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Laboratory-Scale Degradation of Crude oil in a Simulated Low Energy Environment

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Abstract:
A 35- day study was carried out to simulate the effect of nutrient release on the rate of crude oil degradation/removal in low energy environments, using granular and capsular slow release fertilizers. Hydrocarbon degradation was simulated in a 500ml capacity Erlenmeyer flask containing 200ml mineral salt solution and 0.5 ml contaminant (crude oil) and seeded with suspensions of a 24-h old pure cultures of bacterial isolates identified as Pseudomonas sp., Bacillus sp. and Micrococcussp. Agar agar (type 1) was used in the production of the slow release fertilizer. During the35-day period of study, a strong positive correlation existed between bacterial biomass and the nutrient source (nitrate nitrogen) (r = 0.530), while a strong negative correlation was observed between bacterial biomass and total hydrocarbon content (r = -0.957) was observed, using the 2-tailed Pearson's correlation model. Also, statistical analysis using the one-way analysis of variance showed there was a significant difference in the mean values of the various treatment options, A – D, compared to the control E (p>0.05). NPK discovered to be a better source of nutrient than urea for the production of slow release fertilizers for use under this condition. Also, the granular slow release fertilizer (SRF) supported more bacterial growth rate and a concomitant hydrocarbon removal than the capsular SRF. Granular SRF is recommended for use under this condition for optimal results. This is an important achievement in environmental biotechnology as the use of marine biopolymer is innovative and the granular SRF is as well a close substitute to direct fertilizer application, having the potentials of sustained supply of nutrient in a controlled fashion.

Keywords: *Crude oil, low energy environment, simulate, biomass, nutrient.*

1. Introduction

Hydrocarbons are naturally derived from petroleum, natural gas, and coal which are mainly products of decomposition of organic matter after a prolonged period of time. They are also referred to as fossil fuel (Osei, 2007). Oil pollution also comes from aviation fuel spill (John *et al.*, 2012), transportation process, sales, breakages and spills from storage tank (Obire and Wemedo, 1996). Most of the pollution cases in the Niger delta, Nigeria are due to exploitation and exploration practices as well as the vandalization of oil pipelines (Salau, 1999). Some petroleum industries in Nigeria deliberately discharge waste oil in the soil or water.

The environmental impact of hydrocarbon pollution has become very critical as it impacts negatively on microbial diversity. The degree of impact is largely dependent on several factors ranging from the concentration and composition of the hydrocarbon containing compound to the community structure and function of the resident microorganisms at that given time of contamination. Microbial populations having the ability to utilize hydrocarbons are widely distributed in nature, and this accounts for reasons why some have a large hydrocarbon assimilatory potential.

Microorganisms require different levels of nutrients, in addition to hydrocarbon which serves as carbon and energy source. The availability of nutrients is a major factor affecting the rate of hydrocarbon removal in the polluted environment. The major sources of nutrient elements are mainly phosphorus, nitrogen, sulfur, potassium, calcium, magnesium, iron, sodium, vitamins, amino acids, etc. In the case of underground bioremediation, nitrogen and phosphorus are frequently in short supply among these nutrients (EPA, 1985). However, it has been recognized that a low concentration of N (approximately 300 mg/kg soil) is more realistic due to toxicity considerations (Wibowo, 1996).

Hydrocarbon breakdown is relative to nutrient availability, degree of oil spill at the spill site and natural bioremediation taking place (Bragg *et al.,* 1994).In most cases, most of the fertilizers applied are sparingly available following leaching and dissolution. The loss of the applied fertilizers is dependent on the solubility of the fertilizers and the method used in the application: spraying, broadcasting,

embedding, etc. This leaching or washout usually results in eutrophication and pollution of underground, surface and sub-surface water (Omar, 1989).

Most Niger Delta aquatic environments including ponds, lakes, sea water etc. are characterized by fewer amounts of nitrogen and phosphorus and this has made the addition of crude oil-degrading microorganisms almost ineffective in the bioremediation of crude oil (Olivieri *et al.*, 1976). The effectiveness of nitrogen and phosphorus compounds as fertilizers for petroleum biodegradation has been established in laboratory experiments (Okpokwasili and Oton, 2006), through because of their rapid dissolution, these salts have little or no effect when applied in the sea.

In this study the potentials of slow-release fertilizers as well as the effect of nutrient concentration on bioremediation rate in a simulated low energy environment was evaluated, in order to determine the nutrient requirements as well as energy efficiencies associated with the bioremediation of crude oil in such environments.

2. Materials and Methods

2.1. Preparation of Slow-Release Fertilizers (SRFs)

2.1.1. Capsular SRF

Plastic Petri dishes were used to prepare coatings of the capsular slow release fertilizer. Three grams (3g) agar powder,type 1 (by Titan Biotech LTD) was mixed in 200ml distilled water (1.5%) and autoclaved at 121^{o}C for 15 minutes. The molten agar was then poured into the Petri dishes to a depth of 2mm at reduced temperature of about 37- 42^{o}C. It was allowed to solidify and the solid agar film was used to encapsulate commercial NPK fertilizer (15:15:15) and urea fertilizer (46% N). The SRFs were wrapped in sterile aluminum foil papers prior to use.

2.1.2. Granular slow-release fertilizer (SRF)

Three grams (3g) of agar powder, type 1 (by Titan Biotech LTD) was well mixed with 2g granular fertilizer (NPK and also for Urea (46% N) in 200ml distilled water and autoclaved at 121^{o}C for 15 minutes. The molten agar was then poured into plastic Petri dishes at reduced temperature of about 37- 42^{o}C. It was allowed to solidify and then cut into granules. The SRFs were wrapped in sterile aluminum foils prior to use.

2.2. Experimental Design

2.2.1. Crude oil Concentration

A 0.25% concentration of crude was achieved by introducing 0.5ml crude oil into a 500ml capacity Erlenmeyer flask containing 200ml mineral salt solution.

2.2.2. Preparation of Bacterial consortium

The bacterial consortium was prepared by inoculating serially diluted hydrocarbon polluted soil on a mineral salt agar plate through a vapour phase transfer technique. Suspensions of 24-h old pure cultures of bacterial isolates identified as *Pseudomonas* sp., *Bacillus* sp. and *Micrococcus*sp. were used to inoculate the set-ups as shown below. This was achieved by adjusting the turbidity of each suspension using McFarland's standard. The setups were however inoculated with 0.5ml of each bacterial suspension (*Pseudomonas* sp., *Bacillus* sp. and *Micrococcus*sp) in a 500ml capacity conical flask containing 200ml mineral salt solution and 0.5 ml contaminant (crude oil).

2.2.3. Sample Identity

Sample A = 200ml MSS + 2g NPK Capsular SRF + Biomass
Sample B = 200ml MSS + 2g NPK Granular SRF + Biomass
Sample C = 200ml MSS + 2g UREA Capsular SRF + Biomass
Sample D = 200ml MSS + 2g UREA Granular SRF + Biomass
Sample E = 200ml MSS - No fertilizer + Biomass: Control 1
Sample F = 200ml MSS - No fertilizer, no Biomass: Control 2

***MSS = Mineral Salt Solution**

Study duration: The experimental setup was monitored for a 35-day period during which changes in bacterial biomass, nutrient (nitrate) concentration and total hydrocarbon content (THC) was investigated at seven (7)-day intervals.

Precautions taken: In order to avoid sampling error, suicidal sampling technique was employed in the setup: each setup was sacrificed after a particular period (day) of analyses. Also, the results were taken in triplicates.

2.3. Preparation of Mineral Salt Solution (MSS)

The medium was prepared by dissolving 10g NaCl, 0.42g $MgSO_4.7H_2O$, 0.29g KCl, 125g KH_2PO_4 and 0.42g NH_4NO_3 in one (1) liter of distilled water. The medium was sterilized by autoclaving the medium at 121^{o}C for 15 minutes.

2.4. Estimation of Total Hydrocarbon Content (Oil and Grease Method)

The crude oil in the water sample was extracted using an organic solvent, xylene. After extraction, the sample extract was added some anhydrous sodium sulphate to remove any water collected along the side the extract. It was allowed to stabilize on a spectrophotometer for 15 minutes. The absorbance of the extract was read at 420nm wavelength through a 1cm glass cuvette.

2.5Estimation of Nutrient Utilization: Determination of Nitrate-nitrogen by Distillation Method

One milliliter water sample was weighed into distillation flask. This was followed by the addition of 20ml distilled water and 0.4g magnesium oxide. The distillation flask was held on a retort stand on a distillation hot plate. This was connected to a receiver flask via a Liebig condenser. To this receiver, was added10ml 2% boric acid with few drops of double indicator. The flask was heated to distil out the ammonia as distillate in the receiver. The boric acid indicator changed to greenish from purple and was titrated with 0.1N HCl to purple as back titration.

$$\%NH_4^+ = \frac{\text{titre value x inoculums volume of sample x 10}}{Weight\ of\ sample}$$

3. Results and Discussion

Different nutrient formulations were used in this investigation. They included granular NPK SRF (Sample A), capsular NPK SRF (Sample B), granular urea SRF (Sample C), capsular urea SRF (Sample D), which were compared to a control set-up E. At the beginning of the investigations (zero hour), the set-ups had an initial nutrient concentration of 1.95±0.05 mg/L nitrate nitrogen. The results were taken at seven days' interval for a period of thirty-five (35) days. The variations in nutrient concentration were 59.7±0.19, 46.2±0.07, 8.3±0.15, 20.41±0.20and 0.99±0.0105mg/L nitrate nitrogen for samples A, B, C, D and E, respectively on day 35.

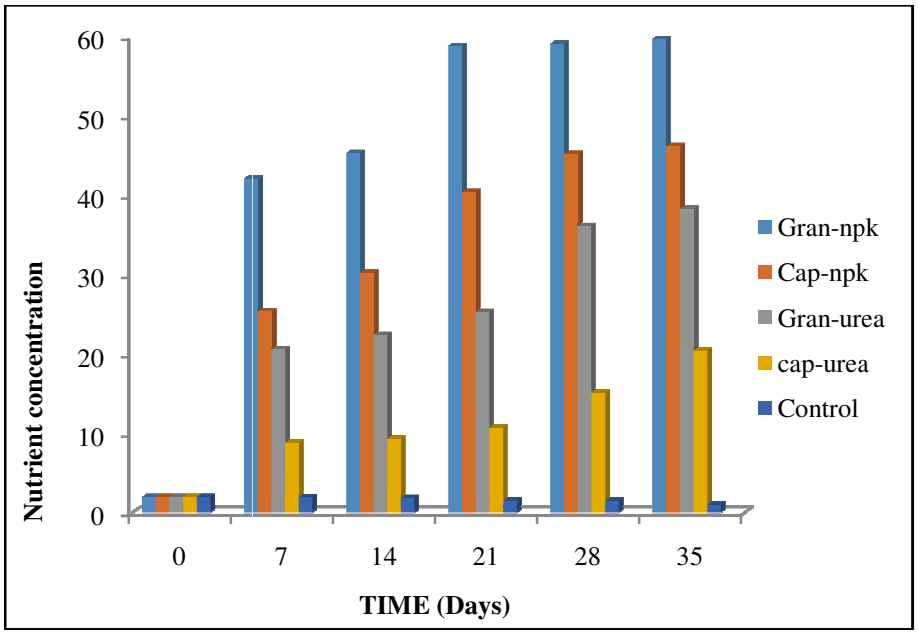

Figure 1: Variation in nutrient concentration

Code: Gran-npk = Granular NPK; Cap-NPK = Capsular NPK; Gran-urea= Granular urea; Cap-urea = Capsular urea

Figure 1shows that the setups witnessed gradual increase in nutrient concentration during the period of study, depicting continuous supply of nutrient.

Statistical analysis indicated that while there was a positive correlation between the nutrient source (nitrate nitrogen) and bacterial biomass (r = 0.530), there was a negative correlation between the nutrient source (nitrate nitrogen) and total hydrocarbon content (r = -0.441), using the two tailed Pearson's correlation model.

A 0.25% concentration of crude was achieved by introducing 0.5ml crude oil into a 500ml capacity Erlenmeyer flask containing 200ml mineral salt solution. The setups: granular NPK SRF (Sample A), capsular NPK SRF (Sample B), granular urea SRF (Sample C) and capsular urea SRF (Sample D), was enriched with slow-release fertilizers (SRFs) containing 2g of respective fertilizer (NPK, Urea) while the controls1 and 2 were without nutrient. The set-ups A –E were however seeded with 0.5ml of each bacterial suspension and studied for 35 days while control 2 (sample F) was without bacterial consortium. The results were taken at seven-day interval for the entire period of study.

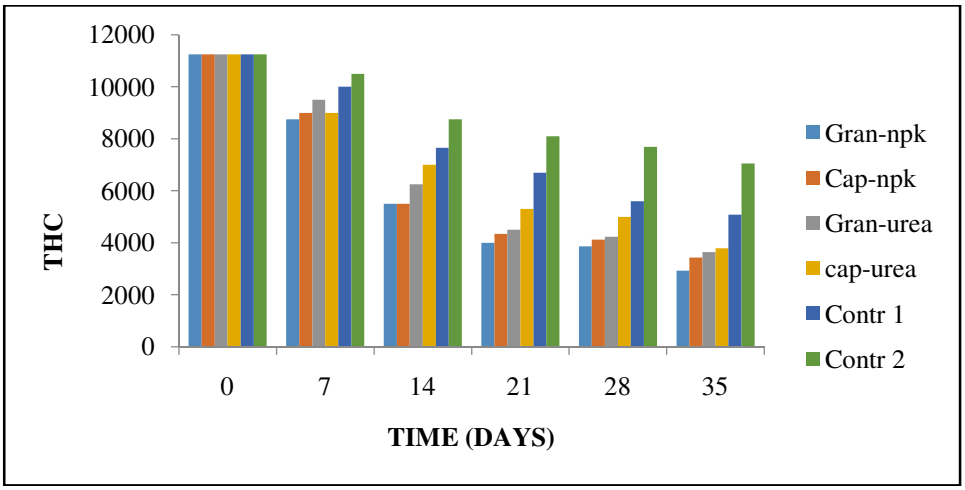

Figure 2: Change in total hydrocarbon content
Code: Gran-NPK = Granular NPK; Cap-NPK = Capsular NPK;
Gran-urea= Granular urea; Cap-urea = Capsular urea; Contr-1 = Control 1; Contr-2 = Control 2

At the end of the investigation, the bacterial consortium was able to appreciably degrade the crude oil as measured by the oil and grease method, to determine the total hydrocarbon content. The initial crude oil concentration was 11250±0.20 mg/l on day 0. Figure 2shows that the various treatment options were effective as the residual total hydrocarbon content after a 35-day period of investigation was observed to have reduced in all the treatments.

A one-way analysis of variance showed there was a significant difference in the mean values of the various treatment options ($p>0.05$, $f(4,36) = 92.70$, PV = 0.00).

The percentage residual hydrocarbon was calculated and it was revealed that after a 35-day period that the treatment options were effective in the descending order; Gran-npk (26%) >Cap-npk (30.6%) >Gran-urea (32.4%) >Cap-urea (33.7%) compared to the two controls E (45.2%) and F (62.7%).

SAMPLE	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
Gran-npk	100	77.8	48.9	35.6	34.4	26
Cap-npk	100	80	48.9	38.7	36.7	30.6
Gran-urea	100	84.4	55.6	40	37.6	32.4
Cap-urea	100	80	62.2	47.1	44.4	33.7
E	100	88.9	68	59.6	49.8	45.2
F	100	93.3	77.8	72	68.4	62.7

Table 1: Percentage residual hydrocarbons (%)
Code: Gran-npk= Granular NPK; Cap-NPK = Capsular NPK; Gran-urea= Granular urea;
Cap-urea = Capsular urea; E = Control 1; F = Control 2

The study was also carried out to determine the changes in bacterial growth and as well examine the rate of hydrocarbon loss under these conditions. The initial cell count at the start of the experiment (zero hours) was $4.9\pm0.03 \times 10^4$ cfu/ml. After a 35–day period of investigation, the bacterial count for the various set-ups was as follows: Sample A (Granular NPK) had $2.23\pm0.07 \times 10^8$ cfu/ml on day 7 and its highest count of $2.29\pm0.09 \times 10^{18}$ cfu/ml on day 35. Sample B (Capsular NPK) had $1.80\pm0.04 \times 10^7$ cfu/ml on day 7 and its highest count of $1.82\pm0.04 \times 10^{16}$ cfu/ml on day 35. Sample C (Granular urea) had $2.00\pm0.02 \times 10^7$ cfu/ml on day 7 and its highest count of $1.91\pm0.07 \times 10^{17}$ cfu/ml was on day 35. Sample D (Capsular urea) had $1.62\pm0.03 \times 10^7$ cfu/ml on day 7 and $1.29\pm0.03 \times 10^{14}$ cfu/ml on day 35. The control set-up E had $2.51\pm0.02 \times 10^6$ cfu/ml on day 7 and its highest count of $1.32\pm0.10 \times 10^{11}$ cfu/ml on day 35.

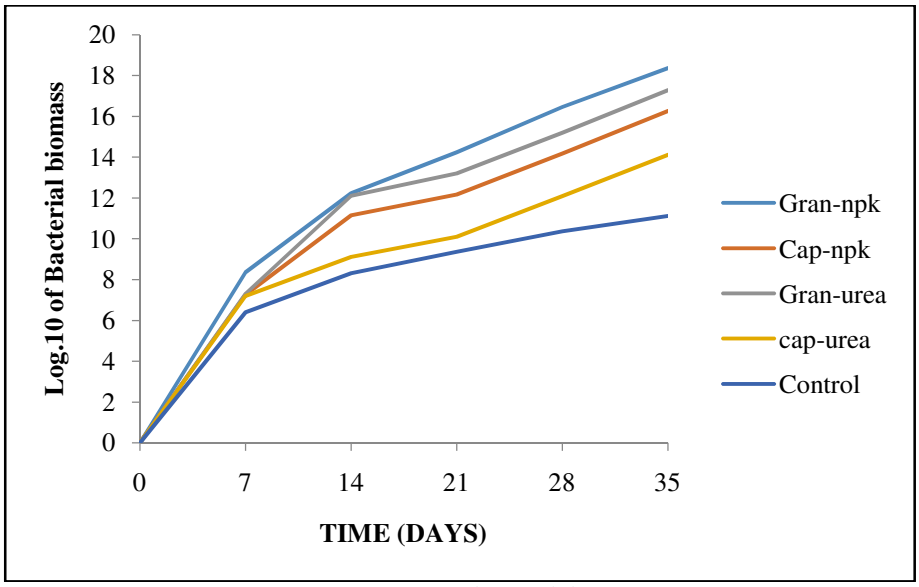

Figure 3: Bacterial growth kinetics
Code: Gran-npk = Granular NPK; Cap-npk = Capsular NPK; Gran-urea= Granular urea; Cap-urea = Capsular urea

The result revealed that the granular NPK SRF (sample A) supported the highest growth rate. The next was the granular urea SRF which performed better than the capsular NPK SRF. The capsular urea SRF (sample D) was observed to be the least inducer of bacterial growth under these conditions. The control setup E however witnessed a rather slow growth rate compared to the treated samples.

Statistical analysis using the one-way analysis of variance showed there was a significant difference in the mean values of the various treatment options, A – D, compared to the control E ($p>0.05$,$f(4,36) = 34.378$, PV = 0.00). However, while there was a positive correlation between bacterial biomass and the nutrient source (nitrate nitrogen) ($r = 0.530$), there was a strong negative correlation between bacterial biomass and total hydrocarbon content ($r = -0.957$), using the Pearson's two-tailed correlation model.

From the results,set-ups E and F had the least hydrocarbon removal rates which imply that crude oil degradation is greatly influenced by bacterial growth and nutrient availability. However, the control experiment E had the least rate of hydrocarbon removal.

It has been well documented that the fate of crude oil pollutants is dependent on physical, chemical and biological factors.Where the rate of hydrocarbon removal was influenced by nutrient concentration in treatments A, B, C and D, a 37.3% loss of hydrocarbons (day 35) was noticed in the control setup (without fertilizer). This is probably due to natural attenuation taking place in the unfertilized setup. Chikere *et al.* (2012) observed a hydrocarbon loss in the heat-killed control signifying that abiotic factors could as well contribute to hydrocarbon attenuation in the environment.

However, the difference in the rate of biodegradation of crude oil observed between the fertilized and unfertilized sample accounts for the role of fertilizers in hydrocarbon bioremediation. The application of slow-release fertilizers at optimal rates and conditions may be very vital in correcting nutrient imbalance and supply nutrients for sustained microbial activities in the environment (Riser-Roberts, 1992).

Several reports have been documented regarding the use of alternative sources of nutrients to provide optimal conditions necessary for bioremediation. Ijah *et al.* (2008) reported that NPK fertilizer caused more enhancement of the crude oil biodegradation than chicken droppings. Ebuchi *et al*. (2005) reported the use of NPK fertilizer for laboratory and field scale remediation of crude oil-contaminated soil in Rumuekpe, Rivers State, using the method of enhanced natural attenuation (RENA).

Different slow-release formulations have been reported to include sulphur coated SRF, paraffin supported SRF, Osmocote etc. Xu *et al.* (2003) found out that an addition of 0.8% of slow-release fertilizer, Osmocote™ consisting of 18, 4.8, and 8.3% N-P-K (w/w) to oil-polluted sediments was sufficient to maximize metabolic activity of the microbial biomass and the biodegradation of straight-chain alkanes (C10-C33); and application of 1.5% rate resulted in optimal biodegradation of recalcitrant branched-chain alkanes, such as pristane and phytane.

Low energy environments like lakes, ponds, slow moving water bodies, etc. are characterized by reduced oxygen, low nutrient content, little or no tidal action. These factors potentially determine the fate of crude oil in these aquatic environments. Appropriate measures must therefore, be put in place while addressing nutrient imbalance in these peculiar Niger Delta environments, as excessive amount of the elements might be toxic to the microbial population or might as well pose further environmental challenges (like eutrophication, surface water pollution) while minute quantity might become ineffective. The need to study the nutrient requirement for an optimal bioremediation process is therefore pertinent (Wang *et al.,* 2011; Dibble and Bartha, 1979).

This study has however shown the fate of crude oil in these environments and at the same time shown the potentials of slow release fertilizer in the bioremediation of hydrocarbons, in the event of oil spills involving ponds, lakes, and other slow-moving water bodies. Also, the effect of nutrient concentration was demonstrated as the granular NPK fertilizer had the highest hydrocarbon removal rate. However, direct fertilizer application in these environments is highly discouraged due to ecological concerns. From the study NPK fertilizer was seen to be a more effective source of nutrient for slow release fertilizer production and application in these low energy

environments. Also, the granular formulation stimulated more biomass increase compared to the capsular form. Therefore, the granular slow release fertilizer should be applied for the bioremediation of crude oil under these conditions as it has the potentials of controlled release and at the same time stimulating appreciable level of biomass increase and thereby giving rise to concomitant hydrocarbon removal. This is an important discovery in the field of environmental bioremediation owing to the paucity of information regarding the application of slow release nutrient sources using a biopolymer as its control matrix. This biopolymer (agar) is environmentally friendly and has also been found to be effective in the bioremediation of hydrocarbon-impacted soil (Sampson and Okpokwasili, 2014).

4. Conclusion

This research has provided some very important pieces of information about bioremediation and its performance at oil spill sites and has consequently provided a plat-form and an easy frame work for the development and optimization of slow-release fertilizers, for the bioremediation of oil spills in the Niger Delta wetlands.

Slow-release fertilizers have promising potentials in bioremediation. The application of marine product (agar) to mitigate environmental challenges following oil exploration is innovative. Agar agar is an environmentally friendly marine biopolymer which has shown promising potentials in the production of slow release nutrient formulation for crude oil bioremediation. They are recommended for surface, sub-surface water as well as sediment bioremediation. The granular slow release fertilizer is recommended as aclose alternative to direct fertilizer application.

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