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Research Article

Effect of Kerosene on the Growth of Nitrifying Bacteria Isolated From Soil

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ABSTRACT

The environment is being subjected to an increasing amount of stress due to the alterations caused by the pollution of refined petroleum products such as kerosene. These changes might be substantial, which would have a big effect on the environment and, in turn, the farm output. Determining the potential toxicities of kerosene dosage response relationships to sensitive species, like soil microbes, is crucial. This investigation assessed how kerosene affected the nitrifying bacteria that were taken out of farmland's soil. We observed the population changes of the two nitrifying bacteria that were isolated from soil samples after they were exposed to varying concentrations of kerosene (0.5%, 1%, 2%, 5%, and 10%) for duration of 120 hours. Using a mineral salts media, the effects of kerosene on the two nitrifying bacteria were investigated. Samples were taken from the medium every 24 hours to gauge the growth of the bacteria, and a spectrophotometer was used to quantify the turbidity at 600 nm. The result showed that as kerosene concentrations were exposed to these bacteria over longer periods of time, the survivability of *Nitrosomonas* sp. and *Nitrobacter* sp. decreased. The toxicity studies' findings demonstrated that the degree of kerosene's toxicity to *Nitrosomonas* and *Nitrobacter* species depended on the quantity of pollutants present and the length of the contact period.

Keywords: Kerosene, Nitrifying Bacteria; Kerosene Toxicity; Soil Bacteria; *Nitrosomonas sp*. and *Nitrobacter sp*.

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INTRODUCTION

The growing number of oil pollution incidents, which can be attributed to vandalism, leaks from corroding pipes, spills, or other factors, has drawn attention to the effects of crude oil and its fractions. According to Eze (2019), most research on the effects of oil pollution in aquatic settings only looks at the effects of complete crude or refined components. Since fish can move readily from polluted areas to free zones, the majority of these studies concentrate on fish (Amakini, 2019). Crude oil and its byproducts penetrate the aquatic ecology and inflict significant harm to the aquatic environment. One way to do this is to restrict the amount of oxygen that aquatic plants and animals have access to (Willey, 2014). Another is to directly disrupt the organism in contact's physiological and biochemical functions (Jie, 2003).

Given its utility in contemporary culture, kerosene is employed as a source of both energy and money. But our ecosystems are starting to be threatened by kerosene seeping into the environment (Njoku et al.,

2016). The contamination of refined petroleum products and kerosene is causing changes that are placing an increasing degree of stress on the ecology. If these changes are considerable, they could have a big effect on the ecology (Ikhajiagbe and Anoliefo, 2013). Numerous sources can discharge crude oil and its refined products into the environment, causing harm to both land and water (Jan, 2010).Fractional distillation, a technique that separates crude oil into fractions that include motor oil, diesel, kerosene, gasoline, and many other petroleum products, is how refined petroleum products are made (Raina et al., 2019). In lieu of this crude oil leak, there might be other incidents of refined petroleum products and crude oil spilling on land connected to the transportation of petroleum-based products and crude oil, as well as spills that happen in transit, storage tank leaks, and the disposal of spent motor oil (Raina *et al*., 2019).

Kerosene can contaminate our farmlands and other arable land, and it may negatively impact the growth and activity of soil bacteria, according to John *et al*. (2017). This pollutant might include substances, such kerosene, which are detrimental to these microbes. Because of this, they might be somewhat poisonous to the development of naturally occurring nitrifying bacteria, which could affect the bacteria's ability to function in the soil.

For soil fertility, nitrifying bacteria must be present. During the nitrification process, nitrifying bacteria mainly give plants access to nitrogen, a common soil nutrient element that plants require in large amounts as nitrate ions (Bona *et al.*, 2019). Given the significance of nitrifying bacteria for soil fertility in our ecosystem and waste water treatment plants, it is necessary to look at how these bacteria react to pollution stress caused by refined petroleum fractions like kerosene. The evaluation entailed measuring the pollutant's toxicity levels and exposing the nitrifying bacteria that were isolated from soil to various kerosene concentrations.

MATERIALS AND METHODS

Sample Collection and Preparation

Kerosene sample was purchased into sterile plastic container from an Independent Petroleum Marketer in Sokoto. The samples were taken to laboratory for use. Fresh soil samples were collected at different locations from agricultural soil free from kerosene pollution within the Usmanu Danfodiyo University, Sokoto, Nigeria. The fresh soil samples were collected at a depth of 0-15cm, using sterile hand trowel and transferred into sterile polyethene bags and transported to the laboratory for use.

After the soil samples were gathered, they were well mixed and run through a mesh sieve with a 2 mm hole size to eliminate big particles. After that, 45 ml of sterile phosphate buffer containing 139 mg of K2HPO4 and 27 mg of KH2PO4 per liter was used to dissolve 5 g of the soil sample. In agreement with Sugumaran *et al*. (2014), the mixture was shaken in a rotary shaker at 100 rpm for two hours at room temperature to release the organisms into the liquid medium after the pH was adjusted to 7.0.

Media Used

The media used in this study were Nutrient agar, Winogradsky agar and broth. All the media were prepared and sterilized according to manufacturer's specifications.

Microbiological Analysis

The methods outlined by Adesemoye *et al*. (2006), Oyeleke and Manga (2008) and Rabah *et al.* (2008) were used to conduct the microbiological analysis. One gram of the soil sample was measured and then serially diluted up to 10^6 times. Next, using the spread plate approach to inoculation, 0.1 ml aliquots from the 104 tubes were aseptically injected onto nutritional agar plates that had already been prepared. For a whole day, the nutrient agar plates were incubated at 37 °C. Plates containing different colonies were enumerated and recorded as cfu/g after the incubation time. To obtain pure isolates, the colonies were also repeatedly subcultured onto new nutrient agar media. According to Cheesbrough (2003), the bacterial isolates were recognized and described by the use of standard biochemical, morphological, and cultural techniques. The tests that were used were the spore test, oxidase test, glucose, lactose and sucrose utilization tests, urease activity, methyl red test, voges-proskaeur test, motility, citrate utilization test, indole synthesis, urease activity, and H_2S and gas generation.

Effects of Kerosene on the Growth of the Isolates

The population changes of the two nitrifying bacteria that were isolated from soil samples was observed after they were exposed to varying concentrations of kerosene (0.5%, 1%, 2%, 5%, and 10%) for a duration of 120 hours. Using mineral salts medium with the following composition (g/l): (NH4)2S04, 1.0 g; KH2P04, 1.0 g; K2HP04, 1.0 g; MgS04, 0.2 g; CaCl2, 0.02 g and FeCl3.6H20, 0.004 g, the effects of

kerosene on the two nitrifying bacteria were investigated. After that, the mineral basal medium (MBM) was divided into each of the 250 ml Erlenmeyer flasks, containing 100 ml, 99.5 ml, 99 ml, 98 ml, 95 ml, and 90 ml, respectively, and sterilized at 121 C for 15 minutes at 15 pressure. The control was set up without kerosene. Carefully, 2 milliliters of the inoculum were added to each flask holding the mineral salts medium. After giving them a good shake to combine them, they were all appropriately labeled and incubated aerobically for 120 hours at room temperature. On a rotary shaker, all of the flasks containing sterile MBM supplemented with various kerosene concentrations and 2 ml of the inoculum were shaken for 120 hours at room temperature at 180 rpm. Every 24 hours, samples were taken out of the medium to monitor growth, and a spectrophotometer was used to quantify turbidity at 600 nm. In order to measure the absorbance of the turbidity at 600 nm using a spectrophotometer, representative samples from each flask were taken out at intervals of 24 hours, 48 hours, 72 hours, 96 hours, and 120 hours during the incubation period.

Effective Concentrations (Ec50) and Lethal Times (Lt50) of Kerosene for the Isolates

Probit regression analysis was used to determine the effective concentrations (EC) and lethal times (LT) of kerosene for isolates A and B, with a 95% confidence level. SPSS was used to evaluate the various growth data (the number of cells that survived after growth) that were acquired after growing the isolates at various kerosene concentrations (0.5%, 1%, 2%, 5%, and 10%) for 24, 48, 72, 96, and 120 hours of exposure time. This software application was used to determine the various lethal times (LT_{50}) at various concentrations (0.5%, 1%, 2%, 5%, and 10%) of kerosene as well as the various effective concentrations (EC_{50}) of kerosene for isolates A and B at the various exposure times. The lines of greatest fit were found using the Probit regression technique. By creating a horizontal line from the 50% point on the Y-axis to the intersection of the lines of best fit on the graph, the effective concentrations (EC_{50}) and the lethal time (LT_{50}) were determined. By drawing a vertical line across the abscissa, the point of crossing was extrapolated, yielding the EC_{50} and LT_{50} of kerosene for the organisms at various exposure times and concentrations, respectively. The graphs were created using the kerosene EC_{50} and LT_{50} values that were found for the two isolates. Therefore, the metrics used to assess kerosene's hazardous potential on the two nitrifying bacteria were EC_{50} and LT_{50}.

Data Analysis

Two-way ANOVA was used to analyze and compare the effects of kerosene on the growth of the isolates. The EC50 and LT50 estimation of the pollutants were calculated using a comparative approach based on Probit regression analysis at confidence limit of 95% using a computer program IBM Spearman correlation analysis (SPSS) version 20. The calculation of EC50 and LT50 values at different concentrations and times as well as comparison of the curve and their corresponding straight lines obtained from the regression analysis of the data was performed at 95% confidence interval based on the ranges of concentrations used in this experiment.

RESULTS AND DISCUSSIONS

The isolated bacteria were morphologically rod-shaped, gram-negative, catalase-positive, indole-negative, and nonfermentative in the presence of lactose and glucose. One isolate was able to convert ammonium ions to nitrite, while the second isolate was able to oxidize nitrite to nitrate, according to the results of the biochemical tests performed on the isolates. *Nitrosomonas sp*. was discovered as the isolate that could oxidize ammonium to nitrite, while *Nitrobacter sp.* was identified as the isolate that could oxidize nitrite to nitrate. Table 1 displays the outcomes of the test performed on the two isolates.

The concentrations of viable cells in the original cultures of the isolates (expressed as colony forming unit per ml or CFU/ml) was calculated from the plate counts on the pour plate to produce standard inoculum size of each isolate that was used during the experiment as shown in the **Table 2**.

Table 1. Morphological and Biochemical Characteristics of the Isolated from Soil Sample

Test	Isolate A	Isolate B
Morphology	Rods	Rods
Gram Reaction	Negative	Negative
Ammonia Oxidation	Positive	Negative
Nitrite Oxidation	Negative	Positive
Methyl Red	Negative	Negative
Voges-Proskauer	Negative	Negative
Catalase	Positive	Positive
Citrate Utilization	Negative	Negative
Nitrate Reduction	Negative	Negative
Urease	Negative	Negative
Coagulase	Negative	Negative
Indole	Negative	Negative
Glucose	Negative	Negative
Lactose	Negative	Negative
Identified Bacteria	*Nitrosomomas* sp.	*Nitrobacter* sp.

Table 2. Total Viable counts of Bacterial isolated from soil

S/N	Isolates	Plate Counts	cfu/g
1	A	53	$5.3x10^6$
2	B	78	$7.8 x10^6$

Effects of Concentrations of Kerosene on the Growth of *Nitrosomonas sp.*

According to the findings of the investigation into how different kerosene concentrations affected the growth of Nitrosomonas sp., the organism grew over the course of 120 hours at 0% (control experiment), 0.5%, 1%, and 2% kerosene concentrations. As seen in Fig. 1, the control experiment showed the largest rise in organism growth, followed by 0.5%, 1%, and 2%, respectively, despite an increase at these concentrations. Over the course of the exposure period, the organism's growth steadily declined at pollutant concentrations of 5% and 10%.The mean growth of *Nitrosomonas sp.* at the various concentrations and the mean growth of *Nitrobacter sp.* at the comparable kerosene concentrations and exposure durations differed significantly ($p<0.05$). Put differently, at different kerosene concentrations and exposure times, the mean growth of *Nitrosomonas sp.* was higher ($p<0.05$) than the mean growth of *Nitrobacter sp.* at the same comparable kerosene concentrations and exposure times.

Effects of Concentration of Kerosene on the Growth of *Nitrobacter sp.*

The growth of *Nitrobacter sp.* increased steadily in the control experiment with no kerosene and at 0.5% concentration. At 1% concentration of kerosene, increase in growth was observed up to 72 hours of exposure after which the growth of the organism begin to decline. At the 2%, 5% and 10% concentrations of kerosene, the growth of *Nitrobacter sp.* declined steadily with increase in contact time and concentration of the pollutant as depicted in **Fig. 2**. Thus, a decrease in number of survivors of the organism with increase in exposure time and concentrations was the general pattern. It was observed that the pH of the growing medium fluctuates between 6.9 and 7.1 throughout the exposure time. There was a significant difference ($p<0.05$) between mean growth of *Nitrobacter sp.* at the different concentrations and the mean growth of *Nitrosomonas sp.* at the equivalent concentrations of kerosene and at the same equivalent exposure times. In other words, the mean growth of *Nitrobacter sp.* at different concentrations of kerosene and at different exposure times were lower ($p<0.05$) than the mean growth of *Nitrosomonas sp.* at the same equivalent concentrations of kerosene and at the same equivalent exposure times.

The effect of varying kerosene concentrations on the isolates' growth revealed that lower concentrations of kerosene had less or no inhibitory effects on the organisms' growth during the test period, whereas higher concentrations of kerosene may have had a negative impact on the two organisms' growth. This demonstrates that while Nitrosomonas sp. and *Nitrobacter* sp. may both grow well at very low kerosene concentrations, they become poisonous to the organisms at greater kerosene concentrations. According to research by Odokuma *et al*. (2003) and Kobeticova *et al.* (2012), it has been found that Nitrosomonas sp. and *Nitrobacter* sp. can flourish at kerosene concentrations below 5% (v/v) of kerosene to media agar. Laboratory investigations by Eze *et al*. (2013) showed that nitrifying bacteria: *Nitrosomonas* sp. and *Nitrobacter* sp. could utilize kerosene, diesel oil, and jet fuel and engine oil as carbon source. But their capacities to use refined petroleum products and crude oil differ in terms of development profile and utilization rates (John and Okpokwasili, 2012). Enzyme inactivation at these higher kerosene concentrations may be the cause of these organisms' poor growth at kerosene concentrations above 1% (v/v). The fact that the organisms experienced a sudden shock effect from exposure to high kerosene concentrations rather than a gradual buildup may also be responsible for the negative effects of kerosene concentrations on the nitrifying bacteria, as these organisms were isolated from soils that had not previously experienced kerosene contamination, as reported by Alrumman (2015) when observing the effects of hydrocarbons contamination on soil microbiomes. This may be part of the reason why these isolates could not grow well at concentrations above 1% (v/v) shown in figure 1 and 2 respectively.

The isolates were harmful to kerosene at concentrations greater than 1% (v/v). The observed pH and temperature ranges aligned with the parameters that are ideal for growth. Therefore, the poisonous effects of kerosene concentrations rather than pH or temperature could not have caused the two species' development to decline. The ideal pH range of 6.6 to 8.0 has been shown to support the rapid growth of the Nitrosomonas and *Nitrobacter* species (Rainer et al, 2009). As shown in figure 3, it was discovered that *Nitrobacter* sp. was more susceptible to kerosene than Nitrosomonas sp.

A decrease in the number of survivors of *Nitrosomonas* sp. and *Nitrobacter* sp. with increase in the exposure time and concentrations of kerosene was a general pattern as observed in figure 1 and figure 2. As in *Nitrosomonas* sp. and *Nitrobacter* sp., the effect was more evident at higher concentrations (2%, 5% and 10%) of the fluids. Controls showed an increase in the population of viable cells (apparent absence of mortality) with increase in exposure time.

Similar to this, as Figures 1 and 2 demonstrate, there was a continuous rise in the two nitrifying bacteria's growth at a 0.5% kerosene concentration during the course of the experiment. This demonstrates that Nitrosomonas sp. and *Nitrobacter* sp. can both thrive at extremely low kerosene concentrations. Kerosene poisoned the organisms at increasing doses.

This demonstrated the bactericidal characteristics of the kerosene fluids, which were demonstrated by the two bacteria's reduced survival rate as contact time (exposure period) and pollutant concentration increased. Similar findings were reported by Okpokwasili and Odokuma (2012) and Nwoba (2014), who found that when *Nitrobacter* sp. was exposed to three spill dispersants and five household detergents, the percentage log survival decreased with increases in contact time and concentrations. The soluble surfactant that may be present in the pollutant and dissolve out the lipid component of the cell membrane, causing the cell contents to leak out, may be the cause of kerosene's harmful effects on Nitrosomonas and *Nitrobacter* spp. (Willey, 2014). It has also been reported by Okpokwasili and Odokuma (2012) that increasing concentrations of some drilling fluids reduced total viable count of marine nitrifying bacteria.

Effective Concentrations (EC_{50}) of Kerosene for *Nitrosomonas* sp.

The effective concentrations (EC_{50}) of kerosene for *Nitrosomonas* sp*.* based on Probit regression calculations were found to decrease with increase in contact time. The EC_{50} values of kerosene for *Nitrosomonas* sp. at 24 ,48 ,72 ,96 and 120 hours exposure time were 70.92 mg/L, 30.84 mg/L, 22.42 mg/L, 14.28 mg/L and 2.23 mg/L respectively (Fig. 3). The 24 h EC_{50} of kerosene for *Nitrosomonas* sp. was 70.92 mg/L while the 96 h EC_{50} of the pollutant for the organism was 2.23 mg/L. The EC_{50} values of kerosene for *Nitrosomonas* sp. at 24, 48, 72, 96 and 120 h exposure period showed that there was a decrease in EC_{50} of kerosene with increase in exposure time for *Nitrosomonas* sp. The EC_{50} of the pollutant showed that the pollutant exerted higher toxicity effects on *Nitrosomonas* sp. when the organisms were exposed to the pollutants for a

longer period of time. Thus, the number of survivors decreased as the exposure period of the organisms to the pollutants increased.

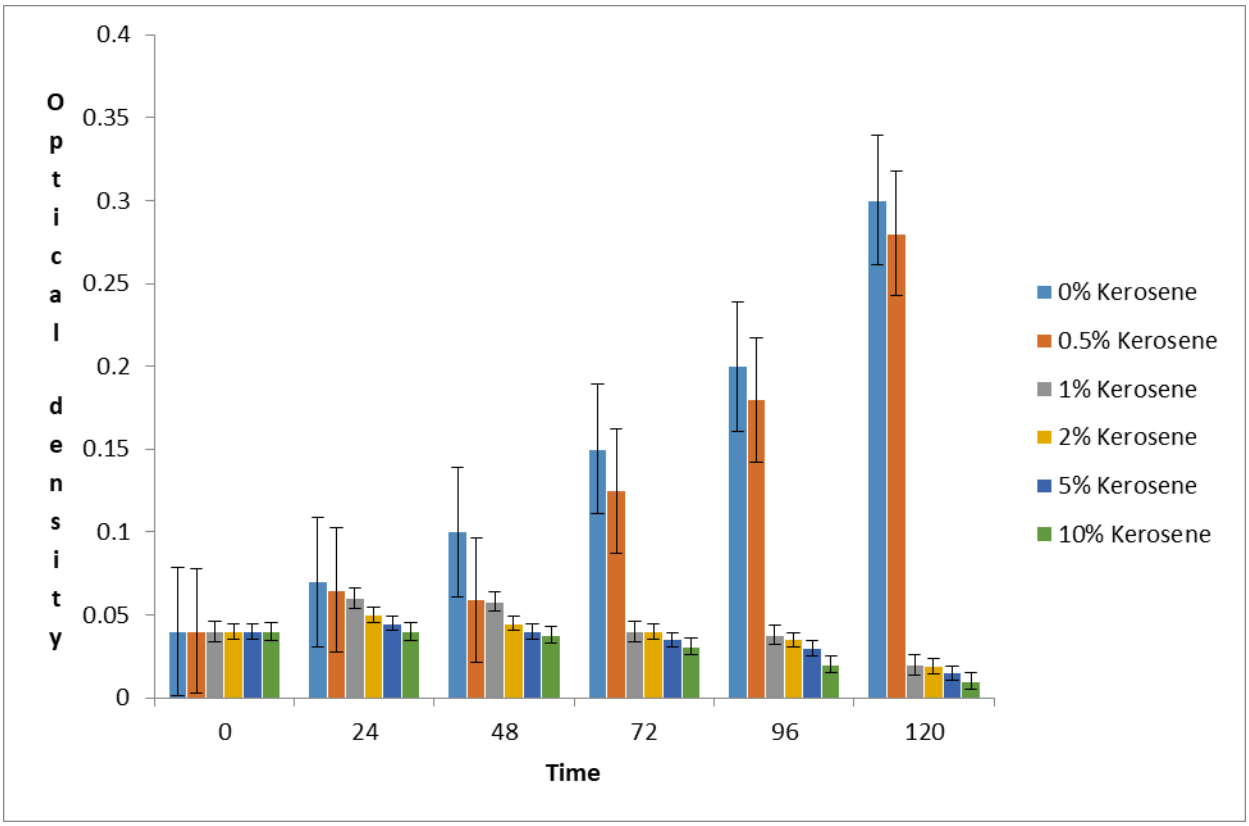

Figure 1. Effects of Concentrations of Kerosene on the Growth of *Nitrosomonas sp.*

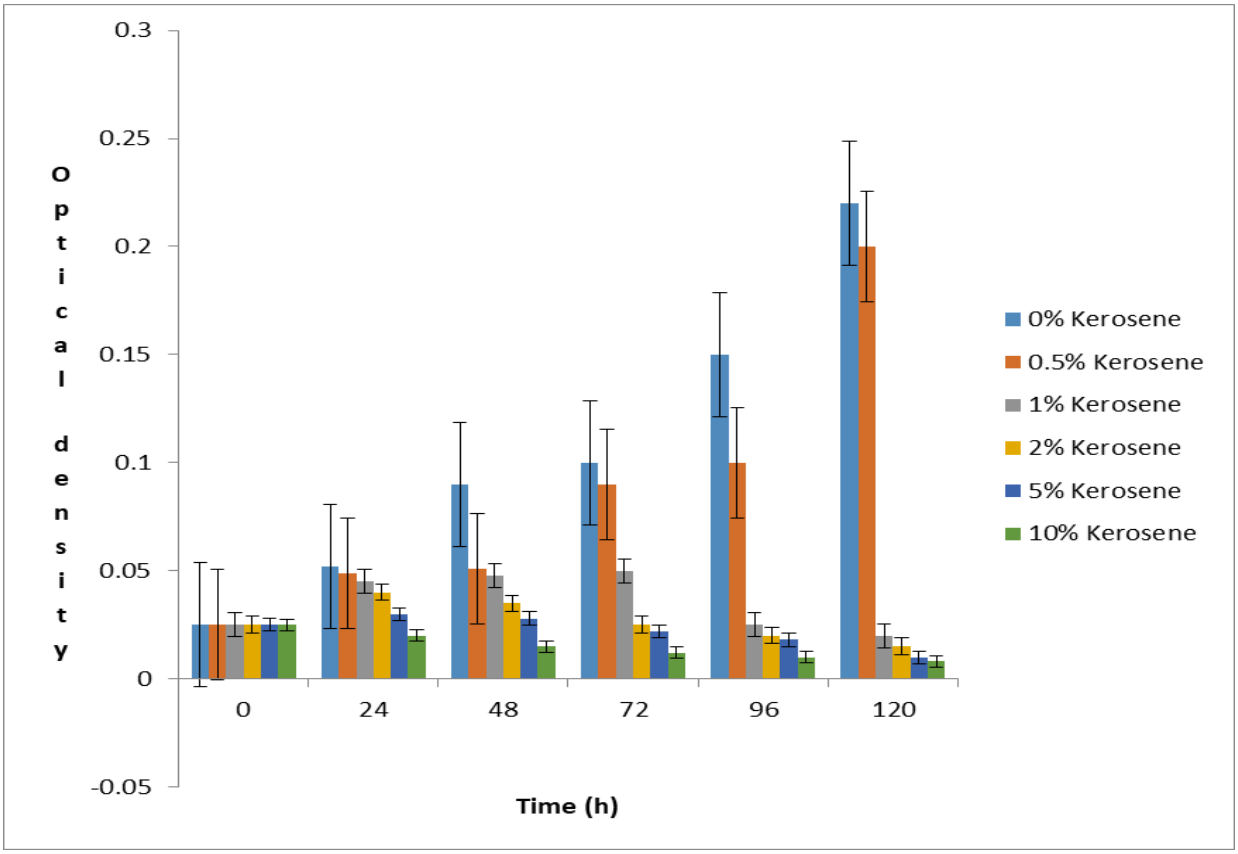

Fig. 2. Effects of Concentrations of Kerosene on the Growth of *Nitrobacter sp.*

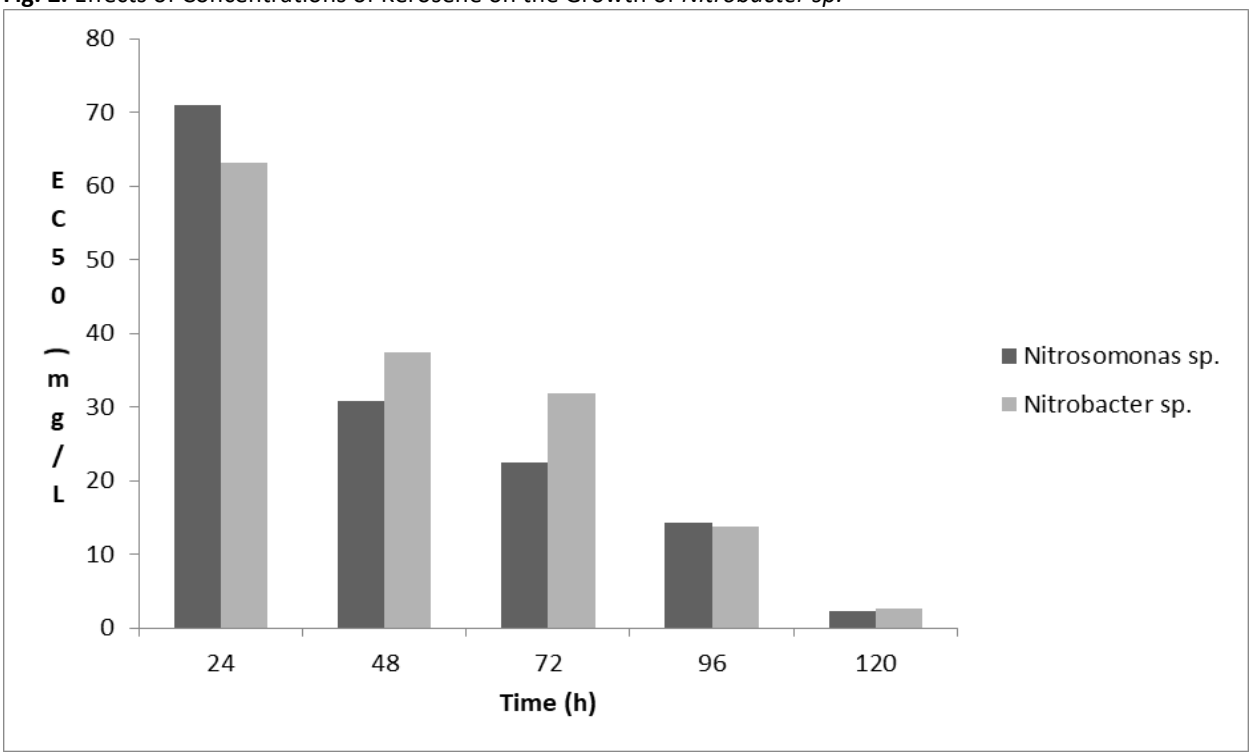

Figure 1. Effective concentration (EC50) of *Nitrosomonas sp.* and *Nitrobacter sp.*

Lethal Times (LT_{50}) at Different Concentrations of Kerosene for *Nitrosomonas* sp.

The LT_{50} (hours) at different concentrations of kerosene on the growth of *Nitrosomonas* sp. showed

that the LT_{50} decreased as concentrations increased. At 0.5% concentration of kerosene, the LT_{50} was 269.02 hours for *Nitrosomonas* sp. while at 10% kerosene concentration, the LT_{50} was 34.31 hours. The LT_{50} (hours) of kerosene on the growth of *Nitrosomonas* sp. at the concentrations of 0.5%, 1%, 2%, 5%, and 10% of kerosene, the LT_{50} of the kerosene for *Nitrosomonas* sp. were 269.02 hours, 173.21 hours, 112.72 hours, 55.43 hours and 34.31 hours respectively (Fig. 4).

The results of toxicity studies showed that the toxicity of the kerosene on *Nitrosomonas* sp. and *Nitrobacter* sp. depended on the contact time and pollutant concentrations.

When exposure time and kerosene concentrations increase, so do the EC_{50} and LT_{50}, which are employed as toxicity indexes. as depicted in Figs. 3 and 4, in that order. Additionally, some researchers have noted that the water solubility, chemical composition, concentrations, and genetic components of the bacteria investigated all had an impact on the toxicity of some of the fluids (Zheng*et al.,* 2003). Kerosene toxicity to *Nitrosomonas* and *Nitrobacter spp.* could be attributed to suppression of their respiratory processes. Because they are aerobic organisms, *Nitrosomonas sp.* and *Nitrobacter sp*. need oxygen to breathe. This happens in the organisms' cell membranes (Bona *et al.,* 2011). Solubilization of the pollutants in the test liquid medium could enable the pollutants come in contact with the respiratory enzymes present in the cell membrane of the organisms, thus, interfering with the process.

After a 72-hour period, data analysis revealed that the EC50 of the kerosene for Nitrosomonas and *Nitrobacter* sp. dropped. Additionally, it showed that *Nitrobacter* sp. was more inhibited by kerosene within the same exposure time limit than Nitrosomonas sp. Likewise, when the quantities of the pollutant under examination increased, so did the LT_{50} values for Nitrosomonas sp. and *Nitrobacter* sp. Time-dependent increases in water solubility could be the cause of the decline in EC_{50} and LT_{50} with concentrations and time. It's possible that the pollution affected the activities of the enzymes. The pollutant's suppression of enzyme activity, as shown by studies conducted by Castaldi *et al*. (2009).

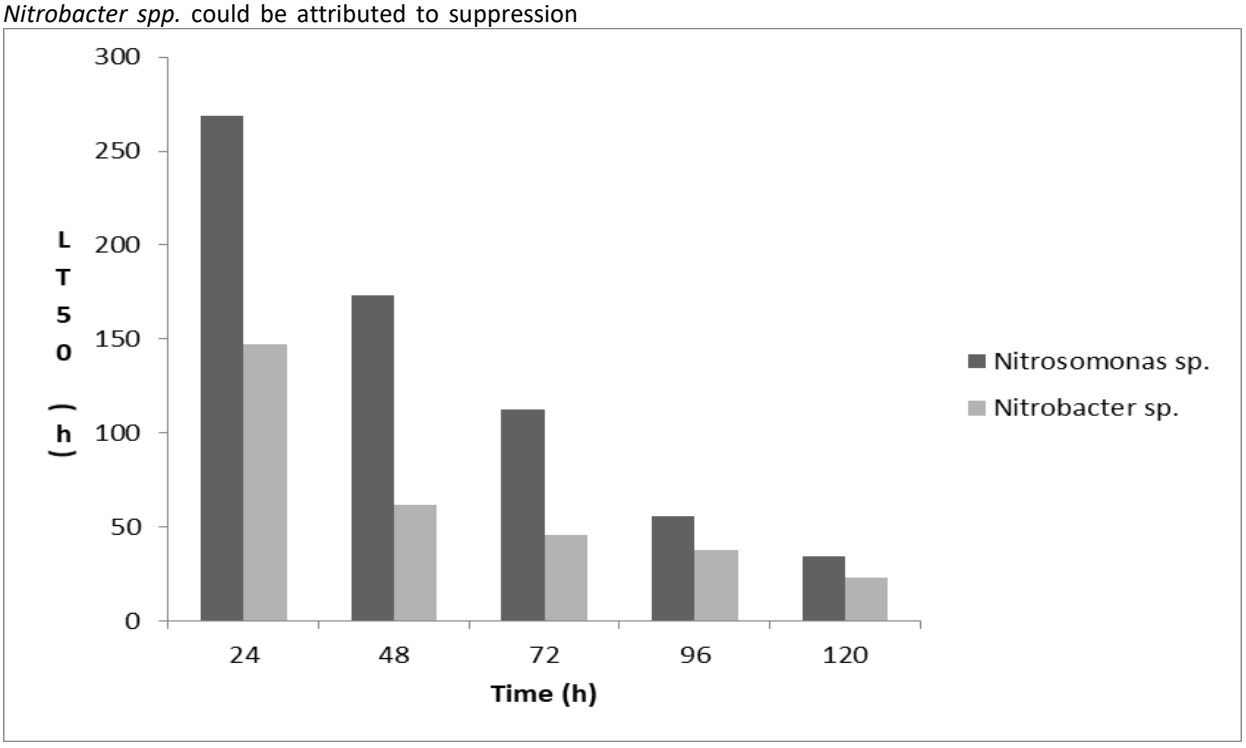

Figure 2. Lethal Time (LT_{50}) of *Nitrosomonas sp.* and *Nitrobacter sp.*

CONCLUSION

The primary productivity of the affected ecosystems may be reduced by high and persistent concentrations of kerosene resulting from improper disposal and fluid spills. This is because these concentrations may disrupt the nitrogen cycle and food chain, which are important food sources for aerobic bacteria like nitrifying bacteria.

The findings from this study shows that kerosene has a toxic effect on nitrifying bacteria isolated from the farmland soil. The potential environmental impacts of kerosene spills in our ecosystems can be seen with the help of information gathered from this study. The results of this study to be used to support a government regulatory and policies efforts by demonstrating why soil that has been exposed to specific amounts of kerosene spills shouldn't be utilized for agricultural activities.

Finding nitrifying bacteria that can break down kerosene and other refining products through their biological capacity and metabolism is thought to be one of the most promising ways to improve soil fertility in an environment that has been impacted by these pollutants.

Therefore, it is crucial to investigate how these organisms react to kerosene and other refined products like diesel and crude oil, specifically how they affect nitrification and growth. This will help develop less toxic and more easily biodegradable fluids, particularly if the ones currently in use are persistent and toxic, failing to meet environmental regulations regarding pollution effects.

CONFLICTS OF INTEREST

Authors declared that there is no conflict of interest

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