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Impacts of bioremediation schemes on bacterial population in naphthalene-contaminated marine sediments

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Abstract

Microcosm experiments were conduced in which the surface of marine sediment was contaminated with naphthalene and subjected to either of three different bioremediation schemes, i.e., biostimulation (BS) by supplementing with slow-release nitrogen and phosphorus fertilizers, bioaugmentation (BA) by inoculating with *Cycloclasticus* sp. E2, an aromatics-degrading bacterium identified to play an important role for aromatic-hydrocarbon degradation in marine environments and combination (CB) of BS and BA. These three schemes were found to be similarly effective for removing naphthalene, while naphthalene disappearance in sediment without any treatment (WT) was slower than those in the treated sediments. Shifts in bacterial populations during and after bioremediation were analyzed by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments. It was found that the *Cycloclasticus* rRNA type occurred as the strongest bands in the course of naphthalene degradation. Clustering analysis of DGGE profiles showed that bacterial populations in the WT, BS and CB sediments differed consistently from those in the uncontaminated control, while the profile for the BA sediment was finally included in the cluster for uncontaminated control sediments after a 150-day treatment. The results suggest that bioaugmentation with ecologically competent pollutant-degrading bacteria is an ecologically promising bioremediation scheme.

Abbreviations: BA – bioaugmentation; BS – biostimulation; CB – combination of biostimulation and bioaugmentation; DGGE – denaturing gradient gel electrophoresis.; WT – without treatment

Introduction

Bioremediation has been recognized as an attractive decontamination strategy for a variety of polluted environments and considered to be advantageous over physical and chemical treatments due to its relatively low cost and little disturbance to the biosphere (Head & Swannell 1999; Morgan & Watkinson 1989; Swannell et al. 1996). Two bioremediation schemes have been known, i.e., biostimulation and bioaugmentation; among them, biostimulation accelerates indigenous pollutant-degrading capacities by supplementing with nutrients and/or inducers for the degradative capacities, while bioaugmentation introduces exogenous pollutant-degrading microorganisms. Currently, most of bioremediation practices employ biostimulation mainly for the following two reasons; (i) it is generally difficult to effectively utilize exogenous microorganisms under a variety

of biotic and abiotic constraints in the environment, and (ii) workers are concerned about risks (e.g., deleterious ecological consequences) associated with the use of non-resident organisms. In experimental bioremediation trials for petroleum-contaminated sandy beaches, however, microbial communities after biostimulation by supplementing with fertilizers differed significantly and persistently from those in uncontaminated control zones (MacNaughton et al. 1999; Röling et al. 2002).

Marine beaches and sediments, particularly those in industrial areas, are frequently polluted with petroleum hydrocarbons as the results of accidental spills, industrial and urban runoffs and shipping activities (Bossert & Compeau 1995). Among petroleum hydrocarbons, polycyclic aromatic hydrocarbons (PAHs) are of primary environmental concern due to their toxicity and persistence in the environment (Angerer et al. 1997). Reports have also documented that bioaccumulation of PAHs in marine higher organisms has occurred as a consequence of the food web initiated by benthic small organisms feeding PAH-contaminated sediment materials (Meador et al. 1995). PAH biodegradation in the marine environment has thus been studied extensively, showing that PAHs can be degraded in aerobic (Geiselbrecht et al. 1998; Kasai et al. 2002; Melcher et al. 2002) and anaerobic zones (Coates et al. 1997).

We are currently investigating bioremediation schemes applicable to PAH-contaminated marine environments, and the present study conducted microcosm experiments using marine sediments contaminated with naphthalene under controlled environmental conditions. The purpose of the experiments was to comparatively assess different bioremediation schemes from the viewpoints of decontamination efficiency and ecological impacts; bioremediation schemes tested included biostimulation by supplementing with slow-release nitrogen and phosphorus fertilizers (BS), bioaugmentation by inoculating with a marine PAH-degrading bacterium, *Cycloclasticus* sp., E2 (BA) and the combination of BS and BA (CB). In particular, we were interested in examining the utility of the ecologically competent PAH-degrading bacterium, *Cycloclasticus* sp. E2, that has been suggested to play the primarily role for PAH degradation in bioremediation of petroleum-polluted marine environments (Kasai et al. 2002). Since this bacterium has been shown to predominantly occur during PAH degradation and disappear rapidly after PAHs were degraded (Kasai et al. 2002), we anticipated that impacts of bioaugmentation with this bacterium on recipient marine biota could be small compared to that of biostimulation with fertilizers. Since a bioremediation strategy should be selected based on both the pollutant-removal efficiency and ecological impacts, results of the present study will contribute to the future development of bioremediation strategies for PAH-contaminated marine environments.

Materials and methods

Preparation of marine-sediment microcosms

Sandy sediment was obtained in December 2000 from the Kamaishi bay (Iwate, Japan). The sediment was stored in reservoir tanks (2 m^3 in capacity and filled with 1 m^3 of sediment) under continuous flow of natural seawater at a rate of 200 ml min^{-1}. A fraction of the stored sediment (15,000 cm^3) was put into a temperature-controlled microcosm tank (30 cm × 50 cm × 30 cm in size [Japan Aqua Tec]) and stabilized under continuous flow of natural seawater for a week or more before starting an experiment. Unless otherwise stated, the flow rate of seawater was 100 ml min^{-1}, and the temperature was kept at 20 °C.

Bioremediation experiments

Naphthalene was dissolved in ethanol at a concentration of 50,000 mg l^{-1}. After the water depth in a tank was reduced down to approximately 2 cm, 100 ml of the naphthalene solution was added to the seawater. Immediately after that, the water and sediment (top 1 cm) was vigorously mixed for several minutes in order for the added naphthalene to be adsorbed onto sediment sands. The sediment was then settled for several hours, before the tank was again filled with seawater and started to continuously supply seawater. Bioremediation experiments were started one day after contamination with naphthalene.

(i) *BS*. A microcosm tank was supplemented with two types of slow-release particle fertil-

izers, 100 g of nitrogen, fertilizer (Super IB, Mitsubishi Kasei; nitrogen content, approx. 33%) and 22 g of phosphorus fertilizer (Linstar 30, Mitsubishi Kasei; phosphorus content, approx. 30%). These amounts were determined according to results of previous spilled-oil bioremediation experiments (Ishihara et al. 1996). The fertilizers were uniformly dispersed at the surface of sediment.

(ii) *BA*. *Cycloclasticus* sp. E2 was grown in 500 ml of artificial seawater supplemented with 200 mg l^{-1} naphthalene (Kasai et al. 2002), After 1-week cultivation, cells were harvested by centrifugation at 10,000× g for 10 min. Cells were suspended in seawater and introduced into a microcosm tank. The surface sediment was gently agitated for several minutes in order for the introduced cells to be incorporated into the sediment sands.

(iii) *CB*. The above-described fertilizers and *Cycloclasticus* sp. E2 were introduced simultaneously as described above.

Bioremediation experiments were conducted two times, and the reproducibility of results was ascertained.

Measurement of naphthalene concentration in sediment

A small core was collected in triplicate from the surface sediment (approximately 1 cm from the top) using a narrow tube (approximately 2 cm in diameter) and used for chemical and molecular analyses. A portion of the collected sediment was suspended in 3 ml of tetrahydrofuran in a 15-ml tube, and the suspension was shaken for 1 min by hand before 3 ml of hexane was added. This mixture was shaken using a SR-II recipro shaker (Taitec) at the maximum speed for 1 h. The tube was centrifuged at 2000× g for 1 h at room temperature for recovering the solvent phase. After the solvent was treated with approximately 0.1 g of anhydrous $NaSO_4$, it was subjected to gas chromatography-mass spectrometry (GC-MS) (GC/MS-QP5000; Shimadzu) equipped with a DB-5 column (length 30 m, ID 0.25 mm, film 0.1 μm [J & W scientific]) according to the method described elsewhere (Wang et al. 1998). Naphthalene was quantified by the SIM analysis ($m/z = 128$).

DNA extraction from marine sediment

DNA was extracted from the sediment (0.5 g) by using a method described elsewhere (Zhou et al. 1996) with following modifications. Three cycles of the freeze–thaw treatment (Sprott et al. 1994) were performed after the initial sodium dodecylsulfate lysis step. Final DNA purification was conducted using a hydroxyapatite column (Steffan et al. 1988) followed by a treatment using Microcon microconcentrators 100 (Millipore). The quality and quantity of the extracted DNA were checked by measuring the UV absorption spectrum (Sambrook et al. 1989).

PCR and denaturing gradient gel electrophoresis

The variable V3 region of the bacterial 16S rRNA gene (corresponding to positions 341–534 in the *Escherichia coli* sequence) was analyzed by denaturing gradient gel electrophoresis (DGGE) after PCR amplification with primers P2 and P3 (Muyzer et al. 1993). The PCR conditions used have been described previously (Watanabe et al. 1998). DGGE was performed with a D-Code system (Bio-Rad Laboratories) used according to the manufacturer's instructions. Ten percent (wt vol^{-1}) polyacrylamide gels with a 25%–60% denaturant gradient (Muyzer et al. 1993) were used, and electrophoresis was performed for 3.5 h at 200 V at 58 °C. Subsequently, the gels were stained with SYBR Gold (FMC Bioproducts) used according to the manufacturer's instructions, and gel images were obtained by using the Gel Doc 2000 system (Bio-Rad Laboratories). Nucleotide sequences of DGGE bands were determined as described previously (Watanabe et al. 1998).

Clustering analysis of DGGE profiles

A DGGE image was converted to computer digital profile using the Multianalyst software (Bio-Rad Laboratories) installed in the Gel Doc 2000 system, and background treatment was conducted for each profile. The profile was divided into approximately 100 parts and imported into Excel files. In the Excel file, band position adjustment was conducted by referring to a standard profile. For this standard, a DGGE profile for seawater (obtained from the Kamaishi bay; SW) was used, which had been prepared in

Table 1. Sequence analysis of major DGGE bands

Band (Accession no.)	Length (bp)	Related organism, accession number (% identity)	Phylogenetic group
Fertilizer addition (Figure 1)			
1(AB167413)	161	Uncultured *Gammaproteobacterium*, AF534243 (91)	*Gammaproteobacterium*
2 (AB167414)	137	Uncultured *Alphaproteobacterium*, AY239004 (94)	*Alphaproteobacterium*
3 (AB167415)	152	*Oceanospirillum pusillurn*, AB006768 (99)	*Alphaproteobacterium*
4 (AB167416)	134	Uncultured bacterium, AB094797 (95)	*Chloflexi*
Bioremediation experiments (Figure 4)			
5 (AB167417)	160	*Cycloclasticus* sp. E2, AB080112 (100)	*Gammaproteobacterium*
6 (AB167418)	157	*Cytophagales* str., MED10, AF025553 (97)	*Bacteroidetes*
7 (AB167419)	135	*Alphaproteobacterium* PWB3, AB106120 (100)	*Alphaproteobacterium*
8 (AB167420)	135	Uncultured Gram-positive bacterium, AY211678 (99)	*Firmicutes*
9 (AB167421)	135	Uncultured *Alphaproteobacterium*, AB459686 (100)	*Alphaproteobacterium*
10 (AB167422)	155	*Cycloclasticus spirillensus*, AY026915 (96)	*Gammaproteobacterium*
11 (AB167423)	140	*Rhodococcus* sp. 17, AY177354 (99)	*Actinobacteria*
12 (AB167424)	161	*Desulfuromonas acetexigens*, U23140 (96)	*Deltaproteobacteria*

one time with a sufficient quantity for using it throughout the study. This standard sample was loaded to all DGGE gels. After the band-position was adjusted, the sum of band areas was normalized to be 1, and a distance (D) between two DGGE profiles were calculated according to an equation; $D = \sqrt{\sum (I_i - I_j)^2}$, where *Ii* and *Ij* are the intensities of profiles *i* and *j* at one part. The distances of two profiles were calculated for all DGGE profiles and used for clustering analysis. A dendrogram showing clustering of DGGE profiles was constructed by the UPGMA method (Sokal & Michener 1958).

Results

Stability of sediment bacterial populations

Before starting the bioremediation experiments, fluctuations in sediment bacterial populations irrespective of pollution and bioremediation were assessed by changing several environmental parameters including temperature and flow rate of seawater (Figure 1). The microcosms were operated under respective conditions for over one month, before being analyzed. In addition, influences of the fertilizers used for biostimulation were

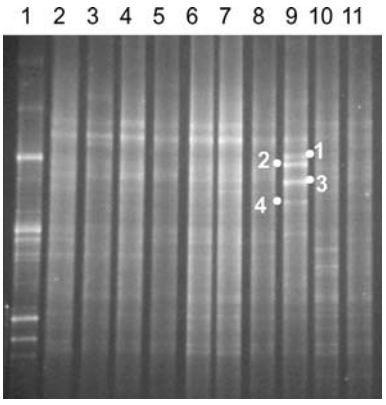

Figure 1. DGGE profiles of marine-sediment bacteria in microcosms affected by flow rates of seawater, temperature and supplementation with fertilizers. Lanes: 1, SW (the reference profile); 2, temperature 10 °C; 3, temperature 20 °C; 4, temperature 30 °C; 5, flow rate 0 ml min^{-1}; 6, flow rate 200 ml min^{-1}; 7, flow rate 1000 ml min^{-1}; 8, before fertilizer supplementation; 9, 11 days after fertilizer supplementation, 10, 46 days after fertilizer supplementation; 11, 74 days after fertilizer supplementation. Bands marked with white dots were excised and sequenced (Table 1).

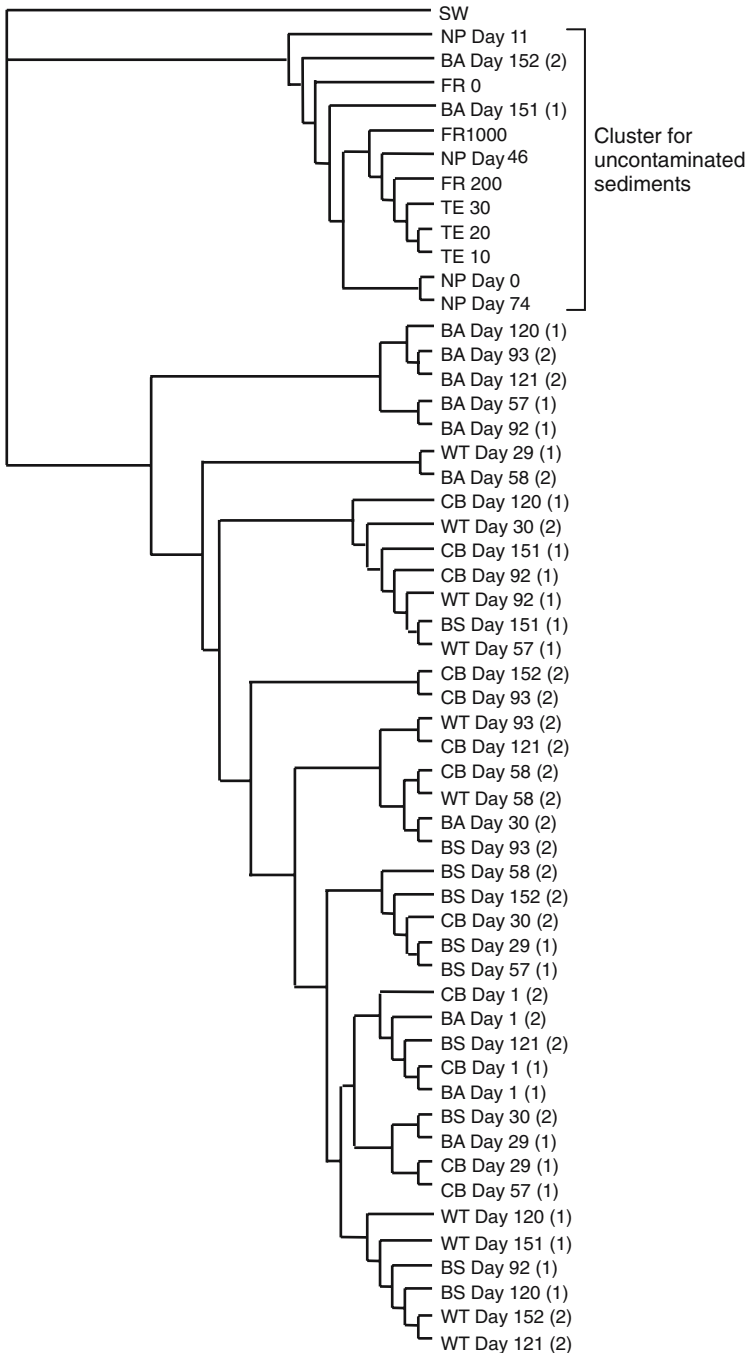

Figure 2. A dendrogram showing the similarities in DGGE profiles for sediment bacterial populations after different treatments. The SW profile was used as outgroup. TE10, temperature at 10 °C; TE20, temperature at 20 °C; TE30, temperature at 30 °C; FR0, flow rate at 0 ml min^{-1}; flow rate at 200 ml min^{-1}; flow rate at 1000 ml min^{-1}; NP, fertilizer supplementation without contamination with naphthalene. Profiles obtained in two bioremediation experiments were included; (1) experiment 1 and (2) experiment 2.

also examined without contamination with naphthalene (Figure 1). As shown in Figure 1, bacterial populations were stable and not significantly affected by the changes in the environmental parameters. Bacterial populations were temporally changed after supplementation with fertilizers (on

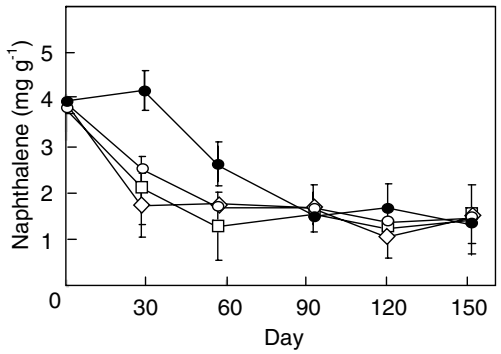

Figure 3. Changes in naphthalene concentrations in the top sediment in the BS (open circle), BA (open square), CB (open diamond) and WT (closed circle) microcosms.

day 11), whereas this shift was not detected on day 46. These trends were reproducible in two independent experiments. Several relatively strong bands appearing on day 11 in the fertilizer-supplemented sediment were found to represent bacterial populations affiliated with *Alphaproteobacteria* and *Gammaproteobacteria* (Table 1).

The DGGE profiles in Figure 1 were subjected to the clustering analysis in order to know a range of fluctuation occurring irrespective of pollution and bioremediation (Figure 2). In the analysis, the profile for seawater bacteria (SW) served as the reference profile, which was always loaded on a DGGE gel and used in the clustering analysis for correcting gel-to-gel variations in the band positions. The clusters formed by the DGGE profiles in Figure 1 was considered to represent the range of variation observed in uncontaminated sediment and was used for comparative purposes to identify DGGE profiles from the bioremediation treatments that were out of the range of natural variation and could be associated with the bioremediation treatments.

Naphthalene degradation

Four microcosms were prepared, in which sediments were contaminated with naphthalene. Among them, three microcosms were subjected to three different schemes of bioremediation; i.e., BS, BA and CB. Effects of these bioremediation treatments were assessed by periodically measuring naphthalene concentrations in the sediments (Figure 3). As shown in this figure, naphthalene concentrations in the three bioremediated sediments were similarly decreased, and the decreases were more rapid than the decrease in the untreated control microcosm (WT). After day 90, however, naphthalene concentrations in the four microcosms became constant at approximately

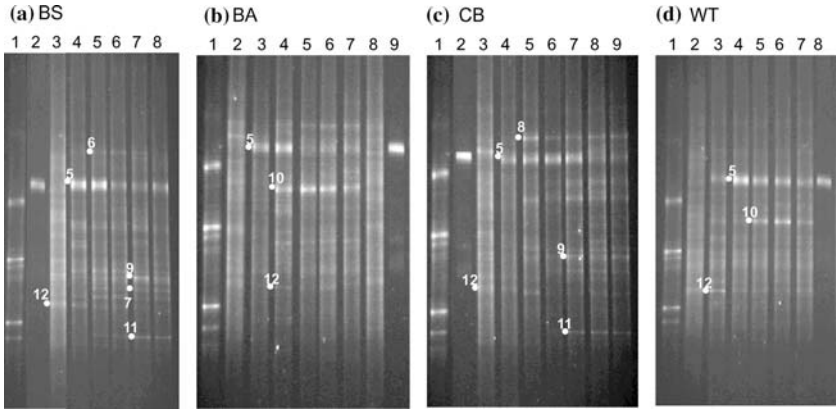

Figure 4. DGGE profiles of marine-sediment bacteria in microcosms affected by naphthalene contamination and bioremediation treatments. (A) BS microcosm. Lanes: 1, SW (the reference profile); 2. *Cycloclasticus* sp. E2; 3, day 0 (before fertilizer supplementation); 4, day 29; 5, day 57; 6, day 92; 7, day 121; 8, day 151. (B) BA microcosm. Lanes; 1, SW (the reference profile); 2, day 0 (before inoculation with E2); 3, day 1; 4, day 29; 5, day 57; 6, day 92; 7, day 121; 8, day 151, 9, *Cycloclasticus* sp. E2. (C) CB microcosm. Lanes: 1, SW (the reference profile); 2, *Cycloclasticus* sp. E2; 3, day 0 (before inoculation with strain E2 and fertilizer supplementation); 4, day 1; 5, day 29; 6, day 57; 7, day 92; 8, day 121; 9, day 151. (D) WT microcosm. Lanes: 1, SW (the reference profile); 2, day 0; 3, day 29; 4, day 57; 5, day 92; 6, day 121; 7, day 151, 8, *Cycloclasticus* sp. E2. Bands marked with white dots were excised and sequenced (Table 1).

1.5 mg g^{-1}, and further degradation was not observed during the period of experiments. We assumed that this was due to the presence of undegraded naphthalene at a deeper part of the sampled sediment core where oxygen was depleted. Actually, when thin surface sediment was scraped from a wide area after the bioremediation was terminated, naphthalene was not detected by the GC analysis (data not shown). It is therefore likely that naphthalene in the surface aerobic zone was mostly degraded and this degradation was accelerated by the bioremediation treatments. For the termination of naphthalene degradation, we also need to consider loss of nutrients and changes of physical conditions in the sediment (such as, pH).

DGGE analysis of bacterial populations

Shifts in sediment bacterial populations after naphthalene contamination and bioremediation were assessed by the DGGE analysis (Figure 4). The changes in DGGE patterns were reproducible in two independent experiments. Major bands in this figure were excised and sequenced (Table 1). When changes in DGGE patterns were assessed in correlation to naphthalene degradation (Figure 3), band 5 was found to appear strongly when naphthalene degradation proceeded. This band was identical in position to the band for *Cycloclasticus* sp. E2, and its sequence was also identical to that of strain E2, indicating that band 5 in the BA and CB profiles immediately after inoculation represented the inoculated E2. Band 5 appearing in the BS and WT sediments, however, should be an indigenous population in the Kamaishi bay. Since *Cycloclasticus* sp. E2 had been isolated from seawater from the Kamaishi bay after the enrichment on phenanthrene (Kasai et al. 2002), we deduced that *Cycloclasticus* populations closely related to strain E2 occurred in the BS and WT microcosms.

In addition to band 5, some other bands also occurred commonly in some of these microcosms, including band 10 that was closely related to *Cycloclasticus spirillensus*. It is interesting that this band most strongly appeared in the BA profiles and second most in the WT profiles. Some bands (9 and 11) appeared only in the fertilizer-supplemented microcosms; these bands may have been indicators for the influences of the fertilizer supplementation.

As shown in Figure 4, no prominent band was seen in the DGGE profile for the BA sediment on day 151, whose pattern was similar to those of uncontaminated sediment (Figure 1) and sediment at the starting point of the bioremediation experiments (day-0 profiles in Figure 4). On the other hand, the day-151 profiles of the other microcosms (BS, CB and WT) included some stronger bands (including band 5), allowing us to consider that effects of contamination/bioremediation remained in the BS, CB and WT microcosms even on day 151.

In order to more clearly examine the recovery of bacterial populations after contamination and bioremediation, we conducted the clustering analysis of DGGE patterns (Figure 2). This figure includes data obtained in two bioremediation experiments and foregoing control experiments (Figure 1). Similar approaches have been employed in several studies (El Fantroussi et al. 1999; Stephen et al. 1999), suggesting that this method can provide valuable information as to the endpoint of bioremediation. It is clearly shown in Figure 2 that the DGGE patterns for the BA sediment on day 151 (experiment 1) and on day 152 (experiment 2) were grouped into the cluster for uncontaminated sediment, indicating that the bacterial populations in the BA sediment were recovered. DGGE profiles for the other treatments at the same time points are grouped into a cluster that also includes the profiles for earlier days of the bioremediation experiments (Figure 2), indicating that bacterial populations were not recovered by the other treatments. In the WT sediments, the persistent change of bacterial populations may have been ascribable to the slower decrease of naphthalene than those in the other microcosms.

Discussion

The present study carried out microcosm experiments in order to assess the effectiveness of different bioremediation schemes for the cleanup of PAH-contaminated marine sediments. This type of assessment under well-controlled experimental conditions is essential for developing the fundamental strategy for bioremediation, while further practical consideration is also necessary for applying the recommended technology to the field bioremediation. According to the result of the

microcosm experiments, we will proceed to field studies of bioaugmentation with the *Cycloclasticus* strain, in which restricted areas of contaminated sediment will be treated after enclosed with walls. PAH-degrading *Cycloclasticus* strains have been isolated from seawater of different locations in the world, e.g., Alaska (Dyksterhouse et al. 1995) and gulf Mexico (Geiselbrecht et al. 1998), and have been suggested to be ubiquitously present in the marine environment (Kasai et al. 2002). It is therefore likely that the *Cycloclasticus* strains have the wide utility for PAH bioremediation in the marine environment.

Supplementation with fertilizers is at present a common practice in bioremediation (MacNaughton et al. 1999; Röling et al. 2002), in which slow-release agricultural fertilizers that gradually release nitrogen and phosphorus (such as those used in the present study) have been used. Although it has been generally accepted that ecological impacts of bioaugmentation is relatively large compared to biostimulation, this study showed for the first time that the ecological impact of the slow-release fertilizers on bacterial populations in marine sediment was more persistent than that of the bioaugmentation treatment. This should have been due to the use of the indigenous degradative bacteria (i.e., *Cycloclasticus* sp. E2) for the bioaugmentation trail. Previous studies have also shown that bacterial populations in sandy beaches artificially polluted with petroleum were not recovered after long periods of bioremediation (MacNaughton et al. 1999; Röling et al. 2002); hence, we are also interested in using the *Cycloclasticus* strain in sandy-beach bioremediation experiments.

In conclusion, the results of the present study suggest that bioaugmentation with ecologically competent pollutant-degrading bacteria is an ecologically promising bioremediation scheme. Identification, isolation and collection of such organisms for a variety of pollutants and for a range of environments will be important subjects in future bioremediation studies (Watanabe 2001).

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