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Response of Pollution Bio-monitors: *Pseudomonas* and *Bacillus* species to Local "Kpo-fire diesel" and Industrial Refined Diesel in Freshwater Environment

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Abstract

This paper reports the response of pollution bio-monitors such as *Pseudomonas* and *Bacillus* species to local and industrial diesel in freshwater environments of the Niger Delta, Nigeria. The illegal local refined diesel is popularly referred to as 'Bunkery diesel' or 'Kpo-fire diesel' in Niger Delta, Nigeria. It uses simply three major step/process; cooking or 'boiling' of crude oil, distillation and extraction at different temperatures into Petrol, Diesel and Kerosene but majorly Diesel and Kerosene are the final products. Standard ecotoxicological bioassay using pollution bio-monitors as indicator tools with different concentrations of local and industrial refined diesel were employed. Local and Industrial refined diesel concentrations of 0, 1.625, 3.25, 6.5, 12.5 and 25 mg/L were tested on *Pseudomonas* and *Bacillus* species at exposure intervals of0, 4, 8, 12 and 24 hours for twenty four (24) separate set-ups. Percentage (%) log mortality (derived from log survival of the test organism) expressed as Median Lethal Concentration (LC_{50} mg/L) was used as indices to monitor toxicity. The research shows that local and industrial refined diesel cause cell mortality. The 24 h LC_{50} of the diesel were *Bacillus* +*Loca*l refined Diesel (20.54 mg/L) >*Pseudomonas* + Local refined Diesel (21.36 mg/L) >*Bacillus* +Industrial refined Diesel (21.88 mg/L) >*Pseudomonas* + Industrial refined Diesel (40.90 mg/L) (noting; the lesser the LC_{50} the more toxic the toxicant). Comparatively, Local refined diesel is more toxic than Industrial refined diesel to all the test organisms. Also *Bacillus* sp. is more susceptible than *Pseudomonas* sp. to both local and industrial refined diesel. Based on these findings, the Local "illegal" diesel popularly called 'Bunkery diesel' or 'Kpo-fire diesel' refining should be discouraged in the Niger Delta of Nigeria especially in freshwater environment. As an industrial environment with high population which is prone to diesel spill impact, toxicity to *Pseudomonas* and *Bacillus* species could become a simple and fast bioassay for monitoring ecosystem response to these pollutants.

Keywords: Local refined diesel, 'Kpo-fire diesel", "Bunkery diesel", Ecotoxicity, Freshwater, *Pseudomonas*, *Bacillus* sp.

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Introduction

Diesel like all other fossil fuels primarily consists of complex mixture of molecules called hydrocarbons, which is composed of 75% alkenes or saturated hydrocarbons (primary paraffin's including n-, Iso-, and cycloparaffins), and 25% of aromatic compounds (including Nepthalenes and alkybenzenes). This is obtained from middle distillate fraction between 200°C and 350°C at atmospheric pressure, resulting in a mixture of carbon chains that typically contain between 8 and 21 carbon atoms per molecule during petroleum separation (Collins, 2007). Diesel is a petroleum-based fuel for diesel engines with chemical formula $C_{12}H_{23}$. It is a thick light oily fuel that has its color varies from colorless to brown (Sakar et al., 2005).

The ability to isolate high or low proportion for certain oil utilizing microorganism from an environment depends on the nutrients available in the contaminated site, and the age of the contamination (Atlas and Bartha, 1998; Nrior and Odokuma, 2017). The most abundant and active petroleum hydrocarbon utilizing microorganisms belong to the following genera; *Pseudomonas, Staphylococcus, Streptococcus, Gordonia, Aeromicrobium, Dietzia, Sarcina, Vibrio, Flavobacteruim, Micrococcus, Bacillus* ((Choi, 2013; Nrior and Mene, 2017; Nrior and Odokuma, 2017).

Petroleum is still the principal source of energy for industries and industrial uses, even for some domestic uses. Despite its importance in the society, petroleum is a major source of pollution in the environment. Diesel exerts toxic effects because some of the chemical compounds in diesel oil are slightly soluble in water. Its elevated concentration of the lower molecular weight aromatic hydrocarbons i.e, monoaromatics hydrocarbon (MAHs) and polynuclear aromatic hydrocarbons (PAHs) are absorbed by water organisms. The principal acute toxic effect caused by these compounds is narcosis (Milinkovitoh and Godefery, 2011). The harmful effect that chemicals have upon individual organism depends on many different factors, not only on the fresh water organisms but also in the form in which population occurs (Richard et al., 2011). Microorganisms found in fresh water environments such as bacteria, viruses and protozoa; influence the fresh water ability to sustain life on earth (Futherman, 2004).

Microbial monitoring specifically for petroleum hydrocarbon is the concurrent stimulation and inhibition effect of petroleum hydrocarbons on bacteria, which are used for toxicity assessments (Macnaughton et al., 1999; Nrior and Obire, 2015; Smalla et al., 2007). Some microorganism, like the bacteria are able to utilize petroleum hydrocarbons in the environment as their sole source of carbon and energy (Bundy et al., 2002). Bacteria have been considered as one of the predominant hydrocarbon utilizing agent found in the environment (Venosa et al., 2001). The abundance of petroleum hydrocarbons utilizing bacteria could be used as bio-indicator reflecting the level of ecosystem pollution by petroleum hydrocarbon (Leahy and Cowell, 1990; Nrior et al, 2017).

The aim of this study therefore was to assess the response of pollution bio-monitors like *Pseudomonas* and *Bacillus* species to Local "Kpo-fire diesel" and Industrial Refined Diesel in freshwater ecosystem in Niger Delta. As an industrial environment with high population which is prone to diesel spill impact, toxicity to *Pseudomonas* and *Bacillus* species could become a simple and fast bioassay for monitoring ecosystem response to these pollutants.

Materials and Methods

Source of Sample

Water sample was collected with sterile sample bottles (container) from a fresh water stream at Ibaa in Emohua Local Government Area in Rivers State, Nigeria. The container was rinsed thrice with the water sample to be collected at site before collection was made. The bottle cap was replaced after collection and the mouth of the bottle was faced up, labeled and taking to the laboratory for analysis within two (2) hours of collection.

Identification of bacterial isolates

The isolates were obtained and subjected to various characterization procedures. Pure isolates of bacteria were identified on the basis of the colonial, morphological, microscopic examination, physico-chemical and biochemical characteristics (Buchanan and Gibbons, 1974; Cowan, 1974). The following standard characterization tests were performed: Gram's staining reaction, motility test, oxidase test, catalase test, coagulase test, starch hydrolysis, methyl red and voges-proskauer tests, indole test, Nitrate reduction test, sugar fermentation test (Glucose, Sucrose, Lactose, and Maltose). Morphological studies were carried out on different colonies on media plates. Pure colonies were isolated based on colony size, shape, pigmentation, elevation and texture of the individual organisms after 48hours of growth at 30°C. The morphology was determined by examination of the plates directly under the microscope at low power (10x).

Physico-chemical parameters of Freshwater

Parameters such as pH was determined using pH meter, Total Dissolved Solids using TDS meter (Jenway 3015 method), Dissolved Oxygen (DO) and Biochemical Oxygen Demand (BOD) were determined by modified Winkler method (APHA, 1998), Chemical Oxygen Demand (COD) was determined by permanganate oxidation method from the biodegradation set-up on various days 1, 7, 14, 21 and 28 days.

Toxicity test procedure for Pseudomonas and Bacillus species

The acute toxicity bioassays were carried out for a total duration of 24hrs according to the guideline provided by APHA (1992) and the Department of Petroleum Resources (DPR, 2002). The tests were carried out in separate test tubes containing sterile habitat water (freshwater) for the different toxicant concentrations preparation.

Illustrative toxicity set-up procedure of Industrial and Local Refined Diesel with *Pseudomonas* sp. and *Bacillus* sp. in freshwater is shown in Tables 1 and 2 respectively. A total of 24 set-ups were carried out; 12 for each organism. The toxicant (local and industrial diesel) concentrations were prepared by setting up six test tubes per set, aseptically covered with cotton wool. The test was carried out in six separate test tubes containing appropriate filtered water (freshwater from the habitat of the organism) separately. In six test tubes per set, were toxicant concentrations (%); Control 0, 1.625, 3.25, 6.5, 12.5, 25 added separately. The test tubes were covered with cotton wool; the control consists of freshwater from the habitat of the organism. After which about 1ml of the test organism was added to separate toxicant concentrations and plated out immediately after inoculation on appropriate media agar plate. An aliquot (0.1 ml) of each concentration was then inoculated onto nutrient agar plates, this is known as the zero hour (0 h), and these processes were then repeated after 4 h, 8 h, 12 h, and 24 h for different set-ups. All the plates after each inoculation were incubated for 24 h for survival colony count.

Table 1: Illustrative toxicity set-up procedure of Industrial and Local Refined Diesel with Pseudomonas sp. in freshwater.

Set-up S/N	**Set-up ID**	**Concentration (mg/L)**	**Toxicant (ml)**	**Diluent (Sterilized Freshwater) (ml)**	**Test Organism (**Pseudomonas **sp.) (ml)**
			Industrial Refined Diesel		
1	IRD+Pse1	Control (0)	0	10	1
2	IRD+Pse2	1.625	0.16	9.84	1
3	IRD+Pse3	3.25	0.33	9.67	1
4	IRD+Pse4	6.5	0.65	9.35	1
5	IRD+Pse5	12.5	1.25	8.77	1
6	IRD+Pse6	25	2.5	7.5	1
			Local Refined Diesel		
7	LRD+Pse1	Control (0)	0	10	1
8	LRD+Pse2	1.625	0.16	9.84	1
9	LRD+Pse3	3.25	0.33	9.67	1
10	LRD+Pse4	6.5	0.65	9.35	1
11	LRD+Pse5	12.5	1.25	8.77	1
12	LRD+Pse6	25	2.5	7.5	1

IRD=Industrial Refined Diesel, LRD=Local Refined Diesel, Pse=Pseudomonas sp.

Table 2: Illustrative toxicity set-up procedure of Industrial and Local Refined Diesel with Bacillus sp. in freshwater.

Set-up S/N	Set-up ID	Concentration (mg/L)	Toxicant (ml)	Diluent (Sterilized Freshwater) (ml)	Test Organism (Bacillus sp.) (ml)
			Industrial Refined Diesel		
13	IRD+Bac1	Control (0)	0	10	1
14	IRD+Bac2	1.625	0.16	9.84	1
15	IRD+Bac3	3.25	0.33	9.67	1
16	IRD+Bac4	6.5	0.65	9.35	1
17	IRD+Bac5	12.5	1.25	8.77	1
18	IRD+Bac6	25	2.5	7.5	1
			Local Refined Diesel		
19	LRD+Bac1	Control (0)	0	10	1
20	LRD+Bac2	1.625	0.16	9.84	1
21	LRD+Bac3	3.25	0.33	9.67	1
22	LRD+Bac4	6.5	0.65	9.35	1
23	LRD+Bac5	12.5	1.25	8.77	1
24	LRD+Bac6	25	2.5	7.5	1

IRD=Industrial Refined Diesel, LRD=Local Refined Diesel, Bac=Bacillus sp.

The Percentage Log survival and mortality of the bacterial isolates in Diesel

The percentage log survival and mortality of the bacterial isolates for *Pseudomonas* and *Bacillus* species in the local and industrial refined diesel used in the study was calculated using the formula adopted by Williamson and Johnson (1981) and Nrior et al (2017). The percentage log survival of the bacterial isolates in the local diesel was calculated by obtaining the log of the count in each toxicant concentration (log C), dividing by the log of the count in the zero toxicant concentration (log c) and multiplying by 100 (equation i).

Percentage log mortality was obtained by subtracting percentage log survival of test toxicant from 100 (equation ii)

Thus:

$$\% \text{ log survival} = \frac{\log C}{\log c} x\ 100 \quad \text{(i)}$$

$$\% \text{ log mortality} = 100 - \% \text{ log survival} \quad \text{(ii)}$$

Median Lethal Concentration (LC_{50}) of the pollution bio-monitors

The Median Lethal Concentration (LC_{50}) was computed from mean % log mortality and sum of dose difference using standard statistical analysis using the formula (equation iii) below:

$$LC_{50} = LC_{100} - \frac{\text{Sum of Dose diff. X Mean \% log Mortality}}{\text{\% Control}} \quad \text{(iii)}$$

Results and Discussion

The 24h Median Lethal Concentration (LC_{50} mg/L) of the diesel (Industrial and Local refined diesel) on the test organisms *Pseudomonas* and *Bacillus* species in freshwater is presented in Fig. 1. The results showed that Local refined diesel were more toxic to the test organisms than the Industrial refined diesel. The 24hlethal concentration of local refined diesel were 20.54 and 21.36 mg/L greater than (more toxic) Industrial refined diesel 21.88 and 40.90mg/L to *Bacillus* and *Pseudomonas* respectively. The diversity and the number of bacteria at a given site may help to characterize that site with respect to the toxicity of the hydrocarbons, the concentration of the hydrocarbons and the age of the contamination (Nrior and Odokuma, 2015)

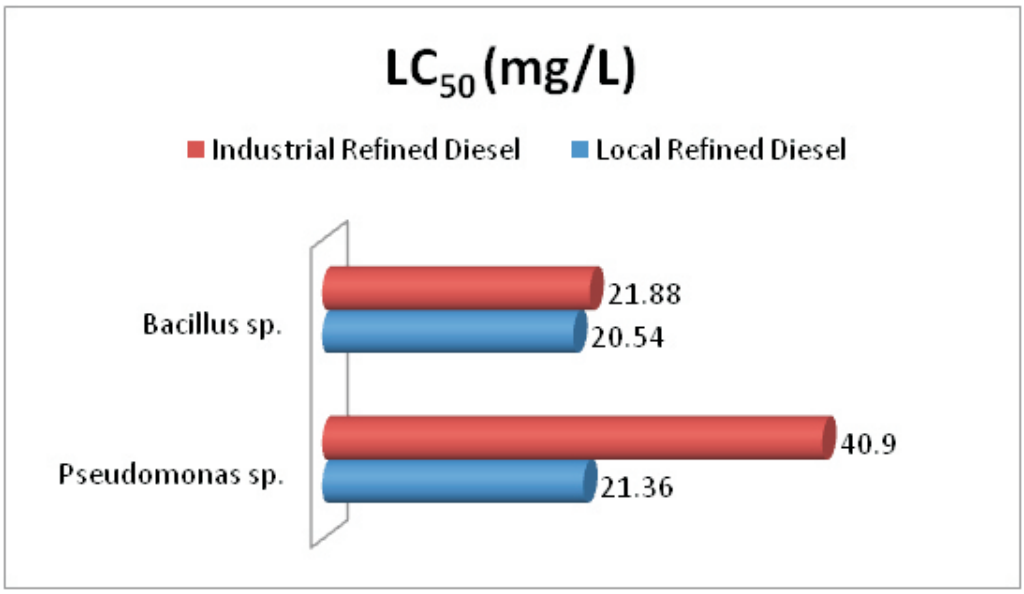

Fig. 1: The Median Lethal concentration (LC_{50} mg/L) of Industrial and Local refined diesel on the test organisms in freshwater

Generally, the toxicity quotient (noting that the lower the LC_{50} the more toxic the toxicant) revealed increasing toxic levels as follows; Industrial refined diesel, the results show that *Pseudomonas* sp. (40.90%) <*Bacillus* sp. (21.88%) while for local refined diesel, *Pseudomonas* sp. (21.36%) < *Bacillus* sp. (20.54%). The log survival count of the test organisms *Bacillus* and *Pseudomonas*, taken during toxicity for Local and Industrial refined diesel in freshwater are shown in Tables 3-4. The harmful effect that chemicals have upon individual organism depends on many different factors, not only on the fresh water organisms but also in the form in which population occurs.

Table 3: Log Survival Count of *Pseudomonas* sp. with Industrial and Local refined Diesel at different concentrations**.**

Set-up S/N	Set-up ID	Conc.(mg/L)	Log survival count of Pseudomonas sp. with exposure time				
			0 h	4 h	8 h	12 h	24 h
	Industrial Refined Diesel						
1	IRD+Pse1	Control (0)	1.833	1.602	1.531	1.398	1.301
2	IRD+Pse2	1.625	1.699	1.556	1.398	1.301	1.255
3	IRD+Pse3	3.25	1.623	1.505	1.342	1.301	1.176
4	IRD+Pse4	6.5	1.580	1.398	1.255	1.255	1.079
5	IRD+Pse5	12.5	1.398	1.301	1.000	1.176	1.000
6	IRD+Pse6	25	1.301	1.301	1.461	1.000	0.903
	Local Refined Diesel						
7	LRD+Pse1	Control (0)	1.778	1.740	1.602	1.531	1.398
8	LRD+Pse2	1.625	1.740	1.716	1.556	1.447	1.342
9	LRD+Pse3	3.25	1.716	1.653	1.477	1.398	1.301
10	LRD+Pse4	6.5	1.681	1.580	1.398	1.301	1.255
11	LRD+Pse5	12.5	1.653	1.477	1.301	1.301	1.176
12	LRD+Pse6	25	1.544	1.447	1.204	1.176	1.000

Table 4: Log Survival Count of *Bacillus* sp. with Industrial and Local refined Diesel at different concentrations**.**

Set-up S/N	Set-up ID	Conc.(mg/L)	Log survival count of Bacillus sp. with exposure time				
			0 h	4 h	8 h	12 h	24 h
	Industrial Refined Diesel						
13	IRD+Bac1	Control (0)	1.813	1.748	1.699	1.653	1.544
14	IRD+Bac2	1.625	1.748	1.681	1.653	1.544	1.477
15	IRD+Bac3	3.25	1.663	1.740	1.602	1.477	1.301
16	IRD+Bac4	6.5	1.544	1.447	1.544	1.398	1.301
17	IRD+Bac5	12.5	1.447	1.398	1.398	1.301	1.255
18	IRD+Bac6	25	1.342	1.301	1.301	1.176	1.146
	Local Refined Diesel						
19	LRD+Bac1	Control (0)	1.833	1.699	1.623	1.544	1.477
20	LRD+Bac2	1.625	1.740	1.681	1.556	1.477	1.398
21	LRD+Bac3	3.25	1.716	1.544	1.447	1.447	1.301
22	LRD+Bac4	6.5	1.653	1.447	1.415	1.380	1.301
23	LRD+Bac5	12.5	1.602	1.398	1.301	1.301	1.204
24	LRD+Bac6	25	1.544	1.342	1.176	1.204	1.176

This result shows that with increasing exposure time at a constant concentration of a particular toxicant leads to increase in the mortality rate as reflected in the deleterious effect on the microbial biota. This result conforms to the findings of Nrior et al. (2017) that local and industrial kerosene toxicity on *Nitrobacter* was a function of both the contact time and concentrations.

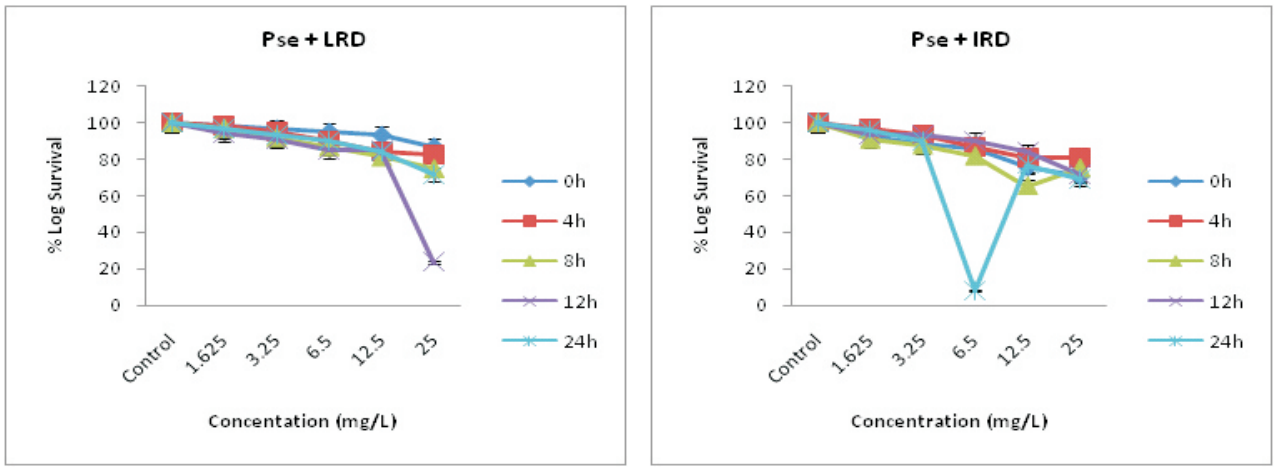

Fig. 2 Percentage (%) mortality of *Pseudomonas* sp. to Industrial Refined Diesel in freshwater

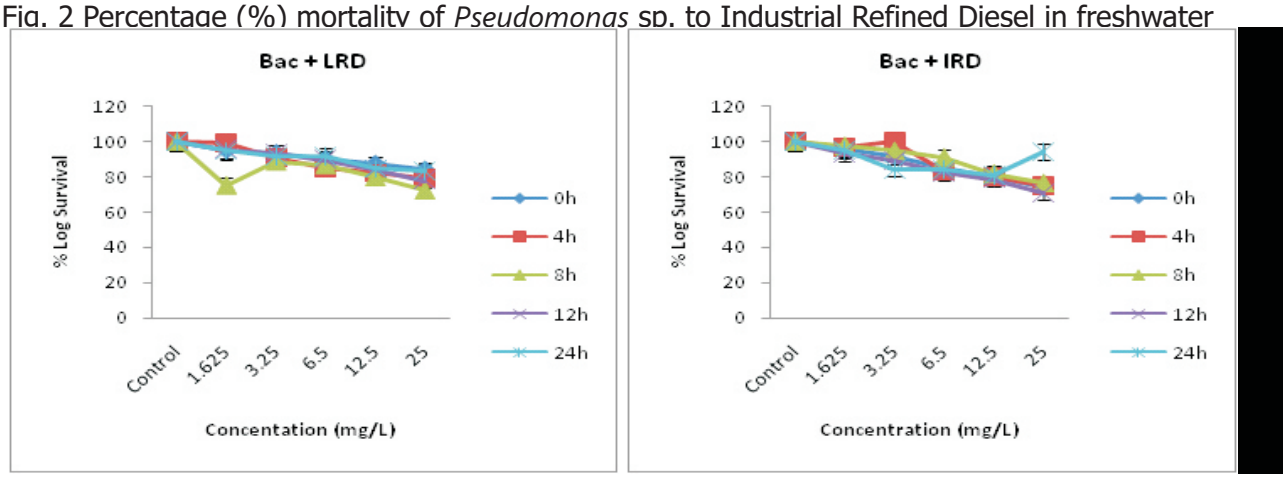

Fig. 3 Percentage (%) mortality of *Bacillus* sp. to Industrial Refined Diesel in freshwater

The values of some physical and chemical properties of the freshwater used for the study are presented in Table 5. The general appearance of the water were clear, odour unobjectionable, colour 2.00 Hazan were within World Health Organisation (WHO) limit, but the pH 5.70 which is outside WHO limit - standard of pH 6.5-8.5 for drinking water. However, the acidic pH range observed does not favour the test organisms in the microcosms. These physico-chemical factors were particularly important for the survival of petroleum product-utilizing microbial consortium in the aquatic systems. These findings corroborated with the findings of Okpokwasili and Olisa (1991). With respect to sensory evaluation: General appearance of the habitat freshwater was clear; Odour was unobjectionable, Total Dissolved solids, Total iron and Lead were above limits. This could probably be due to oxidation of petroleum and other organic matter discharged on daily basis into the water body, since the aquatic area of study serve multiple purposes for oil and gas industry, manufacturing and fabrication industries etc. This could also be attributed to the presence and amount of organic matter present in the sample at the point of collection (Nrior and Odokuma, 2015).

Table 5: Physico-chemical Analysis of Habitat water (Freshwater)

S/N	PARAMETER	LABORATORY RESULTS	EXPECTED WHO SPECIFICATION	REMARK
1	General appearance	Clear	Clear	WL
2	Odour	Unobjectionable	Unobjectionable	WL
3	Taste	Unobjectionable	Unobjectionable	WL
4	Colour	2.00	15 Hazen Units	WL
5	pH	5.70	6.5-8.5	OL
6	Conductivity	20.00	1000 uS/cm	WL
7	Turbidity	1.00	5 NTU	WL
8	Total Hardness	13.60	100 mg/L	WL
9	Total Alkalinity	10.20	200 mg/L	WL
10	Chloride	8.00	250 mg/L	WL
11	Total Suspended Solids	49.40	30 mg/L	OL
12	Total dissolved solids	10.00	500 mg/L	WL
13	Total solids	59.40	500 mg/L	WL
14	Free residual chlorine	0.00	0.2 mg/L	WL
15	Nitrate	1.75	10 mg/L	WL
16	Nitrite	0.08	0.02 mg/L	OL
17	Oil and Grease	<0.001	0.01 mg/L	WL
18	COD	5.20	40 mg/L	WL
19	BOD_5	1.73	15 mg/L	WL
20	Sulphate	23.30	250 mg/L	WL
21	Calcium	6.60	70 mg/L	WL
22	Magnesium	0.80	30 mg/L	WL
23	Total iron	0.38	0.3 mg/L	OL
24	Lead	0.03	0.01 mg/L	OL
25	Copper	0.001	1.040 mg/L	WL
26	Reactive Silica	25.45	40 mg/L	WL

COMMENT: This Water Sample under the same condition does not conform with the stated WHO Specification for Potable Water in all the parameters remarked OL (Out of the Limit of Specification). Appropriate treatment for these OL (Out of the Limit of Specification) parameters is therefore recommended.

This result shows that the petroleum products from Industrial and local refined diesel were inhibitory to microbial growth. Certain petroleum hydrocarbon are carcinogenic and mutagenic (Boonchan et al., 2000; Samanta et al., 2002), 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 ecosystems (Alvarez et al., 1991; Nrior and Odokuma, 2015). Many toxic compound such as polycyclic aromatic hydrocarbon, benzene compounds, and cycloalkane rings causes great deleterious effect on microbial biota (Franco et al., 2004; Nrior et al., 2017). Microorganisms found in freshwater such as bacteria, viruses and *Proteus* can influence the fresh water ability to sustain life on earth (Futherman, 2004). This suggests that the mode of action of industrial and local diesel is not limited to inhibition of the organism.

Conclusion

Diesel is obtained in the fractional distillation of crude oil between 250°C and 350°C at atmospheric pressure, and the purpose of this study was to observe the toxicity of both industrial and local diesel in the microorganisms. There is an evidence to show that diesel can be toxic to microorganisms; it has been observed that industrial diesel is more toxic to *Pseudomonas* sp. (40.90%), *Bacillus* sp. (21.88%) than locally refined diesel *Pseudomonas* sp. (21.36%), *Bacillus* sp. (20.54%). This study therefore shows that industrial refined diesel has more toxic effect on *Bacillus* and *Pseudomonas* species than the locally refined diesel.

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