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EFFECT OF CRUDE OIL POLLUTION ON SOIL AND AQUATIC BACTERIA AND FUNGI

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ABSTRACT

Crude oil and its derivatives are among the most potent contaminants of the environment, affecting both the biotic and abiotic components of the ecosystem. The present study was undertaken to evaluate the effects of crude oil contamination on terrestrial and aquatic microorganisms. Eight different concentrations of crude oil (Bonny light) were used to contaminate soil and water samples obtained from pristine environments. Both the control and polluted samples were organized in triplicates and the studies carried out by plate count procedures using nutrient agar and sabouraud dextrose agar for bacteria and fungi respectively. Effect of the crude oil on bacterial and fungal counts was significantly ($P<0.05$) inhibitory and dose-dependent with 15.0% and 20.0% levels of pollution having the highest impact on the microbial counts. In the control soil samples, bacterial numbers varied between 2.32×10^9 to 2.80×10^9 cfu/g while their numbers varied between 2.00×10^8 to 2.77×10^9 cfu/g in the test samples. For the fungi, numbers varied from 1.02×10^7 to 1.39×10^7 cfu/g in the control soil while it was reported 1.60×10^5 to 1.18×10^7 cfu/g for the test samples. Results showed that both bacteria and fungi were significantly affected by crude oil contamination, among tested microorganisms marine microorganisms demonstrated some tolerance against crude oil contamination.

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1 Introduction

Since the discovery of petroleum as an energy source it has remained the mainstay of many national economies. In spite of this crude oil and its products have been shown to be toxic to living organisms as well as arable lands (Eze et al. 2013; Xue et al., 2015; Wang et al., 2018; Abdullah & Peramaiyan, 2019). Oil spill incidents in Nigeria are major environmental issues especially in the oil-producing Niger Delta region. Nigeria has had over 4000 oil spills ranging from minor spills of a few hundred barrels to over half a million barrels in a single incident. Releases of petroleum into the environment occur naturally from seeps as well as from human sources. These spillages have caused much destruction of flora, fauna and arable lands in Nigerian environment (Ekpo & Udofia, 2008). On the whole, natural and human sources introduce about 380 million gallons of oil into the marine environment annually (National Research Council, 2002). About 55% from this arises from human sources via petroleum production and transportation while the remainder comes from natural seeps.

Present day technology is inadequate to handle such large spills. However, techniques employed include mechanical containment with booms and removal using suction equipment and sorbents, chemical treatment with detergents, and physical removal (National Oceanic and Atmospheric Administration, 1992). Natural processes account for the removal of a large percentage of petroleum spills from the environment. Natural removal of petroleum from water takes place through evaporation, photo-oxidation, microbial degradation and utilization (Yan et al., 2018; Xingjian et al., 2018; Xinxin et al., 2019). Some microorganisms involved in crude oil degradation, detoxication and bioremediation of polluted environments. Notwithstanding, petroleum hydrocarbons have been shown to have deleterious effects on microorganisms through reduction of cell membrane permeability due to its hydrophobicity leading to reduced water and nutrient absorption (Pezeshki et al., 2000) as well as oxygen exchange between soil and the atmosphere (Adedokun & Ataga, 2007). Even though general reports abound on the negative effects of crude oil on living organisms and arable land, there is paucity of research work on its effects on microorganisms specifically. This research work was undertaken to evaluate the relative impact of crude oil on bacterial and fungal populations by checking its effects on their numbers, soil microbial respiration and phospholipids content.

2 Materials and Methods

Bonny light crude oil, uncontaminated sandy loam soil, marine and freshwater samples are the materials which were used in current study. The crude oil was supplied by the Nigerian National Petroleum Corporation (NNPC), Port Harcourt, Rivers State,

Nigeria. Sandy loam soil was obtained from Botany Garden, University of Nigeria.

2.1 Evaluation of the Impact of Crude Oil on Microbial Numbers in Soil

Non-petroleum contaminated sandy loam soil was air dried, sieved and measured in 0.5kg portions into twenty-seven plastic buckets (13cm x 12cm). The buckets were arranged in triplicates and each triplicate set apart from the control was contaminated with one of the following concentrations of crude oil i.e. 0.5%, 1.0%, 2.0%, 2.5%, 5.0%, 10.0%, 15.0% or 20.0%v/w. After crude oil addition the soil in each bucket was thoroughly mixed. Microbiological assay was done with 1.0g of soil from each bucket every week for eight weeks. The population of viable microbial cells (bacteria and fungi) in each soil sample was determined by the spread plating technique as described by Wistreich (1997) using nutrient agar and sabouraud dextrose agar for bacterial and fungal cultivations respectively.

2.2 Evaluation of the Impact of Crude Oil on Microbial Numbers in Water

Marine and freshwater samples were each measured in 200ml volumes into twenty-seven 500ml flasks grouped in triplicates. Each triplicate set excluding the controls was contaminated with one of the following concentrations of crude oil viz., 0.5%, 1.0%, 2.0%, 2.5%, 5.0%, 10.0%, 15.0% or 20.0% v/v. Uncontaminated marine and fresh water were used as control samples. Microbiological assay was carried out with 1.0ml of water from each flask every week for eight weeks. The spread plating technique was also used for the determination of microbial numbers and the media were nutrient agar and sabouraud dextrose agar for bacteria and fungi respectively.

2.3 Determination of the Effects of Crude Oil on Soil Microbial Respiration

This was carried out by the method of Isermeyer (1952) which quantified the level of carbon di oxide (CO₂) evolved from the soil microbes. Fifty grams of each soil sample was weighed in duplicate into beakers placed inside jars with air-tight covers. A 25 ml volume of 0.05M NaOH was introduced into each jar and the jars were instantly sealed with rubber rings. Controls for both contaminated and uncontaminated soil samples consisted of three jars, each containing 0.05M NaOH without soil. All jars were incubated at 25°C for 3 days.

Following incubation, the beakers were brought out and their external surfaces washed with CO₂-free water. Subsequently, 5ml of 0.5M barium chloride solution was introduced into each jar and few drops of phenolphthalein indicator were also added. This was followed by the addition of few drops of hydrochloric

acid (0.05M) with continuous stirring until the colour changed from red to colourless. The rate of microbial respiration in soil was calculated with the following formula devised by Isermeyer (1952):

$$\text{CO}_2 \text{ (mg) / SW/t} = \frac{(\text{V}_0 - \text{V}) \times 1.1}{\text{DWT}}$$

Where SW is the amount of soil dry weight in grams, T is the incubation time in hours, V_0 is the volume of HCl used for blank titration (average value) in milliliters. V is the volume of HCl used for the soil sample (average value), DWT is the dry weight of 1g moist soil and 1.1 is the conversion factor (1ml 0.05M NaOH equals 1.1 mg CO_2).

2.4 Determination of Microbial Phospholipid Phosphate (PLP)

This was done by the method of Frostegard et al. (1991) with some modification. Glass containers used for estimation of PLP were washed with methanol and 15% HNO_3 ; this was followed by the rinsing twice with tap water and thrice with deionized water. Phospholipids were extracted using a chloroform : methanol : citrate buffer solvent in a ratio of 1 : 2 : 0.8v/v/v. The citrate buffer was made up of 0.015M citric acid and 0.15M trisodium citrate at a ratio of 5.9 : 4.1v/v to give a pH of 4.0. A 1.0g wet weight of soil was put in a Mc Cartney bottle containing 11.65ml of the extraction solvent. The ratio of chloroform to soil was 3:1 as prescribed by Frostegard et al. (1991). Two hours later, 3.1ml of citrate buffer and 3.1ml of chloroform were added with

intermittent shaking for another hour to enhance extraction. The solutions were left overnight to dissociate into two partitions. A 6.4ml volume of the lower chloroform partition was removed using a syringe and put into a vial, taking care not to include any soil particles. An aliquot (0.1-1.0ml) was transferred to a 5ml bijou bottle and dried with nitrogen.

Digestion and phospholipid assay were carried out using the method of Findlay et al. (1989). Lipid extracts and glycerol phosphate standards were treated with 1.8ml of acidified potassium persulphate ($\text{K}_2\text{S}_2\text{O}_8$) solution (5g to 100ml of 0.35N H_2SO_4) for 24h at 95°C . The mixture was still hot, this was followed by the addition of 0.4ml of ammonium molybdate solution and allowed to stay for 10 min before the introduction of 1.8ml of malachite green solution. Optical absorbance at 610nm was read after 30min. Distilled water was used for the zero.

2.5 Statistical analysis

Data analysis was carried out using a two-way analysis of variance (ANOVA) and the difference done by comparing tests with $P < 0.05$.

3 Results

3.1 Effects of Crude Oil on the Colony Counts of Soil and Aquatic Bacteria and Fungi

Effects of different crude oil concentrations on microbial numbers in soil and water are presented in Figures 1 and 2. The pristine soil

Figure 1 Effects of varying levels of crude oil on total colony count of soil bacteria

Figure 2 Effects of varying levels of crude oil on total colony count of soil fungi

Figure 3 Effects of crude oil on the total colony count of marine bacteria

sample (0% pollution) experienced increase in bacterial numbers from the first week to the eighth week. At low concentrations (0.5-2%) the crude oil did not have significant negative effects on the cell number of the soil bacteria. The growth curves (Figure 1) at lower concentrations (0.5-2%) was slower for first week, after which they increased progressively till the eighth week. So, when it compared to the control (0%), there is no significant difference between them. On the contrary, crude oil at high levels (2.5%-20% v/w) had a significant ($P < 0.05$) negative effect on bacterial cell numbers. There was a sharp reduction in bacterial populations at 2.5% crude oil level from the first week to the fourth week. Afterwards a gradual increase in population was noted from the fifth week to eighth week.

The fungi seemed to be more prone to the toxic effects of crude oil. This is evidenced by the progressive decrease in fungal numbers with time at the different concentrations of crude oil (Figure 2). Maximum inhibition in fungal growth was reported at 15 and 20% (v/w) concentration of crude oil.

Figures 3 and 4 showed the effects of crude oil on the cell numbers of marine and freshwater bacteria. Like soil bacteria, reduction in the cell numbers of these bacteria was reported at the first week, this was followed by the gradual increase in bacterial numbers with the exposure time increased (Figure 3 & 4). Freshwater bacteria were more susceptible to crude oil toxicity than their marine

counterparts as evidenced by the higher population recorded in marine than freshwater bacteria. Contrary to the bacteria, exposure time of the fungi to crude oil did not enhance their population (Figures 5 and 6). The same trend also occurred in the fungi (Figure 5, 6). In all the aquatic bacteria and fungi the toxic effects of crude oil were highest at 15% and 20% pollution levels.

3.2 Impact of Crude Oil on Microbial Respiration and Phospholipid Phosphate (PLP)

Results of these analyses are shown in Figures 7 to 9. Microbial respiration was evaluated from the quantity of Carbon dioxide emitted from the soil over a specific period. According to the results, high concentrations of crude oil (15% and 20%) significantly ($P < 0.05$) decreased CO_2 emission (Figure 7) as well as phospholipid phosphate in the soil samples. The length of time microorganisms were exposed to the crude oil (especially low to moderate levels) increased the levels of CO_2 and PLP in the soil (Figure 7 and 8). Mean maximum levels of CO_2 and PLP in the test samples occurred at 0.5% and 1.0% crude oil contaminations. This trend was also observed with the PLP and maximum levels also occurred at 0.5% and 1.0% crude oil pollution levels. There was a positive correlation (Pearson's Correlation Model) between soil CO_2 and PLP (correlation coefficient = 0.74).

4 Discussion

Result of current study clearly indicated that crude oil contamination negatively affected the microbial population, in this manner results of current study are in agreement with the findings of previous researchers (Boethling & Alexander, 1979; Long et al., 1995), this is only manifest at high oil concentrations (Ma et al., 2015). Results of this study show low levels of crude oil (0.5- 2.0%) did not have any negative effect on microbial population. This might be because at low levels hydrocarbonoclastic microorganisms very easily metabolize crude oil as carbon source for their growth (Adedokun & Ataga, 2007).

Figure 4 Effects of crude oil on the total colony count of fresh water bacteria

Figure 5 Effects of crude oil on the total colony count of marine fungi

Figure 6 Effects of crude oil on the total colony count of fresh water fungi

Figure 7 Effects of Crude oil on soil respiration

Figure 8 Effects of varying levels of crude oil on soil microbial phospholipids (nm/g of soil)

Figure 9 Relationship between microbial phospholipid and carbon (IV) oxide evolution from crude oil-contaminated soil samples.

The sharp decline observed in soil bacterial numbers from the first week up to the fourth week and subsequent gradual rise in population from the fifth to the eighth week might be the result of a decrease in the non-hydrocarbonoclastic bacterial population caused by the oil pollutant (Ebuehi et al. 2005). The heterotrophic microorganisms (non-hydrocarbonoclastic) usually outnumber hydrocarbon degraders in unpolluted habitats and any introduction of a hydrocarbon pollutant will seriously reduce their numbers. On the contrary, the degradation facilities of hydrocarbonoclastic microorganisms are activated in the presence of hydrocarbon contaminants leading to utilization of the substrate and an increase in their population (Aniruddha & Hermen 2010; Das & Chandran, 2011; Owabor et al. 2011).

Both the aquatic and soil microorganisms had similar sensitivity patterns to crude oil toxicity. In general, the water bacteria demonstrated higher adaptive capacity to the oil than water fungi. This might be because of important role of bacteria in crude oil biodegradation than fungi. Further bacteria are more flexible as a result of the presence of more adaptation features. Furthermore, marine microorganisms more resisted to crude oil toxicity than their freshwater counterparts. This might be result of innate traits of the organisms (Chen et al., 2017) or the development of adaptive characteristics occasioned by pre-exposure of marine organisms to petroleum pollutants (Amanchukwu et al. 1989). These factors plays a significant role in pre-adaptation of marine

organisms to hydrocarbons because of oil spills on the high seas caused by oil tanker accidents, oil leakages from motorized seafaring vessels and other offshore incidents that introduce petroleum into the sea.

The crude oil also had dose-dependent effects on soil microbial respiration and phospholipids content. Crude oil at high concentrations significantly ($P < 0.05$) decreased the amounts of CO_2 and PLP in the soil samples. This is because of its lethal effects on microbial cells. Phospholipids are a constituent of all cell membranes and their levels are always proportional to the bacterial biomass and they disappear soon after the death of the cell (Peterson et al. 1991). Therefore it follows that whatever affects microbial cell number will invariably affect the phospholipid phosphate level. According to Frostegard et al. (1991), phospholipid levels are used to measure total microbial biomass, activity and metabolic status.

Conclusion

The toxic effects of crude oil on microorganisms generally are more perceptible at high levels of the oil. On the other hand very low levels of crude oil enhance the growth of some groups of microorganisms. Additionally, marine microorganisms exhibit a higher tolerance to crude oil than their freshwater counterparts. More research work is needed to unravel and fully elucidate the

reasons for the discrepancy between the reactions of marine and freshwater microorganisms to crude oil pollution.

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Conflict of Interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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