BIOREMEDIATION OF OIL-CONTAMINATED SEAWATER AND SEDIMENT BY AN OIL-DEGRADING BACTERIAL CONSORTIUM

(石油分解細菌コンソーシアムを用いた石油汚染海洋環境の微生物修復)

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ABSTRACT

This study describes a continuum of research aimed at developing a bioremediation technology for oil-contaminated marine environments. First, isolation and identification of bacterial strains with oil-degrading potential from previously oil-contaminated tropical soil (Guimaras Island, Philippines) was done. Eighteen bacterial strains were isolated and identified by 16S rRNA gene sequence analysis. The results indicated that the isolates belonged mostly to Gammaproteobacteria and Alphaproteobacteria with most strains able to utilize *n*-alkanes and heavy oil as a carbon source but only few were able to grow in phenanthrene. Strains closely related to *Pseudomonas aeruginosa, Marinobacter mobilis*, *Halomonas* sp. and *Gaetbulibacter* sp. were selected for further *in vitro* oil-degrading analysis and immobilized individually or as a consortium by attaching to a cocopeat biocarrier or by encapsulating into sodium alginate gel. Results revealed higher oil reduction in treatments receiving the consortium than individual strains in both free-living and immobilized forms.

Once the effectiveness of the consortium to degrade heavy oil was established, its bioremediation potential in seawater was examined. Oil-degrading ability of the immobilized bacterial consortium in cocopeat, rice hull powder and sodium alginate capsules was compared with that of the free-living form and natural microflora in the seawater. Scanning electron microscopy revealed colonization and strong attachment of bacterial cells on the surface of the cocopeat and rice hull powder, yielding significantly higher ($p<0.05$) oil reduction compared to the treatments supplemented with the same consortium in free living and encapsulated forms in a 60-day *in vitro* seawater bioremediation trial. Higher cultivable bacterial counts and significantly higher degradation ($p<0.05$) of both aliphatic and aromatic fractions were obtained in the treatments augmented with carrier-immobilized consortia. The developed immobilized cells showed sustained activities and viabilities during storage in low temperature for six months.

In the next study, the efficiency of cocopeat-immobilized bacterial consortium to

remediate the sediment artificially contaminated with heavy oil was determined. A 3x2 factorial microcosm experiment was conducted to investigate the effect of the different combination of the bacterial cells and inorganic nutrients on degradation of hydrocarbons and bacterial community shifts in the contaminated sediment. Temporal changes in residual heavy oil components, total cultivable and oil-degrading bacterial counts and bacterial community dynamics in the different treatments within 60 days were determined. Results showed positive influences of nutrient and bacterial cell addition to the heavy oil degradation. Addition of the immobilized cells combined with supplementation with inorganic nutrients resulted in the significantly highest reduction ($p<0.05$) in total petroleum hydrocarbons (68.9%) and high numbers of oil-degrading and heterotrophic bacteria throughout the experimental period were sustained.

The effects of the different remediation agents to the bacterial community shifts were determined by PCR-DGGE. Statistical analysis of the DGGE profile revealed differences in responses and adaptation mechanisms of indigenous microflora to oil contamination and addition of different remediation agents. Immobilization of the bacterial consortium improved survival of the augmented strains. Addition of inorganic nutrients promoted growth of bacterial groups closely related to those with hydrocarbon-degrading capacity. Persistence of the augmented cells and growth of the oil-degrading bacterial groups might contribute to enhanced oil degradation.

INTRODUCTION

As the principal source of energy for industry and daily life worldwide, oil and petroleum products are being transported across the world frequently. As such, the potential for oil pollution coming from accidental spills, routine shipping operations and terrestrial run-offs on a global basis is significant (Etkin, 2001; Islam and Tanaka, 2004). In the case of tanker accidents, the impact of the massive amount of oil to the environment warrants fast removal of the pollutant. Common methods of cleaning up the spill include physical removal of oil by burning, scooping and spraying of chemical dispersants (Ghannam and Chaalal, 2003; Lessard and DeMarco, 2000). However, success of these techniques are limited by the nature of the contaminated site, the magnitude of the spill and the environmental safety issues involved with their applications (Al-Majed et al, 2012).

A safe and environment-friendly alternative cleanup approach is the utilization of degrading capability of the natural microbial community or biodegradation (Atlas, 1995; Harayama et al., 1999; Hoff, 1993). The biodegradation process is, however, dependent on many factors such as composition of the oil substances, nature of the microbial community and environmental factors such as pH, temperature and nutrients available to microbial growth (Venosa and Zhu, 2003). Acceleration of biodegradation can be accomplished by biostimulation (addition of nutrients or oxygen), bioaugmentation (addition or seeding of pre-selected microorganisms) or a combination of both (Mrozik and Piotrowska-Seget, 2010; Tyagi et al., 2010). The most important point which facilitates bioremediation attempt is to establish an environment that could satisfy the growth and activity of the target microorganisms. However, these bioremediation technologies cannot necessarily be used with equal effectiveness in every oil spill scenario.

Although several bioremediation techniques have already been described, recent studies have been geared towards achieving higher efficiency and the practicability of applying such technology on site without compromising the safety of the environment and new approaches have

been developed with understanding the underlying mechanisms including catabolic pathways and dynamics of the microbial community (Mair et al., 2012; Pelaez et al., 2013; Szulc et al. 2014). In the present study, a new approach to develop bioremediation technology applicable to oil-contaminated marine environments was tried as follows: 1) isolation, identification and characterization of bacterial strains with oil degrading potential from tropical coastal environments contaminated with heavy oil, 2) selection of isolates with a wide range of hydrocarbon-utilization capabilities, 3) investigation of the effectiveness of the selected isolates and their consortium to degrade heavy oil *in vitro*, 4) immobilization of the bacterial consortium on the surface of organic biocarriers and in sodium alginate beads to improve remediation and application efficiency, 5) determination of the ability of the immobilized cells to degrade heavy oil in seawater microcosms and their stability and viability during storage, 6) elucidation of the hydrocarbon degradation mechanisms of the immobilized cells and its effect on the bacterial community dynamics of a sediment artificially-contaminated with heavy oil in microcosm experiments, and 7) exploration of possible combination of bioremediation techniques that can maximize heavy oil degradation rates.

Overall, results of this study will lead to better understanding of the whole process of bioremediation of oil-contaminated marine environments including physical, biological and chemical aspects. This information will be vital to clarify the universal applicability of the developed bioremediation technology as well as its limitation.

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CHAPTER ONE

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF OIL-DEGRADING BACTERIAL STRAINS FROM GUIMARAS, PHILIPPINES FOR BIOREMEDIATION APPLICATION

ABSTRACT

This study describes isolation and identification of bacterial strains with oil-degrading potential from a tropical soil previously contaminated with heavy oil in terms of their application to bioremediation. Eighteen bacterial strains were isolated from Guimaras Island, the Philippines and identified by 16S ribosomal RNA gene sequence analysis, and their hydrocarbon-utilizing capability was tested. The results indicated that the isolates belonged mostly to Gammaproteobacteria and Alphaproteobacteria. Most isolates were able to utilize *n*-alkanes and heavy oil as a carbon source but only few were able to grow weakly in phenanthrene. Based on the hydrocarbon-utilizing characteristics, strains closely related to *Pseudomonas aeruginosa, Marinobacter mobilis*, and *Gaetbulibacter* sp. were selected to estimate the effect of immobilization on oil degradation. The bacterial cells were immobilized on the surface of a cocopeat baiocarrier individually or as a consortium, or encapsulated into sodium alginate gel. The results of the *in vitro* oil degradation studies revealed higher oil reduction in treatments receiving the consortium than the individual strains in both free-living and immobilized forms. The findings of this study are useful in developing *in situ* techniques for remediation of oil-polluted sites.

INTRODUCTION

Worldwide industrialization expands transportation and utilization of oil and its products as an energy source. As such, the potential for oil spills into the ocean, coming from terrestrial runoffs, offshore oil production and shipping activities, has been becoming significant. Among them, huge oil spills caused by tanker accidents receive much public concern because of the magnitude of their impact on the marine environment, disturbing the coastal ecosystems such as mangroves and sea grass beds (Swanell et al., 1996). One such case is the M/T Solar 1 tanker accident that discharged 220 kl of C-grade heavy oil into the coastal area of Guimaras Island, the Philippines on August 11, 2006. It was reported to be the worst oil spill accident in the country's history, affecting not only the coastal environments of Guimaras but also those of some towns in neighboring islands like Iloilo and Negros Occidental (Paringit and Santillan, 2009).

Despite the several clean-up efforts including physical methods such as manual removal, wiping with absorbents, booming and skimming, and chemical methods like dispersants, emulsifiers, and solidifiers, the coastal environments have been still contaminated with the oil residues. In natural marine environments, microorganisms play a most important role to mineralize contaminated hydrocarbons: although they are less dominant in pristine environments, the oil degraders which degrade petroleum hydrocarbon constituents like alkanes and polycyclic aromatic hydrocarbons are widespread and successively dominate in oil-contaminated intertidal area (Harayama et al., 2004). In addition, more than 20 genera of hydrocarbonoclastic bacteria have been described so far (Roling et al., 2002), and the mechanisms of their oil degradation have been studied extensively (Harayama et al., 1999, Yakimov et al., 2007). Thus, bioremediation, which utilizes the microbiological processes to remove the contaminants, is expected as the second option after the conventional physical and chemical measures.

The aim of this study is to isolate oil-degrading microorganisms and to establish a method to apply these oil-degrading bacteria for bioaugmentation of oil-contaminated marine environments.

We isolated microorganisms from the oil-polluted coastal regions of Guimaras Island, and their potential assimilation of oil constituents was observed. In addition, an *in vitro* experiment was conducted to estimate the effect on the microbial oil degradation by immobilizing on a biocarrier matrix or encapsulating into calcium alginate gel.

MATERIALS AND METHODS

Sample collection

Oil and sand samples were taken from impacted areas in Taklong Island National Marine Reserve (TINMAR), Nueva Valencia, Guimaras, the Philippines (Fig. 1-1) in November 2008. The samples were collected randomly from different sources, such as spilled heavy oil collected and kept in metal drums, contaminated beach sand, contaminated sediment from mangrove areas, and uncontaminated dock sand. All samples were transported and stored at room temperature until bacterial isolation.

Enrichment and isolation of hydrocarbon-degrading bacteria

The bacterial cells were enriched using the Bushnell Haas mineral salt (BHMS) medium supplemented with 2% NaCl and 1% heavy oil (type A; density, 0.8796 g cm^{-3}), containing (per liter): $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 0.02 g, KH_2PO_4, 1 g; K_2HPO_4, 1 g; NH_4NO_3, 1 g; and $FeCl_3$, 0.05 g. The samples (1 g) were inoculated in 100 ml of the sterilized medium. The culture was incubated at room temperature for 14 days with shaking at 125 rpm. After incubation, 1 ml of the culture was serially diluted and spread on the Zobell 2216E agar plates (natural seawater, 750 ml; distilled water, 250 ml; polypeptone, 5 g; yeast extract, 1 g) to isolate the enriched bacteria. The agar plates were incubated for 7 days. Individual colonies exhibiting different distinct morphologies were isolated by repeating the single colony isolation procedure several times. The isolates were maintained in the Zobell 2216E agar and kept at room temperature until further analysis.

Identification of the isolates by phylogenetic analysis of 16S ribosomal RNA gene

The bacterial isolates were grown in Zobell 2216E medium at room temperature for 48 h with shaking at 125 rpm. One milliliter of the bacterial culture was used to extract the genomic DNA using Wizard Genomic DNA Purification Kit (Promega, Madison, USA). Universal bacterial primer set 8F (5'-AGAGTTTGATCATGGCTCAG-3') (Amman et al., 1995) and 1492R (5'-GGCTACCTTGTTACGACTT-3') (Liu et al., 2002) were used to amplify 16S ribosomal RNA genes (16S rDNA) by PCR. Amplification was performed in a thermal cycler (iCycler, Bio-Rad Laboratories, Hercules, CA, USA). The thermal cycling consisted of 25 cycles of denaturing at 95˚C for 1 min, primer annealing at 54˚C for 1 min, and extension at 72˚C for 2 min, with a final extension at 72˚C for 3 min.

The 5' region corresponding to 10–400 in the *Escherichia coli* 16S rDNA numbering system was determined by the BigDye Terminator Cycle Sequencing method using the 8F primer and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). The strains were identified by finding closely related sequences in the genetic sequence database GenBank using the basic local alignment search tool (BLAST) program (Altshul et al., 1990). Phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987) using MEGA Version 4 (Tamura et al., 2007).

Characterization of hydrocarbon-degrading properties of the isolates

Growth of the isolates on different representative hydrocarbons or heavy oil were tested in 10 ml of the BHMS medium supplemented with 2% NaCl and 1 g l^{-1} of *n*-tetradecane as a short chain alkane, *n*-tetracosane as a long chain alkane, phenanthrene as a polycyclic aromatic hydrocarbon (PAH), or heavy oil. The isolates were cultivated at room temperature for 15 days with shaking at 125 rpm. The medium without inoculation was served as a control. The growth was evaluated by comparing turbidity with the control through visual inspection.

Preparation of oil-degrading consortium

The selected strains were sub-cultured separately in the Zobell 2216E broth and grown for 24 h. After incubation, the cells were harvested, washed with 75% sterile natural seawater, and resuspended in 0.8% NaCl solution. Equal volumes of the suspension containing the different bacterial strains were mixed to form the consortium.

Preparation of coconut peat biocarrier immobilizing the oil-degrading bacteria

Raw materials for the production of coconut peat (cocopeat) were obtained from local industries in the Philippines. Cocopeat powder was derived by mechanically grinding the dried coir (middle fibrous coat) and sieved to obtain the particle size of less than 1 mm. The powder was sterilized by autoclaving and kept at room temperature until use.

About 0.3 g of the sterilized cocopeat powder was aseptically transferred into 100 ml of the Zobell 2216E broth in 250 ml Erlenmeyer flasks. One milliliter of the bacterial strains, cultivated in the BHMS broth supplemented with crude oil as a sole carbon source, or the bacterial consortium was inoculated into the cocopeat-containing broth. After 4 days of incubation, the cocopeat powder was harvested, washed by sterile 75% natural seawater, and air-dried.

Preparation of encapsulated bacterial cells

Immobilization of the bacterial cells in sodium alginate gel was done using the method described by Rahman et al. (2006). Briefly, 24 h culture of the bacterial strains/consortium (approximately 10^9 CFU ml^{-1}) were harvested by centrifugation (15,000 x *g*, 10 min), rinsed three times with sterile saline, and resuspended in 150 ml of 3% sodium alginate. The suspension was stirred and the resulting alginate/cell mixture was dripped into ice-cooled, sterile 0.2 M $CaCl_2$, which generated beads of approximately 2 mm in diameter. The beads were then hardened in fresh $CaCl_2$ for 2 h.

in vitro oil degradation by the bacterial strains

The oil-degrading activity of the bacterial strains and the consortium, both in the free and immobilized forms, were determined in an *in vitro* experiment. One and a half ml of the cell suspension of approximately 10^9 CFU ml^{-1}, 1.5 g of the cocopeat with the immobilized bacterial cells of approximately 10^9 CFU g^{-1}, or 1.5 g of the sodium alginate-encapsulated cells of approximately 10^9 CFU g^{-1} were added to 150 ml of the BHMS medium supplemented with 2% NaCl. Heavy oil (type A) was added at a concentration of 1 g l^{-1}, and the setups were incubated at room temperature for 30 days with shaking at 125 rpm.

At the end of the incubation, the residual oil was extracted from each flask using the US-EPA Gravimetric Method (1999). Briefly, a sample containing oil was collected in a clean 500-ml separatory funnel. The pH was brought down to $\leq$ 2 by adding 1:1 HCl in order to hydrolyze oil and prevent sodium sulfate interference. Oil in the samples was exhaustively extracted twice with *n*-hexane. Then, the *n*-hexane layer was dehydrated by passing through a funnel containing anhydrous sodium sulfate and collected in a dry, pre-weighed receiving flask. The remaining solvent in the solution was then evaporated in a rotary evaporator and by flushing nitrogen gas with heating at 70˚C. Finally, the flask containing the concentrated oil was cooled and dried in a desiccator and the residual oil recovered was determined gravimetrically. The reduction of oil concentration was calculated as $(A\text{-}B)/B$, where A is the initial weight of heavy oil added, and B is the weight of the residual oil.

Nucleotide sequence accession numbers

The 16S rDNA sequences obtained from the 18 isolates were deposited in the DNA Data Bank of Japan (DDBJ) database under accession numbers AB649098 to AB649115.

Data analysis

Significant differences of the results in the *in vitro* oil degradation tests were analyzed by the one-way ANOVA followed by Duncan's Multiple Range Test (DMRT) using the statistical program

Statistical Package for Social Sciences (SPSS) version 19.0.

RESULTS

Isolation, identification and phylogenetic analysis of the isolates

The 14-day enrichment in the Bushnell Haas mineral salt (BHMS) medium and the following single colony isolation on the Zobell 2216E agar plates yielded a total of 18 bacterial isolates from the collected soil samples. The isolates and their closest relatives in the GenBank database inferred from 16S ribosomal RNA gene (16S rDNA) sequences are listed in Table 1-1.

The isolates were phylogenetically categorized into three distinct groups: Gammaproteobacteria, *Cytophaga-Flavobacterium-Bacteriodes* (CFB) group, and Alphaproteobacteria (Fig. 1-2). Gammaproteobacteria were the most abundant with 10 isolates including four strains (B1, B2, B3, and B4) closely related to *Pseudomonas aeruginosa*, and one strain (D1) affiliated to *Marinobacter mobilis*, both genera of which include hydrocarbon degrader species (Das and Mukherjee, 2007, Gauthier et al., 1992). The other isolates were related to *Pseudomonas pseudoalcaligenes* (strain A2), *Microbulbifer hydrolyticus* (strain C2), *Gallaecimonas pentaromativorans* (strain C3), and *Vibrio* sp. (strain D4). For the Alphaproteobacteria group, the isolates were related to *Salipiger* sp. (strain C1), *Rhodobacter* sp. (strain D6), *Brevundimonas aurantiaca* (strain A1), *Halomonas* sp. (strain A4), *Labrenzia aggregata* (strain C4), and *Stappia kahanamokuae* (strain D2). The CFB is the least abundant group with only two strains, both of which were closely related to *Gaetbulibacter* sp. (strains D3 and D5).

Hydrocarbon-utilizing characteristics of the bacterial isolates

The capability of the bacterial isolates to degrade different kinds of hydrocarbons were tested by evaluating their growth in media containing representative *n*-tetradecane, *n*-tetracosane, phenanthrene, and heavy oil. The results showed that most of the bacterial strains were able to utilize one or more kinds of hydrocarbon as a carbon source (Table 1-2). Out of the 18 isolates, seven

strains (A3, B1, B2, B3, B4, D5, and D6) grew well in the medium supplemented with both *n*-tetradecane and *n*-tetracosane. Strains A1 and A2 grew well in *n*-tetradecane but not in *n*-tetracosane, while strain D1 grew weakly in *n*-tetradecane and grew well in *n*-tetracosane. Although the growth was weak, strains A4, B1, D1, D2, D3, D5, and D6 were able to assimilate phenanthrene. Most of the strains that were able to grow in at least one of the hydrocarbons exhibited positive growth in heavy oil, except for strains A1, A2, A3, C1, and C4.

in vitro oil degradation of the bacterial isolates and their consortium

The comparison of oil reduction among selected individual strains and a bacterial consortium in free and immobilized forms was done through *in vitro* degradation tests. In order to construct the consortium, ability of the isolates to grow in a wide range of the hydrocarbon substrates was considered. Thus, based on the results of the hydrocarbon-utilization tests, strains B1, D1, and D5, which were able to grow under in the media with *n*-alkanes, polycyclic aromatic hydrocarbon (PAH), and heavy oil, were selected. Strain A4, closely related to *Halomonas* sp., was also added to the consortium as a possible biosurfactant producer (Satpute et al., 2010).

The results of the gravimetric determination of residual oil in the different treatments are shown in Figs 1-3 and 1-4. In a free-living form (Fig. 1-3), significantly higher oil reduction ($p<0.05$) was found in the setup containing the bacterial consortium, compared with the treatments to which the isolates were added individually. In the treatments augmented with the immobilized cells (Fig. 1-4), oil reduction was significantly higher ($p<0.05$) for the bacterial consortium than for the individual isolates, which is the same trend as in a free-living form. In addition, oil reduction was generally higher for the cocopeat immobilization than for the alginate gel encapsulation. On the other hand, the treatments of the individual isolates revealed no significant differences ($p>0.05$). The exception is that strain D1 immobilized on the surface of cocopeat exhibited significantly higher ($p<0.05$) reduction than the other treatments.

DISCUSSION

Microbial degradation is a major process that results in decontamination and complete mineralization of pollutants in an oil spill incident. It is generally believed that microorganisms capable of degrading xenobiotic pollutants such as oil are often isolated from environments that have been previously exposed to the same contaminants (Chaerun et al., 2004). Spilled oil entrained in marine sediment may have supported the growth of oil-degraders thereby allowing their isolation. It was reported that residual petroleum hydrocarbons were still within a detectable level in coastal sediments of some heavily impacted areas in Guimaras one year and two years after the oil spill (Pahila et al., 2010). The present study revealed that samples collected from the oil-contaminated sites contained indigenous bacterial communities that are capable of assimilating diverse hydrocarbon compounds, resulting in the success of isolation of the oil-utilizing bacteria.

Identification based on the 16S ribosomal RNA gene (16S rDNA) sequences showed that the isolates were affiliated to the phylum Proteobacteria and distributed mainly within the classes Gammaproteobacteria and Alphaproteobacteria. These findings are in agreement with previous studies that reported on the dominance of Proteobacteria in oil-contaminated areas (Brito et al., 2006, Said et al., 2008). The dominance of Gammaproteobacteria from these sources is known as the "γ-shift" phenomenon which occurs under a condition of nutrient oversupply (Popp et al., 2006). Increase in bacterial numbers of Gammaproteobacteria over the other classes which are normally abundant in pristine soils may result from the degradation of contaminants at a higher level.

Most of the isolated strains under the class Gammaproteobacteria were related to *Pseudomonas aeruginosa*. This is not surprising considering the frequency of the genus *Pseudomonas* among bacterial strains isolated from sites of tanker accidents and other hydrocarbon-contaminated sites. Some species belonging to the genera *Pseudomonas* and *Marinobacter* are classified as obligate hydrocarbonoclastic bacteria (OHCB) and has been shown to play a significant role in the removal of hydrocarbons from polluted marine waters (Das et al., 2007,

Gauthier et al., 1992). Interestingly, no *Alcanivorax*-related strain was isolated from the soil samples. In most cases, *Alcanivorax* spp. are alkane-degraders that are the first to bloom following an influx of hydrocarbons. However, the samples were taken two years after the spill where considerable degradation of oil, especially an aliphatic fraction, had already taken place. The *Alcanivorax* population may have been succeeded by the metabolically more versatile *Pseudomonas* and *Marinobacter* populations, although less abundant at the onset of hydrocarbon degradation (Kostka et al., 2011, Yakimov et al. 2007).

Some members of the class Alphaproteobacteria, including the genera *Labrenzia* and *Rhodobacter*, were reported to degrade polycyclic aromatic hydrocarbons (PAHs) in pure culture (Lai et al., 2009, Rajasekhar, 2000). Furthermore, the genus *Halomonas* are known to produce biosurfactants in the form of exopolysaccharides (EPS) that emulsify hydrocarbon compounds (Satpute et al., 2010). The presence of Alphaproteobacteria, such as C4, D6, and A4, which are phylogenetically related to the taxa mentioned above, indicates that some contaminated areas in Guimaras during the sampling period had already depleted of alkanes and that succession to the bacterial groups more specialized for breakdown of recalcitrant PAHs had already began.

Investigating the growth of the strains in the medium supplemented with heavy oil as a carbon source is a simple and fast way of evaluating their capacity to degrade oil components. In the present study, the bacterial isolates were cultivated in the Bushnell Haas mineral salt medium supplemented with hydrocarbons as well as heavy oil. Of the 20 isolates, nine grew well and five grew weekly under the presence of *n*-alkane. Among aliphatics, *n*-alkanes are the most biodegradable form and attacked by more microbial species than aromatics (Chaerun et al., 2004), and shorter C_8-C_{15} alkanes are more preferentially degraded compared with longer C_{16}-C_{36} alkanes (Sathishkumar, 2008). In terms of PAH utilization, no isolates grew well in the medium containing phenanthrene and only seven isolates grew weakly after 15 days incubation, although low-molecular-weight PAHs such as phenanthrene is readily degraded aerobically in a natural ecosystem, where synergistic co-metabolism within the microbial community is possible.

Interestingly, the isolates that grew weakly in phenanthrene exhibited better growth in the heavy oil-enriched medium. It can be assumed that the isolates may utilize components such as alkanes, paraffins, asphaltenes, or resins rather than PAHs in heavy oil.

In order to find bacterial strains suitable for bioremediation, oil-degrading capacity of the strains B1 (*P. aeruginosa*), D1 (*Marinobacter mobilis*), and D5 (*Gaetbulibacter* sp.), all of which showed utilization of all the hydrocarbons used, were chosen as well as A4 (*Halomonas* sp.), a possible biosurfactant producer, and effect of bacterial immobilization on hydrocarbon degradation was investigated. Although the individual isolates degraded heavy oil, their degradation was significantly lower than that of the consortium. This indicates the presence of synergistic co-metabolism (Guo et al., 2005) in the consortium. The cooperation among the microorganisms is significant in degradation of complex mixtures such as heavy oil (Wang et al., 2010). In this context, mixed cultures composed of bacterial strains with diverse degrading capability is more desirable for bioremediation.

Immobilization of the oil-degraders on the surface of cocopeat showed significantly higher oil reduction, compared to encapsulation in sodium alginate gel. Although some studies shows improvement in oil degradation by alginate encapsulation (Karamalidis et al., 2010, Larsen et al., 2009, Rahman et al., 2006), limitation of diffusion of hydrophobic oil into a hydrophilic gel matrix may explain the lag in oil degradation. In addition, the encapsulated bacterial cells might not be allowed to replicate inside the alginate matrix and subsequent release into the medium (Cunningham et al., 2004). On the other hand, higher oil reduction by the cocopeat-immobilized cells is presumably due to highly sustained microbial population attached to surface of the biocarrier. This is supported by similar studies (Gentili et al., 2006, Obuekwe and Al-Muttawa, 2001), where immobilization greatly improved viability of the oil-degrading cells, thus rendering higher degradation rates. In addition to providing protective niche, the porous nature of cocopeat might allow efficient substrate diffusion, slow release of nutrients, and acceleration of oxygen transfer, thus providing a favorable niche for hydrocarbon utilization (Liang et al., 2009). Furthermore, the high

C/N ratio of cocopeat (Abad et al., 2002, Hernandez-Apalaoza and Guerrero, 2008) makes it a possible carbon source for bacteria.

In conclusion, the present study confirmed the presence of oil-degrading bacteria in the samples from Guimaras, the Philippines two years after the oil spill incident. The bacterial consortium composed of the 4 isolates obtained from the Guimaras samples indicated high oil degradation in the *in vitro* experiment. In addition, immobilization on a biocarrier, cocopeat improved the efficiency. The findings of this study are useful for future investigations on biodegradation processes and development of *in situ* bioremediation to clean up oil-contaminated sites.

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TABLES AND FIGURES

Table 1-1. List of the bacterial strains isolated from the soil samples

Strain codes	Closest relatives in the GenBank database (accession numbers of 16S rDNA sequences)	Homology (%)	Taxonomic groups
A1	*Brevundimonas aurentiaca* (EU434579)	100	Alphaproteobacteria
A2	*Pseudomonas pseudoalcaligenes* (FJ472857)	97	Gammaproteobacteria
A3	*Pseudoxanthomonas* sp. (FJ55563)	99	Gammaproteobacteria
A4	*Halomonas* sp. (GQ169077)	99	Alphaproteobacteria
B1	*Pseudomonas aeruginosa* (GU726841)	100	Gammaproteobacteria
B2	*Pseudomonas aeruginosa* (HQ162487)	100	Gammaproteobacteria
B3	*Pseudomonas aeruginosa* (HQ162486)	100	Gammaproteobacteria
B4	*Pseudomonas aeruginosa* (HQ164439)	100	Gammaproteobacteria
C1	*Salipiger* sp. (EU440999)	98	Alphaproteobacteria
C2	*Microbulbifer hydrolyticus* (AJ608704)	99	Gammaproteobacteria
C3	*Gallaecimonas pentaromativorans* (FM955225)	99	Gammaproteobacteria
C4	*Labrenzia aggregata* (EU440961)	99	Alphaproteobacteria
D1	*Marinobacter mobilis* (GQ214550)	99	Gammaproteobacteria
D2	*Stappia kahanamokuae* (EF101503)	100	Alphaproteobacteria
D3	*Gaetbulibacter marinus* (EF108219)	99	*Cytophaga-Flavobacterium-Bacteriodes*
D4	*Vibrio* sp. (FJ 457375)	100	Gammaproteobacteria
D5	*Gaetbulibacter* sp. (FJ360684)	100	*Cytophaga-Flavobacterium-Bacteriodes*
D6	*Rhodobacter* sp. (EU697077)	100	Alphaproteobacteria

Table 1-2. Growth of bacterial strains under the existence of different hydrocarbons

Strain codes	Growth under the existence of hydrocarbons*			
	n-tetradecane	*n*-tetracosane	phenantherene	Heavy oil
A1	+	-	-	-
A2	+	-	-	-
A3	++	+	-	-
A4	-	-	$+_w$	$+_w$
B1	+	+	$+_w$	+
B2	+	+	-	+
B3	+	+	-	+
B4	+	+	-	+
C1	$+_w$	-	-	-
C2	-	-	-	-
C3	-	-	-	-
C4	$+_w$	-	-	-
D1	$+_w$	+	$+_w$	+
D2	$+_w$	-	$+_w$	+
D3	$+_w$	-	$+_w$	+
D4	-	-	-	-
D5	+	+	$+_w$	++
D6	+	+	$+_w$	+

*$+_w$, weak growth; ＋, growth; ＋＋, strong growth; －, no growth.

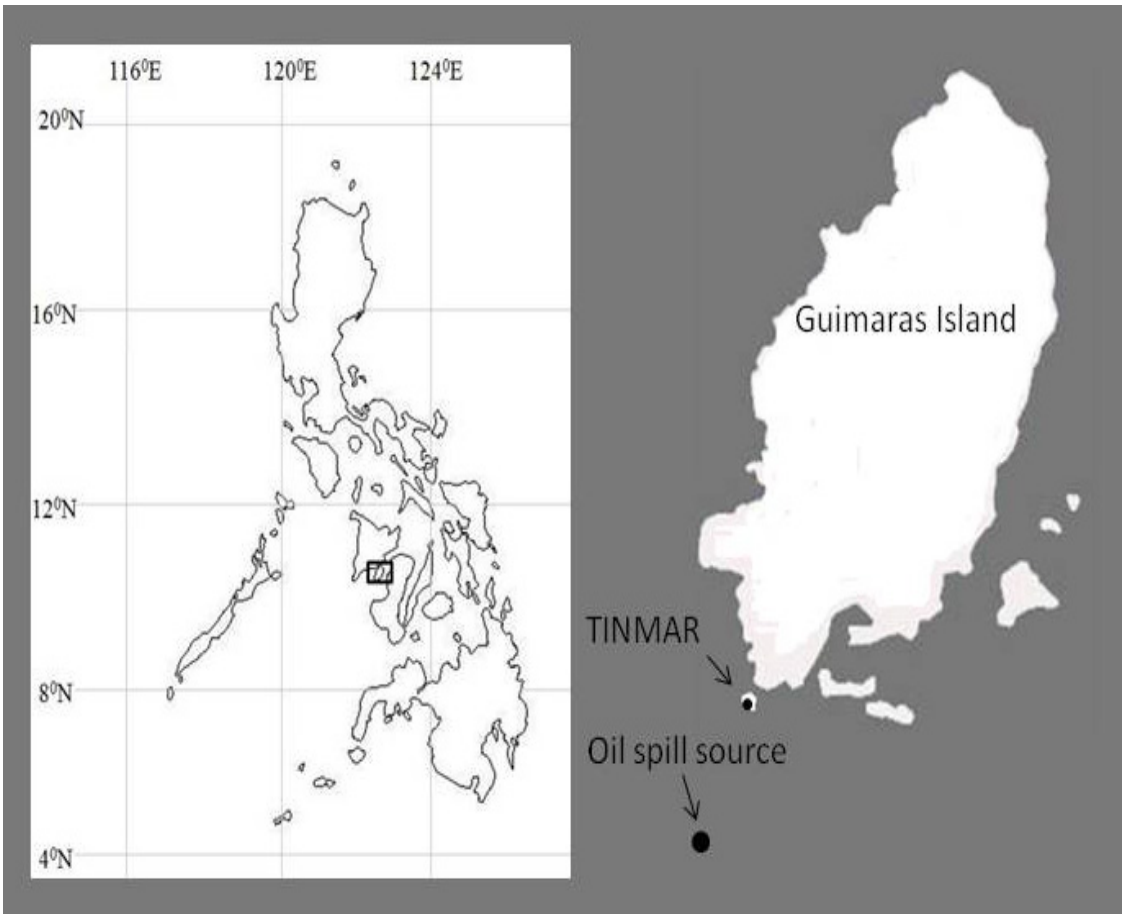

Fig. 1-1. Location of the sampling site (Taklong Island National Marine Reserve) in Nueva Valencia, Guimaras, Philippines.

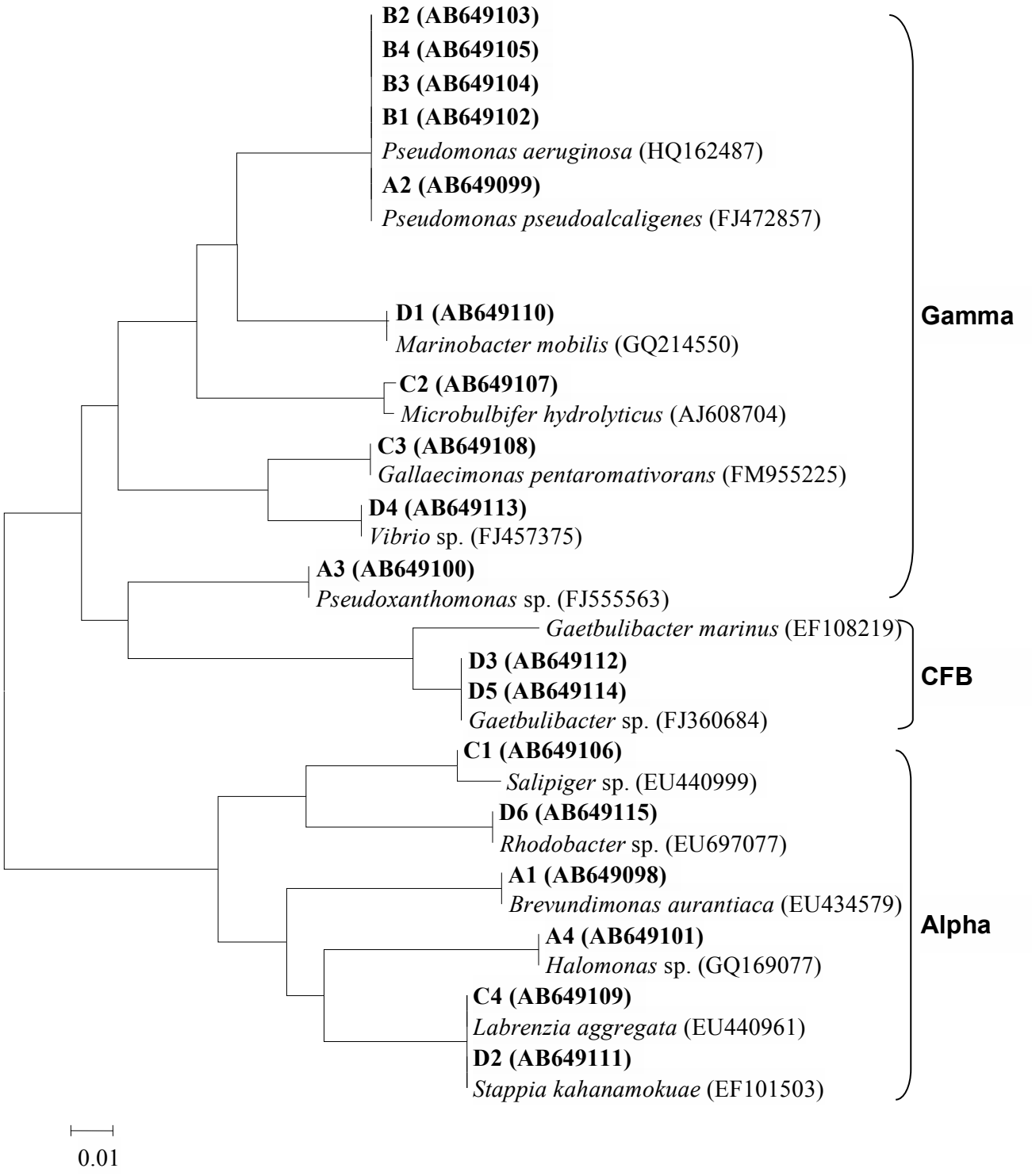

Fig. 1-2. Unrooted phylogenetic tree based on the 16S rDNA sequences of the isolated bacterial strains. The accession numbers are shown in parentheses. Alpha, Alphaproteobacteria; CFB, *Cytophaga-Flavobacterium-Bacteroides*; Gamma, Gammaproteobacteria.

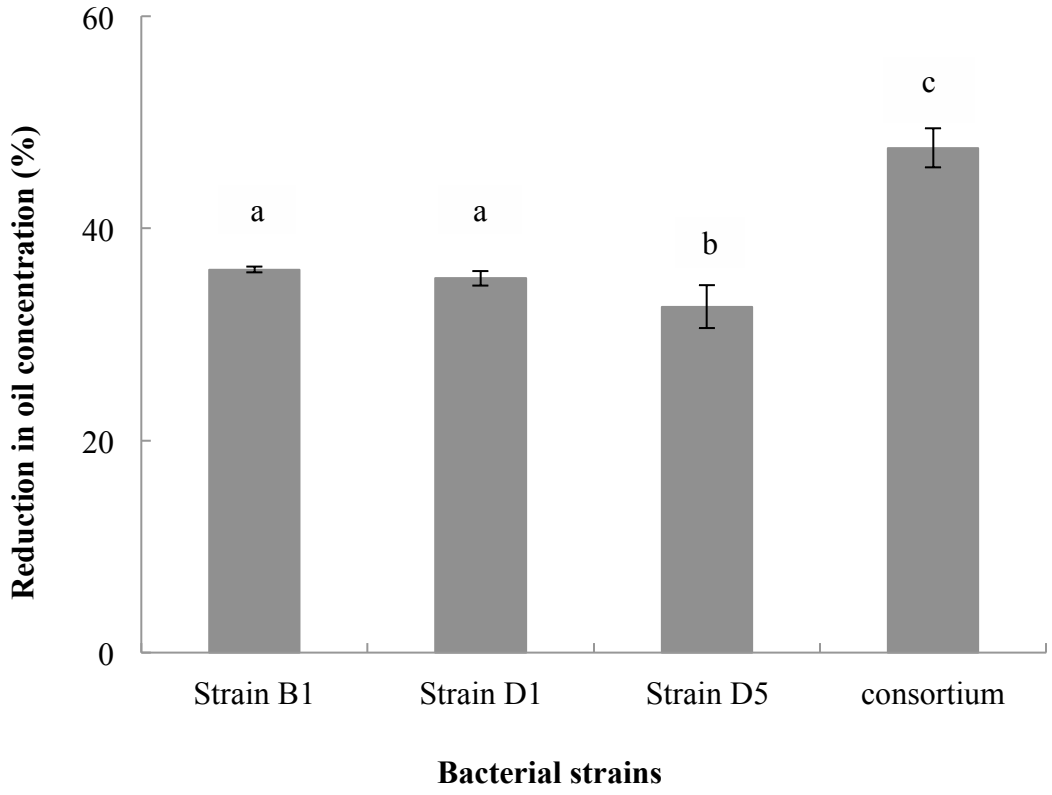

Fig. 1-3. Gravimetric determination of residual heavy oil in the BHMS media to which the bacterial strains or their consortium were added as a free-living form. The values represent the mean of three replicates after 30 days of incubation. The error bars show the standard deviation. The letter above each bar indicates a significant difference at $p<0.05$.

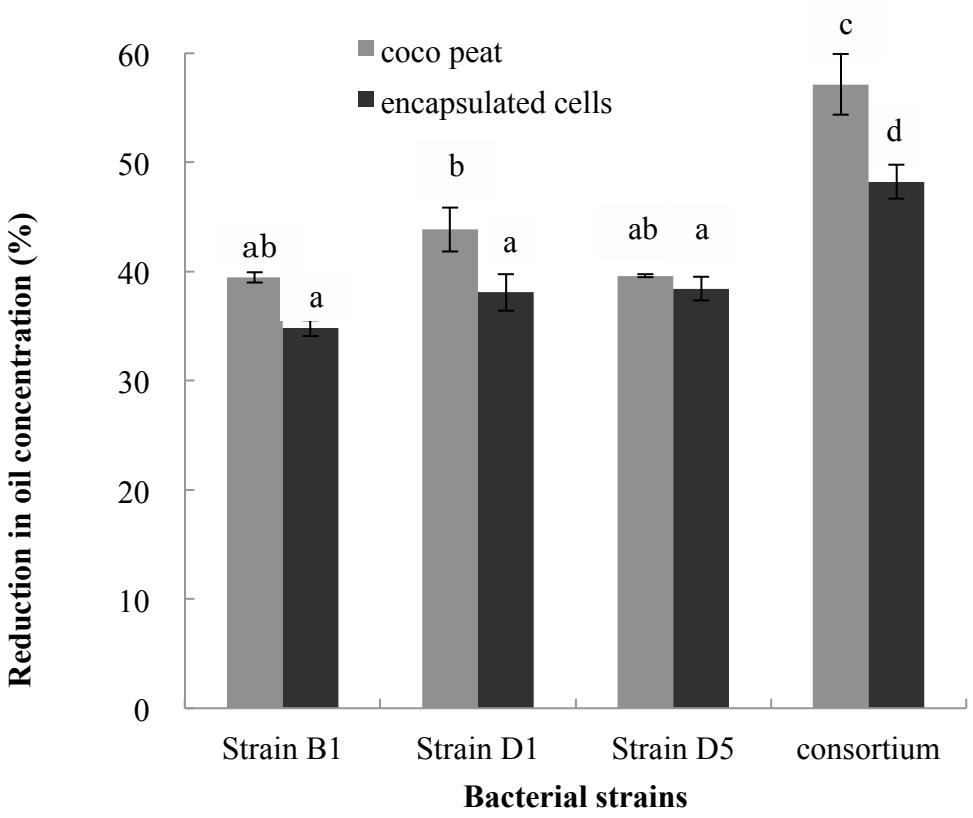

Fig. 1-4. Gravimetric determination of residual heavy oil in the BHMS media to which the bacterial strains or their consortium were added as an immobilized form. The values represent the mean of three replicates after 30 days of incubation. The error bars show the standard deviation. The letters above each bar indicate a significant difference at $p<0.05$.

CHAPTER TWO

BIOREMEDIATION OF HEAVY OIL-POLLUTED SEAWATER BY BACTERIAL CONSORTIUM IMMOBILIZED IN COCOPEAT AND RICE HULL POWDER

ABSTRACT

We examined the effectiveness of cocopeat and rice hull powder obtained from agricultural wastes as biocarriers for an oil-degrading bacterial consortium. Scanning electron microscopy revealed colonization and strong attachment of bacterial cells on the surface of both carriers. Results of a 60-day *in vitro* seawater bioremediation trial showed significant oil reduction and high cultivable bacterial counts in treatments augmented with the carrier-attached bacterial consortia compared to treatments supplemented with the same consortium in free living and encapsulated forms. Significant degradation in both aliphatic and aromatic fractions was obtained in treatments augmented with carrier-immobilized consortia. The developed immobilized cells showed sustained activities and viabilities during storage in low temperature for six months. Results of this study demonstrated that inexpensive waste materials can be utilized as biocarriers of an oil-degrading consortium and that immobilization on biocarriers can enhance bioremediation of oil-contaminated seawater.

INTRODUCTION

Oil contamination of marine water is a continual and almost chronic ecological problem worldwide especially in highly industrialized places. Although oil contaminants are weathered by photooxidation and evaporation, complete degradation relies on the metabolic activities of the inherent microbial population (Harayama et al., 1999). Thus, bioremediation techniques involve manipulation of conditions such as addition of rate-limiting nutrients and oxygen to promote the growth and activity of native oil-degraders. However, the number and degrading activities of the indigenous microorganisms are subsequently decreased by various factors such as diffusion of nutrients, depletion of preferred substrates and production of toxic metabolites among others (Bento et al., 2005; Boopathy, 2000; Ruberto et al., 2003).

In open seas, biodegradation of oil contaminants by indigenous oil degraders is made more challenging by relatively lower microbial numbers compared to the shore or sediment and by limitations of other nutrients necessary for microbial growth and metabolic activities. Addition of nutrients is not effective all the time because such supplements are moved by water current and constant tidal action from the location of the oil (Nikolopoulou and Kalogerakis, 2009). An alternative approach is to add allochthonous microorganisms to supplement the indigenous populations. Bacterial strains or consortium are usually added in liquid culture. However, degrading activities of the augmented bacterial cells is hampered by its limited contact with the oil as the added free-living cells often disperse into the water column and are not confined to the location of the spilled oil. Furthermore, the augmented cells in some cases do not survive due to competition with the native microbial populations which are more adapted to the drastic change of environmental conditions (Alexander, 1999; Kauppi et al., 2011).

To improve the survival and retention of the bioaugmentation agents in the site of the spill, bacterial cells are added as immobilized cultures. Immobilized cells have been extensively used in the production of useful chemicals, treatment of wastewaters and bioremediation of pollution due to

its longer operating lifetime and enhanced stability and survival of the cells (Cassidy et al., 1996). In cold climates, the immobilization matrix may also act as a bulking agent in contaminated soils where freezing and increased viscosity may block oxygen, water and nutrient transfer (Podorozhko et al., 2008).

A number of bioaugmentation agents are already commercially available but have no strong scientific evidence demonstrating their effectiveness (Simon et al., 2004; Zhu et al., 2004). On the other hand, scientific studies evaluating the potential of sawdust, Styrofoam and wheat bran (Obuekwe and Al-Muttawa, 2001); chitin and chitosan (Gentili et al., 2006); peanut hull (Xu and Lu, 2010); and zeolite and activated carbon (Liang et al*.,* 2009) showed improved oil biodegradation by increasing and sustaining high number of oil-degraders. However, preparation of some of these materials requires the use of chemicals and sophisticated technology.

In this study, we present the use of cocopeat (coconut peat) (CP) and rice hull powder (RH) as biocarriers of a hydrocarbon-degrading bacterial consortium. Cocopeat is a powdery material that comes from ground coconut husk while RH is a by-product of rice milling. These agro-industrial wastes are good candidates for biocarriers because of their abundance, cost-effectiveness and biodegradable nature. Crude preparation of these materials for bioaugmentation purposes was accomplished by a two-step procedure of grinding and sieving, hence eliminating additional costs and possible waste by-products.

The general aim of this study is to develop an effective immobilized oil-degrading bacterial consortium that can be applied as "ready-to-use" seeds for various bioremediation needs. To attain this goal, the specific objectives of this study are to determine: (1) the suitability of the coconut peat and rice hull powder as biocarriers, (2) the hydrocarbon-degrading efficiencies of the developed immobilized cells compared to the free-living and sodium alginate-encapsulated forms (Karamalidis et al., 2010; Larsen et al., 2009; Rahman et al., 2006) as another mode of bacterial immobilization, and (3) the storage stabilities of the developed immobilized cells in different temperatures.

MATERIALS AND METHODS

Preparation of oil-degrading consortium

The bacterial strains were isolated from bunker oil-contaminated coastal sediments of Taklong Island, Guimaras, Philippines (Nuñal et al., in press). Four strains were selected to comprise the consortium based on their ability to grow in Bushnell-Haas Mineral Salts (BHMS) medium supplemented with different hydrocarbons as carbon source. The list of strains, their phylogeny and hydrocarbon-utilizing properties are shown in Table 2-1.

The selected strains were sub-cultured separately in the Zobell 2216E broth and grown for 24 h. After incubation, the cells were harvested, washed with 75% sterile natural seawater, and resuspended in 0.8% NaCl solution. Equal volumes of the suspension containing the different bacterial strains were mixed to form the consortium.

Preparation of biocarrier-attached immobilized consortium

Raw materials for the production of cocopeat (CP) and rice hull (RH) powder were obtained from local industries in the Philippines. Both powder were derived by mechanically grinding the dried coir (middle fibrous coat) to produce CP and whole rice hulls to produce RH powder. The ground powder was sieved to obtain a homogenous particle size of approximately 1 mm. About 0.3 g of the powder was dispensed in vials and sterilized by autoclaving at 120 psi for 15 min and kept at room temperature until use.

About 0.3 g of the sterilized CP and RH powder was aseptically transferred into 100 mL of the Zobell 2216E broth in separate 250 mL Erlenmeyer flasks. One milliliter of the bacterial consortium was inoculated into the CP- and RH powder-containing broths. After four days of incubation, the powder was harvested, washed by sterile 75% natural seawater, and air-dried.

Preparation of encapsulated bacterial consortium

Immobilization of the bacterial cells in sodium alginate gel was done using the method

described by Rahman et al. (2006). Briefly, 24 h culture of the bacterial strains/consortium (approximately 10^9 CFU mL^{-1}) were harvested by centrifugation (15,000 x *g*, 10 min), rinsed three times with sterile saline, and resuspended in 150 mL of 3% sodium alginate. The suspension was stirred and the resulting alginate/cell mixture was dripped into ice-cooled, sterile 0.2 M $CaCl_2$, which generated beads of approximately 2 mm in diameter. The beads were then hardened in fresh $CaCl_2$ for 2 h.

Scanning electron microscopy

Samples for scanning electron microscopy (SEM) were prepared as described by Nakano et al. (2008). Briefly, the immobilized cells and sterile biocarriers were placed in a 0.05 μm Nuclepore filter (Whatman, Kent, UK). The filter was rinsed with 0.1 M phosphate buffer solution (pH 7.4). The cells were then subjected to dehydration using a series of graded ethanol solution (60 to 90%, 2 x 15 min; then 99%, 3 x 20 min) and a mixed solution (ethanol and 99% 3-methylbutyl acetate, 1:1, for 10 min), and 99% 3-methybutyl acetate (3 x 10 min) with gentle periodic agitation. Critical point drying was performed with HCP-2 dryer (Hitachi High-Tech, Tokyo, Japan). The fixation and dehydration step were omitted for carriers without the bacteria. All the samples were sputter-coated with the ion sputter E-1020 (Hitachi High-Tech, Tokyo, Japan) and observed by the scanning electron microscope S-4000 (Hitachi High-Tech, Tokyo, Japan).

in vitro seawater bioremediation

Efficiency of heavy oil bioremediation by the developed immobilized cells was determined in seawater microcosm trials. Seawater samples were collected from Ise Bay, Mie Prefecture, Japan. Six 250-mL Erlenmeyer flasks containing 150 mL of seawater and 0.75 mL of heavy oil were prepared for each treatment representing three replicates for each sampling period. The different treatments and the contents of each representative flask are shown in Table 2-2. The flasks were incubated at 26˚C, covered with perforated rubber caps and shaken twice a day to provide aeration.

At the end of 30- and 60-day incubation, three flasks for each treatment were sampled for residual oil content and bacterial counts.

Total petroleum hydrocarbon (TPH) extraction

At the end of the 30- and 60-day incubation, the residual oil was extracted from each flask using the US-EPA Gravimetric Method (1999). Briefly, a sample containing oil was collected in a clean 500-mL separatory funnel. The pH was brought down to $\leq$ 2 by adding 6 N HCl in order to hydrolyze oil and prevent sodium sulfate interference. Oil in the samples was exhaustively extracted twice with *n*-hexane. Then, the *n*-hexane layer was dehydrated by passing through a funnel containing anhydrous sodium sulfate and collected in a dry, pre-weighed receiving flask. The remaining solvent in the solution was then evaporated in a rotary evaporator and by nitrogen gas with heating at 70˚C. Finally, the flask containing the concentrated oil was cooled and dried in a desiccator and the residual oil recovered was determined gravimetrically. Reduction in oil concentration (%) was calculated as $(A\text{-}B)/A$ x 100, where A is the initial weight of heavy oil concentration and B is the weight of the residual heavy oil. Degradation of residual hydrocarbon components was analyzed by the gas chromatograph 6890N with the mass selective detector 5973 Network (Agilent technologies, Palo Alto, CA, USA).

Determination of bacterial counts during incubation

Total cultivable bacterial counts in seawater microcosms were determined by conventional plate count technique. One mL sample from each flask was collected at the end of 30- and 60-day incubation. The sample was serially diluted and 0.1 mL of each dilution was spread on Zobell 2216E agar. Plates were then incubated at 26˚C and bacterial counts were determined after 3-5 days of incubation.

Determination of storage stabilities of the immobililized cells

The viability of immobilized consortia during storage at different temperatures was

determined using the method of Gentili et al. (2006) with some modifications. The immobilized cells were stored at three different temperatures: (a) room temperature, (b) 0˚C and (c) -30˚C. Viable counts were determined every 30 day, by spreading 1 g of immobilized cells on Zobell agar, as previously described. For oil-degrading activities, 0.1 g of the immobilized cells were inoculated in 5 mL of seawater supplemented with 25 µL heavy oil and incubated at 26˚C in a rotary shaker with a speed of 150 rpm for seven days. The cultures showing turbidity and visible changes in crude oil compared with controls were considered positive.

Data Analysis

Significant differences of the data in the *in vitro* oil degradation tests were analyzed by the one-way ANOVA followed by Duncan's multiple range test (DMRT) using the statistical program Statistical Package for Social Sciences (SPSS) version 19.0.

RESULTS

Bacterial attachment on biocarriers

Fig. 2-1 shows the scanning electron microscopic images of cocopeat (CP) and rice hull (RH) powder with and without attached bacterial consortium. Results revealed that CP is characterized by pores and crevices while RH powder contains a flat surface, which are both suitable for bacterial adherence. High microbial colonization on the surfaces of the biocarriers were observed when the consortium and carriers were incubated for 96 h. Monolayer cell adherences were seen in both biocarriers but a more compact aggregate of cells forming a biofilm was observed in the CP powder especially in crevices which accommodated more bacterial cells than the RH powder.

Oil-degrading efficiency of the immobilized consortium

Highest oil degradation was found in the treatments receiving the two biocarrier-immobilized cells after 30 days of seawater bioremediation trial (Fig. 2-2). CP and RH

treatments showed significantly higher oil reduction ($p<0.05$) of 59.8% and 55.7%, respectively. Differences in the oil reduction between the treatments with the encapsulated cells (ENC, 25.1%) and with the bacterial cells in free-living form (FC, 24.5%) were not significant. Natural attenuation of oil shown in the PC treatment recorded 22.6% oil reduction after the 30-day incubation and was not significantly different ($p>0.05$) with the treatment augmented with consortium in free-living form. NC treatment which accounts for the abiotic changes in the heavy oil showed relatively unchanged concentration at 4.6% reduction.

At the end of the 60-days incubation, gravimetric determination of residual heavy oil revealed that CP treatment showed the significantly highest ($p<0.05$) degradation among all the treatments with 86.6 % reduction (Fig. 2-2). Oil reduction in the ENC treatment markedly increased by the end of the 60-day experimental time with 64.9% reduction which is significantly higher than the FC (51.2%) and PC (46.5%) treatments. No significant differences ($p>0.05$) were found between the total petroleum hydrocarbon (TPH) reductions of the FC and PC treatments but both reductions were significantly higher than the abiotic control (NC, 19.9%).

Reduction in different hydrocarbon components

Figures 2-3, 2-4 and 2-5 show the reduction of *n*-alkanes, polycyclic aromatic hydrocarbons (PAHs) and alkyl PAHs, respectively, in the different treatments. Generally, more than 50% of the degradation of the hydrocarbon components was achieved in the first 30 days. All flasks augmented with the bacterial consortium either in immobilized or free-living form showed significantly higher *n*-alkane degradation ($p<0.05$) compared to the control. Significantly highest degradation ($p<0.05$) was found in flasks augmented with carrier-attached consortium (Fig. 2-3, RH and CP). At the end of the 60-day incubation the highest degradation in short chain- (89.94%) mid-length chain- (81.64%), and long chain-alkanes (63.81%) were achieved in the CP treatment, but were not statistically significant compared to degradation found in RH treatment (Table 2-3).

Augmentation with immobilized cells, both in encapsulated and attached forms yielded

significantly higher PAH reduction than FC and PC treatments (Fig. 2-4). The highest reduction in PAHs were found in RH treatment achieving 74.35% reduction after 60 days but no significant difference ($p>0.05$) was found when compared to the CP treatment (71.79%). Significantly higher reductions ($p<0.05$) in 2-4 ring PAHs such as naphthalene, acenapthylene, acenapthene, flourene, phenanthrene, anthracene, and pyrene were obtained in RH and CP treatments (Table 2-4). Degradation of benzo[*a*]anthracene, benzo[*b*]flouranthene and benzo[*k*]flouranthene was detected in all bioaugmented treatments but not in CP treatment. Degradation of high molecular weight PAHs such as Benzo[*g,h,i*]perylene, dibenzo[*a,h*]anthracene and indeno[1,2,3-*c,d*] pyrene were not detected in all treatments.

Alkyl PAHs are least biodegradable with the highest reduction observed in CP treatment yielding only 45.36% (Fig. 2-5). Augmentation with encapsulated and free-living forms of the consortium did not yield any significant difference ($p>0.05$) with the control (PC), in which indigenous oil degraders degraded only about 21.59% of alkyl PAHs after 60 days. Significantly higher ($p<0.05$) degradation of alkyl naphthalenes, alkyl phenanthrenes, 1-methylflourene, 2-methylflouranthene and 4-methylchrysene were obtained in CP and RH treatments (Table 2-5). High molecular weight alkyl PAHs such as 4,6-dimethyldibenzothiophene, 1-methylanthracene, 7,12-dimethylbenzo[*a*]anthracene, perylene and 7-methylbenzo[*a*]pyrene were not degraded in all treatments.

Changes in cultivable bacterial counts during incubation

The cultivable bacterial count in the different treatments during the course of bioremediation is shown in Fig. 2-6. Initial bacterial counts at Day 0 were high in FC, CP and RH (more than 10^8 CFU mL^{-1}) treatments while PC and ENC treatments started with a low cultivable bacterial count at about 10^4 and 10^6 CFU mL^{-1}, respectively. In the first 30 days, decline in bacterial numbers were observed in the FC treatment while almost no change in cultivable numbers were observed in ENC, RH and CP treatments. At the end of the 60-day incubation, total cultivable bacterial counts in the

treatments containing both the biocarrier-attached cells dropped from the original concentration but were still higher than in FC and PC treatments. In contrast, bacterial counts in the PC treatment slightly increased while a dramatic increase was observed in ENC treatment at day 60.

Survival of immobilized cells under storage

Bacterial cell recovery from the immobilized cells during storage in room temperature, 0˚C and -30˚C is shown in Fig. 2-7. In the 3 schemes of immobilization, viable cell counts in RH powder- and CP-immobilized cells decreased by about 2 orders of magnitude after 1 month of storage in the three temperatures tested. Immobilized cells stored at lower temperature (0˚C and -30˚C) exhibited better survival compared to those stored at room temperature. The cultures stored at 0˚C and -30˚C yielded fairly stable viable counts at 10^6 CFU g^{-1} until the end of the 6 months storage period. The stored immobilized cells, both encapsulated and carrier-attached, scored positive oil-degrading activities in all the sampling period (data not shown).

DISCUSSION

The present study attempts to address the limitations of bioaugmentation by immobilizing a bacterial consortium in organic biocarriers to improve the survival of added strains and to develop a more clear-cut application of remediation agents to oil pollutants. The main findings of this study can be summarized as follows: (1) selection of the suitable bacterial species to comprise the oil degrading-consortium as bioaugmentation agents; (2) the effectiveness of biocarriers for attachment of the formulated oil-degrading consortium; (3) the efficiencies of the developed carrier-attached immobilized cells in improving degradation of oil; and (4) the storage stabilities of the developed immobilized cells ready for use when sudden need arises. In this study, the use of cocopeat (CP) and rice hull (RH) powder as possible biocarriers was explored. These materials were selected because of their organic, nontoxic and nonpolluting nature and their ubiquity makes the application possible in a

global perspective. In addition, using CP and RH as biocarriers creates another significant purpose to utilize these waste products, thus contributing an economically and ecologically sound remediation technology. When co-cultured with the bacterial consortium, colonization of the bacteria and formation of compact aggregates on the surface of both materials occurred. Results of the scanning electron microscopy (SEM) revealed that the attachment of the bacterial cells formed on the surfaces of both CP and RH powder was sufficiently strong to withstand washings with seawater during the preparation. Monolayer cell growths forming a biofilm on the surfaces of both biocarriers were observed. This is an important finding as previous studies show that only bacteria within 10-20 μm-thick surface layer can actually participate in the metabolic activities because of the limitations of substrate and oxygen diffusion (Levinson et al., 1994; Obuekwe and Al-Muttawa, 2001).

The results of the degradation of oil components in the *in vitro* seawater bioremediation tests were expressed as the percent reduction in oil concentration relative to the negative control (NC) which accounts for the abiotic losses. This signified that decreases in oil concentration in the treatments could only be attributed to either biotic degradation or removal by adsorption on biocarriers. Although the presence of autochthonous oil degraders in the seawater was confirmed in the PC treatment, bioaugmentation using both free-living and immobilized forms of the cells render improvement in oil reduction, indicating the effectiveness of the mixture of selected strains in enhancing oil degradation.

Comparing all the bioaugmented treatments, significantly higher reduction of total petroleum hydrocarbons (TPH) was observed in the treatments where the biocarrier-attached consortia were augmented (Fig. 2-2). Lower reduction in treatments added with liquid culture was due to loss in microbial biomass and shorter survival of the cells in seawater as shown by the decreasing cultivable bacterial counts during the course of remediation (Fig. 2-6). On the contrary, the immobilization of the consortium on the RH powder and CP biocarriers greatly improved the survival of the added cells, thus sustaining higher microbial population (Gentili et al., 2006; Obuekwe and Al-Muttawa, 2001). Although TPH reductions were not statistically different between

RH and CP treatments during the first 30 days, longer incubation for 60 days yielded a more pronounced difference. This result is supported by higher bacterial counts and SEM images showing more cells attached to the CP than in the RH powder.

One important observation that may have contributed to enhanced degradation in the treatments receiving carrier-attached consortia in this study is the oil-adsorbing capacity of both CP and RH powder as some particles remain suspended in the water column. Although not quantified in the present study, visual inspection suggested adherence of oil onto the surface of RH powder and CP. The liquid-adsorbing capacities of RH and coconut husk have already been extensively studied as these materials are widely used in bioreactors and as biosorbents (Anirudhan et al., 2008; Chuah et al., 2005; El-Shafey, 2007; Namasivayam and Sureshkumar, 2008; Wan Ngah and Hanafiah, 2008). This characteristic of the biocarriers increases the probability of the bacterial cells to be in contact with the hydrocarbon substrate, an important factor for efficient degradation. Furthermore, both organic carriers are known to contain high amounts of phosphorus and nitrogenous nutrients (Abad et al., 2002; Abbas et al., 2012; Hernandez-Apaolaza and Guerrero, 2008) which may enhance growth of the indigenous and augmented oil-degrading cells.

When the formulated bacterial consortium is encapsulated into sodium alginate gel, there was lag in the oil degrading activity: significant reduction was only observed after 60 days. The low degradation in the first 30 days coincided with low cultivable bacterial counts while the improved reduction occurred when bacterial counts were high at day 60. The limitation of diffusion of the hydrophobic oil into hydrophilic sodium alginate matrix may cause the delay in the degrading activities of encapsulated cells. Longer incubation period thus proved to be beneficial to allow sufficient time for cell replication inside the sodium alginate matrix and the eventual release of the cells into the external seawater environment (Cunningham et al., 2004; Qi et al., 2006). This finding revealed that carrier-immobilized cells are better alternative than encapsulated cells if immediate and fast removal of oil contaminants is desired.

In all bioaugmented treatments, degradation was greater in the aliphatic than aromatic

fractions. As the most degradable hydrocarbons, straight chain alkanes from C8 to C33 were degraded in all treatments including the control which contained only indigenous oil degraders. Some members of the genera *Pseudomonas* and *Marinobacter* show abilities of degrading aliphatics whose chain length is ranging from mid-range (C12), long (C34) (Chaerun et al., 2004; Karamalidis et al., 2010; Wentzel et al., 2007) to very long (C36 to C40) (Hasanuzzaman et al., 2007; Zhang et al., 2011). Aromatics with two or three rings were also biodegraded in all treatments but degradation of the more resistant polycyclic aromatic hydrocarbons (PAHs) such as pyrene, benzo[*a*]anthracene, chrysene, benzo[*a*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, albeit in low numbers of rings, were detected only in treatments added with the formulated consortium. A number of documented degrading abilities of *Pseudomonas* and *Marinobacter* species for a wide range of recalcitrant substrates including more complex aromatic compounds are already in place (Karamalidis et al., 2010; Liu et al, 2010; Seo *et* al., 2009; Tian et al., 2008), hence supporting these results.

In general, studies on the bacterial degradation of PAH with alkyl substitutions are relatively scarce. Alkyl PAH degradation is more difficult to achieve due to the presence of the alkyl branch that inhibits the accessibility of bacterial catabolic enzymes to the PAHs (Seo et al., 2009). In the present study, degradation of alkyl PAHs follows the two general principles: (1) decreasing degradation with increasing molecule size and (2) decreasing degradation with increasing alkylation within a homologous series (Budzinski et al., 1998; Jimenez et al., 2006; White et al., 2005). When considering the degradation of compounds with the same number of alkylation in the present study, it appears that the position of methyl substitution did not affect the degradation rates. The microbial communities in the treatments, as a mixture of bacterial groups possessing different catabolic pathways, did not show selectivity based on the position of the alkyl group. Alkyl PAHs with complex molecular structures proved to be more resistant with no detected degradation at all in the different treatments (Table 2-5). This would suggest restrictions in the metabolic capabilities of the microbial population which may also be influenced by other factors such as absence of other

essential inorganic nutrients and shift to degrading by-products as an alternate carbon source (Bamforth and Singleton, 2005; Leahy and Colwell, 1990; Wang et al., 2011).

The other members of the consortium have no documented oil-degrading properties. Members of the genus *Halomonas*, however, have abilities to produce exopolysaccharides which plays an important role in biosurfactant-mediated cell contact with hydrocarbons, making them an important component of most oil-degrading bacterial communities (Nakamura et al., 2007; Uad et al., 2010). Neither oil-degrading nor biosurfactant-producing properties have reported on *Gaetbulibacter* species. In our preliminary experiment (Nuñal et al., in press), although it showed weak growth in the presence of phenanthrene in the medium, it was able to utilize C14 and C24 as a carbon source and showed strong growth when heavy oil was supplemented. To our knowledge, this is the first report of the capacity of the *Gaetbulibacter* species to utilize hydrocarbon for growth. However, further study is required to elucidate the range of degradable hydrocarbon substrate as well as possible utilization of resin and asphaltene fractions of heavy oil. Insofar as the present study is concerned, its cooperative action with the other members of the consortia as well as with the indigenous population of the seawater to degrade the oil contaminant was established.

Analysis of viable counts and oil utilization of immobilized cultures demonstrated the stability and resilience of the cells under storage for six months. The percentage recovery of cells stored in the 3 temperatures appeared fairly stable (Fig. 2-7) in the three immobilization schemes. Actual microbial number of stored carrier-attached cells may actually be higher than the obtained bacterial counts, as strongly anchored bacterial cells are difficult to dislodge from the solid carrier matrix for bacterial count analysis (Obuekwe and Al-Muttawa, 2001). The oil-degrading activities of the cells that survived after storage showed that the immobilized cells can be stored without losing its metabolic activities. The immobilized cells can therefore be stored at low temperatures and be readily used in a sudden event of oil pollution.

The success of bioaugmentation lies on the survival and activity of the added microorganisms to a hostile environment such as marine water. In the present study, a formulated

oil-degrading microbial consortia immobilized on carrier materials were presented. The consortium was able to improve degradation of both the aliphatic and aromatic fractions of heavy oil in *in vitro* seawater bioremediation. When cells are immobilized in biocarriers, significant reductions in oil concentration were observed and higher bacterial counts during incubation were obtained compared to liquid culture and control. Storage of up to six months has minimal effect on the viability and oil-degrading activities of the immobilized cells. Overall, the results of this study showed successful use of available and cheap organic materials as biocarriers and the improvement of biodegradation by immobilizing the cells. Although these results may not solely predict field performance, the strategy used in this study can be very useful in developing carrier-based inoculants applicable to a wide variety of bioremediation scenarios.

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TABLES AND FIGURES

TABLE 2-1. List of bacterial strains comprising the consortium and their growth in the different hydrocarbon-enriched media

Strain code	Closest relatives[a] (Acc. No.)	Genbank Acc. No.	Phylogenetic group	Hydrocarbon utilization[b]			
				Tetradecane (C14)	Tetracosane (C24)	Phenanthrene	Heavy oil
A4	*Halomonas* sp. (GQ1690777)	AB649101	Alpha-Proteobacteria	-	-	+	+
C1	*Pseudomonas aeruginosa* (GU72841)	AB649102	Gamma-Proteobacteria	+	+	+	++
D1	*Marinobacter mobilis* (GQ214550)	AB649110	Gamma-Proteobacteria	+	+	+	+
D5	*Gaetbulibacter* sp. (FJ360684)	AB649114	Cytophaga-Flavobacterium-Bacteroides (CFB)	+	-	+	++

[a]Closest relatives were retrieved from the nucleotide sequence databank GenBank based on the homology search BLASTN of 16S ribosomal RNA genes.

[b]+= growth, ++=strong growth, -=no growth

TABLE 2- 2. Contents of f the different treatments for the *in vitro* seawater bioremediation

Treatments	Code	Seawater	Heavy oil	Bacterial community present[a]
1. Natural attenuation	PC	Natural	Unautoclaved	Indigenous
2. Augmented with bacterial cells in free-living form	FC	Natural	Unautoclaved	Indigenous + consortium in free living form
3. Augmented with encapsulated cells	ENC	Natural	Unautoclaved	Indigenous + encapsulated consortium
4. Augmented with rice hull powder-immobilzed cells	RH	Natural	Unautoclaved	Indigenous + rice hull powder-immobilzed cells
5. Augmented with cocopeat-immobilzed cells	CP	Natural	Unautoclaved	Indigenous + cocopeat-immobilzed cells
6. Abiotic changes in oil	NC	Sterile	Sterile	None

[a]Immobilized and free-living bacterial consortium were added to a final concentration of $1x10^7$ CFU mL^{-1}.

TABLE 2-3. Reduction in concentration (%) of *n*-alkanes in the different microcosms after 60 days of incubation

n-alkanes	Treatments				
	PC	FC	ENC	RH	CP
C8-C15	61.72 ± 0.95^{a}	64.30 ± 3.87^{ab}	78.9 ± 3.68^{b}	77.7 ± 2.74^{c}	89.94 ± 0.09^{c}
C16-C25	54.11 ± 4.87^{a}	57.48 ± 4.47^{a}	60.50 ± 0.98^{a}	81.38 ± 3.18^{b}	81.64 ± 2.74^{b}
C26-C33	24.86 ± 3.14^{a}	44.62 ± 5.36^{b}	55.06 ± 6.90^{bc}	63.45 ± 2.95^{c}	63.81 ± 8.65^{c}

The means of three replicates are shown with the standard deviation. Different superscript letters indicate significant difference at $p<0.05$. Abbreviations of the treatments are shown in Table 2-2.

TABLE 2-4. Reduction in concentration (%) of 16 priority PAHs (US-EPA) in the different microcosms after 60 days of incubation

PAHs	Treatments				
	PC	FC	ENC	RH	CP
Naphthalene	64.60 ± 1.35[a]	58.76 ± 8.36[a]	78.49 ± 0.55[b]	95.21 ± 5.48[c]	93.83 ± 2.85[c]
Acenapthylene	22.77 ± 3.60[a]	32.02 ± 3.92[a]	34.44 ± 6.90[a]	77.76 ± 9.92[b]	63.15 ± 5.44[b]
Acenapthene	22.98 ± 1.34[a]	24.90 ± 7.34[a]	66.30 ± 3.73[b]	79.06 ± 1.57[c]	59.96 ± 0.54[b]
Fluorine	20.04 ± 1.25[a]	26.33 ± 6.75[a]	66.35 ± 0.64[b]	78.07 ± 1.91[c]	76.91 ± 1.48[c]
Phenanthrene	25.89 ± 2.03[a]	23.39 ± 1.40[a]	68.31 ± 0.30[b]	79.12 ± 0.72[c]	73.31 ± 1.35[d]
Anthracene	25.22 ± 4.02[a]	54.98 ± 1.45[a]	74.88 ± 27.74[c]	71.76 ± 31.63[c]	71.79 ±28.79[c]
Flouranthene	22.17 ± 10.66[a]	39.73 ± 9.62[ab]	53.15 ± 9.35[b]	54.15 ± 6.73[b]	43.39 ± 4.45[ab]
Pyrene	11.42 ± 0.75[a]	12.76 ± 2.49[b]	4.85 ± 2.32[a]	12.59 ± 4.80[b]	12.40 ± 0.00[b]
Benzo[*a*]anthracene	N.D.	1.66 ± 0.64[a]	4.59 ±2.03[a]	5.80 ±3.73[a]	6.92 ±3.84[a]
Chrysene	12.08 ± 1.89[a]	15.36 ±1.03[b]	15.91 ±2.1[b]	16.47 ± 1.81[b]	17.94 ± 1.64[b]
Benzo[*b*]flouranthene	N.D.	4.87 ± 1.12[a]	7.70 ± 2.12[ab]	11.63 ± 2.90[b]	12.65 ± 3.22[b]
Benzo[*k*]flouranthene	N.D.	6.78 ± 0.16[a]	7.89 ± 1.41[a]	12.41 ± 2.92[a]	12.62 ± 3.22[a]
Benzo[*a*]pyrene	7.43± 1.27[a]	10.69 ± 7.86[a]	6.41 ± 1.81[a]	12.51 ± 1.10[a]	17.05 ± 0.37[b]
Benzo[*g,h,i*]perylene	N.D.	N.D.	N.D.	N.D.	N.D.
Dibenzo[*a,h*]anthracene	N.D.	N.D.	N.D.	N.D.	N.D.
Indeno[1,2,3-*c,d*]pyrene	N.D.	N.D.	N.D.	N.D.	N.D.

The means of three replicates are shown with the standard deviation. Different superscript letters indicate significant difference at $p<0.05$. Abbreviations of the treatments are shown in Table 2-2. N.D., the reduction was not detected.

TABLE 2-5. Reduction in concentration (%) of alkyl PAHs in the different microcosms after 60 days of incubation

Alkyl PAHs	Treatments				
	PC	FC	ENC	RH	CP
2-methylnaphthalene	34.68±0.23 [a]	46.65±7.69 [a]	41.13±3.45 [a]	81.69±2.63 [b]	82.75± 0.64 [b]
1-methylnaphthalene	33.10±0.30 [a]	44.87±13.15 [a]	53.23±15.90 [ab]	75.92±6.61 [bc]	81.46±1.85 [c]
1,2-dimethylnaphthalene	27.36±4.34 [a]	26.72±3.09 [a]	29.20±5.12 [a]	48.46±7.88 [b]	64.55±1.21 [c]
1-methylfluorene	33.47±1.23 [a]	40.06±1.93 [b]	43.56±0.83 [c]	47.19±2.28 [c]	46.73±0.52 [c]
2,3,5-trimethylnaphthalene	18.92±4.08 [a]	20.19±0.55 [ab]	16.89±5.85 [ab]	21.43±0.28 [ab]	27.43±4.16 [b]
Dibenzothiophene	32.13±1.46 [a]	32.24±4.55 [a]	33.40±1.78 [a]	35.33±7.59 [a]	59.12±3.48 [b]
4-methyldibenzothiophene	20.09±13.38 [a]	23.83±15.01 [a]	28.86±15.42 [a]	26.39±1.89 [a]	37.30±12.18 [a]
4,6-dimethyldibenzothiopehene	N.D.	N.D.	N.D.	N.D.	N.D.
1-methylanthracene	N.D.	N.D.	N.D.	N.D.	N.D.
2-methylphenanthrene	25.26±3.16 [a]	29.29±0.29 [ab]	23.74±0.65 [ab]	22.38±5.79 [ab]	31.09±0.05 [b]
1-methylphenanthrene	35.20±0.81 [a]	35.80±6.02 [a]	26.21±0.47 [b]	33.08±0.89 [ab]	33.36±3.20 [ab]
2,3-dimethylanthracene	3.23±0.08 [a]	5.94±1.17 [a]	3.22±1.51 [a]	3.95±2.63 [a]	3.77±2.66 [a]
2-methylfluoranthene	24.32±3.71 [a]	24.32±2.55 [a]	21.88±1.91 [a]	38.50±5.90 [b]	43.47±0.65 [b]
1-methylpyrene	N.D.	N.D.	N.D.	26.22±4.01 [a]	41.71±3.75 [b]
1-methylbenzo[*a*]anthracene	N.D.	N.D.	N.D.	20.81±0.33 [a]	32.57±1.34 [b]
4-methylchrysene	1.60±1.11 [a]	6.36±0.36 [a]	7.50±0.95 [a]	17.10±2.40 [ab]	29.33±13.23 [b]
7,12-dimethylbenzo[*a*]anthracene	N.D.	N.D.	N.D.	N.D.	N.D.
Perylene	N.D.	N.D.	N.D.	N.D.	N.D.
7-methylbenzo[*a*]pyrene	N.D.	N.D.	N.D.	N.D.	N.D.

The means of three replicates are shown with the standard deviation. Different superscript letters indicate significant difference at $p<0.05$. Abbreviations of the treatments are shown in Table 2-2. N.D., the reduction was not detected.

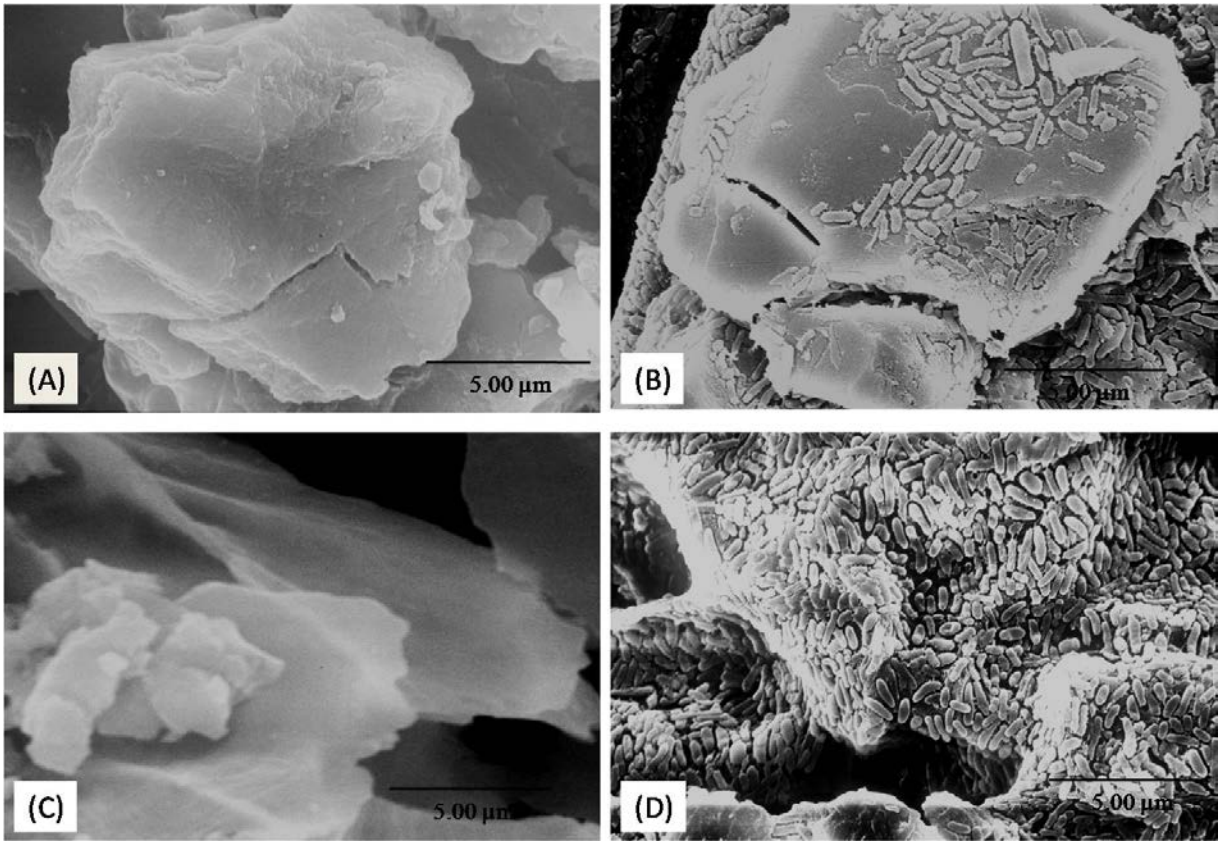

FIG. 2-1. Scanning electron microscopic images of the biocarriers without the bacteria (A, rice hull powder; C, cocopeat) and the consortium colonizing the surface of the biocarriers (B, rice hull powder; D, cocopeat).

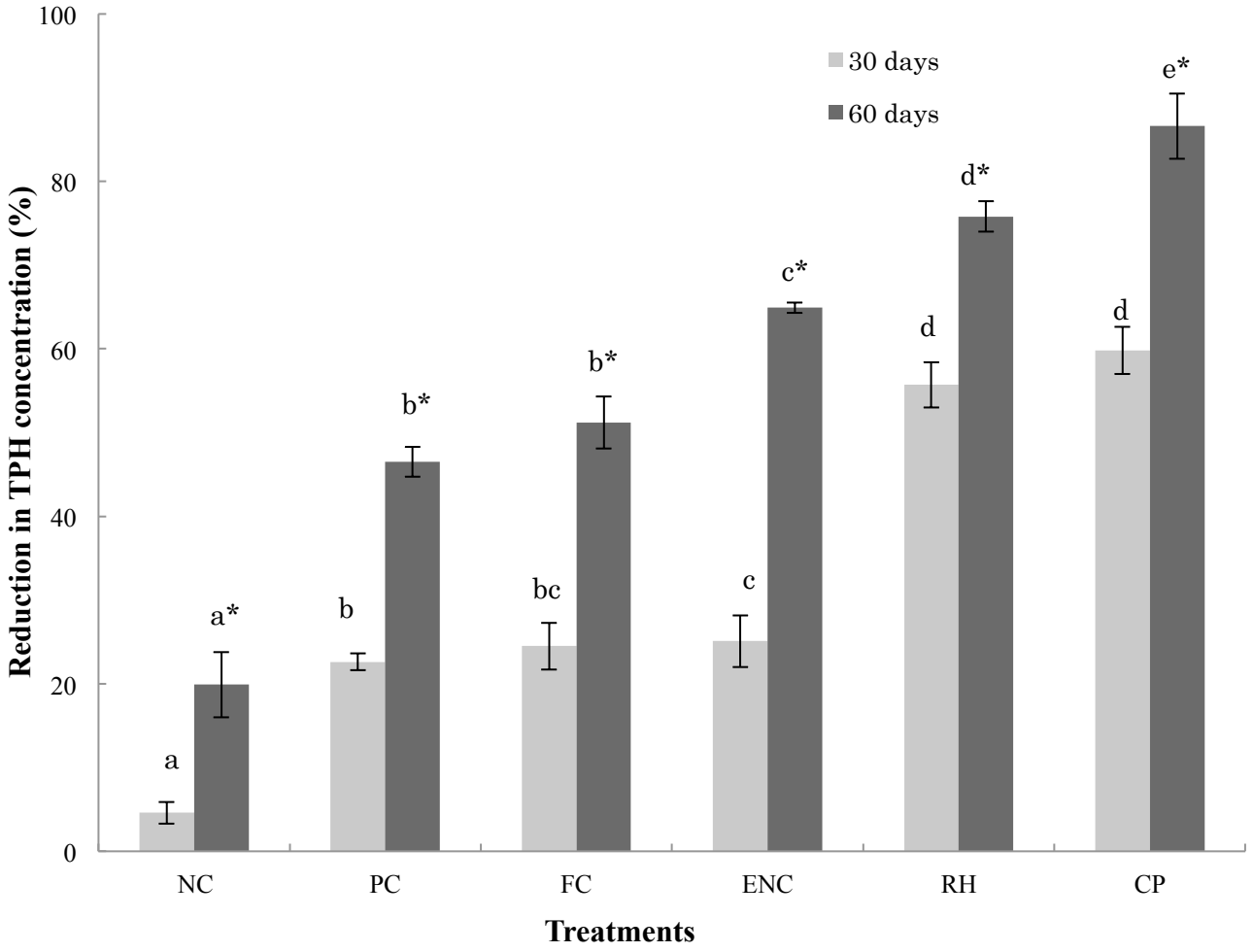

FIG. 2-2. Gravimetric determination of reduction in total petroleum hydrocarbon concentration of different seawater microcosms after 30 and 60 days of incubation. Values represent mean of three replicates. Error bars show the standard deviation. Different letters above the bars indicate significant difference at $p<0.05$. Significant differences of day 60 measurements are indicated by letter with asterisk (*) above the bars. Abbreviations of the treatments are shown in Table 2-2.

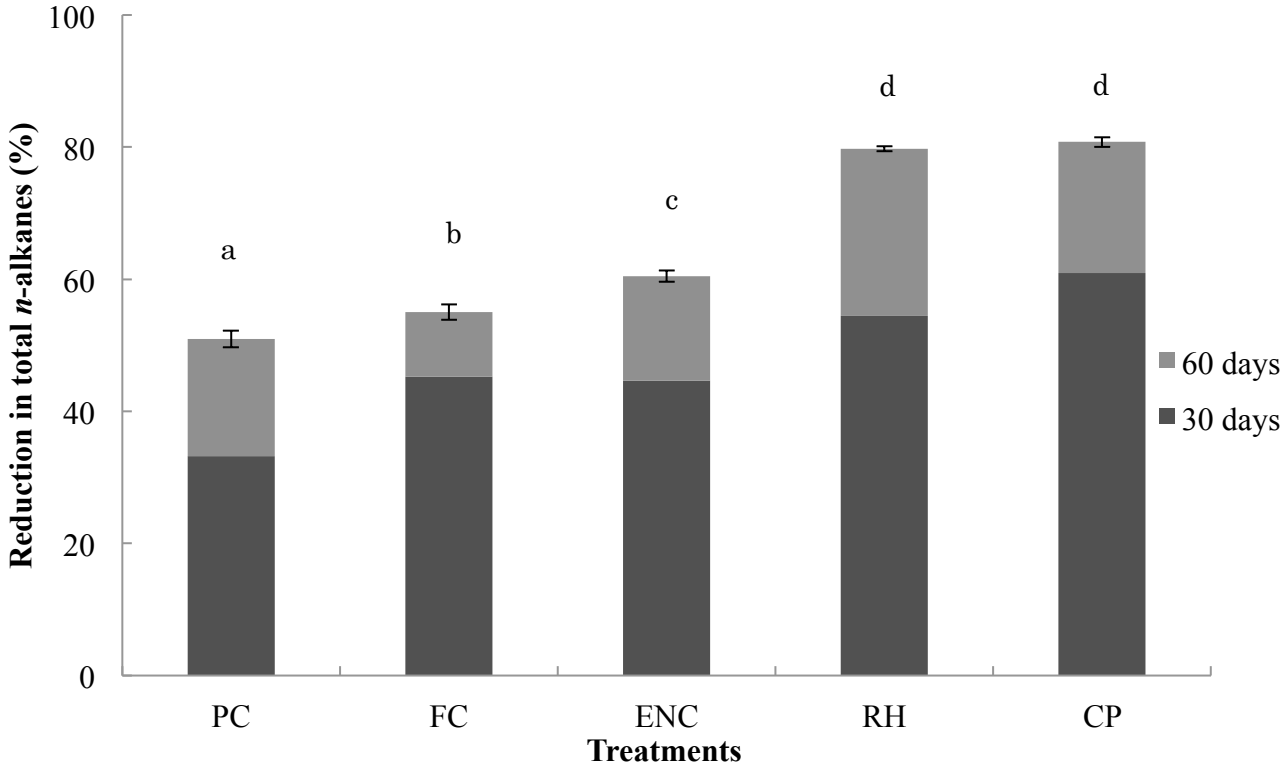

FIG. 2-3. Reduction in total *n*-alkanes in the different microcosms relative to abiotic control (NC) after 30- and 60-days incubation. Values represent the mean of three replicates. Error bars show the standard deviation. Different letters above the bars indicate significant difference at $p<0.05$. Abbreviations of the treatments are shown in Table 2-2.

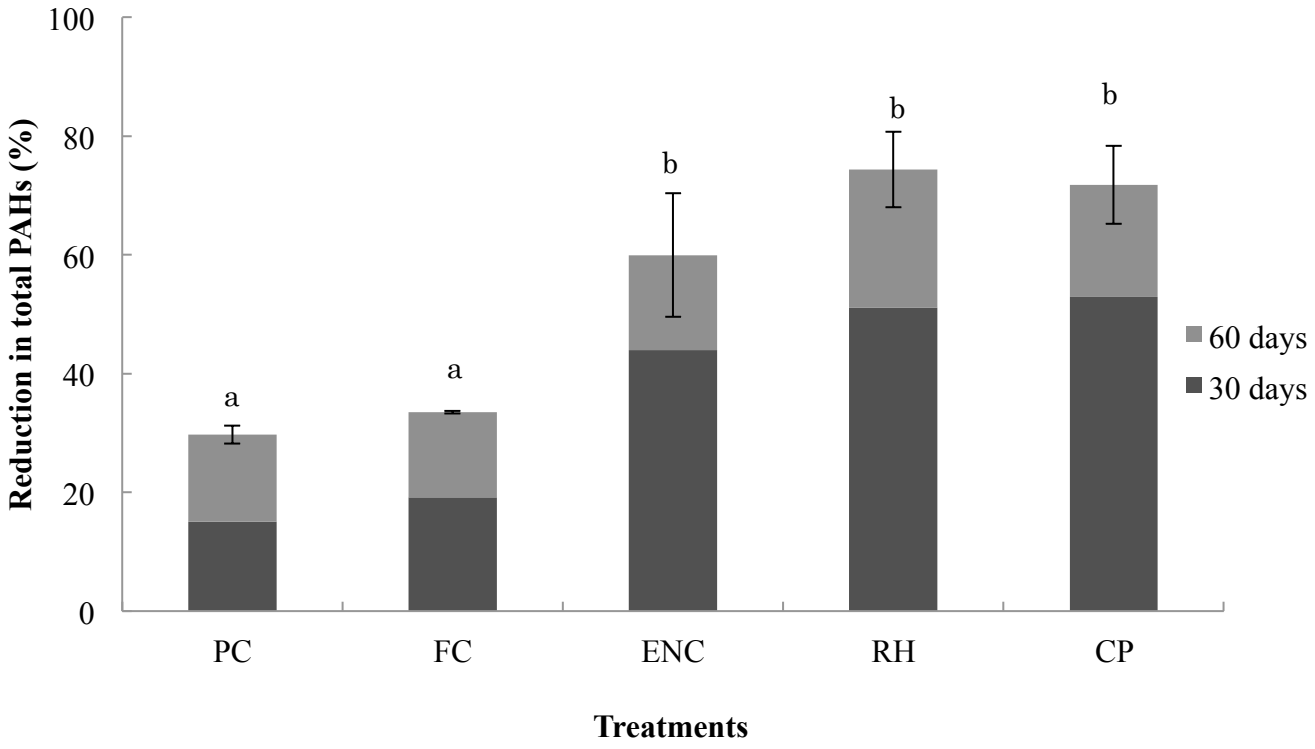

FIG. 2-4. Reduction in total polycyclic aromatic hydrocarbons in the different microcosms relative to abiotic control (NC) after 30- and 60-days incubation. Values represent the mean of three replicates. Error bars show the standard deviation. Different letters above the bars indicate significant difference at $p<0.05$. The treatments indicated by the abbreviated names are shown in Table 2-2.

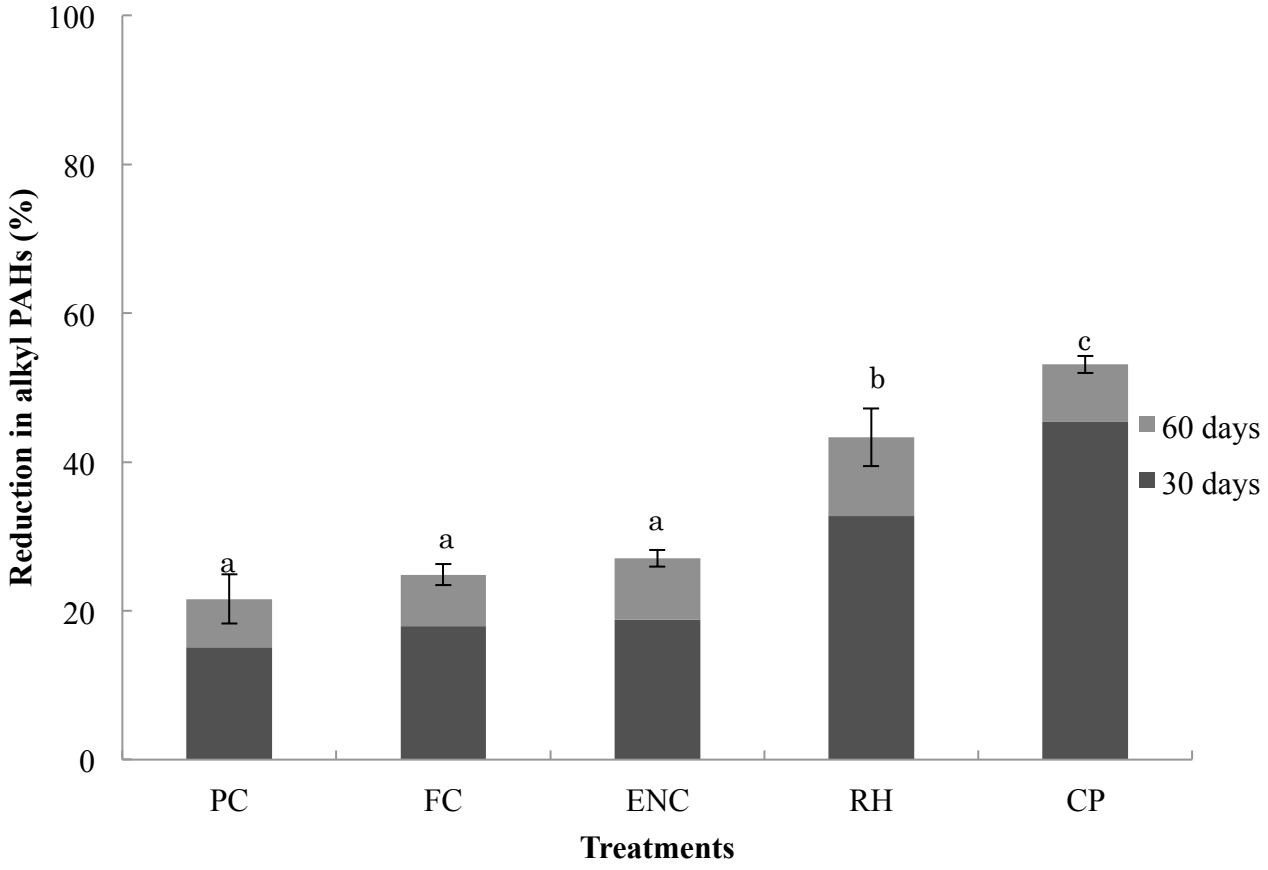

FIG. 2-5. Reduction in total alkyl polycyclic aromatic hydrocarbons in the different microcosms relative to abiotic control after 30- and 60-days incubation. Values represent the mean of three replicates. Error bars show the standard deviation. Different letters above the bars indicate significant difference at $p<0.05$. Abbreviations of the treatments are shown in Table 2-2.

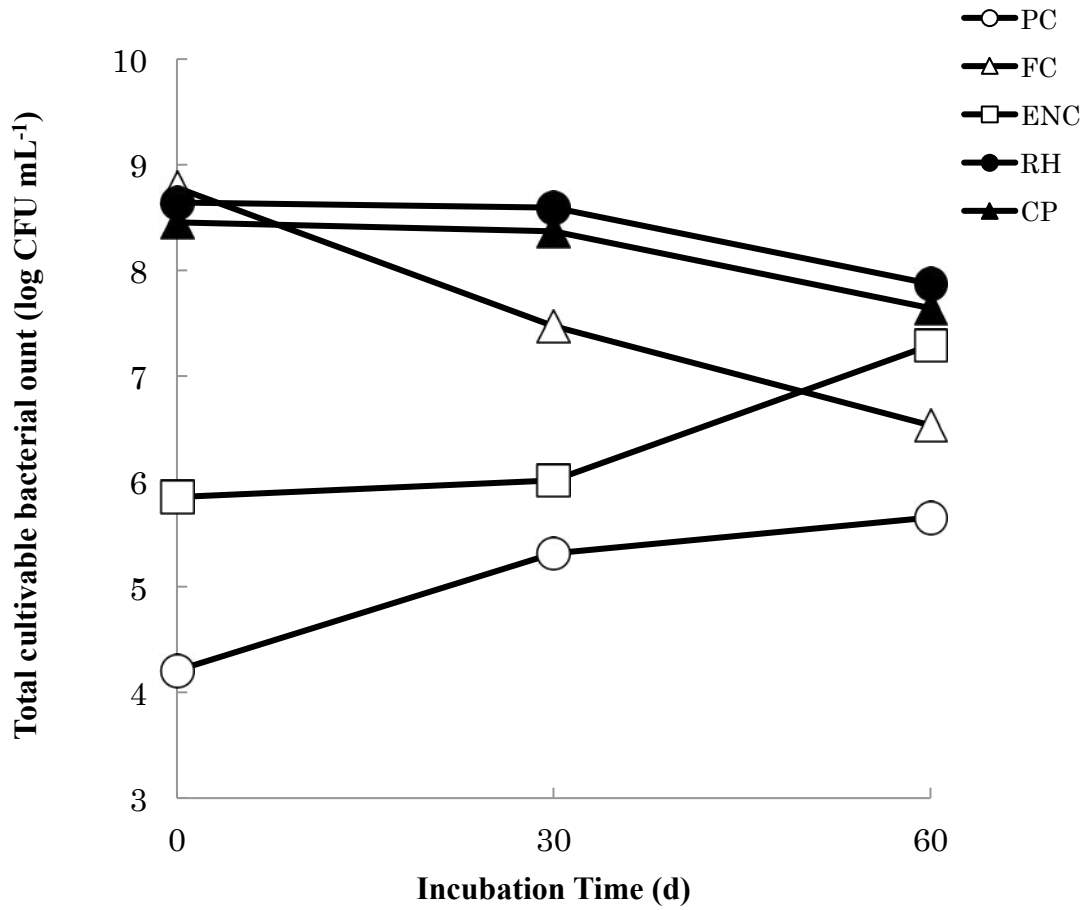

FIG. 2-6. Changes in the total cultivable bacterial counts in the different treatments at the onset, and after 30 and 60 days of remediation. Values represent the mean of three replicates. Abbreviations of the treatments are shown in Table 2-2.

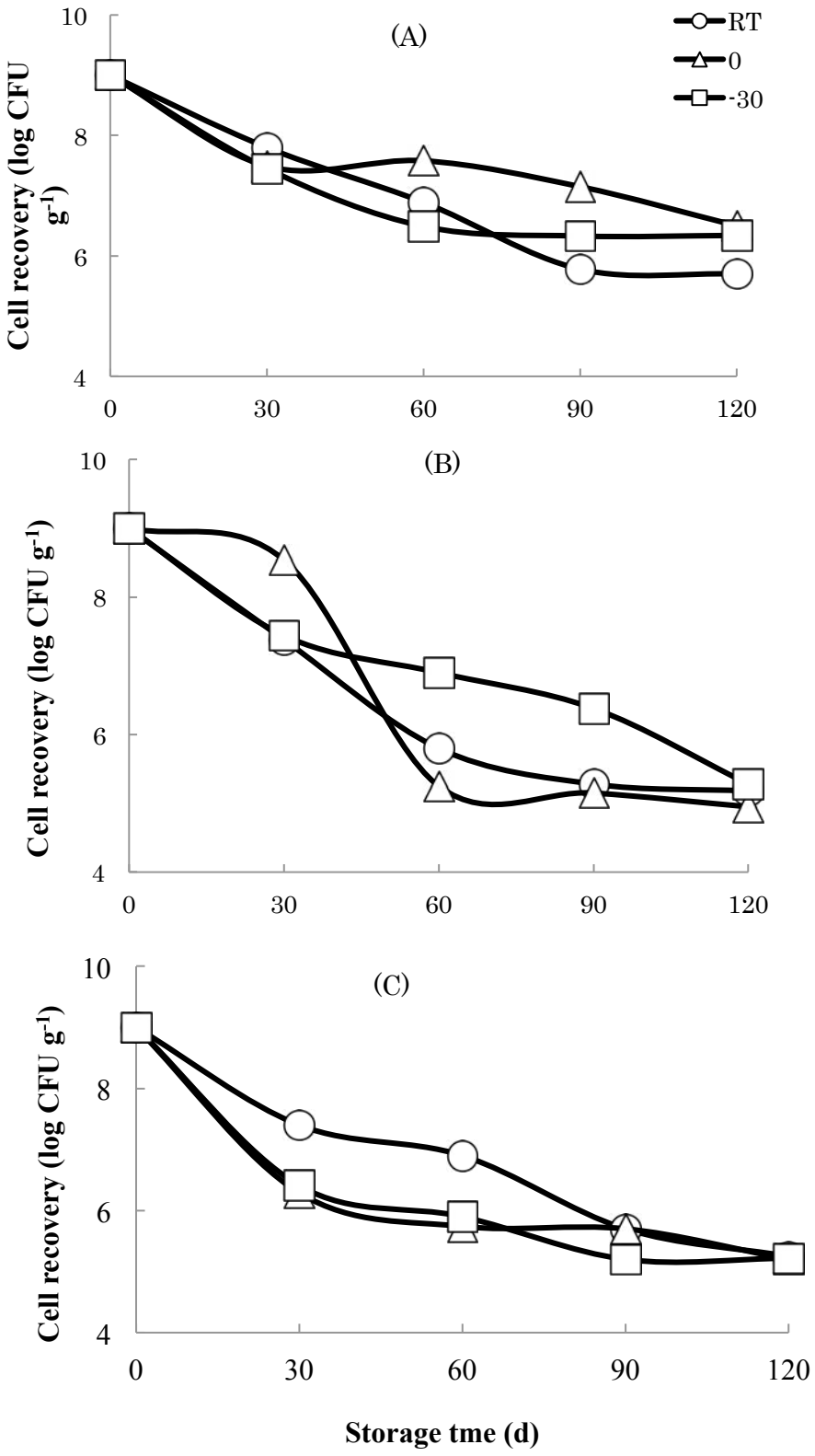

FIG. 2-7. Time course of the changes in viable counts obtained from the immobilized consortia (A, rice hull; B, cocopeat; C, encapsulated) stored in different temperatures, room temperature (open circles), 0˚C (open triangles), and -30˚C (open squares).

CHAPTER THREE

BIOREMEDIATION OF SEDIMENT ARTIFICIALLY-CONTAMINATED WITH HEAVY OIL BY IMMOBILIZED OIL-DEGRADING BACTERIAL CONSORTIUM

ABSTRACT

In bioremediation, the decision to implement bioaugmentation, biostimulation, or both should be supported by laboratory trials evaluating the efficiency under controlled conditions and identifying the limitations. In this study, the efficiency of cocopeat-immobilized bacterial consortium on remediating sediment artificially contaminated with heavy oil was determined. A 3x2 factorial microcosm experiment was conducted to investigate the effects of the addition of a cocopeat-immobilized bacterial consortium, with and without nutrient supplementation, on the remediation of the contaminated sediment. Temporal degradation of the heavy oil components and changes in total cultivable and oil-degrading bacterial counts within 60 days of incubation was determined. Results showed positive influences of nutrient and bacterial cell addition to the degradation of heavy oil. Statistical analysis revealed that addition of nutrients has greater effect to remediation than addition of bacterial cells. Supplementation of inorganic nutrients promoted and sustained growth of oil-degrading and heterotrophic bacteria throughout the experimental period. Highest reduction in total petroleum hydrocarbons (68.9%), and of its components, *n*-alkanes (98.9%), polycylic aromatic hydrocarbons (PAHs) (71.8%) and alkyl PAHs (53.5%), were obtained in the microcosms receiving inorganic nutrients and immobilized bacterial cells. It is concluded that combining biostimulation and bioaugmentation can achieve maximum degradation of heavy oil.

INTRODUCTION

When oil is spilled in the sea, it undergoes changes in form brought about by wave action, sunlight and inherent microbial activity through the passing of time (Mackay and McAuliffe, 1988; Kingston, 2002). Some components of the spilled oil disperse into the water column while some settle on the bottom through the process of sedimentation. Once the oil is interspersed on the surface, it can penetrate into the sediment, making its removal difficult, thus it can persist for a longer time (Amadi et al., 1994; Colombo et al., 2004; Reddy et al., 2002). The oil affects the benthic flora because it contains toxic compounds such as polycyclic aromatic hydrocarbons (PAHs) and their alkylated homologues in high concentration. Aerobic catalytic processes by the natural microbial community in the seawater and sediment can, however, break down the hydrocarbons which can be completely mineralized into CO_2 (Harayama et al., 1999).

In a case of a tanker accident in the sea, the large oil influx causes imbalance in the carbon/nitrogen ratio which retards the growth of bacteria and subsequent utilization of the carbon sources (Yakimov et al., 2007). Furthermore, the nutrients are also quickly assimilated by the microorganisms, thus depleting the nutrient reserves. This limitation in nutrient sources and dissolved oxygen, toxicity of hydrocarbons and their degradants to the bacterial community, and low numbers of the principal oil degraders in the sediment and seawater can affect the rate and effectiveness of natural biodegradation (Boopathy, 2000).

Although natural biological remediation at an oil-polluted site happens, several techniques may be applied to achieve faster and more efficient biodegradation. Several bioremediation studies focused on interventions that aims at enhancing the degradative capacities of the indigenous microbial populations such as supplementation of nutrients or oxygen (biostimulation) and adding microbial oil-degraders (bioaugmentation) (Bento et al., 2005; Liang et al., 2009; McKew et al., 2007; Simons et al., 2012; Thavasi et al., 2011; Tyagi et al., 2010). Different combination of these techniques have also been done in some studies and found to significantly accelerate bioremediation

(Kauppi et al., 2011; Nikolopoulou et al., 2013; Rahman et al., 2002; Ruberto et al., 2003). The *in situ* applicability of any developed bioremediation technologies, however, should be validated through a laboratory-scale trial where hydrocarbon degradation capacities are quantified periodically.

In our previous study, we described isolation, identification and characterization of bacterial strains with potential use in bioremediation of oil pollution (Nuñal, *in press*). A bacterial consortium was prepared by mixing these strains, immobilized in different carrier matrices and evaluated for its oil-degrading capacity in an *in vitro* seawater experiment. The results showed that significantly higher degradation of heavy oil was obtained when the consortium was attached to cocopeat, an organic powdery material that comes from ground coconut husk. It was hypothesized that mechanisms for enhancement of degradation by immobilized cells involved maintaining high microbial population, increasing contact of bacterial cells to oil as a carbon source, and supplying rate-limiting nitrogen (N) and phosphorus (P) sources. The main objective of the present study is to validate this technology, including investigation of the effectiveness of the developed immobilized cells on heavy oil degradation in sediment-seawater microcosms. Furthermore, possible methods to improve its degradation efficiency through addition of inorganic N and P sources were also explored. Temporal degradation of hydrocarbon components of heavy oil and growth of oil-degraders and other heterotrophic bacteria were measured in a 3x2 factorial experiment involving different combinations of remediation agents.

MATERIALS AND METHODS

Sampling

Sediment samples were collected from 2 locations in Yamagawa Bay, Kagoshima, Japan (Location 1, 31°12'44.48"N, 130°38'15.57"E; Location 2, 31°12'34.66"N 130°39'24.28"E) and mixed in equal proportions. Initial soil quality indices such as moisture content (gravimetric method), grain size (sieve analysis), pH, redox potential, total organic carbon (TOC) and total organic nitrogen

(TON) content (CHNS analyzer FLASH EA112, Thermo Fisher Scientific, Massachusetts, USA), and total organic phosphorus (TOP) content (Concentrated H_2SO_4 and dilute base extraction method; Bowman, 1989) were measured. Results of the physico-chemical characterization are presented in Table 3-1.

Preparation of consortium and immobilized cells as bioaugmentation agents

The bacterial consortium with oil-degrading capacity was prepared as we had previously described elsewhere (Nuñal, *in press*). The consortium was composed of strains related to *Pseudomonas aeruginosa*, *Marinobacter mobilis*, *Gaetbulibacter* sp. and *Halomonas* sp. (Accession numbers of their 16S ribosomal RNA gene sequences are AB649102, AB649110, AB649114, and AB649101, respectively). Preparation of the bacterial consortium in liquid culture (free-living form) and cocopeat-immobilized cells were also described previously (Nuñal, *in press*).

Microcosm set-up

The sediment mixture was equilibrated at 26˚C for seven days prior to the microcosm assembly. The equilibrated sediment was then placed in 100 ml Erlenmeyer flasks covered with paper caps Steristopper (Heinz Herenz, Hamburg, Germany) to allow aerobic conditions. For each treatment, 12 flasks were prepared. Each flask contained 50 g sediment and 10 ml natural seawater obtained from the same sampling site were added to avoid desiccation, both of which included indigenous microbial populations. The flasks were wrapped in aluminum foil to prevent photooxidation. After the microcosm assembly, the flasks containing the seawater-sediment mixtures were equilibrated again at 26˚C for seven days. Flasks were spiked with 1% heavy oil at Day 0. The contents of the six treatments are shown in Table 3-2. Bacterial cells were added at 10^8 CFU ml^{-1} for the free living cell treatments and 10^8 CFU g^{-1} for the immobilized cell treatments. Nutrient addition was done by adding 310 µl of 3 M NH_4Cl and 120 µl of 1 M KH_2PO_4 in each flask to achieve the 100:5:1 ratio based on the initial C:N:P content of the sediment. All flasks were incubated at 26˚C

and gently shaken once daily for 60 days. At days 0, 3, 7, 15, 30 and 60, two replicate flasks from each treatment were analyzed for residual oil compositions while three replicate flasks were used for bacterial counts.

Determination of cultivable bacterial counts

Total cultivable bacterial counts in both seawater and sediment were determined by conventional plate count technique. One gram of the sediment collected from each flask was suspended in 9 ml 75% natural seawater (NSW). The sediment suspensions or seawater samples were then serially diluted and 0.1 ml of each dilution was spread on Zobell 2216E agar. The plates were then incubated at 26˚C and bacterial counts were determined after 3-5 days of incubation.

Determination of most probable number (MPN) of oil-degrading microorganisms

Most probable numbers (MPN) of oil-degraders in seawater and sediment were enumerated using the Sheen Screen method as described by Brown and Braddock (1990). For seawater, 1 ml sample from each replicate was added to 9 ml of 75% NSW and was serially diluted. The same procedure was applied for the sediment samples using 1 g sample from each replicate. Three aliquots (0.1 ml) of each serially diluted sample were then inoculated into sterile 24-well microtiter plates containing 1.75 ml of sterile Bushnell Haas medium per well. Following inoculation, 0.15 ml of heavy oil was applied to each well to cover the whole surface with oil. The plates were incubated without agitation at 26˚C for 21 days. After incubation, wells were scored positive when oil emulsification was indicated by the disruption of the oil sheen. Oil-degrading populations were then estimated using a standard MPN table.

Extraction of residual oil and GC-MS analysis

Sediment samples from the microcosms were dried at room temperature until obtaining constant weight. From each replicate, residual oil was extracted using 5 g of the dry sediment by using the method described by Uno et al. (2010). The sediment was ultrasonically extracted twice with

dichloromethane–hexane mixture (1:1, v/v) for 15 min. The extracts were pooled and concentrated to 0.2 ml under a nitrogen gas stream. The concentrated extract was added to 6 ml of 1 M potassium hydrate-ethanol and saponified under 90˚C for 1 h. Then the solution was added to 20 ml of distilled water and 10 ml of dichloromethane-hexane mixture (1:3, v/v), shaken and centrifuged at 760x*g* for 10 min. The procedure was repeated twice with collecting the upper organic phase each time. The collected solution was combined, added with 5 ml hexane and centrifuged at 2000 rpm for 5 min. The organic solvent layer was collected and transferred to a 50 ml tube. Hexane addition and centrifugation was repeated to the remaining solution and the organic solvent layer was added to the first extracted solution. Final extraction was done using 10 ml hexane:ethanol solution (1:1, v/v) and the organic solvent layer was added to the hexane extracts. The tubes containing the combined extracts were filled with milliQ water to the rim, hand-shaked for two min and centrifuged at 2000 rpm for 5 min. The upper organic layer was collected, dehydrated with sodium sulfate and concentrated to 2-3 ml. Clean-up was performed by loading on a silica-gel column packed in a Pasteur pipette. The column was washed and eluted with 10 ml of hexane twice and then with 10 ml of 1% acetone–hexane. The eluents were combined, spiked with internal standard (pyrene-d10) and concentrated until 0.2 ml with nitrogen gas stream.

Measurements of *n*-alkanes, PAHs and alkylated PAHs in the extracted residual oil were conducted using a gas chromatograph (Agilent 6890; Agilent Technologies, USA) equipped with a mass spectrometer (Agilent 5973 MSD; Agilent Technologies, USA). One microliter of the sample was injected by an Agilent 7683B automatic sampler（Agilent Technologies, USA） into a DB-5MS capillary column (0.25 mm i.d. × 60 m; film thickness, 0.25 μm; J&W Scientific, USA). The temperature was programmed as follows: 60˚C for 1 min, 60˚C to 200˚C at a rate of 20˚C min^{-1}, maintained at 200˚C for 1 min, 200˚C to 290˚C at a rate of 3˚C min^{-1}, and then maintained at 290˚C for 5 min. The mass selective detector was operated in the SIM mode.

Statistical analysis

The microcosm experiment was set up as a 3x2 factorial design consisting of six treatments found in Table 3-2. The influence of the independent variables, bacterial cell addition (three levels: not added, free-living cells and immobilized cells) and nutrient addition (two levels: added and not added), to the degradation of hydrocarbons was quantified after 60 days incubation. Significant differences at $p<0.05$ among the means of the different treatments were analyzed by one-way ANOVA followed by Duncan's Multiple Range Test (DMRT) using the statistical program Statistical Package for Social Sciences (SPSS) version 19.0.

The two-way factorial ANOVA which tested the effect of the 2 independent variables (bacterial cell addition and nutrient addition) on the dependent variable (total petroleum hydrocarbon degradation) and the interaction between the independent variables was also conducted using the SPSS program. The analysis was done using Univariate General Linear Model followed by DMRT. An absolute value of the effect was estimated by the following general formula: $Effect = \Sigma Y_+/n_+ - \Sigma Y_-/n_-$, where Y_+ refers to the means of the treatments receiving the variable, while Y_- refers to the means of the treatment without the variable, and the n_+ and n_- refer to number of the data points at each level.

RESULTS

Bacterial growth

Temporal changes in viable bacterial counts in seawater and sediment are shown in Fig. 3-1. In the seawater, control and nutrient microcosms started with lower bacterial counts at $8.0x10^4$ and $2.6x10^5$ CFU ml^{-1} respectively at Day 0, than the other four microcosms with added bacterial cells in free and immobilized forms which have initial counts at $>10^6$ CFU ml^{-1}. Bacterial counts in the control microcosms dropped slightly at day 3 but increased gradually until it reached peak at day 15, then dropped dramatically on day 30. All the other microcosms showed increased bacterial counts from day 3 to day 15. The three microcosms receiving nutrient supplementation exhibited higher

bacterial counts which were maintained until the end of the experimental trial. On the other hand, the two bioaugmented microcosms without nutrient addition showed slight drop at day 30 and continuous decrease until day 60.

In the sediment, the highest initial bacterial count was recorded for the microcosms of immobilized cells, nutrients+free cells and nutrients+immobilized cells, followed by the treatment of free cells, and the lowest was found in the microcosms of nutrients and control. After 3 days incbation, increases in bacterial counts were observed in the microcosms added with remediation agents but a drop in bacterial counts was detected in the control treatment. Thereafter, all the microcosms showed increasing bacterial counts from day 3 to day 15. Bacterial counts reached peak at day 15 for the treatments of control, free cells and immobilized cells. Thereafter, gradual decreases in these treatments were observed until the end of the 60-day incubation except for the control. The three biostimulated microcosms showed continuous increases in bacterial counts until day 30.

Changes in the bacterial counts of oil degraders

Bacterial counts of oil degraders in seawater and sediment in the different microcosms are shown in Fig. 3-2. In the seawater, higher counts ($>10^3$ cells ml^{-1}) of the oil degraders in the four bioaugmented microcosms (free cells, immobilized cells, nutrients+free cells and nutrients+immobilized cells) were observed compared with the treatments of control and nutrients. The most probable numbers (MPN) continuously increased from day 0 to day 7 in all the treatments, with the treatment of nutrients+immobilized cells attaining the highest counts. Continuous increases in MPN were observed in all treatments until day 30. Thereafter, decreases in the oil-degraders were observed in all the treatments in day 60 but their numbers were higher than the values at the start of the experiments. The highest numbers were sustained in the treatments of nutrients+immobilized cells after day 30.

In the sediment, the same initial trend as the seawater was observed: the treatments augmented with the bacterial consortium started with higher MPN values than control and nutrients.

Continuous increases in MPN until Day 15 were observed in all the treatments. For the treatments of nutrients, free cells and immobilized cells, peak of the oil-degrading populations were at Day 15, but for control, nutrients+free cells and nutrients+immobilized cells the numbers still increased until Day 30. MPN values declined in all the treatments but were also higher than the initial counts. The highest MPN in the sediment at the end of incubation was found in the treatment of nutrients+immobilized cells.

Influence of nutrient and bacterial cell addition on total petroleum hydrocarbon (TPH) reduction

In order to have a better understanding of the effect of biostimulation, bioaugmentation and their combination, the univariate general linear model analysis of variance was conducted as shown in Table 3-3. Results showed positive effect when nutrients and bacterial consortium (free or immobilized) were added separately. Higher values for the effect estimate was obtained for nutrient addition compared to bacterial cell addition. Significant interaction ($p<0.05$) of nutrients and bacterial cell addition was also detected.

Reduction of total petroleum hydrocarbons (TPHs) in the different treatments after 60 days incubation are shown in Fig. 3-3. All the treatments receiving a bioremediation agent were significantly higher ($p<0.05$) than the control. No significant difference ($p>0.05$) in TPH reduction was observed between the treatment of free and immobilized cells but the treatments of nutrient, nutrients+free cells and nutrient+immobilized cells were significantly different ($p<0.05$) from one another. The highest TPH reduction of 68.95% was found in the treatment of nutrients+immobilized cells .

Degradation of *n*-alkanes

Degradation data of *n*-alkanes in the different treatments are shown in Fig. 3-4 and Table 3-4. In all the treatments, degradation of *n*-alkanes decreased with increasing chain length (Table 3-4). Almost complete degradation of all *n*-alkane components was achieved in microcosms

augmented with nutrients and immobilized bacterial consortium (C8-C15, 98.6%; C16-C25, 99.2%; C26-C33, 97.4%). All the microcosms receiving remediation agents showed significantly higher total *n*-alkane degradation than the control ($p<0.05$, Fig. 3-4). Temporal analysis showed that reduction in *n*-alkanes already occurred as early as 3 days in all the treatments (Fig. 3-5). At day 15, dramatic drop in the residual *n*-alkanes were observed in the 3 treatments with nutrient supplementation in which more than 75% of the oil was degraded. Further degradation in all treatments occurred until the end of the incubation at 60 days with significantly highest values observed in the microcosm of nutrients+immobilized cells.

Degradation of polyaromatic hydrocarbons (PAHs)

Results of degradation in polyaromatic hydrocarbons (PAHs) in the different treatments are shown in Fig. 3-6 and Table 3-5. PAHs were less biodegradable than *n*-alkanes with the significantly highest degradation ($p<0.05$) of 71.74% in the treatment of nutrients+immobilized cells (Fig. 3-6) after 60 days. In all the treatments, higher degradation occurred in 2-4 ring PAHs including naphthalene, acenapthylene, acenapthene, flourene, phenanthrene, anthracene and flouranthene (Table 3-5). For these compounds, more than 50% were decomposed in the microcosms receiving biological and nutritional interventions, except for acenaphthene in the free cell- and nutrient-treated microcsms and fluoranthene in the free cell microcosm, while significantly lower degradation was observed in the control treatment with the exception of naphthalene. Generally lower degradation was observed in high molecular weight (HMW) PAHs in all the treatments. Degradation of benzo[g,h,i]perylene and indeno[1,2,3,-c,d]pyrene was not detected in the control microcosms. Bioaugmentation and biostimulation brought significant differences ($p<0.05$) from natural attenuation in pyrene, benzo[a]anthracene and benzo[k]flouranthene degradation. Addition of nutrients significantly improved degradation ($p<0.05$) of benzo[b]flouranthene as well as pyrene. Increasing degradation of chrysene proved to be significant in the combined nutrient and bacterial supplementation ($p<0.05$). For more complex HMW PAHs such as benzo[a]pyrene,

benzo[g,h,i]perylene, dibenzo[a,h]anthracene and indeno[1,2,3-c.d]pyrene, significant differences in degradation among the treatments were not found ($p>0.05$). Temporal changes in total residual PAHs showed gradual but steady degradation in all the treatments (Fig. 3-7). The three microcosms added with nutrients showed faster degradation rate than those without their supplementation particularly in the first 15 days. Thereafter, the degradation rates in these three treatments started to diminish but still progressed until the end of the 60-day incubation.

Degradation of alkyl polyaromatic hydrocarbons (alkyl PAHs)

PAHs with alkyl substitution proved to be the least biodegradable among the heavy oil fractions. Results of the degradation and the temporal residual total alkyl PAHs are shown in Fig. 3-8 and Table 3-6. All the bioremediation interventions resulted in significantly higher alkyl PAH degradation than natural attenuation ($p<0.05$). Addition of nutrients alone yielded significantly higher degradation ($p<0.05$) than the two treatments receiving only bacterial cell supplementation but lower than the two treatments with combination. Significantly highest reduction in total alkyl PAHs ($p<0.05$) was observed in the nutrients+immobilized cells at 53.52% after 60 days.

Nutrient and bacterial cell supplementations significantly improved degradation ($p<0.05$) of the following alkyl PAHs: all alkyl napththalenes, dibenzothiophene, 4-methyl dibenzothiophene and 1- and 2-methyl phenanthrene and 7-methyl benzo[a]pyrene (Table 3-6). Among the measured alkyl PAHs, the highest degradation of more than 70% were observed in 1-methyl phenanthrene in the nutrient-supplemented microcosms. Biostimulation also enhanced significantly ($p<0.05$) the degradation of 1-methyl fluorene, 1-methyl anthracene and 4,6-dimethyl benzothiophene compared to the control and the treatment of bioaugmentation alone. Although degradation of 1-methyl benzo[a]anthracene was detected in all the treatments, no significant differences ($p>0.05$) were observed among the values. Degradations of the following alkyl PAHs were not detected in any treatments: 2,3-dimethyl anthracene, 2-methyl flouranthene, 1-methyl pyrene, 4-methyl chrysene, 7,12-dimethyl benzo[a]anthracene and perylene.

Slow and gradual degradation of alkyl PAHs was observed during the course of the 60-day incubation in all the treatments (Fig. 3-9). By the end of the incubation period, more than 50% of the alkyl PAHs was still present in the treatments of control, free cells, immobilized cells and nutrients.

DISCUSSION

The present study was conducted to determine the bioremediation potential of a cocopeat-immobilized oil-degrading bacterial consortium as well as its improvement by inorganic nutrient addition in oil-contaminated marine envirionments. The results showed that hydrocarbon degradation was enhanced by addition of remediation agents: after 60-day incubation, higher *n*-alkanes, polyaromatic hydrocarbons (PAHs) and alkyl PAHs degradation were observed in the treatments amended with inorganic nutrients or bacterial cells than their natural attenuation.

The statistic analysis revealed higher degradation of petroleum hydrocarbon components in the treatment of nutrients compared to the bacterial cell addition. The finding is supported by several studies pointing out that indigenous microbial populations which are allowed to grow optimally through nutrient supplementation can degrade hydrocarbons more effectively compared to anthropogenic microbial introduction. It was also reported that, in environments where physicochemical factors such as temperature and pH were constant and optimal, the limiting factor of organic pollutant degradation was shortage of nitrogen (N) and phosphorus (P) (Das and Chandran, 2010; Welander et al., 2005). However, care must be taken since excessive addition of N and P may promote growth of microbial populations which outgrow the oil degraders in the biostimulated environments (Chaineau et al., 2005; Wang et al., 2010). Furthermore, superfluous inorganic N can inhibit microbial growth due to production of toxic ammonium (Agarry and Ogunleye, 2013; Chaillan et al., 2006). Amendment of N and P at a C:N:P ratio of 100:5:1 used in the present study proved to be functional to promote the growth of the indigenous heterotrophic and added oil-degrading microbial population.

Biostimulation implemented in this study, however, did not sustain oil degraders in the microcosms: decline in the oil-degrading populations at the latter half of the 60-day experimental period, especially in seawater, was observed, which might be attributed to depletion of available carbon sources as well as the added inorganic N and P. Because of this limitation, complementing biostimulation with bioaugmentation will become beneficial, since in the late stage of remediation recalcitrant contaminants and their derivatives persist in the sediment, which are expected to be degraded by the bioaugmented bacteria. It was confirmed by sustained higher numbers of total bacterial and oil-degrader counts as well as continuous hydrocarbon degradation until the end of the experimental period in the microcosms receiving conbined bioargumentation and biostimulation.

Comparing the bioaugmented treatments, significantly higher oil-degradation and higher microbial counts were found in the treatment with the immobilized cells. Immobilization was proven to maintain high numbers of microbial population by virtue of providing solid attachment to the cells, resulting in robust biofilm formation. Porous biocarriers such as cocopeat also serve as bulking agents that facilitate diffusion of dissolved oxygen and contaminated hydrocarbons into the particles and may provide nutrients to the adhered bacterial cells (Liang et al., 2009; Simons et al., 2012; Wu et al., 2011; Xu and Lu, 2010).

The highest oil-degrading efficiency found in the treatment receiving nutrients+immobilized cells can be explained by synergism between these complementary biostimulation and bioaugmentation processes. Mechanisms of the enhancement can be hypothesized as follows: (1) addition of nutrients accelerate growth and activity of indigenous and added microbial population during the early stage of degradation (Day 0-15); (2) the indigenous and augmented cells degraded hydrocarbons synergistically with rapid decline in *n*-alkanes and gradual reduction in PAHs and alkyl PAHs with maintaining their high bacterial counts; (3) environmental conditions suitable for microbial growth achieved by supply of nutrients and their immobilization as well as oxygen in porous cocopeat biocarrier; and (4) attachment of bacterial cells in the cocopeat sustaining high bacterial numbers through the experimental period, allowing degradation of more recalcitrant

petroleum compounds. Different mechanisms of the cooperative interaction are also described in previous studies (Fan et al., 2013; Gertler et al., 2009; McKew et al., 2007; Straube et al., 2003).

Degradation of *n*-alkanes showed significant differences among the 6 treatments, which signified that each intervention brought differences in the capacity of their degradation. Almost complete reduction was achieved where nutrients+immobilized cells were applied. Faster and higher *n*-alkane degradation was also reported for the remediation in combination of bacterial consortium, rhamnolipid and NPK fertilizer (Rahman et al., 2002) and *Pseudomonas* sp. and nutrients (Stallwood et al., 2005).

Polyaromatic hydrocarbons and alkyl PAHs are more recalcitrant than *n*-alkanes, thereby making them more reliable indices of effectiveness of bioremediation intervention. Although relatively slower than that of *n*-alkanes, gradual decrease of PAHs and alkyl PAHs were observed in all the treatments, mainly attributed to degradation of PAHs with 2-4 rings. Addition of the bacterial consortium brought about degradation of high molecular weight (HMW) compounds such as benzo[g,h,i]perylene, indeno[1,2,3-c,d]pyrene, and 1,2-dimethyl naphthalene which were not degraded by the indigenous population. This may indicate co-metabolism of the added bacteria with the indigenous population (Jacques et al., 2007; Teng et al., 2010). Addition of nutrients and bacterial consortium facilitate reduction of less degradable alkyl PAHs, although almost half of the substances was left even in the case of the combined treatment of nutrients+immobilized cells. In addition, degradation of several HMW alkyl PAHs were not detected. This may be due to several factors, including recalcitrance of the alkyl PAHs, depletion of nutrients and limitations of the hydrocarbon-degrading abilities of the indigenous and the added consortium (Makkar and Rockne, 2003; Xu and Obbard, 2004).

In conclusion, this study shows that addition of remediation agents such as inorganic nutrients and bacterial consortium significantly enhances natural biodegradation of heavy oil in a sediment-seawater microcosm trial. Combination of the nutrients and immobilized oil-degrading cells yielded superior degradation of the hydrocarbon components by way of promoting and

maintaining the growth of amended oil-degrading and indigenous heterotrophic bacterial populations. These findings indicate successful implementation of the bioremediation strategy to overcome limitation of the natural biodegradation processes.

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TABLES AND FIGURES

Table 3-1. Physico-chemical characteristics of the sediment used in the bioremediation microcosms.

Variables	Values
Moisture content (%)	41.82 ±0.88
Grain size (sand, %)	81.9 ± 2.6
Grain size (silt/clay, %)	18.1 ± 2.6
pH	8.0
Redox potential (mV)	105 ± 7.5
Total organic carbon (%)	1.72
Total organic nitrogen (%)	0.06
Total organic phosphorus (%)	0.01

Table 3-2. Matrix of the microcosm design (3x2 factorial) for the remediation of the oil-contaminated sediment.

Treatments	Factors	
	Nutrients*	Bacterial cell addition**
control	-	-
free cells	-	+1
immobilized cells	-	+2
nutrients	+	-
nutrients+free cells	+	+1
nutrients+immobilized cells	+	+2

* -, C:N:P ratio was not adjusted; +, C:N:P ratio was adjusted to 100:5:1 by adding NH_4Cl and KH_2PO_4.

**-, indigenous populations only; +1, the bacterial consortium in a free-living form was added; +2, the bacterial consortium immobilized in cocopeat was added.

Table 3-3. Summary of the univariate general linear model analysis for influences of nutrient and bacterial cell addition on the petroleum hydrocarbon degradation in the different microcosms.

Factors	Values					
	Effect estimate (Absolute value)	Sum of squares	Degrees of freedom	Mean Squares	*F*-value	*P*-value
Bacterial cell addition (*A*)	11.65	374.319	2	187.160	2251.543	0.00*
Nutrients (*B*)	17.11	885.285	1	885.285	10650.050	0.00*
A x B	22.83	60.294	2	30.147	362.669	0.00*

*Effect is significant at $p<0.05$.

Table3- 4. Reduction in the concentration (%) of *n*-alkanes in the different microcosms after 60 days of incubation

n-alkanes	Treatments					
	Control	Free cells	Immobilized cells	Nutrients	Nutrients+free cells	Nutrients+ immobilized cells
C8-C15	86.30±0.18[a]	87.14±0.71[ab]	87.88±0.47[b]	91.37±0.32[c]	90.31±0.43[c]	98.62 ±0.90[d]
C16-C25	54.15±1.06[a]	66.93±0.35[b]	71.21±1.26[b]	86.07±0.29[c]	89.46±0.16[d]	99.16 0.15±[e]
C26-C33	30.54±1.74[a]	48.92±0.91[b]	50.59±0.73[b]	74.73±0.49[c]	79.37±0.60[c]	97.42±1.17[d]

The means of two replicates are shown with the standard deviation. Different superscript letters indicate significant difference at $p<0.05$.

Table 3-5. Reduction in the concentration (%) of 16 priority polyaromatic hydrocarbons (PAHs) (US-EPA) in the different microcosms after 60 days of incubation

PAHs	Treatments					
	Control	Free cells	Immobilized cells	Nutrients	Nutrients+ free cells	Nutrients+ immobilized cells
Naphthalene	89.17±3.30^{a}	90.7±4.36^{a}	90.32±1.74a ^{a}	92.26±1.79^{a}	92.40±1.70^{a}	94.23±0.52^{a}
Acenaphthylene	34.09±0.10^{a}	69.8±1.22^{b}	69.51±0.35^{b}	76.71±0.33^{cd}	75.79±0.74^{c}	77.88±0.46^{d}
Acenaphthene	37.71±0.34^{a}	35.1±0.81^{b}	50.35±0.06^{c}	48.20±0.02^{d}	59.14±0.66^{e}	60.73±0.53^{f}
Fluorene	72.43±0.31^{a}	74.9±0.45^{b}	75.31±0.40^{b}	79.81±0.63^{c}	74.29±1.15^{b}	95.80±0.06^{d}
Phenanthrene	39.64±1.70^{a}	70.0±0.16^{b}	92.11±0.23^{c}	91.84±0.17^{c}	93.22±0.16^{c}	94.42±0.12^{d}
Anthracene	58.76±0.42^{a}	63.1±0.93^{b}	67.22±1.46^{c}	69.96±1.25^{d}	69.03±0.34^{cd}	73.20±0.12^{e}
Fluoranthene	52.83±4.74^{ab}	44.9±4.85^{a}	53.86±2.27 ^{ab}	55.50±1.37^{ab}	60.59±3.28^{bc}	63.48±3.24^{c}
Pyrene	8.75±0.65^{a}	25.0±0.14^{b}	18.71±0.67^{c}	14.47±0.34^{d}	35.81±0.11^{e}	35.99±0.53^{e}
benzo[a]anthracene	25.14±5.15^{a}	31.8±0.37^{b}	32.12±3.47^{b}	36.36±1.82^{bc}	41.00±1.41^{c}	41.62±0.14^{c}
Chrysene	18.04±2.14^{a}	27.7±9.96^{ab}	28.77±1.59^{ab}	28.23±5.25^{ab}	31.54±1.00^{b}	31.74±1.92^{b}
benzo[b]fluoranthene	19.69±0.01^{a}	20.4±0.33^{ab}	22.08±1.57^{b}	25.58±0.46^{c}	27.83±0.26^{d}	27.83±0.96^{d}
benzo[k]fluoranthene	10.79±3.33^{a}	28.4±1.75^{b}	28.11±0.68^{b}	31.80±2.60^{b}	32.82±4.97^{b}	33.68±1.18^{b}
benzo[a]pyrene	13.99±8.25^{a}	20.7±0.74^{ab}	20.54±6.04^{ab}	23.25±1.30^{ab}	23.35±0.40^{ab}	25.95±0.47^{b}
benzo[g,h.,i]perylene	N.D.	9.8±5.53^{a}	21.29±5.48^{b}	22.03±3.71^{b}	20.71±1.51^{ab}	28.43±3.84^{b}
dibenzo[a,h]anthracene	22.66±2.31^{a}	21.8±0.88^{a}	22.25±1.17^{a}	22.33±0.35^{a}	22.44±2.89^{a}	22.61±3.10 ^{a}
indeno[1,2,3-c,d]pyrene	N.D.	8.3±4.8^{a}	11.92±5.54^{a}	14.62±0.54^{a}	13.94±0.86^{a}	14.29±0.74 ^{a}

The means of two replicates are shown with the standard deviation. Different superscript letters indicate significant difference at $p<0.05$. N.D., the reduction was not detected.

Table 3-6. Reduction in the concentration (%) of alkyl PAHs in the different microcosms after 60 days of incubation

Alkyl PAHs	Treatments					
	Control	Free cells	Immobilized cells	Nutrients	Nutrients+free cells	Nutrients+immobilized cells
2-methylnaphthalene	30.92±0.71^{a}	32.30±1.7^{a}	37.65±1.87^{b}	39.57±0.89^{bc}	42.22±0.44^{cd}	44.90±1.60^{d}
1-methylnaphthalene	35.21±0.22^{a}	41.74±0.9^{b}	42.96±0.38^{b}	58.78±0.26^{c}	57.48±0.09^{d}	58.73±1.07^{d}
1,2-dimethylnaphthalene	N.D.	37.46±0.8^{a}	36.86±1.98^{a}	40.35±0.97^{b}	52.10±0.04^{c}	54.85±0.70^{c}
1-methylfluorene	N.D.	N.D.	N.D.	29.75±0.09^{a}	29.82±0.46^{a}	30.96±2.77^{a}
2,3,5-trimethylnaphthalene	23.08±0.36^{a}	40.78±0.2^{b}	40.34±0.06^{c}	54.71±0.11^{c}	66.52±0.11^{d}	68.76±0.37^{e}
dibenzothiophene	40.94±2.55^{a}	50.47±0.5^{b}	51.88±1.84^{bc}	52.43±1.20^{bcd}	54.57±0.02^{cd}	55.89±0.66^{d}
4-methyldibenzothiophene	43.32±0.62^{a}	45.43±1.4^{b}	46.73±0.06^{b}	51.95±0.08^{c}	52.14±0.24^{c}	51.34±0.15^{c}
4,6-dimethyldibenzothiophene	41.38±2.21^{a}	44.02±0.5^{a}	44.76±2.24^{a}	51.08±0.57^{b}	51.75±0.47^{b}	52.67±0.46^{b}
1-methylanthracene	54.89±3.13^{a}	54.11±0.4^{a}	54.81±0.84^{a}	58.61±0.21^{b}	60.49±0.61b^{c}	63.32±0.28^{c}
2-methylphenanthrene	35.05±0.22^{a}	40.49±0.7^{ab}	40.85±1.61^{b}	41.77±0.02^{b}	41.69±0.82^{b}	49.75±0.41^{c}
1-methylphenanthrene	25.73±3.13^{a}	31.84±0.9^{b}	34.61±0.07^{c}	71.38±0.29^{d}	72.98±0.47^{de}	75.40±1.58^{e}
2,3-dimethylanthracene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
2-methyl luoranthene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
1-methylpyrene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
1-methylbenzo[a]anthracene	19.75±13.66^{a}	23.54±2.2^{a}	25.54±0.25^{a}	25.79±5.12^{a}	28.72±1.06^{a}	29.28±0.25^{a}
4-methylchrysene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
7,12-dimethylbenzo[a]anthracene	N.D.	N.D	N.D	N.D	N.D	N.D
Perylene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
7-methylbenzo[a]pyrene	14.98±1.41^{a}	17.34±1.43^{b}	18.81±0.34^{b}	18.69±0.49^{b}	18.21±0.57^{b}	18.41±0.06^{b}

The means of two replicates are shown with the standard deviation. Different superscript letters indicate significant difference at $p<0.05$. N.D., the reduction was not detected.

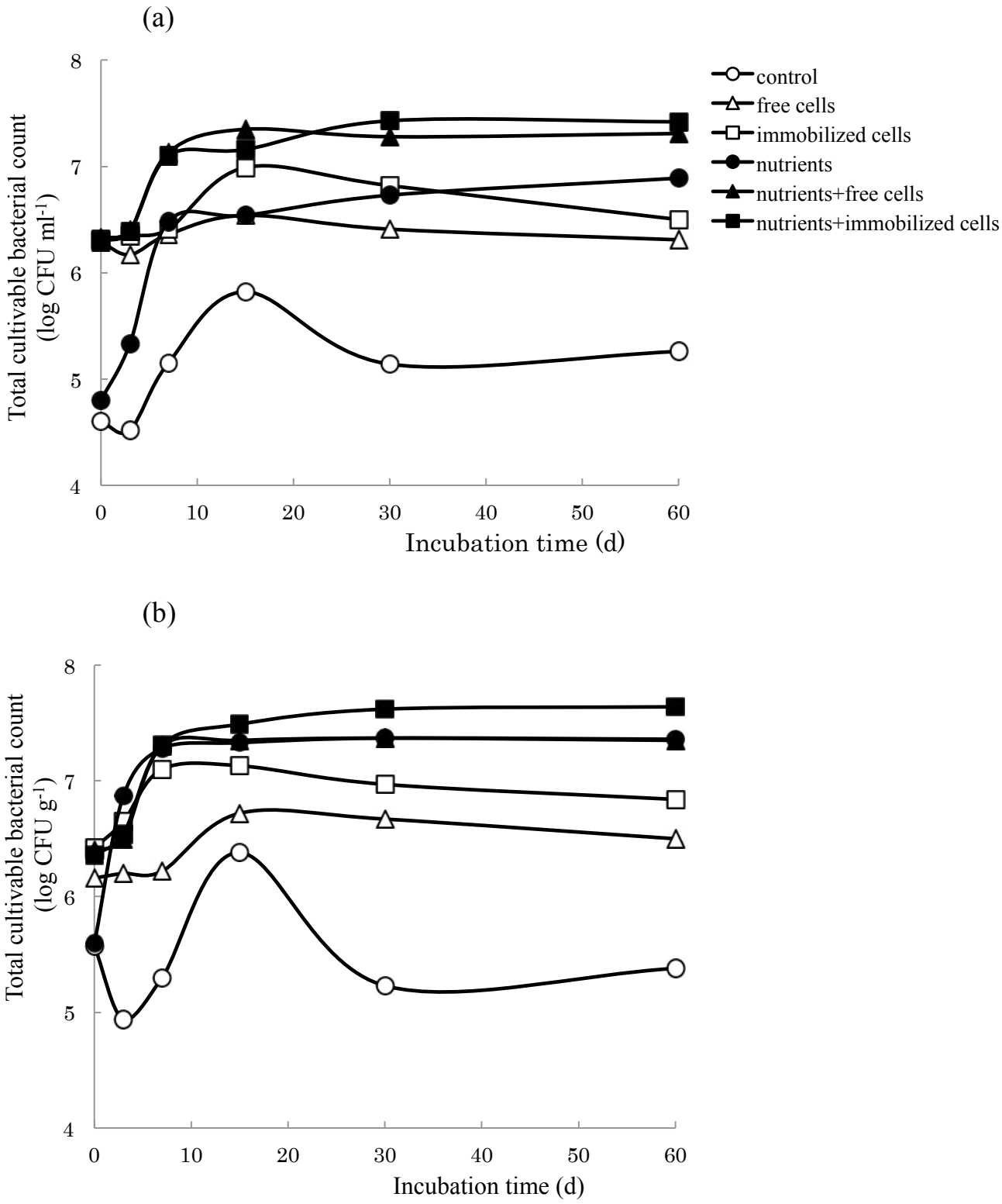
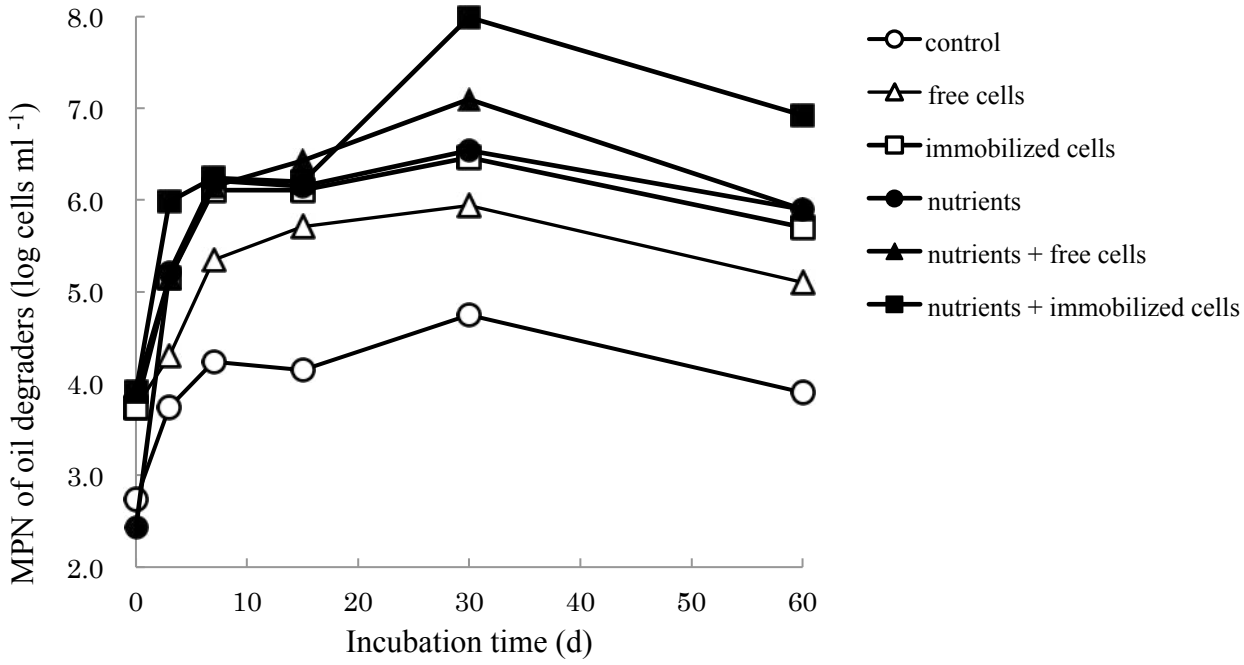


Fig. 3-1. Changes in the total cultivable bacterial counts in (a) seawater and (b) sediment in the different microcosms during 60 days of remediation. Values represent the mean of three replicates.

(a)



(b)

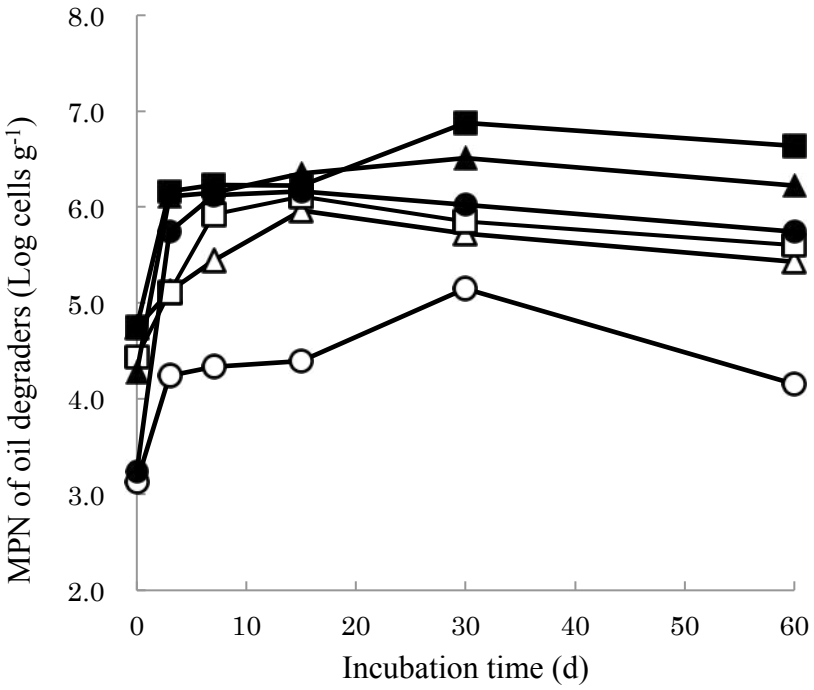


Fig. 3-2. Changes of the most probable number (MPN) of oil degraders in (a) seawater and (b) sediment in the different microcosms during 60 days of remediation. Values represent the mean of three replicates.

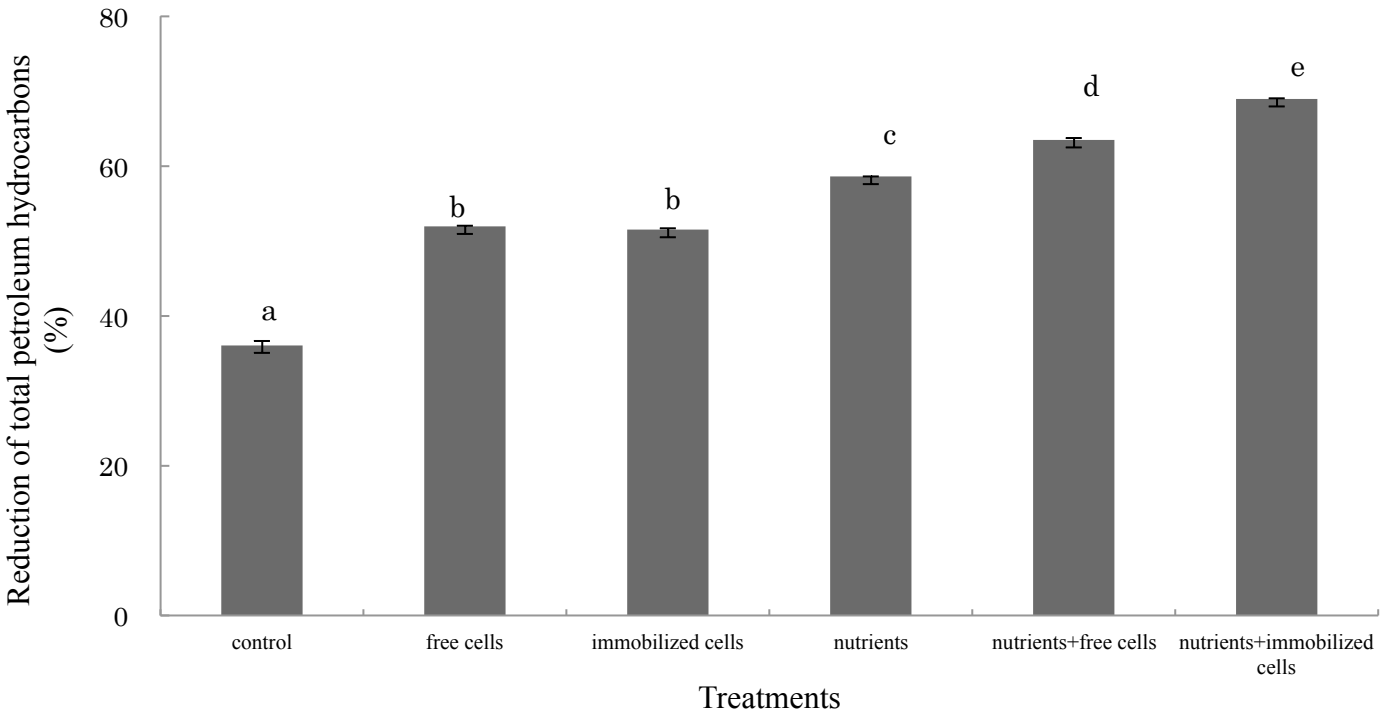


Fig. 3-3. Degradation of petroleum hydrocarbon in the different microcosms after 60 days incubation. Values represent mean of two replicates. Error bars show the standard deviation. Different letters above the bars indicate significant difference at $p<0.05$.

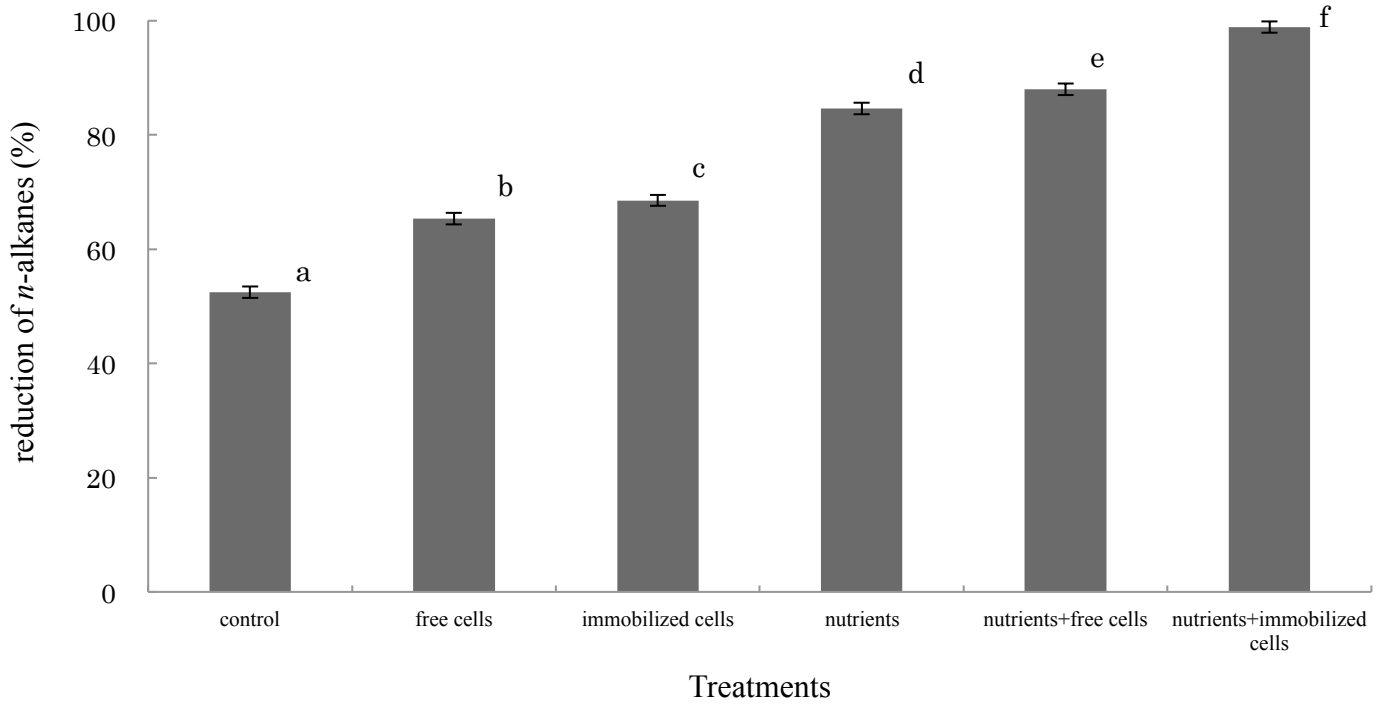


Fig. 3-4. Degradation of total *n*-alkanes in the different microcosms after 60 days incubation. Values represent the mean of two replicates. Error bars show the standard deviation. Different letters above the bars indicates significant difference at $p<0.05$.

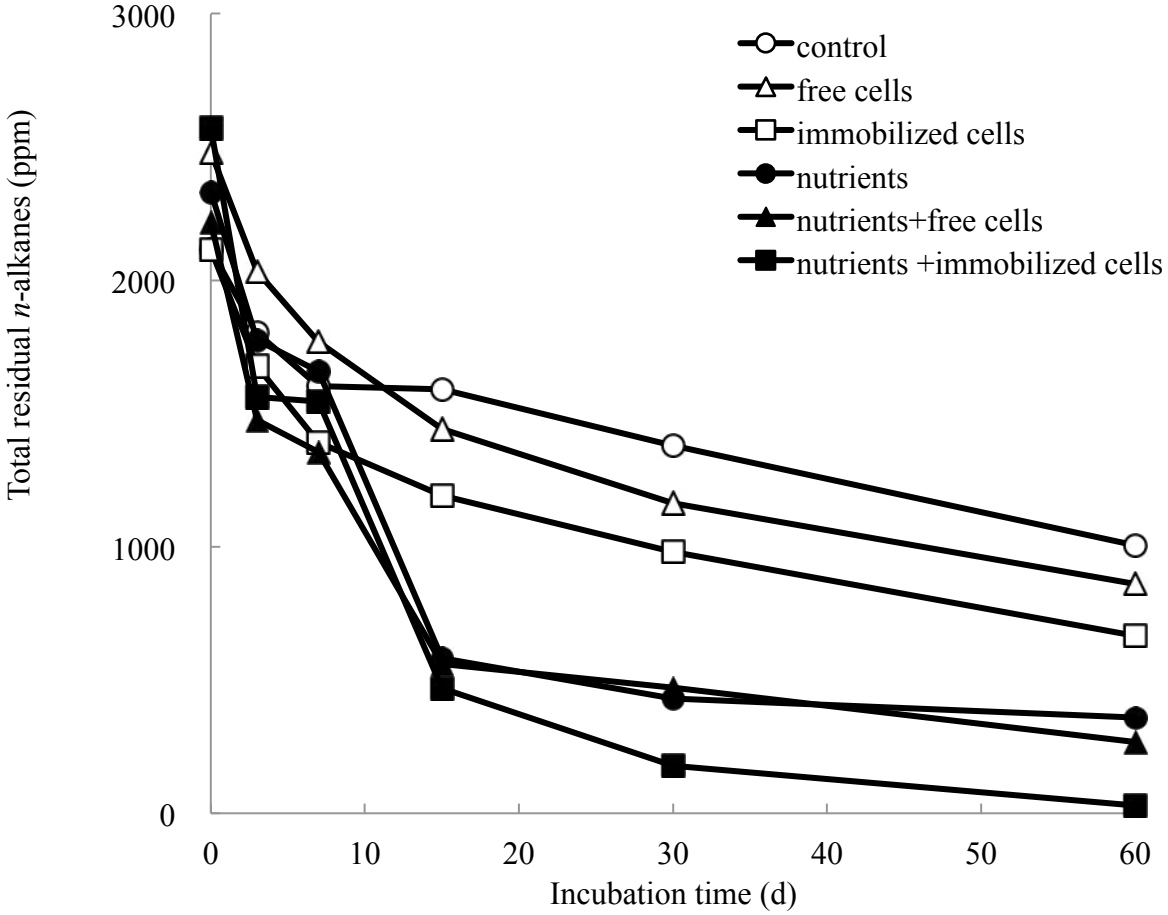


Fig. 3-5. Temporal changes in the total *n*-alkane content of the different microcosms during 60 days of remediation. Values represent the mean of two replicates.

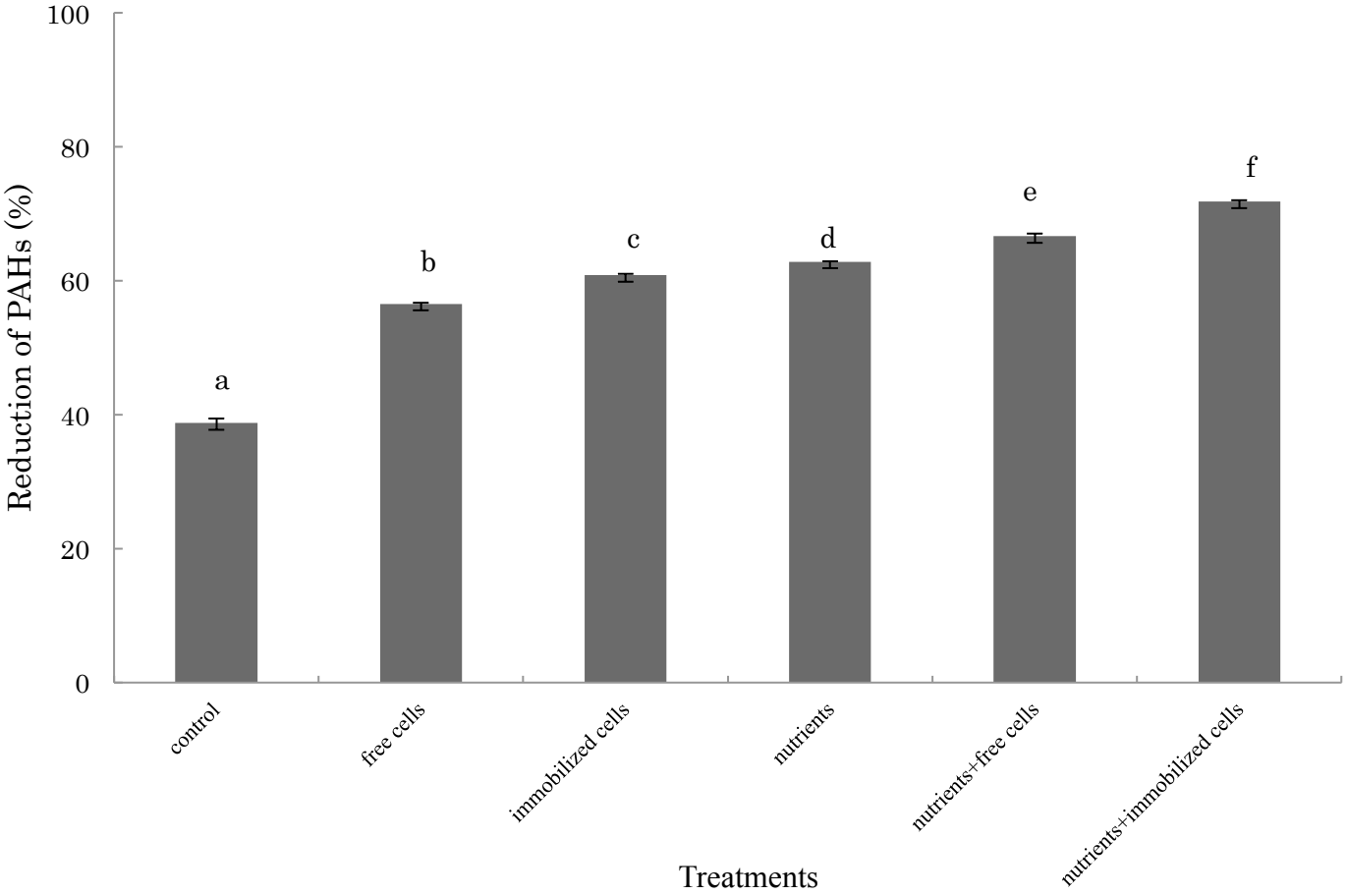


Fig. 3-6. Degradation of polyaromatic hydrocarbons (PAHs) in the different microcosms after 60 days incubation. Values represent the mean of two replicates. Error bars show the standard deviation. Different letters above the bars indicates significant difference at $p<0.05$.

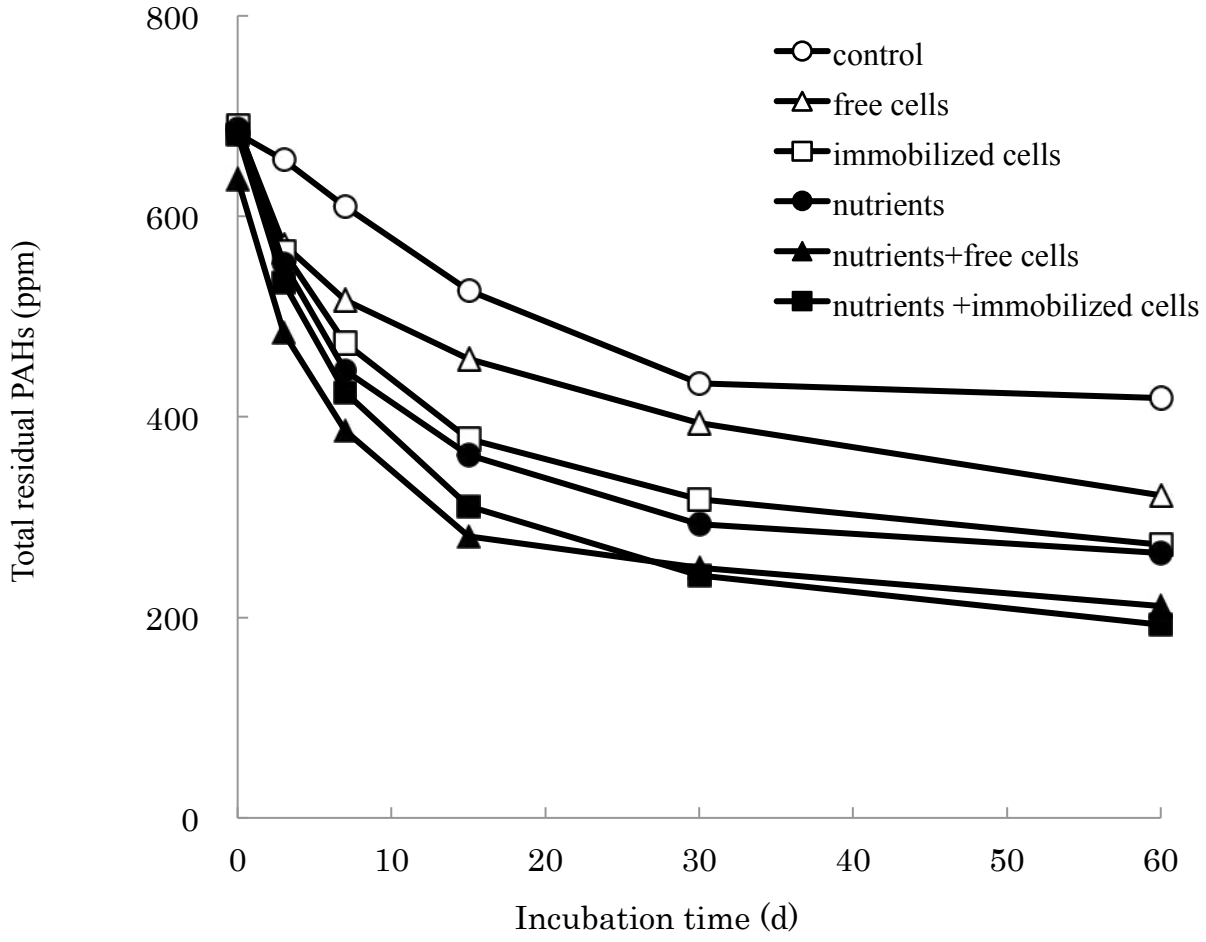


Fig. 3-7. Temporal changes in the total polyaromatic hydrocarbon (PAHs) content of the different microcosms during 60 days of remediation. Values represent the mean of two replicates.

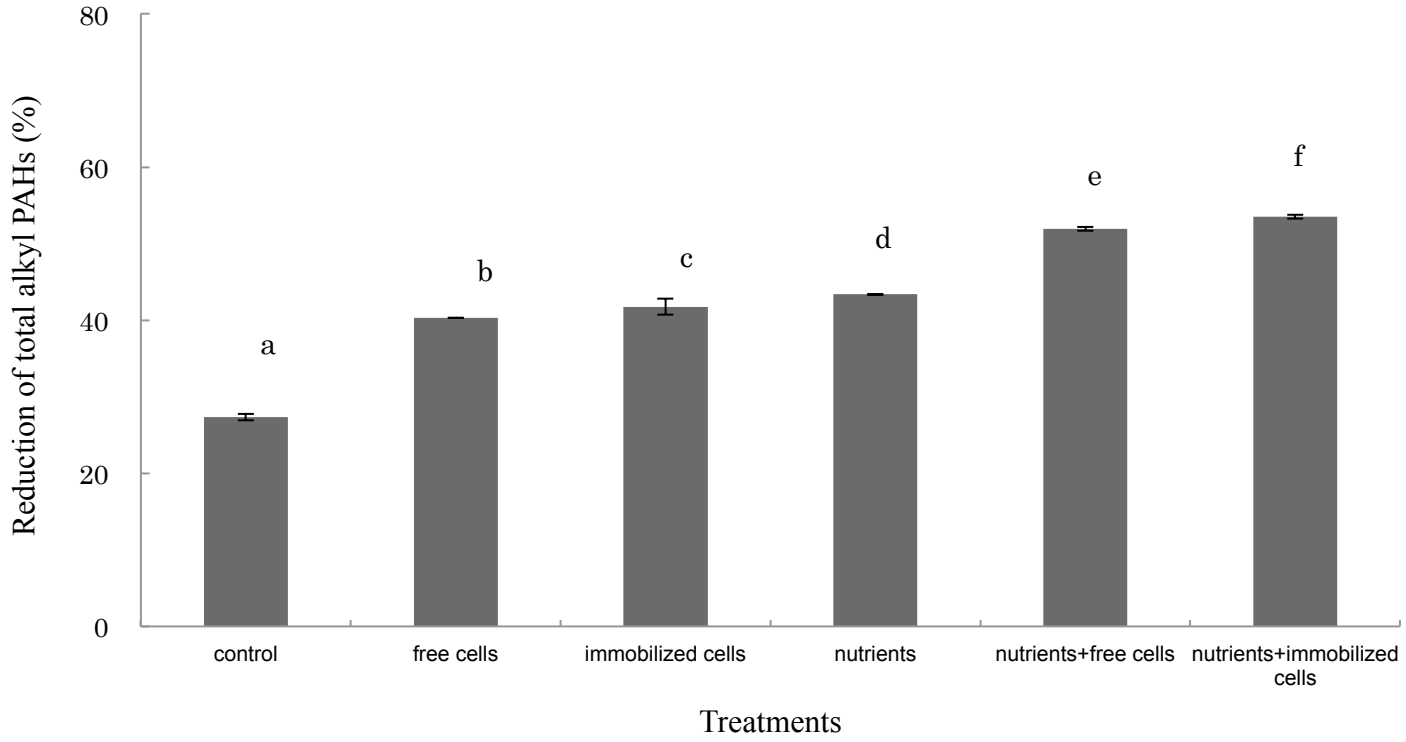


Fig .3-8. Degradation of alkyl PAHs in the different microcosms after 60 days incubation. Values represent the mean of two replicates. Error bars show the standard deviation. Different letters above the bars indicates significant difference at $p<0.05$.

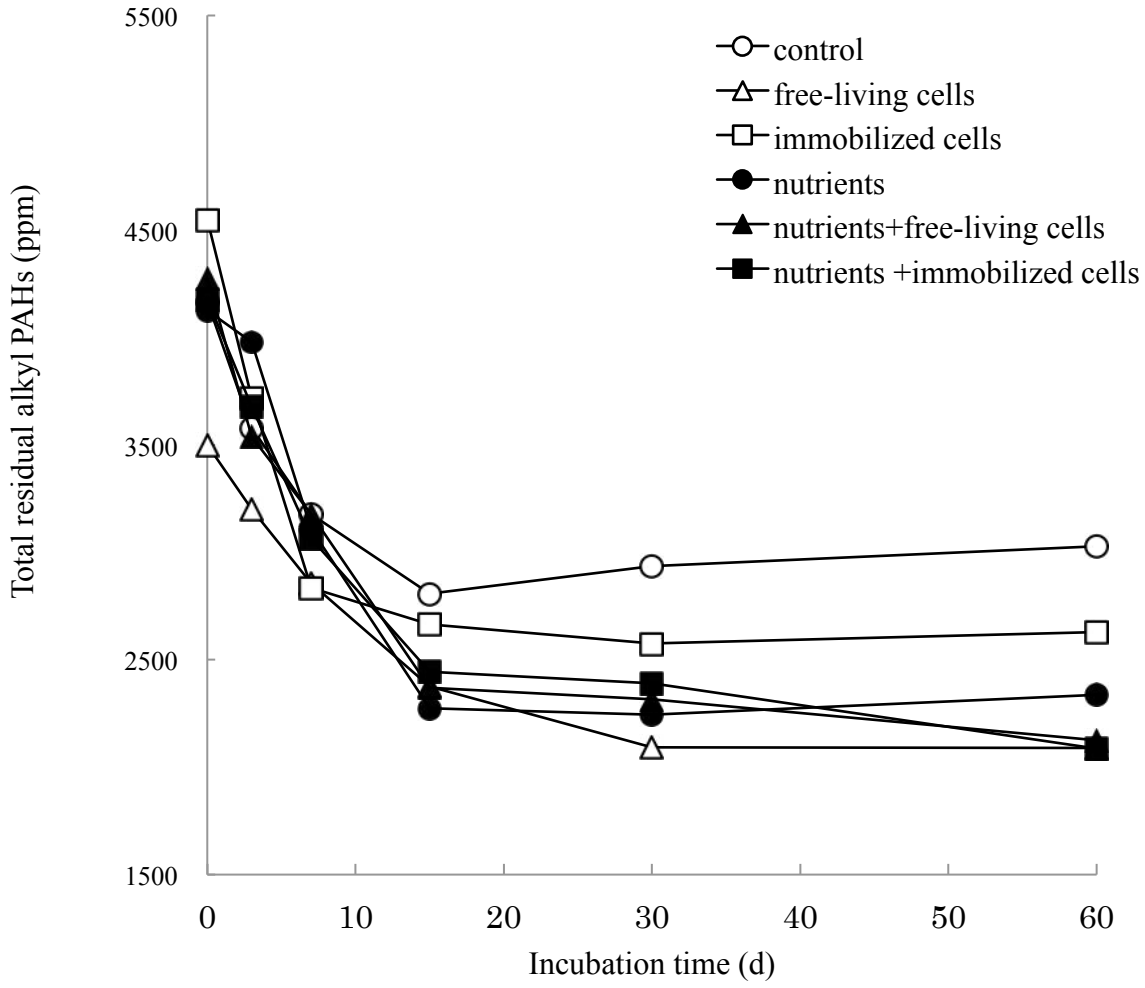


Fig. 3-9. Temporal changes in the total PAH content of the different microcosms during 60 days of remediation. Values represent the mean of two replicates.

CHAPTER FOUR

BACTERIAL COMMUNITY DYNAMICS DURING BIOREMEDIATION OF SEDIMENT ARTIFICIALLY-CONTAMINATED WITH HEAVY OIL

ABSTRACT

Changes in the bacterial community during remediation of marine sediment artificially-contaminated with heavy oil were monitored by denaturing gradient gel electrophoresis (DGGE) of the PCR-amplified 16S rDNA fragments. Addition of inorganic nutrients and bacterial cell consortium was done in a sediment-seawater microcosm experiment and temporal variations in the bacterial community structure were determined. Results revealed different responses of the bacterial community to the addition of different remediation agents. Shifts in the bacterial communities in the seawater were more dynamic than in the sediment in the presence of oil and the remediation agents. DGGE banding patterns revealed that the bacterial community present in seawater was clearly different from that of the sediment. Furthermore, survival of the augmented bacterial consortium was found to be improved by immobilization to cocopeat. Phylogenetic analysis based on the 16S rDNA sequences obtained from the bands excised from the DGGE profiles revealed presence and enrichment of aerobic and anaerobic organisms related to bacterial groups involved in different bioremediation processes. These findings suggested possible simultaneous aerobic and anaerobic degradation of heavy oil in the different microcosms.

INTRODUCTION

To understand the impact of oil pollution to marine environment, it is important to determine microbial responses both at metabolic and genetic levels. Bioremediation studies mostly employ empirical approaches involving either nutrient or bacterial cell amendment or both in laboratory setups. As the extent of biodegradation are often limited by the low availability of nitrogen (N) and phosphorus (P), addition of inorganic N and P has proven to be an effective technique in a number of bioremediation treatments (Yakimov et al., 2007). Augmentation with preselected strains, on the other hand, was done when the metabolic capabilities of the indigenous microflora was not enough to carry out complete mineralization of the contaminants (Gentry et al., 2004). These human interventions are known to impact the natural processes occurring within the different bacterial communities in the marine environment (Tyagi et al., 2011; Vogel, 1996). Better understanding of the degradative processes and related shifts in the bacterial community structure can be helpful in the development of more rational bioremediation strategies.

The major bottleneck in studying microbial communities is the fact that many environmental bacteria cannot yet be cultured by conventional laboratory techniques (Briones and Raskin, 2003; Ranjard et al., 2000). Thus several advanced molecular methods have emerged to provide more powerful tools to study bacterial community dynamics by culture-independent techniques (Yoshida et al., 2006). One of the most widely employed DNA fingerprinting techniques for analyzing phylogenetic diversity is the polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) (Muyzer et al., 1993). PCR-DGGE allows profiling of the bacterial community through the banding patterns by which its diversity can be analyzed quantitatively. Furthermore, the intensity of bands provides a rough estimate of the relative abundance of each species, and nucleotide sequencing of the excised bands provides a phylogenetic and taxonomic interpretation of the obtained amplicons.

This study is designed to determine the effect of addition of inorganic nutrients and bacterial cell consortium to the natural succession of microbial communities affected by oil contamination. Furthermore, this study also aims to determine the fate of the augmented bacterial consortium when applied in free and immobilized forms. In this study, sediment and seawater samples were obtained from the microcosms in the 3x2 factorial experiment described in Chapter Three. Environmental DNA in the seawater and sediment at the defined time intervals was extracted and subjected to PCR-DGGE. Banding patterns were analyzed quantitatively by using nonmetric multidimensional scaling (NMDS) to describe relationships of the microbial community in the different microcosms. Prominent bands were also excised and sequenced to determine their taxonomy.

MATERIALS AND METHODS

Microcosm set-up

The microcosm setups used in this chapter were the same as ones of the Chapter Three.

DNA extraction

Environmental DNA was extracted from both sediment and seawater samples obtained from the different microcosms. For sediment, DNA was extracted using PowerSoil DNA Isolation Kit (MOBIO Laboratories, Inc., Carlsbad, CA, USA) following the manufacturer's instructions. For seawater, samples were pre-filtered with a mixed cellulose ester membrane filter (pore size, 0.20 μm; diameter, 25 mm; Advantec, Japan) to trap the bacteria. The filter was then transferred into a 6-well microplate. DNA extraction was then performed using DNEasy Plant Mini Kit (Qiagen, Hilden, Germany). Presence of the extracted DNA was then confirmed by agarose gel electrophoresis using 4 μl DNA samples.

PCR amplification of 16S rDNA

The 16S ribosomal RNA gene (16S rDNA) was amplified using PCR primers -357F

(5'-CCTACGGGAGGCAGCAG-3') with a GC clamp (Muyzer et al., 1993) and 907r (5'-CCGTCAATTCCTTTGAGTTT-3') (Yu and Morrison, 2004). The 20 µl PCR reaction mixture contained ExTaq buffer, 100 µM each of dNTP mixture, 0.5 µM of the forward and reverse primers, and 0.025 units μl^{-1} of ExTaq DNA polymerase (Hot Start Version, Takara Bio, Otsu, Japan). Amplification program used was touch-down PCR with thermal cycling conditions as follows: initial denaturation of 95˚C for 1 min, 19 cycles of denaturation at 95˚C for 1 min, annealing at 62˚C for 1 min with a decreasing temperature of -0.8˚C at every cycle, and extension at 72˚C for 1 min, followed by 9 cycles of 95˚C for 1 min, 57˚C for 1 min, and 72˚C for 1 min, and a final extension of 72°C for 10 min. PCR amplifications were performed with the ASTEC PC320 thermal cycler (ASTEC, Fukuoka, Japan). The PCR products were visualized by agarose gel electrophoresis with staining of Gel Red™ (Biotium Inc., Hayward, CA, USA).

Denaturing gradient gel electrophoresis and direct nucleotide sequencing of 16S rDNA

Amplified 16S rDNA fragments were run in denaturing gradient gel electrophoresis (DGGE) with the D-Code System (Bio-Rad, Hercules, CA, USA). DGGE was performed in a 6% polyacrylamide gel with denaturing gradient of 25-55% (where 100% denaturing gels contain 7 M urea and 40% formamide in 0.5X TAE buffer). Electrophoresis was run at a constant voltage of 60V for 16 h at 60˚C. Gels were stained with SYBR Gold (Invitrogen, Carlsbad, CA, USA) and viewed with Safe Imager™ 2.0 blue light transilluminator (Invitrogen, Carlsbad, CA, USA)

The DGGE bands were excised from the gels using 200 µl pipette tips. Excised gels were suspended in 100 µl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM ethylenediamine tetraacetic acid) in 1.5 ml microtubes and placed in 20˚C overnight to elute the amplified DNA. The eluted DNA fragments were re-amplified and subjected to DGGE with the same conditions as described above to confirm the recovery based on comparison with the first DGGE. The resultant single bands were re-excised, recovered, and re-amplified in the reaction volume of 50 µl. The PCR products were then applied onto 1.5% agarose gel electrophoresis at 100V. The bands were excised using a sterilized

surgical blade and purified using the MonoFas DNA Purification Kit I (GL Science, Tokyo, Japan) with an elution volume of 30 μl. Thermal cycle nucleotide sequencing of the PCR-amplified 16S rDNA was run using the ABI PRISM BigDye Terminator Cycle Sequencing Kit Ver. 3.1 (Applied Biosystems, Carlsbad, CA, USA) with ≤5 ng template DNA and a -357F primer without a GC clamp. The products were purified using the BigDye XTerminator Kit (Applied Biosystems, Carlsbad, CA, USA) and analyzed by the ABI PRISM 3500xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA).

Cloning and sequencing of 16S rDNA PCR products with heterogeneous sequences

Excised bands which yield heterogeneous sequences with direct sequencing were subjected to cloning using the pGEM®-T Easy Vector System cloning kit with JM109 High-Efficiency Competent Cells (Promega Corp., Madison, WI, USA). The cloning protocol of the manufacturer was followed. The cloned DNA fragments were amplified by colony PCR with primers -21M13 Control Primer (Applied Biosystems, Carlsbad, CA, USA) and BcaBEST Sequencing Primer RV-P (Takara Bio, Otsu, Japan). Nucleotide sequences of the amplified products were determined as described above.

Phylogenetic and statistical analysis

The closest relatives of the 16S rDNA sequences were determined by the basic local alignment search tool (BLAST). The DGGE banding pattern in each lane was converted into a binary matrix indicating the presence and absence of each band detected on all the lanes. Similarity was then examined using the nonmetric multidimensional scaling (NMDS) conducted using PRIMER (version 5) software (PRIMER Enterprises, Plymouth, UK). The degree to which the plots matched was assessed using Kruskal's stress wherein the stress should be less than 0.15 and ideally less than 0.10 for configurations of objects to be considered reliable (Quinn and Keough, 2002).

RESULTS

Temporal changes of the bacterial population structures in sediment and seawater of the microcosms

The denaturating gradient gel electrophoresis (DGGE) profiles in Figs 4-1 to 4-6 show the temporal changes in the bacterial community structures of the different microcosms amended with or without remediation agents, while the quantitative analysis using NMDS are shown in Figs 4-7 to 4-12. For the control treatment (Figs. 4-1 and 4-7), drastic changes of the community structure occurred in the seawater environment from day 0 to day 15. The bacterial community in the seawater stabilized from day 15 to 30 and shifted again at Day 60. Bacterial structure in the sediment remained fairly stable from Day 0 to Day 60. For the free cell treatment (Figs 4-2 and 4-8), bacterial community changes in the seawater occurred at the following intervals: Day 0 to Day 3, Day 7 to Day 15 and Day 30 to Day 60. The sediment bacterial community, on the other hand, showed gradual changes but the positions on the NMDS map were located close to one another, indicating similarity in the band profiles. The treatment with the immobilized cells showed only two drastic shifts in the seawater bacterial community occurring from Day 0 to Day 3 and Day 30 to Day 60 (Figs 4-3 and 4-9). Bacterial community shifts from Day 3 to day 30 were minimal. The sediment samples showed almost unchanging bacterial community structures throughout the experimental period.

Nutrient addition resulted in temporal changes in bacterial community in both the seawater and the sediment (Figs 4-4 and 4-10). Three major shifts in the seawater occurred as follows: from Day 0 to Day 3, Day 7 to Day 15 and Day 30 to Day 60. Changes in the sediment occurred only after Day 3 and changed gradually until Day 60. Combination of nutrient and bacterial cell addition produced different changes in bacterial community structure when the bacteria was added in free living and immobilized forms. When the cells were added in a free-living form, apparent shifts occurred in both the seawater and the sediment (Figs 4-5 and 4-11). In the seawater, the bacterial communities drastically changed in the first 15 days but became more stable thereafter. Changes were more gradual in the sediment in the first 7 days but were more drastic afterwards until Day 60.

In the treatment of nutrients+immobilized cells (Figs 4-6 and 4-12), noticeable changes in bacterial community were only observed in the seawater which occurred in from Day 3 to Day 7 and Day 15 to Day 30. The bacterial community in sediment remained fairly the same throughout the incubation.

Survival of the amended bacterial strains in the bioaugumented microcosms

The oil-degrading bacterial consortium added to the four microcosms showed different responses. In the treatment of free cells (Fig. 4-2), *Gaetbulibacter* sp. and *Halomonas* sp. had better survival, lasting until Day 60 in the sediment. *Gaetbulibacter* sp. was detected in the seawater until Day 7. *Pseudomonas* sp. and *Marinobacter* sp. survived in the sediment only until Day 15 but were not detected in the seawater.

In the treatment of immobilized cells (Fig. 4-3), *Marinobacter* sp. and *Gaetbulibacter* sp. showed better survival than in the free cells treatment which was detected from Days 0 to 60 in the sediment and Day 0 in the seawater. *Pseudomonas* sp. stayed suspended in the water column and was shown to be present in the seawater from Day 3 to Day 30, while it was only detected in the sediment at Day 3 and 30.

When nutrients were amended, the free-living cells were detected as follows: *Gaetbulibacter* sp., from Day 0 to 60 in the sediment and Day 0, 3 and 60 in the seawater; *Pseudomonas* sp., from Day 0 to 30 in the sediment and Day 0-3 in the seawater; *Marinobacter* sp., from Day 0 to Day 30 only in the sediment; *Halomonas* sp., from Day 0 to 60 only in the sediment (Fig. 4-5).

In the treatment of nutrients+immobilized cells, the added cells generally persisted longer than all the other treatments. *Gaetbulibacter* sp. was present in both the sediment and the seawater throughout the experimental period except for Day 7 in the sediment. *Pseudomonas* sp. was detected from Day 0 to 60 in the sediment and in Day 0 and 3 in the seawater. *Marinobacter* sp. and *Halomonas* sp. survived until Day 60 in both the sediment and the seawater (Fig. 4-6).

Taxonomy of the 16S rDNA sequences obtained from the excised DGGE bands

The results of the 16S ribosomal RNA genes (rDNA) sequencing analysis of the excised DGGE bands obtained from all the microcosms are shown in Tables 4-1 to 4-6. The excised bands were affiliated to 28 genera distributed among the different taxonomic groups such as alpha-, beta-gamma- and deltaproteobacteria, Cytophaga, Flavobacteria, Bacteriodetes, Firmicutes, Deferribacteres, Chloroflexi and Spirochaetes. A number of genera were commonly found in more than one microcosm. Generally, changes in the community composition in the seawater were more dynamic than in the sediment in all microcosms. Greater diversities were found in the treatments added with remediation agents compared to the control.

In the control treatment, fewer distinct bands can be found; therefore, lesser DNA sequences were retrieved. The bands related to *Chlorobium phaeobacteriodes* (A1) and uncultured Chloroflexi (A2), both green non-sulfur phototrophic bacterial groups, were detected in both the sediment and seawater from Day 0 to Day 60. The band associated to *Bacillus* sp. (A6) was found from Day 0 to Day 7 but disappeared thereafter. Bands that appeared during the later stages were related to *Marinobacter mobilis* (A5) and uncultured Deltaproteobacteria (A3). In the seawater, temporal bacterial community composition was also characterized by appearance and disappearance of certain bands. The bands related to *Sulfitobacter* sp. (A10), a sulfite-oxidizing bacterium, and *Pseudomonas monteilli* (A12) were both present only at Day 0 then disappeared subsequently. Bands appearing after day 0 were related to the following: uncultured Deltaproteobacterium (A7, Day 15-60), *Bacillus* sp. (A8, Days 3 and 7), *Thalassobius* sp. (A9, Days 7-60) and *Bacillus firmus* (A11, Days 15-60).

In the two bioaugmented treatments (free cells and immobilized cells), bands found in both the sediment and the seawater throughout incubation were affiliated with the following species: *Chlorobium phaeobacteriodes* (B1 and C2) and Uncultured Chloroflexi (B3, C4 and C5). Furthermore, common organisms found between the two treatments were *Bacillus firmus* (B20 and C12) and *Muricauda* spp. (B13, B15, B16, C9 and C16). Notable bacteria include *Erythrobacter* sp. (B14), a genera associated with bacterial communities in oil-contaminated sediments, and

Alcanivorax sp. (B18), a known alkane degrader, both found in the free cell microcosms.

When the microcosms were supplemented with nutrients, the number of identified genera was greater compared to the control and bioaugmented treatments. The most common organisms found in the three nutrient-amended microcosms were the bacteria related to *Bacteriodetes* (D3, E3, E11, F4 and F9), *Muricauda* spp. (D11, E14, F12, F14, F17), *Roseovarius pacificus* (D14 and F11) and *Bacillus* spp. (D16, D19, E9, E16, and F16). Bands related to *Chlorobium phaeobacteriodes* (D2 and E2) and uncultured Chloroflexi (D12 and E6) were found to be persistent, appearing in both the seawater and the sediment from day 0 to 60, in the treatments of nutrients and nutrients+free cells but were not detected in the treatment of nutrients+immobilized cells. Enrichment of bacteria related to *Idiomarina* sp. (D17 and E13), a known polycyclic aromatic hydrocarbon (PAH) degrader, was also found to persist in the seawater of these two microcosms. Bands related to genera that have hydrocarbon-utilizing members were *Marinobacter mobilis* (D15), *Thalassospira profundimaris* (E10) and *Alcaligenes* sp. (F15).

DISCUSSION

In hydrocarbon-contaminated marine areas, natural succession of bacterial communities, including increase of hydrocarbon-degrading microorganisms, is observed (Harayama et al., 1999; Pengerud et al, 1994; Yu et al., 2011). Subsequently, bacterial groups that metabolize more recalcitrant oil compounds replace the groups that have limited hydrocarbon-degrading ability. In this study, microcosms simulating oil-contaminated marine environments showed a shift in the bacterial communities after addition of heavy oil and remediation agents. Results of the non-metric multidimensional scaling (NMDS) analysis revealed that: (1) responses of the bacterial communities varied to addition of the different remediation agents; (2) bacterial communities in the seawater changed more dynamically than in the sediment in response to oil and remediation agent inputs; and (3) based on the denaturing gradient gel electrophoresis (DGGE) banding patterns, compositions of

the bacterial communities in the seawater and the sediment were clearly different.

Although their numbers are usually very low and hardly detected (Atlas, 1981; Leahy and Colwell, 1990), marine sediment and seawater are known to harbor hydrocarbon-degraders. Even if no intervention was made, natural succession of bacterial communities in the control microcosm occurred as indicated by the temporal appearance and disappearance of bands in the DGGE profile, although their diversity was lower than the other microcosms. The most notable shift was observed at the later part of the experimental period, where the bands related to *Marinobacter* sp. and uncultured deltaproteobacteria appeared between Day 15 and 60, with the disappearance of a distinct band related to *Bacillus* sp. The genus *Marinobacter* is known to include species that can degrade both aliphatic and aromatic hydrocarbons (Yakimov et al., 2007; Zhuang et al., 2002). In the seawater, a more dynamic shift occurred in a short time with a disappearance of bands related to non-hydrocarbon degraders such as *Sulfitobacter* sp. (Pukall et al., 1999) and *Pseudomonas monteilli* (Eloimari et al., 1997), and with an appearance of *Bacillus firmus* and uncultured deltaproteobacterium.

One notable species whose DGGE band was unique in the treatment of nutrients+immobilized cells was related to *Chlorobium phaeobacteroides*, a green non-sulfur bacterium which possesses both aerobic and anaerobic respiratory pathways. Although direct evidences concerning its hydrocarbon-degrading capacity have not yet been documented, the bacterium was found in anaerobic microbial communities in groundwater, capable of degrading aromatic hydrocarbons (Yagi et al., 2010). The degradation metabolism involved anaerobic consumption of aromatic compounds coupled to dissimilatory nitrate reduction. Uncultured *Chloroflexi*, also green non-sulfur bacteria, were also found to be abundant and persistent in almost all the microcosms. They are known to include diverse members with both aerobic and anaerobic phenotypes. *Chloroflexi* have been reported to dominate in activated sludge (Bjornsson et al., 2002). Due to their ubiquity, it can be assumed that they may also play an important role in hydrocarbon degradation in the microcosms, although underlying mechanisms need to be elucidated.

Addition of the oil-degrading consortium and nutrients expanded the microbial diversity of the sediment and seawater in microcosms, causing more pronounced shift in the bacterial community structure than the control. Most of the bacterial species included or found in oil-contaminated environments were affiliated to the groups that have oil-degrading capacity, such as *Alcanivorax* sp. (Kasai et al., 2002; Yoshikawa et al., 2010), *Oceanicola* sp. (Yuan et al., 2009), *Peptostreptococcaceae* (Sherry et al., 2013; Sun et al., 2014), *Thalassospira* sp. (Zhao et al., 2009; Shao et al., 2010), *Erythrobacter* sp. (Chung and King, 2001, Liu and Liu, 2013), *Idiomarina* sp. (Zhao et al., 2009; Kleinsteuber et al., 2006) and *Alcaligenes* sp. (Weissenfels et al., 1990). Several facultative or strictly anaerobic bacterial groups with oil-degrading capability, such as *Muricauda* sp. (Hwang et al., 2009), *Desulfubulbaceae* (Savage et al., 2010), *Roseovarius* sp. (Vila et al., 2010), *Tenericutes* (Cardinali-Rezende et al., 2012) and *Thiomicrospira* sp. (Voordouw et al., 1996), were also enriched. These findings are controversial since the microcosms were basically kept aerobic. However, heavy oil added to the microcosm setups covered on the surface of seawater or sediment layers, limiting penetration of oxygen and resultant transition to an anoxic condition in the subsurface layers (Berthe-Corti and Bruns, 2001). Furthermore, initial attack to hydrocarbon substances by aerobic microorganisms consumes dissolved oxygen. In such cases, biodegradation of hydrocarbons is carried out by strict or facultative anaerobic bacteria using NO_3^-, SO_4^{2-}, $Fe(III)^{3-}$, and CO_2 as electron acceptors (Diaz, 2004; Jones et al., 2008). In the present study, several DGGE bands from the different microcosms were affiliated to sulfate reducers (*Desulfubulbaceae*), nitrate reducers (*Muriicola* sp., *Terasakiella* sp.) and methanogens (*Tenericutes*), which were dominant in the sediment. These results, therefore, suggest that both aerobic and anaerobic degradation of hydrocarbons occurred in all the microcosms.

Survival of the augmented strains also has an impact to the bacterial community shift. In the two treatments receiving the bacterial consortium in a free-living form, poor survival of the amended bacteria lead to the appearance of oil-degrading bacterial groups related to *Alcanivorax* sp., *Idiomarina* sp. and *Thalassospira* sp. (Wang et al., 2008). This signifies that enhanced degradation in

this microcosm is attributed to indigenous microflora rather than the augmented strains.

The highest reduction in heavy oil in the treatment of nutrients+immobilized cells (refer to Chapter Three) can be attributed to the survival of the augmented oil degraders. The cocopeat biocarrier remained suspended in the seawater, although some of the particles settled onto the sediment. Thus, the added strains were present in both the sediment and seawater throughout the experimental period. The immobilized bacterial cells were proven to sustain high abundance, therefore conducting faster hydrocarbon degradation (Gentry et al, 2004, Simons et al., 2012). Anaerobic degradation may also play an important role in the remediation as indicated by presence of facultative anaerobes such as *Tenericutes*, *Muricauda* sp. and *Spirochaeta*. In addition, detection of *Thiomicrospira chilensis*, a methanogenic bacterium allows us to speculate a possible anaerobic degradation of hydrocarbons to carbon dioxide and methane (Brinkhoff et al., 1999; Chang et al., 2005).

The PCR-DGGE analysis in the present study indicated a shift in bacterial communities caused by oil contamination and different bioremediation agents. Sequencing of the excised bands revealed presence of both aerobic and anaerobic bacterial groups in the microcosms that were responsible for degradation of heavy oil. It should be, however, noted that elucidation of the bacterial community dynamics based on PCR-DGGE is limited by a bias of the PCR-amplification efficiency among different bacterial species. Despite this limitation, the results show the differences in the adaptation mechanisms of the microbial communities in response to the bioremediation of oil contamination.

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TABLES AND FIGURES

Table 4-1. Closest relatives of 16S rDNA sequences obtained from the excised DGGE bands of the control microcosm based on BLAST homology search.

Band No.	Closest relatives in database	GenBank Acc. No.	Homology	Taxonomic groups
A1	*Chlorobium phaeobacteriodes* BS1	NR074363	100%	Chlorobi
A2	Uncultured Chloroflexi bacterium	JQ579888	98%	Chloroflexi
A3	Uncultured delta proteobacterium	AF229449	100%	Deltaproteobacteria
A4	*Muriicola* sp.	KC839612	97%	Flavobacteria
A5	*Marinobacter mobilis* B17	GQ214550	96%	Gammaproteobacteria
A6	*Bacillus* sp.	JQ030918	100%	Firmicutes
A7	Uncultured delta proteobacterium	AM882608	94%	Deltaproteobacteria
A8	*Bacillus* sp.	JQ030918	99%	Firmicutes
A9	*Thalassobius* sp. D7011	FJ161323	99%	Alphaproteobacteria
A10	*Sulfitobacter* sp.	KC689814	99%	Alphaproteobacteria
A11	*Bacillus firmus* strain KJ-W9	KF011491	100%	Firmicutes
A12	*Pseudomonas monteilii* strain SeaH-As4w	FJ607352	98%	Gammaproteobacteria

Table 4-2. Closest relatives of 16S rDNA sequences obtained from the excised DGGE bands of the microcosm augmented with the bacterial consortium in the free-living form based on BLAST homology search.

Band No.	Closest relatives in database	GenBank Acc. No.	Homology	Taxonomic groups
B1	*Chlorobium phaeobacteriodes* BS1	NR074363	100%	Chlorobi
B2	Uncultured deltaproteobacterium	AM882608	94%	Deltaproteobacteria
B3	Uncultured Chlorofexi bacterium	JQ579888	99%	Chloroflexi
B4	Uncultured Chlorofexi bacterium	DQ811889	96%	Chloroflexi
B5	Uncultured Bacteriodetes clone	GQ249615	95%	Bacteriodetes
B6	Desulfobulbaceae bacterium	HQ400773	94%	Deltaproteobacteria
B7	Uncultured Bacteriodetes clone	DQ394963	98%	Bacteriodetes
B8	*Roseovarius* sp. AMV6	FN376425	98%	Alphaproteobacteria
B9	Desulfobulbaceae bacterium	HQ400825	97%	Deltaproteobacteria
B10	*Sphingobacterium* sp. BF02-S7	DQ677866	99%	Bacteriodetes
B11	Uncultured Firmicutes bacterium clone	JQ516410	94%	Firmicutes
B12	*Marinifilum* sp. ONE-10	KF650761	94%	Bacteriodetes
B13	*Muricauda aquimarina* PR54-6	EU440979	99%	Flavobacteria
B14	*Erythrobacter* sp. MON004	KF042026	100%	Alphaproteobacteria
B15	*Muricauda aquimarina* PR54-6	EU440979	99%	Flavobacteria
B16	*Muricauda ruestringensis* DSM	NR074562	99%	Flavobacteria
B17	Rhodobacteraceae bacterium	DQ486502	98%	Alphaproteobacteria
B18	*Alcanivorax* sp. TK-23	KC161579	99%	Gammaproteobacteria
B19	*Robiginitalea* sp. ZH09	FJ872534	98%	Flavobacteria
B20	*Bacillus firmus* KJ-W9	KF011491	99%	Firmicutes

Table 4-3. Closest relatives of 16S rDNA sequences obtained from the excised DGGE bands of the microcosm augmented with the cocopeat-immobilized bacterial consortium based on BLAST homology search.

Band No.	Closest relatives in database	GenBank Acc. No.	Homology	Taxonomic groups
C1	uncultured bacterium clone	GQ413594	97%	Unclassified
C2	*Chlorobium phaeobacteriodes* BS1	NR074363	99%	Chlorobi
C3	Uncultured organism clone	JN486445	98%	Unclassified
C4	Uncultured Chloroflexi bacterium clone	JQ580303	99%	Chloroflexi
C5	Uncultured Chloroflexi bacterium clone	JQ580303	99%	Chloroflexi
C6	Tenericutes bacterium	JQ411296	99%	Tenericutes
C7	Uncultured *Sphingobacterium* sp. clone SV-8	GU233832	96%	Bacteriodetes
C8	Desulfobulbaceae bacterium enrichment clone	HQ400825	94%	Deltaproteobacteria
C9	*Muricauda* sp.	JN594619	99%	Flavobacteria
C10	Desulfobulbaceae bacterium enrichment clone	HQ400825	94%	Deltaproteobacteria
C11	Uncultured Bacteriodetes clone	HQ242576	97%	Bacteriodetes
C12	*Bacillus firmus* KJ-W9	KF011491	100%	Firmicutes
C13	Uncutured *Cytophaga* sp.	AB238986	95%	Bacteriodetes
C14	*Terasakiella* sp.	EF587999	99%	Alphaproteobacteria
C15	*Oceanicola* sp.	JQ895010	99%	Alphaproteobacteria
C16	*Muricauda olearia* CL-SS4	NR044579	98%	Flavobacteria
C17	Uncultured Chloroflexi bacterium clone	JQ580303	99%	Chloroflexi
C18	Desulfobulbaceae bacterium	HQ400825	97%	Deltaproteobacteria
C19	*Gaetbulibacter* sp.	FJ360684	100%	Gammaproteobacteria

Table 4-4. Closest relatives of 16S rDNA sequences obtained from the excised DGGE bands of the microcosm supplemented with inorganic nutrients based on BLAST homology search.

Band No.	Closest relatives in database	GenBank Acc. No.	Homology	Taxonomic groups
D1	Peptostreptococcacea bacterium	GU194175	97%	Firmicutes
D2	*Chlorobium phaeobacteriodes*	NR074363	99%	Chlorobi
D3	Bacteriodetes bacterium	JQ683778	99%	Bacteriodetes
D4	*Marinifilum* sp. ONE-10	KF650761	95%	Bacteriodetes
D5	*Flexibacter* sp. cu2i-19	JN594614	100%	Flavobacteria
D6	*Myxosarcina* sp. NTIA-05	EU583792	97%	Cyanobacteria
D7	*Coccinistipes vermicola* IMCC1411	EF108212	97%	Bacteriodetes
D8	*Idiomarina* sp. YCWA67	FJ984796	98%	Gammaproteobacteria
D9	*Virgibacillus* sp. B6B	HQ433456	98%	Firmicutes
D10	Uncultured Chloroflexi bacterium	JQ579888	99%	Chloroflexi
D11	*Muricauda ruestringensis*	NR074562	99%	Flavobacteria
D12	Uncultured Chloroflexi bacterium	AB433096	99%	Chloroflexi
D13	*Chlorobium luteolum* DSM 273	NR_074096	98%	Bacteriodetes
D14	*Roseovarius pacificus* IMCC17041	KC593284	99%	Alphaproteobacteria
D15	*Marinobacter mobilis* HH2	HQ284162	100%	Gammaproteobacteria
D16	*Bacillus* sp. HWE-119	JQ723721	99%	Firmicutes
D17	*Idiomarina* sp. YCWA67	FJ984796	98%	Gammaproteobacteria
D18	*Marinobacterium* sp. IC961	AB196257	99%	Gammaproteobacteria
D19	*Bacillus* sp. Asd14	JQ030918	99%	Firmicutes

Table 4-5. Closest relatives of 16S rDNA sequences obtained from the excised DGGE bands of the microcosm added with inorganic nutrients and bacterial consortium in the free-living form based on BLAST homology search.

Band No.	Closest relatives in database	GenBank Acc. No.	Homology	Taxonomic groups
E1	Tenericutes bacterium	JQ411296	99%	Tenericutes
E2	*Chlorobium phaeobacteroides* BS1	NR074363	100%	Chlorobi
E3	Bacteroidetes bacterium FH5	JQ683778	98%	Bacteriodetes
E4	*Coccinistipes vermicola* IMCC1411	EF108212	97%	Bacteriodetes
E5	*Flexibacter* sp. cu2i-19	JN594614	100%	Bacteriodetes
E6	Uncultured Chloroflexi bacterium	AB644877	95%	Chloroflexi
E7	*Maritimibacter* sp. PR58-5	EU440991	99%	Alphaproteobacteria
E8	Uncultured delta proteobacterium clone	EU702903	97%	Deltaproteobacteria
E9	*Bacillus* sp. PP12	KF554099	99%	Firmicutes
E10	*Thalassospira profundimaris* strain S8-2	KC420685	100%	Alphaproteobacteria
E11	Bacteroidetes bacterium	JQ683778	99%	Bacteriodetes
E12	*Alcanivorax* sp. CC-AMWY-24	KC169810	99%	Gammaproteobacteria
E13	*Idiomarina* sp. A19	JX298543	100%	Gammaproteobacteria
E14	*Muricauda* sp. cu2i-38	JN594619	99%	Flavobacteria
E15	Uncultured bacterium clone	JX983878	98%	Unknown
E16	*Bacillus* sp. PP12	KF554099	100%	Firmicutes

Table 4-6. Closest relatives of 16S rDNA sequences obtained from the excised DGGE bands of the microcosm added with inorganic nutrients and cocopeat-immobilized bacterial consortium based on BLAST homology search.

Band No.	Closest relatives in database	GenBank Acc. No.	Homology	Taxonomic groups
F1	Tenericutes bacterium	JQ411296	99%	Tenericutes
F2	*Thiomicrospira chilensis* Ch-1	NR028680	99%	Gammaproteobacteria
F3	*Rhodobacter sphaeroides*	AM983574	96%	Alphaproteobacteria
F4	Uncultured Bacteroidetes bacterium	FJ024309	93%	Bacteriodetes
F5	Uncultured Deferribacteres bacterium clone	JQ580221	94%	Deferribacteres
F6	*Rhodobacter sphaeroides* strain JA334	AM983574	96%	Alphaproteobacteria
F7	Erysipelotrichaceae bacterium	HQ133039	94%	Firmicutes
F8	*Marinifilum* sp. ONE-10	KF650761	98%	Bacteriodetes
F9	Bacteroidetes bacterium G22	JQ683777	95%	Bacteriodetes
F10	*Maritimibacter* sp. PR58-5	EU440991	99%	Alphaproteobacteria
F11	*Roseovarius pacificus* IMCC17041	KC593284	99%	Alphaproteobacteria
F12	*Muricauda aquimarina* strain PR54-6	EU440979	99%	Flavobacteria
F13	Uncultured *Spirochaeta* sp.	FN687104	99%	Spirochaetes
F14	*Muricauda ruestringensis* DSM 13258	NR074562	99%	Flavobacteria
F15	*Alcaligenes* sp. isolate CCUG 36768	AJ133493	100%	Betaproteobacteria
F16	*Bacillus firmus* strain KJ-W9	KF011491	100%	Firmicutes
F17	*Muricauda aquimarina* strain TVG01-C001	KF500397	96%	Flavobacteria

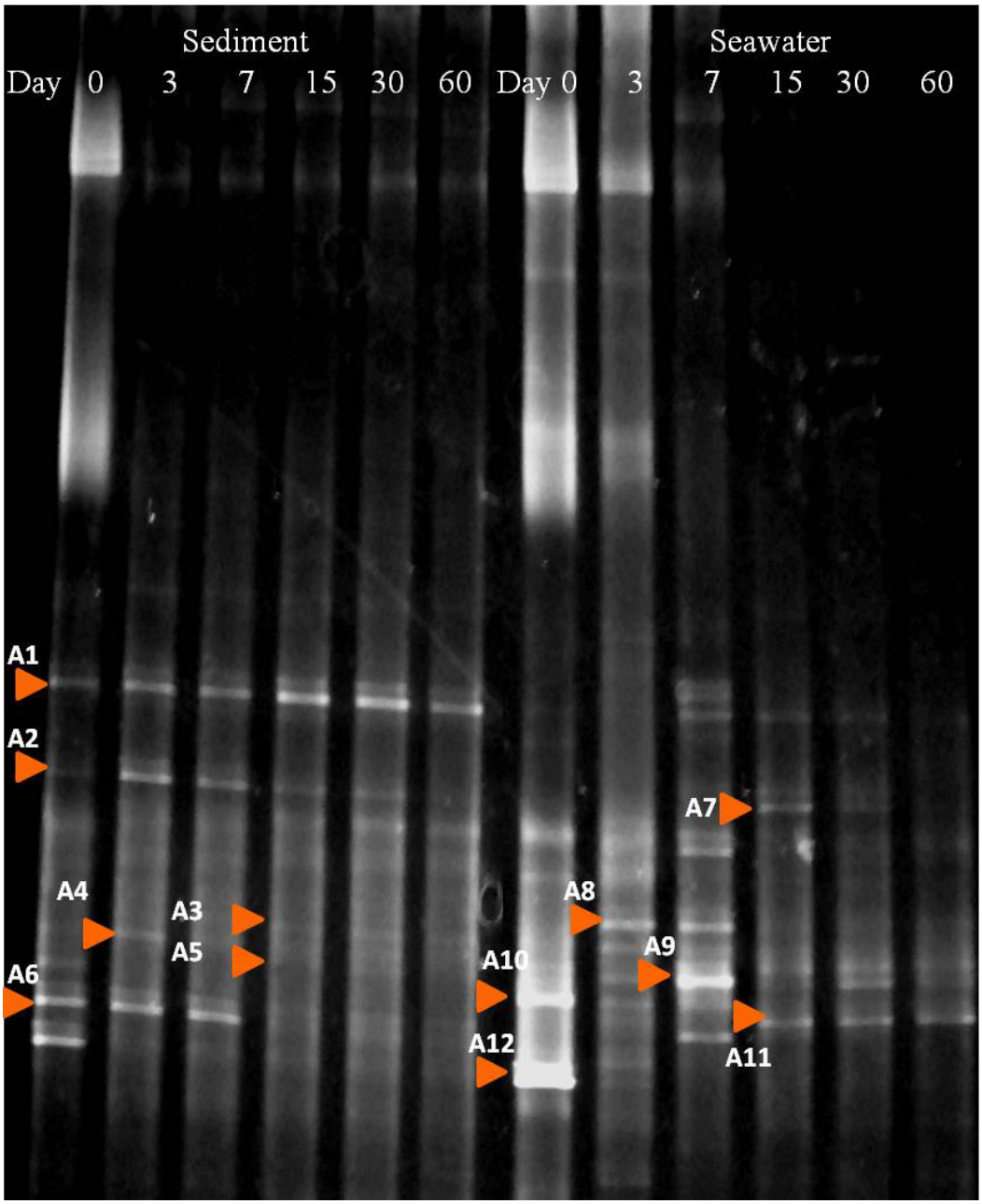

Fig. 4-1. DGGE analysis of the amplified 16S rDNA fragments from sediment and seawater samples of the control microcosm. The labels above the lane indicate the sampling days. The bands subjected to nucleotide sequencing were indicated by the orange arrowheads.

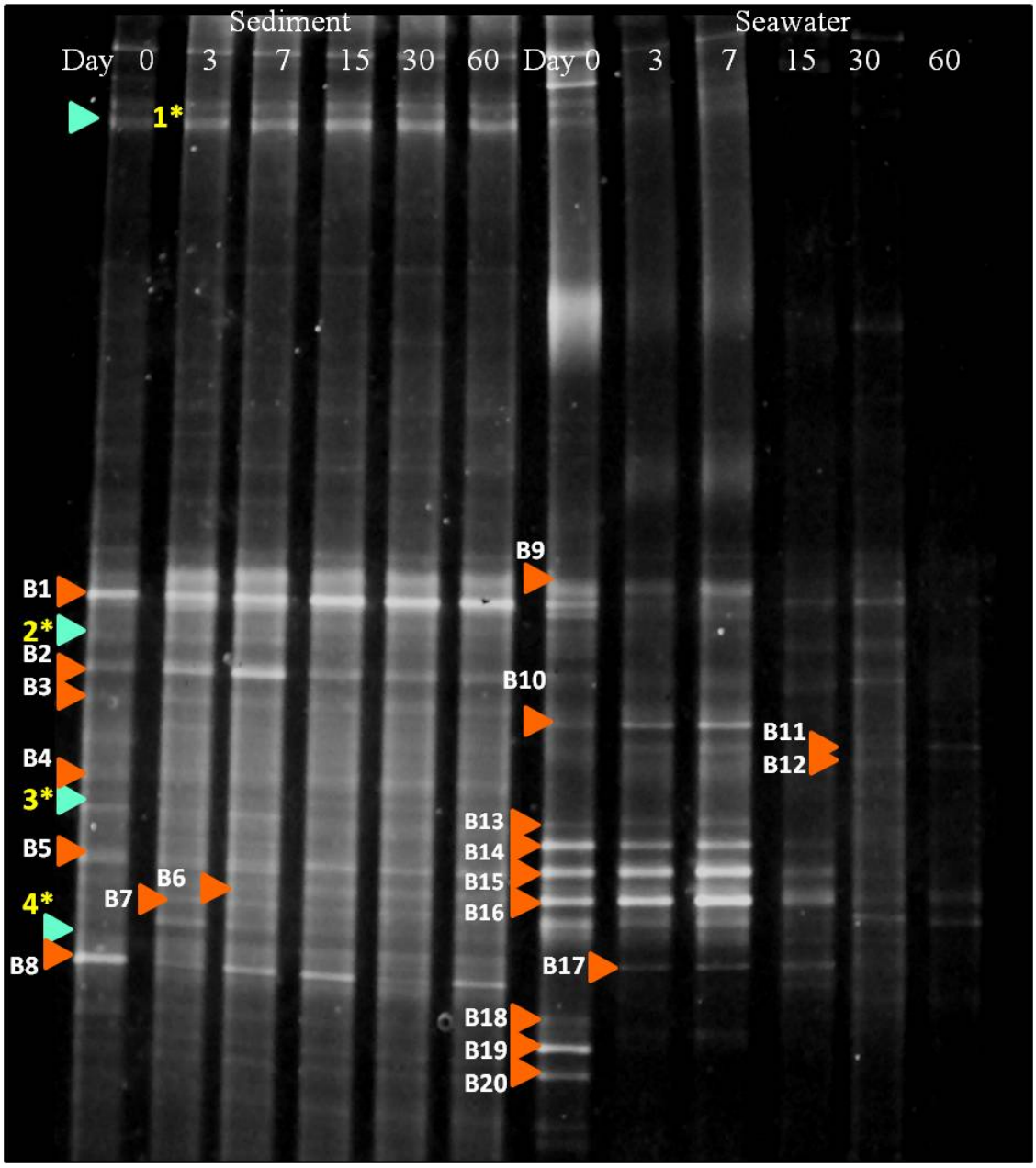

Fig. 4-2. DGGE analysis of the amplified 16S rDNA fragments from sediment and seawater samples of the microcosm augmented with the bacterial consortium in the free-living form. The labels above the lane indicate the sampling days. The bands with the blue arrowheads indicate the position of augmented bacteria as follows: 1, *Gaetbulibacter* sp.; 2, *Pseudomonas* sp.; 3, *Marinobacter* sp.; 4, *Halomonas* sp. The bands subjected to nucleotide sequencing were indicated by the orange arrowheads.

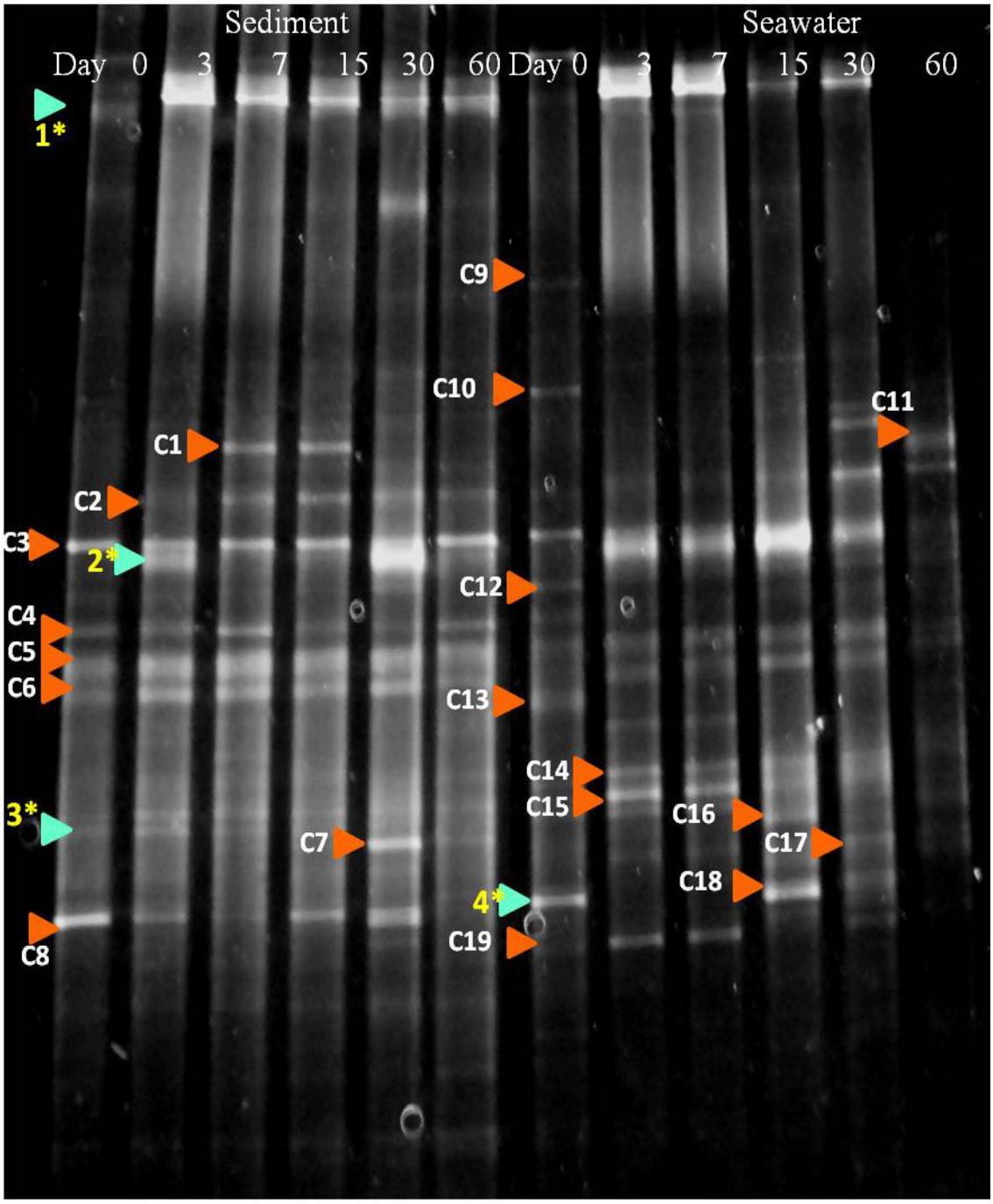

Fig. 4-3. DGGE analysis of the amplified 16S rDNA fragments from sediment and seawater samples of the microcosm augmented with the cocopeat-immobilized bacterial consortium. The labels above the lane indicate the sampling days. The bands with the blue arrowhead indicate the position of augmented bacteria as follows: 1, *Gaetbulibacter* sp.; 2, *Pseudomonas* sp.; 3, *Marinobacter* sp.; 4, *Halomonas* sp. The bands subjected to nucleotide sequencing were indicated by the orange arrowheads

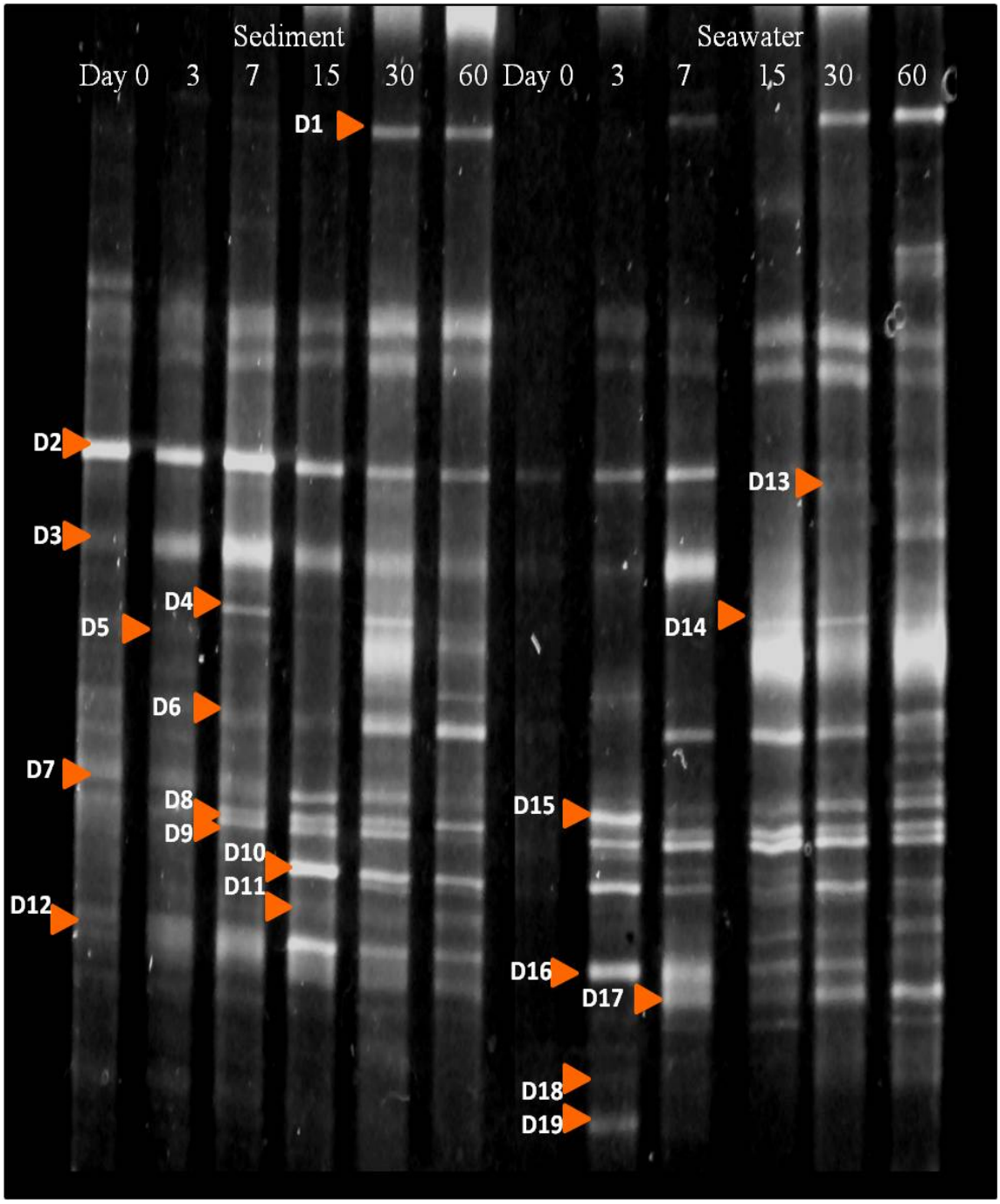

Fig. 4-4. DGGE analysis of the amplified 16S rDNA fragments from sediment and seawater samples of the microcosm supplemented with inorganic nutrients. The labels above the lane indicate the sampling days. The bands subjected to nucleotide sequencing were indicated by the orange arrowheads.

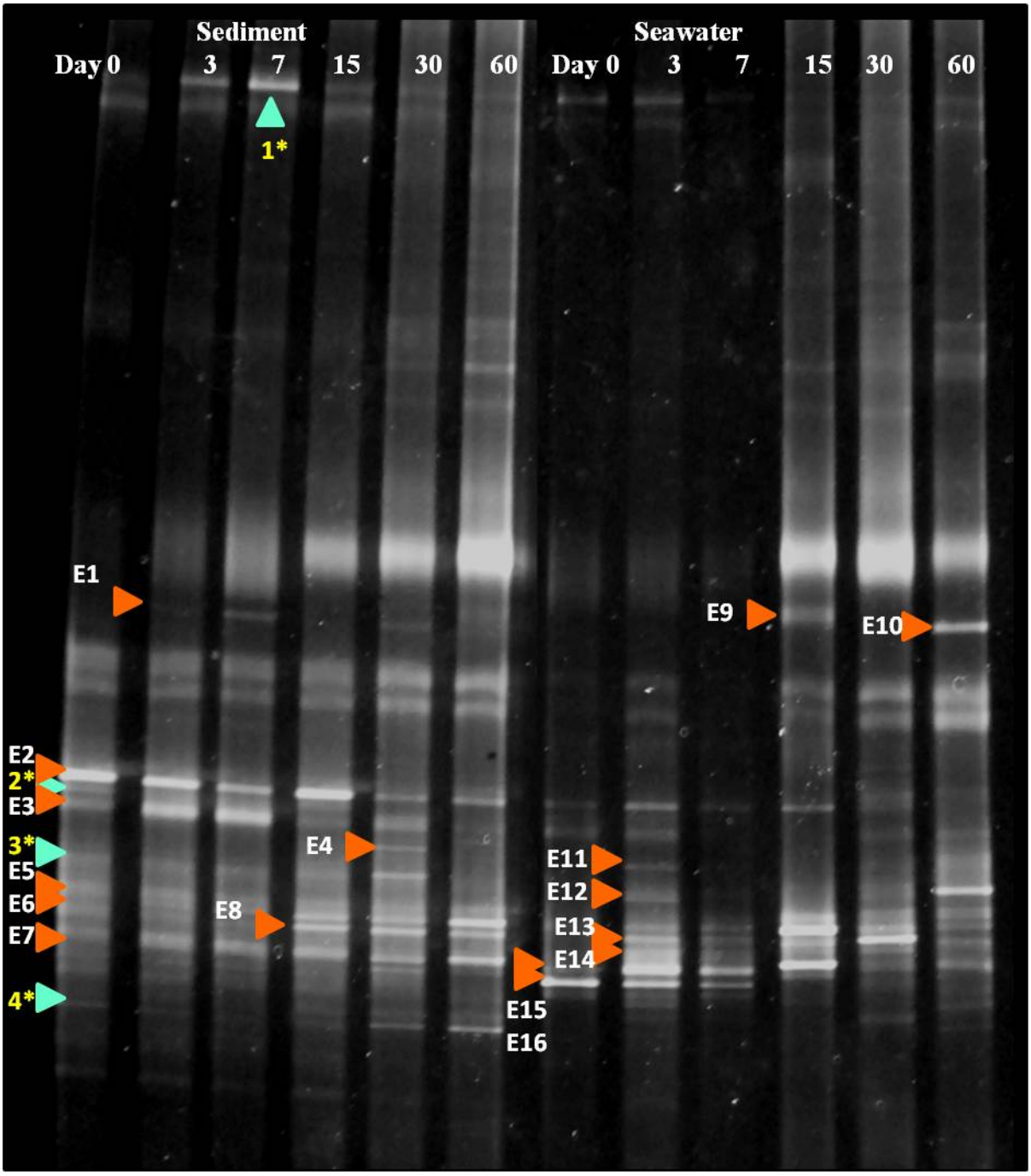

Fig. 4-5. DGGE analysis of the amplified 16S rDNA fragments from sediment and seawater samples of the microcosm added with inorganic nutrients and bacterial consortium. The labels above the lane indicate the sampling days. The bands with the blue arrowheads indicate the position of augmented bacteria as follows: 1, *Gaetbulibacter* sp.; 2, *Pseudomonas* sp.; 3, *Marinobacter* sp.; 4, *Halomonas* sp. The bands subjected to nucleotide sequencing were indicated by the orange arrowheads.

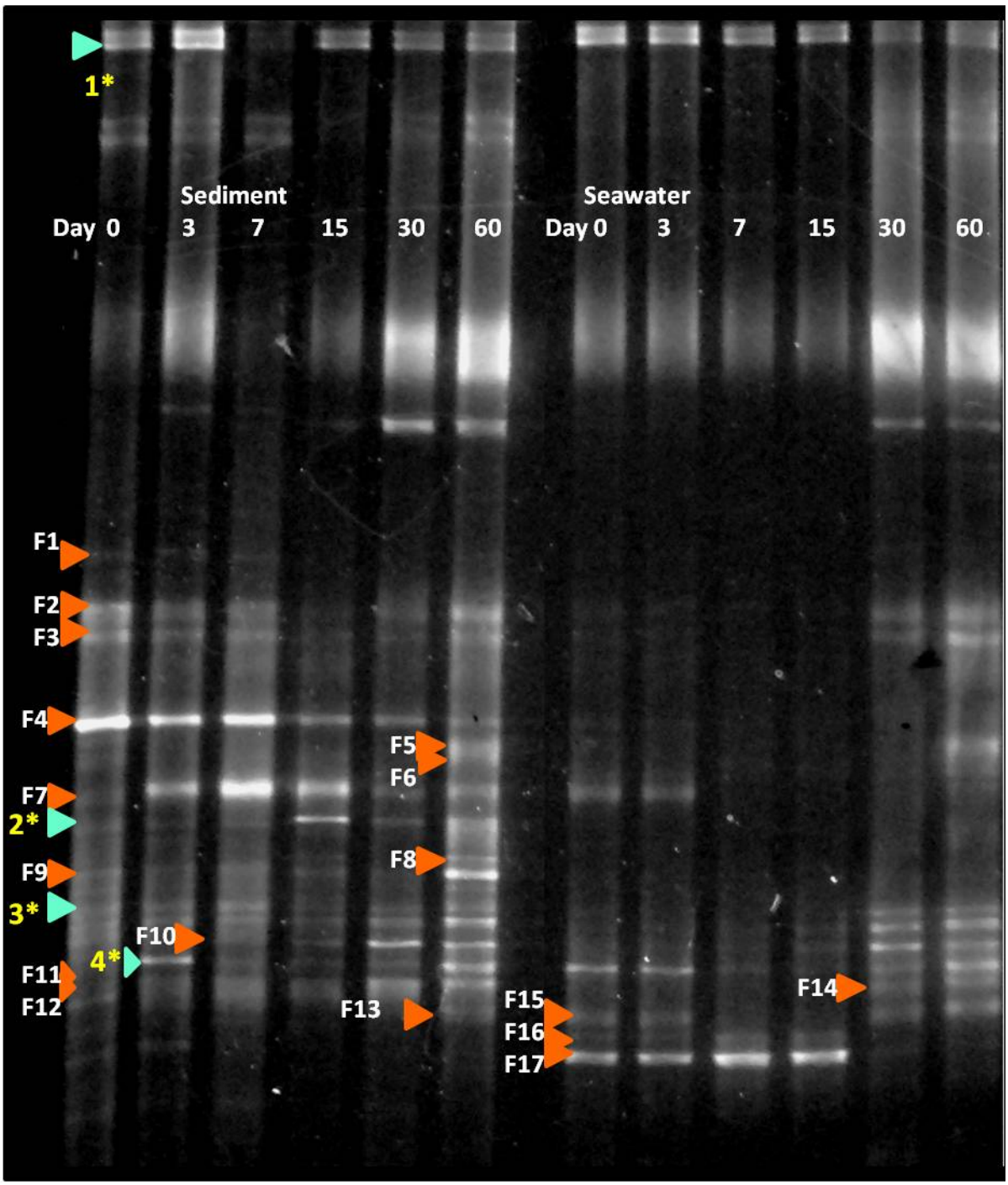

Fig. 4-6. DGGE analysis of the amplified 16S rDNA fragments from sediment and seawater samples of the microcosm added with inorganic nutrients and cocopeat-immobilized bacterial consortium. The labels above the lane indicate the sampling days. The bands with the blue arrowheads indicate the position of augmented bacteria as follows: 1, *Gaetbulibacter* sp.; 2, *Pseudomonas* sp.; 3, *Marinobacter* sp.; 4, *Halomonas* sp. The bands subjected to nucleotide sequencing were indicated by the orange arrowheads.

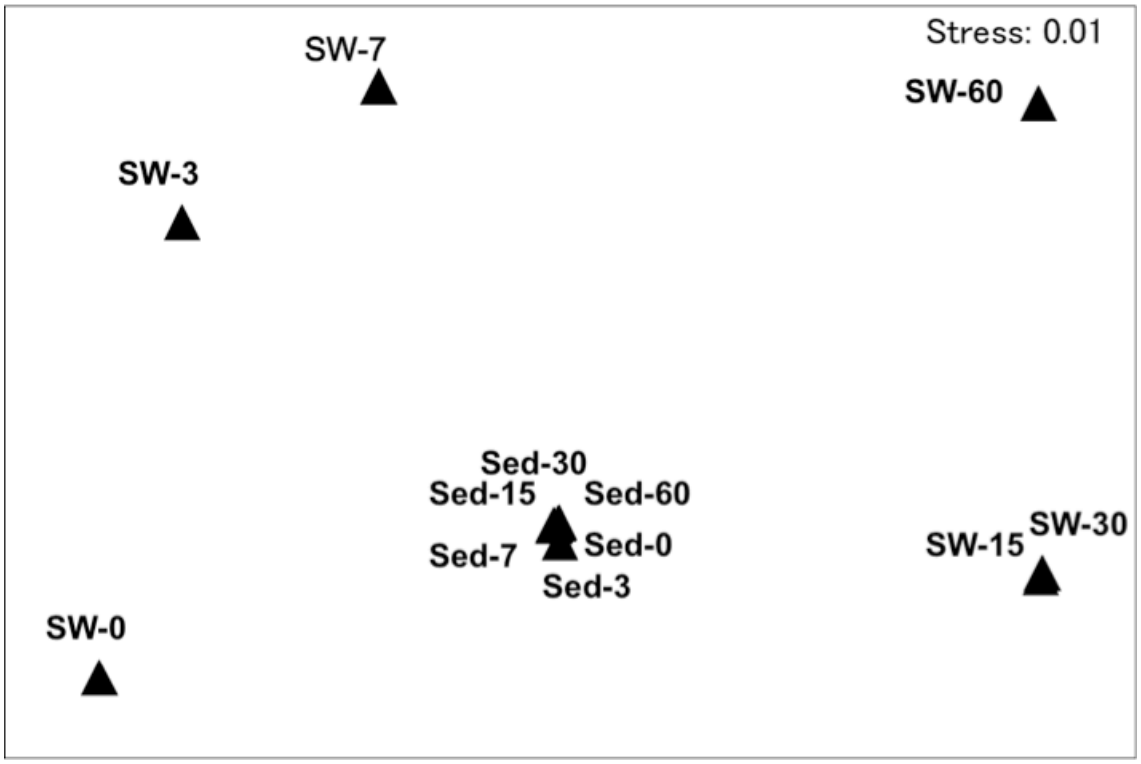

Fig. 4-7. Nonmetric multidimensional scaling analysis (NMDS) map of the banding patterns from sediment and seawater samples of the control microcosm. SW, the band profiles of the seawater samples; Sed, the band profiles of the sediment samples. The sampling days are also shown as numbers following “SW” or “Sed”.

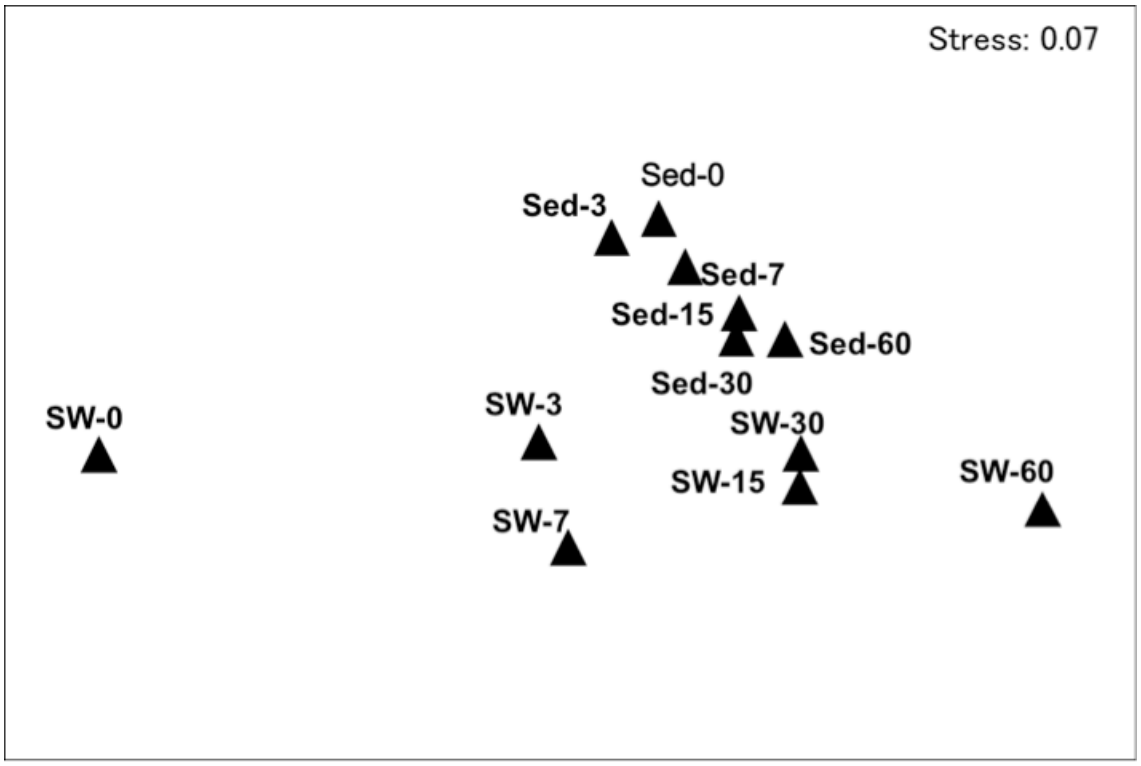

Fig. 4-8. Nonmetric multidimensional scaling analysis (NMDS) map of the banding patterns from sediment and seawater samples of the microcosm augmented with the bacterial consortium in the free living form. Abbreviations shown in the panel are the same as Fig. 4-7.

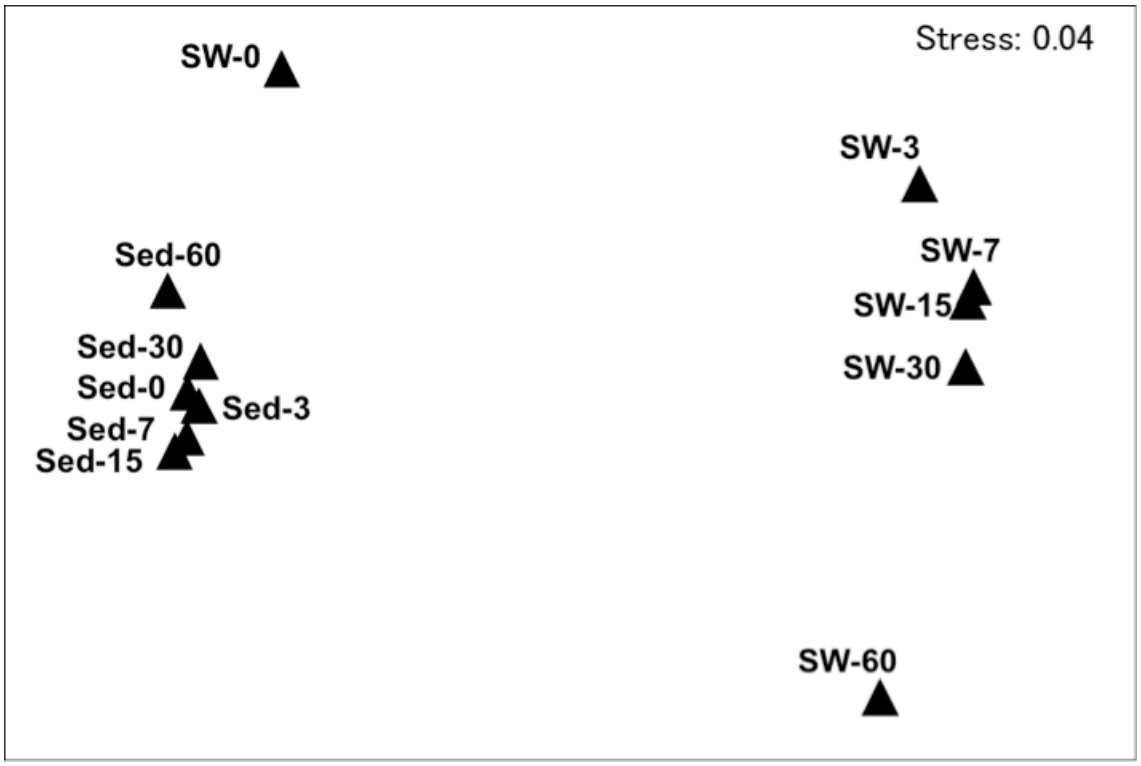

Fig. 4-9. Nonmetric multidimensional scaling analysis (NMDS) map of the banding patterns from sediment and seawater samples of the microcosm augmented with the bacterial consortium immobilized on the surface of cocopeat. Abbreviations shown in the panel are the same as Fig. 4-7.

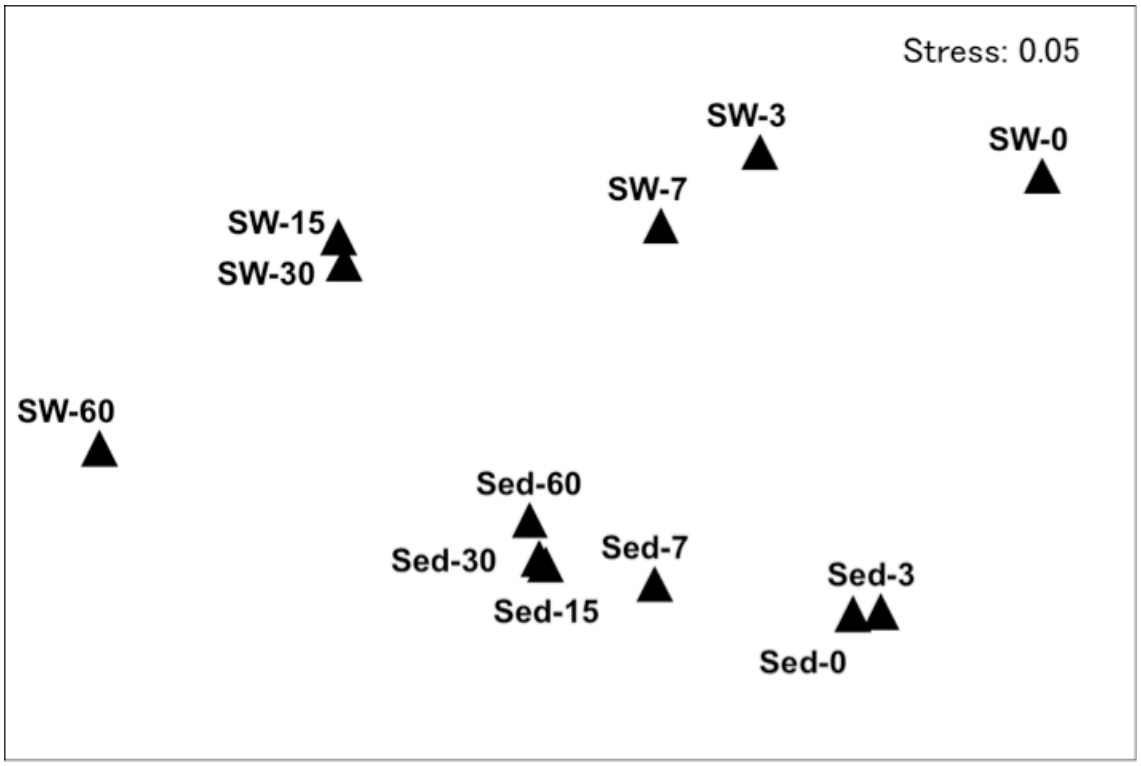

Fig. 4-10. Nonmetric multidimensional scaling analysis (NMDS) map of the banding patterns from sediment and seawater samples of the microcosm added with inorganic nutrients. Abbreviations shown in the panel are the same as Fig. 4-7.

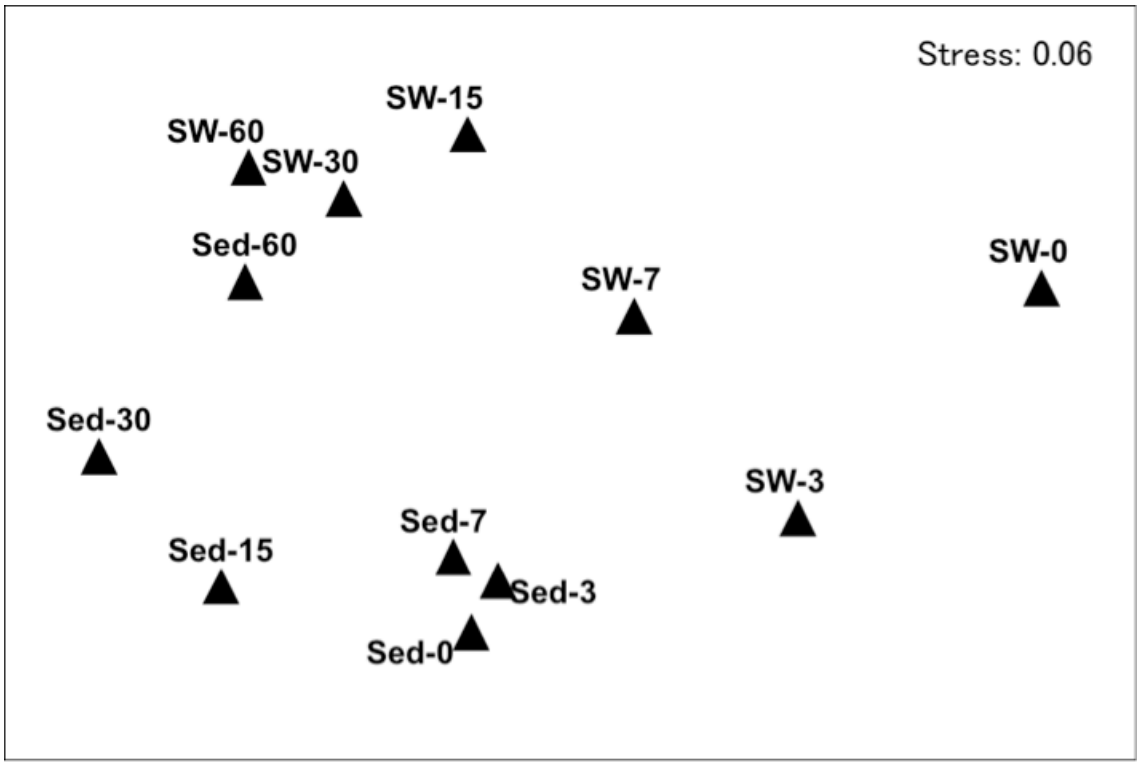

Fig. 4-11. Nonmetric multidimensional scaling analysis (NMDS) map of the banding patterns from sediment and seawater samples of the microcosm added with inorganic nutrients and bacterial consortium in the free-living form. Abbreviations shown in the panel are the same as Fig. 4-7.

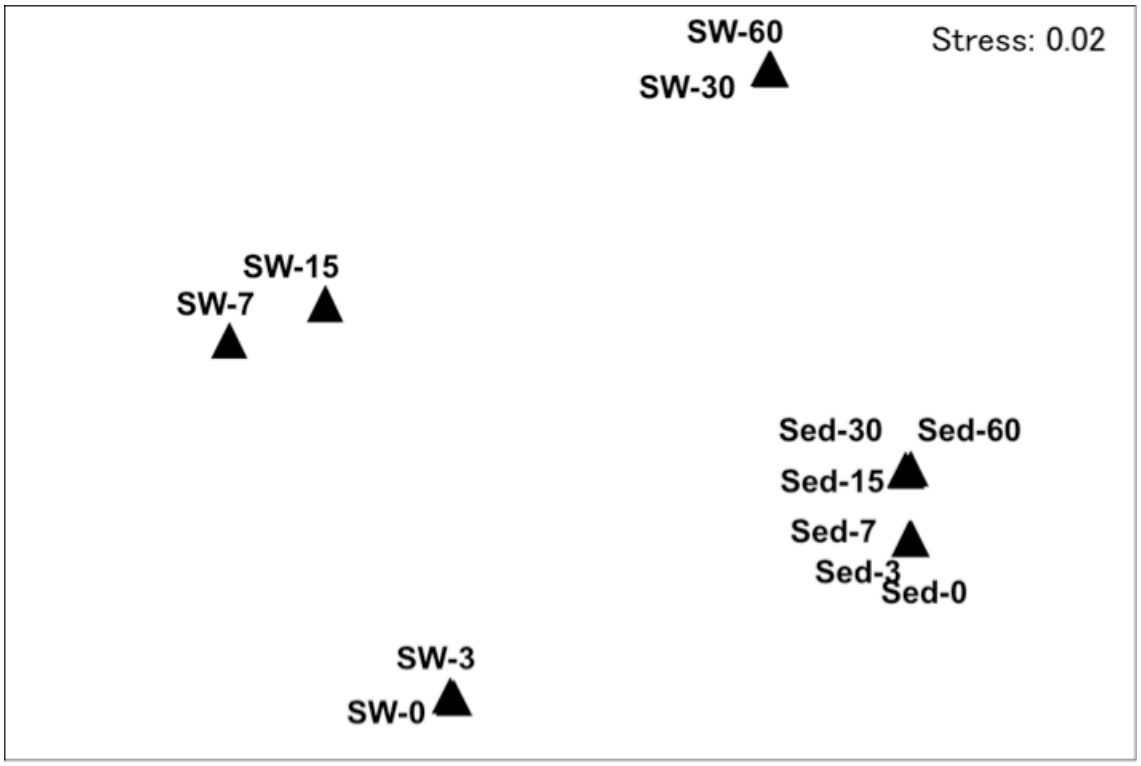

Fig. 4-12. Nonmetric multidimensional scaling analysis (NMDS) map of the banding patterns from sediment and seawater samples of the microcosm added with inorganic nutrients and bacterial consortium immobilized on the surface of cocopeat. Abbreviations shown in the panel are the same as Fig. 4-7.

GENERAL CONCLUSION

The presence and cultivability of oil-degrading bacteria from tropical soil (Guimaras, Philippines) previously contaminated with heavy oil was confirmed in the present study. The isolation and identification of the bacterial strains revealed that most isolates belonged to Gamma- and Alpha-proteobacteria. Evaluation of their hydrocarbon-utilizing capability showed that the isolates could utilize either *n*-alkane, PAHs or heavy oil. A consortium composed of the four strains showing good growth in a medium with different hydrocarbons was formulated and its effectiveness in degrading heavy oil was revealed in the *in vitro* experiment. The consortium was then immobilized on biocarrier materials and tested in an *in vitro* seawater bioremediation. The immobilized cells showed significant reductions in oil concentration and higher bacterial counts during incubation were obtained compared to the free-cell forms and control. The consortium was able to improve degradation of both the aliphatic and aromatic fractions of heavy oil. Storage of up to six months had minimal effect on the viability and oil-degrading activity of the immobilized cells.

Highest oil-degrading efficiency was found in a cocopeat-immobilized bacterial consortium, thus it was further tested to remediate sediment artificially contaminated with heavy oil. A 3x2 factorial microcosm experiment was conducted to investigate effect of the addition of a cocopeat-immobilized bacterial consortium, with and without nutrient supplementation, to the remediation of the contaminated sediment. Positive influences of nutrient and bacterial cell addition to the degradation of heavy oil were observed. Supplementation of inorganic nutrients promoted and sustained growth of oil-degrading and heterotrophic bacteria throughout the experimental period. Maximum reduction in total petroleum hydrocarbons their derivatives were achieved when inorganic nutrients and immobilized bacterial cells are applied. Changes in the bacterial communities during remediation of the artificially contaminated sediment revealed different responses of the bacterial community to the addition of different remediation agents. Shifts in the bacterial communities in the

seawater were more dynamic than in the sediment in the presence of oil and remediation agents. Banding patterns of denaturing gradient gel electrophoresis suggests that the bacterial community present in the seawater was clearly different from that of the sediment. Furthermore, survival of the augmented bacterial consortium was improved by immobilization to cocopeat.

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