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Environmental Impact Statement

Oil spills and the dispersant application increase the concentration of dissolved petroleum hydrocarbons in water column which are potentially toxic to marine ecosystem. In this paper, simulated bioremediation experiments on oil spill-polluted marine shoal were conducted in open custom-designed devices. The impact of different substrates on microalgae followed a decreasing order: the microbial consortium plus Tween-80 > the microbial consortium > Tween-80. The acute toxicity effects of different substrates on microalgae illustrated that the microalgae in the biotreated seawater was recruited significantly during 96 h, suggesting the oil spill-polluted seawater quality was obviously improved by the bioremediation.The simulation and assessment of the ecological response to enhanced bioremediation can facilitate the better management of future oil spill restoration.

Bioremediation of oil spill polluted marine intertidal zone and its toxicity effect on microalgae

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Abstract: Custom-designed device with 0.6 m (*L*)×0.3 m (*W*)×0.4 m (*H*) and a microbial consortium were applied to simulate bioremediation on oil spill-polluted marine intertidal zone. After the bioremediation, the removal efficiency of *n*-alkanes and polycyclic aromatic hydrocarbons homologues in crude oil evaluated by GC-MS were higher than 58% and 41% respectively. Besides, the acute toxicity effects of crude oil on three microalgae, i.e. *Dicrateria sp.*, *Skeletonema costatum* and *Phaeodactylum tricornutum*, varied with concentration. The effects of microbes and surfactant treated water on three microalgae followed a decreasing order: the microbial consortium plus Tween-80 > the microbial consortium > Tween-80. During 96 h, the cell densities of the three microalgae in treated seawater increased from 4.0 $\times 10^5$, 1.0×10^5 and 2.5×10^5 cells•mL^{-1} to 1.7×10^6, 8.5×10^5 and 2.5×10^6 cells•mL^{-1} respectively, which illustrated that the quality of seawater contaminated by crude oil was significantly improved by the bioremediation.

Keywords: bioremediation, marine oil pollution, microalgae, toxicity effect

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assessment

1 Introduction

Petroleum is one of the most important energy resources and raw materials for the chemical industry.Rising global energy demand has resulted in an increase in the extraction and transportation of crude oil in the sea, making marine environments especially susceptible to increased risk of crude oil spills. The latest examples of large-scale marine oil spills are the "Deepwater Horizon" blowout in Gulf of Mexico on 20th April 2010 and the oil pipeline explosion in Dalian, China on 16th July 2010. Therefore, huge quantities of spilled oils and petroleum products might enter the sea leading to serious environmental problems and extensive damages to marine life, human health and ecological resources and systems.[1-2]

Bioremediation which is economical and environmental friendly is considered to be one available and effective technique to eliminate oil pollution from contaminated environments and reduce damages caused by oil spills in comparison with other physical and chemical methods.[3-5] Crude oil is an extremely complex mixture containing thousands of different hydrocarbon compounds such as *n*-alkanes, aromatics, resins, asphaltenes and many other chemical compounds.[6] Many microorganisms are known to be able to attack and degrade some specific oil components and/or certain classes of oil hydrocarbons.[7,8] At present, about 70 genera and more than 200 species of microorganisms have been found to utilize and degrade one or more hydrocarbons. In addition, it has been reported that bioremediation can

remove oil hydrocarbons from oil contaminated environments successfully and effectively.[9, 10]

During a bioremediation process, oil biodegradation efficiency may be limited by several factors such as substrate, oxygen, nutrient concentration, environment sensitivity and the abundance of oil-degrading microorganisms themselves.[11] Bioaugmentation and biostimulation by adding microorganisms, nitrogen, phosphorus and surfactant have been employed to improve and enhance bioremediation efficiency.[12, 13] However, most of the experiments have been performed in microcosm and few studies have been dedicated to investigate bioremediation on marine intertidal zone.[14, 15] In order to study bioremediation performance of oil-degrading microorganisms in natural circumstances, it is necessary to simulate the bioremediation condition in amplified models.

Phytoplankton is one of primary producers, which sustains the pelagic food chains in the aquatic ecosystems. One of the factors supporting the use of algae in bioassays is their niche in the aquatic ecosystem.[16-18] If these organisms are adversely affected by a toxicant, the surrounding ecosystem may also be implicated, either directly or indirectly, due to the lack of a food source. Thus, phytoplankton being sensitive to any change of seawater quality is a good bioindicator. Extensive investigations on toxicities of crude oil, polycyclic aromatic hydrocarbons (PAHs) and metals to a variety of fish and mollusk species have been conducted.[19-21] Pasternak and Ko1wzan[22] had reported that microtox toxicity changes during biodegradation of carbazole by newly isolated methylotrophic strain

methylobacterium sp. GPE1 without microalgae. However, little attention has been paid to the toxicity effect of a bioremediation process on marine microalgae and the evaluation of the ecological response to bioremediation. In addition, previous studies only covered the impact of oils on algae without biodegradation.[16, 23]

In this paper, open custom-designed devices were employed to simulate oil-spill bioremediation on marine intertidal zone. We also characterized the predominant bacterial strains isolated from petroleum-contaminated marine sediments. This study evaluated the hydrocarbon-utilizing capability of microbial consortium and surfactant and their oil removal performance by bioaugmentation and biostimulation in mesocosm on contaminated marine intertidal zone. In addition, the toxicity effects of different substrates on *Dicrateria sp.*, *Skeletonema costatum* and *Phaeodactylum tricornutum* in the bioremediation process were evaluated. This work will provide a support for the practical applied feasibility of the microbial consortium by enhanced bioremediation in marine oil-spill environment.

2 Materials and methods

2.1 Materials

2.1.1 Chemicals

All chemicals used in this work were analytical grade and obtained from various commercial sources. The crude oil which was purchased from Shengli oilfield (Dongying, Shandong, China) was dissolved in the hexane and filtered to remove un-dissolved precipitate (such as colloids and asphaltenes), and it had a viscosity of 22.2 mPa·s (50 ºC, 50 RPM) and a density of 0.855 g·cm^{-3}.

2.1.2 Microbial consortium and microalgae

A microbial consortium was isolated from petroleum-contaminated sediments in Bohai Sea and then was recultivated in the laboratory conditions using only oil as the carbon source, which has enhanced the capacity of degrading crude oil. Previous studies in our laboratory had shown that the microbial consortium exhibits strong biodegradation potential, i.e. crude oil degradation efficiency reached up to 50% at the optimum growth conditions of pH 6-9, temperature 20-30 °C and salinity 10‰-35‰. The microbial consortium was cultivated in beef extract peptone medium at 30 ºC with shaking at 120 rpm for 24 h before used.

Marine microalgae (*Dicrateria sp.*, *Skeletonema costatum* and *Phaeodactylum tricornutum*) used in toxicity assessment experiments were obtained from The First Institute of Oceanography, SOA (Qingdao, Shandong, China). The microalgae were cultivated at 20 ± 1 ºC, with an irradiance of 3000-4000 lux cold light source and a 12 h light, 12 h dark photoperiod. The algae were sampled at a certain time and fixed with Lugol's solution. Then algal cells were counted using a hemocytometer under a light microscope.

2.1.3 Seawater and sand samples

Seawater and sand used for the simulated bioremediation experiments were collected from Shilaoren beach (Qingdao, Shandong, China). Seawater with pH of 8.1 and salinity of 32‰, was filtrated with 0.45 μm pore-size filters before used in microalgae toxicity assessment experiments.

2.1.4 Media

Nutrient (N, P and Si) solution was composed of (1 L filtered seawater) 75 g $NaNO_3$, 5 g $NaH_2PO_4 \cdot H_2O$ and 30 g $Na_2SiO_3 \cdot 9H_2O$. Trace elements solution was composed of (1 L filtered seawater) 2.45 mg $CuSO_4 \cdot 5H_2O$, 19.9 mg $Na_2MoO_4 \cdot 2H_2O$, 22 mg $ZnSO_4 \cdot 7H_2O$, 10 mg $CoCl_2 \cdot 6H_2O$, 180mg $MnCl_2 \cdot 4H_2O$, 1.3 mg H_2SeO_3, 2.7 mg $NiSO_4 \cdot 6H_2O$, 1.84 mg Na_3VO_4 and 1.94 mg K_2CrO_4. Vitamins solution was composed of (1 L filtered seawater) 1.0 g VB_{12}, 0.1 g VH and 0.2 g thiamine hydrochloride. Final medium for cultivating marine microalgae was composed of 1 L filtered seawater to which 1 mL of nutrient, 1 mL trace elements and 0.5 mL vitamins solutions were added. These media were prepared for cultivating marine microalgae.

Crude oil medium used for bioremediation experiments was composed of 20 g NaCl, 0.7 g $MgSO_4 \cdot 7H_2O$, 3.0 g KH_2PO_4, 1.5 g Na_2HPO_4, 0.1 g anhydrous $CaCl_2$, 1.0 g yeast extract powder and 5.0 g crude oil, the substances to make the medium were added to 1 L of distilled water. Beef extract peptone medium was composed of 10 g peptone, 20 g NaCl and 3.0 g beef extract. The pH of crude oil medium and beef extract peptone medium were adjusted to 7.0-7.5 with either HCl or NaOH solutions, then they were sterilized in an autoclave at 121 ^{o}C for 20 min.

2.2 Identification of bacteria isolates

2.2.1 Biochemical characterization

Biochemical tests such as gram staining, catalase reaction, methyl red test, V-P, indole and the hydrolysis of cellulose were performed to identify the morphological, physiological and biochemical characterization of the isolates, according to manual of common bacterial identification.[24]

2.2.2 DNA extraction, PCR amplification and sequences analysis

Total DNA was extracted from the bacterial strains using a TIANamp Bacteria DNA extraction Kit (Tiangen Biotech Co., Ltd, Beijing, China). PCR (Polymerase chain reaction) was performed on the DNA extracts of the isolates using a pair of PCR universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-AAGGAGGTGATCCAGCCGCA-3'). The reaction mixture (50 μL) contained 10 ×PCR buffer (5μL), 1.5 mmoL·L^{-1} $MgCl_2$ (3μL), 2.5mmoL·L^{-1} dNTP (1μL), 0.4 U of Tap DNA polymerase, 10 μmoL·L^{-1} 27F (1μL), 10 μmoL·L^{-1} 1492R (1μL) and 0.8 μL template DNA. Amplification program was performed with initial denaturation step at 94 ^{o}C for 5 min; followed by 32 cycles of 1 min denaturation step at 94 ^{o}C, 1 min annealing step at 55 ^{o}C and 90 s elongation step at 72 ^{o}C; and a final extension step at 72 ^{o}C for 15 min. The amplified products were purified and cloned. The DNA sequences were determined with the chain-termination method on a DNA sequencer. All sequences were aligned in the GenBank database using the BLASTN program and subjected to a similarity search on the NCBI website (http://www.ncbi.nlm.nih.gov). Many relevant available 16S rDNA gene sequences were selected as the references from GenBank. The sequence alignment was carried out with ClusterX (version 2.0). Phylogenetic trees were constructed using the neighbor-joining (NJ) method by software MEGA 4.0. The scale bar indicated the average number of amino acid substitutions per site.

2.3 Simulated bioremediation experiments on oil spill-polluted marine shoal

The simulated bioremediation experiments on oil spill-polluted marine intertidal

zone were conducted in five custom-designed tanks with 0.6 m (*L*) × 0.3 m (*W*) × 0.4 m (*H*). Each tank was filled with 41 kg sand, 205 g crude oil and 20 L seawater to simulate oil-spill marine shoal. Enhanced bioremediation technology influenced by microbes, surfactant and nutrient was employed. To test the impact of nitrogen (N)-source, phosphorus (P)-source, surfactant and microbes on components of crude oil biodegradation, different experimental designs were applied whose details are shown in Table 1. The tanks were named from 1# to 5#. Tank 1#, 3#, 4# and 5# were supplemented with 20 g yeast powder and 60 g KH_2PO_4. Tween-80 (15mL) was added into tank 4# and 5#. Tank 3# and 5# were spiked with 5% (*v*/*v*) of the microbial consortium. All tanks were put in dry and ventilated places at ambient temperature (25 ^{o}C) for 21 d. The seawater was added in the tank following the sand covered on the bottom of the tank. Marked the seawater level on each tank after the water claming. The seawater was drained after heated oil (approximately 50 ^{o}C) spread evenly on it with a slow flow to make the oil attaching on the sand naturally after the oil stabilizing on the water surface. After standing 12 h, the seawater was added to the calibration recorded. Moreover, the aeration equipment was employed to cycle the oxygen in the seawate.

2.4 Petroleum hydrocarbons analysis

Analyses for *n*-alkanes, PAHs and biomarker compounds were performed on a Shimadzu (Kyoto, Japan) GC-MS-QP2010. A DB-5MS capillary column (30 m×0.25 mm I.D.) was used for GC-MS. Helium was employed as a carrier gas. System control and data acquisition were achieved with a GC-MS solution software. Detailed

chromatographic conditions and quality control were described in the papers of Wang[25] and Sun.[26]

2.5 Toxicity effect of biotreated seawater on microalgae

The acute toxicity effects of dissolved crude oil, microbial consortium, surfactant (Tween-80) and biotreated seawater on three microalgae were assessed in three batch experiments. In the first experiment, dissolved crude oil adjusted to 0, 10, 20, 40, 60 and 80 mg•L-1 in f/2 medium was used. In the second experiment, the effects of f/2 media spiked with 5% (v/v) of the microbial consortium and/or 0.075% (v/v) of surfactant were evaluated.In the third experiment, biotreated seawater was collected from tanks 1# to 5# after 21 d of the simulated bioremediation. This water was inoculated with three microalgae and then incubated in photochemical incubator.

Toxicity effect on algae in all three experiments was assessed by measuring cell densities at different time points during 96 hours.

3 Results and discussion

3.1 Characterization and identification of the isolates

Three bacterial strains composing the utilized microbial consortium were named as S-1, S-2 and S-3. Their morphological, physiological and biochemical characteristics were shown in Table 2. The colonies of S-1, S-2 and S-3 were milky-white, white and orange colors, respectively. All bacteria were gram-positive and found to be baculine by electron microscope. They were oxidized form in the glucose oxidation test. Strains S-1 and S-3 had spores, while S-2 did not have spores.

Based on GenBank database, the 16S rDNA gene sequences of the three bacterial strains were aligned and checked their similar strains manually. The 16S rDNA sequences of strains S-1, S-2 and S-3 had 97%, 98% and 99% similarity to *Bacillus cereus* (GenBank ID: GU369810.1), *Lysinibacillus fusiformis* (GenBank ID: JQ900546.1) and *Rhodococcus* sp. (GenBank ID: KC200263.1) are respectively shown. The three genera were isolated from petroleum-contaminated environment and employed in bioremediation processes.[27-30] The phylogenetic trees of the three strains were respectively shown in Fig. 1 a-c. Sequence analysis of the 16S rDNA gene, BLAST sequence comparison and phylogenetic analysis confirmed that the bacteria S-1, S-2 and S-3 (GenBank IDs) were respectively affiliated with *Bacillus cereus* (KF033125), *Lysinibacillus* sp. (KF033126) and *Rhodococcus rubber* (KF033127).

3.2 Biodegradation of crude oil

To investigate the biodegradation efficiency of crude oil with different enhanced bioremediation agents on oil spill-contaminated marine shoal, the degradation process in oil-amended mesocosm with the microbial consortium, *N*-source, *P*-source and Tween-80 was assessed by studying the changes in the chemical composition of crude oil using pristane and phytane as internal conservative markers. During the remediation process, crude oil in tanks with the microbial consortium and/or Tween-80 were emulsified and gradually turned into small pellets or patches in different degrees, which has consistent results with the reports of Zahed et al.[31] The seawater became more turbid due to the cell growth and dissolved crude oil than the corresponding control.

3.2.1 *n*-alkanes

The crude oil samples in different tanks showed similar changes for the *n*-alkanes in the C9 to C30 range. The degradation depletion was calculated by the different value divided by the initial value, while the different value was between the amount of crude oil before and after the experiments. The degradation of *n*-alkanes decreased as the carbon number increased. It appeared that the *n*-alkanes (from C_9 to C_{30}) were removed in different degrees(Fig. 2 a). *n*-alkanes ranging from C_9 to C_{24} were highly degraded compared with *n*-alkanes ranging from C_{25} to C_{30} in tank 3# and 5#, while in tank 2# and 4# *n*-alkanes ranging from C_9 to C_{15} were fluctuated degraded. Most of the short chain *n*-alkanes (i.e., C_{11}-C_{24}) were degraded by day 21. However, the content of the long chain *n*-alkanes (C_{25}-C_{30}) in tank 1#, 2#, 4# and 5# and the short chain *n*-alkanes (C_9 and C_{10}) in tank 1# and 2# were increased after day 21. This resulted in the bacteria consumed the short chain *n*-alkanes (C_{11}-C_{24}) first to much shorter *n*-alkanes (C_9 and C_{10}), the longer chain *n*-alkanes (C_{25}-C_{30}) have not been consumed by the bacteria, however the total *n*-alkane is reduced, compared to the beginning of the control group, the percentage is relatively negative. Comparing tank (3# and 5#) with tank (1#, 2# and 4#), the *n*-alkanes (from C_9 to C_{24}) were significantly removed, which may be caused by the biodegradation of the microbial consortium. The enhanced bioremediation efficiency of *n*-alkanes was the most significant in tank 5# due to the addition of the N-, P-source and Tween-80.Rahman[32] et al had reported significant positive effects on the bioremediation of n-alkane in petroleum sludge by adding bacterial consortium, rhamnolipid biosurfactant and

nitrogen, phosphorus and potassium solution.In tank 5#, the shorter chain *n*-alkanes of C_9-C_{12} were completely degraded, the *n*-alkanes of C_{13}-C_{24} were degraded by 60%-70%. However, the longer ones of C_{26}-C_{30} were resistant to degradation. The degradation ratio of total *n*-alkanes in tank 5# was higher than 58% on average. Dastgheib et al.[33]have isolated a halotolerant *Alcanivorax* sp. Strain Qtet3 which degrades a wide range of *n*-alkanes (from C_{10} to C_{34}) with considerable growth on C14 and C16 with the highest hydrocarbon degradation of 26.1% observed at 2.5% NaCl. Two strains growing on crude oil from hypersaline sabkhas in Kuwait also utilized Tween 80 and a wide range of individual aliphatic hydrocarbons (C_9–C_{40}) and the oil biodegradation *M. sedimentalis* and *M. flavimaris* was 76 – 90 and 71 % respectively [34]. The oil degradation in this paper is just between the results of the two studies above, therefore further research should be conducted to rise the degradation.

3.2.2 PAHs

Five targeted alkylated PAH homologues (naphthalene, phenanthrene, diphenylazolethiophene, fluorene and chrysene) were selected to represent aromatic hydrocarbon compounds. The degradation depletion on the distribution of 2-4 ringed aromatics were shown in Fig. 2 b. The alkylated PAHs were almost removed above 32% in all tanks, however the weathering effect on degradation was weak. The total biodegradation depletion of phenanthrene, chrysene and their alkyl derivatives in tank 5# were greatly higher than those in tank 1#-4#, reached 30% and 60% respectively. The total removal efficiency of all targeted alkylated PAH homologues by biodegradation was higher than 41% on average in tank 5#. These biodegradation

removal are significantly higher than the reported results of a recent study on the degradation of the *Prestige* fuel oil by a highly specialized PAH-degrading microbial consortium that showed a 22-30% degradation of alkyl fluoranthenes/pyrenes and a 22-25% degradation of alkyl chrysenes.[35] Acevedo et al[36] isolated a white-rot fungus *Anthracophyllum discolor* which had a high removal capability for phenanthrene(62%), anthracene(73%), fluoranthene (54%), pyrene (60%) and benzo(a)pyrene (75%). These biodegradation percentages are substantially higher than our research, so suitable conditions must be found to promote the biodegradation percentage of the PAHs. Vila et al[37] isolated a marine microbial consortium proved highly efficient in removing three to five-ring polycyclic aromatic hydrocarbons [PAHs; including anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, and benzo(a)pyrene] (30-100%), and shown to considerably degrade their alkyl derivatives. The biodegradation of *n*-alkanes and PAHs could be possibly explained that the surfactant stimulated crude oil dissolved in formation and microbial consortium promoted the biodegradation of petroleum. Campo[38] et al reported oil biodegradation in the Gulf of Mexico was not inhibited by dispersants and that Corexit EC9500A can increase the extent of oil degradation under some circumstances for some hydrocarbon classes.

3.3 Acute toxicity effect on marine microalgae

3.3.1 Acute toxicity effect of crude oil on marine microalgae

The acute toxicity effects of dissolved crude oil with different concentrations on the growth of three microalgae are depicted in Fig. 3. The cell densities of *Dicrateria*

sp. and S*keletonema costatum* were higher than the individual control on 24 h when the concentration of dissolved crude oil was lower than 20 $mg \cdot L^{-1}$, but their growth was inhibited with increasing exposition time and remained unchanged at 80 $mg \cdot L^{-1}$ of crude oil concentration. From these results, it is possible to infer that lower concentration of dissolved crude oil could stimulate the growth of the two microalgae in a shorter time, while inhibited the growth in a longer time. The growth of *Phaeodactylum tricornutum* increased with the increasing concentration of the dissolved oil (0-80 $mg \bullet L^{-1}$.) These results demonstrated that *Dicrateria* sp. and *Skeletonema costatum* were sensitive to dissolved crude oil, while *Phaeodactylum tricornutum* had a certain resistance to crude oil. Physiological acclimatization was considered as a plausible explanation for the microalgae diversity in different concentration gradients of crude oil and crude oil inhibited the photosynthesis of microalgae which led to the decrease of algae density.[39]Fabregas et al. indicated that toxicity for marine microalga *Tetraselmis suecica* was increased with higher concentrations, and longer extension of the lag phase and lower cellular density in the stationary phase occurred.[40]Hing et al. designed a continuous culture conditions to model the effect of diesel on the growth behaviour of natural phytoplankton communities in the oceans in the event of an oil spill and the results showed the effect of diesel on steady state growth differed among species and the number of cells affected was largely dependent on the cell concentration at steady state. The higher the cell concentration at steady state, the lower the effect of diesel on the phytoplankton.[41] Chao et al. verified the acute toxicity of four fuel oils including F120,

F180, F380 and No.-20 to the marine microalgae *Chlorela spp.* (Chlorophyta) and *Skeletonema costatum* (Bacillariophyta) and showed that F180 was the most toxic to both microalgae, and the 96 h EC_{50} value for *Skeletonema costatum* and *Chlorela spp.* was 9.41 and 13.63 mg/L expressed in concentration of total petroleum hydrocarbons, respectively.These studies are focused on the toxicity of crude oil, oil spill and fuel for marine microalgae, not considering the remediation effects of microorganism on the contaminated environment, while in this paper this effect was concerned.

3.3.2 Acute toxicity effect of microbes and surfactant on marine microalgae

Fig. 4 shows the effect of the microbial consortium and/or surfactant Tween-80 treated seawater on the growth of the three microalgae. The impact of different substrates on three microalgae followed a decreasing order: the microbial consortium plus Tween-80 > the microbial consortium > Tween-80. The effect of the microbial consortium plus surfactant Tween-80 on the three microalgae was the most significant and became prominent along the time, which probably resulted because of the biological competition for the nutrients between the microbial consortium and microalgae and the toxicity of Tween-80 to the microalgae. Tween-80 is a non-ionic surfactant and may be adsorbed into the cell membrane to affect the growth of microalgae. Previous studies reported that adsorbed surfactant LAS could exert toxicity on cells through the denaturation and the binding of proteins in the cell wall and consequently the alteration of membrane permeability to nutrients and chemicals.[42] Rial et al[43] assessed the effects of four cleaning agents on microalgal growth kinetics and conducted that spill-treating agents had different toxicity,

especially Agma OSD 569. The results from above suggested Tween-80 might produce toxicity effect on microalgae in a similar way as surfactant LAS and Agma OSD 569 did.

3.3.3 Acute toxicity effect of biotreated seawater on marine microalgae

To assess the ecological response to the enhanced bioremediation of marine oil spill, the toxicity effect on microalgae in the bioremediation process was evaluated in many reports[44,45]. Fig. 5 showed the acute toxicity effect of the repaired seawater on the cell densities of the three microalgae. The three microalgae in tank 1# grew more vigorously than those in tank 2#, which indicated that nutrients could stimulate the growth of microalgae even in the toxic environment. Comparing tank (1#, 2# and 4#) with tank (3# and 5#), the cell densities of three microalgae with the microbial consortium were lower than those without the microbes, which was similar to the result described in **section 3.3.2**. In addition, the holistic impact of nutrients, surfactant and microbes on three microalgae in tank 5# was the most obvious. These results were mainly due to the changes of phytoplankton magnitude, the group involved in food web and the physical characteristics of the water, such as concentrations of dissolved organic compounds and nutrient loading affected the growth of marine microalgae and further impacted the marine ecosystems.[46] However, these three microalgae were dead in the seawater. During 96 h, the cell densities of *Dicrateria* sp., S*keletonema costatum* and *Phaeodactylum tricornutum* in the biotreated seawater by 21-day-bioremediation were boosted along the time, which were increased from 4.0×10^5, 1.0×10^5 and 2.5×10^5 cells·mL^{-1} to 1.7×10^6, 8.5×10^5 and

351 2.5×10^{6} cells·mL^{-1} respectively. Moreover, the petroleum-resistant mutants were
352 undeveloped in a short time.[16] These results suggested that the seawater contaminated
353 by oil spill was significantly improved during the bioremediation process.

354 ## 4 Conclusions

355 Simulated bioremediation experiments on oil spill-polluted marine intertidal
356 zone were conducted in open custom-designed devices using a microbial consortium
357 of *Bacillus cereus* S-1, *Lysinibacillus* sp. S-2 and *Rhodococcus ruber* S-3. This
358 microbial consortium exhibited exceptional ability of degrading crude oil. GC-MS
359 analysis showed that removal efficiencies of *n*-alkanes and PAH homologues in crude
360 oil were higher than 58% and 41% using enhanced bioremediation. Moreover, the
361 acute toxicity effects of different substrates on three microalgae were estimated.
362 During 96 h, the cell densities of *Dicrateria* sp., S*keletonema costatum* and
363 *Phaeodactylum tricornutum* in the biotreated seawater showed a significant growth
364 trend, suggesting the oil spill-polluted seawater quality was obviously improved by
365 the bioremediation. The simulation and assessment of the ecological response to
366 enhanced bioremediation with bacteria used in this study can facilitate the better
367 management of future oil spill pollution.

368

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Table 1 The Details of Enhanced Bioremediation Condition

Tank Number	Crude oil	Hydrocarbon degradation bacteria	Nutrients	Surfactant (Tween-80)
1#	+	-	+	-
2#	+	-	-	-
3#	+	+	+	-
4#	+	-	+	+
5#	+	+	+	+

"+"represents that the substrates exist in the tank, while "-" represents that the substrates do not exist in the tank.

Table 2 Morphological, Physiological and Biochemical Identification of the Three Bacterial Isolates

Item	S-1[a]	S-2[a]	S-3[a]
Colony color	Milky-white	white	Orange
Colony surface	Wet smooth	Wet smooth	Wet smooth
Sharpe of cells	Rod	Short rod	Short rod
Gram staining	+	+	+
Spore staining	+	-	+
Catalase reaction	+	+	+
Methyl red test	-	-	+
V-P test	+	-	-
Gelatin liquefaction	+	+	-
Amylolysis test	-	-	-
Hydrate cellulose	-	-	-
Glucose oxidation	Oxidized form	Oxidized form	Oxidized form

[a] Results of substrate-utilizing test were interpreted after 24 h incubation at 36 ºC in bacteriological incubator.

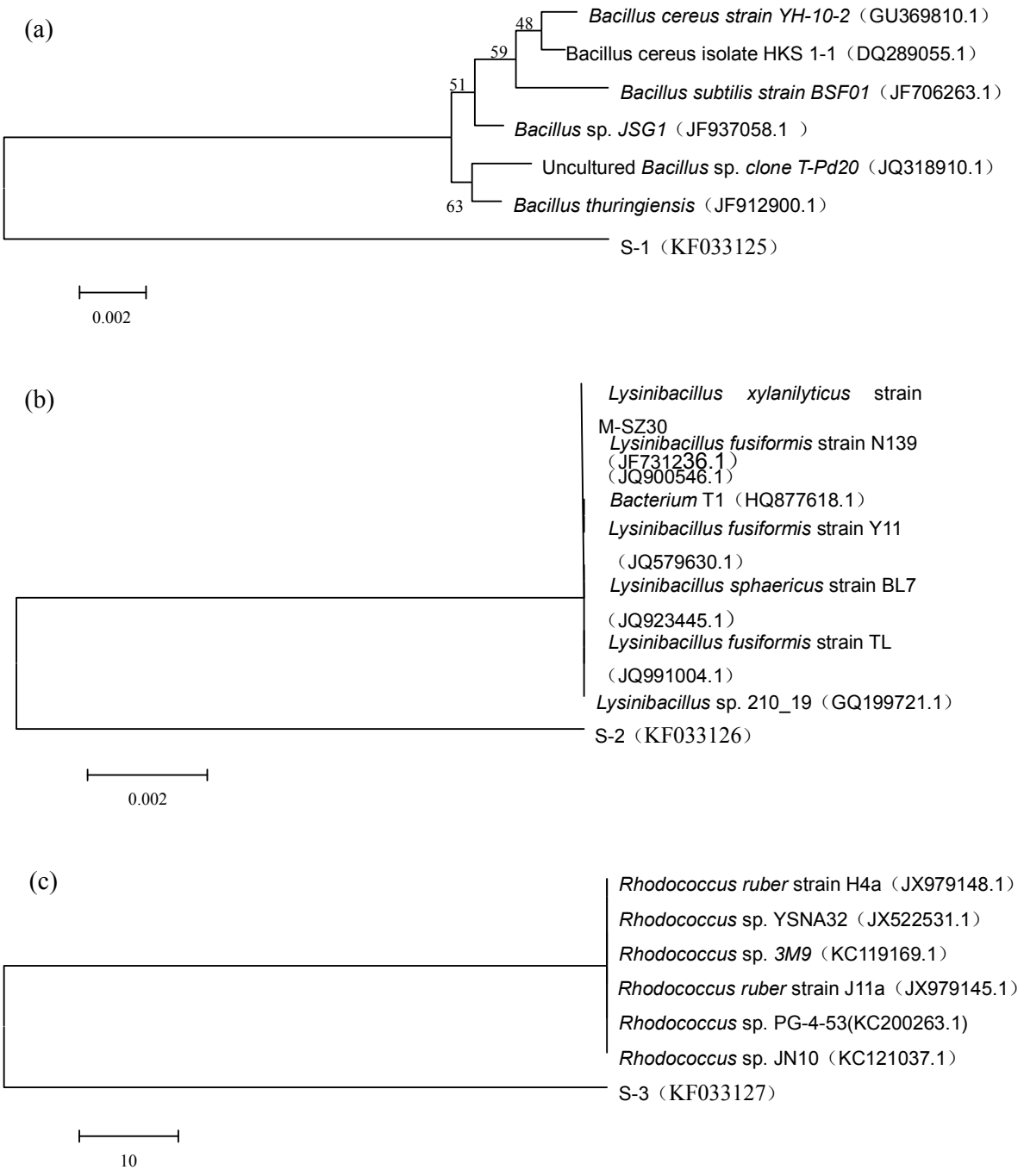

Fig. 1 Phylogenetic trees of the three strains.(a) S-1; (b) S-2; (c) S-3.

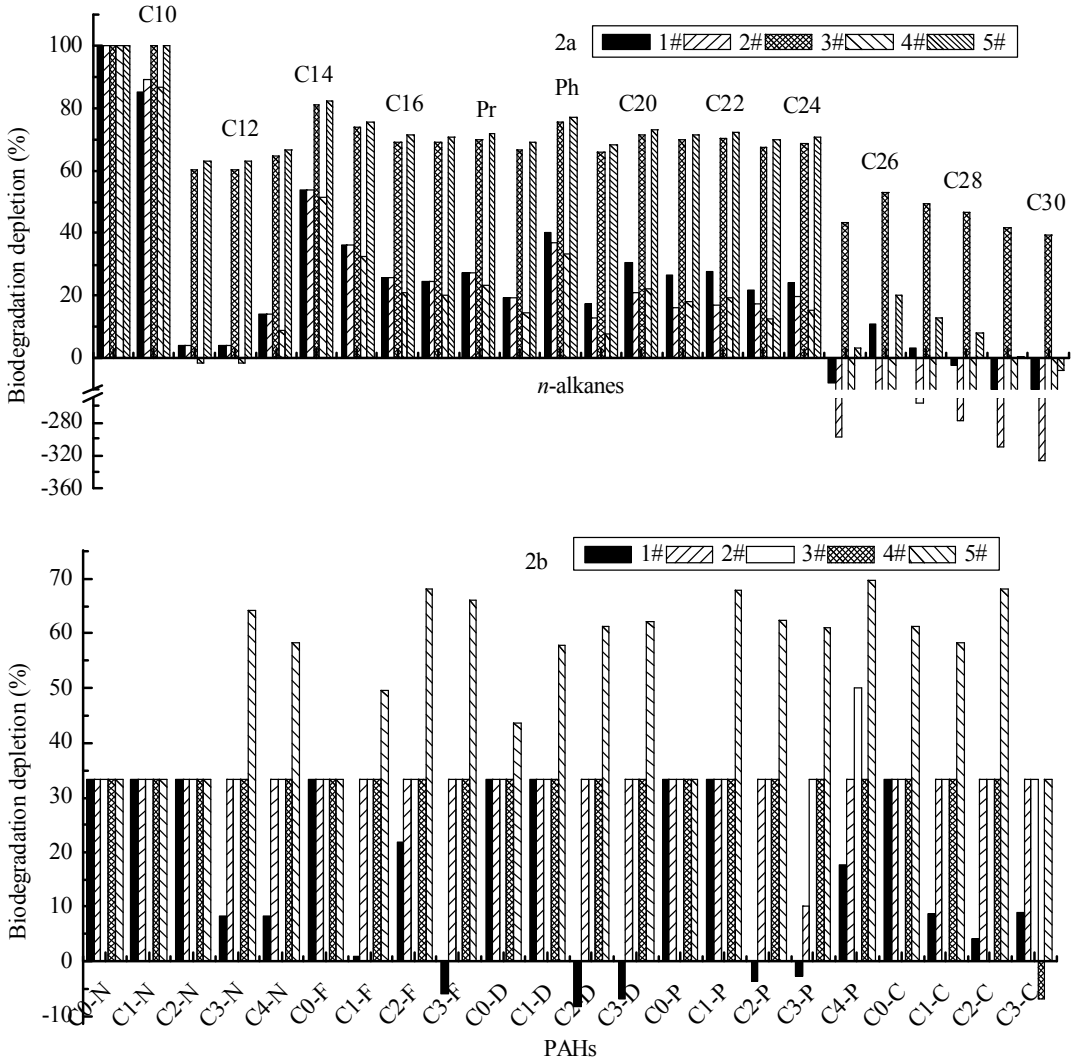

Fig. 2 Biodegradation rate of petroleum hydrocarbons. (a) n-Alkane (Including Pristane and Phytane); (b) PAHs

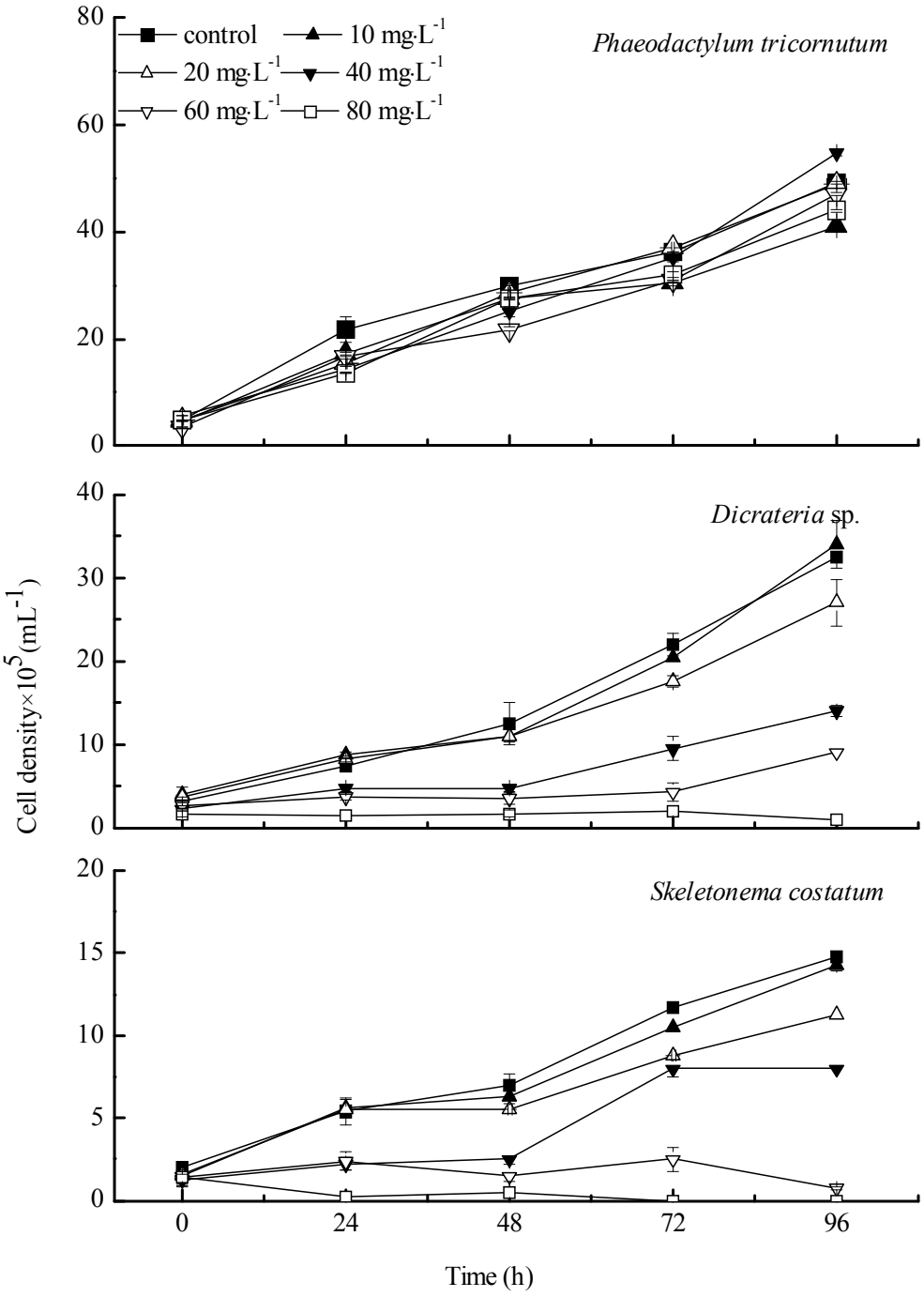

Fig. 3 Acute toxicity effect of soluble crude oil on microalgae.

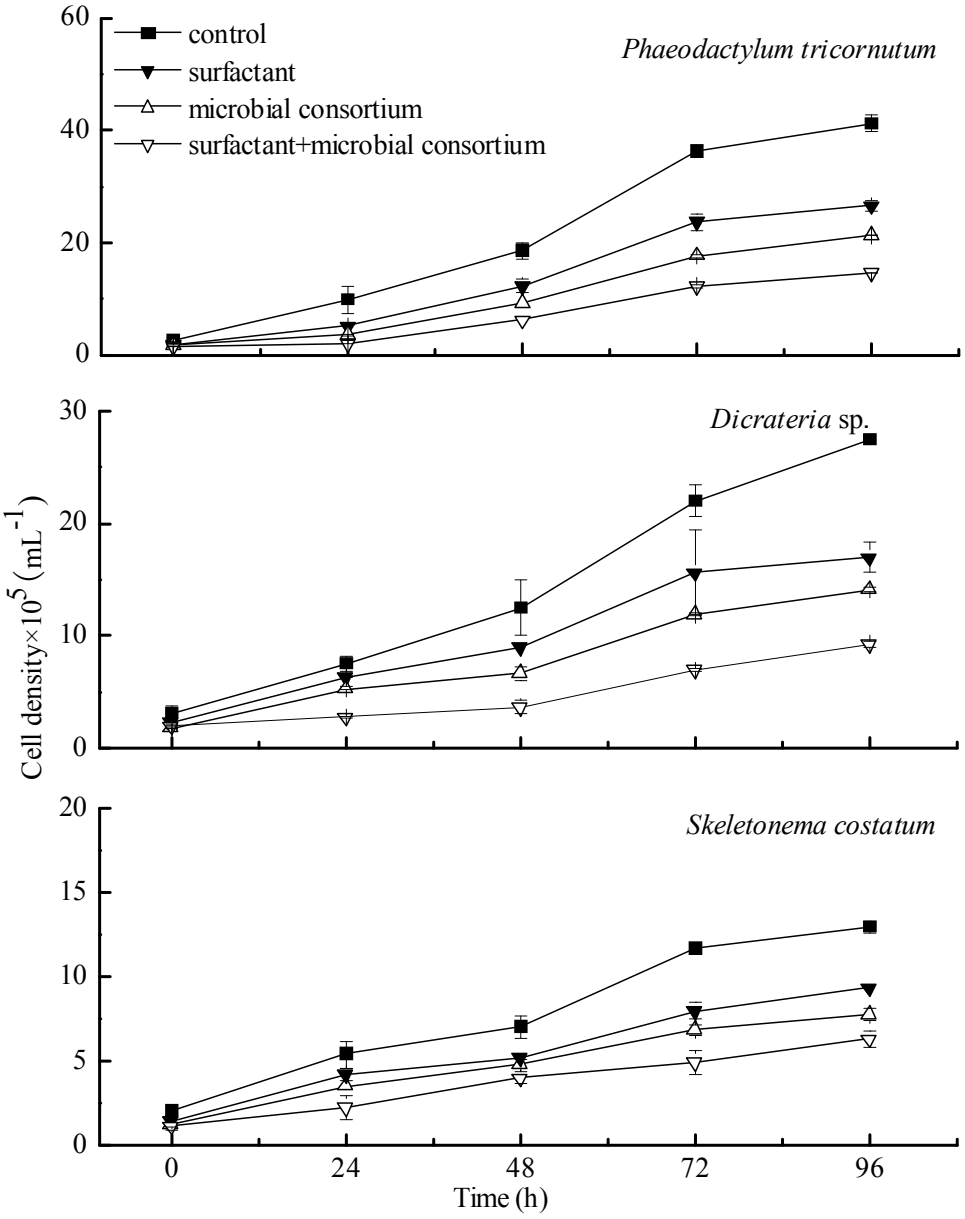

Fig. 4 Effect of microbial consortium and surfactant on microalgae.

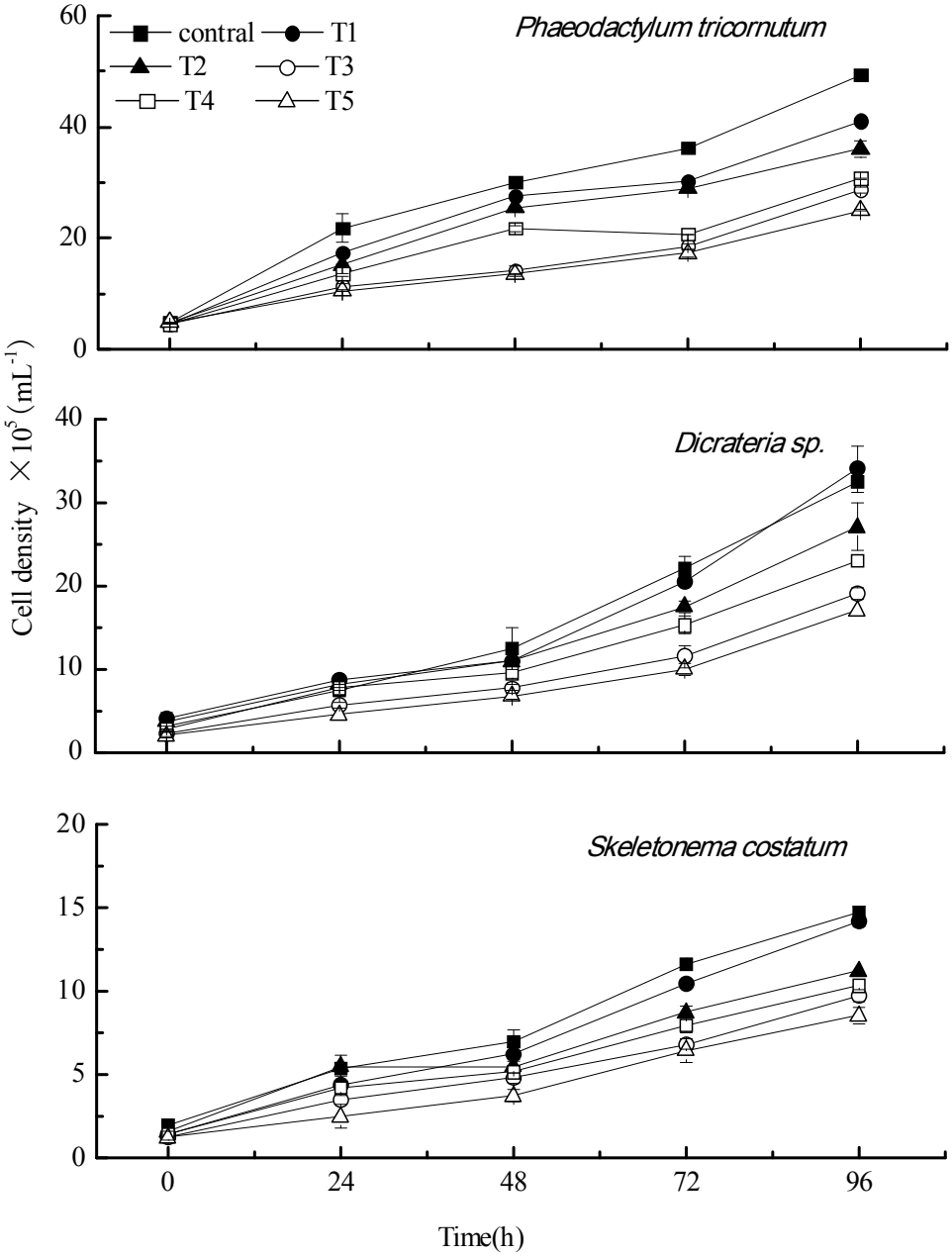

Fig. 5 Effect of biotreated seawater on microalgae.