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Mesocosm experiment on the succession of microbial community in response to oil contamination to coastal seawater.

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Abstract: The influence of oil contamination on marine bacterial communities was examined with a pair of meso-scale tanks filled with 5 tons of surface seawater from a semi-enclosed bay, Hamana-ko, Japan. One of the tanks was contaminated with the water-soluble fraction of Rank-A residual oil, while the other was kept uncontaminated for comparison. Surface seawater in the two tanks was periodically taken to monitor microbial abundance and community structure. Bacteria in the oil tank proliferated within a day, which was followed by an increase in viral abundance. Then, the number of bacterial cells and viral particles returned to the initial levels within three days. Denaturing gradient gel electrophoresis of bacterial 16S-rDNA amplicon showed distinctive differences in band patterns between the oil and control tanks. The following conclusions can be made from the experiments. The succession of oil-exposed microbial community was made up of three steps, as follows: (i) Bacteria increased in number within a day with a slight increase of 16S rDNA sequence types. (ii) After three days, bacterial cell numbers returned to the initial levels, but sequence types increased to the maximum. (iii) Finally, bacterial numbers stayed put with the decline in sequence types.

Keywords: *oil pollution, marine bacteria, mesocosm and DGGE*

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1. Introduction:

Oil pollution in the ocean, which ruins the spot area and inflicts a loss of marine resources on the neighboring environments, is one of the most devastating disasters caused by human activities. In aquatic environments, some oil compounds are well known as toxic to plants and animals (ALBERS, 1995). In addition, it has been evident that polycyclic aromatic hydrocarbons (PAHs) exhibited the effect of growth inhibition upon marine and freshwater microalgae (OKUMURA *et al.*, 2003; DJOMO *et al.,* 2004).

Part of these aromatics are finally doomed to be broken down and transformed into non-toxic forms through the processes of microbial degradation. As hydrocarbon degradation hinges on enzyme activities of indigenous oil-degraders and on environmental imbalances, such as, oxygen, nitrogen, phosphate, temperature, salinity levels (LEAHY and COLWELL, 1990;

ATLAS and BARTHA, 1992), it is important to understand relationship between microbial populations and hydrocarbon degradation activities under natural environmental conditions upon oil degradation in the ocean. Mesocosm has been considered suitable for monitoring the impacts of oil-contamination upon aquatic microbes because it impounds fairly large amounts of water, which include all sorts of microbial assemblage playing the leading part of primary production and catabolism of organic substances (LACAZE, 1974; BAK and NIEUWLAND, 1987; DELILLE and SIRON, 1993; MAKI *et al.*, 1999). By using 5-ton-capacity mesocosm tanks, we examined the fate of soluble fractions of discharged rank-A heavy oil, the chemical fate of toxic constituents, and their effects upon marine coastal organisms (OHWADA *et al.*, 2003; YAMADA *et al.*, 2003). It has been shown in the previous report that bacterial growth rates quickly accelerated in response to the oil contamination, and their abundance and growth rates appeared to vary with the growth of other trophic levels (TOYODA *et al.*, 2005). Although biomass fluctuation of microbes was disclosed in previous studies, little was known about the effect of oil contamination in seawater on the structure of microbial community. In our most recent survey on microbial community change, a Denaturing Gradient Gel Electrophoresis (DGGE: MUYZER *et al.*, 1993) and multidimensional scaling analysis has been undertaken (YOSHIDA *et al.*, 2006).

In this experiment, the microbial community change was simply deduced from the band numbers of 16S rDNA sequence types. The impact strength of oil pollution and other biological factors controlling microbial community structure were discussed.

2. Materials and Methods:

2.1 Mesocosm experiment:

Meso-scale experimental tanks were built on the seaside of Hamana-ko, a semi-enclosed bay located on the Pacific coast of central Japan. Details of the mesocosm facilities have previously been shown (OHWADA *et al.*, 2003). First, seawater was pumped from the surface water of Hamana-ko into the two reservoirs. Secondly, the reserved seawater was equally distributed to two experimental tanks. Tank tops were covered with transparent acrylic plates to let the sunlight in. Before conducting a series of experiments, it was confirmed that general environmental conditions among four experimental tanks did not show any remarkable differences. The present experiment was conducted from Oct. 26 to Nov. 6 in 2000. For the microbial experiments shown herein, water samples were collected periodically from days (0, 1, 2, 3, 4, 5, and 6). Water samples for genetic analysis were collected on Day 1, Day 3 and Day 6. The reservoir water of Day 0 was also taken for comparison. The experiment was further carried out for chemical analyses (data not shown).

2.2 Preparation of water-soluble fractions of heavy oil and operation of experimental tanks:

Rank-A heavy residual oil was obtained through the courtesy of Showa-Shell Co. Water-soluble fractions were prepared by mixing the oil vigorously at 800 rpm with a twenty-fold volume of seawater for two hours. The water-oil mixture was held stationary for an hour. Then thirty liters of water soluble fraction were poured into one of the experimental tanks, and then the tank water was stirred with the blades until the added oil became well mixed. We envisioned the mesocosm designed to simulate a slight oil pollution, which can be seen in a port facility and littoral industrial zone. Further information on the oil pollution level is contained in the previous report (OHWADA *et al.*, 2003). The other tank stayed uncontaminated to work as a negative control. This experiment was conducted, in the first place, with the aim of examining anti-proliferative effect of oil components. The experimental tanks were amended with KNO_3 and K_3PO_4 at 720mg/m^3 and 44mg/m^3 respectively so that nutrient deficiency could not be a growth limiting factor in surface water enclosed.

2.3 Determination of salinity, temperature, dissolved oxygen and chlorophyll *a:*

A portable STD system (YSI Model 610-DM) was used to obtain vertical profiles of salinity, temperature, and dissolved oxygen. Water was

sampled at a 50-cm depth and pumped into bottles with a handmade sampler assembled by stainless pipe and Tefron tubing. Fifty-milliliters of water were filtered through a glass fiber membrane (GF/F). Then the pigments were extracted in N, N-dimethylformamide (SUZUKI and ISHIMARU, 1990), and chlorophyll *a* concentrations were measured using a Turner-designed fluorometer.

2.4 Bacterial and viral counts:

Microscopic examination revealed bacterial and viral abundance in the water sampled at a 50-cm depth. Ten-milliliter samples were fixed with formalin at the final concentration of 2 % (vol/vol). Bacteria were stained with DAPI according to the method by PORTER and FEIG (1980). Then, they were concentrated on a 0.2 μm-pore black filter, and enumerated on epifluorescence microscopy (Nikon Eclipse E-800). Dividing cells were differentiated and enumerated in the same field of vision, thereby estimating frequency of dividing cells (FDC). Another ten milliliters were fixed with 2% of formalin for viral particle count. Viral particles were stained with SYBR Green-I, concentrated on a 0.02 μm-pore filter and enumerated under the Nikon microscope (NOBLE and FUHRMAN, 1998).

2.5 PAH analysis:

Water sampled at a 50-cm depth was filtered with a pre-baked glass fiber filter (GF/F, Whattman). Solid-phase extraction of oil components were performed by using a disposable column (SEP-PAK C18 plus environmental cartridge, Waters). Extracts were further purified and fractionated through an activated silica gel column and oil components were finally obtained. All the oil components were analyzed by means of gas chromatography and mass spectrometry (GC-MS) as has been previously reported (YAMADA *et al.*, 2003).

2.6 DNA extraction and Denaturing Gradient Gel Electrophoresis (DGGE):

One liter of water sampled at a 50-cm depth was pre-filtered with a 5.0 μm-pore polycarbonate filter and introduced into a cylindrical filter unit (Sterivex-GS : Millipore Corp.). Bacterial concentration and subsequent DNA extraction were performed by the method of SOMERVILLE *et al.* (1989) with modification of STEWARD and AZAM (1999). Two primers, GC341F and 907R were used for DGGE analysis (MUYZER *et al.*, 1995). Gene segments of bacterial 16SrDNA were amplified by using HS-taq polymerase (TAKARA Inc.) according to the supplier's instruction. A hot-start and touch-down PCR was performed as follows. In the first 19 cycles, the annealing temperature was initially set at 65℃ and decreased 0.5℃ every cycle. Then it was set at 55℃ for a further 9 cycles. Cycling was followed by 10 min of incubation at 72℃. PCR product concentrations were determined by means of fluorodensitometry, and 250ng of the product was loaded onto an each lane of the DGGE gel. The DGGE fingerprinting was carried out on the Dcode system (BIO-RAD Inc.) according to the manufacturer's instruction and previous reports (MUYZER *et al.* 1993; SCHAFER *et al.* 2000). Gel images were captured with a FLA 2000 and analyzed by using a Science lab 2001 (FUJIFILM Inc.).

3. Results and Discussion:

3.1 Environmental and microbial parameters

The temperature dropped from 21℃ to 19℃ due to a cooling of cold north winds during the 7-day experiment. Salinity was rather constant (ca. 30.5). Figure 1 summarizes changes of dissolved oxygen, chlorophyll *a*, PAHs, FDC, bacterial cells and viral particles. Dissolved oxygen in the oil tank gradually decreased up to 4mg/liter, whereas that of the control tank remained constant (Fig.1-A).

Oil components decreased quickly during the first two days. Changes of two major low molecular oil components, naphthalene and phenanthlene, are shown in Fig.1-B. Alkans decreased in the same way as naphthalene and phenanthrene. Hopane, a representative indecomposable component, decreased to 70% of the initial amount in the first day, and to 40% after five days (data not shown). The concentration of chlorophyll *a* in the oil tank rose dramatically and dropped after Day 3, while in the control tank showed a bimodal increase and reached a peak value of 5.7mg m^{-3} on Day 6 (Fig.1-C). Nutrient assays revealed that dissolved inorganic nitrogen, phosphate and

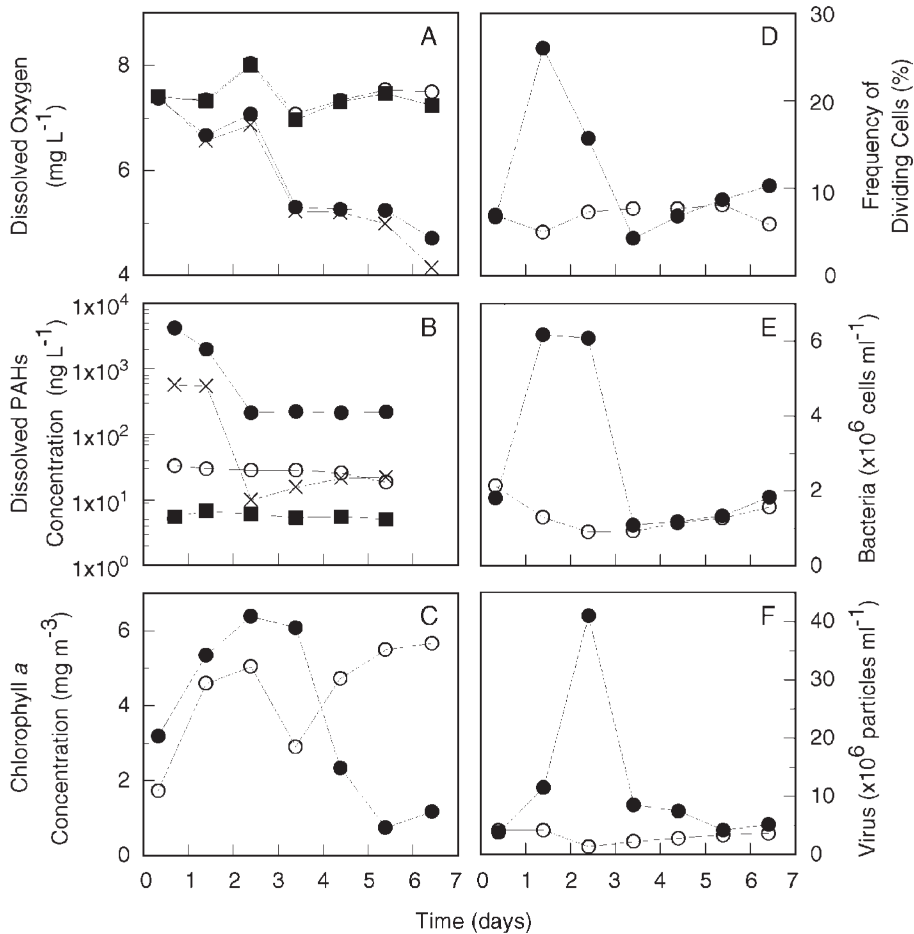

Fig.1 Time courses of environmental and microbial parameters in oil and control tanks
Panel-A: Dissolved oxygen measured at the surface and near the bottom of tanks
(○) control tank_ 0.5m deep, (●) oil tank_0.5m deep, (■) control tank_2.5m deep, (×) oil tank_2.5m deep.
Panel-B: Dissolved PAHs concentration in water samples collected at 0.5m
(○) control tank_ naphthalene, (■) control tank_phenanthrene, (●) oil tank_naphthalene,
(×) oil tank_phenanthrene.
Panel-C: Chlorophyll *a* concentration in water samples collected at 0.5m
(○) control tank_0.5m, (●) oil tank_0.5m.
Panel-D: FDC in water samples collected at 0.5m
(○) control tank_0.5m, (●) oil tank_0.5m.
Panel-E: Bacterial cells in water samples collected at 0.5m
(○) control tank_0.5m, (●) oil tank_0.5m.
Panel-F: Viral particles in water samples collected at 0.5m
(○) control tank_0.5m, (●) oil tank_0.5m.
Dotted lines represent the time of oil addition

silicate stayed high, over the lapse of the experiment (data not shown). This result implies that the algal fall had nothing to do with nutrient depletion. In the oil tank, there was a considerable increase in the number of bacterial cells and viral particles and the FDC percentage, while no remarkable changes were found in the control tank. Then, the number of bacterial cells and viral particles and the FDC percentage returned to the initial levels within

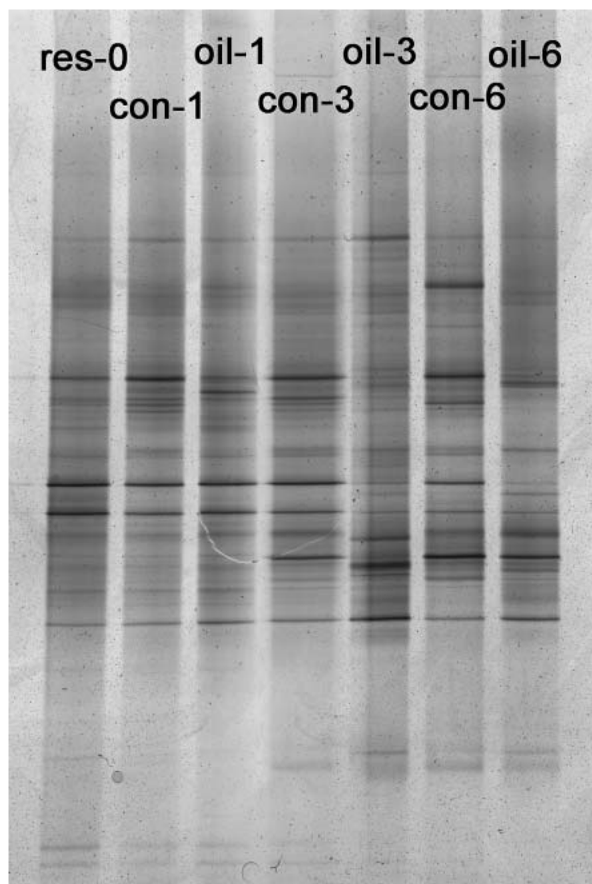

Fig.2 Gel image of DGGE analysis of 16S rDNA fragments amplified from water samples collected from oil and control tanks
res-0; reservoir of Day 0.
con-1; control of Day 1. oil-1; oil-contaminated of Day 1.
con-3; control of Day 3. oil-3; oil-contaminated of Day 3.
con-6; control of Day 6. oil-6; oil-contaminated of Day 6.

three days, respectively (Figs. 1-D, 1-E, 1-F). If we consider a rapid decrease in dissolved oxygen in the oil tank was caused by microbial activity during the decomposition of major oil components, a big difference of dissolved oxygen between the two tanks (Figs. 1-A, 1-D, 1-E) is reasonable. We also infer from the figures 1-B and 1-D that the oil components would have been utilized, as a carbon source for the bacterial growth. Viral particles proliferated one day later than bacterial growth in the oil tank (Fig.1-E, 1-F). It is difficult at the present time to determine whether those viruses were algae-infectors or bacteriophages, as we did not conduct culture experiments to confirm the host organisms.

3.2 DGGE analysis of microbial community structure during oil contamination

Figure 2 shows the band patterns of DGGE analysis. The three lanes from the left (res-0, con-1 and oil-1) show little difference in the band patterns. This suggests that the microbial community structure in experimental tanks did not substantially change during the 24 hours after the introduction of oil components. Although a drastic increase of bacterial cells took place up to Day 1 (Fig. 1), a major change of the microbial community appeared after a delay of a few days (Fig. 2; con-3 and

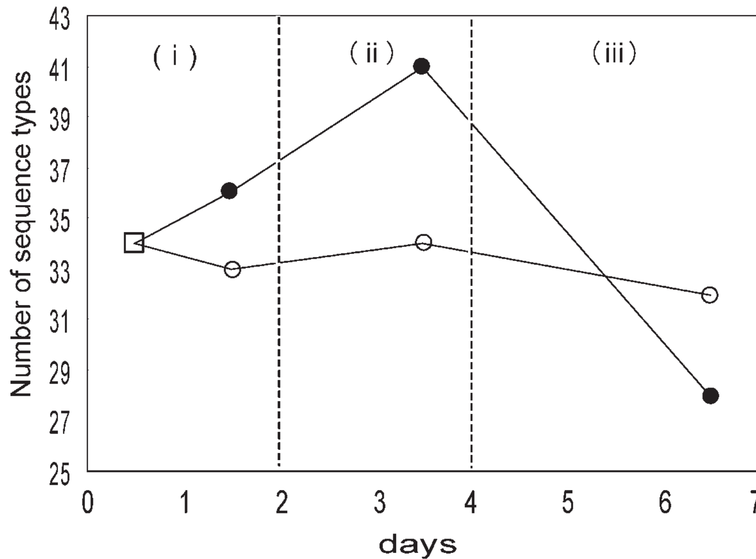

Fig.3 Changes of the number of sequence types on the basis of DGGE analysis shown in Fig.2
(○) control tank, (●) oil tank. (□) Day-0 reservoir tank.
Microbial change of each phase(i , ii , iii) are mentioned in the last paragraph of the text.

oil-3). This phenomenon was observed again in the following experiment conducted in 2001 (YOSHIDA *et al.*, 2006). The fact that the increase of bacterial cell number was not coincident with the change of the DGGE band patterns suggests that the fast-growing oil degraders were composed of a few limited numbers of species.

Figure 3 shows the number of bands visible in each sample lane of the DGGE gel. The band number in the oil tank reached up to 41 on Day 3, and decreased to 28 at the end of the experiment, while in the control tank, it ranged from 34 at the start, and to 32 at the end. This implies that the growth rate of other groups of bacteria in the oil tank was also accelerated during the 2nd and 3rd days, and several species of bacteria present in the tank disappeared after Day 3.

In conclusion, it seems reasonable to consider that the fluctuation of oil-exposed microbial community was made up of three steps as follows: (i) Bacteria proliferated and increased in number quickly within a day with a slight increase of 16S rDNA sequence types. (ii) After a few days of the experiment, bacterial numbers returned to the initial level, but the sequence types increased to the maximum. (iii) Then bacterial numbers stayed put with the decline of sequence types.

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