BIODEGRADATION OF FRACTIONATED CRUDE OIL BY ESTUARINE BIOFILMS IN THE WETLAND

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ABSTRACT

Four separate mesocosm experiments (A-D) were carried out over a period of four months involving impactation of microalgal mats in cores at two levels of petroleum hydrocarbons (PHC) concentrations and nutrient enrichment (using nitrate and phosphate) at three levels of concentrations in the light and in the dark. The non-enriched and oil spiked biofilms indicated a state of increased sequestration or 'shock' probably due to the added oil in the absence of added nutrients. The effect of periodic variation at low and high oil-spiking in the light and in the dark, indicated that there was decrease in PHC towards the 14th day ($P = < 0.001$) in the controls treatments. The overall assessments indicated that the treatment of choice after correction to baseline status, at low oil impactation was in the dark. This was at minimal nitrate and moderate phosphate enrichment which was 60% of baseline value. At higher oil impactation it was also in the dark at moderate nitrate and maximum phosphate enrichment which was approximately 110% of the baseline value on the 14th day. From these observations it was suspected that a negative effect could develop from excessive enrichment with nitrate for maximum biodegradation of PHC in the environment during bioremediation. There is presently no standard for the enrichment regimes for bioremediation

exercises. There was however an indication of increased dependence on nitrate and especially phosphate for more effective oil bioremediation in the dark and this dependence appears to increase with the concentration of spiked oil. What triggers accelerated metabolism in the dark is still largely unknown; nevertheless, it is an important factor for the survival of the primary producers in an increasingly oil-polluted marine ecosystem. It is most likely that this is probably one of the most important mechanisms in the protective cascade used by the biofilms (especially when challenged with high concentrations of pollutants such as PHC) to evade destruction from toxic contaminants.

KEY WORDS: Petroleum hydrocarbon, poly aromatic compounds, sequestration, biofilms, total fluorescence materials, petrogenic hydrocarbons.

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1.0 INTRODUCTION

Assessment of biodegradation potential of the estuarine biofilms on PHC was based on the manipulative parameters and treatments viz. the amount of added oil and nutrients; the effect of continuous light and dark phases on the degradative capability of the biofilms. The data was collected at set times on the loss or gain of PHC.

2.0 MATERIALS AND METHODS

2.1. Gross assessment of environmental PHC pollutants

The experimental design and mesocosms setup are as shown in the General Materials and Methods and General Introduction. The use of the shake spectrofluorimetry techniques for the assessment of oils and greases was used. Petroleum hydrocarbons, especially the poly aromatic

hydrocarbons (PAH's), have been monitored using the spectrofluorimetry techniques. There is increasing interest in the use of simple spectrofluorimetric methods for the widespread continuous and real-time monitoring of oil-pollution. Spectrofluorimetric methods have been used in various applications in the assessment of the quantity and quality of petroleum hydrocarbons in water and sediment. Vandermeulen *et al*., (1979) and Law (1978), after the *Amoco Cadiz* Oil-spillage, applied spectrofluorimetric methods for the assessment of inshore freshly spilled oil. Picer and Hocenski, (1994), used a simple and rapid fluorescence procedure based on PAHs, to estimate PHC in organisms and marine sediment. Vo-Dinh and White, (1986), used sensitized luminescence method (based on anthracene) to detect trace amounts of PAH. Morrel *et al,* 1991, compared various methods used for PHC assessment. The methods include the Synchronous Scanning Fluorescence Spectroscopy (SSFS); High-Performance Liquid Chromatography (HPLC) on C-18 reversed-phase and NH_2 normal-phase columns with UV and fluorescence detectors; Gas Chromatography on fused-silica capillary columns (GC) with flame ionization detector (FID), mass spectrometer (MS) and Flame Photometric Detector (FPD); and High-Resolution Molecular Spectrofluorimetry in Shpol'skii matrix at 10 K (HRSS). The different methods were found to give complementary information. SSFS was useful for fast evaluation and preliminary assessment of oil pollution. In this study, the ambient temperature spectrofluorimetric technique was applied to enable continuous and real time assessment of PHC.

2.2. Ambient temperature assessment of fractionated crude oil

The ambient temperature technique was applied for the quantitative and qualitative assessment of sedimentary petroleum hydrocarbons in the mesocosms. Assessment of petroleum hydrocarbons was carried after 0 hour, and on days 3, 7 and 14. Each time, 0.1g freeze-dried sediment from the top 2-3 mm of the cores in triplicates were made into slurry in washed centrifuge tubes using 2 ml of 2% saline to which was added 0.5 ml of solvent (dichloromethane). The slurry was mixed thoroughly for 2 min on a stirrer and then centrifuged at 4200g for 15 minutes; the

solvent phase was carefully siphoned into clean glass test tube using Pasteur pipette. The extract was covered with aluminium foil and allowed to dry at ambient temperature in a fume chamber. The Total Fluorescent Mixture (TFM) and Total Non–polar Fluorescent Mixture (NFM i.e. petrogenic PHC) were determined as follows: To each dried extract was added 5.0 ml *n*-hexane; and the TFM fluorescence measured using the LS50 Perkin-Elmer spectrofluorimeter. Using 1.0 cm quartz curvette; the excitation wavelength was set at 300 nm while the emission was set at 395 nm and the slit widths set at 10 nm for a linear concentration response in the range of 0.1-10 µg ml^{-1} of PHC in *n*-hexane. A predetermined blank value was corrected for all values in the assessments. Caution was taken to avoid siphoning of water along with the solvent phase in order to avoid delay in the drying process in the fume chamber. The TFM was then eluted through a column of silica-gel (40-80 Å mesh, i.d. = 5 cm; l =15 cm) using two instalments of 5 ml *n*-hexane. The fluorescence of the pooled eluent which is the total non-polar fluorescence mixture was measured as the petrogenic fraction with highest intensity between 310-374 nm using Nigerian crude oil (Keizer and Gordon, 1973; Parsons, 1985). This procedure was repeated at 0 hour, and on the 3rd, 7th and 14th day.

3.0 RESULTS

3.1 Petroleum hydrocarbon.

The petroleum hydrocarbon baseline status of each mesocosm setup is shown in Fig 1; the effect of periodic variation in the mesocosm PHC assessment in the light and in the dark is as shown in Fig 2 while correction of results as percent baseline value for each mesocosm is as shown in Fig 3.

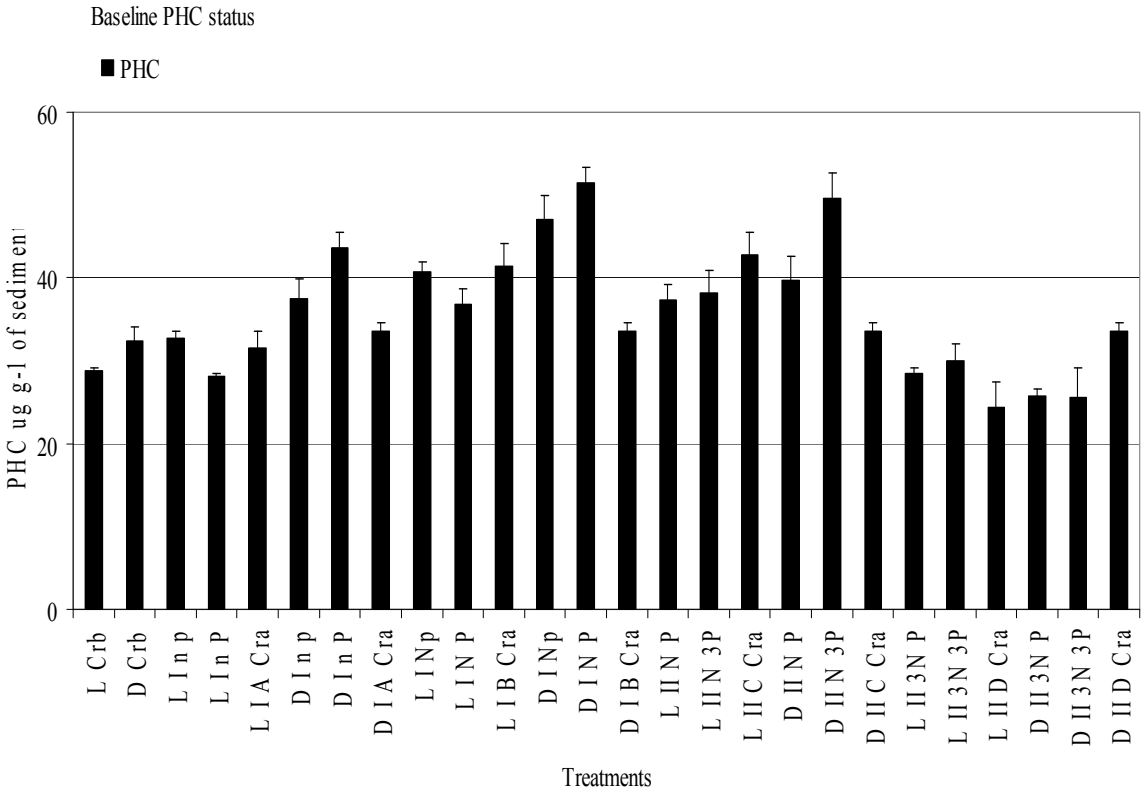

Fig. 1: Baseline petroleum hydrocarbon status (µg g^{-1} of sediment) of mesocosm sediment samples.

LCrb & DCrb: Baseline PHC status (in the light & dark respectively) of mesocosms.
Experiment A: *LInp, LInP, LIACra; DInp, DInP, DIACra (P = <0.001)*
Experiment B: *LINp, LINP, LIBCra; DINp, DINP, DIBCra (P = <0.001).*
Experiment C: *LIINP, LIIN3P, LIICCra; DIINP, DIIN3P, DIICcra (P = <0.001).*
Experiment D: *LII3NP, LII3N3P, LIIDCra; DII3NP, DII3N3P, DIIDCra (ns);* Mean (± S.E; n = 3).

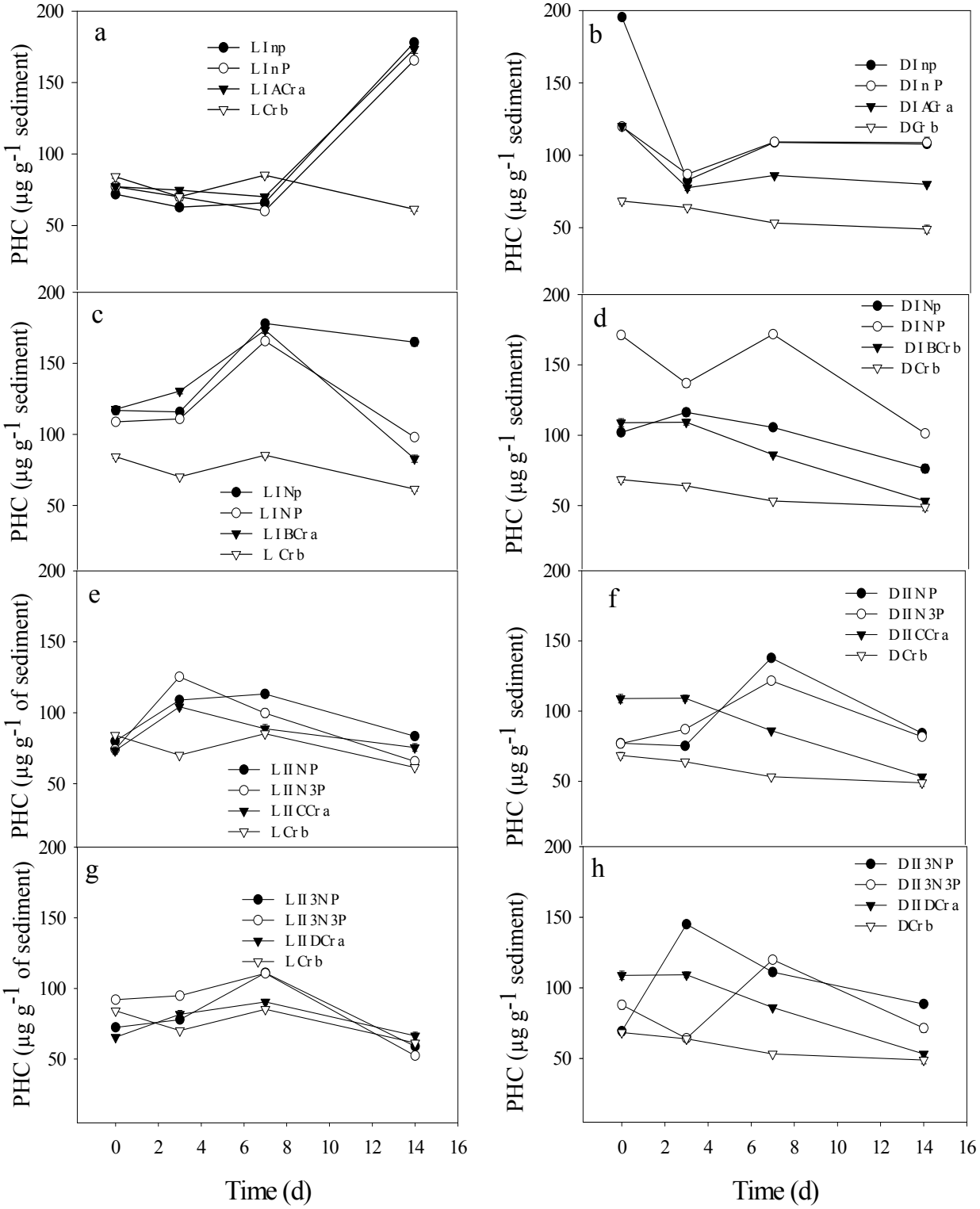

Fig 2: Effect of periodic variation on estuarine biofilms PHC degradation.

Experiment A: *LInp; LInP; LIACra; DInp; DInP; DIACra;*
Experiment B: *LINp; LINP; LIBCra; DINp; DINP; DIBCra;*
Experiment C: *LIINP; LIIN3P; LIICCra; DIINP; DIIN3P; DIICCra;*
Experiment D: *LII3NP; LII3N3P; LIID Cra; DII3NP; DII3N3P; DIICCra;* Mean (± S.E; n = 3).

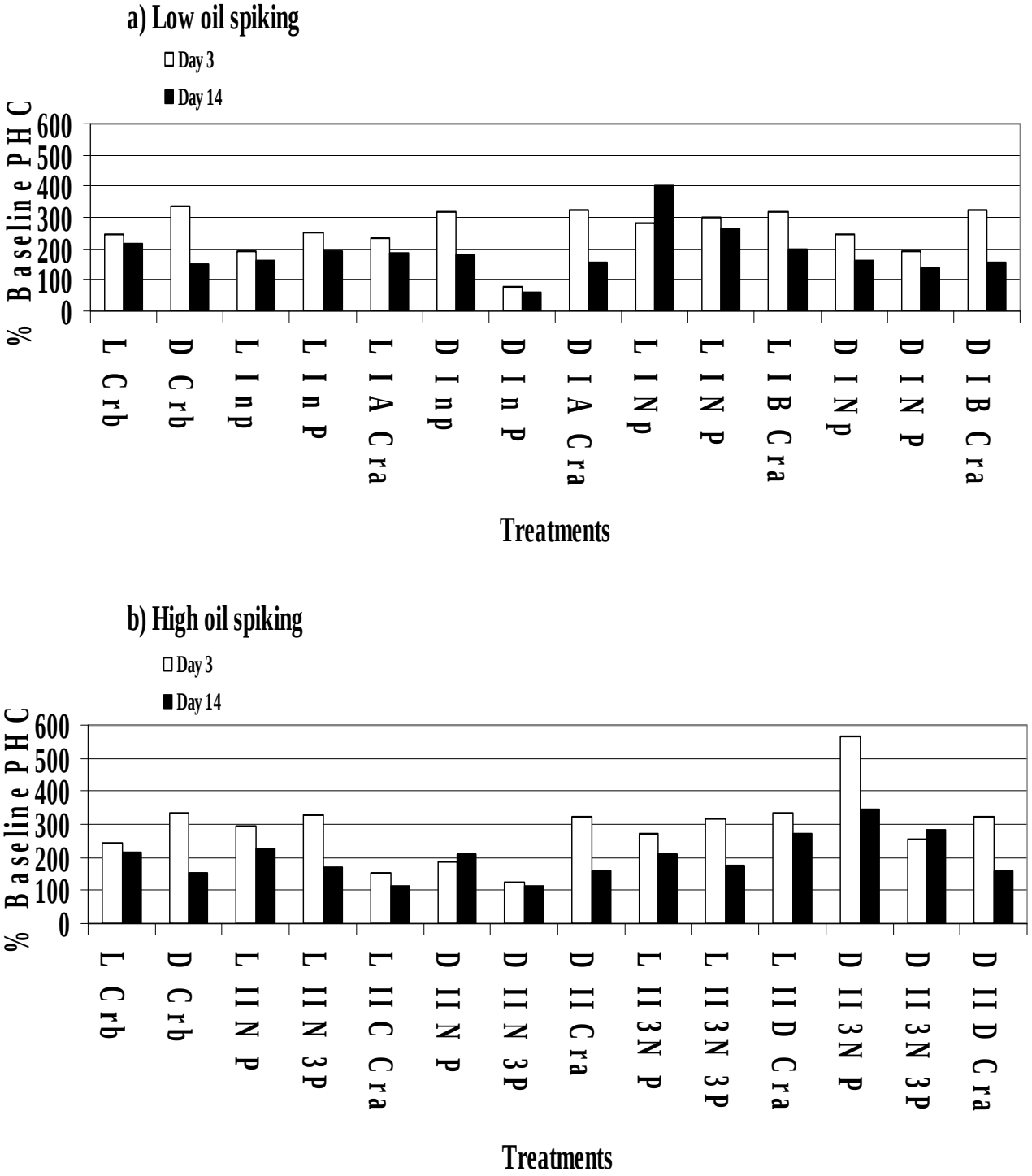

Fig. 4: Percent correction to baseline values of PHC at a) low oil and b) high oil spiking:
LCrb & DCrb: Baseline PHC status (light & dark) of mesocosms before PHC was added.
Experiment A: *LInp, LInP, LIACra; DInp, DInP, DIACra*
Experiment B: *LINp, LINP, LIBCra; DINp, DINP, DIBCra*
Experiment C: *LIINP, LIIN3P, LIICCra; DIINP, DIIN3P, DIICcra*
Experiment D: *LII3NP, LII3N3P, LIIDCra; DII3NP, DII3N3P, DIIDCra*

3.2. Assessment of biofilms bioremediation potential of residual and spiked oils.

Four batches of experiments (A, B, C and D) were setup according to the schedule in Table 1. PHC biodegradation by the estuarine biofilms, with or without added nutrients and the effect of continuous light and dark treatments on the degradation capability of the biofilms was assessed. The baseline value of the mesocosms (Fig 1) indicated significant differences in the background PHC in three of the Experiments (A, B and C, P = <0.001) before treatment was started. Results were therefore corrected as percentages of their baseline values (Fig 3).

3.2.1. Biofilms Response I & II (Crb & Cra)

The effect of periodic variation at low oil-spiking in Experiment A (Fig 2 a), in the light indicated that all the oil impacted treatments including the control *Cra*, were significantly higher in oil than the residual oil (*Crb*) at day 14 (P = <0.001). In the dark, in Fig 2 (b), at day 7 however, there was apparent release of oil, probably due to increase in the bioavailability of PHC, followed by decrease towards the 14th day. Oil was significantly more reduced (P = < 0.001) in the controls *Cra* and *Crb*. A comparison of the effect of light and dark on the control *Crb* and *Cra* indicated that there were significant differences in PHC at day 14 (P = <0.001) and this was lower in the dark.

At higher oil-spiking (Experiments C & D in Fig 2 e-h) the effect of high oil spiking and high nutrient enrichment was assessed for biofilms oil degradation. There was apparent initial sequestration in the light but from days 3 to 14, there was steady decrease in PHC. In the light in the presence of limited nutrient enrichment, the Biofilm Response I was similar to that at low oil impaction in which the control treatments *Crb* and *IICra* were lower than the other treatments in PHC.

3.2.2. Biofilms Response III

A comparison of the treatments effect after correction to baseline values for each treatment in Experiment A as shown in Fig 4 (a) appears to indicate that the treatment *DInP* at low oil spiking was the choice treatment in which oil was reduced from 80% on day 3, to 60% of the baseline

residual oil within 14 days. With increased nutrient enrichment in the light, at higher oil-spiking, the treatments *LII3N3P* and *LII3NP* were lower than the control treatments *Crb* and *IICra* in PHC. The response at higher oil-spiking in the dark was similar to that at low oil spiking indicating the enormous bioremediative effect of nutrient enrichment over the *status quo* in the non-enriched naturally occurring bioremediative activity in the dark.

After correction to baseline value in Fig 3 b, the treatment of choice in the light was *LIIN3P* which was 180% baseline value. In the dark (Fig 2 f) from day 7 to 14, there was steady decrease in PHC; in which the control *LCIICra* and the treatment *DIIN3P* were lowest (105% each) on the 14th day; PHC ranging from 49.0- 137.9 μg g^{-1} of sediment. The oil spiked and enriched treatment of choice in the light and dark phases at high oil spiking was *DIIN3P*, and this was 105% of the baseline value.

5.0 Discussion

The reduced PHC assessed in the baseline status (or residual Response I) of the biofilms in the light was not unexpected since the other treatments were spiked with oil. The oil spiked biofilms without enrichment (in the biofilms Response II) appear to indicate lower oil concentration thus indicating a state of increased sequestration in the dark since the biofilms might have been 'shocked' due to the added oil in the absence of added nutrients. The fundamental response of biofilms in the absence of added nutrient is to exhibit '*shock*' followed by the exudation of much EPS especially under pollutant concentrations that are toxic in the environment (Karthikeyan et al., 1999; Kigigha and Underwood, 2010). The occurrence of this phenomenon during oil-bioremediation exercises in the field could be seen as a 'mystery' because while initial clean-up might have correctly observed and recorded reduction in impacting oil (arising from sequestration), assessment of the same field at a later time (in less than one week) could indicate increased assessed oil (due to bioavailability) in attempting to verify the results of the initial clean-up. Furthermore, this observation became evident

because of comparison of the light and dark responses of the biofilms. The effect of nutrient enrichment in greatly improving reduction in impacting oil in the environment was very evident (Lessard *et al*., 1995). This appears to suggest that under conditions of low or high oil impactation, enrichment would be required for the most effective bioremediation in the dark especially. The effect of darkness in enhancing storage and metabolism of carbohydrates had been observed in plants and the microphytobenthos (Underwood and Smith, 2000).

Environmental and ecological factors have been known to affect contaminant biodegradation. In the dark however, there was evidence of biodegradation which indicated that some non-photosynthetic probably heterotrophic organisms may be also involved in PHC biodegradation. Furthermore, this would suggest a probable diauxic response in which there was a switch to utilize carbon that was available in the added PHC by heterotrophic biofilms population or from the environment in the absence of carbon derived from the light reactions of photosynthesis. Alternatively it could infer increased toxicity in the light due to release of photo-oxidation subunits of PHC which are probably more toxic to the photosynthetic biodegradation organisms but less so to the heterotrophic population (Miles, 1994).

At low oil impactation in the presence of increased nutrient enrichment sequestration appears to have been reduced. In the light at maximum enrichment at low PHC, the treatment *LINP* was the choice treatment for biodegradation in the light after correction to baseline values in which PHC, was reduced from about 200% on day 3, to about 160% on the 14^{th} day. This was in agreement with the observation by a number of authors in which nitrate and phosphate enrichment caused 3 to 4 times increase in the population of bacteria and has been the reason for the use of oleophilic fertilizers in oil-spill bioremediation (Lessard *et al*., 1995). The treatment *DInP* (60% of baseline value) was the overall choice treatment both in the light and in the dark at low oil impactation. In the dark there was a clearer indication of increased biodegradation of PHC by the estuarine biofilms under natural conditions. At day 14, there was no significant difference between low oil spiking

without added nutrients and the control in which only residual oil in the sediment was degraded within the period of experimentation. Bacteria from sites that had history of previous oil contamination have been shown to demonstrate enormous potential of (about hundred-fold) degrading "or probably sequestering" freshly-added oil when compared to pristine sites. How this is brought about is still largely speculative especially for the enriched treatments (Atlas, 1981a; Gunkel and Gassman 1980; Carman et al., 2000). This appears to point to the vaguely understood typical mode of existence of the biofilms in which they elaborate copious exudation of EPS (Smith and Underwood, 2000); elaborate PHA (Rothermich et al, 2000) in the dark. What triggers this accelerated metabolism in the dark is still largely unknown; nevertheless, it is an important factor for the survival of the primary producers in an increasingly oil-polluted marine ecosystem. It is most likely that this is probably one of the most important mechanisms in the protective cascade used by the biofilms (especially when challenged with high concentrations of pollutants such as PHC) to evade toxic contaminants (i.e. to sequester them for decades and gradually degrade them).

At increased oil impactation with limited enrichment, there was sequestration of PHC till the 14th day. The biofilms were clearly aided to significantly reduce PHC with increasing enrichment in which the treatment *LIIN3P* after correction to baseline value was the treatment of choice in the light. Enrichment has been known to reduce bio-availability period of contaminants in the ecosystem. It appears to imply that the biofilms were hindered in the light and that increased enrichment was needed to improve their PHC biodegradation ability (Lessard *et al*., 1995). In this study, also this was further confirmed in the dark in which the treatment *DIIN3P* (which was approximately 110% of the baseline value on the 14th day) after correction to baseline value was the overall choice treatment in the dark and in the light.

After correction to baseline status, the treatment *LIIN3P* caused the lower biodegradation of PHC in the light. Nevertheless, the overall assessments indicated that the treatment of choice after correction to baseline status, at high oil impactation was *DIIN3P* which was

approximately 110% of the baseline value on the 14th day. Even at low oil impactation it was the treatment *DInP* (60% of baseline value) that was the overall treatment of choice in the light and in the dark. From these observations it is suspected that a negative effect could develop from excessive enrichment with nitrate for maximum biodegradation of PCH in the environment during bioremediation. There is presently no standard for the enrichment regimes for bioremediation exercises. There was however an indication of increased dependence on phosphate for more effective oil bioremediation in the dark and this dependence appears to increase with the concentration of spiked oil.

A summary of the biodegradation activity of the estuarine biofilms on fractionated crude oil in the wetland appear to suggest the following:

1. *That baseline response indicated that biodegradation of PHC was more effective in the dark than in the light.*
2. *That while nutrient enrichment indicated obvious reduction in PHC in the light and in the dark; observed reduction in the oil-spiked non-enriched control treatment appears to have been caused by sequestration due the probable presence of EPS and or due to other unknown factors in the sediment.*
3. *That interpretation of field assessment bioremediation should be done cautiously in view of the effects of estuarine sedimentary and biofilms sequestration and probable bioavailability (with time) of oil in the wetlands.*
4. *That at low oil-spiking, PHC biodegradation appears to require low nitrate and moderate phosphate enrichment in the dark; for higher oil-spiking nutrient application regime increased to moderate nitrate to high phosphate enrichment also in the dark.*

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