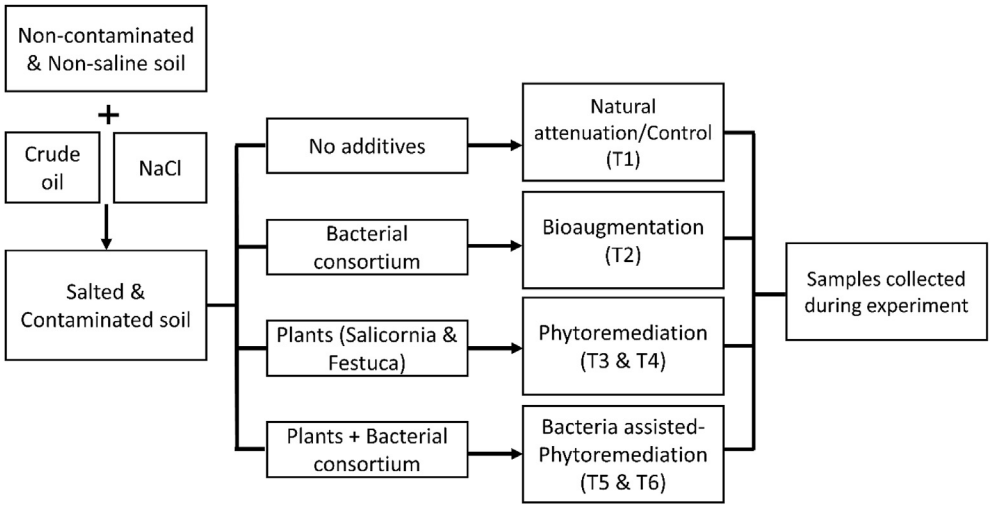

Fig. 1. The experimental design. Non-contaminated, non-saline soil was dosed with varying amounts of crude oil and/or NaCl. Each treatment was triplicated. T1 - no additives; T2 - 10^6 CFU/g soil of *P. aeruginosa*; T3, T4 - three seedlings of, respectively, salicornia and tall fescue; T5, T6 – a combination of respectively, T3+T2 and T4+T2.

2.6. Dehydrogenase activity (DHA)

DHA was measured using the triphenyl tetrazolium chloride reduction method (Tang et al., 2010). Briefly, 2 g soil samples were mixed with 2 mL 4% (w/v) triphenyl tetrazolium chloride and incubated at 30 °C for 24 h in the dark. The resulting triphenyl formazan generated was acetone-extracted and quantified colorimetrically (absorbance wavelength 485 nm). DHA was expressed in the form mg triphenyl formazan formed per g soil per h.

2.7. Determination of total petroleum hydrocarbon (TPH) content of the soil

The TPH content of each soil sample was determined after its ultrasonic extraction in a 1:1 (v/v) mixture of hexane and acetone (extraction method EPA 3550b). A 2 g sample of soil was mixed with 1 g anhydrous Na_2SO_4 and then extracted at 20 °C in 15 mL of the solvent with the aid of an ultrasonic device delivering 250 W (Branson M8800, www.bransonic.com). The resulting suspension was centrifuged (10,000 g, 5 min) and the supernatant set aside. The pellet was re-extracted in the same solvent and re-centrifuged, and the resulting supernatant combined with the initial one. The solvent was evaporated using a Vacufuge plus concentrator (Eppendorf, Hamburg, Germany), and the residual amount of TPH determined gravimetrically (Li et al., 2012). The residual TPH was dissolved in 30 mL n-hexane to separate the insoluble asphaltene fraction from the soluble fraction. The soluble fraction was loaded onto a glass column (15 × 200 mm) filled with activated silica gel of mesh size 70. The aliphatic fractions present were separated using 60 mL n-hexane as the eluting solvent (Peng et al., 2009) and the separated aliphatic hydrocarbons were then subjected to gas chromatography–mass spectroscopy (GC-MS) analysis using a Varian 4000 device (Agilent Technologies, Santa Clara, CA, USA) coupled with a VF-5ms capillary column (30 m, 0.25 mm, 0.25 μm). The initial column temperature of 80 °C was held for 1 min, and was then increased to 120 °C at the rate of 15 °C min^{-1} and held for 1 min, followed by an increase of 7 °C min^{-1} to 290 °C, where it was held for 5 min. The injector temperature was set at 310 °C and the injector was operated in the splitless mode. The detector temperature was programmed at 300 °C. MS data were acquired in electron ionization mode (70 eV). The individual components in the alkane and aromatic fractions were determined by matching their retention time with that of authentic standards and/or by reference to a MS database.

2.8. Phytotoxicity assay

The soils' phytotoxicity was evaluated using a lettuce seed germination/root elongation test. Lots of 20 seeds were sown in 50 g air-dried soil, which was then brought to 75% water holding capacity. After holding in the dark at 25 °C for 120 h, the number of germinated seeds was counted and the seedling root lengths were measured. A root elongation inhibition index was then calculated following Hamdi et al. (2012).

2.9. Statistical analysis

The experiments were all run in triplicate and the data subjected to a standard analysis of variance. Means were compared using the Duncan's multiple range test ($P < 0.05$). Statistical calculations were carried out using routines implemented in SPSS v17.0 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. The effect of petroleum contamination and salinity on the germination of salicornia and tall fescue seed

The ability of the two test species to germinate successfully in the variously treated soils is summarized in Fig. 2. Under non-saline conditions, the germination of salicornia was increasingly inhibited as the crude oil load was raised, while that of tall fescue was unaffected. In contrast, in the absence of crude oil, the germination of tall fescue but not that of salicornia was compromised as the level of soil salinity was raised from 0 to 150 mM NaCl. For both species, the ability of the seed to germinate was greatly reduced at the highest level of salinity tested: in the presence of 30 g/kg crude oil, salicornia completely failed to germinate, and the same was the case for tall fescue in the presence of both 10 g/kg and 30 g/kg crude oil.

3.2. Plant biomass

The materials transplanted into salinized soil all survived throughout the 120 day experimental period: their accumulated shoot and root dry weights are summarized in Fig. 3. In the soils not inoculated with *P. aeruginosa* ("non-bioaugmented" soils), the aerial biomass of salicornia was boosted by the presence of salinity and was not influenced by the presence of crude oil, while its accumulation of root biomass was unaffected by the treatment. In contrast, the dry weight of the shoot material produced by tall fescue was significantly compromised by salinity increasing at 1% oil concentration level; the addition of crude oil to the soil severely reduced shoot growth. Root growth was similarly retarded by both salinity and crude oil. The bioaugmented soils supported a greater growth of salicornia plants (T5): the most favorable treatment with respect to the accumulation of shoot dry matter was 150 mM NaCl plus 10 g/kg crude oil. In the presence of 30 g/kg crude oil, inoculation with *P. aeruginosa* improved performance with respect to biomass accumulation, but not significantly so. The bioaugmentation treatment had no positive effect on the biomass accumulation of tall fescue (T6) and similar tendency was observed with non-bioaugmented soil (T4).

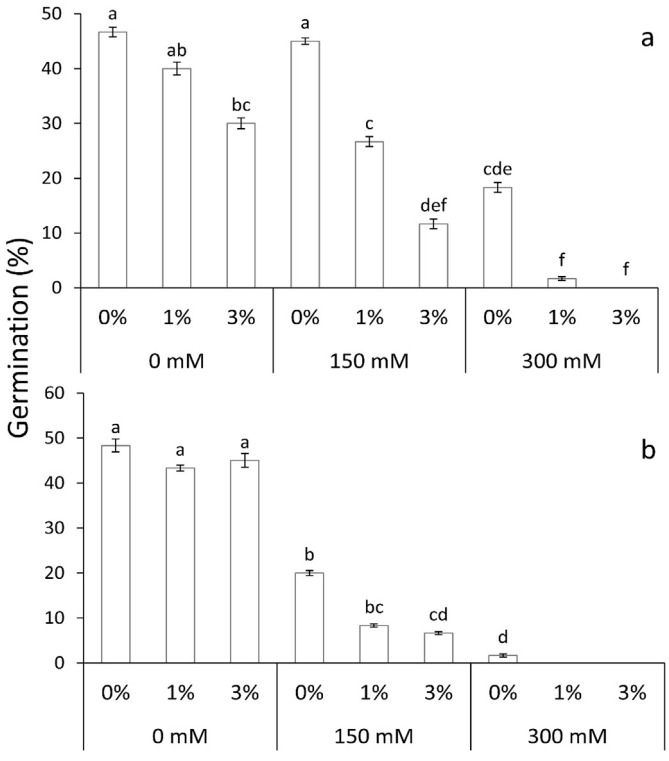

Fig. 2. Germination of (a) salicornia and (b) tall fescue seed in soils containing a variable concentration of NaCl (0, 150 and 300 mM NaCl) and crude oil (0, 10 or 30 g/kg). Values expressed in the form of mean ± S.E. ($n = 3$). Columns marked by the same lower case letter indicate means not differing significantly from one another according to Duncan's multiple range test ($P = 0.05$).

3.3. The abundance of P. aeruginosa in the treated soils

The MPNs measured in the soil subjected to the various treatments are shown in Fig. 4. The addition of crude oil to the soil stimulated the size of the bacterial population, while salinity had a suppressive effect, except where the treatment included salicornia plants. The bacterial population size in T2 soil was significantly higher than in T1 soil (no treatment control), irrespective of the level of salinity or the concentration of crude oil present. MPN was significantly higher in the soils subjected to treatments T3 and T4 than in T1-treated soil when sampled at 60 days, but was lower than in the soil inoculated with *P. aeruginosa* (T2). The largest population of hydrocarbon-degrading bacteria in both salinized and non-salinized soils was developed in the two bioaugmentation treatments T5 and T6, where their abundance was enhanced by some tenfold.

3.4. DHA

The measured DHAs in the soils exposed to the various treatments are represented in Fig. 5. Across all of the treatments, DHA increased gradually over the first 60 days subsequently declining. It was markedly higher in soils loaded with 30 g/kg crude oil than in those containing 10 g/kg; the effect of increasing the soil's salinity was to depress DHA, unless salicornia plants were present. Compared to T1 soil, DHA was higher in each of the treatments at all levels of salinity and crude oil loading. In the non-bioaugmented soils, the highest DHA values, under both saline and non-saline conditions, obtained where either salicornia (T3) or tall fescue (T4) plants were grown. Among the non-salinized bioaugmented soils, DHA reached its highest value in T6, representing about 3.5 fold the level measured in T1 soil, and 1.3 fold the level in T2. In contrast, in the salinized, bioaugmented soils, the highest measured DHA value recorded in T5 soil and equivalent to 3.6 fold that in T1 and 1.5 fold that in T2 soil.

3.5. TPH degradation in the soil

The progress of crude oil degradation over the course of the experiment is summarized in Table 3. The extent of degradation after 120 days was least in the T1 soil, and highest in the bioaugmented ones. Under non-saline conditions, the concentration of TPH in soils loaded with 10 g/kg crude oil decreased by 50% in the T2 treatment, by 63% in the T4 treatment and by 74% in the T6 treatment; the respective reductions in the soils loaded with 30 g/kg crude oil were 45%, 58% and 75%. In the salinized soils, the rate of TPH degradation following these same three treatments (none of which included salicornia plants) was significantly reduced. The highest rate occurred in the T5 treatment (salicornia/*P. aeruginosa*): the reduction was 61% in soil loaded with 10 g/kg crude oil and 60% in soil loaded with 30 g/kg crude oil. The extent of degradation following the T2 and T3 treatments was, respectively 43% and 57% in soil loaded with 10 g/kg crude oil and 36% and 55% in soil loaded with 30 g/kg crude oil.

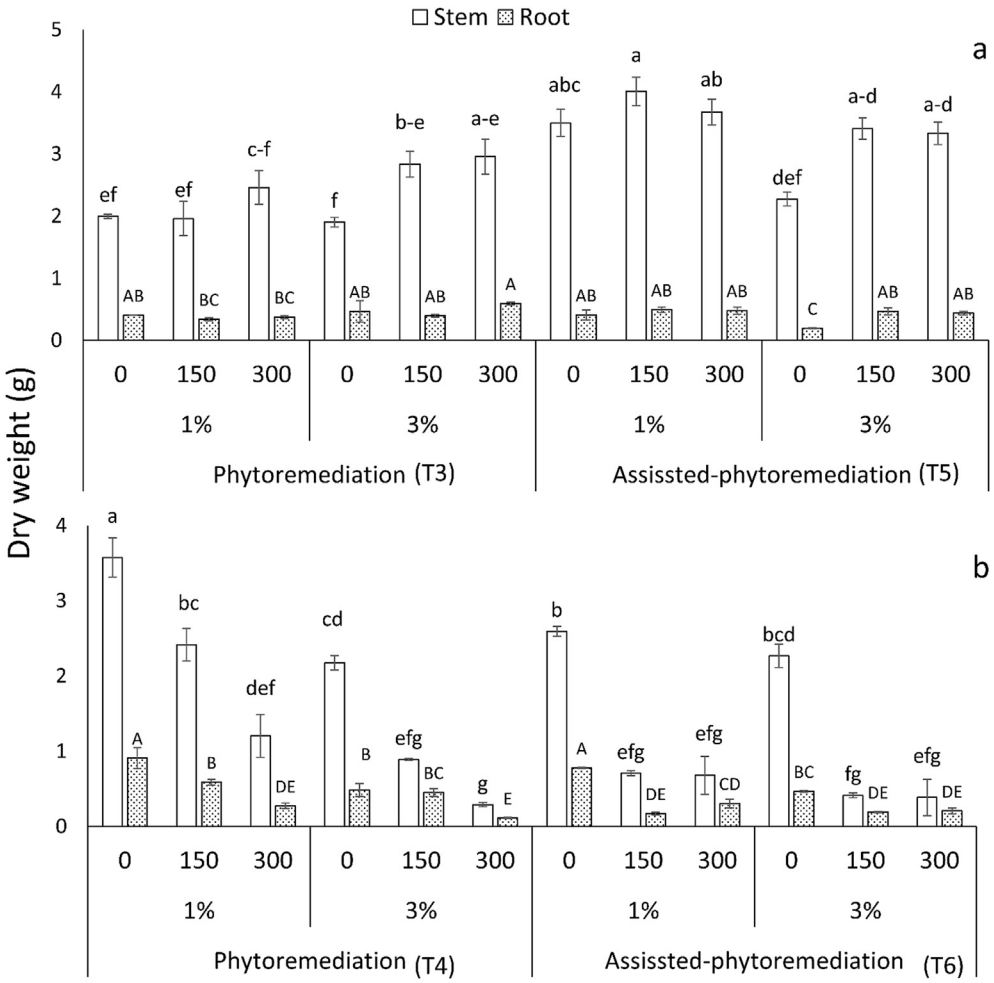

Fig. 3. Shoot and root biomass accumulated by (a) salicornia and (b) tall fescue in non-bioaugmented soil (treatments T3 and T4) and in bioaugmented soils (T5 and T6) at various levels of salinity and initial crude oil load. Values expressed in the form of mean ± S.E. ($n = 3$). Columns marked by the same letter (lower case: shoot samples; upper case: root samples) indicate means not differing significantly from one another according to Duncan's multiple range test ($P = 0.05$).

3.6. Alteration in the profile of aliphatic hydrocarbons

The profile of aliphatic hydrocarbons present in the residual crude as analyzed by GC-MS is shown in Fig. 6. By the end of the measurement period, almost no short chain (up to C13) alkanes remained at a detectable level in the soil sampled from treatments T2 through T6, but they persisted in the T1 soil. The relative abundance of C13-C16 alkanes declined more steeply in the T2-T6 than in the T1 soils, and especially so in T2 soil. Although the relative abundance of longer chain (above C16) molecules was higher than in T1, that of C20 and above ones declined in both T5 and T6 soil.

3.7. Phytotoxicity assay

The treated soils were sampled after 120 days, and their phytotoxicity was determined by a bioassay based on the germination of lettuce seed and subsequent root elongation (Fig. 7). In soil loaded with 10 g/kg crude oil, the level of phytotoxicity was reduced in all of the treatments T2-T6 compared to the level displayed by T1 soil: the decrease was 92% as a result of T6 and 76% as a result of T5. In soil loaded with 30 g/kg crude oil, there was no marked decrease in phytotoxicity as a result of T2; at this level of contamination, the ameliorative effect of growing salicornia was greater than that of growing tall fescue. The most strongly improved soil (46% reduction in phytotoxicity) resulted from the bioaugmented salicornia treatment (T5).

4. Discussion

A critical bottleneck for any phytoremediation-based strategy aimed at the removal of contaminating crude oil from a saline soil is the ability of the plants to germinate and establish a sustainable stand. Both a high level of soil salinity and a heavy load of crude oil strongly inhibited the germination of both salicornia and tall fescue. The suppression of germination exerted by salinity has been well documented for both turfgrass species (Harivandi et al., 1982) and various halophytes (Ameixa et al., 2016; Gul et al., 2016; Khan et al., 2000). Similarly, the presence of crude oil is known to inhibit germination in various plant species (Eze et al., 2013; Macoustra et al., 2015; Wei et al., 2014). According to the present data, stand establishment in a saline crude oil-contaminated soil appears to be more readily achievable via the transplantation of seedlings raised under non-stressed conditions than by sowing seed directly into contaminated soil.

A successful phytoremediation exercise requires that the plants accumulate a substantial volume of biomass. In this respect, salicornia seems to be a favorable candidate for use in saline soils, since it is well adapted to growing in an environment where the level of soil salinity is as high as half strength sea water (Aghaleh et al.,

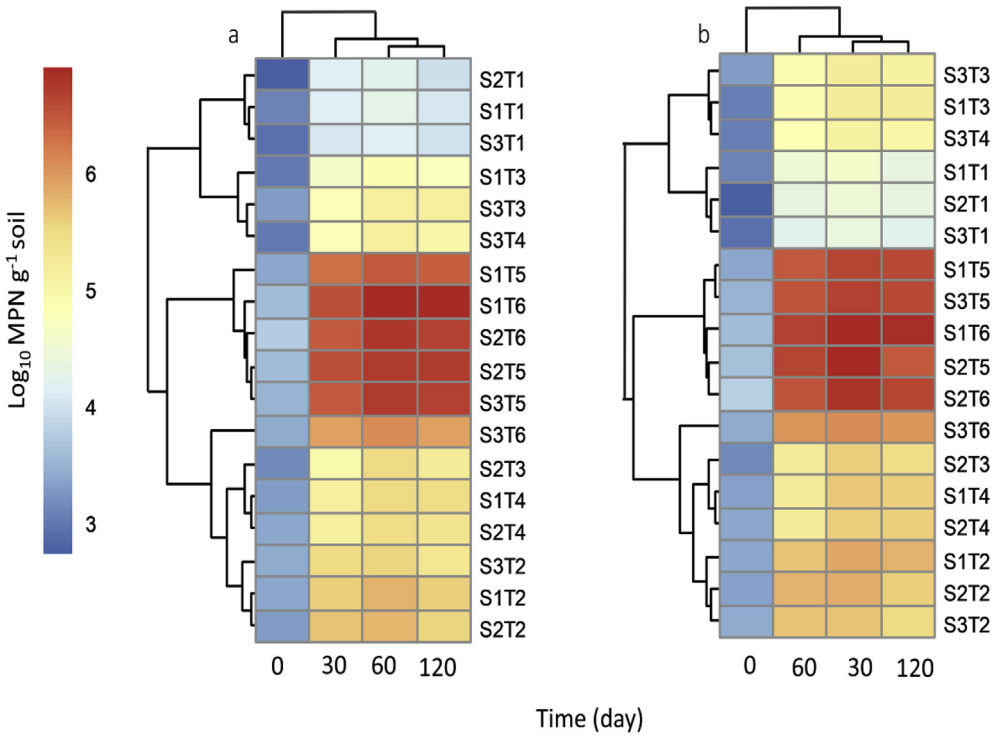

Fig. 4. Most probable number (MPN) of crude oil-degrading bacteria present in the soils exposed to the various treatments: T1 - no additives; T2 - 10^6 CFU/g soil of *P. aeruginosa*; T3, T4 - three seedlings of, respectively, salicornia and tall fescue; T5, T6 – a combination of respectively, T3+T2 and T4+T2. The NaCl load was (S1) 0 mM, (S2) 150 mM, (S3) 300 mM, and the initial crude oil load was (A) 10 g/kg, (B) 30 g/kg.

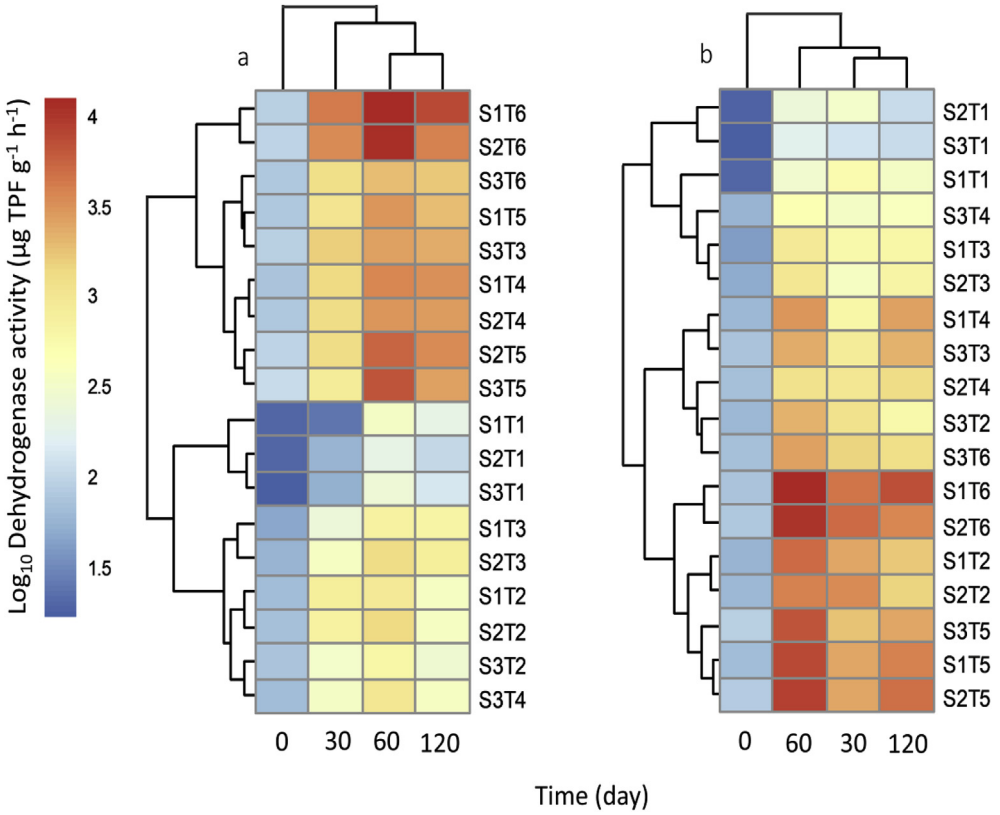

Fig. 5. Alteration in DHA during the bioremediation process, assayed using triphenyl formazan reduction following the various treatments: T1 - no additives; T2 - 10^6 CFU/g soil of *P. aeruginosa*; T3, T4 - three seedlings of, respectively, salicornia and tall fescue; T5, T6 – a combination of respectively, T3+T2 and T4+T2. The NaCl load was (S1) 0 mM, (S2) 150 mM, (S3) 300 mM, and the initial crude oil load was (A) 10 g/kg, (B) 30 g/kg.

Table 3
Residual crude oil content following the bioremediation treatments: T1 - no additives; T2 - 10^6 CFU/g soil of *P. aeruginosa;* T3, T4 - three seedlings of, respectively, salicornia and tall fescue; T5, T6 – a combination of respectively, T3+T2 and T4+T2. The initial crude oil load was either 10 g/kg (left) or 30 g/kg (right). Shared lower case letters indicate means not differing significantly from one another according to Duncan's multiple range test (P = 0.05).

Time (day)	Salinity (mM)	10 g/kg						30 g/kg					
		T1	T2	T3	T4	T5	T6	T1	T2	T3	T4	T5	T6
30	0	9.5^{b}	7.5^{hi}	8.7^{c}	7.8^{fg}	7.8^{fg}	7.0^{i}	27.3^{bc}	24.6^{gh}	26.5^{cd}	23.6^{j}	24.8^{fgh}	21.5^{k}
	150	9.7^{ab}	7.8^{fg}	8.1^{def}	7.9^{efg}	7.3^{ij}	7.2^{ij}	28.5^{a}	25.5^{efg}	24.7^{f-i}	24.9^{efg}	23.7^{ij}	23.5^{j}
	300	9.9^{a}	8.2^{de}	8.3^{d}	8.7^{c}	7.7^{gh}	8.3^{d}	28.4^{a}	24.7^{f-i}	25.9^{de}	27.9^{ab}	23.8^{hij}	25.7^{def}
60	0	8.6^{b}	6.6^{efg}	7.7^{c}	5.9^{ij}	6.3^{f-i}	5.2^{k}	26.3^{ab}	21.6^{de}	24.5^{bc}	18.1^{f}	19.8^{ef}	14.6^{g}
	150	9.1^{a}	6.7^{def}	6.7^{def}	6.1^{hij}	6.2^{ghi}	5.6^{ik}	27.6^{a}	21.7^{de}	21.5^{de}	20.2^{def}	18.4^{f}	18.5^{f}
	300	9.3^{a}	7.1^{d}	6.8^{def}	7.8^{c}	6.4^{fgh}	7.0^{d}	28.0^{a}	22.7^{cd}	20.5^{def}	25.8^{ab}	19.2^{ef}	22.5^{cd}
120	0	7.1^{a}	5.0^{cd}	6.5^{b}	3.7^{fg}	4.7^{de}	2.6^{h}	23.3^{b}	16.4^{ef}	20.5^{cd}	12.5^{ghi}	14.6^{fgh}	7.4^{j}
	150	8.3^{a}	5.3^{c}	4.9^{cde}	4.2^{ef}	3.3^{gh}	3.5^{fg}	26.7^{a}	18.0^{de}	15.0^{fg}	14.4^{fgh}	11.9^{hi}	10.8^{i}
	300	8.5^{cde}	5.7^{b}	4.3^{ef}	6.8^{b}	3.9^{fg}	5.5^{cd}	26.8^{a}	19.2^{d}	13.4^{ghi}	22.9^{bc}	11.9^{hi}	18.3^{de}

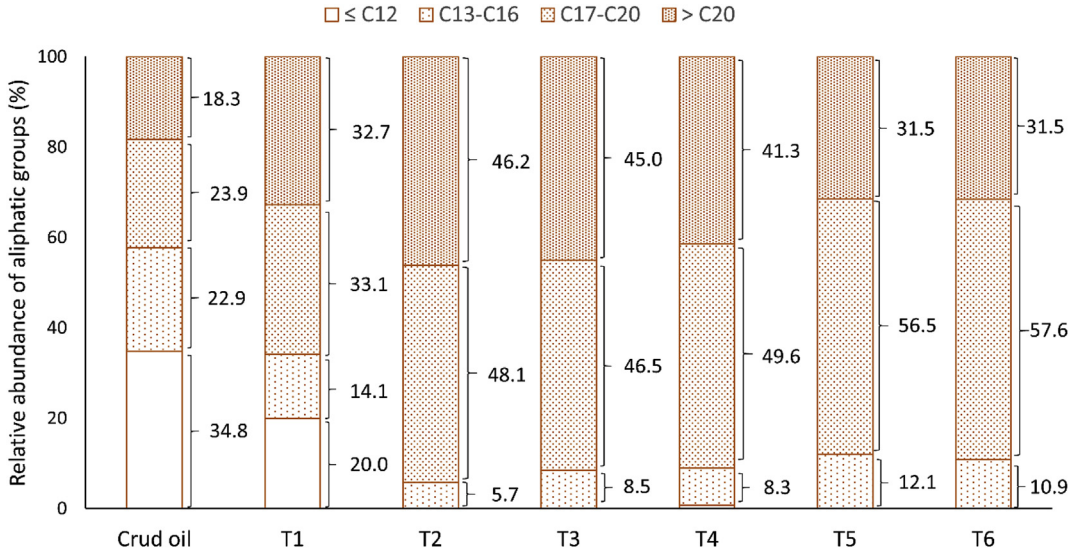

Fig. 6. Relative abundance of aliphatic hydrocarbon of various chain lengths (below C12 and above C20) in the contaminating crude oil and in the residual oil following the various treatments: T1 - no additives; T2 - 10^6 CFU/g soil of *P. aeruginosa*; T3, T4 - three seedlings of, respectively, salicornia and tall fescue; T5, T6 – a combination of respectively, T3+T2 and T4+T2.

2011; Khan et al., 2001). Unlike tall fescue, which responded poorly to high levels of salinity with respect to its biomass accumulation, salicornia grew readily, as has also been noted by Manuchehri and Salehi (2015). Inoculating the rhizosphere with *P. aeruginosa* was able to boost the growth and productivity of the salicornia plants, possibly because the bacteria helped to degrade the shorter chain hydrocarbons, thereby reducing the phytotoxicity of the soil (Afzal et al., 2014). However, the inoculation did not appear to exert any positive effect on the growth of tall fescue plants.

The rate at which petroleum hydrocarbons are biodegraded depends strongly on the size of the microbial population. The MPNs of *P. aeruginosa* were significantly higher in the bioaugmented than in the non-bioaugmented treatments, under both saline and non-saline conditions. Agnello et al. (2016) have reported that inoculation with *P. aeruginosa* can boost the population size of other crude oil-degrading bacteria, thereby accelerating the degradation of TPHs. Inoculation of saline, contaminated soils with a salt tolerant *P. aeruginosa* consortium was able to alleviate the inhibition imposed by salinity on microbial growth and activity (Ebadi et al., 2017a). The abundance of these bacteria appeared to be enhanced by the presence of plant roots, as has also been noted elsewhere (Liu et al., 2013; Ribeiro et al., 2014; Soleimani et al., 2010); the basis of this enhancement is typically through growth-promoting exudates delivered by the roots into the rhizosphere (Liu et al., 2013). Towards the end of the present experiment, the size of the hydrocarbon-degrading bacterial populations had begun to decline, possibly reflecting either a fall in either the concentration of TPHs and/or the level of carbon bioavailability (Cubitto et al., 2004).

DHA has been widely used as an indicator of microbial activity in bioremediation situations (Qin et al., 2012). Here, the inoculation of the *P. aeruginosa* consortium had the effect of raising DHA in both saline and non-saline soils. Qin et al. (2012) showed that DHA was boosted by the inoculation of a bacterial consortium in a crude oil-contaminated saline soil. The presence of both salicornia and tall fescue plants served to increase DHA, the former in saline soil and the latter in non-saline soil. The scale of the increase in DHA was larger in the bioaugmentation treatments (T5 and T6) than in treatments involving only plants or only bacteria. The likely basis for the synergistic effect of plant and bacterium is that the environment generated in the rhizosphere is favorable for the growth of hydrocarbon-degrading bacteria (Nanekar et al., 2015). Given that salicornia thrives under high levels of soil salinity, it is likely to maintain a high level of root exudation under these conditions (Wang and Zhao, 2004), resulting in the observed boost to DHA in salinized soils supporting salicornia plants. As also noted by Silva-Castro et al. (2013), the level of DHA rose during the early part of the experiment, but later fell away; this phenomenon is thought to reflect degradation processes, or of changes in the biodiversity of microbial communities.

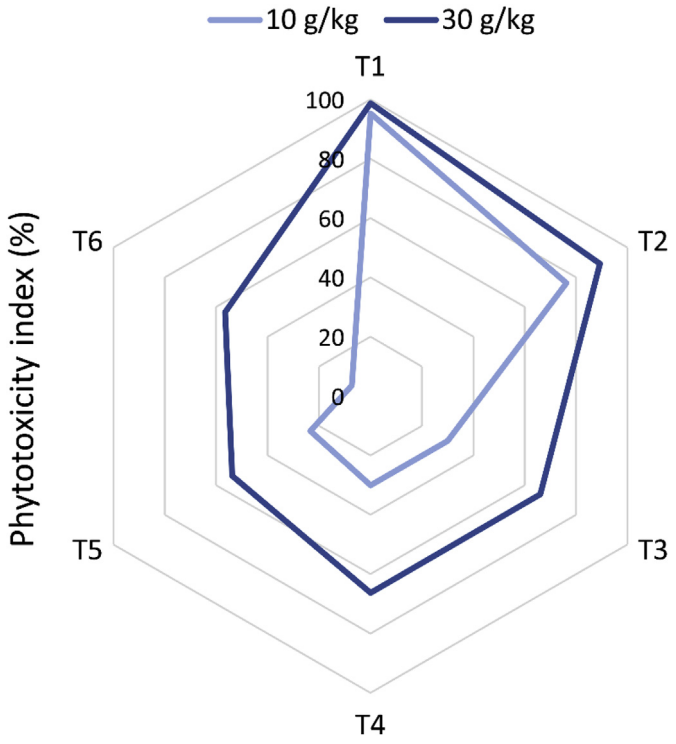

Fig. 7. Phytotoxicity test of the soils, based on the germination and seedling root elongation of lettuce. T1 - no additives; T2 - 10^6 CFU/g soil of *P. aeruginosa*; T3, T4 - three seedlings of, respectively, salicornia and tall fescue; T5, T6 – a combination of respectively, T3+T2 and T4+T2.

A more direct means of assessing the effectiveness of bioremediation aimed at crude oil spillage is to quantify the breakdown of TPHs. Here, they were degraded more rapidly in the early stages of the experiment, with most of the degradation occurring within the first 60 days. The most readily degraded fraction was the short chain compounds, resulting in a rise in the relative abundance of the heavier compounds, which tend to be more refractory to breakdown (Cai et al., 2016; Shen et al., 2016). TPH degradation was inhibited by salinity, except where salicornia plants were present. The suppressive effect of salinity on microbial growth and activity, in conjunction with a decline in root growth, acts to reduce the rate of TPH degradation (Cai et al., 2016; Qin et al., 2012; Xun et al., 2015). This decline was mitigated by planting salicornia. Thus, the highest rate of TPH degradation achieved under saline conditions was in the bioaugmentation treatment in the presence of salicornia, while under non-saline conditions, it was the bioaugmentation treatment in the presence of tall fescue. Previous studies have similarly shown that bioaugmentation enhances the rate of TPH degradation (Agnello et al., 2016; Andreolli et al., 2013; Nanekar et al., 2015). In addition to their acting as a source of carbon and energy to rhizosphere bacteria, root exudates, due to their structural similarity with certain hydrocarbon compounds, can both induce co-metabolism effects and up-regulate bacterial genes encoding TPH-degrading enzymes (Gkorezis et al., 2016). The GC-MS analysis of residual crude oil revealed that the longer chain alkanes further degraded in the bioaugmentation treatments, which is of interest, given that the other treatments enhanced the degradation of only the small chain molecules. According to Li et al. (2016), the observed increase in the relative abundance of intermediate length hydrocarbons probably reflects the enhanced degradation of the long chain ones.

Due to the complexity of the bioremediation process, neither chemical analysis nor the quantification of TPH degradation are able to fully describe the efficacy of a phytoremediation intervention. Since the main objective of bioremediation is to restore the soil's capacity to support plant growth, a bioassay based on plant survival represents the ultimate test of whether the intervention has been effective (Shen et al., 2016). The lettuce germination/seedling root elongation assay employed here showed that the bioaugmentation treatments involving salicornia in saline soil and tall fescue in non-saline soil were both able to markedly reduce the soil's phytotoxicity. Nevertheless, in the more heavily contaminated soil (30 g/kg of crude oil); even though these treatments substantially reduced the quantity of TPHs present, the level of phytotoxicity remained high. The implication is that the bioremediation of heavy spills would require a longer treatment time than 120 days. Note that some evidence suggests that the intermediate metabolites formed during the bioremediation process are themselves responsible for increased phytotoxicity (Shen et al., 2016), but other researchers have established that the phytotoxicity of a crude oil-contaminated soil can be greatly ameliorated through bioremediation (Graj et al., 2013; Hamdi et al., 2012).

5. Conclusion

The bioremediation of crude oil-contaminated saline soils is particularly difficult given the simultaneous presence of salt and crude oil as two independent environmental stress agents. While the effectiveness of bioaugmentation and phytoremediation based on tall fescue was compromised 29–36% by soil salinity, TPH degradation was not inhibited by salinity when it was replaced by salicornia. The bioaugmentation approach was particularly effective in removing the higher molecular weight alkanes. Both DHA and *P. aeruginosa* abundance were stimulated respectively 3.5 and 10 fold by the presence of a phytoremediant, resulting in 46–76% reduction in the soil's phytotoxicity in a saline soil. Thus, a treatment based on combining a halophyte with a salinity tolerant bacterial consortium appears to be a promising strategy to address the remediation of crude oil-contaminated saline soils.

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Declarations of interest

None.

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