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Comparative Ecotoxicological Evaluation of Locally Refined Diesel and Kerosene on *Rhizopus stolonifer* in Rivers State, Nigeria

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Authors' contributions

This work was carried out in collaboration between all authors. Author RRN designed the study, author NPA performed the statistical analysis, wrote the protocol, author AW wrote the first draft of the manuscript. Authors NPA, RRN and AW managed the analyses of the study. Author AW managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To compare the effect of locally refined diesel and kerosene on *Rhizopus stolonifer* in tri aquatic bodies.
Study Design: The study employs experimental assay and statistical analysis of the data and interpretation.
Place of Study: Freshwater, brackish water, and marine water samples were collected in sterile bottles from Ugama Ekede Stream, Ugama Ekede River and at the foot of the Atlantic ocean in Udun Ama all in Andoni Local Government Area Rivers State, using sterile sampling bottles. These samples were transported to the microbiological laboratory with ice pack within 24 hours for both isolations of test organisms and toxicity.
Methodology: Standard microbiological techniques were used; toxicity testing procedures were carried out by preparing locally refined diesel and kerosene at concentrations of 0%, 5%, 10%, 25%, and 50%, tested for durations of 0 h, 24 h, 48 h, 72 h, 96 h. The cultures were incubated at 35ºC for 48 hours. LC_{50} was determined.
Results: The results indicate that logarithm of mortality of *Rhizopus stolonifer* increases with

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increased toxicants concentration and exposure time. The median lethal concentration (LC_{50}) of the locally refined diesel and kerosene increases in the following order: (Note: the higher the LC_{50}, the lower the toxic effect. *Rhizopus stolonifer* in locally refined diesel in fresh water (41.88%) <*Rhizopus stolonifer* in locally refined kerosene in fresh water (41.64.5%) < *Rhizopus stolonifer* in locally refined diesel in brackish water (43.28%) < *Rhizopus stolonifer* in locally refined kerosene in brackish water (33.41%) < *Rhizopus stolonifer* in locally refined diesel in marine water (40.17%) <*Rhizopus stolonifer* in locally refined kerosene in marine water (36.10%).
Conclusion: Locally refined kerosene in brackish water (LC_{50} = 33.41%) is the most toxic, having the lowest LC_{50} while locally refined diesel in brackish water (LC_{50} = 43.28%) have the lowest toxicity effect. These results show that locally refined diesel and kerosene can inhibit the growth of *Rhizopus stolonifer* in an aquatic ecosystem.

Keywords: Locally refined diesel and kerosene; toxicity; Rhizopus stolonifer; freshwater; brackish water; marine water; ecosystem.

1. INTRODUCTION

Petroleum products such as diesel and kerosene are widely used in commercial and domestic sectors for various purposes [1] such as running of gas plants, cooking, aircraft gas turbines and jet fuel, fuels for vehicles, boats and machines, heating oil, and as spray oil to combat insects on citrus plants [2]. Nigeria being an oil producing nation with a population of 170 million people utilises an average of 35 million litres of kerosene and diesel, and as a result of this high demand especially in the rural areas, these products have been subjected to locally refined processes to meet demand [3]. Poverty is another determining factor for the adoption of locally refined means of diesel and kerosene in the Niger Delta region [4]. The management of diesel and kerosene has been an issue of significant concerns in developing countries, especially in Nigeria. This is large as a result of oil spills and illegal oil refining. According to [5], kerosene is one of the most commonly spilt petroleum products.

Diesel like every other fossil fuel primarily consists of complex mixtures of molecules called hydrocarbons [6]. Its constituents are 75% alkenes or saturated hydrocarbon and 25% of aromatic compounds (including naphthalene and alkybenzenes), obtained from middle distillate fraction between 200ºC and 350ºC at atmospheric pressure, resulting in a mixture of carbon chains that typically contain between 8 and 21 carbon atom per molecule during petroleum separation. Diesel is a petroleum-based fuel that has its colour varies from calories to brown [6]. Although diesel is a commonly used fuel for vehicles, boats and machines, it is recognised as a serious threat to ecosystems [7]. Diesel can accumulate in food chains at various levels where they disrupt biochemical or physiological processes of many organisms [7].

Kerosene is used to refer to the kerosene fraction removed during the distillation of petroleum at boiling point 145-300ºC [5]. Kerosene was first processed from crude oil in the early Pennsylvania oil fields [5]. Kerosene is a complex mixture of branched and straight-chain compounds, which can be generally categorized into three classes: paraffin (55.2%), naphthenes (40.9%), and aromatics (3.9%) [5].

Although some microorganisms have been employed in bioremediation processes based on their metabolic activities [8], ecotoxicological studies have emerged to assess the degree to which a substance can damage an organism. The adverse effects of these substances may be lethal (cause death) or sub-lethal (cause negative effects that damage the organism in some way, but do not cause death) [9]. Exposure is a function of the concentration of the substance and period of time for which the organism is exposed to it. Exposure can be acute-exposure to a high concentration of a substance for a short period of time, or chronic-exposure to low concentrations of a substance for prolonged periods.

2. MATERIALS AND METHODS

Freshwater sample was collected in sterile bottles from Ugama Ekede Stream, while brackish water was collected from Ugama Ekede River, a marine water sample was collected in sterile sampling bottles at the foot of the Atlantic ocean in Udun Ama all in Andoni Local Government Area Rivers State. These samples were transported to the microbiological

laboratory with ice pack within 24 hours for both isolations of test organisms and toxicity. All samples collections were carried out at weekly interval for 4 months.

2.1 Choice of Fungus

The choice of *Rhizopus stolonifer* is because of its role in degrading petroleum hydrocarbons by producing extracellular enzymes which allow for digestion of energy sources in their surroundings. The organism is characterised by their aggressive growth, higher biomass production and extensive hyphal growth [10]. The high surface-to-cell ratio of this filamentous fungus (*Rhizopus stolonifer*) makes them better degraders under specific niches, and *Rhizopus stolonifer* can especially handle breaking down some of the most abundant molecules present [11].

2.2 Microbiological Analysis

2.2.1 Total heterotrophic bacteria (THB)

Total heterotrophic bacteria for each water samples were enumerated using spread plate method. An aliquot (0.1 ml) of the dilution of 10^{-6} were aseptically transferred into properly dried nutrient agar plates in duplicate, spread evenly using bent glass rod and incubated at 35-37^{0}C for 24 to 48 hours. After incubation, the bacterial colonies that grew on the plates were counted and sub-cultured unto fresh nutrient agar plates. Further identifications were carried out on the discrete colonies on the plates [12]. Total Heterotrophic bacte ... sample ...] below:

...ulate ... ria ount ... or eacl ... a by [13

$$\text{...ctial population} \left(\frac{Log10cfu}{ml}\right)$$

N of Colonies x Reciprocal of dilution facto... ...e inoculums

2.2.2 Total heterotrophic fungi

The total fungi in each of water samples were enumerated using spread plate method. An aliquot (0.1 ml) of the dilution of 10^{-4} dilution was aseptically transferred into properly dried Potato Dextrose Agar plates containing antibiotic (Tetracycline and Penicillin) to inhibit bacterial growth, in duplicate, spread evenly using bent rod and incubated at 25-27ºC for 3 days, pure culture of fungal isolates were counted and sub-cultured unto Potato Dextrose Agar slant in bijou bottles for preservation and identification as described by [13].

2.2.3 Identification of fungus

Macroscopic: Examination of growth was done by observing the colonial morphology, color of colony, texture, shape and surface appearance.

Microscopy: This was done by using the wet prep (needle mount) and slide culture characteristics like sexual and asexual reproduction structures like the conidial head, sporangia, the vegetative mycellia, septate or non septate hyphae [13].

2.3 Toxicity Test Procedures

The toxicity tests were done as described by [14]. Setting up thirty 100ml conical flasks aseptically covered with cotton wool. The test was carried out in five (5) separate conical flask containing appropriately autoclaved water samples from fresh, marine and brackish water from the habitat of the organism separately. In each of the conical flask, the four toxicant concentrations (5%, 10%, 25%, 50%) were added separately. while the control consists of fresh, marine and brackish water without toxicants respectively. One millilitre (1 ml) of broth containing the test organism was added to each toxicant concentration in the conical flask containing (5%, 10%, 25%, 50%, and control respectively). Then an aliquot (0.1ml) from each of the concentrations of the effluent were then plated out using spread plate technique on Potato dextrose agar immediately after ... zero (0) hour, inoculation and ...tinues after 24, 48, 72 and 96hours ...nd was incubated for 48 hours at ...ature (37± 2ºC). After which the colonies on the plates were counted [14].

Toxicity test of fungus *Rhizopus stolonifer* in locally refined diesel and kerosene.

The log survival of the *Rhizopus stolonifer* in the locally refined diesel and kerosene effluent were calculated according to the formula used by [13].

$$Fungal\ population - \frac{log10sfu}{ml}$$

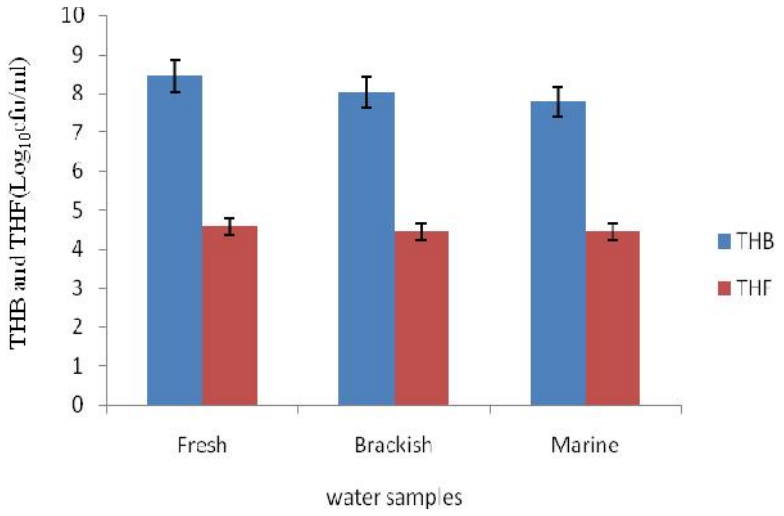
$$- No.of\ spores\ x\ reciprocal\ of\ dilution\ factor\ x\ size\ of\ inoculum$$

3. RESULTS AND DISCUSSION

The total heterotrophic bacterial and fungal counts in the three aquatic bodies are presented in Fig. 1. The result shows that microbial load in fresh water is higher than the rest followed by brackish water and marine water respectively. Possible organisms isolated include; *Bacillus* species, *Pseudomonas* species, *Rhizopus stolonifer*, *Mucor* species, *Aspergillus* species, *Penicillium* species, similar results were recorded by [15].

The logarithm counts of *Rhizopus stolonifer* exposed to locally refined diesel and kerosene toxicants in fresh, brackish and marine water are revealed in Tables 1 and 2 respectively.

There was a significant ($p \leq 0.05$) reduction in the log survival count of *Rhizopus stolonifer* with an in increase in the concentrations of the toxicant when compared with the control (Table 1). This agrees with [16], that high concentration of hydrocarbon can be inhibitory at the initial stage. At 10% concentration, there was a little increase in the population this could be seen as the acclimation period [17]. The effect of the toxicant became highest at 50% in Marine water samples this could be due to the combined effect of the toxicant and salinity in marine water. From Table 2, at 10% concentration, there was a significant increase in the population, [2] also reported a significant gain in fresh weight of *Rhizopus stolonifer,* at 10% kerosene concentration.



Key: THB= Total heterotrophic bacteria, THF= Total heterotrophic fungi

Fig. 1. Variations in total heterotrophic bacteria and total heterotrophic fungi in three aquatic environment

Table 1. Effect of different concentration of locally refined diesel in Fresh, brackish and marine water on *Rhizopus stolonifer* population (Log_{10}sfu/ml) during 96 hours of exposure period

Conc. of diesel (%)	Treatments		
	F+D+R	B+D+R	M+D+R
0 (control)	3.07 ± 0.01^{c}	3.08 ± 0.02^{b}	3.07 ± 0.02^{c}
5	2.47 ± 0.22^{a}	2.39 ± 0.29^{a}	2.37 ± 0.21^{ab}
10	2.67 ± 0.15^{ab}	2.78 ± 0.22^{ab}	2.67 ± 0.19^{b}
25	2.64 ± 0.14^{ab}	3.03 ± 0.57^{b}	2.66 ± 0.22^{b}
50	2.80 ± 0.19^{b}	2.84 ± 0.16^{b}	2.15 ± 0.52^{a}

**Means with the same alphabet across the column shows no significant difference at ($p \geq 0.05$)*
key: D=diesel, F=fresh water, B=brackish water, M= marine water, R= Rhizopus stolonifer

Table 2. Effect of different concentration of locally refined Kerosene in fresh, brackish and marine water on *Rhizopus stolonifer* population (Log_{10}sfu/ml) during 96 hours of exposure period

Conc. of kerosene	Treatments		
	F+K+R	B+K+R	M+K+R
0(Control)	3.09±0.02^{c}	3.06±0.02^{c}	3.07±0.01^{b}
5%	2.44±0.22^{a}	1.79±0.72^{a}	2.01±0.54^{a}
10%	2.70±0.15^{b}	2.19±0.10^{ab}	2.48±0.22^{a}
25%	2.66±0.06^{ab}	2.29±0.31^{ab}	2.41±0.29^{a}
50%	2.81±0.12^{b}	2.51±0.15^{bc}	2.40±0.25^{a}

**Means with the same alphabet across the column shows no significant difference at ($p \geq 0.05$)*

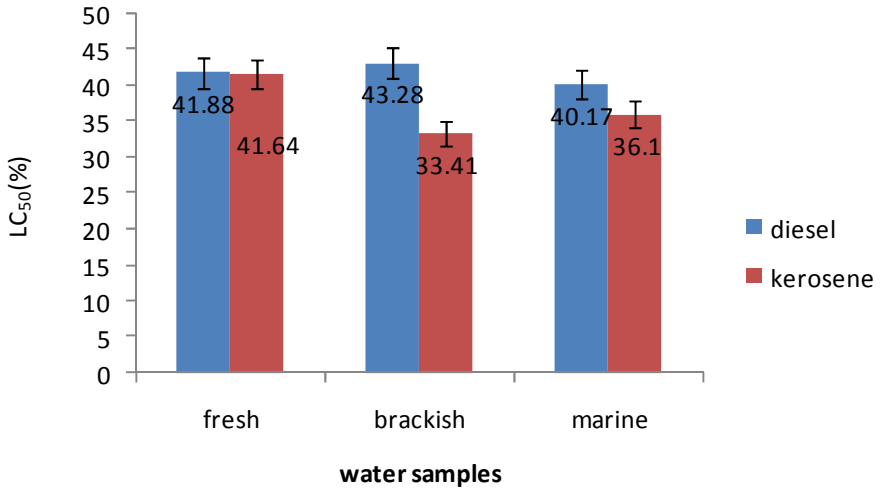


Fig. 2. Median lethal concentration (LC_{50}) from log mortality of *Rhizopus stolonifer* in diesel and kerosene in three aquatic environments

Rhizopus stolonifer mortality expressed as median Lethal concentration (LC_{50}) was used as indices to monitor toxicity [15 and 18]. The sensitivity of this fungus to the toxicity of the different concentrations of petroleum products (locally refined diesel and kerosene) with the different water samples (fresh, brackish water and marine). The median lethal concentration (LC_{50}) of the petroleum products used increased in the following order: as shown in Fig. 2, (Note: the higher the LC_{50}, the lower the toxic effect and vice-versa). Locally refined diesel in fresh water (41.88%) <Locally refined kerosene in fresh water (41.64%) <Locally refined diesel in brackish water (43.28%) <locally refined kerosene in brackish water (33.41%) <locally refined diesel in marine water (40.17%) <locally refined kerosene in marine water (36.1%).

The log survival of *Rhizopus stolonifer* during 0hr, 24hr, 48hr, 72hr and 96hr exposure periods to different concentrations (0%, 5%, 10%, 25%, 50%) 0f locally refined diesel and kerosene in fresh, brackish and marine environments as represented in Tables 1 and 2, respectively shows that both locally refined diesel and kerosene exhibited little effect on the test organism in fresh water than brackish water and marine water. This may be due to the saline nature of the brackish and marine water. Hence, the results of this study suggest that both toxicants caused cell death which resulted in a reduction in viable counts, this however corresponded with [15,16 and 18].

The results of this research revealed that different concentrations of the toxicants have adverse effect on the survival rate of the test organism which shows that the constituents of these petroleum products diesel; 75% alkenes and 25% of aromatic compounds (including naphthalene and alkybenzenes) and kerosene; paraffins (55.2%), naphthenes (40.9%), and

aromatics (3.9%), directly affect *Rhizopus stolonifer* which play an important role in bioremediation and carbon cycle as a results of its ability to degrade substances in the ecosystem [2] and also in the industrial production of ethyl alcohol (most important fermentation product) [16]. Also it has been observed by [19] that a long-term exposure to kerosene and diesel oil can result in excess of lung cancer, restlessness, convulsions, coma and death in humans.

4. CONCLUSION AND RECOMMENDATION

Conclusively, locally refined kerosene in brackish water (LC_{50} = 33.41%) is the most toxic, having the lowest LC_{50} while locally refined diesel in brackish water (LC_{50} = 43.28%) have the lowest toxicity effect. These results show that locally refined diesel and kerosene can inhibit the growth of *Rhizopus stolonifer* in aquatic ecosystem although the organism can degrade petroleum products at specific concentrations.

Therefore it is recommended that illegal oil refining activities and other indiscriminate human activities that result in oil spill especially into the water bodies be checkmated.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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