

Original Research Article

TOXICOLOGICAL EFFECTS OF ~~NIGERIAN~~ PETROLEUM REFINERY SLUDGE ON THE TERRESTRIAL ENVIRONMENT USING BACTERIA AND EARTHWORM AS BIOINDICATORS

ABSTRACT

Aim: The aim of the study is to assess the sublethal (chronic) ecotoxicological effects of ~~Nigerian~~ petroleum refinery sludge pollution on the biota of the terrestrial environment using the effects on bacteria nitrogen transformation activity and growth rate of the soil fauna, Earthworm (*Aporrectodea longa*) as bioindicators.

Place and duration of study: Department of Environmental Management and Toxicology, Federal University of Petroleum Resources, Effurun, between 2017 and 2018.

Methodology: The OECD TG 216 (2000) test method was used to determine the chronic effects (% inhibition and EC₅₀) of the petroleum sludge on the nitrogen transformation activity of *Nitrobacter sp.* in the soil, while the methods of Sandoval *et al.*, 2001 and OECD, 207 was used for the chronic effects of the sludge on the growth rate of the earthworms. Bioconcentration factor (BCF) of the sludge in the earthworms was also determined. Both tests were conducted for 28 days. ~~Results from the test with multiple concentrations was~~ Results from the test with multiple concentrations were analyzed using a regression model (ANOVA). EC₅₀ was also determined using the Probit analytical software. Analyses were done using ASTM methods.

Results: Analysis of the Nigerian petroleum refinery sludge used for this research indicated that the sludge was acidic with a pH value of 5.91 and had a high TPH content of 340,000 mg/kg made mainly of between 10-40 carbon unit compounds. There was a progressive increase in the percentage (%) inhibition of the nitrogen transformation activities of the bacteria, from 18.0 % (3125 mg/kg) to 79.30 % (5000 mg/kg) with increase in concentration and time. The effective concentration that decreased 50 % (EC₅₀) of the nitrogen transformation ~~activity~~ activity was 13761.059 mg/kg. The growth rate of the test earthworms decreased from 9.19 x 10³-g/day (375 mg/kg) to 3.55 x 10³-g/day (3000 mg/kg) for the test period. The BCF decreased from 5.168 (375 mg/kg) to 1.22 (3000 mg/kg) in the earthworms, while the EC₅₀ for the earthworms

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was 825.02 mg/kg as the concentration of the sludge increased with time.

Conclusion: The sludge inhibited the nitrogen transformation activity of *Nitrobacter sp.* because it exceeded the 25% inhibition limit for chemicals with the potential to cause chronic effects on soil microbial activities as specified in OECD TG 216. Similarly, the sludge reduced the growth rate of the earthworm progressively as the sludge concentration increased in relation to the control. Bioaccumulation factor also increased with increasing sludge concentration and time.

Keywords: *Petroleum*, *Refinery Oily Sludge*, *Chronic effects*, *Bioindicators*, *Environment*.

1.0. INTRODUCTION

Petroleum sludges are oily and viscous residues, which are formed during production, transportation, refining of petroleum and storage and are composed of basically oil, water and solids (Ururahy *et al.*, 1998). Due to their characteristics, such as varied composition, their neutralization become difficult and confer on them high recalcitrance. This recalcitrance can be ascribed to the presence of aromatics, polycyclic aromatic hydrocarbons (PAHs) and complex compounds such as asphaltenes. Some of these compounds act as solvents of microbial membranes and could impair biodegradation (Prince *et al.*, 1993).

The oil industry is responsible for the generation of high amounts of oily sludge as waste by-product. However; one of the problems faced by the oil industry is the safe disposal of the oily waste generated. It is estimated that approximately 1% of the total oil processed in a refinery is discarded as oily sludge (Ururahy, 1998). These oily wastes are expensive to store or destroy and previously contaminated areas have required expensive remediation processes to minimize contaminant dispersion. Improper disposal leads to environmental pollution, particularly soil contamination, and poses a serious threat to groundwater. Many of the constituents are carcinogenic and immunotoxicants (Prospt *et al.*, 1999). The polynuclear aromatic hydrocarbons (PAHs) have also been known to impair chemoreceptors functions in aquatic lives and hence lead to extinction of some species. They have also being known to bioaccumulate up the food chain, resulting in cancers and other genetic malfunctioning in man and other higher animals (Atlas and Bartha, 1992).

Information about in situ toxicity of the bioavailable pools of adsorptive soil pollutants is a prerequisite for proper ecological risk assessment in contaminated soils. Petroleum sludge is known to have strong adsorptive properties in soil and the bioavailability and in situ toxicity of such a pollutant to the soil microorganisms may thus be affected differently by dissolved and adsorbed pools in the soil (Knaebel *et al.*, 1994). On the other hand, many recent reports have

suggested that the adsorbed pollutant pool may also be at least partially available for the microorganisms, both in terms of causing toxic effects (Alexander, 2000, Doherty, 2001; Ronnpagel *et al.*, 1998) and serving as substrates for biodegradation (Knaebel *et al.*, 1994).

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Toxicity tests are used to expose test organisms (fish, shrimps, microorganisms, earthworm) to a medium—water, sediment, or soil—and evaluate the effects of contamination on the survival, growth, reproduction, ~~behavior~~ behaviour and/or other attributes of these organisms. Chronic toxicity tests generally are longer-term tests that measure the effects of exposure to relatively lower, less toxic concentrations. For a chronic toxicity test the measurement endpoint concerns a sublethal effect or both lethal and sublethal effect. Sublethal effects may include growth reduction, reproductive impairment, nerve function impairment, lack of motility, ~~behavioral~~ behavioural changes, inhibition of enzyme activities and the development of terata, which are structural abnormalities.

Microorganisms play an important role in the breakdown and transformation of organic matter in fertile soils with many species contributing to different soil fertility through biogeochemical cycles such as nitrogen cycle. Any long-term interference with these biochemical processes could potentially interfere with nutrient cycling and this could alter soil fertility (OECD, 2000). Nitrogen transformation occurs in all fertile soil. This process can be used to detect long-term (Chronic test) adverse effects of a substance such as petroleum sludge in aerobic surface soils (OECD, 2000).

Sentinels are biological indicators that can help define the ecotoxicological effects of environmental contaminants (USEPA, 2004). These are sensitive organisms in the environment that indicate early warnings to measure pollutant effects and area affected and thus transplant them into waste pollution gradients (Beeby, 2001). Sentinels could be microorganisms, shrimp, fish, gastropods among others.

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The suitability of earthworms as sentinel in soil toxicity is largely due to the fact that they ingest large quantity of the soil and are in full contact with the substrate they consume (OECD, 1984; Sandoval *et al.*, 2001). Earthworms are superb 'barometers' or 'sentinels' providing an early warning of deterioration in soil quality. Earthworms have been recommended as a critical (suitable) representative of soil organisms and an indicator of soil health (Culy and Berly, 1995).

Nitrobacter hamburgensis is a gram-negative bacterium. ~~The main types of environments they~~ which inhabit ~~are in~~ soil, building sandstone, and sewage sludge. They are mostly pear-shaped.

N. hamburgensis gains energy from oxidation of nitrite to nitrate via the enzyme nitrite oxidoreductase (NOR).

2.0 .MATERIALS AND METHODS

2.1. Sample collection

2.1.1. ~~Collection.~~ **Collection** of Petroleum Refinery Sludge

The petroleum sludge used for this research was collected from the Petroleum sludge holding tank of Warri Refinery and Petrochemical Company Ltd, Warri, Ekpan, Delta State, Nigeria in 2 L glass bottles and preserved at 4 °C until required for use.

2.1.2. Collection of *Nitrobacter sp.*

The *Nitrobacter sp.* used was isolated from Aladja River, Aladja, Delta State, in Southern Nigeria. DSMZ heterotrophic nitrobacter medium was used for the isolation of the bacteria. Isolates that are grayish, mucoid, flat, Gram negative, pear shaped and aerobic were selected according to the scheme of Colwell and Zambruski, 1972. Subcultures were made into slants of DSMZ Nitrobacter agar and stored at 4 °C until required for use.

2.1.3. Collection of Earthworms (*Aporrectoda longa*)

The earthworm, *Aporrectoda longa* commonly found in southern Nigeria was collected from a farm at Ubogo, Delta State, Southern Nigeria. The worms were collected according to the method describe by Terhivo *et al.* (1994) and Spiegel (2002).

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2.2. 1. Sublethal (chronic toxicity) Effects of Petroleum sludge Pollution on Nitrogen Transformation Activity in the Soil

The OECD TG 216 (2000) test method was used for this test. This test was used to detect long-term (chronic) adverse effects of Petroleum sludge to the process of nitrogen transformation activity in the soil. The soil was dried, sieved and amended with 5 g/kg compost and treated with five concentrations (3125 mg/kg, 6250 mg/kg, 12500 mg/kg, 25000 mg/kg, and 50000 mg/kg) of Petroleum sludge or left untreated (control). After day 0, 7, 14 and 28, treated and control composite samples were extracted and analyzed for ammonia, nitrate and *Nitrobacter sp.* counts. The rate of ammonia nitrate formation in treated soil was compared with the rate in the controls and the percent deviation of the treated from control was calculated. ~~Results from the test with multiple concentrations was~~ Results from the test with multiple concentrations were analyzed using a regression model (ANOVA) and the EC₅₀ was calculated. The bioconcentration factor (BCF) of the TPH in the earthworms was also determined. All analyses were done by ASTM Method. Three replicates for both treatments and control were used.

The ~~rate of nitrate formation in treated samples were~~ rates of nitrate formation in treated samples were compared with the rate in the controls, and the percent deviation/Inhibition of the treated from the control was calculated after 28 days using the formula below (Grunditz and Dalhammar, 2001).

$$\% \text{ inhibition} = \frac{C_{ref} - C_{sample}}{C_{ref}} \times 100$$

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Where, C_{ref} is concentration of nitrate formed in control,

C_{sample} , concentration of nitrate in samples.

2.2.2. Chronic Toxicity Effects of Petroleum sludge on the Growth and Survival of the Earthworm, *Apporectoda longa*

Experimental procedure for this test was conducted in accordance with the procedures detailed in Sandoval *et al.*, 2001; OECD, 2007. The selected worms were acclimatized for 1-7 days in the soil from the organism's habitat. During this period the worms were fed with cellulose.

The test medium and control were analysed for pH, TPH content, at the start of the experiment and weekly for 28 days. In addition to death, weight loss, behavioural symptoms and pathological symptoms were recorded. Each test and control chamber was checked for dead or affected earthworms and observations recorded weekly for 28 days. The sublethal effects (weight loss) data were used to plot concentration-response curves and calculate the EC_{50} value. ANOVA was used to test for significant differences between treatment means and the control. The worms were also analysed for bioaccumulation of TPH at the end of the test.

3.0. RESULTS AND DISCUSSION

3.1. RESULTS

3.1.1. Chronic effect of petroleum sludge pollution on nitrogen transformation in soil.

The petroleum refinery sludge used for the experiment was acidic with a pH value of 5.81, and had a high TPH content of 340,000 mg/kg made mainly of between 10-40 carbon unit compounds (fig 1). The chronic toxicity effects of petroleum sludge to nitrogen transformation

activities in soils showed that the percentage (%) inhibition of nitrogen transformation in petroleum sludge contaminated soils in relation to the control increased with increasing sludge concentration. It ranged from 18.0% to 79.37% from the lowest concentration of 3125 mg/kg to the highest concentration of 50000mg/kg respectively (fig 2). As stipulated in the test guideline, OECD TG 216 (2000), since the difference between the lowest and highest ~~%-percentage (%)~~ inhibition is greater than 25%, the sludge has the potential to inhibit nitrogen transformation. Chronic toxicity profile of nitrogen transforming bacteria exposed to petroleum sludge at day 28 recorded an EC₅₀ of 13761.059 mg/kg (Table 1). This concentration resulted in 50% inhibition of the nitrogen transformation activity of the *Nitrobacter sp.* in the test soil samples.

UNDER PEER REVIEW

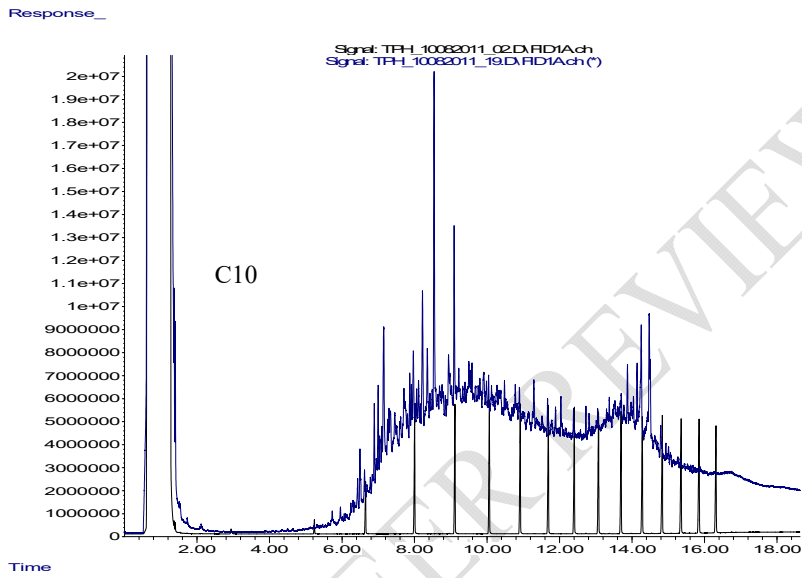


Fig 1: Overlay of Petroleum sludge and a Chromatogram of a mixture of even numbered n-alkanes between C₁₀ and C₄₀.

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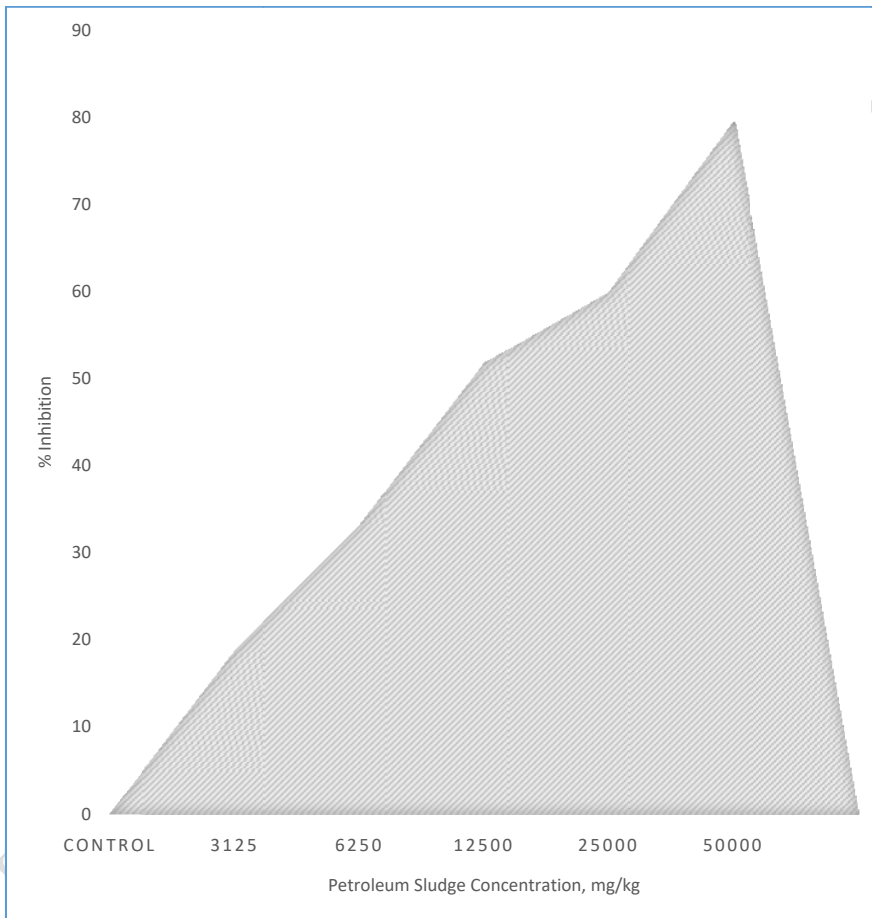


Fig 2: Percentage (%) inhibition of nitrogen transformation in petroleum refinery sludge contaminated soils

Table 1: Chronic toxicity profile of nitrogen transforming bacteria exposed to petroleum refinery sludge for 28 Days

Days	EC₅₀(mg/kg)	Confidence limit	Probit Equation	Slope
28	13761.059	11244.102 to 16961.715 mg/kg	0.544 + 1.340 x LOG(conc)	5.484

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3.1.2. Chronic toxicity test of petroleum sludge on Earthworm (*Apporectoda longa*)

The Earthworm (*Apporectoda longa*) was successfully used as a bioindicator to determine the chronic effect of petroleum sludge on terrestrial fauna by exposure to four sludge concentrations (375_mg/kg, 750_mg/kg, 1500_mg/kg, 3000 mg/kg). The growth rate decreased progressively from 9.19×10^3 g/day (375_mg/kg) to 3.55×10^3 g/day (3000_mg/kg) at the end of the test period (Fig 3). The % growth inhibition also increased from 37.91% (375_mg/kg) to 76.01% (3000 mg/kg), while 825.02 mg/kg was obtained as the EC_{50} at the end of 28days (Table 2). This concentration reduced the growth rate of the test earthworms by 50_%. In comparison with toxicity rating of chemicals to Earthworms (OECD_ 2003), the EC_{50} value indicates the sludge as slightly toxic (Table 2). The reduction of growth at higher concentrations showed it reduced growth progressively as the sludge concentration increased and could eventually lead to death. Bioconcentration factor (BCF) of TPH in the test Earthworms was also determined. The BCF decreased as the sludge concentration increased; an indication of higher bioaccumulation at higher sludge concentration (Table 3). It decreased from 5.168 (375_mg/kg) to 1.22 (3000 mg/kg). The BCFs obtained were much lower than the EPA stipulated limit of 1000 for persistence of chemicals.

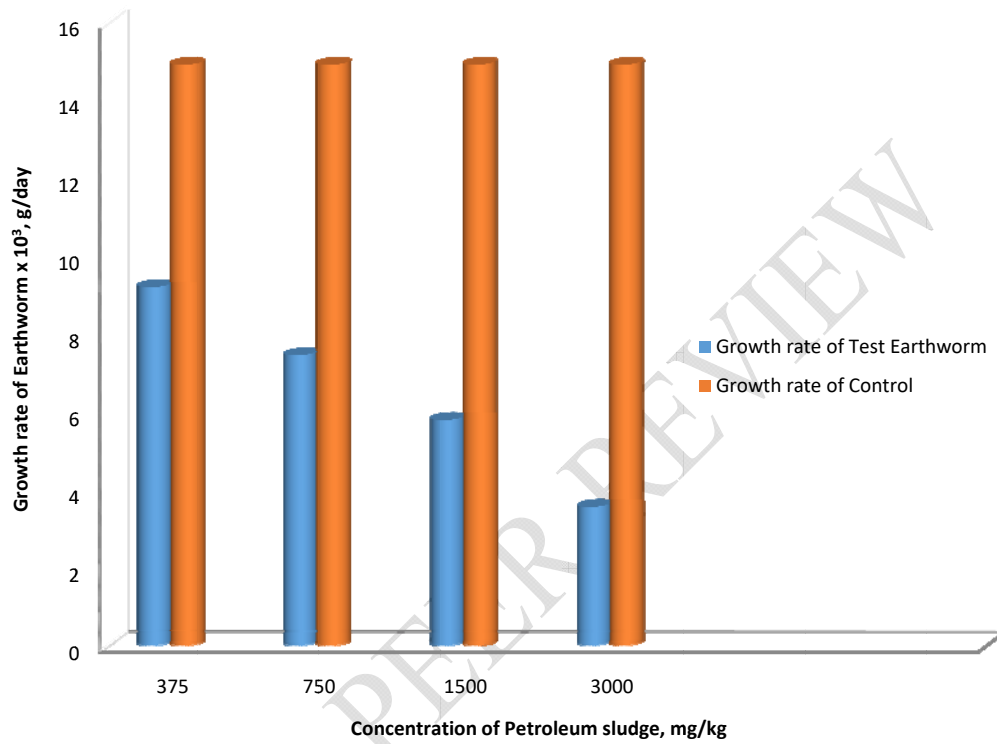


Fig 3:Growth rate of Earthworms exposed to petroleum sludge at day 28.

Table 2: Chronic toxicity profile of petroleum sludge on the growth rate of *Apporectoda longa*

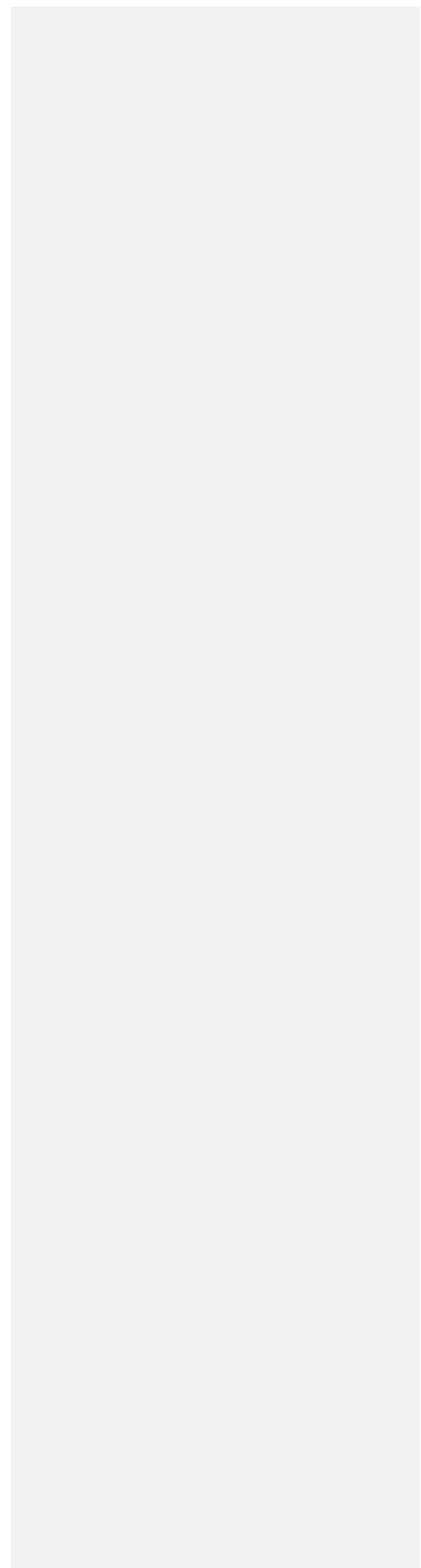
Test sample	Time (days)	EC ₅₀ , mg/kg	OECD(2003) Earthworm Toxicity		
			Rating	Designation	EC50(mg/kg)
Petroleum Sludge	7	Cannot be determined not up to 50% deaths	1	Super toxic	< 1.0
Petroleum Sludge	14	Cannot be determined not up to 50% deaths	2	Extremely toxic	1.0 – 10
Petroleum Sludge	21	1655	3	Very toxic	10- 100
Petroleum Sludge	28	825.024	4	Slightly toxic	100 -1000
			5	Practically non-toxic	>1000

UNDER PEER REVIEW

Table 3: Bioconcentration factor of TPH in Earthworm exposed to different concentrations of petroleum sludge for 28 days

TPH concentration, mg/kg in soil	TPH concentration in Earthworm	BCF
375	1938	5.168
750	2052	2.738
1500	2660	1.773
3000	3651	1.2173

UNDER PEER REVIEW



3.2. DISCUSSION

3.2. 1. *Nitrobacter* bioassay

The ~~discharge of untreated petroleum refinery sludge into the environment has~~ discharge of untreated petroleum refinery sludge into the environment has been shown to have acute and chronic effects on the biotic and abiotic components of the aquatic and terrestrial environments (Wang and Reed, 1983; Ma *et al.*, 2002; Tang *et al.*, 2012).

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Nitrobacter strains are ubiquitous in nature and have been found in several environments including soil, freshwater and sewage sludge (Chartrain *et al.*, 1983; Bock *et al.*, 1990). The genus *Nitrobacter* belongs to a variety of nitrite -oxidizing bacteria which are responsible for the second step of the nitrification process (oxidation of nitrite to nitrate) in the treatment of wastewaters (Daims *et al.*, 2001; Dionisi *et al.*, 2002; Maron *et al.*, 2003). This second step of nitrification is particularly sensitive. Inhibition of this step under uncontrolled conditions may lead to accumulation of nitrite nitrogen which is toxic (Dokaniakis *et al.*, 2005). Chronic effect of petroleum sludge pollution on nitrogen transformation in soil was determined since microorganisms play an important role in break-down and transformation of organic matter in fertile soils and any long-term interference with these biogeochemical processes could potentially interfere with nutrient cycling and this could affect soil fertility (OECD, TG 216, 2000). Percentage (%) inhibition of nitrate formation ranged from 18.0% to 79.37% from the lowest concentration (3750 mg/kg) to the highest concentration (50000 mg/kg) respectively. The observed increase in inhibition of transformed nitrogen as the concentration of petroleum sludge increased could be due to the increase of some physicochemical properties of the sludge such as PAHs, TPH and metals (Wilde *et al.*, 1983; Okpokwasili and Odokuma, 1997). High TPH concentrations have also been shown to be lethal to microbial activity, thus limiting their biodegradation potential (Admon *et al.*, 2001). Similar findings by Suschka *et al.* (1996), showed that the presence of PAHs (BTEX) which

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is a component of TPH produced a negative effect on the efficiency of the nitrification process. It could also be due to the toxic effect of accumulated nitrite nitrogen as a result of the inhibition of the nitrification process as the sludge concentration increased (Maron *et al.*, 2003). The EC₅₀ obtained (13761.059 mg/kg) is higher than the DPR Intervention limit (5000 mg/kg) for hydrocarbon contaminated soils.

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3.2.2. Earthworm bioassay

Earthworms are associated with a healthy soil and their absence is an indication of poor soil health (Doube and Schmidt, 1997; Edwards and Shipitalo, 1998; Parmelee *et al.*, 1998).

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Earthworms have been used in environmental risk assessment as good indicator organisms for toxicity (Callahan *et al.*, 1991; Dorn and Salanitro, 2000; Eijsackers, 1998; Reinecke and

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Reinecke, 1998) as they ingest large quantities of soil and are in full contact with the substrate they consume. In addition, they constitute up to 92% of the invertebrate biomass of soils and participate in many food chains, acting as a food source for a variety of organisms (Ireland 1983; ASTM E1676-95).

The chronic effect studies of petroleum sludge on earthworms showed that the sludge led to a reduction of growth progressively as the concentration of the sludge increased. Growth rate was inhibited from 37.91% (375 mg/kg) to 76.01% (3000 mg/kg) at the end of 28 days. Similar findings were recorded by Tang *et al.* (2011), who observed that body

weight of earthworms reduced to 48.91% on exposure to 1.5% TPH. The mechanism of toxicity of hydrocarbons to earthworms was observed to be based on the ability of hydrocarbons to bind at the polar regions in biogenic membranes and to disorganize them (Krab *et al.*,

2000). Furthermore, being lipophilic, they act as endocrine disruptors and disrupt processes

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related to the organism's growth, immune system function, sexual development, reproduction and malformation (Sandoval *et al.*, 2001).

The values obtained as bioconcentration factor (BCF) for TPH concentration in the earthworms ranged from 0.122 to 0.517, from the lowest to highest sludge concentration respectively. This indicates that the sludge would be bioaccumulated into the tissues of terrestrial organisms as the sludge concentration increases. Similar observations were recorded by MacGeer *et al.* (2003) who observed an inverse relationship between BCF and exposure concentrations of the test chemical and attributed this to the lipophilic nature of the sludge.

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4.0. Conclusion

Results obtained from the chronic effect studies for both the bioassays on nitrogen transformation activity of the bacteria *Nitrobacter sp.* and the earthworm indicated that the petroleum refinery sludge pose serious risks to the biota of the terrestrial environment as well as the biogeochemical cycles that ensure a sustainable ecosystem at high concentrations.

Due to its effects on terrestrial biota as observed in the bioindicators studied, biodegradation/treatment of petroleum refinery sludge needs to be done before its disposal. This could be done *ex situ* in bioreactors, where it could be used to generate biogas that could be employed to increase the energy mix between fossil fuel and sustainable source of energy such as biogas. Alternatively, it could be treated by landfarming, but the sludge should be introduced at concentrations below the EC_{50} so as to protect the fragile and sensitive biota of the terrestrial environment.

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COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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