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Effectiveness of Bioremediation on Oil-Contaminated Sand in Intertidal Zone

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Abstract Bioremediation technologies were applied to experimental microcosms, simulating an oil spill in a lower intertidal area. Three treatments (oil only, oil plus nutrients, and oil plus nutrients and microbial inocula) were applied, and each microcosm was repeatedly filled and eluted with seawater every 12 h to simulate tidal cycles. To minimize washing-out of the inoculum by the tidal cycles, microbial cells were primarily immobilized on diatomaceous earth before they were applied to the oiled sand. Oil degradation was monitored by gravimetric measurements, thin layer chromatography/flame ionization detector (TLC/FID) analysis, and gas chromatography (GC) analysis, and the loss of oil content was normalized to sand mass or nor-hopane. When the data were normalized to sand mass, no consistent differences were detected between nutrient-amended and nutrient/inoculum-amended microcosms, although both differed from the oil-only microcosm in respect of oil removal rate by a factor of 4 to 14. However, the data relative to nor-hopane showed a significant treatment difference between the nutrient-amended and nutrient/inoculum-treated microcosms, especially in the early phase of the treatment. The accelerating effect of inoculum treatment has hardly been reported in studies of oil bioremediation in the lower intertidal area. The inoculum immobilized on diatomaceous earth seemed to be a very effective formulation for retaining microbial cells in association with the sand. Results of this study also suggest that interpretation of the effectiveness of bioremediation could be dependent on the selection of monitoring methods, and consequently the application of various analytical methods in combination could be a solution to overcome the limitations of oil bioremediation monitoring.

Key words: Bioremediation, crude oil, slow release fertilizer, intertidal zone, nor-hopane

When oil spills reach a coastline, intertidal regions receive most of the oil. The fate of oil stranded in the intertidal regions is determined by a variety of biotic and abiotic factors. Whereas biodegradation represents a natural process whereby microorganisms alter and break down organic molecules into other substances, eventually producing fatty acids and carbon dioxide, bioremediation has emerged as a promising restoration technology for oil-contaminated shorelines, especially in sensitive environments, as well as remote locations where access is difficult and costly. Bioremediation is the most environmentally friendly countermeasure against oil spills. However, it should not be used indiscriminately, in isolation from other procedures, or without adequate knowledge of the environment in which it is to be employed. Therefore, successful bioremediation programs require application methodologies specifically tailored to the environmental parameters at each contaminated site.

During field bioremediation studies, the observed losses in bulk total hydrocarbons may not be the result of biodegradation processes, but rather are due to dilution, leaching, or volatilization. Dilution may occur when heterogeneous oily soils are tilled so that clumps of soil with higher oil content are mixed with less oily soil. Leaching may occur during irrigation or heavy rainfall. Volatilization of lighter hydrocarbons may occur during tilling or warm summer months. Accurately quantifying biodegradation losses in the field is also often complicated by the spatial variability of hydrocarbons at spill sites. Therefore, chemical analysis plays a crucial role in evaluating the fate of oil in the contaminated intertidal environments. To predict biodegradation in the field, the compositional changes have been compared in oils [16] or oil content loss compared to conserved internal biomarkers such as nor-hopane [17α(H),21β(H)-30-norhopane], pristane and phytane [1, 9, 20, 25]. Among the biomarkers, nor-hopane has been used as the most prominent biomarker compound in the oil due to its resistance to biodegradation [21], presence in all crude oil, and its sufficient concentration (440 μg/g-oil) in oil [20].

A microcosm study was conducted to evaluate oil biodegradation in oil-contaminated sand augmented with

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oil-degrading microorganisms and/or inorganic nutrients. Oil biodegradation was assessed by gravimetric measurement, TLC/FID (thin layer chromatography/flame ionization detector), and GC/FID (gas chromatography/flame ionization detector) using nor-hopane as a biomarker, and the results were compared to find the best way of evaluating the effectiveness of bioremediation.

MATERIALS AND METHODS

Experimental Design

Polyethylene boxes (19 cm×24 cm×13 cm) were used as microcosms. Commercially available sea sand was purchased and sieved prior to use. The sand was homogeneously mixed with Arabian light crude oil (3%, v/v), which consisted of 30% of aliphatics, 55% of aromatics, and 15% of asphalts on weight basis. Three treatment strategies were tested on the oiled sand: oil-only control (microcosm I), addition of inorganic nutrients (microcosm II), and addition of inorganic nutrients and oil-degrading microorganisms (microcosm III). As a source of inorganic nutrients, a slow-released fertilizer (SRF, Chobi Ltd., Ulsan, Korea) was added to the sand to make a C:N:P ratio of 100:10:3 [3, 18]. In microcosm III, the sand was then homogeneously mixed with the following petroleum-degrading microorganisms; *Corynebacterium variabilis* IC 10 [13], *Yarrowia lipolytica* CL180 [10, 11], and *Sphingomonas* sp. KH3-2 [23], at respective concentrations of 1.0×10^6 cells/cm^3-sand. To simulate intertidal conditions, the oiled sand was treated with artificial tidal cycles. During the 12-h high-tide period, each microcosm received 250 ml of sea water, which was sufficient to submerge all sand materials. For low-tide conditions, the excess water was removed from the microcosm by gravity through a port positioned at the bottom of each microcosm. Each volume of tidal water was saved to determine the amount of oil residue physically removed from the microcosms.

Preparation of Microorganisms

A mixture of three oil-degrading microorganisms, *Yarrowia lipolytica* CL180, *Corynebacterium variabilis* IC 10, and *Sphingomonas* sp. KH3-2, was used as the inoculum. Each strain was grown in ZoBell 2216e liquid medium at 25°C for 3 days. The cells were centrifuged at 10,000 ×g for 15 min, and washed three times with sterilized water (aged sea water:deionized water, 1:1). The cell pellets were mixed with diatomaceous earth (95% SiO_2, Sigma,) and added to the oil-contaminated sand to make the required cell density.

Physical Factor Analyses

The temperature change in the microcosms was measured using a maximum-minimum monitoring thermometer. The water content was measured by weighing 1 cm^3 of the sand before and after drying overnight at 80°C. Soil particle size distribution was determined by the Pipette method using an automatic particle size analyzer (Sedograph 5100D, Micromeritics, U.S.A.). The content of gravel, sand, and silt in the oiled sand was 1.85%, 96.85%, and 1.31%, respectively.

Microbiological Analyses

One cm^3 of sand sample was added to 20 ml of deionized water and blended in a blender mixer (Waring Co.) at high speed for two 30-sec periods, with 30 sec of cooling in ice between each blending cycle. One ml of the sample was serially diluted and inoculated onto ZoBell 2216e agar medium for the counting of heterotrophic microorganisms (HM), which took place after 1 week of incubation at 25°C. The number of petroleum-degrading microorganisms (PDM) was determined by the Sheen Screen most probable number (MPN) method [2]. The procedure calls for inoculation of five 200 µl aliquots of each diluted sample into sterile 24-well microtiter plates containing approximately 1.8 ml of sterile Bushnell-Hass marine mineral salts broth (Difco Laboratories) per well. Following inoculation, a sheen of sterile Arabian light crude oil (about 20 µl) was applied to each well. Microtiter plates were incubated at 25°C for 2 weeks following inoculation. Wells were scored as positive when oil emulsification was clearly indicated by disruption of the oil sheen.

Microbial Activity

To measure the general activities of a large part of the microbial community, ETS (electron transport system) activity was measured [28]. A 0.1 cm^3 portion of the microcosm sand was incubated with 250 µl of 0.4 M succinic acid, 100 µl of 0.88 mM NADH, 100 µl of 0.25 mM NADPH, and 500 µl of 0.2% (w/v) INT [2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride]. The reaction mixture was incubated for 1 h *in situ*, and then the amount of INT-formazan was determined [26].

Analysis of Residual Oil

The amount of residual oil was analyzed using GC/FID and TLC/FID. Ten g of freeze-dried sand were mixed with 3 g of anhydrous sodium sulfate, 5 mg of copper, and stearyl alcohol (10% of added crude oil, Sigma) and squalene (1% of added crude oil, Sigma) as internal standards for TLC/FID and GC analysis, respectively.

After 4 extractions of the samples with 20 ml of chloroform, the extracts were combined and concentrated to 1 ml using a rotary evaporator (Eyela, Japan). The combined extract was directly used to analyze the residual hydrocarbons by gravimetric measurement and TLC/FID analysis, as described by Goto *et al.* [5] using a latroscan MK-5 (Latron Lab., Inc., Japan). For GC analysis, the concentrated extract was

introduced into a silica-alumina column [29] for separation of the aliphatic hydrocarbon fraction. The aliphatic hydrocarbons were analyzed using a gas chromatograph (HP 5890 II, Hewlett Packard) equipped with a FID and a fused silica capillary column (30 m×0.32 mm×0.25 μm, SPBTM-1, Supelco Inc.). The analytical conditions were: initial temperature, 100°C for 3 min; rate, 3°C/min; final temperature, 320°C; final time, 2 min; detector, 340°C; injector, 340°C; carrier gas (N_2), 40 ml/min.

Use of Nor-Hopane as a Conserved Internal Marker

Due to its resistance to biodegradation, nor-hopane, a multi-ring-saturated hydrocarbon, has been used as a good conserved internal standard in crude oil. To estimate the loss of hydrocarbons via biodegradation, the ratios of total GC-detectable hydrocarbons to nor-hopane (TGCDHC/nor-hopane), total resolvable hydrocarbons (nC_{11}-nC_{38}) to nor-hopane (TRHC/nor-hopane), and TLC/FID-detectable hydrocarbons to nor-hopane (TLC/FID/nor-hopane) were determined.

RESULTS AND DISCUSSION

Microbiological Analyses

Air temperature of the test area varied from 0 to 28°C. Although the air temperature was around 10°C during the first 35 days, oil degradation did not show a temperature dependency.

Fluctuations of microbial population density and their activities in the oiled sands are shown in Fig. 1. There were significant differences between the untreated microcosm (microcosm I) and the treated ones (microcosm II and III) in terms of the numbers of microorganisms and microbial activity. However, little differences in the microbial numbers and activity were observed between microcosm II and microcosm III.

Effect of Nutrient Amendment on Biodegradation

The efficiency of bioremediation in oil-spilled intertidal areas depends on the environmental conditions of the area. In particular, there are significant differences in the efficiencies of bioremediation between the upper and lower intertidal areas. The upper area is least submerged and, therefore, is likely to be exposed to higher aerobic conditions for a longer time period. It is also less subject to the churning action of breaking waves, which occurs more in the lower intertidal area. Thus, added nutrients or microorganisms added would persist longer in the higher intertidal zone and would be more available for continued bioremediation [19, 27].

Nutrients that are particularly required in the field application of bioremediation are nitrogen and phosphorus, whereas minor trace elements are usually present in sufficient

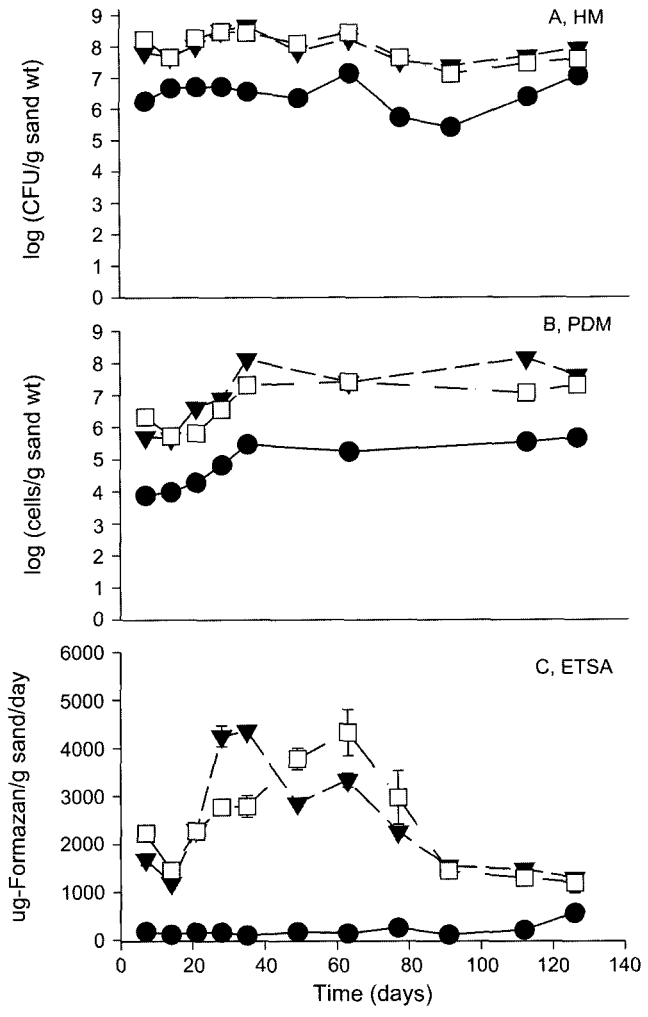

Fig. 1. Fluctuation of heterotrophic microorganism (A, HM), petroleum-degrading microorganism (B, PDM), and dehydrogenase activity (C, ETSA) in microcosms.
●, Microcosm I; ▼, microcosm II; □, microcosm III.

amounts in the natural environment [12]. It appeared logical, therefore, that addition of nitrogen and phosphorus to oil-contaminated sands might possibly enhance *in situ* oil biodegradation. SRF, which was used as the source of nitrogen and phosphorus in this study, was already known to continuously release inorganic nutrients at a relatively constant level for a long period [19]. The addition of SRF greatly increased the number of heterotrophic and petroleum-degrading microorganisms, and stimulated ETS activity (microcosms II and III in Fig. 1). The decrease in the extent of residual oil also clearly shows the stimulation of oil degradation by the addition of SRF. In the gravimetric measurement of residual oil, biodegradation efficiencies after 126 days in microcosms I, II, and III were 12.6% (±0.1), 26.1% (±5.9), and 24.4% (±6.7), respectively (data not shown). Oil degradation rates obtained by TLC/FID (Fig. 2) clearly show that degradation rates of aliphatic and

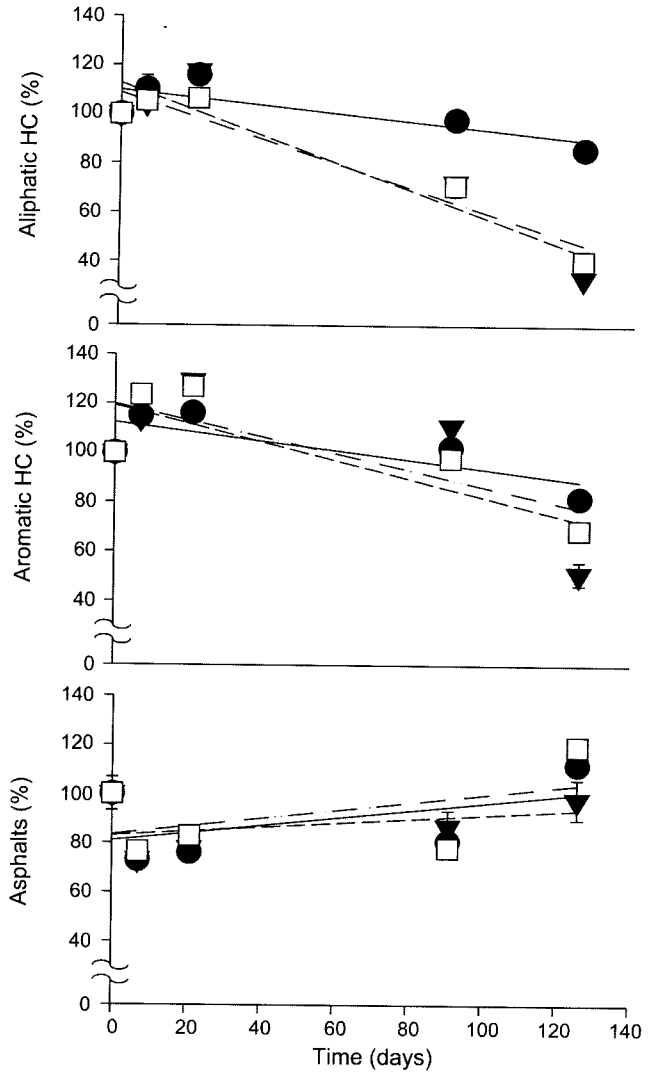

Fig. 2. Fluctuation of residual crude oil recovered from polluted sand analyzed by TLC/FID.
●, Microcosm I; ▼, microcosm II; □, microcosm III.

aromatic hydrocarbons were significantly higher in the SRF-treated sands (microcosms II and III) than in the oil-only control (microcosm I). After 126 days, the biodegradation efficiencies of aliphatic and aromatic hydrocarbons in microcosm II were 66.5% (±3.8) and 48.7% (±4.8), respectively, and the degradation efficiencies in microcosm III were 59.5% (±4.2) and 31.3% (±1.32), respectively. The amount of asphalts slightly increased during the experiment, indicating that polar organic compounds resulting from biotransformation of hydrocarbons had accumulated. Therefore, increase of the amount of asphalts could often serve as a measure of the extent of biodegradation [1, 18, 20]. The effects of nutrients were also clearly shown in the results obtained by GC/FID analysis of aliphatic hydrocarbons, where *n*-alkanes (nC_{13}-nC_{38}) were rapidly degraded to a level below detection within 126 days in SRF-treated microcosms, whereas they were poorly degraded in microcosm I (data not shown). These results implied that nutrient availability was one of the major factors influencing biodegradation of the oil in the sand.

Effect of Inoculation on Biodegradation

In addition to inorganic nutrients, the inoculation of oiled sands with hydrocarbon-degrading microorganisms has also been proposed as a bioremediation approach to improve hydrocarbon degradation, by demonstrating that, under certain conditions, inoculation accelerated the rate and extent of oil biodegradation [6, 7, 8, 12]. However, inoculation of oiled sand with high numbers of hydrocarbon-degrading

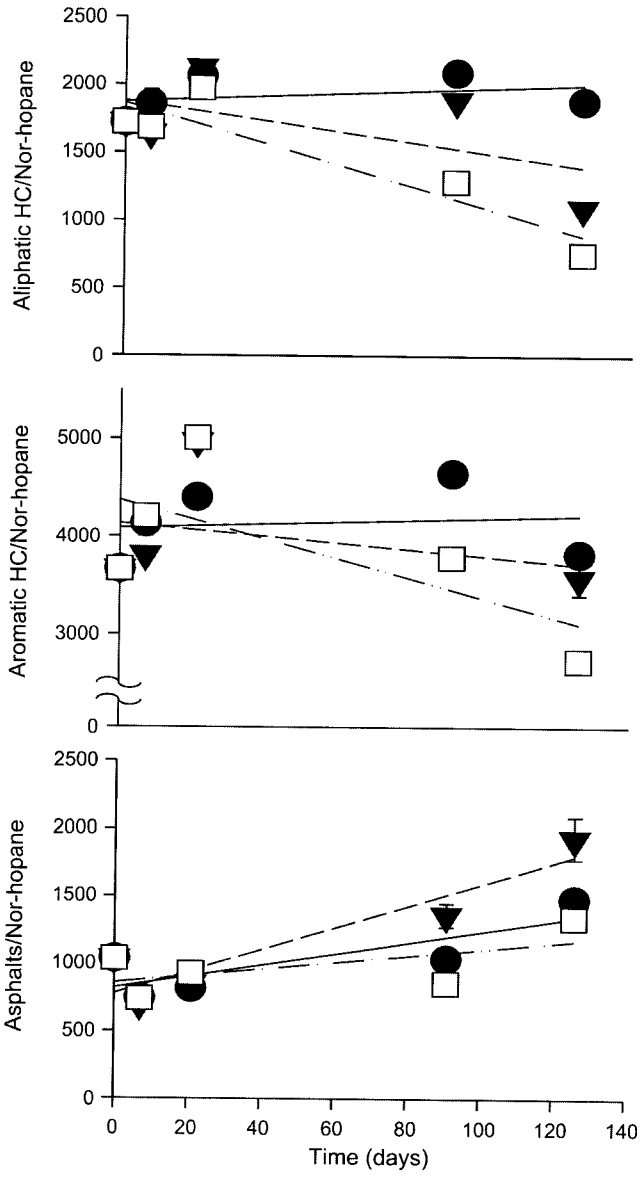

Fig. 3. Fluctuation of residual crude oil recovered from polluted sand analyzed by TLC/FID/nor-hopane.
●, Microcosm I; ▼, microcosm II; □, microcosm III.

microorganisms in intertidal zones commonly resulted in little effect on oil biodegradation, probably due to the washing-out of cells by tidal water [4, 17, 27]. In this study, the inoculum was immobilized on diatomaceous earth, and then added to the sand to minimize the washing-out effect. The hydrocarbon losses, measured by gravimetric or TLC/FID analyses (Fig. 2), did not show any significant difference between microcosms II and III. However, monitoring of hydrocarbon losses relative to nor-hopane clearly confirmed the treatment difference between microcosms II and III (Fig. 3). It is shown that inoculation accelerated the rate of hydrocarbon degradation. The inoculum also seemed to be effective in degradation of the transient polar intermediates, which were maintained at a lower level in microcosm III than in microcosms I and II (Fig. 3C). When hydrocarbons were analyzed by GC/FID, the loss of *n*-alkane showed most clearly the accelerating effect of inoculation on oil biodegradation (Fig. 4). Concerning the biodegradation of resolved *n*-alkanes, Fig. 4 confirms the already known fact that the most common form of degradation is of short chain *n*-alkanes (nC_{17}-nC_{28}) rather than long chain *n*-alkanes (nC_{29}-nC_{38}), as reported by Setti *et al.* [24]. Degradation of *n*-alkanes with chain lengths of 17 to 28 was significantly faster in microcosm III than in microcosm II. Although there was no big difference in the extent of degradation of aliphatic hydrocarbons between microcosms II and III at the end of the experiment, the initial degradation rate (days 0–21) in microcosm III was higher than in microcosm II. When the initial degradation rates of *n*-alkane (C_{17-28}) were compared between the control and the treated microcosms, the rate ratio of microcosm I:II:III was 1:4:12. These results indicate that consideration of only either the presence of fertilizer or the presence of inoculum is not adequate for the oiled sand and both substances must be considered in a bioremediation strategy.

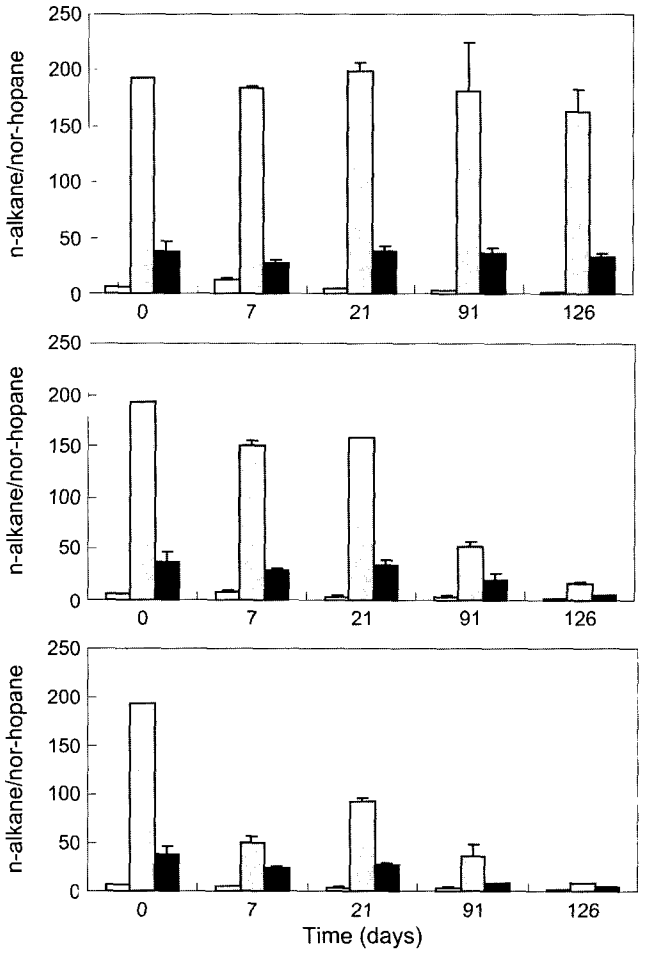

Fig. 4. Changes in the composition of resolved *n*-alkanes, measured with GC/FID, with respect to nor-hopane.
A, Microcosm I; B, microcosm II; C, microcosm III. □ for C_{13-16}; ▨ for C_{17-28}; ■ for C_{29-38}.

Release of Oil Constituents to the Tidal Waters

The total amount of hydrocarbon in the collected tidal water was analyzed using TLC/FID (Fig. 5). Although the actual release of oil during the tidal cycling in microcosm I was little, release of oil to the tidal water started to be detected after 2 months in both microcosms II and III, probably due to the production of polar intermediates and emulsifying agents. However, the release of oil reached a plateau after 4 months in all microcosms. The total amounts of oil released from microcosms I, II, and III during 5 months were 0.1%, 2.9%, and 1.3% of the oil in the sand, respectively. These results indicated that biological treatment might increase the mobility of contaminated oil in the sand. To date, detrimental effects from bioremediation by nutrient enrichment have not been observed in actual field operations [15, 21]. However, for safety assurance,

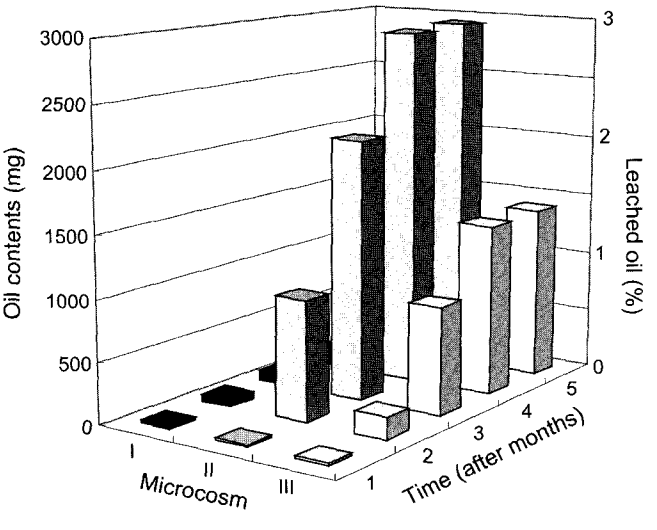

Fig. 5. Cumulative amount of residual crude oil released in tidal water from each microcosm.

the release of oil to tidal water in intertidal environments should be seriously considered in regard to ecotoxicity, and a bioremediation strategy that allows only a minimal possibility of release should be selected.

Significance of Nor-Hopane as an Internal Biomarker

The importance of nor-hopane or hopane as an internal biomarker to confirm oil biodegradation has been proved in several studies [1, 11, 27]. However, the use of these biomarkers has been only used for oil content data obtained by GC analysis. In the present study, it was found that the amount of nor-hopane determined by GC/FID analysis had consistent correlationship with the amount of hydrocarbon fractions measured by TLC/FID (Fig. 6). Based on this result, nor-hopane was applied as an internal biomarker to interpret oil content data obtained by TLC/FID analysis. When oil content data were normalized to sand mass, oil losses by various physical and chemical processes or heterogeneous distribution of oil in the sand resulted in significant oil removal in the oil-only control. However, this was a misinterpretation which could be rectified by normalizing the oil content data to nor-hopane in the TLC/FID analysis (Figs. 2 and 3). Furthermore, monitoring oil chemistry relative to nor-hopane provided a key to determining the significant effects of SRF or inoculum addition on oil biodegradation. The GC/FID data normalized to nor-hopane also showed results similar to TLC/FID data which were normalized to nor-hopane (Fig. 4). The TLC/FID method, which separates hydrocarbons based on characteristic chemical types such as aliphatics, aromatics, and polar compounds, has been widely applied for the routine measurements of crude oil due to its simplicity and good reproducibility [5]. The TLC/FID method has advantages in analyzing high-boiling-point hydrocarbons, including higher molecular weight saturated hydrocarbons, aromatics, resin, and asphaltene, which are not detectable by GC or HPLC analyses. Therefore, the application of nor-hopane in TLC/FID data is suggested as an alternative method to confirm oil biodegradation, and can save time and labor, which are major problems in GC analysis.

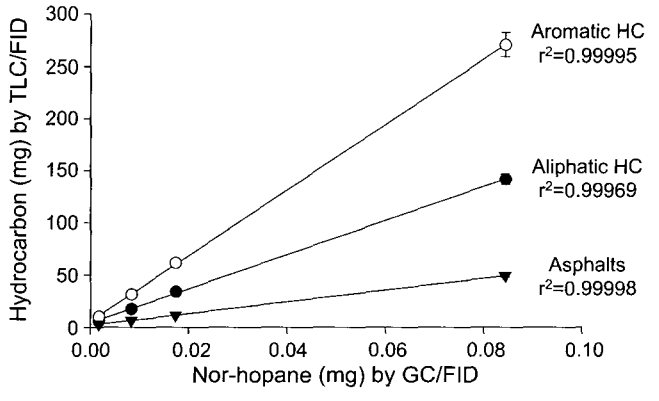

Fig. 6. The relationship between the content of hydrocarbon analyzed by TLC/FID and the content of nor-hopane analyzed by GC/FID.

In conclusion, microbiological monitoring and oil recovery data suggest that the application of microbial inoculum and/or nutrients to oil-contaminated intertidal sand was very beneficial. In lower intertidal environments, SRF and inoculum immobilized on diatomaceous earth seemed to be very effective formulations for the continuous release of nutrients and for remaining in association with the sand, respectively. Results of this study also emphasize the importance of choice of monitoring program. As all bioremediation strategies have some limitations due to lack of defined monitoring standards, interpretation of the effectiveness of bioremediation could depend on the selection of the monitoring program. To overcome such limitations, more reliable monitoring programs, such as application of various combined analytical methods, or normalization of data to conserved markers, should be implemented to validate the effectiveness of bioremediation. When this was accomplished, a site-specific bioremediation strategy with higher efficacy, reliability, and predictability can be established.

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