BIOREMEDIATION OF PETROLEUM CONTAMINATED BEACH SEDIMENTS: USE OF CRUDE PALM OIL AND FATTY ACIDS TO ENHANCE INDIGENOUS BIODEGRADATION

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Abstract. Amendment of simple organic carbon, in the presence of inorganic nutrients, to oil contaminated beach sediments can potentially stimulate the biodegradation of hydrocarbons by the indigenous microbial biomass. The ability of crude palm oil (CPO) and fatty acids, in the presence of soluble inorganic nutrients (C:N:P = 100:10:1), to stimulate biodegradation in sediments amended with Arabian light crude oil was investigated in laboratory microcosms over a 30-day period. The presence of nutrients alone enhanced the most probable number (MPN) of hydrocarbon degrading bacteria by almost 12 times after 30 days, and by 26 times in sediment also amended with CPO, thereby confirming its ability to stimulate the biomass. Addition of individual fatty acids to nutrients-amended sediments resulted in MPN enhancements of up to 170 times, where straight alkanes (i.e., C_{17} and C_{18}) were completely degraded. Amendment with CPO, myristic, oleic, linoleic and palmitic acids (0.5% dry weight equivalent) also enhanced the metabolic activity of the entire microbial biomass, as measured by intracellular dehydrogenase activity, reaching a maximum of 30 μg INTF g dry sed^{-1} h^{-1}. Loss of branched alkanes was significantly enhanced, where loss of pristane was complete and phytane loss reached 98% in sediment treated with myristic acid. This study highlights the considerable benefit of adding readily oxidizable fatty acids for enhancing *in situ* bioremediation of hydrocarbons in nutrient-amended beach sediments.

Keywords: biodegradation, crude oil, dehydrogenase activity, petroleum hydrocarbons, simple organic carbon source

1. Introduction

Bioremediation can be an effective technology to combat oil pollution on marine foreshore environments (Lee and Merlin, 1999). The technique utilizes microorganisms to reduce the concentration and/or toxicity of a range of chemicals found in mineral oils (Korda *et al.*, 1997). Biodegradation is a naturally occurring process where oil-degrading microorganisms are capable, under favorable conditions, of utilizing petroleum hydrocarbons as a metabolic carbon source (Mearns, 1997; Atlas, 1991). Rapid growth in the indigenous microbial biomass can occur in beach sediments immediately after contamination with oil, but subsequent depletion of nutrients and readily degradable carbon can rapidly curtail biodegradation rates (Alexander, 1991; Atlas, 1991). Although the application of water-soluble

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fertilisers to supply inorganic nutrients can stimulate the microbial biomass, this is usually short term due to rapid nutrient washout in the open, inter-tidal environment (Wrenn *et al*., 1997).

The addition of simple organic carbon sources, in addition to inorganic nutrients, to oil contaminated beach sediments has been recently identified as a useful technique for further stimulating the biodegradation of petroleum hydrocarbons by an indigenous microbial biomass. For example, fishmeal and related products have been found to be superior in stimulating biodegradation compared to the commercial oleophilic nutrient formulations, such as Inipol EAP-22 (Santas *et al*., 1999). Methyl derivatives of vegetable oils (or 'biodiesel') are also known to stimulate biodegradation and enhance the bioavailability of hydrocarbons in sediments (Miller and Mudge, 1997; Nelson *et al.*, 1996). Fatty acids are readily degraded by *in situ* microorganisms (Ratledge, 1994), and their presence in bioremediation additives may act as simple carbon sources for microorganisms which then either co-metabolise petroleum hydrocarbons or switch to hydrocarbon metabolism after depletion (Mudge and Pereira, 1999; Sveum *et al.*, 1994). Enhanced nutrient immobilisation into the biomass as a result of readily available carbon mineralisation may also reduce nutrient 'wash-out' in the open inter-tidal zone and sustain the biodegradation process as a result of subsequent biomass attrition and sustained nutrient release (Sveum *et al.*, 1994). Although the entire biomass may not participate directly in the biodegradation of hydrocarbons, it may serve as a labile reservoir of nutrients that is slowly released to hydrocarbon degrading bacteria during biomass attrition and turnover.

This investigation is a follow-up of an earlier field study that successfully demonstrated the inorganic nutrient-enhanced biodegradation of petroleum hydrocarbons by the indigenous microbial biomass in oil-contaminated, inter-tidal beach sediments in Singapore (Mathew *et al.*, 1999). The prospect of using simple carbon substrates to further stimulate hydrocarbon degradation was proposed. Vegetable crude palm oil (CPO) was selected for use on the basis of its rich fatty acid content and its local availability in South East Asia (Palm Oil Research Institute of Malaysia, 1985; Tan and Oh, 1981). This paper reports on the ability of CPO and several component fatty acids to stimulate the indigenous microbial biomass and degrade selected petroleum hydrocarbons in oil-spiked beach sediments under laboratory conditions as a prelude to further field trials in Singapore.

2. Materials and Methods

2.1. Beach sediment, preparation

The sediment used in this experiment was clean a beach sediment collected from St. John's Island, a small island 2 km south of the main island of Singapore. The sediment was screened to remove particulates greater than 5 mm in size and

adjusted to 15% moisture content using reconstituted seawater (Eaton *et al.*, 1995) before spiking with an Arabian light crude oil (ALCO) at 5.5 g per 100 g of sand (dry weight equivalent). The sediment was then weathered for 15 days in darkness at ambient temperature (i.e., 23–34 °C) with manual daily mixing and tilling in order to maintain an aerobic condition. After weathering, the oil content of the spiked sediment decreased to a level of 3.55 g per 100 g of sand (dry weight equivalent) due to loss of volatile organics. Nutrients were added to sediment in the form of ammonium nitrate, di-sodium hydrogen phosphate, and potassium dihydrogen phosphate to obtain a molar ratio of C:N:P = 100:10:1. Sediment treatments included oil-spiked biotic and abiotic controls (i.e., amended with 1% mercuric chloride solution), and oil-spiked sediments amended with either CPO or single fatty acids i.e., myristic, oleic, linoleic or palmitic acid, all at 0.5% sediment dry weight equivalent. All fatty acids, as well as the CPO, no matter if in liquid or solid form, were physically mixed with sediment and no solvent was added to dissolve or dilute them. CPO was obtained from a palm-oil processing plant in Johore, Malaysia. All sediments were prepared in triplicate and placed in aerobic microcosms (500 g of sediment, dry weight equivalent) at ambient temperature. Nutrients were amended to all sediments on days 0, 12 and 20, and sediments were tilled every alternate day to maintain an aerobic condition. Experimental duration was 30 days.

2.2. Sediment sampling

Twenty grams of sediment (wet weight) was collected from each microcosm on days 0, 5, 9, 20 and 30 for determination of microbial dehydrogenase activity (DHA) and GC-MS analysis of selected hydrocarbons. An additional sampling was undertaken on day 2 for DHA analysis. The most probable number (MPN) of petroleum hydrocarbon degrading bacteria in sediments was determined on day 0 and day 30, and in the abiotic control 3 h after the addition of 1% mercuric chloride solution.

2.3. Biological analysis

Hydrocarbon degrading microorganisms were enumerated based on a modified method of Brown and Braddock (1990). Ten mL of 1 M phosphate buffer was added to 1 g of sediment sample in a sterile conical flask. The mixture was then shaken for 30 min on a reciprocal shaker at 200 rpm. Serial dilutions (i.e., 10^{-3} to 10^{-6}) were then prepared in phosphate buffer and 2 mL aliquots of each dilution were then added to separate wells of a 24-well tissue culture plate. Each well, containing the aliquot, was covered with 5 μL of filtered-sterilized ALCO. The plates were then incubated at 30 °C and 120 rpm for 21 days. Wells were scored as positive when dispersion of the oil sheen occurred after incubation. The most probable number (MPN) of hydrocarbon degrading microorganisms was determined by using a standard MPN table (Cochran, 1956) and converting counts to the number of cells g dry sed^{-1}.

The metabolic activity of the entire microbial biomass was determined by measurement of intracellular dehydrogenase activity (DHA) using the method optimized by Mathew and Obbard (2001). DHA is known to be a reliable indicator of microbial metabolic activity in both soils and sediments (Lee *et al.*, 2000). 2.5 mL deionised water and 1 mL 0.75% freshly prepared 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazoliumchloride (INT) solution (pH 7.9) was added 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, INT-formazan (INTF) formed was extracted by the addition of 25 mL methanol. The extract was then filtered through a Whatman® autovial and measured for absorbance at $\lambda_{max} = 428$ nm on a Perkin Elmer UV-vis Spectrometer Lambda 20. The spectrophotometer was calibrated with INTF standards prepared in methanol. DHA was expressed as micrograms INTF g dry sed^{-1} h^{-1}.

2.4. Chemical analysis

Loss of oil from sediments was measured by GC-MS analysis of straight (i.e., C_{17} and C_{18} indicators) 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 C_{30}-17α(H), 21β(H)-hopane was used as the conservative biomarker in this study. This alkane is insoluble in water, is extremely resistant to biodegradation (Venosa *et al.*, 1997) and has been used successfully to provide quantitative information on the extent of hydrocarbon degradation in a range of oil-contaminated environments (Venosa *et al.*, 1997; Butler *et al.*, 1991).

Sediment samples were dried overnight at 60 °C and 5 g of sediment was then extracted with a 165-mL hexane-acetone (1:1, v/v) mixture using soxhlet-extraction. The extract was then cooled and filtered through grease-free glass microfibre filter discs (Whatman®) into a tared flask (USEPA methods 413.3 and 418.1, 1983). The filtrate was rotary evaporated (Eyela®) for solvent removal at 68.8 °C i.e., the boiling point of hexane. The residue was then dissolved in a hexane:acetone mixture (1:1, v/v) prior to GC-MS analysis. Analysis was undertaken for straight (C_{12}-C_{33}) and branched alkanes (i.e., pristane and phytane), and C_{30}-17α(H), 21β(H)-hopane on a Hewlett-Packard (HP) 6890 GC equipped with a HP 6890 Mass Selective Detector (MSD) and HP6890 auto-sampler. An HP 19091S-433, HP-5MS 5% phenyl methyl siloxane 30.0 m × 250 μm i.d. (0.25 μm film) capillary column was used for alkane 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 °C and 300 °C, respectively. The temperature program for straight and branched 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 C_{30}-17α(H), 21β(H)-hopane was set as follows: 2-min hold at 50 °C; ramp to 105 °C at 8 °C min^{-1}; ramp to 300 °C at

5 °C min^{-1}, and 5-min hold at 300 °C. A 1 μL aliquot was injected 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 hopane at *m*/*z* of 191, 177, 412 and 397 (Wang *et al.*, 1994).

3. Results

3.1. MPN OF PETROLEUM HYDROCARBON DEGRADING MICROORGANISM

MPN data of petroleum hydrocarbon degrading bacteria for the controls and for various sediment treatments at day 0 and day 30 are given in Table I.

3.2. DEHYDROGENASE ACTIVITY

DHA levels of the microbial biomass over the 30-day experimental period for the controls and for various sediment treatments are shown in Figure 1.

3.3. LOSS OF STRAIGHT AND BRANCHED ALKANES

The ratios of C_{17} and C_{18} alkanes to the conservative hopane biomarker for each of the sediments over 30 days are shown in Figures 2a and 2b, and similarly for

TABLE I

MPN of hydrocarbon degrading bacteria

	MPN of bacteria cells ($\times 10^3$) (cells per gram of dry sediment)		
Sediment treatment	Day 0 (a)	Day 30 (b)	Relative change (b/a)
Control (biotic)	75.9	891.3	11.7
Control (abiotic)	0	0	0
Crude palm oil	75.9	1 995.3	26.3
Myristic acid	75.9	12 882.5	169.7
Oleic acid	75.9	13 182.6	173.7
Linoleic acid	75.9	6 918.3	91.2
Palmitic acid	75.9	12 589.3	165.9

Note. All sediments were amended with inorganic nutrients. Crude palm oil and fatty acids were added at a concentration of 0.5% (sediment dry weight equivalent).

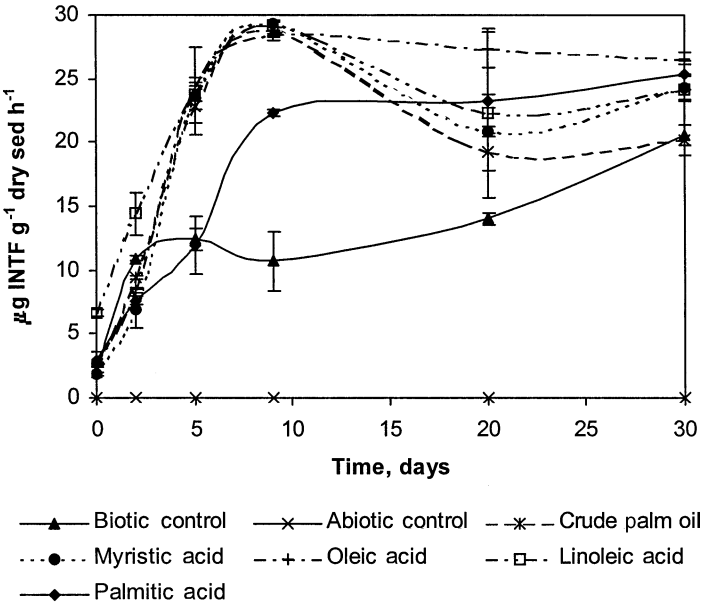


Figure 1. Dehydrogenase activity of the microbial biomass in beach sediments.

phytane and pristane in Figures 3a and 3b. The total percentage losses of C_{17} and C_{18}alkanes, and pristane and phytane are given in Table II.

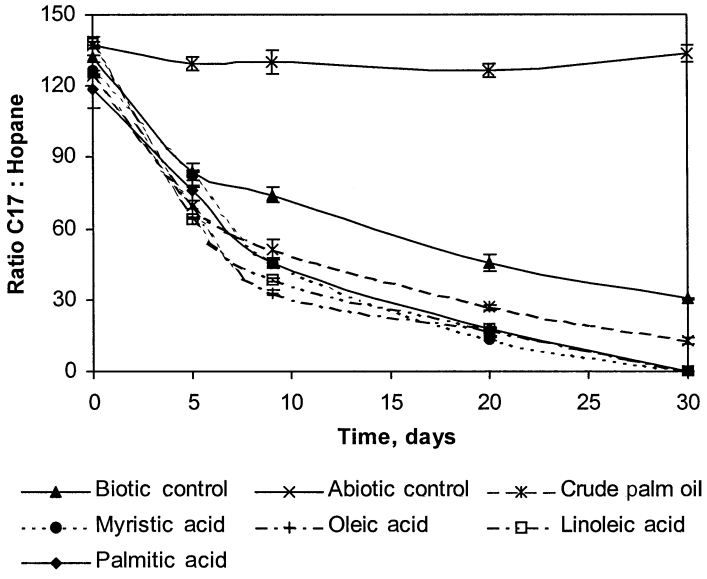
3.4. Statistical analysis

The results were subjected to analysis of variance (ANOVA) to determine if mean values of nutrients, n-alkanes, pristane and phytane to hopane ratios, as well as DHA in the controls and CPO and fatty acids treated sediments differed significantly. Data were considered to be significantly different between two values if $P < 0.01$. All statistical analyses were performed using MINITAB Release 13.2.

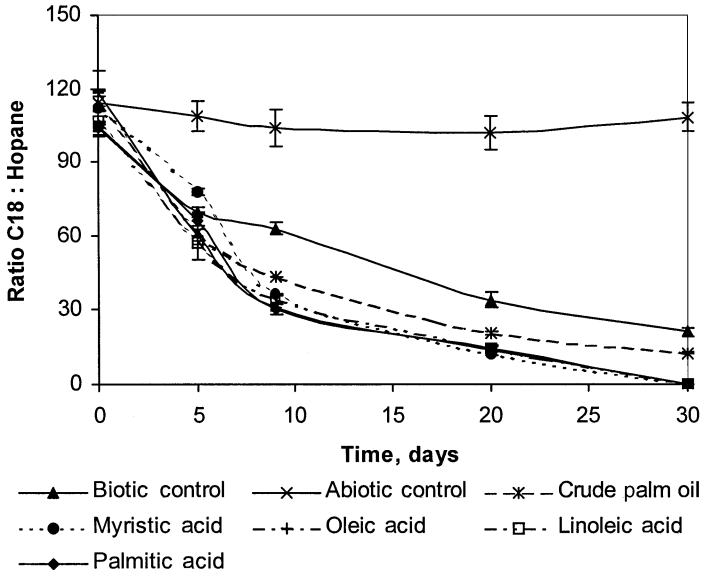
4. Discussion

4.1. MPN of hydrocarbon degrading bacteria

Approximately 7.6×10^4 hydrocarbon degrading bacteria per g dry sed^{-1} (Table I) were present in sediment prior to treatment. Bacteria in the abiotic control were eliminated after treatment with 1% mercuric chloride and were still absent on day 30. The presence of nutrients alone in the biotic control enhanced the MPN of hydrocarbon degrading bacteria by almost twelve times after 30 days. However, MPN



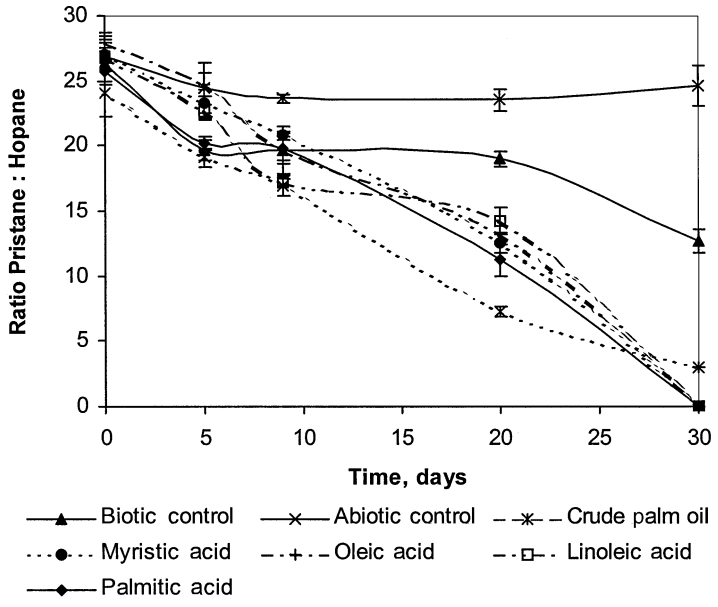
(a)



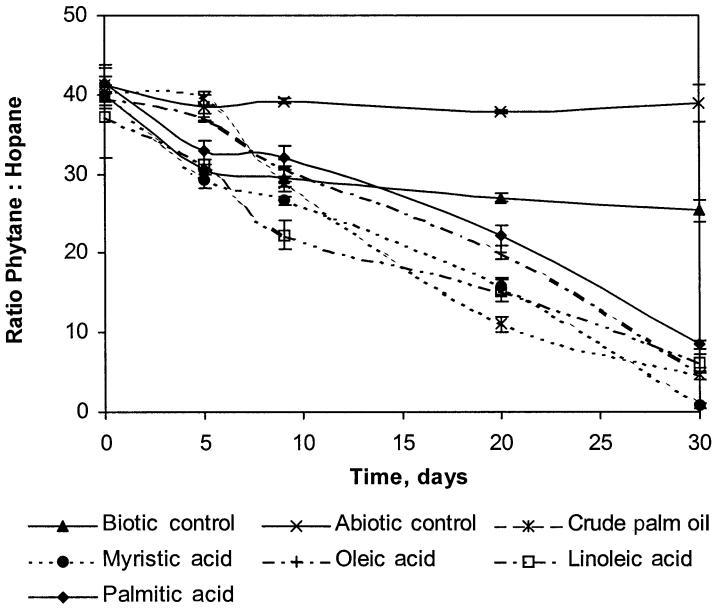
(b)

Figure 2. (a) Ratio of straight alkanes (C_{17}:hopane) for each of the sediments over 30 days. (b) Ratio of straight alkanes (C_{18}:hopane) for each of the sediments over 30 days.

was increased by 26 times in sediment that also received CPO (Table I), thereby confirming the benefits of an additional organic carbon source, in the presence of inorganic nutrients, on stimulating the population size of indigenous hydrocarbon degrading bacteria. However, the addition of single fatty acids alone resulted in the



(a)



(b)

Figure 3. (a) Ratio of branched alkanes (pristine:hopane) for each of the sediments over 30 days. (b) Ratio of branched alkanes (phytane:hopane) for each of the sediments over 30 days.

TABLE II

Loss of C_{17}, C_{18}, pristane and phytane after 30 days. Means and standard deviations of triplicate samples are shown[a]

Sediment treatment	Loss of C_{17}(%)	Loss of C_{18}(%)	Loss of pristane (%)	Loss of phytane (%)
Oil-spiked control (biotic)	77.2 ± 0.9	79.8 ± 1.6	52.0 ± 3.3	36.4 ± 5.4
Oil-spiked control (abiotic)	2.4 ± 0.1	1.4 ± 1.6	1.0 ± 0.9	0.5 ± 1.5
Crude palm oil	89.8 ± 1.2	88.9 ± 0.7	87.4 ± 1.0	88.6 ± 1.5
Myristic acid	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	98.0 ± 0.9
Oleic acid	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	87.8 ± 1.4
Linoleic acid	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	83.5 ± 1.0
Palmitic acid	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	79.6 ± 2.3

[a]All sediments were amended with nutrients at a ratio of C:N:P = 100:10:1.

greatest enhancement in the population size of hydrocarbon degrading bacteria. All fatty acids resulted in an MPN enhancement of approximately 170 times relative to the biotic control, with the exception of linoleic acid that increased MPN by approximately 90 times over the duration of the experiment.

4.2. DEHYDROGENASE ACTIVITY

Microbial DHA in the oil-spiked abiotic control was absent (Figure 1), meaning any alkane loss in this sediment was solely due to abiotic losses. In contrast, DHA in the oil-spiked biotic control continued to increase over the duration of the experiment reaching a peak of approximately 27 μg INTF g dry sed^{-1} h^{-1} on day 30. This increase in metabolic activity was likely a response of the microbial biomass to the presence of inorganic nutrients in the sediment. Additions of CPO, myristic, oleic, linoleic and palmitic acids further enhanced DHA relative to the biotic control, at least up to day 20. Although there was a reduction in DHA in all sediments treated with fatty acids, apart from palmitic acid, on day 20, levels did not significantly differ to levels at day 30. For these treatments, DHA increased rapidly and peaked at approximately 30 μg INTF g dry sed^{-1} h^{-1} on day 10, with the exception of sediment treated with palmitic acid where DHA continued to increase to approximately 25 μg INTF g dry sed^{-1} h^{-1}on day 30.

4.3. STRAIGHT ALKANE LOSS

The ratios of the straight alkanes C_{17} and C_{18}relative to the stable hopane biomarker in the abiotic control were almost constant throughout the experiment (Figures 2a and 2b), meaning that negligible abiotic loss of straight alkanes occurred following

the initial weathering of oil-spiked sediment (Table II). The slight differences in C_{17} and C_{18} levels between different treatments on Day 0 of the experiment were probably due to experimental errors and these differences are not significant ($P > 0.05$). Losses of straight alkanes, C_{17} and C_{18}, in the nutrient-amended biotic control were significantly greater than the abiotic control ($P = 0$ and 0.003, respectively), and a total loss of 77.2% of C_{17} and 79.8% of C_{18} by day 30 indicates the beneficial effect of nutrient addition alone. The losses of C_{17} and C_{18} in the sediment treated with CPO after 30 days were not significantly different from the biotic control ($P = 0.011$ and 0.019, respectively). The addition of CPO increased the DHA of the microorganisms, but did not enhance degradation of *n*-alkanes.

In contrast to CPO amended sediment, C_{17} and C_{18} alkanes were completely degraded in sediments treated with individual fatty acids in the presence of inorganic nutrients (Table II). This enhanced loss is supported by the higher levels of DHA and the MPN of hydrocarbon degrading bacteria in sediments treated with fatty acids alone (Table I and Figure 1). The addition of a relatively low amount (i.e., 0.5%) of fatty acid as a simple carbon source was therefore sufficient to significantly enhance biodegradation of straight alkanes by hydrocarbon degrading bacteria in nutrient-amended sediments. The presence of fatty acids provided an easily assimilative alternative growth substrate that stimulated the indigenous microbial biomass and increased the biodegradation rate of alkanes via co-metabolism. It has been reported that supplemental carbon additions may allow for mixed substrate utilization, increases in overall biomass and reduced per-microbe toxin concentrations, hence enhancing biodegradation rates (Baker and Herson, 1994). Sorption of petroleum hydrocarbons to sediment particles reduces their availability to microorganisms, and hence reduces their biodegradability. The presence of fatty acids can enhance the biodegradation of n-alkanes as the fatty acids act as nonionic surfactants, which increase the oil-water surface area to improve the bioavailability of petroleum hydrocarbons for microbial actions (Bruheim *et al.*, 1997; Riis *et al.*, 2000).

4.4. Branched alkane loss

The relative losses of the branched alkanes (i.e., pristane and phytane) in the nutrient-amended biotic control were significantly lower than in the abiotic control on day 30 ($P = 0.001$ and 0.003, respectively), with losses of pristane and phytane at 52% and 36.4% respectively (Table II). In fact, almost no pristane and phytane was lost throughout the 30-day period in the abiotic control. Biodegradation of pristane and phytane commenced between 5 and 9 days after initiation of the experiment, and the lower relative loss of phytane compared to pristane is most likely due to its longer carbon chain length, and hence recalcitrance (Figures 3a and 3b). Degradation of pristane and phytane in our study confirms their limitation as biomarkers in hydrocarbon degradation studies due to their own susceptibility to biodegradation (Prince *et al.*, 1994).

The amendment of CPO as a simple carbon source to sediment, in the presence of inorganic nutrients, significantly enhanced the degradation of pristane and phytane relative to the biotic control ($P = 0.005$ and 0.006, respectively), and losses over 30 days were 87.4% and 88.6% respectively (Table II). Pristane loss was complete in all fatty acid amended sediments. An increase in the degradation rate of pristane and phytane occurred after day 20 when the rate of C_{17}, C_{18} degradation had slowed and the amount of C_{17}, C_{18} left in the sediment was low (Figures 2 and 3). This may represent a metabolic switch in the utilisation of simple to more complex hydrocarbons by the microbial biomass. The introduction of branching into the hydrocarbon molecule hinders biodegradation (Baker and Herson, 1994) and generally, degradation of branched-chain alkanes is repressed by the presence of straight chain alkanes (Del'Arco and de Franca, 2001). The effects of different fatty acids on the biodegradation of phytane were notable, where the loss of phytane in sediment amended with myristic acid was significantly greater than sediments treated with linoleic or palmitic acid (Table II; $P = 0.004$ and 0.009, respectively). The difference in phytane loss between myristic and oleic acid treatments was not so significant ($P = 0.014$). In contrast, the differences between treatments of oleic, linoleic and palmitic acid were not significant ($P > 0.05$).

Overall, this study has shown that the amendment of CPO, as a readily available carbon source, significantly enhanced the population size of hydrocarbon degrading bacteria and also increased the metabolic activity of the entire indigenous microbial biomass in the oil contaminated sediments under laboratory conditions. The addition of CPO to further enhance biodegradation under tropical conditions is therefore beneficial for a bioremediation program. This is consistent with recent studies that have highlighted the beneficial effects of providing an additional carbon source to the indigenous microbial biomass (Santas *et al.*, 1999; Miller and Mudge, 1997; Nelson *et al.*, 1996). However, the addition of myristic, oleic, linoleic and palmitic fatty acids, in the presence of inorganic nutrients, resulted in higher rates of biodegradation of straight and branched alkanes. Sveum *et al.* (1994) showed that the supply of external carbon in additives was important for the assimilation and retention of essential nutrients in microbial biomass, thereby reducing nutrient loss in an open beach environment. The presence of fatty acids may also accelerate the biodegradation rate of crude oil due to their surfactant properties, and their ability to enhance the bioavailability and dispersion of hydrocarbons (Riis *et al.*, 2000; Nelson *et al.*, 1996). Indeed, a combination of nutritional and surfactant properties in bioremediation additives is recognised as an advancement in petroleum hydrocarbon remediation (Santas *et al.*, 1999).

CPO represents a more complex carbon source than individual fatty acids, and in our study this may have resulted in a delay in hydrocarbon loss compared to sediments treated with component fatty acids alone, as well as a less beneficial effect on the MPN of hydrocarbon degrading bacteria and the DHA of the microbial biomass. Although Sveum *et al.* (1994) showed that microorganisms metabolised both fish-meal additive and hydrocarbons simultaneously in the bioremediation of

oil-spiked sediments, albeit at different rates, a switch to hydrocarbon metabolism was dependent on the total amount of additive supplied to the sediment.

5. Conclusion

In this study, all the fatty acids (i.e., myristic, oleic, linoleic or palmitic acid) and CPO, as a supplementary carbon source, significantly enhanced the MPN and DHA of microbial biomass. All fatty acids also significantly elevated the biodegradation of n-C_{17}, n-C_{18}, pristane, and phytane in oil-contaminated sediments amended with nutrients relative to a biotic and abiotic control ($P < 0.01$), where myristic acid was the most effective among all the fatty acids. CPO significantly enhanced the biodegradation of pristane and phytane ($P < 0.01$). The addition of simpler component fatty acids alone is more effective in stimulating oil biodegradation rates than CPO. Since fatty acids can act as both a co-substrate and a nonionic surfactant (Bruheim *et al.*, 1997; Riis *et al.*, 2000), further investigations are planned to determine their effects on the biodegradation rates of polycyclic aromatic hydrocarbons (PAHs) which are less bioavailable and more recalcitrant than alkanes in oil-contaminated sediments.

References

Alexander, M.: 1991, 'Research needs in bioremediation', *Environ. Sci. Technol.* **25**, 1972–1973.

Atlas, R. M.: 1991, 'Microbial degradation of petroleum hydrocarbons: bioremediation of oil spills', *J. Chem. Technol. Biotechnol.* **52**, 149–156.

Baker, K. H. and Herson, D. S.: 1994, *Bioremediation*, McGraw-Hill, New York, pp. 210–231.

Brown, E. J. and Braddock, J. F.: 1990, 'Sheen Screen, a miniaturized most-probable-number method for enumeration of oil-degrading microorganisms', *Appl. Environ. Microbiol.* **56**, 3895–3896.

Bruhein, P., Bredholt, H. and Eimhjellen, K.: 1997, 'Bacterial degradation of emulsified crude oil and the effect of various surfactants', *Can. J. Microbiol.* **43**, 17–22.

Butler, E. L., Douglas, G. S., Steinhauer, W. G., Prince, R. C., Aczel, T., Hsu, C. S., Bronson, M. T., Clark, J. R. and Lindstrom, J. E.: 1991, 'Hopane, a New Chemical Tool for Measuring Oil Biodegradation', in R.E. Hinchee and R.F. Olfenbuttel (eds), *On-Site Bioreclamation: Processes for Xenobiotic and Hydrocarbon Treatment*, Butterworth-Heinemann, Boston, pp. 515–521.

Cochran, W. G.: 1956, 'Estimation of bacteria densities by means of the 'Most Probable Number'', *Biometrics* **5**, 95–105.

Del'Arco, J. P. and de Franca, F. P.: 2001, 'Influence of oil contamination levels on hydrocarbon biodegradation in sandy sediment', *Environ. Pollut.* **110**, 515–519.

Eaton, A. D., Clesceri, L. S. and Greenberg, A. E.: 1995, *Standard Methods for the Examination of Water and Waste Water*, 19th edn., Maryland-APHA, pp. 5-34–5-35.

Korda, A., Santas, P., Tenente, A. and Santas, R.: 1997, 'Petroleum Hydrocarbon Bioremediation: Sampling and Analytical Techniques, in situ treatments and commercial microorganisms currently used', *Appl. Microbiol. Biotechnol.* **48**, 677–686.

Lee, K. and Merlin, F. X.: 1999, 'Bioremediation of oil on shoreline environments: development of techniques and guidelines', *Pure Appl. Chem.* **71**, 161–171.

Lee, S. M., Jung, J. Y. and Chung, Y. C.: 2000, 'Measurement of ammonia inhibition of microbial activity in biological wastewater treatment process using dehydrogenase assay', *Biotechnol. Lett.* **22**, 991–994.

Mathew, M., Obbard, J. P., Ting, T. P., Gin, Y. H. and Tan, H. M.: 1999, 'Bioremediation of oil contaminated beach sediments using indigenous microorganisms in Singapore', *Acta Biotechnol.* **19**, 225–233.

Mathew, M. and Obbard, J. P.: 2001, 'Optimization of deydrogenase assay for petroleum contaminated beach sediments', *Biotechnol. Lett.* **23**, 227–230.

Mearns, A. J.: 1997, 'Cleaning oiled shores: Putting bioremediation to the test', *Spill Sci. Technol. Bull.* **4**, 209–217.

Miller, N. J. and Mudge, S. M.: 1997, 'The effect of biodiesel on the rate of removal and weathering characteristics of crude oil within artificial sand columns', *Spill Sci. Technol. Bull.* **4**, 17–33.

Mudge, S. M. and Pereira, G.: 1999, 'Stimulating the biodegradation of crude oil with biodiesel preliminary results', *Spill Sci. Technol. Bull.* **5**, 353–355.

Nelson, E. C., Walter, M. V., Bossert, I. D. and Martin, D. G.: 1996, 'Enhancing biodegradation of petroleum hydrocarbons with guanidinium fatty acids', *Environ. Sci. Technol.* **30**, 2406–2411.

Palm Oil Research Institute of Malaysia: 1985, *Palm Oil Factory Process Handbook*.

Prince, R. C., Elmendorf, D. L., Lute, J. R., Hsu, C. S., Halth, C. E., Senlus, J. D., Dechert, G. J., Douglas, G. S. and Butler, E. L.: 1994, '17α(H), 21β(H)-Hopane as a conserved internal marker for estimating the biodegradation of crude oil', *Environ. Sci. Technol.* **28**, 142–145.

Ratledge, C.: 1994, 'Biodegradation of Oils, Fats and Fatty Acids', in C. Ratledge (ed.), *Biochemistry of Microbial Degradation*, Kluwer Academic Publishers, Boston, MA, USA, pp. 89–141.

Riis, V., Brandt, M., Miethe, D. and Babel, W.: 2000, 'Influence of special surfactants on the microbial degradation of mineral oils', *Chemosphere* **41**, 1001–1006.

Santas, R., Korda, A., Tenente, A., Buchholz, K. and Santas, P. H.: 1999, 'Mesocosm assay of oil spill bioremediation with oleophilic fertilizers: inipol, F1 or both?', *Mar. Pollut. Bull.* **38**, 44–48.

Sveum, P., Faksness, L. G. and Ramstad, S.: 1994, 'Bioremediation of Oil-contaminated Shorelines: The Role of Carbon in Fertilizers', in R. E. Hinchee, B. C. Alleman, R. E. Hoeppel and R. N. Miller (eds), *Hydrocarbon Bioremediation*, Lewis, Boca Raton, FL, USA, pp. 163–174.

Sveum, P. and Ramstad, S.: 1995, 'Bioremediation of Oil on Shorelines with Organic and Inorganic Nutrients', in R.E. Hinchee, J.A. Kittel and H.J. Reisinger (eds), *Applied Bioremediation of Petroleum Hydrocarbons*, Battelle Press, Columbus, OH, USA, pp. 201–217.

Tan, B. K. and Oh, F. C. H.: 1981, 'Malaysian palm oil chemical and physical characteristics', *PORIM Technol.* **3**, 2–3.

USEPA Method No. 413.3: 1983, *Oil and Grease, Gravimetric Method.*

USEPA Method No. 418.1: 1983, *Total Recoverable Petroleum Hydrocarbon, Method for Chemical Analysis of Water and Waste*.

Venosa, A. D., Suidan, M. T., King, D. and Wrenn, B. A.: 1997, 'Use of hopane as a conservative biomarker for monitoring the bioremediation effectiveness of crude oil contaminating a sandy beach', *J. Ind. Microbiol. Biotechnol.* **18**, 131–13.

Wang, Z., Fingas, M. and Li, K.: 1994, 'Fractionation of a light crude oil and identification and quantitation of aliphatic, aromatic, and biomarker compounds by GC-FID and GC-MS, Part I', *J. Chromatogr. Sci.* **32**, 361–366.

Wrenn, B. A., Suida, M. T., Strohmeier, K. L., Eberhardt, B. L., Wilson, G. J. and Venosa, A. D.: 1997, 'Nutrient transport during bioremediation of contaminated beaches: evaluation with lithium as a conservative tracer', *Water Res.* **31**, 515–524.