

Effect of Nutrient Amendments on Indigenous Hydrocarbon Biodegradation in Oil-Contaminated Beach Sediments

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ABSTRACT

Nutrient amendment to oil-contaminated beach sediments is a critical factor for the enhancement of indigenous microbial activity and biodegradation of petroleum hydrocarbons in the intertidal marine environment. In this study, we investigated the stimulatory effect of the slow-release fertilizers Osmocote (Os; Scotts, Marysville, OH) and Inipol EAP-22 (Ip; ATOFINA Chemicals, Philadelphia, PA) combined with inorganic nutrients on the bioremediation of oil-spiked beach sediments using an open irrigation system with artificial seawater over a 45-d period. Osmocote is comprised of a semipermeable membrane surrounding water-soluble inorganic N, P, and K. Inipol, which contains organic N and P, has been used for oil cleanup on beach substrate. Nutrient concentrations and microbial activity in sediments were monitored by analyzing sediment leachates and metabolic dehydrogenase activity of the microbial biomass, respectively. Loss of aliphatics (n -C₁₂ to n -C₃₃, pristane, and phytane) was significantly greater (total loss between 95 and 97%) in oil-spiked sediments treated with Os alone or in combination with other nutrient amendments, compared with an unamended oil-spiked control (26% loss) or sediments treated with the other nutrient amendments (28–65% loss). A combination of Os and soluble nutrients (SN) was favorable for the rapid metabolic stimulation of the indigenous microbial biomass, the sustained release of nutrients, and the enhanced biodegradation of petroleum hydrocarbons in leached, oil-contaminated sediments.

IT IS GENERALLY ACKNOWLEDGED that the biodegradation of petroleum hydrocarbons in oil-contaminated marine foreshore environments can be accelerated by the application of nutrients (Atlas, 1991; Mearns, 1997). Such biostimulation of the indigenous microbial biomass is based on the premise that essential metabolic nutrients, including nitrogen and phosphorus, are usually deficient in the open intertidal environment (Pritchard et al., 1992; Sveum and Ramstad, 1995). Levels of soluble nitrogen in sediment pore waters are known to affect oil biodegradation rates, and the presence and fate of nutrients in sediments are often the key factors in determining the overall success of bioremediation (Lee and Merlin, 1999). Both organic and inorganic forms of nutrients may be amended to beach sediments as a single fertilizer or mixtures of several types. The prerequisite for their effective use as a bioremediation nutrient amendment is the ability to stimulate biodegradation within a short period of time following application, combined with an ability to deliver a sustained release of nutrients to the microbial biomass in an aggressive leaching environment (Lee and Merlin, 1999).

Three strategies of nutrient application are generally used for bioremediation purposes:

- Addition of soluble mineral nutrients. Venosa et al. (1996, 1997) showed that approximately 1.5 mg NO₃-N L⁻¹ in interstitial pore water of beach sediments is sufficient to maintain optimal biodegradation activity by the microbial biomass, and this level could be maintained by the daily application of inorganic nutrients (i.e., NaNO₃ and Na₅P₃O₁₀) dissolved in seawater.
- Addition of organic nutrient formulations. Inipol EAP-22 (Ip) is the most widely used oleophilic fertilizer for oil bioremediation in beach sediments (Pritchard and Costa, 1991; Lessard et al., 1995). By emulsifying the oil into droplets, Ip enhances the contact area between the oil and its outside environment including air, water, nutrients, and hydrocarbon-degrading microorganisms, thereby stimulating biodegradation. Chemically altered organic fertilizers are designed to render a portion of the fertilizer insoluble in water. For example, urea has been chemically modified to make the slow-release fertilizer Ureaform (ureaformaldehyde; Barmac Industries, Archerfield, QLD, Australia) (Relf, 1996). Fishmeal and related products, composed mainly of protein, are other types of organic fertilizer that are known to accelerate oil biodegradation (Sveum and Ramstad, 1995; Santas et al., 1999; Santas and Santas, 2000).
- Addition of slow-release inorganic fertilizers (SRIFs). Slow-release inorganic fertilizers have been developed mainly for agricultural use and are slowly dissolved or degraded by continual or intermittent contact with water to provide a sustained release of nutrients (Lessard et al., 1995). They can be divided into two groups based on the process by which the nutrients are released (Relf, 1996). First, pelletized SRIFs consist of relatively insoluble nutrients in pelletized form, where nutrient release is dependent on pellet surface area. Such SRIFs, including Customblen (Sierra Chemical Co., Sparks, NV) (Pritchard et al., 1992; Lessard et al., 1995) and Max Bac (a product derived from the Customblen used in Alaska by Grace-Sierra Chemicals) (Sveum and Ramstad, 1995; Wright et al., 1996; Oudot et al., 1998), have been used successfully in the bioremediation of oil-contaminated sediments. Second, coated SRIF is designed to coat or

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Abbreviations: DHA, dehydrogenase activity; GC-MS, gas chromatograph-mass spectrometry; Ip, Inipol EAP-22; INT, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazoliumchloride; Os, Osmocote; SN, soluble nutrients; SRIF, slow-release inorganic fertilizer; TRPH, total recoverable petroleum hydrocarbons.

encapsulate water-soluble fertilizers in membranes to permit a slow release of inorganic nutrients into the substrate. For example, the commercially available SRIF Osmocote (Os) is comprised of a semi-permeable membrane surrounding water-soluble nitrogen and other essential metabolic nutrients. Water passes through the membrane via osmosis, eventually generating sufficient internal pressure to disrupt the membrane and release the encased nutrients. Altering the thickness of the pellet coat can control the nutrient release rate. Release rate is also dependent on prevailing environmental conditions in the substrate. Recently, Os has been used for the bioremediation of oil-contaminated mangrove sediments (Ramsay et al., 2000) and shorelines (Swannell et al., 1999).

Different nutrient amendments have their own distinct merits, and a combination of different fertilizer types may further enhance the effectiveness of oil bioremediation additives. This is based on the principle that the indigenous microbial biomass, in the presence of petroleum hydrocarbons, benefits from a source of nutrients that can be readily assimilated before the onset of nutrient release from SRIFs or oleophilic fertilizers. For example, Pritchard et al. (1992) demonstrated that oil biodegradation was enhanced when soluble inorganic nutrients were used in conjunction with Ip.

In this study, metabolic activity of the microbial biomass in a beach sediment was determined by dehydrogenase activity (DHA) measurement using the method optimized by Mathew and Obbard (2001). Traditional cultural methods for enumeration of microorganisms suffer large uncertainties from the inherent heterogeneity in distribution of the microbial population and adherence of viable cells to the substrate matrix (Oberbremer and Muller-Hurtig, 1989; Torstensson, 1997). In recent years, DHA has been recognized as a useful indicator of the overall intensity of microbial metabolism as the enzymes are intracellular and are rapidly degraded following cell death (Rossel et al., 1997; Lee et al., 2000).

This investigation was undertaken as a follow-up to an earlier field study that successfully established the ability of an inorganic nutrient-stimulated indigenous microbial biomass to degrade oil in beach sediments in Singapore (Mathew et al., 1999). Singapore's petrochemical industry has a refining capacity in excess of 1.3 million barrels of oil per day (Economics Department, 1999), and contamination of foreshore environments occurs on an intermittent basis. The tropical climate of Singapore, with a diurnal temperature and humidity range of 23 to 34°C and 60 to 100%, respectively (Singapore National Environment Agency, 2002), is well suited to biodegradation processes. In addition, microbial communities have been preexposed to petroleum hydrocarbons as a result of previous oil-spillage events. The aim of this study was to investigate and compare the effect of soluble inorganic nutrients, the organic fertilizer Inipol EAP-22, and the SRIF Osmocote on the biodegradation of oil and selected hydrocarbons under controlled laboratory conditions. In addition, the effect of a combination of soluble

inorganic nutrients, Os, and Ip on biodegradation rates was investigated.

MATERIALS AND METHODS

Experimental Setup

An uncontaminated beach sediment (2–10 cm depth) was collected from Pulau Semakau, a small island located 8 km south of the Singapore main island. The sediment contained 81.1% sand, 18.5% silt, 0.4% clay, and 0.2 g organic C per kg dry sediment. Its pore water contained 0.03 mg L⁻¹ N and 0.002 mg L⁻¹ P and was at pH 7.6. Sediment was transported to the laboratory and spiked with an Arabian light crude oil to achieve a total petroleum hydrocarbon content of 3.22 kg oil per 100 kg sediment (dry weight equivalent). The sediment was then weathered for 3 wk in darkness at ambient temperature (i.e., 23–34°C) with daily mixing to maintain an aerobic condition. After weathering, the oil content of the spiked sediment decreased to a petroleum hydrocarbon level of 2.20 kg oil per 100 kg dry sediment. The sediment was then used to establish a range of treatments, including the application of soluble nutrients (SN) only, Os only, Ip only, as well as the combination of SN and Os (SN + Os), Ip and Os (Ip + Os), and Ip and SN (Ip + SN) to the oil-spiked sediments (Table 1). The addition of nutrients was based on attaining the optimal molar ratio of C to N to P for bioremediation (i.e., 100:10:1; Liebeg and Cutright, 1999). Osmocote 18–11–10 used in this study comprises 7.5% NO₃-N, 10.5% NH₃-N, 11% P₂O₅, 10% K₂O, and a resin coating, giving a molar N to P ratio of 10:1.2, and it does not contain minor nutrients. Inipol EAP-22 comprises 26.2% oleic acid, 23.7% lauryl phosphate, 10.8% 2-butoxy-1-ethanol, 15.7% urea, and 23.6% water. For treatment Ip, the addition of Ip was based on the prescribed dosage of 110 mL per kg of crude oil (Santas et al., 1997, 1999). For SN + Os, Ip + Os, and Ip + SN treatments, the molar ratio of C to N to P also approximated to 100:10:1. In the SN + Os treatment, 10% N and P came from SN, and the remainder from Os. In Ip + Os and Ip + SN treatments, nutrients derived from Ip were negligible, and the dosage of Ip was 10%, with the remainder from the alternative nutrient source.

Following treatment, 5 kg oil-spiked sediment (dry weight equivalent) was placed in an irrigated microcosm, in duplicate. The experimental setup was designed as an "open" irrigation system, where sediments were free-draining following irrigation with reconstituted seawater. This consisted of dissolved natural sea salts in sterile deionized water at a density of 1.023 kg L⁻¹. Each microcosm comprised a seawater spray outlet and flow meter connected to an electric water pump and timer. A schematic representation of the irrigation system is shown in Fig. 1. The flow rate, time, and interval of water spraying were controlled automatically and set at 0.2 L min⁻¹, 10 min, and 12 h, respectively. Each microcosm was fully saturated with seawater on irrigation and was then held in sediments for 1 h, before being left to drain under gravity before the next wetting episode. The residence time was selected for practical reasons and to simulate a period of sediment inundation with seawater in the field environment. The apparatus was placed in the outdoor environment, but sheltered from rainfall. Experimental duration was 48 d, and microcosms were tilled daily throughout the experiment to ensure an aerobic condition. Sediments were sampled for analysis just before irrigation. Sediment sampling for chemical analysis was undertaken on Days 0, 15, 30, and 45, and for biological analysis on Days 0, 2, 6, 12, 21, 30, 38, and 45. Seawater leachate samples were collected from all treatments before irrigation on Days 1, 2, 7, 25, and 45. For each duplicate microcosm,

Table 1. Description of treatments performed in 5 kg oil-spiked beach sediment on Day 0.

Treatment†	Nutrient amendments	Dosage g kg ⁻¹ sediment	Total C to N to P mole ratio
Control	none	0	no nutrient added
Treatment 1 (SN)	soluble nutrients	11.52	100:10:1
Treatment 2 (Os)	Osmocote	18.88	100:10:1
Treatment 3 (Ip)	Inipol	5.5	100:1.1:0.05
Treatment 4 (SN + Os)	soluble nutrients	1.152	100:10:1
	Osmocote	16.99	
Treatment 5 (Ip + Os)	Inipol	0.55	100:10:1
	Osmocote	16.99	
Treatment 6 (Ip + SN)	Inipol	0.55	100:10:1
	soluble nutrients	10.36	

† Ip, Inipol; Os, Osmocote; SN, soluble nutrients.

sediment samples (5 g) were taken from five separate points and then homogenized to form a compound sample (i.e., 25 g) for each treatment duplicate.

Chemical Analysis

Nutrients in seawater leachate were analyzed on a Hach (Loveland, CO) DR2000 spectrophotometer. Total ammonia and organic nitrogen was determined as ammonia nitrogen using a Kjeldahl method (Hach Company, 1995b); nitrate nitrogen using a cadmium reduction method (Hach Company, 1995c); and total phosphorus using a PhosVer 3 method (Hach Company, 1995a) following seawater digestion using acid persulfate (Hach Company, 1995d).

Loss of oil from sediments and leachate was measured by gravimetric weight of total recoverable petroleum hydrocarbons (TRPH), and gas chromatograph–mass spectrometry (GC–MS) analysis of straight (i.e., C₁₂–C₃₃) and branched alkanes (i.e., pristane and phytane). The latter have been used as conservative biomarkers in oil bioremediation studies, but their recalcitrance has been questioned due to their own susceptibility to biodegradation (Prince et al., 1994). Therefore, the more stable polycyclic alkane, C₃₀–17 α (H), 21 β (H)-hopane, was used as the conservative biomarker in this study. This alkane is insoluble in water and extremely resistant to biodegradation (Venosa et al., 1997; Swannell et al., 1996). It has been used successfully to provide valuable quantitative information on the extent of oil degradation in a range of environments (Butler et al., 1991; Venosa et al., 1997).

The TRPH in sediments was measured using USEPA Method 3540 (Eaton et al., 1995, p. 5-34 to 5-35). Sediment samples were dried overnight at 60°C (Korda et al., 1997) and 5 g of sediment sample was extracted with 165 mL hexane–acetone mixture (1:1, v/v) using Soxhlet extraction. The organics in leachate were extracted using dichloromethane by liquid–liquid partitioning, and extract was then filtered through grease-free glass microfiber filter discs (Whatman, Maidstone, UK) into a tared flask (Eaton et al., 1995, p. 5-34 to 5-35). The filtrate from sediments was rotary evaporated (Eyela; Fisher Scientific, Singapore) for solvent removal at 68.8°C (i.e., the boiling point of hexane). The flask with residue was then dried and cooled in a dessicator for 12 h before weighing.

The GC–MS analysis was undertaken for straight (i.e., C₁₂–C₃₃) and branched alkanes (i.e., pristane and phytane), and C₃₀–17 α (H), 21 β (H)-hopane on a Hewlett-Packard (Palo Alto, CA) 6890 GC equipped with a HP 6890 mass selective detector (MSD) and an HP 6890 autosampler. The biomarker standard of C₃₀–17 α (H), 21 β (H)-hopane was purchased from Chiron Laboratories in Trondheim, Norway. The GC–MS samples were prepared by dissolving the residues obtained for TRPH measurement in 100 mL of solvent (i.e., 1:1, v/v, hexane–acetone mixture). An HP 19091S-433, HP-5 MS 5% phenyl methyl siloxane 30.0-m-long $\times$ 250- μ m-i.d. (0.25- μ m film) capillary column was used for hydrocarbon separation, using helium as the carrier gas at a flow rate of 1.6 mL min⁻¹. The injector and detector temperatures were set at 290 and 300°C, respectively. The temperature program for alkanes was set as follows: 2-min hold at 50°C; ramp to 105°C at 8°C min⁻¹; ramp to 285°C at 5°C min⁻¹, and 3-min hold at 285°C. The temperature program for C₃₀–17 α (H), 21 β (H)-hopane was set as follows: 2-min hold at 50°C, then ramp to 300°C at 6°C min⁻¹ and hold 10 min. A 1- μ L aliquot of solvent was injected into the GC–MS using a splitless mode with a 6-min purge-off. The MSD was operated in the scan mode to obtain spectral data for identification of hydrocarbon components and in the selected ion monitoring (SIM) mode for quantification of target compounds. Ions monitored included: alkanes at *m/z* of 71 and 85; pristane at *m/z* of 97 and 268; phytane at *m/z* of 97 and 282; and hopanes at *m/z* of 191, 177, 412, and 397 (Wang et al., 1994).

Biological Analysis

Metabolic activity of the microbial biomass was determined by measurement of dehydrogenase activity using the method optimized by Mathew and Obbard (2001). We added 2.5 mL deionized water and 1 mL 0.75% freshly prepared INT [2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazoliumchloride] solution (pH 7.9) into 5 g (dry weight equivalent) of moist sediment. This sample was incubated in the dark at 27°C for 22 h, and the metabolic product formed, INT-formazan, was extracted by the addition of 25 mL methanol. The extract was

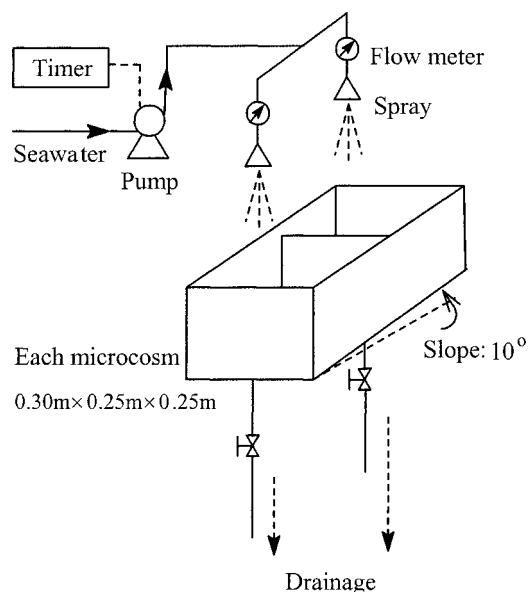


Fig. 1. Schematic diagram of sediment irrigation system.

then filtered through Whatman autovials and measured for absorbance at $\lambda_{\max} = 428$ nm on a PerkinElmer (Wellesley, MA) Lambda 20 UV-vis spectrometer. The spectrophotometer was calibrated with INT-formazan standards prepared in methanol. Dehydrogenase activity was expressed as milligrams INT-formazan formed per kilogram dry weight of sediment per hour.

Statistical Analysis

Tukey's one-way analysis of variance (ANOVA) test at a family error rate of 5% was used to determine the statistical significance of nutrient concentrations in seawater leachate and DHA values of each treatment over time, as well as any difference in TRPH loss and aliphatics loss between treatments. Data were considered to be significantly different between two values if $p < 0.05$. All statistical analyses were performed using MINITAB Release 13.20 (Minitab, 2000).

RESULTS AND DISCUSSION

Nutrients in Seawater Leachate

In this experiment, seawater was maintained in the microcosms for 1 h before gravity drainage of leachate for collection and analysis. Thus, nutrient concentrations measured reflect the levels of nutrient in the sediment pore water in the various microcosms. Nutrient concentrations in seawater leachate from the oil-spiked control and treated sediments are shown in Fig. 2. The total N in Ip leachate was measured using a Kjeldahl method by changing all N in the samples to the form of $\text{NH}_3\text{-N}$ (Fig. 2a).

Nutrient (i.e., $\text{NH}_3\text{-N}$, $\text{NO}_3\text{-N}$, and total P) concentrations in seawater leachate from the oil-spiked control sediment (i.e., with no nutrient additions) were the lowest among the seven treatments (Fig. 2). The concentrations of $\text{NH}_3\text{-N}$, $\text{NO}_3\text{-N}$, and total P in leachate from SN, Ip + SN, and total N and P in Ip dropped significantly in the initial 15 d and were then relatively stable for the remainder of the experiment. Nutrient levels of Ip were significantly lower than those found in leachate from any other treated sediment, but significantly higher than those in unamended oil-spiked control leachates. No statistically significant difference was found between the nutrient (i.e., $\text{NH}_3\text{-N}$, $\text{NO}_3\text{-N}$, and total P) concentrations of SN and Ip + SN leachates during the experiment. Thus, Ip had no effect on the leachability of SN, and the nutrient contribution from Ip was negligible relative to that from added SN. It is possible that Ip was readily leached out of the sediments as it is easily dissolved in water as a surfactant. This phenomenon has also been observed in other laboratory studies and field trials (Lee and Merlin, 1999).

The $\text{NH}_3\text{-N}$ concentration of leachate from Os sediment significantly increased between Days 1 and 15 and then remained stable for the remaining duration of the experiment. Total P concentration also significantly increased from Days 1 to 25 and then stabilized. The $\text{NO}_3\text{-N}$ concentration decreased from Days 1 to 7, then increased gradually until Day 45. Therefore, a substantial release of nutrients from Os-treated sediment was deferred for approximately 15 d. All treatments that

contained SN (i.e., SN, SN + Os, and Ip + SN) produced leachates that were initially high in nitrogen concentrations (Fig. 2a,b), but ammonia and nitrate were quickly leached out of pore waters, especially nitrate. Phosphorous concentrations in leachate from Os + SN were low on Day 1 as the dosage of soluble P was only 10% of that in SN or Ip + SN. The $\text{NH}_3\text{-N}$ concentration in Os + Ip dropped significantly between Days 1 and 2 and then increased gradually up to Day 45. The $\text{NO}_3\text{-N}$ and total P concentration profile in leachate from Os + Ip repeated that of Os and Os + SN, respectively. Therefore, this concentration of Ip in sediments had no obvious effect on nutrient release or leaching from Os-treated sediments.

Comparing nutrient concentrations in leachate from the three Os-treated sediments, the nitrogen ($\text{NH}_3\text{-N}$ and $\text{NO}_3\text{-N}$) concentration in leachate from Os + SN was significantly higher than Os and Ip + Os before Day 7 (Fig. 2a,b). Afterward, $\text{NH}_3\text{-N}$ levels in Os and Os + SN were almost identical, indicating that soluble nutrients were depleted. All three Os-treated sediments generated a more stable and higher level of nutrients in leachates than other sediments after Day 15, meaning that Os had the ability to sustain nutrient levels in the pore water of sediments over the 48-d duration of the experiment. Among the three sediments treated with Ip, the nutrient level in Ip decreased the most quickly in the initial 7 d and was subsequently the lowest among all treatments. Thus, the sediment treated with Ip alone was the most liable to lose nutrients through leaching among all the nutrient additives used in this study.

Dehydrogenase Activity

Dehydrogenase activity of the microbial biomass in the oil-spiked control and treated sediments is shown in Fig. 3. The DHA in the oil-spiked control sediment (no nutrient addition) was the least among all samples and had no significant variance during the 48-d experiment. All sediments amended with nutrient additives significantly enhanced DHA by between two- and three-fold in the first two days relative to the unamended control. The DHA in SN continued to increase significantly until it reached 8.8 mg INT-formazan kg^{-1} dry sediment h^{-1} on Day 12, and subsequently declined to a stable level between 5.0 and 6.2 mg INT-formazan kg^{-1} dry sediment h^{-1} from Day 21 onward. This pattern was repeated for Ip + SN when DHA was only marginally higher than SN over the 48-d period. The DHA of Os increased until it reached 21.9 mg INT-formazan kg^{-1} dry sediment h^{-1} on Day 30 before declining slightly. This pattern was repeated for Os + SN and Ip + Os. There was no significant variation in DHA values of Ip samples over time except in the first two days.

The DHA of Ip + SN was slightly, but significantly, higher than that of SN from Day 7 onward, and DHA of Ip + Os was the highest among all treatments on Day 12. This may be attributed to the presence of Ip, which serves as a surfactant and contains both nutrients and oleic acid. The surfactant properties probably re-

sulted in the dispersion of oil from the sediment particles into microdroplets, thus enhancing the interface area between the oil and water phases.

Overall, DHA in sediments was strongly related to

the nutrient concentrations in leachate and sediments. The DHA in all nutrient-amended sediments was stimulated after two days. After Day 15, leachate nutrient concentrations were in the order of treatments with Os

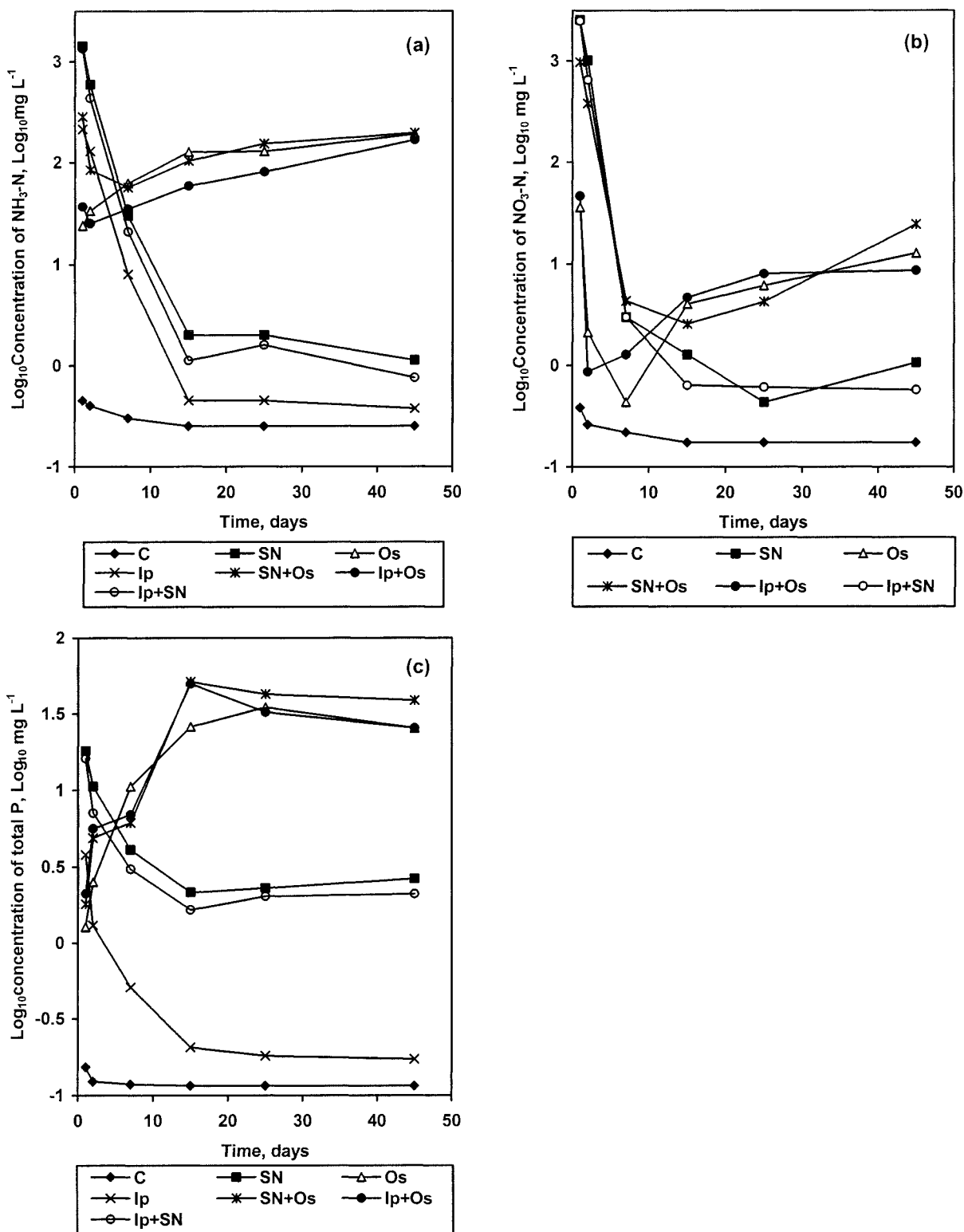


Fig. 2. Concentration of (a) $\text{NH}_3\text{-N}$, (b) $\text{NO}_3\text{-N}$, and (c) total P in leachate from oil-spiked control and treated sediments. For Inipol (Ip) samples treated with Ip only, the total organic nitrogen was determined as ammonia nitrogen using a Kjeldahl method, meaning there is no $\text{NO}_3\text{-N}$ data in (b). C, control; Ip, Inipol; Os, Osmocote; SN, soluble nutrients.

(Os, SN + Os, and Ip + Os) > treatments without Os (SN, Ip, and Ip + SN) > control. Similarly, the DHA levels in all sediments also followed this order indicating that the SRIF Osmocote was superior to Ip in sustaining a high level of metabolic activity in the indigenous microbial biomass, as well as nutrients in the oil-contaminated sediment.

Total Recoverable Petroleum Hydrocarbons Loss

There are three main pathways for loss of hydrocarbons from sediments: evaporation, leaching, and biodegradation. In this study, evaporation was negligible relative to biodegradation as the mixture of the oil and the sediment had been weathered for 3 wk and the TRPH values were stabilized before the experiment was conducted. Normally, it may be supposed that the difference between the TRPH loss from sediment and from leachate is a result of biodegradation. Table 2 gives the initial TRPH values, the total loss of TRPH from sediment and leachate, and the total loss of TRPH due to biodegradation in 45 d in the control and six sediment treatments. These TRPH losses were calculated per microcosm (i.e., per 5 kg dry weight of sediment). Significant differences in TRPH loss from sediment occurred between treatments ($p < 0.05$) with the exception of two pairs of treatments, that is, Os vs. SN + Os and SN vs. Ip ($p > 0.05$). This means that the effect of SN and Ip on TRPH loss was negligible compared with Os. The loss of TRPH from Ip leachate was significantly higher than the other treatments and the control ($p < 0.05$), probably due to the surfactant function of Ip. The TRPH losses from the leachate of the samples treated with Os (Os, SN + Os, and Ip + Os) were significantly higher than the control. This phenomenon can be explained by the high DHA of microbial biomass in these three treated sediments, where the bacteria may also produce biosurfactant to enhance oil bioavailability. As mentioned above, the difference between TRPH loss from sediment and leachate can be regarded as a loss due to biodegradation. This TRPH loss due to biodegradation differed significantly between treatments ($p < 0.05$) except for four pairs of treatments, that is, SN vs. Ip + SN, Os vs. SN + Os, Os vs. Ip + Os, and SN + Os vs. Ip + Os. This indicates that the effect of Ip on TRPH loss due to biodegradation was negligible compared with SN, and the effect of these two nutrients was negligible compared with Os. Generally, the TRPH loss in sediments followed the sequence of treatments with Os (Os, SN + Os, and Ip + Os) > treatments without Os (SN,

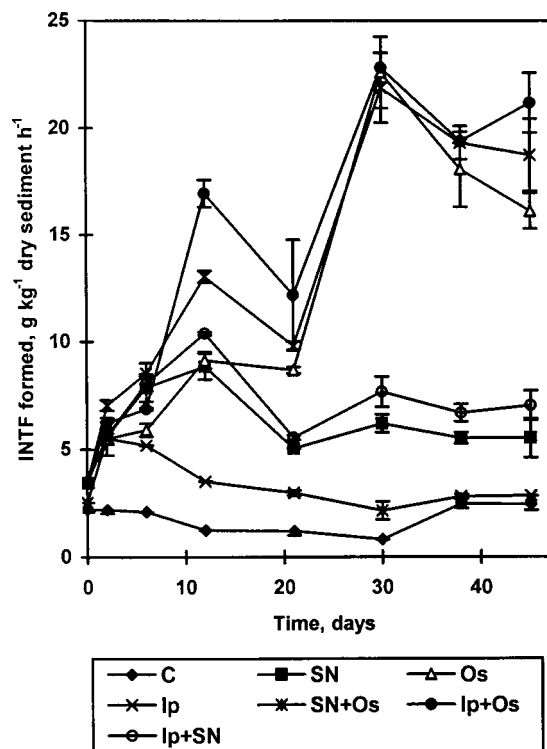


Fig. 3. Dehydrogenase activity of microbial biomass in oil-spiked control and treated sediments. Mean and standard deviation of duplicates are shown. C, control; INTF, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazoliumchloride (INT)-formazan; Ip, Inipol; Os, Osmocote; SN, soluble nutrients.

Ip, and Ip + SN) > control. Thus, the high level of metabolic activity in the indigenous microbial biomass in the three Os-amended sediments was coincidental with a greater total loss of petroleum hydrocarbons due to biodegradation.

The TRPH measurement was based on a gravimetric method that suffers from inherent variability (Butler et al., 1991). Oil residues remaining in the sediment and those leaching out may contain metabolic daughter products, particularly from aromatic compounds. Therefore, from the TRPH results alone, it was possible only to determine the quantity of total organics remaining in the sediments and not the extent of petroleum hydrocarbon biodegradation.

In response, the biomarker $C_{30}-17\alpha(H)$, $21\beta(H)$ -hopane, which is extremely resistant to biodegradation, was used as an internal reference to quantify the depletion of the individual oil components. The ratios of

Table 2. The mean values of initial total recoverable petroleum hydrocarbons (TRPH), TRPH loss from sediment, TRPH loss from leachate, and TRPH loss due to biodegradation (i.e., the difference between the latter two) in 45 d as well as their standard deviation for each treatment. These TRPH losses were calculated per microcosm (i.e., per 5 kg dry weight of sediment).

Treatment†	Initial TRPH	TRPH loss from sediment	TRPH loss from leachate	TRPH loss due to biodegradation
Control	111.24 ± 9.84	18.63 ± 0.39	2.89 ± 0.34	15.74 ± 0.73
SN	111.39 ± 12.41	29.16 ± 0.74	5.25 ± 1.59	23.91 ± 0.84
Os	111.09 ± 7.52	58.15 ± 2.06	9.43 ± 1.60	48.72 ± 0.46
Ip	140.42 ± 21.69	38.53 ± 2.29	33.70 ± 4.40	4.83 ± 2.12
SN + Os	111.12 ± 7.78	59.84 ± 2.62	9.11 ± 0.22	50.73 ± 2.84
Ip + Os	114.21 ± 16.11	67.41 ± 3.43	14.15 ± 2.95	53.26 ± 6.38
Ip + SN	113.73 ± 2.72	36.24 ± 2.51	8.45 ± 1.25	27.79 ± 3.76

† Ip, Inipol; Os, Osmocote; SN, soluble nutrients.

aliphatics to hopane decrease when aliphatics are biodegraded. This is not the case if aliphatic loss is due to by leaching or other physical processes, as the hopane would also be lost from sediment. By combining TRPH measurements with quantitative GC–MS analysis using the hopane biomarker, it was therefore possible to distinguish oil biodegradation from other losses, including leaching.

Gas Chromatograph–Mass Spectrometry Data

Initial composition of the saturated fraction (i.e., n -C₁₂ to n -C₃₃, pristane, and phytane) (Fig. 4) and the bio-

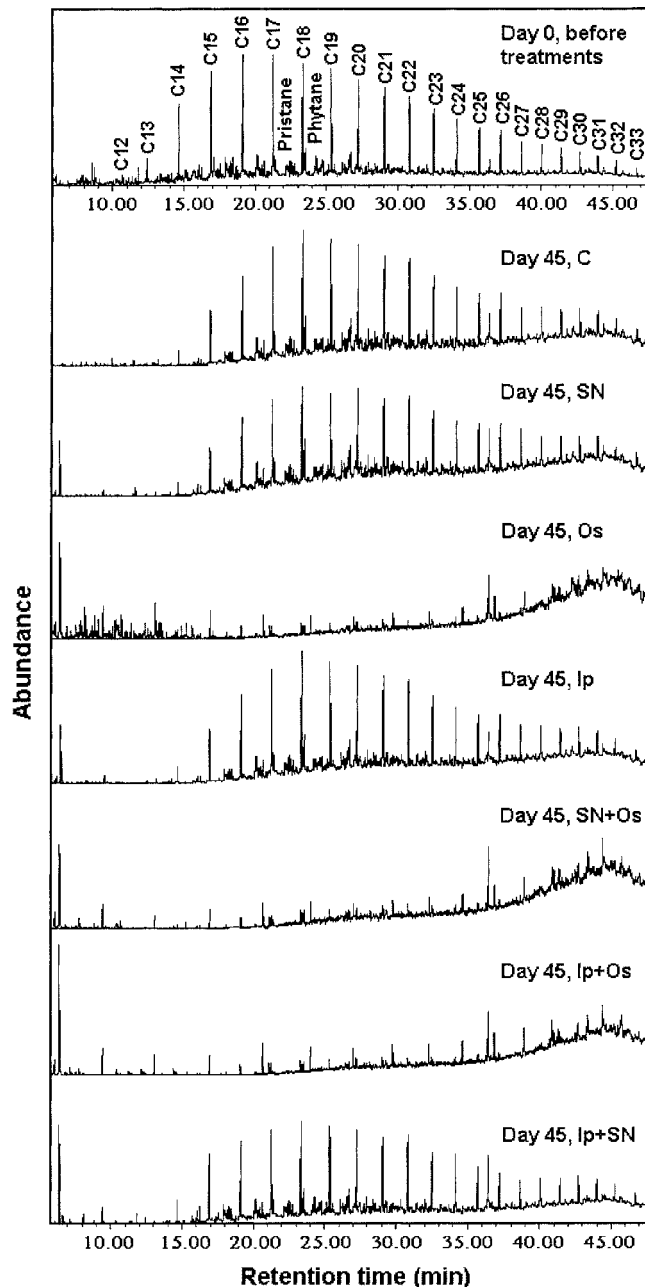


Fig. 4. Gas chromatograph–mass spectrometry (GC–MS) data of oil residue extracted from sediment before experiment on Day 0 and the control and six treated sediments on Day 45. Peak identification of hydrocarbon components is shown in GC–MS data of oil residue on Day 0. The y axes of all graphs are in the same range. C, control; Ip, Inipol; Os, Osmocote; SN, soluble nutrients.

marker, C₃₀–17 α (H), 21 β (H)-hopane (Fig. 5), in three subsamples in the Arabian light crude oil–spiked weathered sediment was determined before sediment treatment with the bioremediation additives. The scan mode GC–MS data of oil residues in the control and six treated sediments on Day 45 are shown in Fig. 4. Biodegradation of the saturated fraction in oil residue was more obvious in Os-treated samples (Os, SN + Os, and Ip + Os) than in the other samples. Light oil components, that is, n -alkanes (C₁₂–C₂₂), were detected in the leachate of the control (Fig. 6) up to Day 7. Subsequently, no obvious oil component was detected from GC–MS data (Fig. 6). Leachates of all treated sediments were similar to the control.

The losses of aliphatics, including n -C₁₂ to n -C₃₃, and pristane and phytane in the oil-spiked control and treated sediments are given in Fig. 7. The total losses of aliphatics as a percentage of the initial concentrations in sediments are shown in Table 3. The proportion of both straight and branched alkanes relative to the conservative biomarker C₃₀–17 α (H), 21 β (H)-hopane decreased in all sediments over the duration of the experiment (Fig. 7). Alkane loss in the control was least among all sediments, representing only a 26% loss on Day 48 (Table 3). The overall fate of aliphatics in Ip sediment was similar to that of the unamended control except

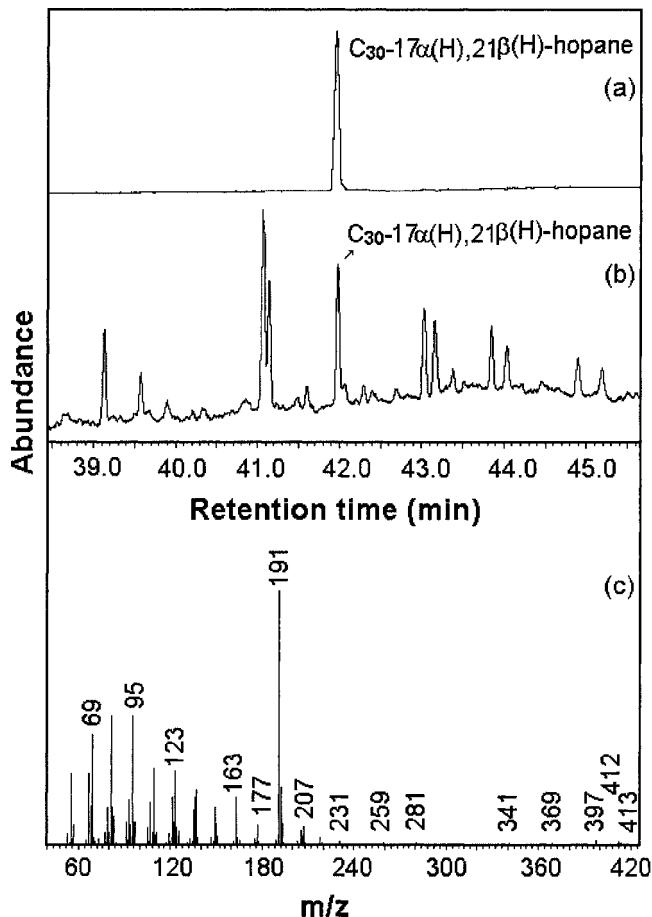


Fig. 5. Gas chromatograph–mass spectrometry (GC–MS) data of the biomarker C₃₀–17 α (H), 21 β (H)-hopane in commercial standard (a) and oil residue (b). The MS spectra of C₃₀–17 α (H), 21 β (H)-hopane is shown in (c).

that oil biodegradation in Ip was significantly enhanced in the first 15 d (Fig. 7). In addition to nutrient limitation, the low degradation in the Ip treatment (Fig. 7) may also be due to the presence of 2-butoxyethanol in Ip, which may be toxic to bacteria.

From Table 4, it can be seen that a significant loss of aliphatics occurs in each 15-d period for each treatment, conforming to the order of $\Delta L_{(0-15)} > \Delta L_{(16-30)} > \Delta L_{(31-45)}$, except for Os. For the unamended oil-spiked control sediment, SN, Ip, and Ip + SN, this phenomenon can be explained by the reduction in nutrient concentrations in sediments over the duration of the experiment. However, nutrient concentrations and DHA values of Os, SN + Os, and Ip + Os in the third 15-d period were equal to or higher than in the other two periods. Therefore, the decrease in the biodegradation of aliphatics in Os-treated sediments may be attributed to the reduction of substrate concentration in sediment.

At the end of the experiment, statistical analysis of aliphatic loss among the treatments was similar to that of TRPH loss due to biodegradation, except for two pairs of treatment, that is, control vs. Ip and SN vs. Ip + SN. Analysis of TRPH losses showed that there was a significant difference between the control and Ip ($p < 0.05$), and a significant difference between SN and Ip + SN ($p < 0.05$). On the contrary, analysis of aliphatic losses showed that there was no significant difference between the control and Ip ($p > 0.05$), and a significant difference between SN and Ip + SN ($p < 0.05$). From the above, it can be seen that the same significant difference between the two treatment pairs, that is, control vs. Ip, and SN vs. Ip + SN, is due to the existence of Ip. Without SN, the biodegradation of aliphatics could not be significantly enhanced by Ip, while the result was opposite in the presence of SN. Therefore, the TRPH loss in Ip sediment was mainly due to the surfactant

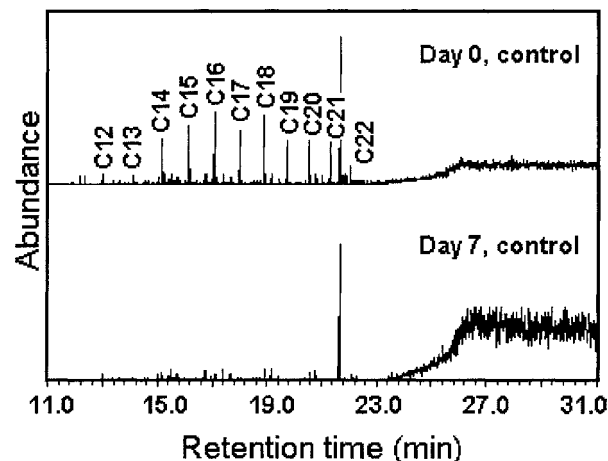


Fig. 6. Gas chromatograph-mass spectrometry (GC-MS) data of oil residue extracted from leachate of control on Days 0 and 7. Peak identification of hydrocarbon components is shown in GC-MS data of oil residue on Day 0.

function of Ip rather than its nutrient properties. For SN and Ip + SN, oil biodegradation was dominant in the first 15 d when nutrient concentrations were sufficient to support oil biodegradation.

In Table 3, oil biodegradation expressed by percentage TRPH loss is compared with the percentage loss of aliphatics for each sediment treatment. It can be seen that the two losses were not significantly different from each other in the control and SN samples, but differed significantly in the other treated samples. In fact, the biodegradation would be low when estimated by TRPH alone due to the presence of metabolic daughter products in the sediments and leachate. The loss of aliphatics overestimates the oil biodegradation, as aliphatics are easier to biodegrade than the more recalcitrant hydro-

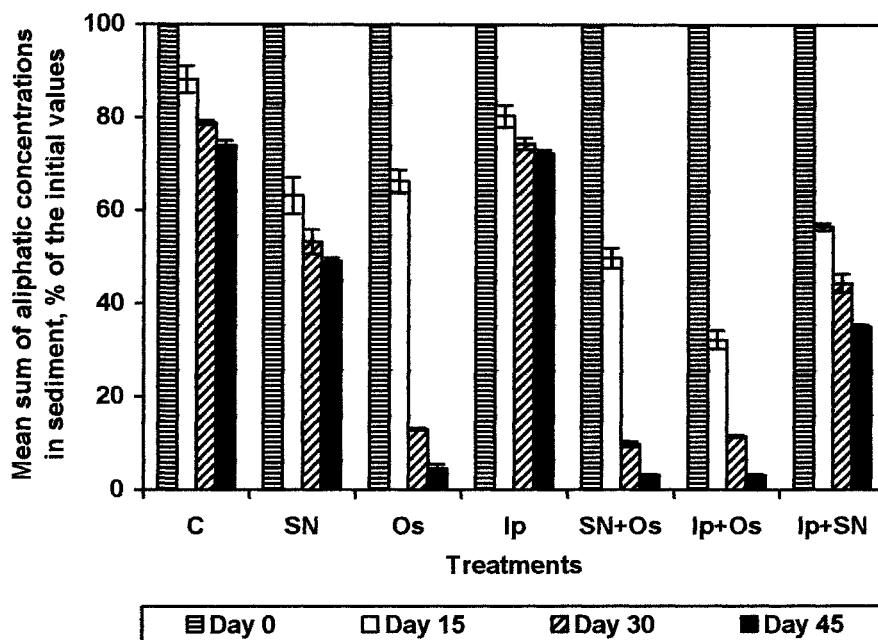


Fig. 7. Total amounts of aliphatics (n -C₁₂ to n -C₃₃, pristane, and phytane) relative to hopane and normalized by initial values in oil-spiked control and treated sediments on Days 0, 15, 30, and 45. Mean and standard deviation of duplicates are shown. C, control; Ip, Inipol; Os, Osmocote; SN, soluble nutrients.

Table 3. Loss of total recoverable petroleum hydrocarbons (TRPH) and aliphatics due to biodegradation in 45 d (mean and standard deviation of duplicates are shown).

Treatment†	TRPH loss due to biodegradation	Loss of aliphatics due to biodegradation
	% of initial	
Control	14.18 ± 0.60	26.05 ± 0.85
SN	21.64 ± 3.17	50.98 ± 1.15
Os	43.94 ± 2.56	95.38 ± 3.37
Ip	3.37 ± 0.99	27.80 ± 1.42
SN + Os	45.67 ± 0.64	96.81 ± 2.88
Ip + Os	46.70 ± 1.00	97.01 ± 2.33
Ip + SN	24.48 ± 3.89	65.00 ± 3.40

† Ip, Inipol; Os, Osmocote; SN, soluble nutrients.

carbons also present in the crude oil. Therefore, it may be summarized that the real scale of oil biodegradation may fall between the two.

In general, soluble inorganic nutrients were readily available to the indigenous microbial biomass and resulted in early metabolic stimulation and biodegradation of hydrocarbons. Venosa et al. (1996, 1997) applied mineral nutrients (NaNO_3 and $\text{Na}_5\text{P}_3\text{O}_{10}$), which were dissolved in seawater, to an oiled beach sand using a sprinkler system in a field trial on the Delaware Bay, and successfully enhanced oil biodegradation. Inipol can simultaneously serve as a surfactant, co-substrate, and nutrient source and is able to enhance the availability of both hydrocarbons and nutrients to the biomass. It has been demonstrated that Ip was capable of dispersing oil to form microdroplets, allowing enhanced biodegradation (Ladousse and Tramier, 1991; Santas et al., 1999). However, the successful use of Ip was only achieved on coarse sand or mixed sand and gravel (Swannell et al., 1996). In this study, its main function was to serve as a surfactant, where nutrient release was negligible, possibly due to the fine-grained sediment used in the experiment. It has been reported that the SRIFs Customblen (Pritchard et al., 1992; Lessard et al., 1995) and Max Bac (Sveum and Ramstad, 1995; Wright et al., 1996; Oudot et al., 1998), have also been used successfully in the bioremediation of oil-contaminated sediments. Our study has demonstrated that Os can successfully maintain the nutrient level and enhance oil biodegradation in fine-grained beach sediment. Ramsay et al. (2000) applied Os to an oil-contaminated mangrove sediment and increased the alkane-degrading microbial population size by three orders of magnitude. Swannell et al. (1999) used Os as a nutrient source to treat oiled shore-

Table 4. The loss of aliphatics in control and six treated sediments in three 15-d periods during the experiment.

Treatment†	$\Delta L\ddagger$		
	1–15 d	16–30 d	31–45 d
	% of the initial concentration		
Control	11.84	9.33	4.88
SN	36.89	9.92	4.17
Os	33.71	53.31	8.36
Ip	19.69	5.96	2.15
SN + Os	50.24	40.01	6.56
Ip + Os	67.80	20.72	8.49
Ip + SN	43.47	12.19	9.34

† Ip, Inipol; Os, Osmocote; SN, soluble nutrients.

‡ Loss of aliphatics in the first, second, and third 15-d periods.

lines and 37% more oil was degraded than in an untreated oil-contaminated control. So far, there have been no reports comparing the effectiveness of SN, Ip, and Os on oil bioremediation. This study has provided relative information on the bioremediation additives in beach sediments in a tropical environment.

CONCLUSIONS

The beneficial effects of both soluble inorganic nutrients and Inipol in our experiment were found to be limited in duration due to their susceptibility to leaching loss from irrigated sediments. After only 15 d, the levels of nutrients and DHA of the indigenous microbial biomass were comparable with that of an unamended control. In contrast, sediments amended with the slow-release inorganic fertilizer Osmocote maintained nutrient levels at a concentration that was beneficial for the bioremediation of oil-contaminated sediments, albeit with a deferred effect before the onset of nutrient release. The presence of Os significantly enhanced the metabolic activity of the microbial biomass, and resulted in the loss of more than 95% of aliphatics (i.e., *n*-C₁₂ to *n*-C₃₃, pristane, and phytane) over a 45-d period. The amendment of SN together with Os remedied the initial deficiency in nutrients before the onset of nutrient release from the SRIF, thereby resulting in an earlier stimulation of the indigenous biomass and the biodegradation of aliphatics. This study has demonstrated that a combination of SN with an SRIF is favorable for a rapid stimulation of the indigenous microbial biomass, sustained release of nutrients, and enhanced biodegradation of petroleum hydrocarbons in a leached, oil-contaminated beach sediment.

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