Original Research Article

TOXICOLOGICAL EFFECTS OF PETROLEUM REFINERY SLUDGE ON THE TERRESTIAL ENVIRONMENT USING BACTERIA AND EARTHWORM AS BIOINDICATORS

ABSTRACT

Aim: The aim of the study is to assess the sublethal (chronic)ecotoxicologicaleffects ofpetroleum 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 EC_{50}) of the petroleum sludge onthenitrogen 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 were analyzed using a regression model (ANOVA). EC_{50} was also determined using the Probit analytical software. Analyses weredone using ASTM methods.

Results: Analysis of the Nigerian petroleum refinery sludge used for this research indicated that the sludge wasacidic with a pH value of 5.91and had a high TPH content of 340,000mg/kg made mainly of between 10-40 carbon unit compounds. There was a progressive increase in the percentage (%) inhibition of the nitrogen transformation activitiesof the bacteria, from 18.0%(3125mg/kg) to 79.30% (5000mg/kg) with increase in concentration and time. The effective concentration that decreased 50% (EC_{50}) of the nitrogen transformation activity was 13761.059mg/kg. The growth rate of the test earthworms decreased from 9.19 x 10^3g/day(375mg/kg) to 3.55 x 10^3g/day (3000mg/kg) for the test period. The BCF decreased from 5.168 (375mg/kg) to 1.22 (3000mg/kg) in the earthworms, while the $EC_{50}$for the

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

Conclusion: The sludge inhibitedthe 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 Oilysludge,* *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 poolsof adsorptive soil pollutants is a prerequisite for proper ecologicalrisk assessment in contaminated soils. Petroleum sludge is known to have strong adsorptive properties in soil and the bioavailability and in situ toxicity of sucha pollutant to the soil microorganisms may thus be affecteddifferently by dissolved and adsorbed pools in the soil (Knaebel*et al.,* 1994).On theother hand, many recent reports have

suggested that the adsorbedpollutant pool may also be at least partially available forthe microorganisms, both in terms of causing toxic effects (Alexander, 2000, Doherty, 2001; Ronnpagel*et al.*, 1995) and serving as substrates for biodegradation (Knaebel*et al.*, 1994).

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, 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, 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.

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).

Nitrobacterhamburgensis is a gram-negative bacterium whcih inhabit 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 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 2L glass bottles and preserved at 4^0C until required for use. It was analyzed for its physicochemical parametersusing standard methods.

2.1.2. Collection of *Nitrobacter sp.*

The *Nitrobacter* sp. used was isolated from Aladja River, Aladja, DeltaState, 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 4^0C 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).

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 5g/kg compost and treated with five concentrations (3125 mg/kg, 6250 mg/kg, 12500 mg/kg, 25000 mg/kg, and 50000mg/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 analyzed using a regression model (ANOVA) and the EC_{5O} 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 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).

% inhibition = $\dfrac{C_{ref} - C_{sample}}{C_{ref}}$ x 100

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 1-7days 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   Physicochemical Characteristics of the Petroleum sludge.

The petroleum refinery sludge used for the experiment was acidic with a pH value of 5.81and had a high TPH content of 340,000mg/kg made mainly of between 10-40 carbon unit compounds (fig 1). The physicochemical and microbial qualities of Nigerian petroleum refinery oily sludge shown in table 1 indicates the sludge has Polyaromatic hydrocarbon (PAH) content of was 0.075 ± 0.02 mg/kg and high values of26.04 ±1.02mg/kg and 21.65 ± 1.21 mg/kg for nitrate and ammonium respectively. The heterotrophic bacteria and fungi counts were 5.86E +

05 and 4.72E + 05 cfu/g, respectively. Hydrocarbon degrading bacteria and fungi counts were 2.85E + 02 and 2.75E + 02 cfu/g, respectively. Zinc recorded the highest concentration of 100.65 ±2.30mg/kg for the metals analyzed.

Table 1: Physicochemical and Microbiological properties
of Nigerian Petroleum refinery oily sludge.

Parameter	Mean (± S.E).
pH	5.81± 0.28
Conductivity, us/cm$_2$	466.65 ± 25.25
Sulphate, mg/kg	4.83 ± 0.64
Nitrate, mg/kg	26.40 ±1.02
Phosphate, mg/kg	7.73 ± 0.88
Total Nitrogen, mg/kg	0.12 ± 0.5
Total Petroleum Hydrocarbon, mg/kg	340,000 ± 50,000
Polyaromatic Hydrocarbon, mg/kg	0.075 ± 0.02
Ammonium, mg/ kg	21.65 ± 1.21
Copper, mg/kg	5.53 ±0.20
Chromium, mg/kg	8.68 ± 0.03
Nickel, mg/kg	3.36 ± 0.02
Cadmium, mg/kg	0.32 ± 0.5
Zinc, mg/kg	100.65 ± 2.30
Barium, mg/kg	0.31 ±0.04
Heterotrophic Bacteria, cfu/g	5.86E +05
Heterotrophic Fungi, cfu/g	4.72E+ 05
Hydrocarbon Degrading Bacteria, cfu/g	2.75E + 05
Hydrocarbon Degrading fungi, cfu/g	2.85E + 02

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

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 3125mg/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 % 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 EC_{50} of 13761.059mg/kg(Table 2). This concentration resulted in 50% inhibition of the nitrogen transformation activity of the *Nitrobacter sp.* in the test soil samples.

Response_

Signal: TPH_10082011_02.D\FID1Ach
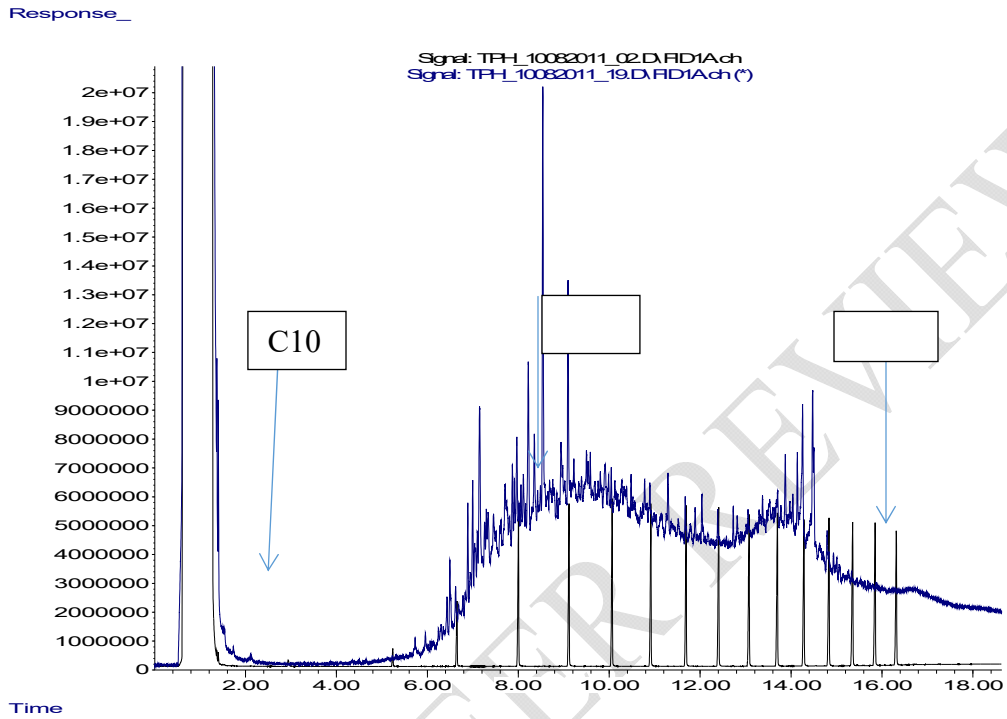Signal: TPH_10082011_19.D\FID1Ach (*)

C10

Time

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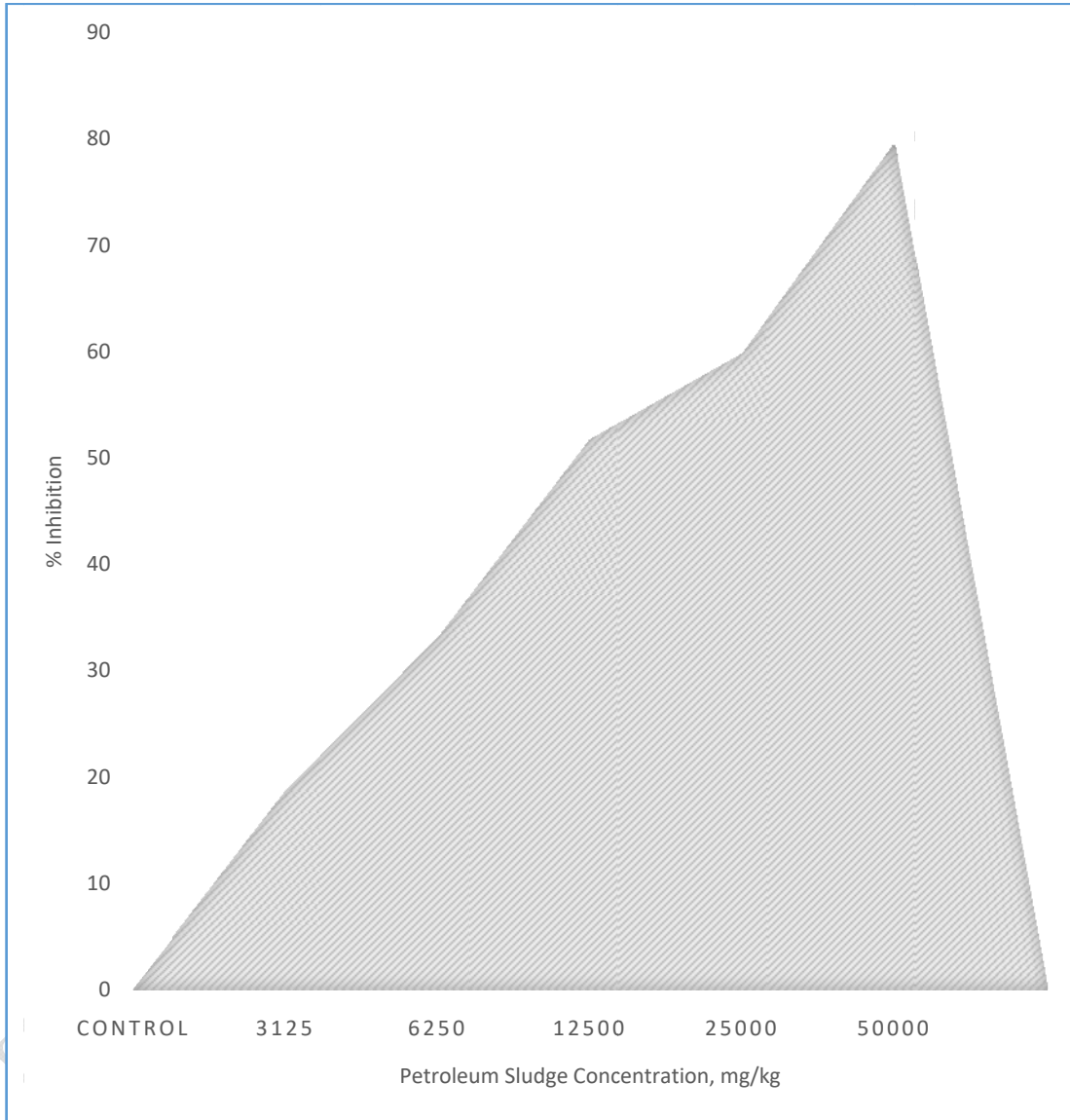
n-alkanes between C10 and C40

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

Table 2: Chronic toxicity profile of nitrogen transforming bacteria exposed to petroleum refinery sludge for 28 Days

Days	EC$_{50}$(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

3.1.3.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 (375mg/kg, 750mg/kg, 1500mg/kg, 3000 mg/kg). The growth rate decreased progressively from 9.19 x 10^3 g/day (375mg/kg) to 3.55 x 10^3 g/day (3000mg/kg) at the end of the test period (Fig 3). The growth rate of the organisms in the control increased at the end of the test duration when compared to those in the sludge contaminated soils.The average weight increased steadily from 7.58 x 10^3g/day to9.76 x 10^{14}g/day (fig 3).The % growth inhibition also increased from 37.91% (375mg/kg) to 76.01% (3000mg/kg), while 825.02 mg/kg was obtained as the EC_{50} at the end of 28days (Table 3). This concentration reduced the growth rate of the test earthworms by 50%. In comparison withtoxicity rating of chemicals to Earthworms (OECD 2003), the EC_{50} value indicates the sludge as slightly toxic (Table 3). 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 4). It decreased from 5.168 (375mg/kg) to 1.22 (3000mg/kg). The BCFs obtained were much lower than the EPA stipulated limit of 1000 for persistence of chemicals.
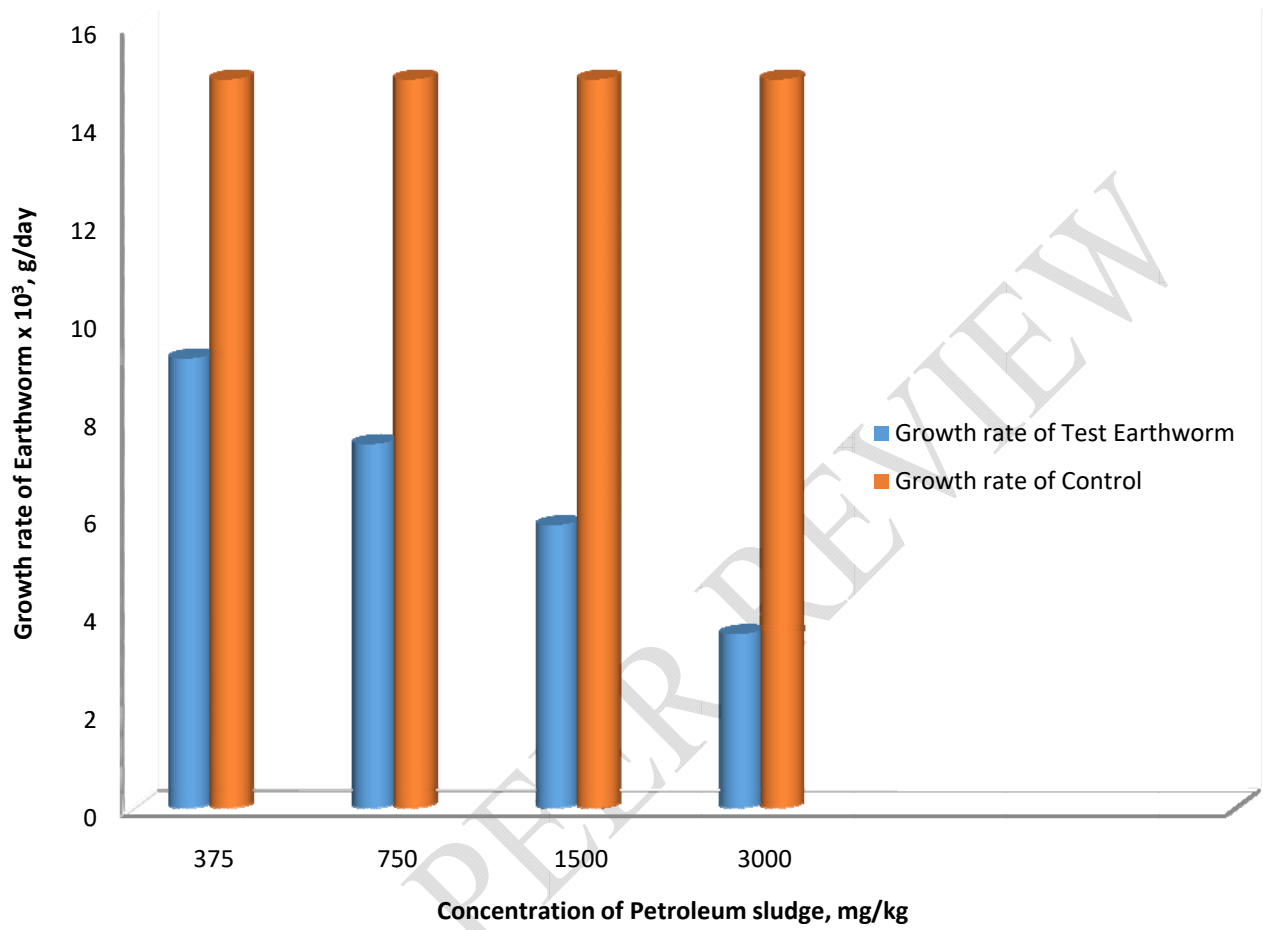
Fig 3:Growth rate of Earthworms exposed to petroleum sludge at day 28.

Table 3: 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

Table 4: 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

3.2. DISCUSSION

The high concentration of TPH analysed in the test sludge (340,000 ±50,000mg/kg)and Zinc concentration (100.00 ±2.30mg/kg) were similar to findings by Prakash *et. al.*,(2015)who recorded a high sludge concentration of >380,000mg/kg and a high Zinc content of 1321.7 ± 529.9 mg kg−1 at variance with the EU guidelines. According to the EU guidelines, the sludge was not allowed to be disposal even in landfills for hazardous waste due to its toxic nature (Kriipsalu*et.al.,* 2008). The hazardous nature of the sludge was hence assessed using sensitive bioindicators in this terrestrial environment.The Nitrobacter*sp.* Is involved in the very important biogeochemical nitrogen cycle, while the earthworm helps to improve the structure and oxygen content of the soil.

3.2. 1. *Nitrobacter bioassay*

The discharge of untreated petroleum refinery sludge into the environment have been shown to have acute and chronic effects on the biotic and abiotic components of the aquatic and terrestrial environments (Wang and Reed, 1984; Ma *et al*., 2002; Tang *et al*., 2012).

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 thesebiogeochemical 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 (3750mg/kg)to the highest concentration

(50000mg/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 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 (5000mg/kg) for hydrocarbon contaminated soils.

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). 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 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% (375mg/kg) to 76.01% (3000mg/kg) at the end of 28days. 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 biogeneous membranes and to disorganize them (Krab*et al.,* 2000). Furthermore, beenlipophylic, they act as endocrine disruptors and disrupt processes 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.

4.0. Conclusion

Results obtained from the chronic effect studiesfor 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 EC50 so as to protect the fragile and sensitive biota of the terrestrial environment.

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