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Comparative evaluation of bioremediation techniques on oil contaminated sediments in long-term recovery of benthic community health

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ABSTRACT

While various bioremediation techniques have been widely used at oil spill sites, the in situ efficiency of such techniques on recovering the benthic communities in intertidal areas has not been quantified. Here, the performance of several bioremediation tools such as emulsifiers, multi-enzyme liquid (MEL), microbes, and rice-straw was evaluated by a 90-days semi-field experiment, particularly targeting recovery of benthic community. Temporal efficiency in the removal of sedimentary total petroleum hydrocarbons (TPH), reduction of residual toxicity, and recovery of bacterial diversity, microalgal growth, and benthic production was comprehensively determined. Concentrations of TPH and amphipod mortality for all treatments rapidly decreased within the first 10 days. In addition, the density of bacteria and microphytobenthos generally increased over time for all treatments, indicating recovery in the benthic community health. However, the recovery of some nitrifying bacteria, such as the class Nitrospinia (which are sensitive to oil components) remained incomplete (13–56%) during 90 days. Combination of microbe treatments showed rapid and effective for recovering the benthic community, but after 90 days, all treatments showed high recovery efficiency. Of consideration, the "no action" treatment showed a similar level of recovery to those of microbe and MEL treatments, indicating that the natural recovery process could prevail in certain situations.

1. Introduction

Oil spills represent one of the worst types of anthropogenic disasters, threatening marine ecosystems through the (in)direct adverse effects associated with various toxic components (Golet et al., 2002). In particular, oil-contaminated sediments in coastal areas might have relatively long-term ecological effects on benthic communities, sometimes with unexpected prolonged recovery (Hong et al., 2014). For example, the *Hebei Spirit* oil spill (HSOS) on the Taean coast (west coast of South Korea) in 2007 has caused adverse biological impacts over the last 10 years, particularly in intertidal mudflats and some sheltered rocky shores (Hong et al., 2014). Recently, Yim et al. (2017) indicated that the recovery of ecosystem damage along the Taean coast would have varied in time cross the habitat types, such as rocky shores, sandy beaches, and mudflats. As another case, the coastal ecosystems affected by the *Exxon Valdez* oil spill (EVOS), which occurred in Prince William Sound, Alaska, in 1989, showed very slow recovery over the last 30 years due to the persistence of residual oils (Shigenaka, 2014).

Environmental factors that control the natural recovery of coastal ecosystems include many aspects, such as initial cleanup methods, the use of purification techniques, biological activity, and

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tidal energy in the given environment (ITOPF, 2010a). For example, the recovery of the Taean ecosystem was generally faster than other sites subject to oil spills, which might be explained by the strong tidal and wave mixing under the macrotidal regime along the open coast on the eastern part of the Yellow Sea (Seo et al., 2014). For the EVOS, recovery was slower in regions that were subject to a mechanical cleanup of hot water flushing during the initial stage, compared to the regions with "no action" (Paine et al., 1996). Thus, the key in recovery efficiency in marine ecosystems at oil spill sites would be the selection of appropriate remediation techniques being maximized at the given oceanographic settings or conditions.

Bioremediation techniques are generally considered as an eco-friendly method to promote the natural recovery of threatened ecosystems by reducing the toxic residual oils through microbial degradation (Atlas and Hazen, 2011; Roy et al., 2014). Various bioremediation techniques have been developed and widely utilized in areas affected by oil spills, including fertilizers, emulsifiers, multi-enzyme liquids (MEL), and microbes (Dave and Ghaly, 2011; Das and Chandran, 2011; Doshi et al., 2018; Lee et al., 2018; Swannell et al., 1996). For example, in the case of EVOS, fertilizers were widely used along the shoreline, resulting in the rapid decrease of total petroleum hydrocarbons (TPH) concentrations by ~1.2% per day (Atlas and Hazen, 2011). During the Nakhodka oil spill, commercial petroleum degrading bacteria (TerraZyme, Oppenheimer Biotechnology) was applied to treat the oil contaminated shoreline, resulting in a general decline in oil cover (Tsutsumi et al., 2000). The mixed usage of bioremediation tools is also regarded as a common measure to remove residual oils at spill sites. For example, a mixture of emulsifiers, nutrients, and microorganisms might increase the degradation rate of oils in contaminated sand (Kim et al., 2005). However, our current understanding of the long-term efficiency of bioremediation techniques to aid the recovery of the benthic community remains limited (Jernelöv, 2010; Prince, 1997).

In particular, it is very difficult to select appropriate indicators/endpoints to evaluate the recovery of ecosystem health in areas affected by oil spills (Board and Council, 2013; ITOPF, 2010b). This issue exists because each target ecosystem has distinct characteristics (e.g., abiotic factors and community structure), with potential variation and/or uncertainty in the indicator species. Several previous studies showed that the sediment microbiome determined by the next-generation sequencing (NGS) technique was quite useful, as the bacterial community was directly related to the chemodynamics of petroleum hydrocarbons (Bourlat et al., 2013; Lee et al., 2019; Mukherjee et al., 2017). The profiling of polycyclic aromatic hydrocarbons (PAHs) degrading bacteria in the oil contaminated environment has provided effective and/or suitable strategies for bioremediation under various environmental conditions (Mukherjee et al., 2017). The potential biases and uncertainties in NGS analysis might be complemented by monitoring floral and/or faunal communities, facilitating the actual recovery of trophic communities in situ. Of note, to date, the elements of ecosystem structure (e.g., macro- and/or meiofaunal taxa) have been widely determined, whereas fewer studies exist on the functional responses (such as benthic primary production and food-web interactions) of recovering ecosystems following oil spills (Baustian et al., 2010; Elliott et al., 2007).

The mesocosm and/or semi-field experiments are vital to understand how ecosystems respond to oil spills because they quantify how oil spill conditions and environmental factors contribute to adverse impacts on ecosystem health (Mohr et al., 2005). In particular, indoor semi-field experiments are preferred, as they minimize the effect of extraneous factors, reducing uncertainty (i.e., environmental contamination by the experiment, sample loss caused by certain environmental factors such as temperature, precipitation, wind, wave, typhoons, and human activity) (Mohr et al., 2005). Mesocosm studies help in interpreting and/or clarifying the information gained through small-scale laboratory experiments and large-scale field research, by reducing the gap between laboratory and field studies (Petersen, 2009). Also, such semi-field experiments require less manpower and/or cost compared to field experiments.

In the present study, we developed a semi-field experimental system, which simulated an artificial tide in the experimental aquariums, with tests being performed under well-controlled tidal conditions. After applying various bioremediation techniques to the oil contaminated sand, we investigated the residual oils (TPH), in vivo toxicity using marine amphipods (*Monocorophium uenoi*), the recovery of bacteria and microphytobenthos (MPB), and benthic primary production over a 90-day period. By comparing the bioremediated treatments (single or combined treatments) with the control (natural clean sand) and "no action" treatment, we aimed to quantitatively identify the optimal recovery time and most efficient bioremediation methods. Finally, we adopted the ratio to negative control approach to address the recovery efficiency of the bioremediation methods in an objective and comprehensive manner. Five endpoints were considered in the evaluation of the bioremediation efficiency: 1) residual TPH; 2) residual in vivo toxicity; 3) diversity and recovery of PAHs sensitive bacteria; 4) MPB biomass; and 5) benthic primary production during 90 days. Our data provide insights on the method-specific and endpoint-dependent recovery efficiencies of marine ecosystem by the bioremediation of areas affected by oil spills.

2. Materials and methods

2.1. Development of a semi-field experimental system

A semi-field experimental system (2 systems, $202 \times 90 \times 250$ cm) was developed to simulate and control the tidal cycles of the intertidal area to evaluate the performance of various bioremediation techniques (Fig. S1 of the Supplementary Materials (S)). Each system consisted of three compartments: 1) one larger water storage tank on top (for thermal and dissolved oxygen control, $200 \times 90 \times 41$ cm, ~700 L); 2) a set of experimental aquariums in the middle (to control water level, tidal cycle, and light condition, $n = 10$ per system, $45 \times 35 \times 40$ cm aquarium); and 3) one wastewater tank on bottom (to provide a reservoir for wastewater after the experiment, $200 \times 70 \times 80$ cm, ~1100 L). Water temperature was maintained by using a temperature control system. A total of 1000 L natural seawater was supplied to the experimental aquariums (2 systems $\times$ 10 aquariums = 20 aquariums, 50 L per aquarium) every 12 h. Seawater was slowly added to the aquarium at a rate of 18 L h^{-1} to prevent disturbance to the bottom sediment. After adding seawater, each experimental aquarium was separated by a valve to prevent mixing between treatments. After 12 h, the seawater was discharged automatically into the wastewater tank. LED lamps (20 W, $n = 8$ per system) were installed in each system and the irradiance of light reaching the sediments was about 380–425 μmol m^{-2} s^{-1}. Of note, two artificial tide control systems were developed and used, which allowed the establishment of 20 aquariums (viz., 10 treatments for each system; Fig. 1) as a back-up, allowing duplication.

2.2. Experimental settings

The experimental design included 10 treatments (shown in Fig. 1), with various combinations of four bioremediation techniques being used (EPA, 2017). The bioremediation methods included 1) an emulsifier: Tween 80, 2) MEL: Oil Spill Eater II, 3)

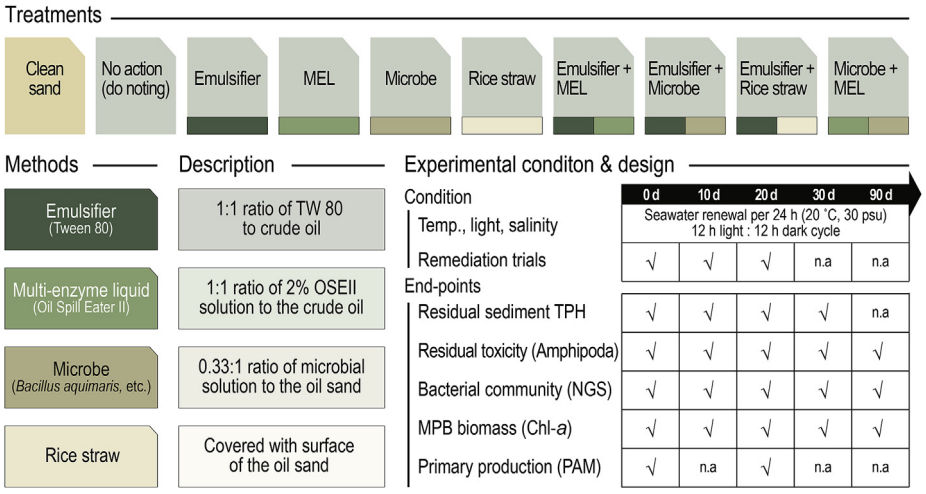

Fig. 1. Schematic diagram showing the bioremediation treatments, methods, experimental conditions, and sampling design conducted in this study. Detailed experimental conditions are present in Tables S1 and S2 (n.a.: not available).

microbes: *Corynebacterium variabilis*, *Sphingomonas yanoikuyae*, *Bacillus aquimaris*, *Kyotococcus sedentarius*, *Novosphingobium*, *Pentaromativorans*, and *Yarrowia lipolytica*, and 4) rice straw. Fertilizer was added to all experimental aquariums to maintain sufficient nutrients during the testing periods. The rice straw was considered as an alternative material for the bioremediation of oil contaminated sediments because it has a large surface area that readily adsorbs oil and contains a variety of microorganisms.

Fresh Iranian Heavy Crude (IHC, details given in Table S1) oil was added to cleaned sandy sediments (<1 mm diameter; Samhangang INC., Incheon, Korea) at a volume ratio of 1:10 and was homogenized for 15 min. Two days before starting the experiment, the same amount of oil contaminated sediment (15 L) was allowed to settle into the experimental aquariums. Fertilizer (oleophilic fertilizer S200) and the bioremediation solution was sprayed on the surface sediment (frequency: 0, 10, and 20 days) in each treatment, and rice straw was added at a thickness of about 1 cm on the surface sediment (details given in Table S2). Details on the subsampling methods during and after the experiments are provided in the following sections to analyze the five endpoints: 1) residual TPH; 2) amphipod mortality; 3) microbial abundance; 4) microalgal biomass; and 5) benthic primary production (Fig. 1).

2.3. Concentrations of residual TPH in sediments

The analytical procedures for TPH in sediments followed that of previous methods (Yim et al., 2005, 2011). In brief, 5 g of wet sediment samples (day 0, 10, 20, and 30) were mixed with anhydrous sodium sulfate (Sigma-Aldrich, St. Louis, MO) to remove water in a Teflon tube and an appropriate surrogate standard (*o*-terphenyl) was added. The samples were extracted with 30 mL of dichloromethane (DCM, Burdick & Jackson, Muskegon, MI) on an ultrasonicator for 15 min. The extracts were concentrated to 1 mL under a gentle stream of nitrogen gas and the internal standard (5α-androstane) was added. The mixture was stored in a GC vial for instrumental analysis using an Agilent 7890 gas chromatograph equipped with a flame-ionization detector (GC-FID, Agilent Technologies, Santa Clara, CA). The instrumental conditions and quantification method for TPH followed that of a previous study (Wang et al., 1994).

2.4. Amphipod toxicity test

The benthic amphipod *Monocorophium uenoi* (provided by NeoEnBiz Inc., Bucheon, Korea) was used as a test animal to evaluate the residual oil toxicity of the sediments. Before the test, the amphipods were fed dry flakes once a day, and were cultured in clean sediment with filtered seawater (GF/F, Whatman, Kent, UK; 35 psμ). The overlying water was continuously aerated under static renewal conditions in a temperature controlled room, maintained at 20 °C. Before the experiment, amphipods were collected by sieving them through a 0.3–0.5 mm mesh, and were then allocated to three 1 L-beakers with a 2 cm-deep layer of each test sediment (about 150 g; day 0, 10, 20, 30, and 90) that was filled with 500 mL filtered seawater. During the test period, salinity, temperature, and dissolved oxygen saturation were maintained at 35 psμ, 20 °C and over 80%, respectively. Amphipods were not fed and the overlying water was not replaced during the test. After 10 days, the live amphipods were collected using a sieve and the mortality rate was obtained (Docurnems, 1992).

2.5. Microbial metagenomic analysis

To analyze the microbial community, total genomic DNA were extracted from sediments (at day 0, 10, 20, 30, and 90) using a PowerSoil® DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA). Sequencing was conducted using the Illumina MiSeq Platform, with 16S, rRNA gene amplicons. The V3 and V4 regions of the bacterial 16S ribosomal gene were amplified using the Illumina primers and barcoded adapters. Quantitative Insights into Microbial Ecology (QIIME) was used to analyze the sequence data. Using UCLUST, sequences were clustered by operational taxonomic units (OTUs) at a 97% identity threshold (Edgar, 2010). Taxonomic information was delegated by aligning sequences against data from the Ribosomal Database Project (RDP) (Cole et al., 2013).

2.6. Measurement of MPB biomass

Surface sediments (at 0–0.5 cm depth) were collected to measure chlorophyll *a* (Chl-*a*) concentrations (indicating MPB biomass) using a syringe corer at day 0, 10, 20, 30, and 90. Chl-*a* was extracted with 10 mL of 100% acetone for 24 h at 4 °C. Samples were centrifuged at 3000 rpm for 5 min. The extract was transferred into a quartz cuvette and Chl-*a* was measured spectrophotometrically (Jenway 7310 spectrophotometer, UK). The Chl-*a* concentration was calculated using the Lorenzen method (Lorenzen, 1967).

2.7. Measurement of benthic primary production

Benthic primary production was measured in each treatment of the experimental aquariums (n = 3 per treatment) during the daytime by using a Diving-PAM fluorometer (Walz, Effeltrich, Germany) at day 0 and 20 (Perkins et al., 2001). The maximum relative electron transport rate ($rETR_m$) was calculated as the product of $F_{q'}/F_{m'}$ ($F_{q'}$: proportion of harvested photons that drive photosynthesis; $F_{m'}$: light-adapted maximum fluorescence) and irradiance (Sakshaug et al., 1997). The $rETR_m$ was determined from repetitive measurements (>3 times) of the light response curve (Long and Hällgren, 1985).

2.8. Data analyses

To evaluate the bioremediation efficiency, we calculated the ratio to negative control values (clean sand) for the five target endpoints; TPH, residual toxicity, bacterial community response (diversity and relative abundance of PAHs sensitive species), MPB biomass, and primary production. As for the recovery state of microbial community, we considered including the increase of relative abundance of PAH-degrading bacteria. However, this might be an indication of an ongoing recovery state of the ecosystem from oil contamination and thus we excluded the increase of PAH-degrading bacteria. The bioremediation efficiencies were calculated as percentages for each endpoint, and described as five-axis plots (detail equations given in Table S3). SPSS 24.0 (IBM, Armonk, NY) software was used to perform statistical analyses. Non-parametric statistics were used to investigate the relationships between the measured log TPH data and bacterial community (Phylum) because the data did not meet the assumption of normality (Table S4). Spearman rank correlation coefficient analysis (r) was used to evaluate the cross-association of the log TPH concentration and bacterial community (Phylum) (Table S5).

3. Results and discussion

3.1. Chemical, toxicological, and biological responses to bioremediation treatments

TPH concentrations in the sediments of all treatments, after applying the various bioremediation techniques, rapidly decreased within the first 10 days, ranging from 84 to 96% of removal of TPH (Fig. 2). Unexpectedly, the TPH concentration in sediments decreased by 85% in the treatment with “no action” (i.e., applied only tidal action). Schratzberger et al. (2003) showed similar results, with 75% of oil removed by tidal currents and wave action after 45 weeks. Our result indicate that the residual sedimentary oil could be almost completely removed in a short period of time (~1 week) only by the exchange of seawater. Similarly, the amphipod toxicity in sediments sharply decreased within 10 days. Thus, the results of the amphipod toxicity test strongly support the sharp reduction in residual TPH concentrations in all treatments after 10 days of remediation. Lee et al. (2013) obtained similar results, whereby the mortality of amphipods was strongly dependent on the concentrations of residual sedimentary oil.

The bacterial OTUs varied depending on the bioremediation methods and time, although the bacterial OTUs had stabilized in the sediments of all treatments after 90 days. Initially, bacterial OTUs was significantly affected by residual oil in sediments, but it gradually recovered through seawater exchange and the applied bioremediation techniques (Fig. 2). In particular, bacterial OTUs tended to be slightly different among the treatments (Fig. 2). The tendency for bacterial OTUs to recover was relatively faster (>3% than others) in treatments containing microbes. After 10 days, the bacterial OTUs were relatively lower in the treatments containing the emulsifier. A previous study suggested that emulsifiers adversely affect the microbial community and/or strain (Hui et al., 2007). Despite these adverse effects, bacterial OTUs seemed to generally recover after 10–20 days (Ron and Rosenberg, 2002). Of note, the “no action” treatment also showed rapid recovery of microbial community at the early stage of remediation, which was consistent with the chemistry and toxicity data. Overall, bacterial OTUs rapidly increased in all treatments in this study, supporting a previous study showing that nutrient-enhanced bioremediation leads recovery of bacterial diversity after >6 days (Röling et al., 2002).

Although the concentration of oil decreased after 10 days, MPB took a long time to recover over the 90 days. In general, MPB biomass (Chl-*a*) in sediments showed slow increasing trends cross the treatments, with accelerated biomass peak in treatments containing the emulsifier, MEL, and microbe + MEL ($p < 0.05$) after 30 days (measured at Day 90) compared to those of rice straw and emulsifier + rice straw treatments (Fig. 2). Overall, the fertilizer itself might increase MPB biomass, except in treatments with rice straw, but might not reduce the recovery time. It should be noted that microalgae are vulnerable to crude oil when nutrient concentrations are not high enough (Özhan et al., 2014a). The recovery of MPB biomass in the “no action” treatment was comparable to other treatments, because seawater might contain enough nutrients; thus, seawater circulation alone could promote the recovery of MPB biomass over the long-term.

3.2. Recovery of microbial community

The relative abundance of the bacterial 16S rRNA gene sequence detected in sediments was clearly distinguished among the bioremediation treatments (Fig. 3). After 10 days, 9 phyla (>0.1%) were recorded in all treatments, including Proteobacteria, Bacteroidetes, Actinobacteria, Cyanobacteria/chloroplast, Planctomycetes, Verrucomicrobia, Firmicutes, Acidobacteria, and Chlamydiae, among others. On average, three phyla (Proteobacteria, Bacteroidetes, and Actinobacteria) represented more than about 80% of the entire bacterial community. During the experiments, the relative abundance of Proteobacteria, Bacteroidetes, and Actinobacteria tended to decrease in all treatments (except for MEL treatment), whereas Cyanobacteria/chloroplasts increased except for the rice straw treatment (Fig. S2).

Microbial diversity indices (Simpson's index: the probability that two randomly selected individuals in a habitat belong to the same species; Shannon index: accounts for the number and evenness of species; and Chao 1: OTU richness estimator) had smaller values in the treatment of emulsifier compared to the others (Fig. 4). Although most of the microbial diversity index values were similar, the dominant genera (>3% of relative abundance) differed among the bioremediation treatments after 10 days. Some genera (>3% of abundance) have already been reported as PAH-degrading bacteria, including *Corynebacterium*, *Dietzia*, *Nocardioides*, *Algoriphagus*, *Pseudomonas*, *Alcanivorax*, *Arenimonas*, *Porticoccus*, *Lysobacter*, *Winogradskyella*, and *Thalassolituus* (Dong et al., 2015; Gutierrez et al., 2012; Hilyard et al., 2008; John et al., 2012; Liu et al., 2017; Noh et al., 2018; Seo et al., 2009; Wang et al., 2014; Young et al., 2007; Yetti et al., 2016). In addition, two genera (*Owenweeksia* and *Lutibacter*) represented over 3% of the relative abundance, suggesting that they degrade oil components.

In the experimental groups treated with microbes (microbe, emulsifier + microbe, and microbe + MEL), specific microorganism (such as *Corynebacterium* sp.) represented over 8% of total relative abundance. In addition, relatively fewer genera (2–3 represented >3% relative abundance) were observed in the treatments

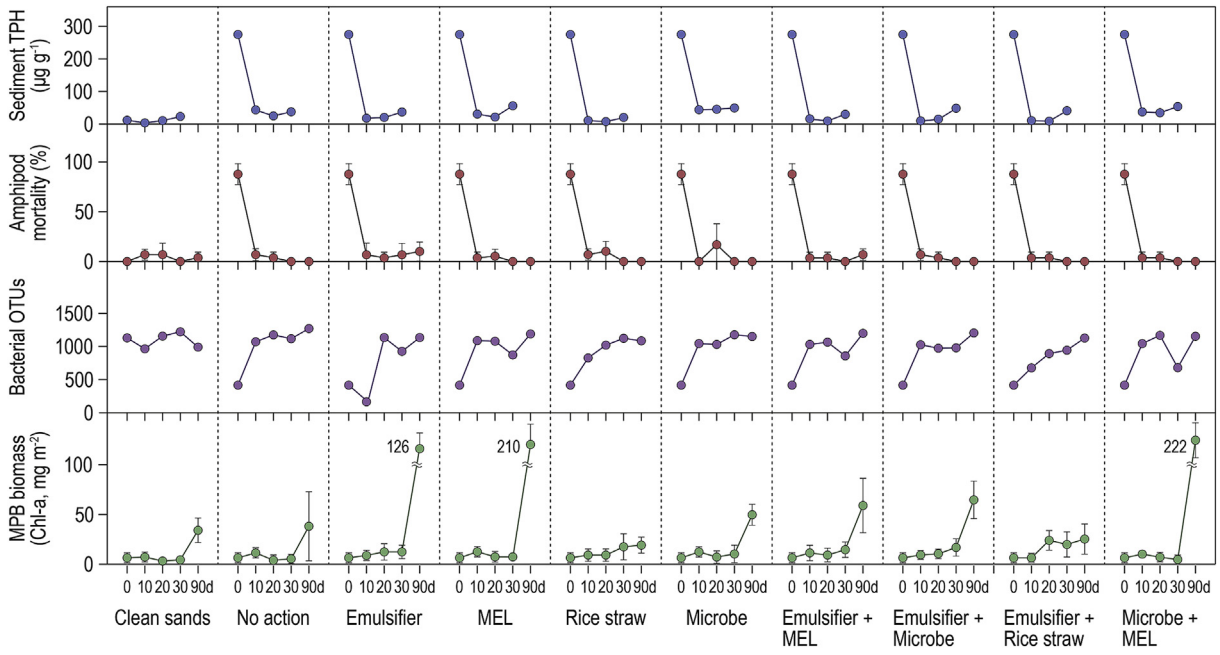

Fig. 2. Temporal variation of sedimentary TPH concentrations, amphipod (*Monocorophium uenoi*) mortality, bacterial OTUs, MPB biomass, and primary production measured in the 90-day bioremediation experiments; a total of 10 treatments including control.

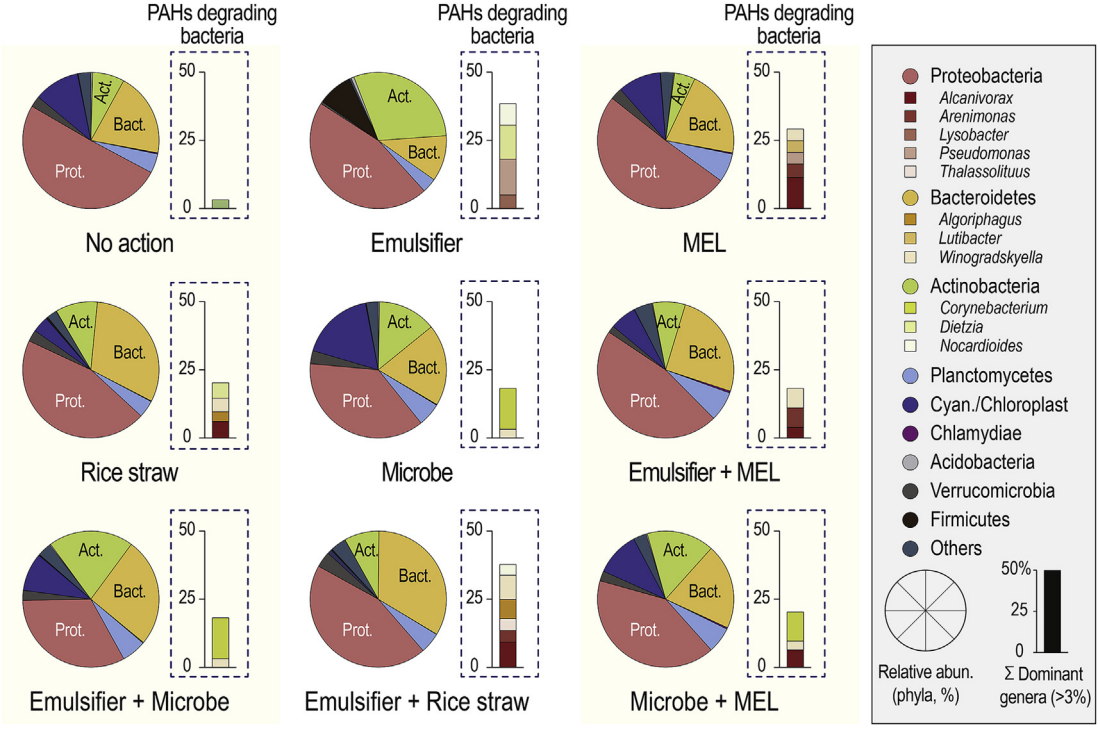

Fig. 3. Structure of the microbial community observed at 10 days after the bioremediation experiment; at genus level. The genus name represents over 3% of the relative abundance to total in each treatment.

containing microbes compared to the other treatments (viz., 3–6 genera with >3% in relative abundance) (Fig. 3). Thus, certain microbial genera (such as *Corynebacterium* sp.) might suppress other PAH-degrading bacteria. The relative abundance of PAH-degrading bacteria (except for *Corynebacterium* sp.) was 20–29% in the remediation treatments lacking microbes, while just 7–13% of relative abundance of other PAH-degrading bacteria was in the treatments containing microbes (Fig. 4). Our results might be explained by a significant difference in the total relative abundance of PAH-degrading bacteria among the purification treatments. McGenity et al. (2012) also suggested this possibility with respect to inter-specific competition for hydrocarbon resources. For example, the impact of antibiotic substances on PAH-degrading bacteria has yet to be determined (i.e., *Alcanivorax* releases antibiotic

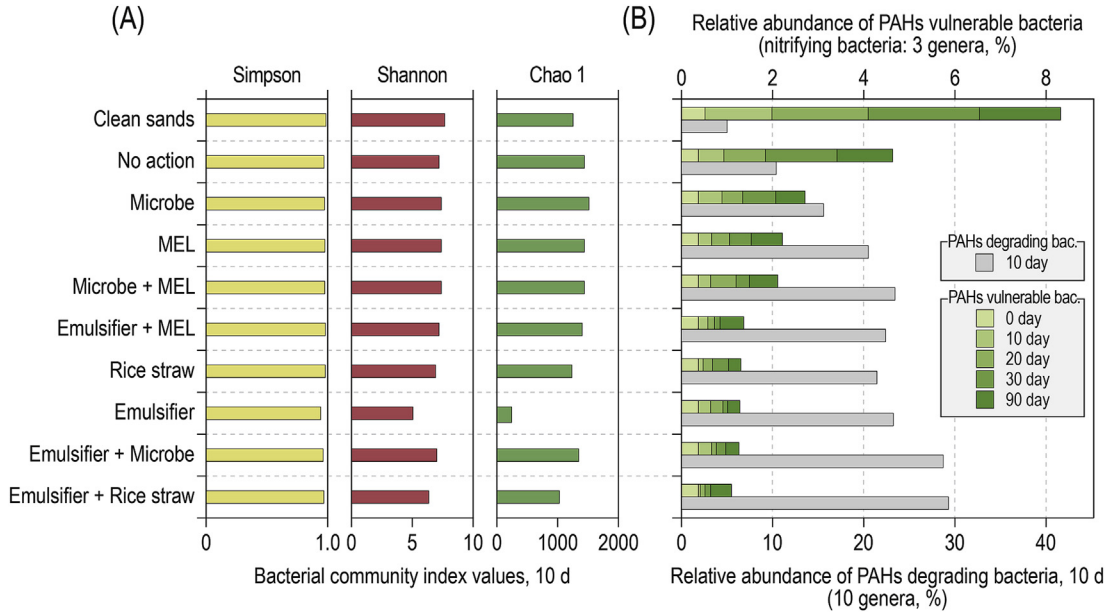

Fig. 4. Comparison of (A) microbial diversity indices (Simpson's index, Shannon index, and Chao 1), and (B) relative abundance of PAH-vulnerable bacteria (the genus *Nitrosospira* and *Nitrospira* or the class Nitrospinia) and PAH-degrading bacteria (10 genera, listed in Table S2) among the treatments.

compounds like alcanivorone to inhibit other competitors) (McGenity et al., 2012). Hazen et al. (2010) showed that hydrocarbon degrading bacteria in the marine environment rapidly respond to oil spills, leading to the possible intrinsic bioremediation of hydrocarbon contaminants.

The microbial solution used in the present study contained six species of PAH-degrading bacteria (*Corynebacterium variabilis*, *Sphingomonas yanoikuyae*, *Bacillus aquimaris*, *Kytococcus sedentarius*, *Novosphingobium pentaromativorans*, and *Yarrowia lipolytica*), but only three microbes (*Corynebacterium variabilis*, *Bacillus aquimaris*, and *Novosphingobium pentaromativorans*) were identified in the treated sediments. The relative abundance of *Corynebacterium* sp. was consistent (4.6–15.6%) throughout the experiments (Fig. S3). Thus, the results supported that: 1) different bacterial communities exist depending on the applied bioremediation methods (i.e., emulsifier, MEL, microbe, and rice straw); 2) certain microbial species constantly exist in oil contaminated environments; and 3) the biomass of these species increases rapidly in response to oil spills.

Hazen et al. (2010) reported that the concentration of nitrate in areas with oil plumes was lower than that in areas lacking plumes following the *Deepwater Horizon* blowout. Also, laboratory scale bioassays showed that nitrifying bacteria are sensitive and/or vulnerable to petroleum toxicants (Urakawa et al., 2012). Thus, the nitrifiers would be useful indicators of oil contamination levels in marine environment. The present study showed that nitrifying bacteria (the genus *Nitrosospira* and *Nitrospira* or the class Nitrospinia) tended to decrease at the beginning of the experiments (between 0 and 10 days), but increased after 20 days (Fig. 4). After 90 days, the relative abundance of nitrifying bacteria had not recovered more than that of the control. Nitrifying bacteria proportion are only 0.1% of the total bacterial community in the marine environment, but play a vital role converting ammonia to nitrates that are essential for phytoplankton growth (Capone et al., 2008). The lack of nitrifying bacteria may reduce the nitrogen availability, ultimately causing problems with the marine nitrogen cycle (Capone et al., 2008). Nevertheless, the "no action" treatment had a significantly greater relative abundance of nitrifying bacteria compared to the other treatments (Fig. 4). The results of the current study showed that the bacterial community that was damaged by oil contamination could be recovered with sufficient seawater circulation alone (if initial cleanup is well conducted).

3.3. Recovery of benthic primary productivity

Benthic primary production ($rETR_m$) showed a 1.5- to 4.3-fold increase in all of the bioremediation treatments over the first 20 days (Fig. 5). The primary production of MPB in the treatments with rice straw was greater than that of the other treatments, but the MPB biomass was lower (Figs. 2 and 5). This phenomenon might be

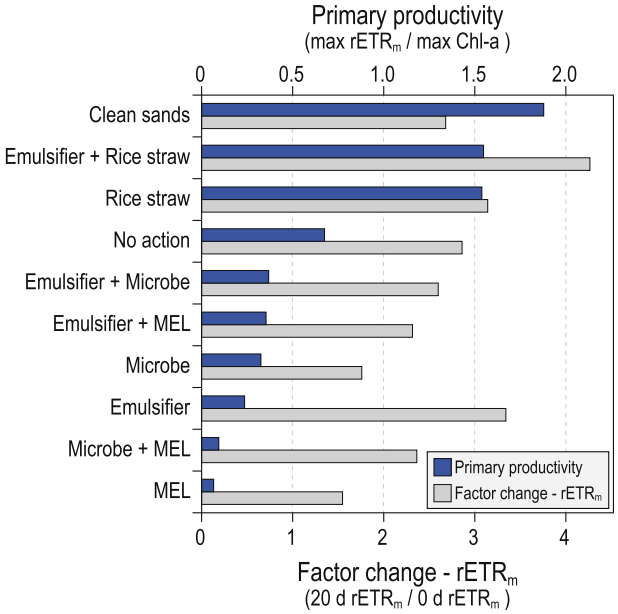

Fig. 5. Comparison of benthic primary productivity among the treatments. Production value refers to the factor change between day 0 and day 20.

because decomposing rice straw supplies a continuous carbon source to MPB for primary production, but it also interrupts MPB growth by blocking out the sunlight. A previous study reported that the degree of benthic primary production is strongly associated with MPB biomass (Colijn and De Jonge, 1984). However, in the present study, there was no clear correlation between MPB biomass and benthic primary production. The complete understanding of the relationship between MPB biomass and primary production is complicated by the presence of other environmental factors, including insufficient light intensity and/or deterioration of physiological conditions by oil contamination, which also hinder benthic primary production.

Several previous studies have reported that phytoplankton are adversely affected by crude oil; however, only a few studies have evaluated the sensitivity of microphytobenthos to oil contamination (Özhan et al., 2014a, 2014b; Riaux-Gobin, 1985). Özhan et al. (2014b) reported that concentrations of crude oil exceeding 1 mg L^{-1} might slightly inhibit the growth of phytoplankton, while concentrations below 1 mg L^{-1} might stimulate phytoplankton growth. Our data showed that the biomass of MPB slightly decreased at 0–10 days after crude oil exposure, but this result was not significant (Fig. 2). MPB exhibit tolerance to crude oil, with measurements of photosynthetic efficiency representing an objective method to demonstrate the adverse effects of oil (Chronopoulou et al., 2013; Özhan et al., 2014b). The primary productivity (max $rETR_m$/max Chl-*a*) values of MPB differed with respect to the bioremediation treatments (Fig. 5). In the bioremediation group supplemented with rice straw (rice straw, emulsifier + rice straw), primary productivity was similar to that of the control (clean sand), and seemed to be caused by lower light input (rice straw covered the sediment surface). Thus, the "no action" treatment represented a good alternative with respect to the recovery of primary productivity (Fig. 5). To our knowledge, the present work is the first to investigate recovery of the MPB assemblage in terms of "functional" response endpoint, viz., production, following oil contamination.

3.4. Recovery integrated comparisons of bioremediation treatment techniques

To improve our understanding of the recovery efficiency of oil contaminated sediments across various bioremediation treatments, the "ratio to negative control values" approach was applied (Fig. 6). This approach provides a reasonable estimate of the overall status of recovery of benthic ecosystem health when integrating these five endpoints, and shows the degree of recovery at each endpoint. Five-angular circular sectors, representing the degree of recovery for the corresponding benthic quality elements were produced for all the 10 treatments. The resulting color of each circle represents the recovery status of the treatments in short-term (light green) and long-term (dark green) aspects (Fig. 6 and Table S3). The results generally indicated that the bioremediation treatments mostly showed good recovery efficiency after 10 days (short-term) and the difference of each treatment was relatively small when long-term (after 90 days) recoveries are compared (Fig. 6 and Table S3). The benthic primary production seemed to be the least sensitive in recovery efficiency but showed fully responded after 90 days for the most of treatments.

In general, the combined application of microbe treatments showed rapid and effective recovery the benthic community, but has some deficiencies: 1) disturbance of indigenous microbial community; 2) low TPH removal efficiency at the initial stage of an oil spill; and 3) difficulty in culturing microorganisms (required longer periods of time, 3–7 days) (McGenity et al., 2012). The combination of emulsifier treatments exhibited rapid TPH removal efficiency, but showed relatively low scores for the recovery of PAHs sensitive bacteria compared to microbial treatments during 90 days. Effendi et al. (2017) reported comparable results to the present study, in that emulsifier could cause negative impact on the microbial community. The rice straw treatments had low recovery efficiencies for MPB biomass and primary production, but showed comparable recovery efficiencies for all the other endpoints, in extremely cost effective manner (Table S6). Rice straw treatment

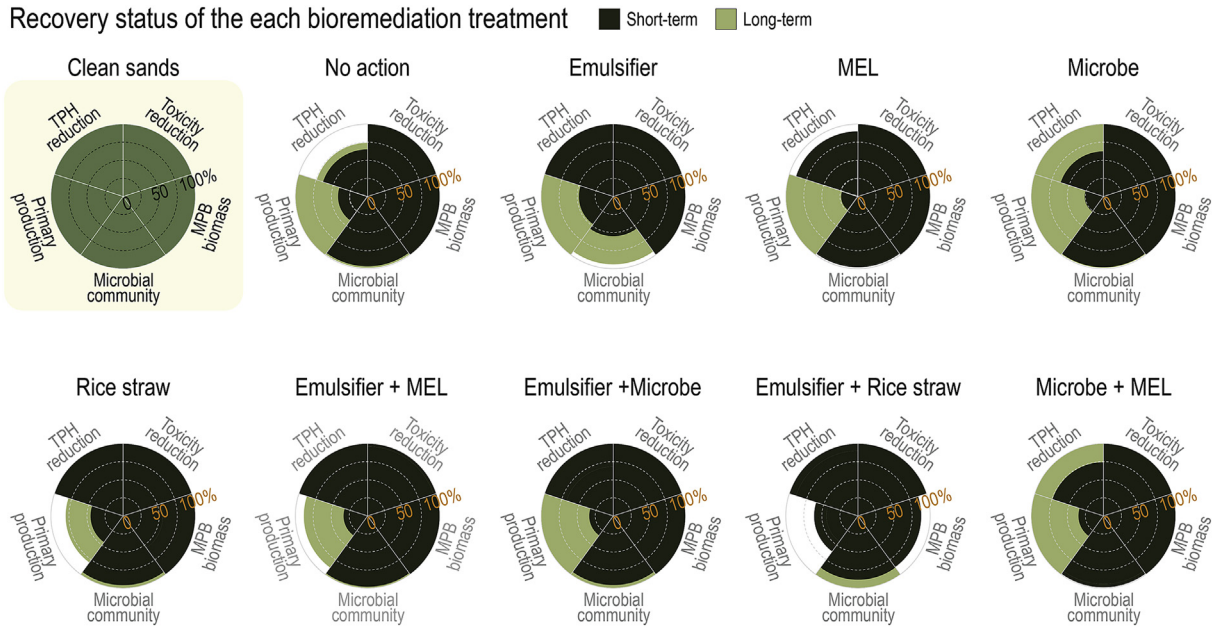

Fig. 6. Comparison of bioremediation efficiency in five target elements; 1) TPH pollution, 2) residual toxicity, 3) bacterial community response, 4) MPB biomass, and 5) primary production relating to each treatment in short-term and long-term exposure periods. Each endpoint was calculated using the ratio to negative control, refer to the details in Data Analyses section and Table S3.

has some limitations: 1) difficulty in installation for the areas with high-wave and strong wind and 2) difficulty in application to a large area. Surprisingly, the "no action" treatment had a good recovery efficiency score, despite only having circulated seawater, for most components. The presented simple additive assessment using a the "ratio to negative control values" method might have certain limitations in that each component is equally considered in scoring, thus further efforts on prioritizing or weighting could be necessary, if appropriate. Altogether, the results suggest that a high recovery efficiency is possible when relatively low concentrations of crude oil in the sediments are exposed to sufficient seawater circulation in coastal areas.

Overall, the current study aimed to determine how the benthic community recovers from oil contaminated sediments by applying different types of bioremediation methods under artificial tidal conditions. All of the bioremediation methods were sufficiently effective to reduce sedimentary TPH residues and toxicity reduction. When considering the recovery time to oil spills and cost, "no action" (i.e., doing nothing, just sufficient seawater circulation) would be one powerful solution without extra cost and/or endeavors. At present, information remains limited on how microbes recover in relation to sediment characteristics and type of crude oils in quantity and composition (such as weathering state etc.). Despite these limitations, the present study provides useful data to establish protocols for application of various cost-effective bioremediation tools in coastal environments.

4. Conclusions

Unexpected oil spills could happen elsewhere and always bring significant environmental issues. In particular, spilled oil lingering in the sediment could cause long-term adverse effects on various benthic organisms. The present study examined the long-term recovery efficiency of various bioremediation techniques, specifically targeting benthic community health following oil contamination in a mesocosm experimental system. Our results generally confirm that presently utilized bioremediation techniques are valid and powerful to remove the residual oils and toxicities. However, the recovery efficiency greatly varied depending on the experimental endpoints, particularly in the short period of time. At the same time, note that the "no action" alternative (i.e. natural bioremediation) may be a potential alternative solution. In addition, the combination of bioremediation techniques did not seem to greatly reduce the recovery time, but interactions between the associated recovery mechanisms may need to be elucidated in future investigations. Overall, the present study successfully addressed the integrated aspects of bioremediation efficiencies being associated with long-term recovery of benthic community health. The present data are useful to establish the application of site- or habitat-specific bioremediation methods in the oil contaminated environments.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.05.100.

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<Supplementary Materials>

Comparative evaluation of bioremediation techniques on oil contaminated sediments in long-term recovery of benthic community health

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Supplementary Tables

Supplementary Figures

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Supplementary Tables

Table S1. General properties of Iranian Heavy Crude oil used in this study.

Properties	Unit	Value
Density (15 °C)	g/ml	0.8756
API Gravity	API	30
Dynamic Viscosity	mPa.s	20
Surface Tension (15 °C)	mN/M	26.1
Interfacial Tension (15 °C, 33psu)	mN/M	22.5
Sulphur Content	% w/w	1.2
Water Content	% w/w	< 0.025
Total Petroleum Hydrocarbons (TPH)	µg/g	300,000
Unresolved Complex Mixture (UCM)	µg/g	190,000
n-Alkane	µg/g	40,000
Total 16 PAHs	µg/g	396
Total alkylated PAHs	µg/g	12,216

Table S2. Summary of the experimental conditions and description of bioremediation techniques tested in this study.

Experimental conditions		
Water temperature (°C)	20	
Salinity (psu)	28-30	
Aeration	Continuous	
DO (%)	> 80%	
pH range	7.9-8.2	
Seawater inlet system	Renewal (100% every 1 day)	
Experimental duration (d)	90	
Bioremediation method application	0, 10, and 20 days	
Monitoring interval	0, 10, 20, and 30 days for TPH 0, 10, 20, 30, and 90 days for amphipod mortality, microbial abundance, and microalgal biomass 0 and 20 days for benthic primary production	
Number of treatment	10	
Number of replicates	2	
Water volume per day	50 L per each treatment, total 1000 L per day	
Sediment size	Φ <1 mm	
Total organic matter contents	~ 6%	
Crude oil	Iranian Heavy Crude oil	
Volume ratio (sediment:oil)	1000:1	
Fertilizer (Oleophilic fertilizer; S200)	15 mL per treatment	
Bioremediation techniques	**Product name**	**Description**
Emulsifier	Tween 80	15 mL per treatment
Multi-enzyme liquid	Oil Spill Eater II	15 mL per treatment
Microbe	Microbial solution (bacteria mixture; *Corynebacterium variabilis*, *Sphingomonas yanoikuyae*, *Kyotococcus sedentarius*, *Bacillus aquimaris*, *Novosphingobium*, *Pentaromativorans*, and *Yarrowia lipolytica*), 50 mL per treatment	
Rice straw	Covered with surface (>1 cm) of oil contaminated sand	

Table S3. Equations used for calculation of bioremediation efficiencies in varying endpoints; the bioremediation efficiencies given in aspects of short-term and long-term, depending on the reliable and/or available data points.

Endpoints	**Variables**	**Equations**	**Data points**	
			Short-term	**Long-term**
TPH reduction	TPH concentration	$E^{*} = (A^{*} / B^{*}) \times 100$	10 day	30 day
Toxicity reduction	Amphipod survival number	$E = (B / A) \times 100$	10 day	90 day
MPB biomass	Chl-*a*	$E = (B / A) \times 100$	10 day	90 day
Microbial community	Microbial diversity index and relative abundance of nitrifier	$E = [[Bd^{**}(B / A) \times 100] + [Rn^{**}(B / A) \times 100]] / 2$	10 day	90 day
Primary production	$rETR_m$	$E = (B / A) \times 100$	20 day	90 day

*E = bioremediation efficiency; A = negative control value; B = treatment value.
**Bd = Mean bacterial diversity index (Simpson, Shannon, Chao 1); Rn = relative abundance of nitrifier
***If the calculated efficiency value is greater than 100%, only 100% is indicated.

Table S4. Shapiro-Wilk normality test results for concentrations of TPH and the bacterial community (phylum).

End-point	Statistic	Degrees of freedom	P value
Log TPH	.948	32	0.13
Acidobacteria	.550	32	< 0.01
Actinobacteria	.868	32	< 0.01.
Bacteroidetes	.967	32	0.42
Chlamydiae	.658	32	< 0.01
Chloroflexi	.334	32	< 0.01
Fusobacteria	.172	32	< 0.01
Gemmatimonadetes	.262	32	< 0.01
Planctomycetes	.980	32	0.80
Proteobacteria	.964	32	0.35
Tenericutes	.172	32	< 0.01
Verrucomicrobia	.893	32	< 0.01
Candidatus Saccharibacteria	.537	32	< 0.01
Cyanobacteria/Chloroplast	.937	32	0.06
Firmicutes	.323	32	< 0.01
Nitrospinae	.334	32	< 0.01
Nitrospirae	.391	32	< 0.01
Parcubacteria	.768	32	< 0.01
Others	.853	32	< 0.01

Table S5. Spearman rank correlation results between TPH concentration and bacterial community (phylum). Values in bold (*r*) indicate a significant correlation Values in bold (*r*) indicate correlation significance at the $p < 0.0l$ level (matched with ++).

	Log TPH	Prot.	Bact.	Acti.	Plan.	Cyan./ Chlo.	Chla.	Acido	Verr.	Firm.	Nitrospinae	Nitrospirae
Log TPH		.006	-.318	.170	.200	.213	.071	.054	-.039	-.376	-.041	-.338
Proteobacteria			-.263	**-.679**	.000	-.240	-.318	.136	.113	.145	-.023	.097
Bacteroidetes				.067	**-.536**	.091	-.084	-.371	.085	.-195	-.128	-.123
Actinobacteria		++			-.239	-.051	.087	-.148	**-.468**	-.041	-.303	-.288
Planctomycetes			++			-.109	**.433**	.167	.069	.-342	.117	-.041
Cyanobacteria/ chloroplast							-.391	-.050	.016	-.160	.362	.082
Chlamydiae					+	+		-.133	.100	-.266	-.227	-.267
Acidobacteria			+						.209	**.556**	.420	**.562**
Verrucomicrobia				++						.019	.271	.369
Firmicutes	+						++	++			.319	**.686**
Nitrospinae					+	+	+	+				**.527**
Nitrospirae							++	++	+	++	++	

++ Significantly correlated at the $p < 0.01$ level (2-tailed).
+ Significantly correlated at the $p < 0.05$ level (2-tailed).

Table S6. The cost of purifying oil-contaminated sediment according each methods.

Treatments	**USD (unit)**	**Cost per unit (USD per m^2)**
Clean sands (negative control)	-	-
No action (positive control)	-	-
Emulsifier (Tween 80)	15 (500 mL)	3.2
Multi-enzyme liquid (Oil Spill Eater II)	1000 (19 L)	5.6
Microbe	0.3 (1 L)	0.1
Rice straw	0.7 (1.1 m^2)	0.7
Emulsifier + Multi-enzyme liquid	-	8.8
Emulsifier + Microbe	-	3.3
Emulsifier + Rice straw	-	3.8
Microbe + Multi-enzyme liquid	-	5.7

Supplementary Figures

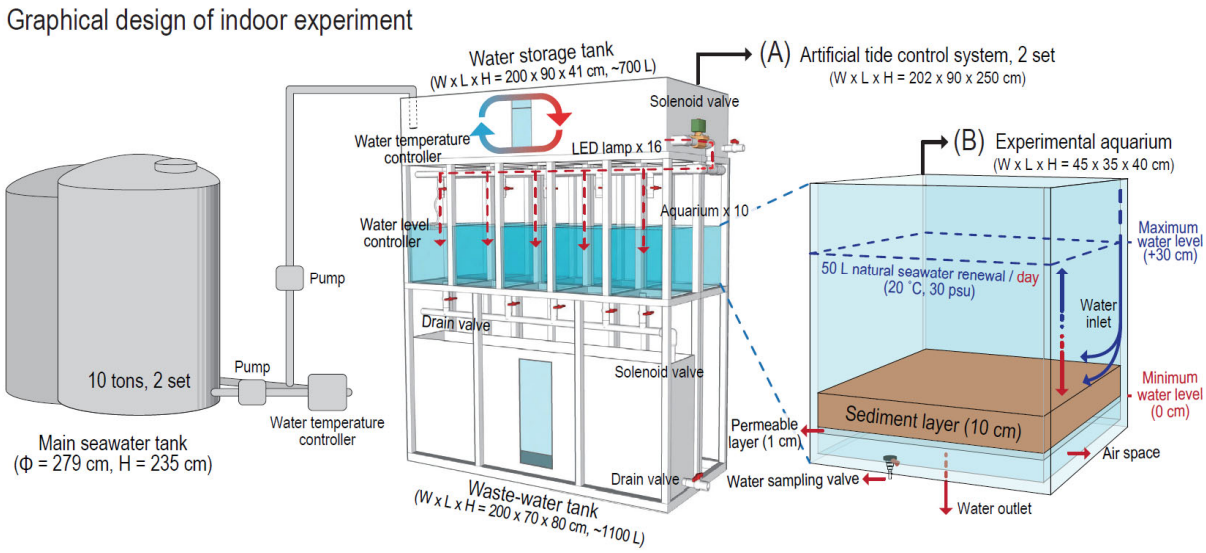

Fig. S1. Schematic of the artificial tide control system. (A) Artificial tide control system, and (B) experiment aquarium. Experimental conditions are detailed in Table 1.

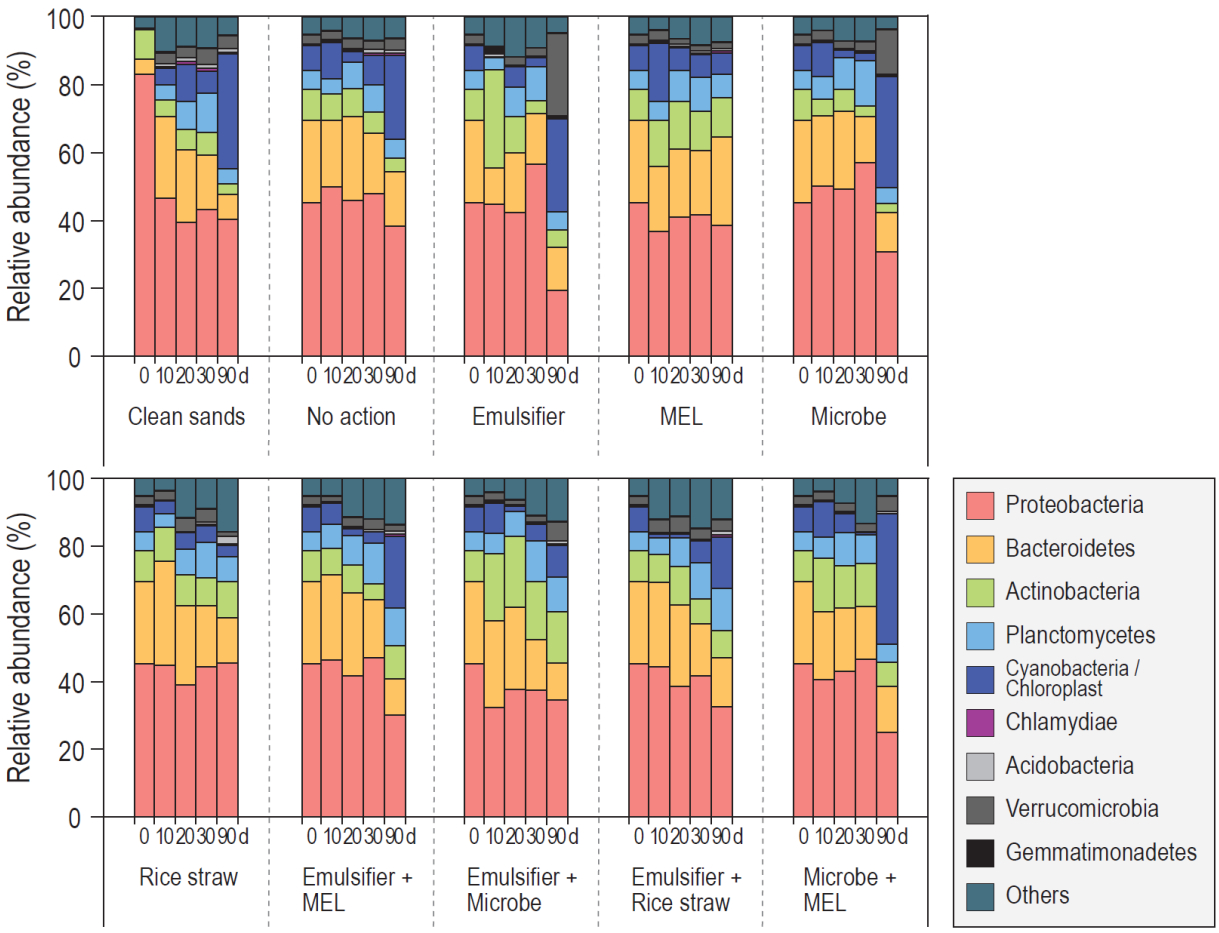

Fig. S2. Relative abundance of the bacterial community (phylum) in the bioremediation treatment during the experiment.

(A) Heatmap of oil-degrading bacteria in the remediation methods (relative abundance)

Class	Genus	Clean sands					No action					Emulsifier					MEL					Rice straw				
		0	10	20	30	90	0	10	20	30	90	0	10	20	30	90	0	10	20	30	90	0	10	20	30	90
Actinobacteria	*Corynebacterium*	0.0	0.0	0.0	0.3	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Dietzia	0.3	0.1	0.0	0.0	0.1	2.7	1.7	1.4	0.8	0.7	2.7	12.6	1.2	0.7	0.4	2.7	1.0	1.0	0.7	0.3	2.7	5.7	3.5	2.7	6.2
	Nocardioides	0.8	0.9	0.1	0.1	0.1	2.7	0.7	0.6	0.4	0.7	2.7	8.0	1.2	0.7	0.4	2.7	0.5	0.7	0.4	0.2	2.7	5.7	3.5	2.1	6.2
Flavobacteriia	*Owenweeksia*	0.0	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.7	0.1	0.0	0.0	0.0	0.7	0.8	0.0	0.0	0.0
Sphingobacterria	*Algoriphagus*	0.3	1.7	0.2	0.0	0.1	1.4	1.1	0.5	0.2	0.2	1.4	0.8	0.4	0.1	0.0	1.4	0.3	0.1	0.0	0.0	1.4	3.7	0.8	0.1	0.2
Planctomycetia	*Planctomyces*	0.0	0.0	0.4	0.4	0.1	1.0	0.6	0.7	0.5	0.2	1.0	0.1	1.4	2.1	0.5	1.0	1.1	1.1	1.3	0.2	1.0	0.7	1.1	0.8	0.6
Gamma-proteobacteria	*Pseudomonas*	6.2	4.5	0.8	0.8	0.5	1.5	0.4	1.0	0.4	0.5	1.5	13.0	0.7	0.8	0.1	1.5	1.9	1.9	1.6	0.5	1.5	4.1	0.8	0.9	1.4
	Alcanivorax	0.1	0.1	0.0	0.0	0.0	4.9	1.5	0.5	0.6	0.2	4.9	0.0	3.8	2.6	0.8	4.9	11.5	2.6	1.9	0.1	4.9	6.2	1.2	0.5	0.3
	Arenimonas	0.9	1.8	0.3	0.2	0.2	2.2	1.5	1.5	0.8	0.8	2.2	1.4	1.3	0.7	0.2	2.2	4.9	2.1	1.9	0.2	2.2	2.4	1.8	1.1	0.2
	Porticoccus	0.9	0.2	2.8	1.1	0.9	2.2	3.3	1.6	2.3	1.7	2.2	0.0	0.9	0.3	0.5	2.2	1.0	1.1	0.7	0.7	2.2	1.8	0.9	2.0	3.3

Class	Genus	Microbe					Emulsifier + MEL					Emulsifier + Microbe					Emulsifier + Straw					Microbe + MEL				
		0	10	20	30	90	0	10	20	30	90	0	10	20	30	90	0	10	20	30	90	0	10	20	30	90
Actinobacteria	*Corynebacterium*	0.0	8.2	8.3	7.9	5.7	0.0	0.0	0.0	0.0	0.0	0.0	15.3	15.6	12.8	6.9	0.0	0.0	0.0	0.1	0.0	0.7	10.9	9.1	11.7	4.6
	Dietzia	2.7	0.7	0.6	0.4	0.9	2.7	2.1	2.1	1.3	2.3	2.7	0.7	0.8	0.6	1.8	2.7	1.6	3.9	1.7	1.4	2.7	0.6	0.5	0.1	0.4
	Nocardioides	2.7	0.6	0.6	0.4	0.6	2.7	2.5	1.0	0.5	1.3	2.7	0.8	0.6	0.6	1.0	2.7	4.1	1.9	0.9	0.8	2.7	1.1	0.5	0.1	0.3
Flavobacteriia	*Owenweeksia*	0.7	0.2	0.0	0.0	0.0	0.7	0.5	0.0	0.0	0.0	0.7	0.5	0.0	0.0	0.0	0.7	1.5	0.0	0.0	0.0	0.7	0.2	0.0	0.0	0.0
Sphingobacterria	*Algoriphagus*	1.4	0.8	0.2	0.1	0.1	1.4	1.9	1.3	0.1	0.1	1.4	2.8	2.2	0.4	0.3	1.4	7.1	2.3	0.3	0.0	1.4	0.1	0.1	0.0	0.0
Planctomycetia	*Planctomyces*	1.0	0.6	0.9	0.7	0.3	1.0	2.0	2.0	2.9	0.9	1.0	0.8	1.4	2.0	1.0	1.0	2.0	2.8	2.1	0.9	1.0	0.8	0.7	0.8	0.2
Gamma-proteobacteria	*Pseudomonas*	1.5	1.2	0.8	0.8	1.1	1.5	1.8	1.0	2.8	0.6	1.5	0.9	0.9	0.8	0.2	1.5	1.2	1.2	0.5	0.2	1.5	1.5	0.7	2.0	1.0
	Alcanivorax	4.9	2.4	1.5	0.8	0.2	4.9	4.1	2.0	1.9	1.2	4.9	2.5	3.1	2.0	1.2	4.9	9.3	2.7	1.5	0.8	4.9	6.4	2.0	3.2	1.5
	Arenimonas	2.2	0.3	0.3	0.2	0.2	2.2	7.1	4.9	2.2	1.0	2.2	2.6	2.9	1.4	1.4	2.2	4.3	4.5	1.5	0.6	2.2	1.1	0.9	0.2	0.4
	Porticoccus	2.2	1.2	1.5	1.6	2.9	2.2	2.4	0.5	0.2	1.4	2.2	2.0	0.8	0.6	2.1	2.2	0.5	0.6	0.3	0.6	2.2	1.7	1.3	1.9	1.1

*PAHs degrading bacteria

(B) Bacterial composition similarity among the remediation methods

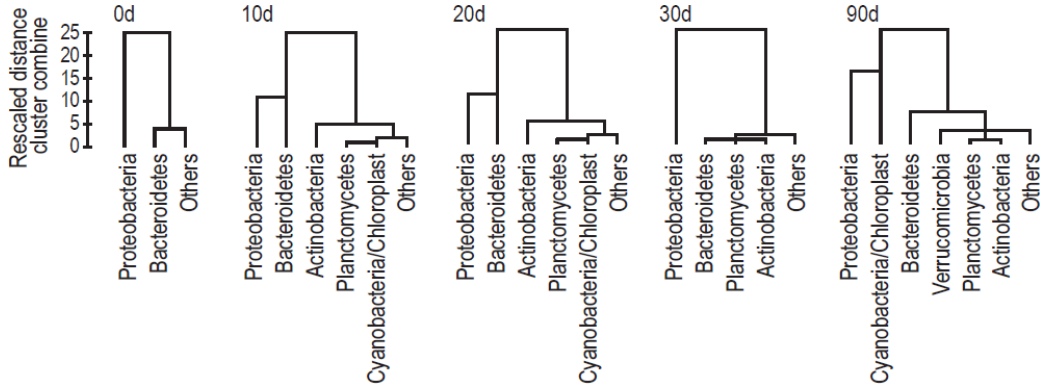

Fig. S3. (A) Comparison of the relative abundance of PAH-degrading bacteria in the bioremediation treatments and (B) similarity in bacterial composition among the bioremediation treatments during the experiment.