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Toxicity of bitumen on soil fertility bacteria - Nitrobacter and Nitrosomonas from Tekulu Waterside, Rivers State, Nigeria

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Abstract

Toxicity of bitumen on Nitrosomonas and Nitrobacter from Tekulu Waterside, Bodo in Gokana Local government area of Ogoni, Rivers State, Nigeria was carried out using standard analytical procedures. The density, API and flash point of the bitumen used in this study at 15°C was 0.9898 g/cm^3, API at 60°F value of 11.46 and >200°C respectively. The total TPH and PAH constituent of the bitumen was 20,549.77 mg/L and 924.09 mg/L respectively. Analysis of the effect of different concentration (50 mg/L, 250 mg/L, 500 mg/L, 750 mg/L and 1000 mg/L) of bitumen on Nitrosomonas sp. over a period (24hrs, 48hrs. 72hrs and 96hrs) revealed that the mortality of the organism increased as the concentration of the toxicant. The mortality was also affected by the duration of exposure. The probit analysis on the lethal concentration (LC_{10}, LC_{20} and LC_{50}) of the bitumen on Nitrosomonas sp. was 223.32 mg/L, 369.682 mg/L and 969.622 mg/L respectively and Nitrobacter sp. 587.837 mg/L, 765.74 mg/L and 1269.841 mg/L respectively, when exposed for 96 hours revealed a varying mortality status. The LC_{50} of the bitumen on Nitrosomonas sp. peaked at 48hrs. The study shows that bitumen was more toxic to Nitrosomonas than Nitrobacter sp. based on the findings of the study, it is recommended that test organisms (*Nitrosomonas* sp. and *Nitrobacter* sp.) could serve as a potential tool for eco-toxicological assay and pollution biomarker.

Keywords: Toxicity; Nitrosomonas; Nitrobacter; Probit; Mortality

1. Introduction

Petroleum is still the principal energy source for industries and industrial uses; even for some domestic uses. Despite the importance in the society; petroleum is a major source of pollution in the environment. Certain petroleum hydrocarbons are carcinogenic and mutagenic [1;2] and pose serious threat to human; plants; and animals health. Pollution of ecosystem is a matter of global concern as it leads to contamination of the food chain. In the environment; microorganisms play a fundamental role in biogeochemical processes including nutrient cycling. One of the microbial groups that play vital functions especially in plant fertility is the nitrogen fixing (nitrifying) bacteria. The abundance of these groups of organisms sometimes are used as indicators of soil fertility status and as bioindicators. Environmental pollution especially those of hydrocarbon-based pollutants; are known to be toxic to nitrifying bacteria and other autochthonous soil microorganisms; thus; influencing their growth and survival in the ecosystem [3]. Nitrobacter is a genus of mostly rod-shaped; gram-negative; aerobic-nitrifying and chemoautotrophic bacteria and cells normally reproduce by budding [4].

Microbial monitoring specifically for hydrocarbon is the concurrent stimulation and inhibition effect of petroleum hydrocarbons on bacteria; which complicates toxicity assessments [5]. The number of organisms that die after the exposure can then be measured and the concentration of a substance that kills half the test population calculated. This is the basis of the 24hour LC_{50} (Lethal concentration that kills 50% of the test population) toxicity test method [3]. The harmful effect of chemicals has on organism depend on many different factors such as the type of organism; age; size;

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population; concentration of the toxicant; etc., [6]. Microorganisms found in fresh water; brackish water and marine water such as bacteria; fungal; viruses and protozoa; can influence the tri-aquatic ecosystem ability to sustain life on earth [7]. Bacteria such as Nitrobacter are also present in the fresh water; brackish water and marine water [3].

Driven by the roles of nitrifying bacteria in soil and water; this study was designed to investigate the toxicity of bitumen on two representatives (Nitrosomonas and Nitrobacter) associated with the brackish water collected from Tekulu Waterside; Bodo in Gokana Local government area of Ogoni; Rivers State; Nigeria.

2. Material and methods

2.1. Sample Collection

Bitumen used for this study was collected from an artisanal refinery located in Bodo town, Gokana Local Government Area of Ogoni, Rivers State, Nigeria. Bodo city is situated on a geographic grid reference of longitude 4°30N and latitude 7°15E. The samples were collected in sterile four (4) litre plastic containers and transported to the laboratory. *Nitrobacter* sp. and *Nitrosomonas* sp. was isolated from a brackish water collected from Tekulu Waterside, Bodo in Gokana Local government area of Ogoni, Rivers State.

2.2. Isolation of *Nitrobacter and Nitrosomonas* (autotrophic bacteria)

Winogradsky broth was used for the isolation of *Nitrobacter* and *Nitrosomonas*. The composition of Winogradsky broth was obtained from Odokuma and Akponah, [8]. Winogradsky broth medium (phase 1) was prepared with the following composition (g/l) in sterile distilled water: $(NH_4)_2S0_4$, 2.0 g; K_2HP0_4, 1.0 g; $MgS0_4.7H_20$, 0.5 g; NaCl, 2.0 g; $FeS0_4.7H_20$, 0.4 g; and $CaC0_3$, 0.01 g. The Winogradsky broth medium (phase 1) was used for the isolation of nitrifying bacteria responsible for nitrification phase 1. Another Winogradsky broth medium (phase 11) used for the isolation of nitrifying bacteria responsible for nitrification phase II was also prepared. The Winogradsky broth medium (phase 11) was prepared with the following composition (g/l): KNO_2, 0.1 g; Na_2C0_3, 1.0 g; NaCl, 0.5 g; and $FeS0_4.7H_20$, 0.4 g. The preparations were sterilized at 121^0C for 15 minutes at 15psi. Winogradsky agar media for nitrification phases 1 and 11 were prepared by adding 15.0 g agar to 1000ml of the fresh broth and sterilized at 121˚C for 15 minutes at 15 psi. The media were allowed to cool to about 45˚C before they were dispensed into sterile Petri dishes and allowed to stay overnight to test for sterility.

All the agar plates were aseptically inoculated with 0.1 ml of the brackish water sample using spread plate technique. The inoculum was spread over the entire surface of the solid agar with a sterile glass rod. All the inoculated petri dishes were incubated aerobically at temperature of 30˚C 72-96 hours. After incubation, the colonies were observed (counted) and discrete colonies from each plated media were sub-cultured into fresh Winograsdky media to obtain the pure culture. The pure cultures were inoculated on slants using bijou bottles and stored at 40°C, this was the stock cultures. Each isolate was picked from their respective stock cultures using a wire loop and inoculated on a fresh media and then incubated at 37°C for 24hrs. A ten-fold (10^{-4} and 10^{-5}) serial dilution was prepared by transferring 1 m into the tubes. From the 10^{-4} and 10^{-5} serial dilutions, 0.1 ml was plated into a sterile Winogradsky medium and were incubated. Plates containing 30 - 80 colonies were chosen for the toxicity test [8].

2.3. Purification and Maintenance of Pure Isolate

Different discrete colonies that developed on the Winogradsky agar media for nitrification phases I and II after incubation period were aseptically sub-cultured repeatedly on freshly prepared Winogradsky agar media, phases 1 and II, respectively. All the plates were incubated aerobically at temperature of 30˚C 72-96hours. The pure isolates were transferred to Winogradsky agar slants and stored in the refrigerator for further use.

2.4. Preparation of Nitrate Reagent (Griess-Ilosvay Reagent)

The preparation of the reagent was done by dissolving 0.6 g of sulphanilic acid in a hot (90°C) 70 ml of distilled water. The solution was kept for a while to cool before adding 20 ml of concentrated HCl. The solution was diluted to 100ml with distilled water and mixed thoroughly. An amount of 0.6 g of alpha-naphthylamine was dissolved in 15 ml of distilled water containing 1 ml of concentrated HCl to produce N-napthyl-ethylene-diamine dihydrochloride (NED). It was again diluted to 100 ml with distilled water while 16.4 g of $CH_3COONa.3H_2O$ (sodium acetate) was dissolved into 70 ml distilled water and diluted again to 100ml with a distilled water. The reagents were stored in dark bottles separately.

2.5. Identification of Isolates

Pure isolated organisms from the appropriate agar slants were characterized and identified using morphological (cell and colonial morphology, shape, motility, and gram reaction) and physiological attributes [9, 10].

2.6. Determination of Total Petroleum and Polycyclic Aromatic Hydrocarbon (PAH) of Toxicants

To determine the total petroleum content in the samples, one hundred (100) ml of the sample was measured into a separating funnel and 10 ml of Dichloromethane: Hexane (1:1) was added into it. The mixture was shaken gently and vented for 5 minutes. The aqueous layer was allowed to separate and was decanted. The extracts were concentrated by rotary evaporator into 1ml. Precisely 1.0 µL of the extracts was injected into a pre-programmed Hewlett-Packard HP 5890 GC-FID. The concentration of TPH was calculated from the peak area of the calibration standards. The GC operational conditions employed for determining the TPH is as follows: Initial oven temp-50 ˚C, Initial Hold time - 2.0 mins, Ramp-10˚C/min to 300˚C, Final Oven Temp - 320˚C, Detector Temp - 340˚C, Injector Temp - 250˚C, Carrier gas – Helium, Ignition gas - Hydrogen and air [11].

To determine the PAH concentration, precisely, 100 ml of the sample was also measured into a separating funnel and 20 ml of dichloromethane was added and shaken for 5 minutes. The extract was concentrated to 2 ml in a rotary evaporator. 20 ml 0.5 M KOH in 100 ml of methanol was added and the mixture was refluxed for 30 minutes in a water bath at 60°C. 10 ml Deionized water was added and extracted with hexane (10 ml). The extract was dried over anhydrous sodium sulphate and concentrated at 60°C in a rotary evaporator to 2 ml. The extract was then passed through a silica gel column which had been pre-conditioned with hexane and eluted with 10 ml of hexane for aliphatic fractions. To same column, 10 ml of dichloromethane was added for the elution of PAHs and the eluent was concentrated to 1 ml and solvent exchanged with 1 ml of acetonitrile. 1 µL of the extract was injected into a pre-programmed HP 5890 GC-FID. The concentration of the PAHs was calculated from the peak area of the calibration standards.

The operational condition for PAH analysis were as follows: Initial oven temp - 100˚C, Initial Hold time - 0.5 minutes, Ramp – 15°C/min to 200°C, then 20°C/min to 300°C, Final Oven Temp - 300˚C, Detector Temp - 340˚C, Injector Temp – 250°C, Carrier gas – Helium, Ignition gas - Hydrogen and air. The PAHs were determined in selective ion-monitoring mode with ionization energy of 70 eV. The m/z peaks corresponding to the molecular masses of individual PAH were used for identification and quantification [11].

2.7. Physiochemical Parameter of Bitumen, API Gravity of Bitumen (Hydrometer Method)

Gravities are determined at 60°F (15.56°C), or converted to values at 60°F, by means of standard tables (ASTM 2012 and ASTM 2026). The thermohydrometer was used. The temperature of the samples was adjusted in accordance with the table below. The hydrometer cylinder was approximately the same temperature as the samples being tested. The samples were transferred into the clean hydrometer cylinder without splashing, this is to avoid the formation of air bubbles and to reduce to a minimum the evaporation of the lower boiling constituents of the more volatile samples. The more volatile samples were transferred to the hydrometer cylinder by siphoning. The bubbles collected on the surface of the samples were removed by touching them with a piece of clean filter paper before inserting the hydrometer. The cylinder containing the samples were placed in a vertical position in a location free from air currents. Precautions were taken to prevent the temperature of the samples from changing appreciably during the time necessary to complete the test. The temperature of the surrounding medium was keenly motored during this period to prevent it from changing beyond 5°F (2°C). The hydrometer was gently lowered into the samples and, when it had settled, it was depressed about two scale divisions into the liquid and then released. The rest of the stem was kept dry, as unnecessary liquid on the stem changes the effective weight of the instrument, and so would affect the reading to be obtained. The samples with low viscosity, a slight spin was imparted to the instrument on releasing assists to bring it to rest, floating freely away from the walls of the hydrometer cylinder. Sufficient time was allowed for the hydrometer to become completely stationary and for all air bubbles to come to the surface. After the hydrometer had come to rest, floating freely, and the temperature of the sample became constant to 0.2°F (0.1°C), the hydrometer was read to the nearest scale division. The correct reading is that point on the hydrometer scale at which the surface of the liquid cuts the scale. This point was determined this point by placing the eye slightly below the level of the liquid and slowly raising it until the surface, first seen as a distorted ellipse, appeared to become a straight line cutting the hydrometer scale. To make a reading, the point on the hydrometer scale was observed to note when the samples rose above its main surface, placing the eye slightly above the plane surface of the liquid. The reading required a correction. The correction was determined for the hydrometer in use by observing the height above the main surface of the liquid to which the samples rose on the hydrometer scale. This was done when the hydrometer was immersed in a transparent liquid having a surface tension like that of bitumen. The temperature of the samples was observed to the nearest 0.25°F (0.1°C) immediately before and after the observation of the gravity, the liquid in the cylinder being thoroughly but cautiously

stirred with the thermometer, and the whole of the mercury thread being immersed. The mean of the thermometer reading was recorded before and after the final hydrometer reading to the nearest 1°F, as the temperature of the test (ASTM 2012 and ASTM 2016).

Using the procedure, the correction was subtracted from the hydrometer reading observed after gravities was observed on opaque liquids. All hydrometer readings were corrected to 60°F (15.56°C), using Tables 1. The corrected hydrometer reading was recorded as degrees API (°API) or as API Gravity. The samples were consistent with the type of samples and necessary limiting conditions shown in the table below.

Table 1 (ASTM 2012)

S/N	Sample Type	Gravity Limits	Initial Boiling Point Limits	Other Limits	Test Temperature
1	Highly volatile	lighter than 70° API			Cool to 35°F (2°C) or lower in original closed container.
2	Moderately volatile	heavier than 70° API	below 250°F (120°C)		Cool to 65°F (18°C) or lower in original closed container.
3	Moderately volatile and viscous	heavier than 70° API	below 250°F (120°C)	Viscosity too high at 65°F (18°C	Heat to minimum temperature for sufficient fluidity.
4	Nonvolatile	heavier than 70° API	above 250°F (120°C)		Any temperature between 0 and 195°F (−18 and 90°C) as convenient. 60 6 0.25°F (15.56 6 0.1°C)
5	Mixtures of nonpetroleum products or essentially pure hydrocarbons				

2.8. Toxicity Test Procedure *Nitrobacter* sp and *Nitrosomonas* sp.

Tubes containing 9ml of appropriate broth for *Nitrobacter* sp. and *Nitrosomonas* sp., and the different concentrations (0.01 ppt, 0.1 ppt, 1 ppt, 10 ppt, and 100 ppt) of the toxicant (bitumen) was set up. 1 ml of the test organism was added to the respective labelled tubes and 0.1 ml of each was plated out immediately on appropriate agar plates. This was to determine the 0 hour count. The inoculated tubes were incubated at appropriate temperature and incubation periods. Aliquot (0.1 ml) of each concentration of the toxicants was then plated out in triplicates at 24 hours, 48 hours, 72 hours and 96 hours on appropriate agar plates and incubated at 30°C 72-96 hours. The colonies were then counted, and average colony taken. The treatments were in duplicates.

2.9. The Percentage log Survival of *Nitrobacter* sp., and *Nitrosomonas* sp., in the Toxicants

The percentage log survival of the isolates in the toxicant used in the study was calculated using the formula adopted from Williamson and Johnson [10]. The percentage log survival of the bacterial isolates in the toxicant was calculated by obtaining the log of the count in each toxicant concentration and dividing the count in the zero toxicant concentration and them multiplying the product by 100.

Therefore % log survival = $Log\ C\ x\ 100/Log\ c$

Where Log C = log count in each toxicant concentration and Log c = log count in the zero-toxicant concentration

The toxicity of different concentration of bitumen (0.01 ppt, 0.1 ppt, 1 ppt, 10 ppt, and 100 ppt) on *Nitrobacter and Nitrosomonas* after 24 hours, 48 hours, 72 hours and 96 hours exposure was determined using standard technique. *Nitrosomonas* and *Nitrobacter* are chemoautotrophic organisms found in soil and water and are responsible for the oxidation of ammonium to nitrite (*Nitrosomonas*) and nitrite to nitrate (*Nitrobacter*). This process, known as nitrification, is important because it can affect plant growth beneficially, but nitrate also contributes to potable water contamination. The process of nitrification occurs in the cell membrane of the organisms. The presence and abundance of these organisms in any ecosystem may be used as a fertility status indicator for such ecosystem. The morphological

and biochemical characteristics of *Nitrobacter* sp. and *Nitrosomonas* sp. isolated from a brackish water from Tekulu Waterside, Bodo in Gokana Local government area of Ogoni, Rivers State agreed with that given on the Bergeys Manual of Determinative Bacteriology. The morphological and biochemical characteristics of the *Nitrobacter* sp. and *Nitrosomonas* sp. isolated from a brackish water from Tekulu Waterside, Bodo in Gokana Local government area of Ogoni, Rivers State is as presented on Table 1.

Table 2 Morphological and biochemical characteristics of *Nitrobacter* and *Nitrosomonas*

Tests Organism A Organism B
Colour of colony Colourless/brownish Greyish/whitish
Shape of colony Circle – like and raised Round and flat
Size of colony Tiny Tiny
Cell shape Straight long rod Pear-shaped short rod
Gram staining _ _
Motility _ _
Nitrate reduction _ _
Ammonia oxidation + _
Nitrite oxidation _ +
Organism suspected *Nitrosomonas* sp. *Nitrobacter* sp

Key: + = Positive reaction; – = Negative reaction.

Bitumen is a mixture of organic liquids that are highly viscous, black, sticky and entirely soluble in carbon disulphide. Although no two bitumen are chemically identical and chemical analysis cannot be used to define the exact chemical composition of bitumen, elemental analysis indicates that most bitumen contains 79-88% carbon; 7-13% hydrogen, traces to 3% nitrogen; 8% sulphur; 8% oxygen by weight [12]. The density of the bitumen used in this study at 15˚C was 0.9898 g/cm^3 with an API at 60˚F value of 11.46 (Table 3). The Flash Point of the bitumen sample was >200˚C. Based on flash point of the bitumen sample, it can be rated as a good bitumen. According to Mumah and Muktar [13], the flash point of a good grade bitumen sample lies in the range of 245-352˚C.

Table 3 Physiochemical Proprieties of Bitumen

Parameter Method Bitumen Result
Density @15°C (g/cm3) ASTM-D4052 0.9898
API @ 60°F (˚API) ASTM -D287 11.46
Flash Point (°C) ASTM D92 >200
Basic Sediment and Water (BS&W) (%) ASTM-D1796 <0.1

Analysis of the total petroleum hydrocarbon constituent (TPH) and Polycyclic Aromatic Hydrocarbon (PAH) of the toxicants (Bitumen) revealed that the total TPH and PAH constituent of the bitumen was 20,549.77 mg/L and 924.09 mg/L respectively. A summary of the TPH and PAH constituent and their concentration is as presented on Figure 1 and 2 respectively.

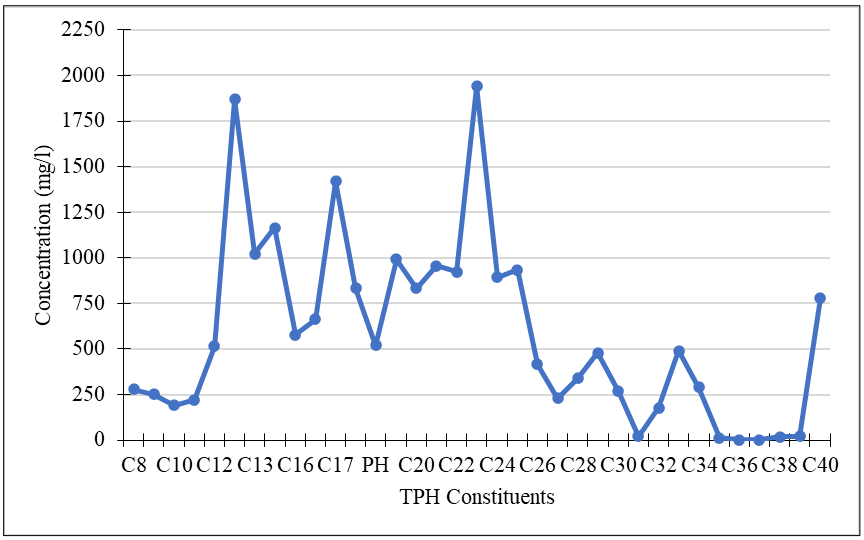

Figure 1 Total Petroleum Hydrocarbon Constituent of the Bitumen

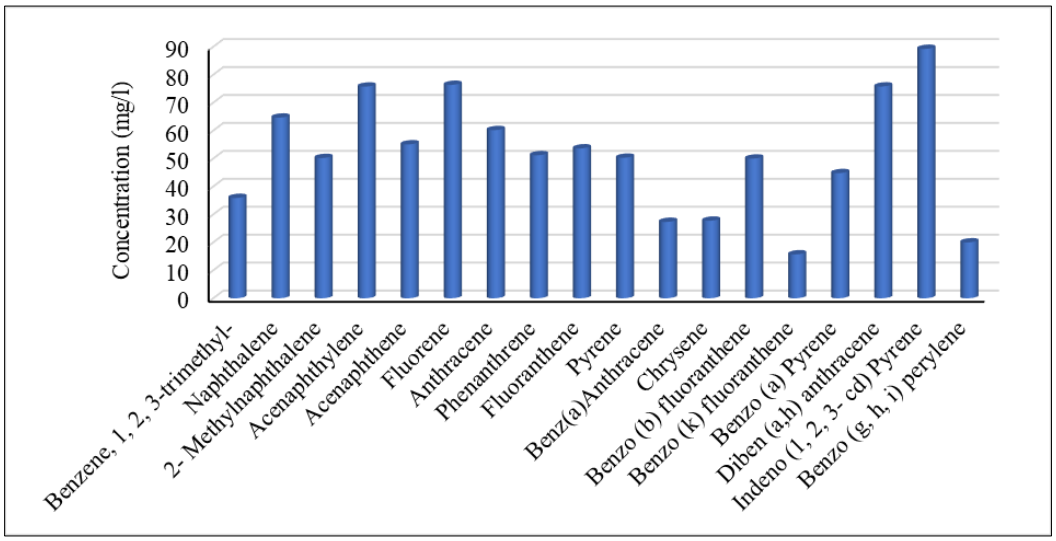

Figure 2 Polycyclic Aromatic Hydrocarbon Constituent of the Bitumen

Analysis of the effect of different concentration (50 mg/L, 250 mg/L, 500 mg/L, 750 mg/L and 1000 mg/L) of bitumen on *Nitrosomonas* sp. over a period (24 hrs, 48 hrs. 72 hrs and 96 hrs) revealed that the mortality of the organism increased as the concentration of the toxicant. The mortality was also affected by the duration of exposure. Mortality observed with 24 hours was lower than that observed after 96 hours. The LC_{10}, LC_{20} and LC_{50} of bitumen to *Nitrosomonas* sp. after exposure to 96 hours is 223.32 mg/L, 369.682 mg/L and 969.622 mg/L respectively. The LC_{50} of bitumen at 969.622 mg/L revealed that bitumen was moderately toxic to *Nitrosomonas* sp. when compared to the GESAMP [27] toxicity grading scale. A summary of this result is as presented on Table 4.

Table 4 Mortality of *Nitrosomonas* sp. when Exposed to Bitumen

Parameters	***Nitrosomonas* sp. exposure to Bitumen (24hr)**	***Nitrosomonas* sp. exposure to Bitumen (48hr)**	***Nitrosomonas* sp. exposure to Bitumen (72hr)**	***Nitrosomonas* sp. exposure to Bitumen (96hr)**
LC10	916.261 mg/L	646.172 mg/L	570.771 mg/L	223.32 mg/L
LC20	1437.923 mg/L	868.083 mg/L	757.108 mg/L	369.682 mg/L
LC50	3405.308 mg/L	1527.014 mg/L	1299.821 mg/L	969.622 mg/L

Analysis of the effect of different concentration (50 mg/L, 250 mg/L, 500 mg/L, 750 mg/L and 1000 mg/L) of bitumen on *Nitrobacter* sp. over a period (24 hrs, 48 hrs. 72 hrs and 96 hrs) revealed that the mortality of the organism increased as the concentration of the toxicant. The LC_{10}, LC_{20} and LC_{50} of bitumen to *Nitrobacter* sp. after exposure to 96 hours is 587.837 mg/L, 765.74 mg/L and 1269.841 mg/L respectively. The LC_{50} of bitumen at 1269.841 mg/L revealed that bitumen was slightly toxic to *Nitrobacter* sp. when compared to the GESAMP [27] toxicity grading scale. A summary of this result is as presented on Table 5

Table 5 Mortality of *Nitrobacter* sp. when Exposed to Bitumen

Parameters	***Nitrobacter* sp. exposure to Bitumen (24hr)**	***Nitrobacter* sp. exposure to Bitumen (48hr)**	***Nitrobacter* sp. exposure to Bitumen (72hr)**	***Nitrobacter* sp. exposure to Bitumen (96hr)**
LC_{10}	1076.008 mg/L	792.043 mg/L	688.848 mg/L	587.837 mg/L
LC_{20}	1705.943 mg/L	1231.291 mg/L	902.929 mg/L	765.74 mg/L
LC_{50}	4119.698 mg/L	2863.711 mg/L	1515.295 mg/L	1269.841 mg/L

The probit analysis on the lethal concentration (LC_{10}, LC_{20} and LC_{50}) of the bitumen on *Nitrosomonas* sp. and *Nitrobacter* sp. when exposed for 96 hours revealed a varying mortality status. The LC_{50} of the bitumen on *Nitrosomonas* sp. was peaked at 48 hrs.

Findings of the study revealed that the mortality of the *Nitrosomonas* sp. and *Nitrobacter* sp. increased as the concentration of the toxicant. The mortality was also affected by the duration of exposure. The findings of the study agree with the observations of Wang [14]; Nrior and Owhonda [15] and Nrior and Okele [16]. This finding agrees with various reports that shows that nitrification is sensitive to environmental stressors and contaminants [17, 18, 19]. Nitrifying microorganisms have been used as bioindicators to assess the health condition of environments and ecosystems [20] as they are ubiquitously found in various natural and manmade environments [21] and play an important role in the marine nitrogen cycle. According to Okpokwasili and Odokuma [22], hydrocarbon concentrations negatively affect nitrite oxidation but does not induce the cell death of *Nitrobacter* sp. Based on the authors report, *Nitrobacter* showed tolerance to Nigerian crude oil and 50% inhibition of nitrification was observed in the range of 60–7500 mg/L of crude oil concentrations.

The ability of Nitrobacter to resist hydrocarbon toxicity may be attributed to the potential of the isolates to utilize the light hydrocarbons as a source of carbon for growth. This assertion is in line with various reports on the potential of Nitrobacter and Nitrosomonas to utilize hydrocarbons. John and Okpokwasilli [23] reported that the organisms could utilize crude oil and its petrochemical products, such as diesel, kerosene, jet fuel and engine oil for growth. This agrees with the reports of Deni and Penninck [24], Okpokwasili and Odokuma [22] and John *et al.* [25, 26] that nitrifying bacteria is an excellent degrader of crude oil and has ability to utilize crude oil and its products.

3. Conclusion

The toxicity of bitumen on *Nitrosomonas* sp and *Nitrobacter* sp. is of importance as it affects the nitrification process which negatively and adversely affects aquatic fauna. The study shows that bitumen was more toxic to *Nitrosomonas* than *Nitrobacter* sp. based on the findings of the study, it is recommended that test organisms (*Nitrosomonas* sp. and *Nitrobacter* sp.) could serve as a potential tool for eco-toxicological assay and pollution biomarker.

Compliance with ethical standards

Disclosure of conflict of interest

The authors have declared that there is no conflict of interest.

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