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Toxicity of Local and Industrial Refined Diesel on *Nitrobacter* Species a Key Environmental Pollution Bio-marker

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Authors' contributions

This work was carried out in collaboration between both authors. Author RRN designed the study. Author CMO performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript, managed the analyses of the study and managed the literature searches under the strict supervision of author RRN. Both authors read and approved the final manuscript.

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ABSTRACT

Aim: Hydrocarbon toxicological effect on nitrogen fixing bacterium *Nitrobacter* sp. is of prime importance as it affects the nitrification process which negatively and adversely affects aquatic flora. In view of the significance of this process, the toxicity of local and industrial refined diesel on a key environmental pollution bio-marker, *Nitrobacter* was investigated.
Study Design: Semi-static ecotoxicological bioassay was used to study the effect of varying concentrations of toxicants local and industrial refined diesel on aquatic bacterium *Nitrobacter* sp.
Place and Duration of Study: Sample: marine water samples were collected from bonny sea, bonny, freshwater from a stream in MuuBagia in BiaraGokana and brackish water from sand-field in Port Harcourt, Nigeria.
Methodology: Winogradsky medium, nutrient agar, and King agar B base was used for the isolation of bacteria species by spread plate techniques. Standard toxicity procedure was carried out using diesel prepared at different concentrations (%) 0, 3.25, 6.5, 12.5, 25 and, 50; tested with

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Nitrobacter sp. for 0 h, 4 h, 8 h, 12 h, and 24 h separately for each toxicant. Median lethal concentration (LC_{50}) was employed to compute the toxicity of different concentration to the test organism.

Results: The median lethal concentration (LC_{50}) of the diesel used were calculated mean mortality of the test organism *Nitrobacter* sp. with industrial diesel in fresh water was (43.85%) >*Nitrobacter* with industrial diesel using brackish water (30.23%) >*Nitrobacter* with industrial diesel in marine water was (15.93%). *Nitrobacter* with locally refined diesel in fresh water (34.76%) >*Nitrobacter* with locally refined diesel in brackish water (26.81%) >*Nitrobacter* with locally refined diesel in marine water (29.77%). [Noting that the lower the LC_{50}, the more toxic the toxicant].

Conclusion: The study shows that local refined diesel has more toxic effect in brackish and freshwater than industrial refined diesel whereas in marine water a reverse trend occurs; industrial refined diesel being more toxic than local refined diesel. In view of the sensitive nature of *Nitrobacter* sp. to slight variation in toxicity quotient and its role in biogeochemical cycle; it could serve as a potential tool for eco-toxicological assay and pollution bio-marker.

Keywords: Toxicity; Nitrobacter sp.; modified Winogradskyagar; local and industrial refined diesel; pollution bio-maker.

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], thus posing a serious threat to human, plants and animals health. Accumulation of petroleum hydrocarbons in animals and plants tissues may cause progeny's death or mutation thus leading to extensive alteration or damage of ecosystem [3]. Petroleum hydrocarbon is a physical substance comprising many toxic compounds such as polycyclic aromatic hydrocarbon, benzene compounds [4].

Diesel fuel is a mixture of more than 2000 compounds which cannot be all separated by chromatography, thus among petroleum hydrocarbon, diesel which is composed of alkanes and aromatic compounds, has been widely used in various industries. The use of hydrocarbons as substrates for microbial growth causes problems to both the microorganism using them as a source of carbon energy and to researchers in the field of petroleum microbiology. The localisation of hydrocarbon oxidising bacteria in natural environment has received considerable attention because of the possibility of utilising their biodegrading potential in the treatment of oil spill [5,6,7].

Microbial monitoring specifically for hydrocarbon is the concurrent stimulation and inhibition effect of petroleum hydrocarbons on bacteria, which complicates toxicity assessments [8]. 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 [7]. The harmful effects that chemicals have upon individual organism depend on many different factors, not only on the organisms but also in the form in which the population occurs [9]. Micro organisms 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 [8]. Bacteria such as *Nitrobacter* are also present in the fresh water, brackish water and marine water [7].

Spillages arising from diesel and kerosene into our environment are becoming a visible problem and may be toxic to nitrifying bacteria and other autochthonous soil microorganisms, thus, influencing their growth and survival in the ecosystem. Microorganisms play a fundamental role in the biogeochemical cycles in nature by re-mineralising organic matter to carbon dioxide, water and various inorganic salts. *Nitrobacter* is a genus of mostly rod-shaped, gram-negative, aerobic-nitrifying and chemoautotrophic bacteria and cells normally reproduce by budding [10,11]. The conversion of ammonia to nitrate is achieved by two groups of nitrifying bacteria; the ammonia-nitrifying bacteria and nitrate-oxidising bacteria and depends on the activities of at least two different genera. The first stage in ammonia oxidation involves *Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosocystis* and *Nitrosogloea* whilst the second stage involves the conversion of nitrite to nitrate by the genera Nitrobacter, Nitrocystis, Nitrococcus, Nitrospina [12]. Nitrobacter and nitrifying bacteria play a very

important role in soil mineralisation and fertility. The nitrogen required in large quantity by plants is supplied in the form of nitrate ion by the activities of nitrifying bacteria through the process of nitrification [13].

Driven by the roles of nitrifying bacteria in soil, water (aquatic plants) and waste water treatment plants, assessment of *Nitrobacter* to pollution stress and tolerance in local and industrial diesel in various aquatic ecosystems becomes imperative. This study was, therefore, designed to assess the tolerance and toxicity levels of Nitrobacter in marine, brackish and freshwater microcosms incorporated with local and industrial diesel in Nigeria.

2. MATERIALS AND METHODS

2.1 Source of Sample

Water Sample: Marine water samples were collected from Bonny River at Bonny with- one liter sterile plastic bottle (container); so is freshwater from a stream at MUU Bagia in BiaraGokana, LG.A and brackish water from sand-field in Port Harcourt, Nigeria. The cap of the sterile sample bottle was removed, containers rinsed with the habitat water at site before collection; the mouth of the bottle placed up after collection and capped, labeled and taking to the laboratory for analysis. This was used within 48 hours of collection for the isolation of *Nitrobacter* sp. employed in the study.

Diesel Sample: Local refined Diesel was purchased from Creek Road depot of the "illegal" refined diesel, in Port Harcourt, Nigeria while the Industrial refined diesel was purchased from Mobil Filling Station, Mile 3 Diobu, Port Harcourt, Nigeria.

2.2 Microbiological Analysis

2.2.1 Media used and identification of *Nitrobacter* sp.

The method used for the isolation of *Nitrobacter* from the river water sample was adopted from Colwell and Zambuski [14] using Winogradsky agar medium modified by Nrior and Odokuma [7]. Note: The new modification by Nrior and Odokuma [7] include the addition of king B Agar and trace quantity of Nutrient agar to Winogradsky formulation. The new formulationenhances proper expression of *Nitrobacter* sp. and reduces the incubation period to 4-5 days instead of 5-7 days usual period.

The composition of the formulated medium were as follows; KNO_2 (0.1 g), Na_2CO_3 (1.0 g), Nacl (0.5 g), $FeSO_4.7H_20$ (0.04 g), King agar (3.0 g), Agar agar (15.0 g), Nutrient agar (5.0 g), Distilled water (1000 ml).

The Winogradsky agar was autoclaved and aseptically transferred to Petri dishes after cooling to about 40℃. The Petri-dishes were then inoculated with the river water samples and incubated aerobically for 4 days at room temperature (30 ± 2℃). Grayish, mucoid of the colonies revealed pear shaped, gram negative organisms indicative of *Nitrobacter* [15]. The colonies were aseptically streaked on fresh winogradsky agar and inoculated for 2 days grayish, mucoid flat colonies were once more obtained and aseptically transferred from the inoculated plates into 200 ml Erlenmeyer flasks containing the growth medium and incubated for 24 hours at room temperature.

2.3 Toxicity Test Procedure for *Nitrobacter* sp

The acute bioassay toxicity was carried out for 24 hours duration according to the guidelines provided by APHA [15] and the Department of Petroleum Resources (formally NNPC inspectorate Division). The tests were carried out in separate test tubes containing the different habitat water (marine, brackish and freshwater).

In each of the experiment set up, the five toxicant concentrations (3.25, 6.5, 12.5, 25 and 50%) and one control were prepared using industrial diesel; same standard were also prepared for local refined diesel. After which 1 ml of the test organism was transferred from the broth culture of *Nitrobacter* sp. into different test tubes containing the various concentrations and control; stirred for 2 minutes to mix properly.

An aliquot (0.1 ml) of each concentration was then inoculated on freshly prepared Winogradsky agar using spread plate technique, incubated at room temperature (28 ± 2℃) for 4-5 days; these processes were repeated after 4 h, 8 h, 12 h and 24 h for the different concentrations (set-ups).

2.4 Percentage (%) Log Survival and Mortality of the Bacterial Isolates in Diesel

The percentage log survival of the bacterium *Nitrobacter* sp., in the local and industrial diesel

used in the study was calculated using the formula adopted from Williamson and Johnson [16]; Odokuma and Nrior [17]; Nrior and Obire [9]. The percentage log survival of *Nitrobacter* sp. in the local and industrial diesel was calculated by obtaining the log of count in each toxicant concentration, divided by log of count in Control, multiplied by 100.

Thus:

$$\% \text{ log survival} = \frac{\text{Log C x 100}}{\text{Log c}}$$

Where;

Log C = Log of the count in each toxicant concentration
Log c = Log of count in Control

% log mortality = 100 – %log survival.

3. RESULTS AND DISCUSSION

Ecotoxicological bioassay was carried out on environmental pollution bio-marker *Nitrobacter* sp. in different aquatic ecosystem (marine, brackish and freshwater) on two different toxicant; local and industrial refined diesel at concentrations of 0, 3.25, 6.5, 12.5, 25 and 50% at 0 h, 4 h, 8 h, 12 h, and 24 h exposure. The results show that certain toxicant were stimulatory while others were inhibitory, similarly observation has been reported [18,19]. Figs. 1-6 shows the toxicity of diesel products (locally refined and industrial) at concentration of 0, 3.25, 6.5, 12.5, 25 and 50% on *Nitrobacter* in fresh, brackish and marine system at 0 4 8 12 and 24 h exposure.

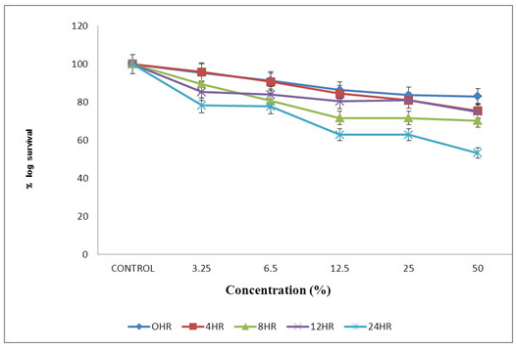

Fig. 1. Lethal toxicity of *Nitrobacter* on industrial diesel using marine water

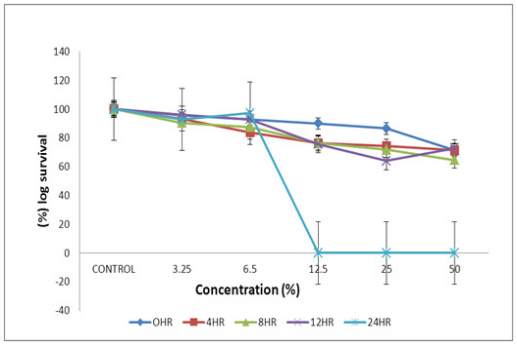

Fig. 2. Lethal toxicity of *Nitrobacter* on locally refined diesel using marine water

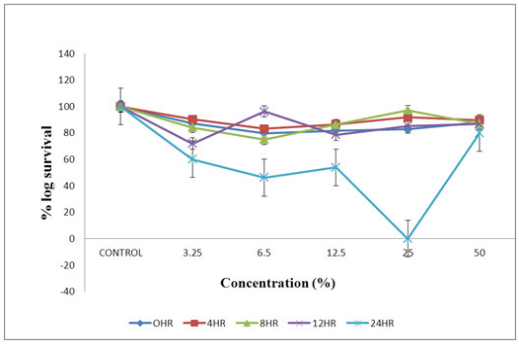

Fig. 3. Lethal toxicity of *Nitrobacter* on industrial diesel using brackish water

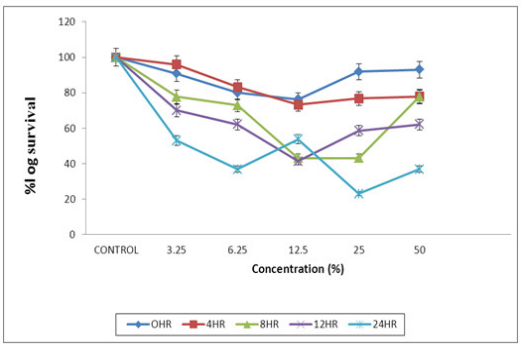

Fig. 4. Lethal toxicity of *Nitrobacter* on locally refined diesel using brackish water

Toxicological evaluation of Median Lethal Concentration (LC_{50}) of local and industrial refined diesel on *Nitrobacter* sp. at concentration of 0, 3.25, 6.5, 12.5, 25 and 50% in marine, brackish and freshwater ecosystem at 0, 4, 8, 12, and 24 h exposure were shown in Tables 1-6.

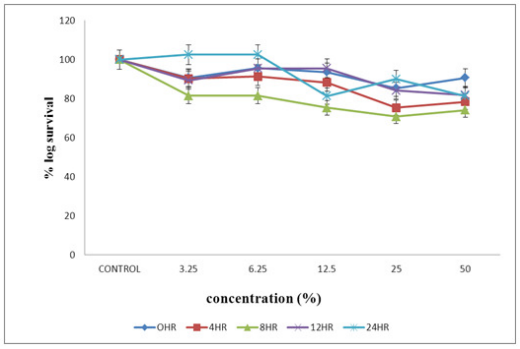

Fig. 5. Lethal toxicity of *Nitrobacter* on industrial diesel using fresh water

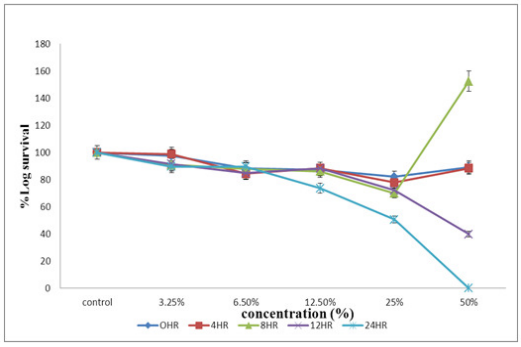

Fig. 6. Lethal toxicity of *Nitrobacter* on locally refined diesel using fresh water

Table 1. LC_{50} of local refined diesel in marine water on *Nitrobacter* sp.

Conc.	%mortality	Mean % mortality	Conc. Diff.	Σ dose diff. x mean % mortality
Control	0	-	-	-
3.25	60.4	12.08	3.25	39.26
6.5	73.06	14.6	3.25	47.45
12.5	116.49	23.30	6	139.8
25	94.79	18.8	12.5	2359.4
50	164.17	32.83	25	820.75
				3406.66

$Lc_{50} = LC_{100} - \frac{\Sigma \text{ dose diff. x mean \% mortality}}{\% \text{ control}}$

$= 50 - \frac{3406.66}{100}$

LC_{50} = 15.934

Table 2. LC_{50} of Industrial refined diesel in marine water on *Nitrobacter* sp.

Conc.	%mortality	Mean % mortality	Conc. Diff.	Σ dose diff. x mean % mortality
Control	0	-	-	-
3.25	43.66	8.732	3.25	28.38
6.5	99.64	19.93	3.25	64.77
12.5	109.87	21.974	6	131.84
25	98.81	19.762	12.5	247.025
50	310.15	62.03	25	1550.75
				2022.765

$Lc_{50} = LC_{100} - \frac{\Sigma \text{ dose diff. x mean \% mortality}}{\% \text{ control}}$

$= 50 - \frac{2022.765}{100}$

LC_{50} = 29.77

Generally there was increase in the loss of *Nitrobacter* with industrial diesel using fresh water (43.85%) >*Nitrobacter* with industrial diesel using brackish water (30.23%) >*Nitrobacter* with industrial diesel using marine water (15.93%) >*Nitrobacter* with locally refined diesel using fresh water (34.76%) >*Nitrobacter* with locally refined diesel using marine water (29.77%) >*Nitrobacter* with locally refined diesel using brackish water (26.81%) (Fig. 7). With increasing exposure time that was observed with locally and industrial diesel.

Table 3. LC_{50} of local refined diesel in brackish water on *Nitrobacter* sp.

Conc.	%mortality	Mean % mortality	Conc. Diff.	Σ dose diff. x mean % mortality
Control	0	-	-	-
3.25	68.11	13.62	3.25	44.27
6.5	93.02	18.60	3.25	60.45
12.5	180.18	36.03	6	216.18
25	206.89	41.38	12.5	517.25
50	296.19	59.24	25	1481
				2319.15

$LC_{50} = LC_{100} - \frac{\Sigma \text{ dose diff. x mean \% mortality}}{\text{\% control}}$

$= 50 - \frac{2319.15}{100}$

LC_{50} = 26.81

Table 4. LC_{50} of Industrial refined diesel in brackish water on *Nitrobacter* sp.

Conc.	%mortality	Mean % mortality	Conc. Diff.	Σ dose diff. x mean % mortality
Control	0	-	-	-
3.25	80.16	16.03	3.25	52.10
6.5	58.37	11.67	3.25	37.93
12.5	70.46	14.09	6	84.54
25	81.03	16.21	12.5	202.63
50	260	52	25	1300
				1677.2

$Lc_{50} = LC_{100} - \frac{\Sigma \text{ dose diff. x mean \% mortality}}{\text{\% control}}$

$= 50 - \frac{1677.2}{100}$

LC_{50} = 30.23

Table 5. LC_{50} of local refined diesel in freshwater on *Nitrobacter* sp.

Conc.	%mortality	Mean % mortality	Conc. Diff.	Σ dose diff. x mean % mortality
Control	0	-	-	-
3.25	55.89	11.18	3.25	36.34
6.5	62.21	12.44	3.25	40.43
12.5	117.49	23.50	6	141
25	127.72	25.54	12.5	319.25
50	197.35	39.47	25	986.75
				1523.77

$LC_{50} = LC_{100} - \frac{\Sigma \text{ dose diff. x mean \% mortality}}{\text{\% control}}$

$= 50 - \frac{1523.77}{100}$

LC_{50} = 34.76

Furthermore, results obtained showed that local and industrial diesel causes cell mortality. This suggests that the modes of action of industrial and local diesel are not limited to inhibition of the organism but causes the death of the test organism [16]. Diesel release toxic break down products from oil that allows toxicity vary with time of exposure and increases as the exposure time increases. This is due to the accumulation of toxicant overtime up to critical tissue concentration that causes mortality.

The Lowest median lethal concentration 15.93% observed in the viable count of *Nitrobacter* with industrial diesel using marine water while the highest was recorded in industrial diesel in freshwater 43.85%. This shows that salinity could be a contributory factor in aquatic toxicity [7].

Table 6.LC $_{50}$ of industrial refined diesel in freshwater on *Nitrobacter* sp.

Conc.	%mortality	Mean % mortality	Conc. Diff.	Σ dose diff. x mean % mortality
Control	0	-	-	-
3.25	44.11	8.82	3.25	28.67
6.5	76.41	15.28	3.25	49.66
12.5	116.91	23.38	6	140.28
25	53.8	10.76	12.5	134.5
50	52.4	10.48	25	262
				615.11

$LC_{50} = LC_{100} - \frac{\Sigma \text{ dose diff. x mean \% mortality}}{\% \text{ control}}$

$= 50 - \frac{615.11}{100}$

LC_{50}= 43.85

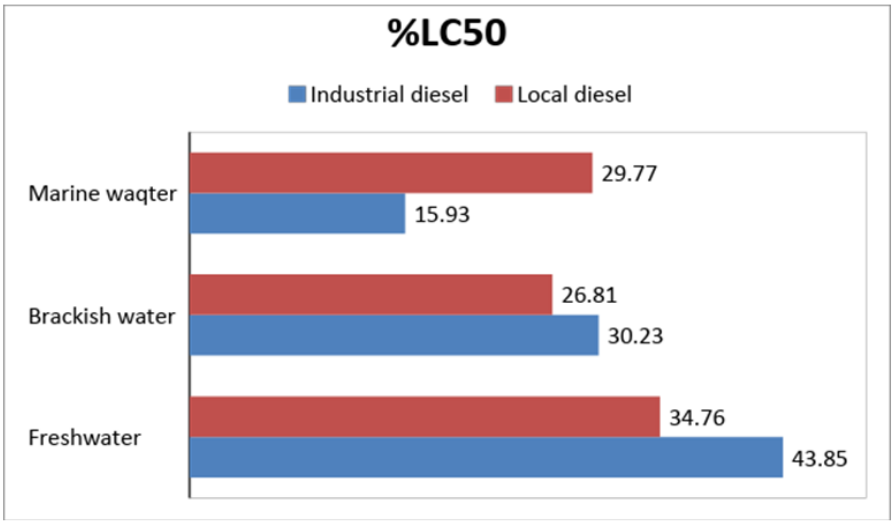

Fig. 7. Percentage (%) Median Lethal Concentration (LC_{50}) of Local and Industrial refined Diesel on *Nitrobacter* in marine, brackish and freshwater

4. CONCLUSIONS AND RECOMMENDATION

Hydrocarbon toxicological effect on nitrogen fixing bacterium *Nitrobacter* sp. is of prime importance as it affects the nitrification process which negatively and adversely affects aquatic fauna. The study shows that local refined diesel has more toxic effect in brackish and freshwater than industrial refined diesel whereas in marine water a reverse trend occurs; industrial refined diesel being more toxic than local refined diesel.

In view of the sensitive nature of *Nitrobacter* sp. to slight variation in toxicity quotient and its role in the biogeochemical cycle; it is recommended that *Nitrobacter* sp. could serve as a potential tool for eco-toxicological assay and pollution bio-marker.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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