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# Evaluation of Fertilizer Additions to Stimulate Oil Biodegradation in Sand Seashore Mesocosms

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**Abstract** Effects of fertilizer additions for oil degradation were examined in sand seashore mesocosms. Within 37 days, up to 85% removal was achieved by the addition of slow-release type fertilizer (SRF) with the initial degradation rate of 423.3 mg oil (kg sand)$^{-1}$ day$^{-1}$. The removal was mostly of biological origin based on the changes of $C_{17}$/pristane and $C_{18}$/phytane ratios from 2.60 to 0.81 and from 3.55 to 1.29, respectively. The addition of oleophilic fertilizer (Inipol EAP22) was less effective and resulted in the removal of 64% of the added oil (3%, v/v) with a lower initial degradation rate. Petroleum-degrading bacteria had achieved a value of $1\times10^8$ CFU (g sand)$^{-1}$ at Day 3 and this peak exactly coincided with the initial degradation in the SRF-treated mesocosm. In this mesocosm, surface tension values were decreased drastically during Days 3 and 8, suggesting that microbially-produced surface-active agents actively enhanced the oil degradation rate and cell proliferation. Although the Inipol-treated mesocosm appeared to show significantly enhanced oil degradation compared to that of the untreated control mesocosm, Inipol was found to be less effective than SRF in enhancing a true oil-degrader when compared under similar experimental conditions.

**Key words:** Degradation, oil, oleophilic fertilizer, slow-release fertilizer, surface tension

Several major oil spills have focused attention on the problem of hydrocarbon contamination in marine and estuarine environments and the potential use of bioremediation through nutrient addition to remove petroleum pollutants. The availability of nitrogen and phosphorus is critical for microorganisms to degrade hydrocarbons, where nutrient deficiencies limit the rate of petroleum degradation. An oleophilic nitrogen and phosphorus fertilizer, originally developed by Atlas and Bartha [3], and commercialized as Inipol EAP22, stimulated oil biodegradation on cobble beaches in Alaska after the Exxon Valdez incident [5, 7, 23]. However, failures of Inipol EAP22 to stimulate oil biodegradation have been reported [14, 24, 25]. As an alternative, nutrient enrichment with agricultural slow-release fertilizer was found to be an effective countermeasure [11, 15]. Nevertheless, the addition of slow-release fertilizer does not always show a significant enhancement of oil degradation [26]. Therefore, it is necessary to analyze this discrepancy by comparing the efficacy of both oleophilic and slow-release type fertilizers on the degradation of oil under controlled experimental conditions.

The aim of the present research was to compare the effectiveness of a slow-release inorganic fertilizer with Inipol EAP22 in stimulating the biodegradation of Arabian light crude oil on an out-door sand seashore.

## Materials and Methods

### Mesocosm Design

The experimental site was the reclaimed area at Sihwa, on the west coast of Korea. Three mesocosms (I, II, and III) were set up on the seashore sand by using bottomless plastic buckets having 360 mm diameter and 250 mm height. To the surface of each mesocosm, 5,000 cm$^3$ sieved sand contaminated with 150 ml of Arabian light crude oil was added so that the final concentration of oil in the surface layer was 3% (v/v). In addition to the mixture of sand and crude oil, mesocosm II was amended with 16 ml (recommended amount by manufacturer) of oleophilic fertilizer Inipol EAP22 (Elf Aquitaine, France). The N/P ratio of Inipol EAP22 is 2.6:1. Mesocosm III was prepared in the same manner except that both a mixed type and a urea type of slow-release fertilizer (SRF; Chosun Fertilizer Co., Korea), 240 g and 170 g, respectively, were added instead of oleophilic fertilizer. The mixed type SRF contained

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N:P:K as 10:11:12 by weight, whereas the urea type comprised only urea (ca. 42%). These SRFs also contained clay mineral as a filler, and Si, Latex as a coating material, as previously described [22]. The amount of SRF added corresponded to 100:10:1 ratio of C:N:P. Mesocosm I was not amended with any fertilizer and served as a control. At days 0, 3, 8, 16, and 37, composite samples were obtained by combining at least 10 grab samples from surface layers of each mesocosms and transported to the laboratory while maintaining at 4°C. After the sample collection, 1:3 (v/v) mixture of aged seawater and distilled water was sprayed on the sand surface of each mesocosm and these were then carefully tilled so as not to mix the surface layer with subsurface layer.

To estimate the concentrations of inorganic nutrients, one $cm^3$ of surface sand sample was mixed well with 50 ml of Milli-Q water and then centrifuged to retrieve pore water. The resulting supernatant was passed through a membrane filter (pore size 0.45 μm, diameter 47 mm) and the filtrate was used for quantitative measurements of $NH_4^+$-N, $NO_2^-$-N, $NO_3^-$-N, and $PO_4^{3-}$-P [1].

After the elimination of oil and other debris including sand particles and microorganisms, surface tension was measured with Tensiomat (Fisher Scientific) according to the method of Mulligan *et al.* [17].

### Oil Degradation Analyses

For the analysis of residual oil, 1 $cm^3$ surface sand samples from each mesocosm were extracted 3 times with 20 ml each of chloroform after the addition of squalene (Sigma Chemical Co., U.S.A.) to normalize the value according to the extraction efficiencies. After extraction, crude oil hydrocarbons were analyzed with a gas chromatograph (GC; HP5890II, Hewlett Packard) equipped with a flame ionization detector and a capillary column (30 m×0.32 mm× 0.25 μm, Ultra-1, Hewlett Packard Co., U.S.A.). The oven temperature was at 100°C for 3 min and then raised to 280°C at 8°C $min^{-1}$ and held at 280°C for 7.5 min. Both injector and detector were at 280°C. $N_2$ was used as carrier gas at 45 ml $min^{-1}$.

As a specific indicator of microbiological degradation of oil hydrocarbons, $C_{17}$/pristane and $C_{18}$/phytane ratios were obtained, as suggested by Atlas [2]. The assessment is based on the difference in degradation rates between linear alkanes having 17 or 18 carbon atoms and highly branched alkanes of similar molecular weights, such as pristine and phytane.

### Microbiological Analyses

Sand samples (1 $cm^3$) from each mesocosm were vortex mixed with 10 ml of distilled water for 5 min to separate microorganisms from sand particles. Serially diluted samples were inoculated either on marine agar medium (Difco) or on oil agar medium for estimating heterotrophic plate counts and petroleum-degrading bacteria, respectively. The oil-agar medium was mineral salts medium ($NH_4Cl$ 0.6 g, $K_2HPO_4$ 0.26 g, agar 15 g, aged seawater 750 ml, distilled water 250 ml) supplemented with 0.2% (v/v) Arabian light crude oil, and counted after 2 weeks of incubation at 25°C.

All data including microbial population size, inorganic nutrients, and residual oil analyses were obtained with triplicated analyses and are presented as based on dry weight of sand.

## RESULTS AND DISCUSSION

### Oil Degradation

The effects of fertilizer additions were evident, when the duration of acclimation periods for oil degradation were compared among the mesocosms (Fig. 1). There was no significant oil disappearance until Day 16 in microcosm I, but the contaminating oil declined rapidly after Day 3 in mesocosm II, to which oleophilic fertilizer was added. There was no observable acclimation period in slow-release fertilizer (SRF)-supplemented mesocosm III. When hydrocarbon contaminates environments, the major factor that limits its degradation is usually inorganic nitrogen and phosphorus since the contaminant itself provides sufficient carbon and energy [21]. A rather rapid oil degradation in both microcosms II and III indicated that the acclimation period was indeed due to the limiting inorganic nutrients and not to a toxic effect of oil contaminants on the existing microorganisms. Nevertheless, inorganic ammonia released from oleophilic fertilizer via urea or directly from soluble fertilizer solution is regarded to be mildly toxic to sensitive marine organisms [27]. A relatively low and constant level

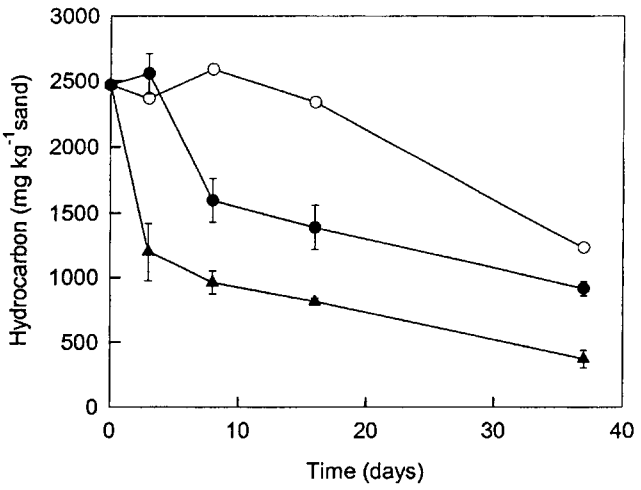


**Fig. 1.** Degradation of hydrocarbon with various treatments during 37 days of mesocosm experiments.
○: no fertilizer control; ●: oleophilic fertilizer; ▲: slow-release fertilizer. Data were obtained with triplicated samples from each mesocosm and were presented by dry weight of sand basis. Error bars represent 1SD.

of ammonia released by the addition of SRF can minimize such unwanted adverse environmental effect, including eutrophication.

After 37 days, the concentration of GC detectable hydrocarbon remaining in mesocosm III was 370 mg (kg sand)$^{-1}$ which corresponded to only 15% of the added amount (Fig. 1). The addition of oleophilic fertilizer (mesocosm II) was less effective, resulting in the degradation of 64% of the added oil. Control mesocosm I, which received no N or P, showed only 50% degradation. The initial degradation rates (mg oil (kg sand)$^{-1}$ day$^{-1}$) which could be calculated from the initial slope of degradation after the onset of degradation were in the order of mesocosm III (423.3)>II (192.2)>I (31.4). These results together with the comparison of the acclimation period suggested that the addition of fertilizer not only increased the extent of degradation but also enhanced the initial oil degradation by 6–13 fold. In addition, slow-release type fertilizer was clearly shown to be more effective than oleophilic fertilizer. Under identical experimental conditions on the sand shoreline, nutrient addition stimulated oil degradation and 40% of the initial amount was recovered after 60 days of incubation [12]. Sveum and Ladousse [25] also reported that the addition of oleophilic fertilizer reduced the oil concentration to less than 10% within 1 year. During our field test, air temperature (15–30°C), pH (8.0–8.5), and other environmental factors seemed to be within the generally accepted favorable range for oil degradation [4]. The results indicated that SRF enhanced the oil degradation rates, especially at the early phase of degradation.

In order to monitor the biological removal of contaminated oil, changes of $C_{17}$/pristane and $C_{18}$/phytane ratios were estimated at Day 0 and Day 16 (Table 1). A slight decrease of the ratios in both mesocosms I and II suggested that the oil removal was mainly by physicochemical phenomena such as photooxidation and evaporation. However, residual oil analysis in mesocosm II showed that more than half of the added oil disappeared within 16 days, whereas the control mesocosm I showed only a marginal decrease (Fig. 1). Therefore, the relatively less dramatic decrease of pristane/phytane ratios than residual oil analysis in mesocosm II is puzzling. A similar discrepancy between oil degradation and pristane/phytane ratios has been observed previously [16]. Either the addition of oleic acid somehow affected the bacterial degradation of $C_{17}/C_{18}$ aliphatic hydrocarbons, or simply the ratios were not suitable as specific indicators of biological oil degradation. Microbial degradation of pristane/phytane is indeed possible and relevant data have been published [6, 18, 19]. Nevertheless, it is clear that the addition of SRF in mesocosm III greatly promoted oil disappearance by enhancing biological activities. With the addition of Inipol EAP22, Ladousse and Tramier [12] reported that $C_{17}$/pristane and $C_{18}$/phytane ratios decreased from 2.2 and 1.7 at the beginning to 1.1 and 1.4 after 42 days, respectively. These results were comparable with our present results of $C_{17}$/pristane and $C_{18}$/phytane ratios, decreasing from 2.60 and 3.55 to 1.93 and 3.02, respectively.

**Table 1.** Values of specific indicators during the biodegradation of Arabian light crude oil in mesocosms after 16-day experiment.

| Mesocosms | Treatments | $C_{17}$/Pristane[a] | $C_{18}$/Phytane[b] |
|---|---|---|---|
| I | oil[c] only | 2.26 | 3.18 |
| II | oil+oleophilic fertilizer | 1.93 | 3.02 |
| III | oil+slow-release fertilizer | 0.81 | 1.29 |

[a,b]Values of $C_{17}$/pristane and $C_{18}$/phytane at Day 0 were 2.60 and 3.55, respectively.

[c]Oil was added at the concentration of 3% (v/v).

The optimal N/P ratio for oil degradation has been known to be 3–10. The ratios actually applied, as recommended by the manufacturer, were 2.6 and 10 for Inipol and SRF, respectively. Although the relative amount of phosphorus was higher in Inipol fertilizer, the actual N/P ratio estimated from the pore water of mesocosm II remained approximately 70, while the ratio in mesocosm III was below 6 throughout the experimental period (data not shown). These results indicated that sufficient phosphorus was not provided compared to the nitrogen source in mesocosm II. Both Inipol and SRF supply N in the form of urea, and thus urease is necessary to liberate N as available ammonia. Since there was no evidence of higher urease activity in mesocosm II than in mesocosm III, the high N/P ratio observed in mesocosm II therefore suggests that either the urea released from the product was assimilated slowly by the microbial community or that the rate of urea release was exceedingly high. In other study, sudden increase of nitrogen concentration on Day 5, accounting for 42.3% of the N added as Inipol EAP22, was observed in the effluent of an Inipol-treated column packed with oiled sand [26]. Therefore, in mesocosms I and II, phosphorus could be a major limiting factor for oil degradation, and this hypothesis may explain not only the high rates and extents of oil degradation in mesocosm III, but also the results obtained by specific indicators for biological oil degradation.

## Microbial Population

All mesocosms showed rapid increase in both heterotrophic plate counts (HPC) and petroleum-degrading bacteria (PDB), following the addition of oil at Day 0 (Figs. 2A and 2B). By Day 3, both the control and Inipol-treated mesocosms reached plateaus of $2\times10^7$ and $2\times10^8$ CFU (g sand)$^{-1}$, respectively. The value of HPC in mesocosm III, however, showed the maximal counts at Day 8 and then decreased to $7\times10^6$ CFU (g sand)$^{-1}$. Similar trends were also observed in the PDB counts (Fig. 2B). An initial sharp increase and gradual decrease thereafter were common in both the control and Inipol-treated mesocosms. The number of

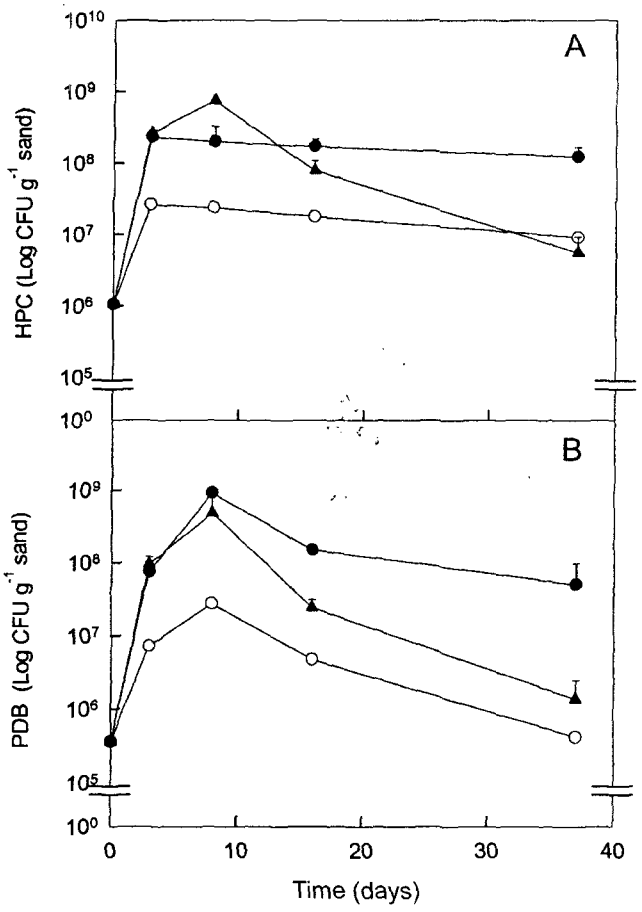


**Fig. 2.** Fluctuations of heterotrophic plate counts (A) and petroleum-degrading bacterial numbers (B) estimated from mesocosms I, II and III.
○: no fertilizer in mesocosm I; ●: oleophilic fertilizer in mesocosm II; ▲: slow-release fertilizer in mesocosm III. Data were obtained with triplicated samples from each mesocosm and were presented by dry weight of sand basis. Error bars represent 1SD.

PDB in the SRF-treated mesocosm showed rather rapid decline after the initial increase. Approximately 1–2 orders of magnitude increase of HPC was often observed in uncontaminated environments after receiving oil, even without any treatment [26]. Amendment of fertilizer, however, further promoted cell proliferation by at least one order of magnitude higher than that of the control. Unexpectedly, cell numbers in the Inipol-treated mesocosm remained relatively constant, whereas the numbers in the SRF-treated mesocosm gradually decreased in this study. The main ingredients in Inipol EAP 22 are oleic acid and urea, along with chemicals to maintain them in a microemulsion [16]. The product was designed to initially stimulate oleic acid-degrading bacteria. Elf Aquitaine, the manufacturer of Inipol, claims that oil biodegradation is thought to commence once the added oleic acid is consumed and many oleic acid-degrading bacteria are known to degrade petroleum hydrocarbon. In the present study, this group of bacteria did not seem to have actually contributed to the oil degradation rate in the mesocosm II. The increase of total HPC largely reflects the increase of number of PDB, demonstrated by the comparison of both the fluctuation pattern and the extent of increase. Communities exposed to hydrocarbons become adapted by selective enrichment and genetic changes, resulting in increased proportions of PDB and bacterial plasmids encoding hydrocarbon catabolic genes [13].

The fluctuation pattern of HPC and PDB in mesocosm III properly explains the degradation of oil in that mesocosm. At Day 3, the PDB level reached a value of $1\times10^8$ CFU (g sand)$^{-1}$ and this peak coincided with the maximal extent of degradation and with the highest rate of degradation as well (Fig. 1). As the cell numbers declined thereafter, the degradation rate also declined. These results suggest that the specific degradation rate (degradation per cell) remained unchanged.

At Day 3, mesocosm II showed the lowest surface tension (Fig. 3), indicating the presence of surface-active agents that most likely originated from Inipol EAP22. Surfactants and oleic acid in the fertilizer formulation of Inipol cause the nutrients to become sequestered to the oil phase, thereby preventing rapid release of the nutrients into the aqueous phase and subsequent washout [27]. However, the subsequent steady increase of the surface tension in mesocosm II suggested that the surfactant inherent in the Inipol was washed out by precipitation and regular watering. This also implied that a shortage of inorganic nutrients could occur in the Inipol-treated mesocosm II. On the other hand, mesocosm III showed a drastic

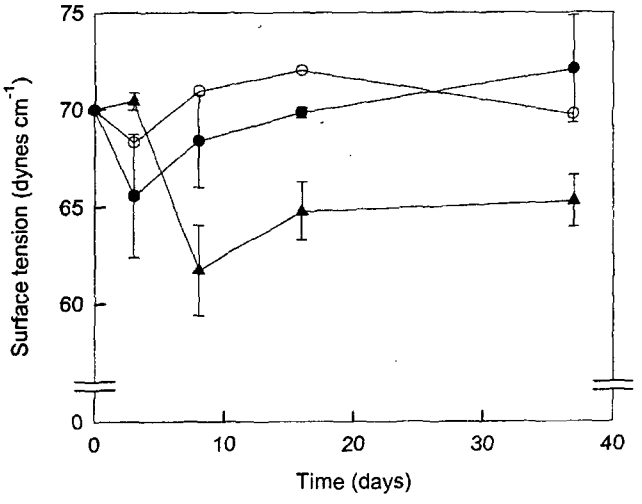


**Fig. 3.** Changes of surface tension values in the sand seashore mesocosms I, II and III.
○: no fertilizer in mesocosm I; ●: oleophilic fertilizer in mesocosm II; ▲: slow-release fertilizer in mesocosm III. Data were obtained with triplicated samples from each mesocosm and were presented by dry weight of sand basis. Error bars represented 1SD.

decrease of the surface tension during the period of Days 3 and 8, which generally reflected the patterns of both oil degradation and microbial population change. It was possible that microbially-produced surface-active agents actively enhanced the oil degradation rate and cell proliferation. There are many reports that self-production or the addition of emulsification agents could accelerate micelle formation and the degradation rate of hydrocarbons [8, 9, 10, 20].

In summary, the effects of fertilizer additions on oil-contaminated sand mesocosms were evaluated. The amendment clearly enhanced both the extent and the initial rates of degradation, suggesting N, P-limitation was critical for oil degradation. Cell numbers counted coincided well with this trend, except for the Inipol-treated mesocosm, possibly due to the lack of enhancing a true oil-degrader. Nevertheless, the Inipol-treated mesocosm showed significantly enhanced oil degradation compared to that of the untreated control mesocosm. SRF, typically used for agricultural purposes, therefore promises to be useful in the treatment of oil-contaminated sand beaches.

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## REFERENCES

1. APHA (1992). *Standard Methods for the Examination of Water and Wastewater*, 18th ed. APHA-AWWA-WEF, Washington, DC, U.S.A.
2. Atlas, R. M. 1981. Microbial degradation of petroleum hydrocarbons: An experimental perspective. *Microbiol. Rev.* **45:** 180–209.
3. Atlas, R. M. and R. Bartha. 1973. Stimulated biodegradation of oil slicks using oleophilic fertilizers. *Environ. Sci. Technol.* **7:** 538–541.
4. Boopathy, R. 2000. Factors limiting bioremediation technologies. *Bioresource Technol.* **74:** 63–67.
5. Bragg, J. R., R. C. Prince, E. J. Harner, and R. M. Atlas. 1994. Effectiveness of bioremediation for the Exxon Valdez oil spill. *Nature* **368:** 413–418.
6. Bregnard, T. P., A. Haner, P. Hohener, and J. Zeyer. 1997. Anaerobic degradation of pristane in nitrate-reducing microcosms and enrichment cultures. *Appl. Environ. Microbiol.* **63:** 2077–2081.
7. Croft, B. C., R. P. J. Swannell, A. L. Grant, and K. Lee. 1995. Effect of bioremediation agents on oil biodegradation in medium-fine sand, pp. 423–434. *In* R. E. Hinchee, G. S. Douglas and S. K. Ong (eds.), *Applied Bioremediation of Petroleum Hydrocarbons.* Battelle Press, Columbus, OH, U.S.A.
8. Foght, J. M., D. L. Gutnick, and D. W. S. Westlake. 1989. Effect of emulsan on biodegradation of crude oil by pure and mixed bacterial cultures. *Appl. Environ. Microbiol.* **55:** 36–42.
9. Humphries, M., F. Jaworzyn, and J. B. Cantwell. 1986. The effect of a range of biological polymer and synthetic surfactants on the adhesion of marine *Pseudomonas* sp. strain NCMB 2021 to hydrophilic and hydrophobic surface. *FEMS Microbiol. Lett.* **22:** 495–503.
10. Kim, T.-H., Y.-S. Oh, and S.-J. Kim. 2000. The possible involvement of the cell surface in aliphatic hydrocarbon utilization by an oil-degrading yeast, *Yarrowia lipolytica* 180. *J. Microbiol. Biotechnol.* **10:** 333–337.
11. Kim, S.-J., J. H. Sohn, D. S. Sim, K. K. Kwon, and T. H. Kim. 1998. The effects of bioremediation on the oil degradation in oil polluted environments, pp. 181–188. *In* Y. Le Gal and H. O. Halvorson (eds.), *New Developments in Marine Biotechnology.* Plenum Press, New York, U.S.A.
12. Ladousse, A. and B. Tramier. 1991. Results of 12 years of research in spilled oil bioremediation: Inipol EAP 22, pp. 577–581. *In: Proceedings of 1991 International Oil Spill Conference.* American Petroleum Institute, Washington, DC, U.S.A.
13. Leahy, J. G. and R. R. Colwell. 1990. Microbial degradation of hydrocarbons in the environment. *Microbiol. Rev.* **54:** 305–315.
14. Lee, K. and E. M. Levy. 1987. Enhanced biodegradation of a light crude oil in sandy beaches, pp. 411–416. *In: Proceedings of 1987 International Oil Spill Conference.* American Petroleum Institute, Washington, DC, U.S.A.
15. Lee, K. and E. M. Levy. 1991. Bioremediation: Waxy crude oils stranded on low-energy shorelines, pp. 541–547. *In: Proceedings of 1991 International Oil Spill Conference.* American Petroleum Institute, Washington, DC, U.S.A.
16. Marty, P. and Y. Martin. 1993. Use of oleophilic fertilizer and selected bacterial communities to enhance biodegradation of crude oil in seawater. *J. Mar. Biotechnol.* **1:** 27–32.
17. Mulligan, C. N., G. Mahmourides, and G. F. Gibbs. 1989. The influence of phosphate metabolism on biosurfactant production by *Pseudomonas aeruginosa. J. Biotechnol.* **12:** 199–210.
18. Nakajima, K. and A. Sato. 1983. Microbial oxidation of isoprenoid hydrocarbons. IV. Microbial metabolism of isoprenoid alkane pristane. *Nippon Nogeikagaku Kaishi* **57:** 299–305.
19. Nakajima, K., A. Sato, Y. Takahara, and T. Iida. 1985. Microbial oxidation of isoprenoid hydrocarbons. V. Microbial metabolism of isoprenoid alkanes phytane, norpristane and farnesane. *Agric. Biol. Chem.* **49:** 1993–2002.
20. Oberbremer, A. and R. Muller-Hurtig. 1989. Aerobic stepwise hydrocarbon degradation and formation of biosurfactants by an original soil populations in a stirred reactor. *Appl. Microbiol. Biotechnol.* **31:** 582–586.
21. Oh, Y.-S., W.-Y. Choi, Y.-H. Lee, S.-C. Choi, and S.-J. Kim. 2000. Biological treatment of oil-contaminated sand: Comparison

of oil degradation based on thin-layer chromatography/flame ionization detector and respirometric analysis. *Biotech. Lett.* **22:** 595–598.
22. Oh, Y.-S., J. Maeng, and S.-J. Kim. 2000. Use of microorganism-immobilized polyurethane foams to absorb and degrade oil on water surface. *Appl. Microbiol. Biotechnol.* **54:** 418–423.
23. Pritchard, P. H. and C. F. Costa. 1991. EPA's Alaska oil spill bioremediation project. *Environ. Sci. Technol.* **25:** 372–379.
24. Safferman, S. I. 1991. Selection of nutrients to enhance biodegradation for the remediation of oil spilled on beaches, pp. 571–576. *In: Proceedings of 1991 International Oil Spill Conference.* American Petroleum Institute, Washington, DC, U.S.A.
25. Sveum, P. and A. Ladousse. 1989. Biodegradation of the oil in the arctic: Enhancement by oil-soluble fertilizer application, pp. 439–446. *In: Proceedings of 1989 International Oil Spill Conference.* American Petroleum Institute, Washington, DC, U.S.A.
26. Swannell, R. P. J., B. C. Croft, A. L. Grant, and K. Lee. 1995. Evaluation of bioremediation agents in beach microcosms. *Spill Sci. Technol. Bull.* **2:** 151–159.
27. USEPA. 1990. Interim Report - *Oil Spill Bioremediation Project*, p. 219.