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'Petroleum Derivatives' Toxicity: Influence on The Growth of Soil Nitrifying Bacteria

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Abstract
Over dependence on crude oil and its derivatives to drive our daily activities for energy and economy results in frequent discharge of petrochemicals into the environment causing pollution of varying magnitude with worrisome ecological and environmental impact of global concern. This study aimed to determine the toxicity of petroleum derivatives on soil nitrifying bacteria. Soil samples were collected into sterile polythene bag and transported to the laboratory for analyses. Microbiological analyses of the soil samples were performed using conventional and standard microbiological techniques. Kerosene, diesel and petrol at 1%, 3%, 5% and 10% concentrations were used as toxicants on nitrogen-fixing bacteria. Results revealed the mean counts of 2.7 x 10^7 (CFU/g), 4.3 x 10^6 (CFU/g), 8.7 x 10^4 (CFU/g) and 1.2 x 10^3 CFU/g of total heterotrophic bacteria, bacterial growth on Okon red, Ashby medium and Yeast extract mannitol agar respectively. The identities of nitrifying bacteria were Azospirillum spp., Azotobacter spp. and Rhizobium spp. Toxicity results revealed that while Azospirillum spp. exhibited complete cell death to petrol from 3%, all the other bacteria showed log survival of 70.29 – 86% to the toxicants and Azospirillum, spp., Azotobacter spp. and Rhizobium spp. had 76.63%, 79.63% and 86% log survival to diesel and 77.76%, 79.63 and 77.6% respectively to kerosene. Significant difference (p-value = 0.000) was observed among the three toxicants across all concentration levels. Therefore, this study concludes that petroleum derivatives are toxic to nitrifying bacteria at any concentration and the microbial response is dependent on concentration and hydrocarbon types. Soil contaminated with petroleum derivatives should of necessity be treated in order to ameliorate the consequences of pollution and restore soil productive capacity.

Keywords: Nitrifying-fixing bacteria, Petroleum derivatives, Pollution, Toxicant, Toxicity.

INTRODUCTION
Crude oil is refined through fractional distillation process into kerosene, petrol, diesel, motor engine oil and other products based on the differences in structure and boiling points (Odeyemi, 2014). Over dependence on crude oil and its derivatives to drive our daily activities for energy and economy result in frequent discharge of petrochemicals into the environment causing pollution of varying magnitude with worrisome ecological and environmental impact of global concern. The negative consequences of hydrocarbon pollution on soil as key receiver of spills have been extensively reported by several authors. Petroleum hydrocarbons can sterilize soil and cause alteration in soil microbiological properties (Digha *et al*., 2017; Sharma and Pathak, 2017), destroy soil richness and reduce soil fertility and render the agricultural soils less productive (Etuk *et al*., 2013) with concomitant adverse effects on the entire ecosystem and all forms of life dependent on the environment.

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Soil provides habitat for micro flora and micro fauna and a dynamic entity where complex biogeochemical cycling of elements takes place (Delgado and Gomez, 2016) and plays vital roles in food chain and ecosystem sustenance. Microorganisms in soil are involved in recycling of nutrients, in the decomposition of plants and animal residues in soil and converting them into organic matter which improves soil structure and influences on the physical, chemical and biological properties. Bacteria are the most dominant of soil microorganisms with a population of 10^6- 10^8 cfu/ g in fertile soil (Suliasih, 2005). Nitrogen is a macronutrient required in large quantity by plant but made available in the form of nitrate ion by the activities of nitrifying bacteria through the process of nitrification (Zewdie and Reta, 2021). Nitrogen fixing bacteria are widely available in most soil types as free – living, symbiotic and associative with plants (Thomas and Singh, 2019). Among nitrogen fixing bacteria isolated from soil and rhizosphere of various crops are *Acetobacter, Arthrobacter, Azoarcus, Azospirillum, Azotobacter, Bacillus, Beijerinckia, Enterobacter, Klebsiella, Pseudomonas* and *Zoogloea* (Thomas and Singh, 2019).

Petroleum hydrocarbons (PHs) are highly toxic environmental pollutants comprising a mixture of several hydrocarbons of different structures such as polycyclic aromatic hydrocarbons, benzene and its substituted and cycloalkane rings (Agarry and Ogunleye, 2012). The introduction of hydrocarbon pollutants into the environment can alter the activities and population of microorganisms (Ikuesan, 2017; Sharma and Pathak, 2017). Thus, any process that affects microbial population dynamics will influence ecosystem function including nitrification. Toxicity is the quantity to which a substance can cause damage or injury to a living organism (Moreno and Gandolfi, 2013). The components of these pollutants exert toxic effects not only to humans, animal and plants but also to soil microorganisms (Heipieper and Martínez, 2010; Moreno and Gandolfi, 2013) including plant growth promoting bacteria indigenous to soil, thereby affecting their activities of nitrogen fixation. Moreno and Gandolfi (2013) stated that toxicants change the function of cells in an organism by interfering with normal processes. Organic pollutants in the environment may persist due to physicochemical properties of the hydrocarbons such as low bioavailability (not accessible for microbial attack or degradation) as well as very high toxicity of the hydrocarbon. Several classes of organic compounds are toxic to living organisms as they accumulate in and disrupt cell membranes (Heipieper and Martínez, 2010). The major reason hydrocarbons are toxic to microbial cells is due to their preferential partitioning into membranes causing an increase in the fluidity of the membrane that leads to its non-specific permeabilization (Heipieper and Martínez, 2010). The rapid decline in the population of many plants and continual depletion in the fertility of agricultural soils in oil rich areas have been observed by ecologists (McOrist and Lenghaus, 1992). This study therefore assessed the toxicity of petroleum derivatives to nitrifying bacteria isolated from agricultural soil in order to ameliorate its negative consequences on food security particularly in the Niger-Delta region where farming is the main occupation of the people.

MATERIALS AND METHODS

Collection of samples

Soil samples were collected from three points 5 m apart from an agricultural farm in Igodan-Lisa, Okitipupa (6° 27′ 0′′N, 4° 47′ 0′′ E), Nigeria using disinfected hand trowel at a depth of 10 – 15 cm into sterile polythene bag and thereafter transferred to the laboratory for analyses. Composite sample of soil was prepared, partially air-dried at 28 ± 2° C and sieved with a 2 mm mesh to remove large particles, debris and stones and stored at room temperature for analyses. Kerosene, diesel and petrol used as toxicants were purchased with a sterile plastic container from an Independent Petroleum Marketer (Abat filling station) in Okitipupa.

Microbiological analysis of the soil sample
All media used in this study were prepared according to manufacturers' specification. The nitrogen fixing media used were Yeast extract mannitol agar (Subba, 1994), Okon medium (Narayan, 2018) and Ashby mannitol agar (Ahmad *et al*., 2016) selective for the isolation of *Rhizobium* spp., *Azospirillum* spp. and *Azotobacter* spp. species respectively. All media were mixed with the aid of magnetic stirrer to homogenize and 20 mL of each medium was dispensed into MacCartney bottles, autoclaved at 121° C for 15 minutes and allowed to cool to 44° C and aseptically poured into well labelled petri dishes and allowed to solidify.

Enumeration of total heterotrophic and nitrogen- fixing bacteria
Ten grams (10 g) of soil sample was rehydrated with 90 mL distilled water in 200 mL holding capacity bottle and then agitated vigorously to dislodge bacteria from the soil particles. The stock was then serially diluted to the sixth dilution. One (1) mL of each dilution was pour plated onto Nutrient agar, Yeast mannitol agar, Okon red agar and Ashby mannitol agar supplemented with antifungal agents (50µg/mL of nystatin and 75µg/mL of cycloheximide) for the enumeration of total heterotrophic bacteria, *Rhizobium* spp., *Azospirillum* spp. and *Azotobacter* spp. respectively. Triplicate culture plates were allowed to set and subsequently incubated at 35° C for 24 hours for Nutrient agar and at 35° C for 48 hours for nitrifying bacteria (John *et al*., 2011). The plates were observed for growth and selected for count and the number of colonies expressed as colony forming unit per gram (CFU/g) of sample.

Identification of bacterial isolates
Nitrogen-fixing bacterial isolates were purified, characterized and identified based on cultural characteristics, Gram staining and biochemical tests using the procedures reported by Holt *et. al.* (1994) and Chessbrough (2006).

Growth response and tolerance of test nitrifying bacteria to petroleum derivatives (petrol, diesel and kerosene).
The method described by Nseabasi and Antai (2012) was adopted and modified to determine the growth response and toxicity of petroleum derivatives on nitrifying bacteria. Kerosene, diesel and petrol were employed as toxicants at different concentrations of 1%, 3%. 5% and 10%. Dilutions 10^{-4} of twenty-four (24) hour old broth culture of *Rhizobium* spp., *Azospirillum* spp. and *Azotobacter* spp. were prepared to tease out bacterial population. The mineral salt medium (Bushnell Hass) used was sterilized by autoclaving at 121° C for 15 mins and supplemented with 1%, 3%. 5% and 10% filter sterilized hydrocarbons (kerosene, petrol and diesel) to serve as the only source of carbon and energy. The Mineral salt medium (MSM) tubes containing varying concentrations of hydrocarbon were seeded with 1 mL from 10^{-4} dilution of test organisms and incubated at 28° C for 24 hours and then 1mL of each MSM culture tube was pour plated on Nutrient agar plate in triplicate and incubated at 28° C for 24 hours. MSM tubes without hydrocarbon source served as control. After the incubation period, tolerance range of the petroleum derivatives at different concentrations on the nitrifying bacteria species were obtained on the basis of their growth (cell count) as response. The number of colonies were counted and expressed in colonies forming unit (CFU/mL).

Percent-log survival test

Percent- log survival for each test nitrogen fixing bacterium was calculated based on the formula of Williamson and Johnson (1981). This was done by obtaining the log of count in each toxicant concentration (Log C) respectively and dividing by log of count in the zero (control) toxicant concentration (Log c) and multiplying by 100 (% log survival) = Log C/ Log c ×100.

Statistical Analysis

Data collected were subjected to analysis of variance to compare sample mean among toxicants across concentration levels at 95% confidence level. All analysis were carried out using Microsoft Excel.

RESULTS AND DISCUSSION

Population and identity of nitrifying bacteria in soil sample

Table 1**:** Population of Bacterial isolates from soil sample

Cultural Medium	Bacterial type		%NFB
	NFB	THB	
Nutrient Agar		$2.7\times10^{7} \pm 0.1$	-
ORA	$4.3 \times 10^{6} \pm 1.2$	-	16.049
AMA	$8.7 \times 10^{4} \pm 1.2$	-	2.008
YEMA	$1.2 \times 10^{3} \pm 0.1$	-	0.004
Total			18.07

Data is expressed as mean ± Standard Error

Legend: ORA = Okon red agar, AMA = Ashby mannitol agar, YEMA =Yeast extract mannitol agar.

Soil provides habitat for diverse groups of micro flora and micro fauna and serves as medium for interactions among its biogeochemical components. Table 1 shows the total heterotrophic and nitrogen fixing bacterial population from the soil sample. The population of *Azospirillum* spp. on Okon red agar ($4.3 \times 10^{6} \pm 1.2$ CFU/ g) was higher than the $8.7 \times 10^{4} \pm 1.2$ (CFU/ g) and $1.2 \times 10^{3} \pm 0.1$(CFU/g) of *Azotobacter* spp. on Ashby mannitol agar (AMA) and *Rhizobium* spp. on Yeast extract mannitol agar (YEMA) respectively. The proportion of the nitrifying bacteria to the heterotrophic population ($2.7\times10^{7} \pm 0.1$ CFU/ gm) was 18.07%. The population of heterotrophic bacteria obtained in this study was within the value range of bacterial population (10^{6}- 10^{8} CFU/ g) for a fertile soil reported by Suliasih (2005). The colonial, morphological and biochemical characteristics (tables 2 and 3) of bacterial isolates obtained from the soil sample on Okon red agar, Ashby mannitol agar and Yeast extract mannitol agar revealed the identity of the nitrifying bacteria as *Azospirillum* spp., *Azotobacter* spp. and *Rhizobium* spp. respectively. This implies that soil sample harboured diverse genera of nitrogen fixing bacteria and therefore corroborates the assertion of Sneha *et al*. (2018) that differential bacterial genera are key components of the soil. Nitrification is natures' way of replenishing soil of depleted nitrogen as essential nutrient for plant growth and crop yield. The population of the presence and population of the three nitrogen-fixing bacterial genera in the soil sample suggest the potential of the soil as a fertile soil for productive agriculture. This probably account for the assertion of Sneha *et al*. (2018) that *Azotobacter* spp. let alone *Azospirillum* spp., *Rhizobium* spp. and other plant growth promoting bacteria increase the growth of agricultural crops by 10 – 12%.

The bacteria genera isolated from the soil sample and used as test bacteria are among such organisms classified as nitrogen-fixing bacteria by different researchers. This finding is in agreement with Suliasih (2005) who reported *Azotobacter* spp. and *Azospirillum* spp. among nitrogen-fixing bacteria living in soil. The growth of these bacteria on nitrogen-free media suggests their potentials in fixing atmospheric nitrogen (Ikuesan and Fajolu, 2022). *Rhizobium* spp., *Azotobacter* spp. and *Azospirillum* spp. isolated from the soil sample are among the differential bacterial genera classified as plant growth promoting bacteria, free living and present in soil with ability to fix atmospheric nitrogen as well as the production of certain metabolites such as auxin, cytokinin, hydrogen cyanide (HCN) among others (Sneha *et al*, 2018).

Table 2: Cultural and morphological characteristics of nitrogen fixing bacteria isolated from soil sample.

Growth medium	Description of cultural and morphological characteristics of isolates
Okon Red Agar	Large, white gray colony, convex with entire margin, Gram negative, motile, non – sporing short rod shaped.
Ashby Mannitol Agar	Slimy white, opaque, raised, convex, smooth, mucoid, Gram negative, non – sporing and motile short rod shaped
Yeast Extract Mannitol Agar	White/ creamy translucent, motile, elongated rod shaped, Gram negative and non – sporing.

Table 3: Biochemical characteristics of nitrogen fixing bacteria isolated from the sampled soil

Test	Isolates from growth medium		
	ORA	AMA	YEMA
Catalase	+	+	+
Oxidase	+	+	+
H_2S Production	-	+	
Citrate	+	+	+
Urease	+	+	
VP	-	+	+
MR	-	-	-
NR	-		+
Fermentation			
Glucose	AG	AG	+
Sucrose	NC	AG	+
Maltose	A	AG	
Lactose	A	AG	+
Mannitol	A	AG	+
Possible organism	*Azospirillum* sp.	*Azotobacter* sp.	*Rhizobium* sp.

Legend: ORA = Okon Red Agar, AMA = Ashby Mannitol Agar, YEMA = Yeast Extract Mannitol Agar, + = Positive reaction, - = Negative reaction; VP= Voges-Proskauer, NR =Nitrate Reduction, A = Acid production only, AG = Acid and gas production, NC = No change.

Growth response and tolerance of test nitrifying bacteria to petroleum derivatives

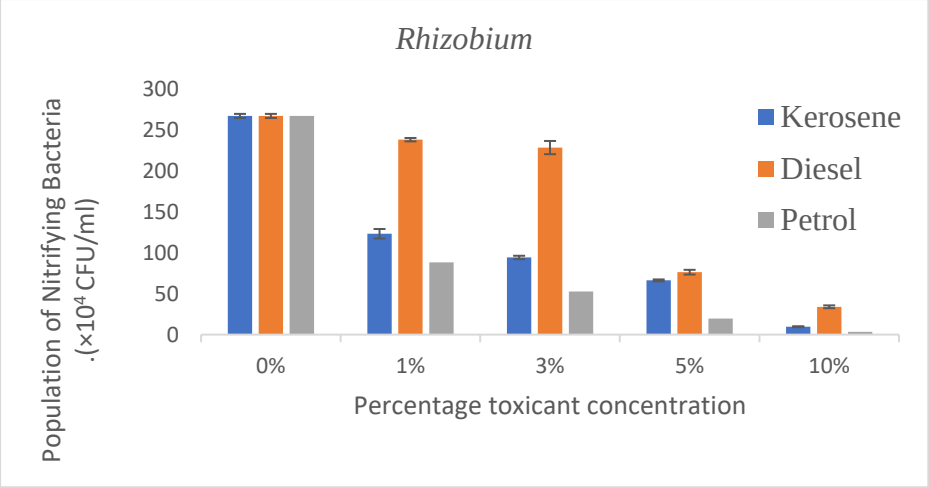

Figure 1: Growth response of *Rhizobium* spp. at different concentrations of toxicant contamination.

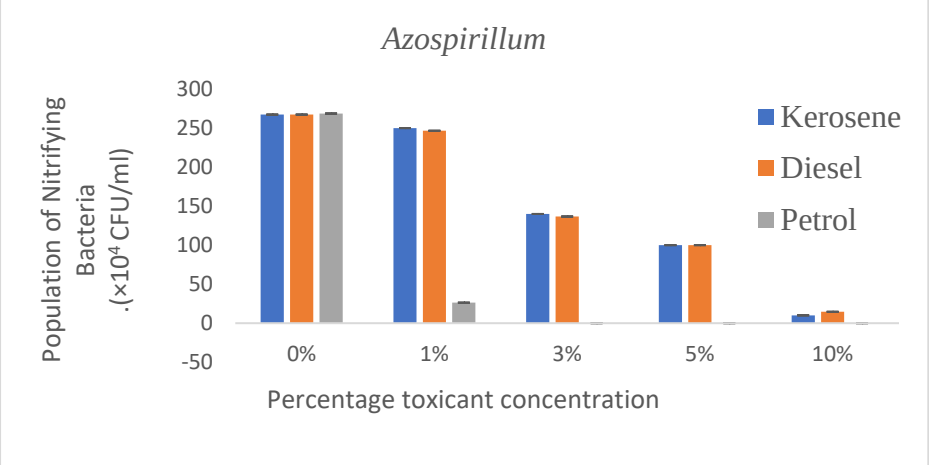

Figure 2: Growth response of *Azospirillum* spp. at different concentrations of toxicant contamination.

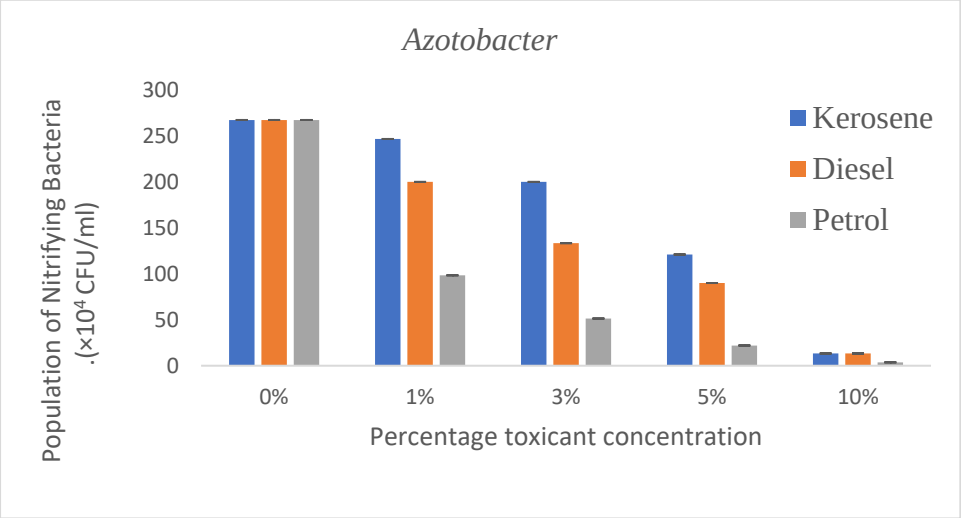

Figure 3: Growth response of *Azotobacter* spp. at different concentrations of toxicant contamination.

Figures 1, 2 and 3 show the responses of the isolated nitrogen-fixing bacteria (NFB) to varying concentrations of kerosene, diesel and petrol. Results revealed a progressive reduction in the population of NFB as the concentration of hydrocarbon toxicants increased with petrol exerting the greatest lethal effect on all the test bacterial isolates. Toxicity results revealed that except *Azospirillum* spp. which exhibited zero tolerance (complete cell death) to petrol from

3%, all the other bacteria showed log survival of 70.40 – 86.14% to all the applied toxicants and *Azospirillum* spp., *Azotobacter* spp. and *Rhizobium* spp. having 80.0%, 84.11% and 86.14% log survival to diesel at 10% concentration. The lethal effects of the petroleum hydrocarbon derivatives ranked as petrol > kerosene ≥ diesel for all the bacteria at 10% toxicant concentration. The result of analysis of variance showed significant difference (p-value = 0.000) among mean population of test nitrifying bacteria across all concentration levels of the toxicants (kerosene, diesel and petrol) used in this study. For each bacterial species, population count reduced with increasing concentrations of toxicants. The result of this study corroborates the work of Nseabasi and Antai (2012) and Babalola *et al*. (2016) which reported that the higher the percentage of petroleum hydrocarbon, the lower the mean count of bacteria species. Stancu (2014) reported that organic solvents are toxic to bacterial cells even at very low concentration of 0.1%. It also agrees with the study of Kucharski *et al*. (2010) who reported that nitrifying bacteria are particularly sensitive to environmental conditions. However, this result is contrary to the submission of Osuji *et al*. (2005) that only until beyond 3% concentration, oil has increasing deleterious effects on soil biota. The result of this study implies that the degree of toxicity of kerosene, diesel, petrol and hence survivability of the microorganisms is largely dependent on the concentration of the toxicants in the medium. Thus, it is expected that an increase in the concentration of the contaminant would result in further decrease in percentage log-survival of these bacterial genera and chronic contamination will result in their elimination. These would consequently result in the absence of certain bacterial genera that can metabolize these derivatives in an extreme pollution case. Statistical analysis on the percentage log-survival showed that there was significant difference in the effect of kerosene, diesel and petrol contamination on bacterial species. Hence, a decrease in microbial counts is indicative of susceptibility to kerosene, diesel and petrol toxicity. This suggests that these nitrifying bacteria do not possess physiological and genetic properties to utilize these petroleum derivatives as carbon and energy source. The results generated from this study indicated that petrol within the exposure time and toxicant concentration inhibited the growth of *Rhizobium* spp., *Azospririllum* spp. and *Azotobacter* spp. more that kerosene and diesel. Clark and Brown (1977) reported that petrol fuel has C_4 – C_{12} carbon atoms and kerosene and diesel fuel with C_{10} – C_{20}. Wyszkowska and Kucharski (2002) reported that lighter hydrocarbons are more harmful to microorganisms than higher molecular weight fraction hydrocarbons (C_{12} – C_{42}). This property probably explains why petrol was more toxic than kerosene and diesel fuel as obtained in this study. This result further corroborates the report of Babalola *et al*. (2016) that low molecular weight hydrocarbons are more toxic than long chain because long chain HCs are less soluble and less bioavailable. The test bacteria varied in their susceptibility to the toxicants. The difference in response of these bacteria to kerosene, diesel and petrol might be due to their genetic differences. Various authors gave account of the mechanism of action of toxicity of hydrocarbons to microbial cells to include the oil inhibits the action of the enzyme ''nitrogenase'', thereby disrupting the process of protein necessary for plants to thrive and survive in the affected ecosystem (John *et al*., 2011), disruption of plasma membranes (Sulaiman, 2015), preferential partitioning into membranes causing an increase in the fluidity of the membrane that leads to its non-specific permeabilization (Heipieper and Martı´nez, 2010) as well as alteration of the functionality of the microbial community and therefore the ecosystem (Chikere *et al*., 2011). It is also known that toxicity of some pollutants is dependent on soil characteristics (Sulaiman *et al*., 2015).

The implication of the result of this study is that pollution of soil with petroleum derivatives even at relatively light concentration will negatively affect the population of nitrifying bacteria and can therefore interfere with natural biogeochemical processes such as nitrification and nutritional chains since microorganisms perform significant roles in these

processes. This submission agrees with Moreno and Gandolfi (2013) who stated that toxicants change the function of cells in an organism by interfering with normal processes and the response is measurable by changes that occur.

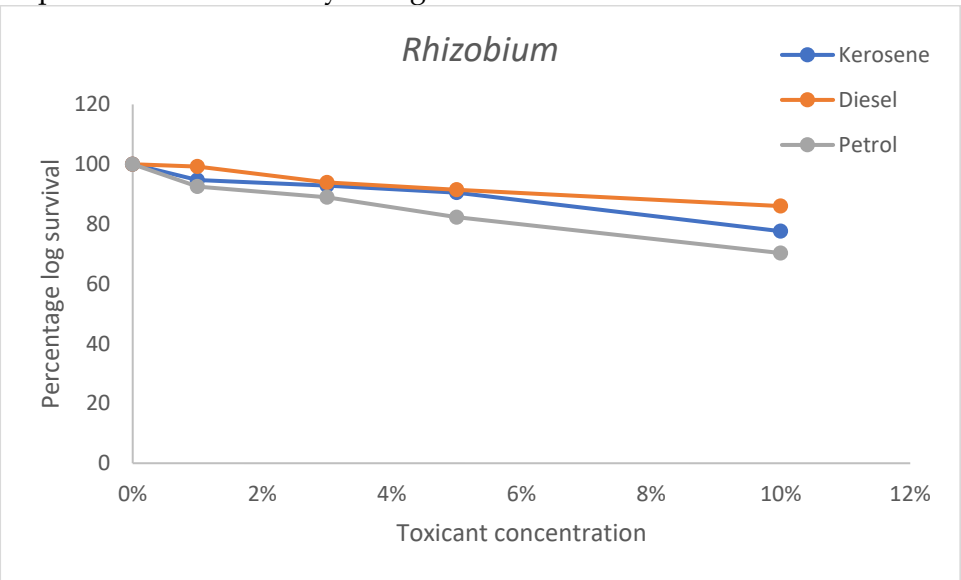

Figure 4: Toxicity of petroleum derivatives on *Rhizobium* spp.

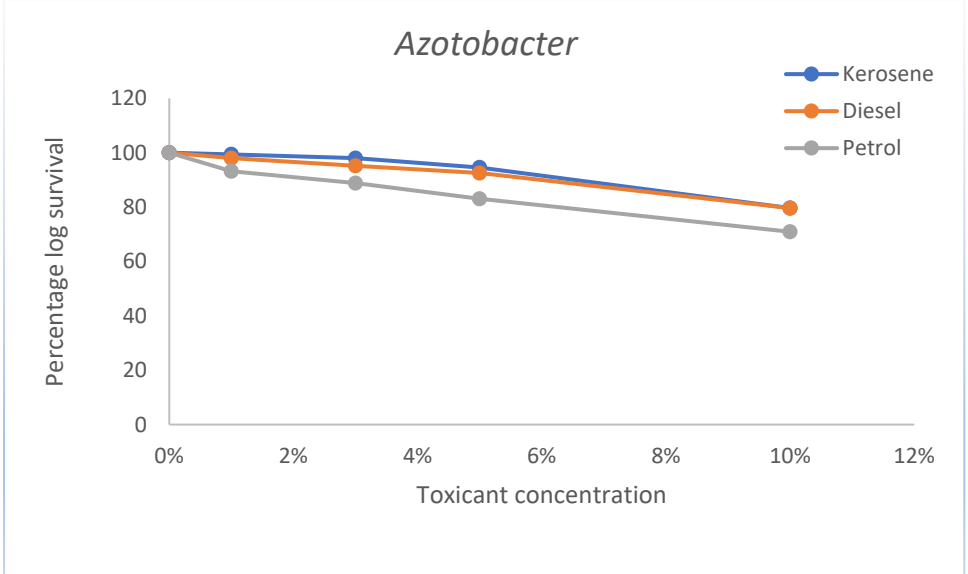

Figure 5: Toxicity of petroleum derivatives on *Azotobacter* spp.

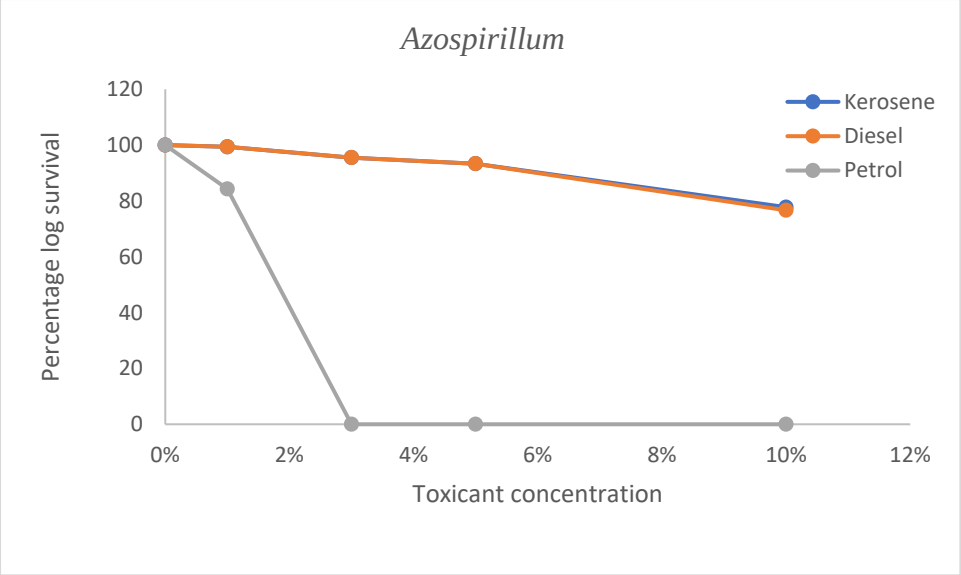

Figure 6: Toxicity of petroleum derivatives on *Azospirillum* spp.

CONCLUSION

From this study, it can be concluded that nitrogen-fixing bacteria are diverse and dominant in the soil. Low molecular weight petroleum derivatives such as kerosene, petrol and diesel fuel are toxic to nitrifying bacteria at all concentrations but increases with increase in concentration which could lead to specie elimination. Therefore, high and persistent concentrations of petroleum derivatives resulting from improper disposal and spills may result in the reduction of primary producers of the affected ecosystems and ultimately destroy ecosystem function due to elimination of nitrogen fixing bacteria with overall effect on soil fertility, plant growth and productive agriculture.

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