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Original Research Article

Ecotoxicity of local and industrial refined kerosene on key environmental pollution monitor, *Nitrobacter* sp. in tri-aquatic systems in Nigeria

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Nitrification process involves *Nitrobacter* species and their growth and activities in the microenvironment when impacted negatively would consequently adversely affect soil fertility. In view of the significance of this process, the toxicity of local refined kerosene (LRK) and industrial refined kerosene (IRK) on a key environmental pollution monitor, *Nitrobacter* was investigated. LRK and IRK were apportioned into six sets for each of the experiments using tri-aquatic systems or microcosms of freshwater (FW), marine water (MW) and brackish water (BW) at percentage concentrations of; 0, 3.25, 6.5, 12.5, 25 and 50 into which the test organism (*Nitrobacter* sp.) was inoculated at intervals of; 0, 4, 8, 12 and 24hours. Toxicity results indicated that the sensitivity of the test organism was a function of both the contact time and concentrations, and also reflected lethal effects of the pollutants/toxicants (kerosene). The outcome of percentage median lethal concentration (%LC_{50}) on *Nitrobacter* sp. in the triaquatic microcosms with pollutants were as follows; in IRK + FW 34.41 < in IRK + MW 37.89 < in LRK + FW 39.43 < in IRK + BW 40.99 < in LRK + MW 41.56 < in LRK + BW 45.35. This study revealed that IRK + FW microcosm was the most toxic (LC_{50}) whereas LRK + BW microcosms was the least toxic. The inability of the organism to thrive well at kerosene concentration above 1% (v/v) is a warning signal of serious environmental pollution problem which could affect aquatic life forms and eventually humans. However, due to high fatality rate inherent from the use of LRK (though not reported here) and its toxicity to microbial life, it is hereby advocated that the public should rather resort to use IRK products.

Key words: Toxicity, Percentage Median Lethal Concentration (%LC_{50}), local and lndustrial refined kerosene, *Nitrobacter*

INTRODUCTION

Kerosene is one of the most predominantly used energy sources for households and bush burning by subsistence farmers in Nigeria. It is one of the refined petroleum products derived from crude oil by fractional distillation (Kobeticova et al., 2012). The number of carbon in kerosene (paraffin) ranges from 10-14 with boiling point of 1650-2000 (Nihad and Mutlak, 2011). Unlike the conventional IRK, LRK production is governed by illegality, lack of expertise and improper processing (i.e., the simple distillation process) resulting in coloured poor quality product which poses serious threat to consumers and equipment. The simple distillation process involves the use of crude oil, wood fire (heat energy source), galvanized pipes (of about one inch are connected to the metal drum as

conductors) immersed in a water bath as condenser. The first distillation product is collected as petrol, then kerosene and lastly diesel, the rest is disposed off as waste. Thus, these mixtures of petrol or diesel in kerosene or diesel in petrol are the resulting by-products. Because it is not fractionalized these coloured by-products are relatively hazardous.

IRK is a clear liquid fuel with a mixture of hydrocarbon containing 6 - 16 carbon atoms in length. It is a middle distillate of petroleum refining process, defined as the fraction of crude oil that boils between 145 and 3000 . It is a complex mixture of branched and straight chained compounds; paraffin (55.2%), naphthalene (40.9%), and aromatics (3.99%) (USEPA, 2011). Refined petroleum products from crude oil fractionalization include kerosene. The main sources of IRK discharge to the environment are leakage from surface or submerged of storage tanks and spillages due to accidental discharge during transportation (Raina et al., 2009). Spillages arising from kerosene especially LRK into our environment is 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-mineralizing 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 (Willey et al., 2011; Holt et al., 1994). The conversion of ammonia to nitrate is achieved by two groups of nitrifying bacteria; the ammonia-nitrifying bacteria and nitrate-oxidizing 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, Nitrospira* (Scragy, 2005).

Nitrobacter and nitrifying bacteria play a very important role in soil mineralization 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 (Bona et al., 2011). Driven by the roles of *Nitrobacter* and nitrifying bacteria in soil and waste water treatment plants, assessment of *Nitrobacter* to pollution stress and tolerance in LRK and IRK products in various aquatic ecosystems becomes imperative. This study was, therefore, designed to assess the tolerance and toxicity levels of *Nitrobacter* in triaquatic microcosms incorporated with LRK and IRK products in Nigeria.

MATERIALS AND METHODS

Source and collection of samples

Three (3) water samples from various sources were collected with one (1L) litre of sterile plastic container and used as the tri-aquatic microcosms. They are Marine water (MW) from Bonny River in Bonny, freshwater (FW) from Muu Bagia Biara, Gokana LGA and Brackish water (BW) from Eagle Island, Nkpolu-Oroworukwo, Port Harcourt City LGA all in Rivers State.

Pollutant/toxicant samples

Local refined kerosene (LRK) was purchased at Nembe waterfront, Creek Road whereas Industrial refined kerosene (IRK) was purchased at NNPC Filling Station, all in Port Harcourt, Rivers State, Nigeria.

Test Bacterium

The test bacterium, *Nitrobacter* species isolated from the river water sample was obtained from a stock culture at the Department of Microbiology, Rivers State University from previous work using Winogradsky agar medium (Odokuma and Nrior, 2015). The compositions of this medium were: KNO_2 (0.1g), Na_2CO_3 (1.0g), NaCl (0.5g), $FeSO_4.7H_2O$ (0.4g), Agar agar (15.0g), Distilled water (1000ml). The Winogradsky agar medium was autoclaved at 121℃ for 15minutes and aseptically transferred to sterile Petridshes after cooling to about 40°C. The Petridishes were then inoculated with the river water and incubated aerobically for 4days at room temperature (30± 2°C). Cultural and morphological characteristics revealed; grayish, mucoid, flat colonies and Gram's reaction of the colonies revealed pear shaped, and other biochemical tests for identification of *Nitrobacter* were carried out as earlier reported (Colwell and Zambuski, 1972; Okpokwasili and Odokuma, 1996). The colonies were aseptically streaked on fresh Winogradsky agar and incubated for 2 days at 30±2°C. Furthermore, the grayish, mucoid and flat colonies were aseptically transferred from the Petridishes into 200ml Erlenmeyer flasks containing the growth medium and incubated for 24hours at room temperature.

A solution of 0.2mg sodium nitrite ($NaNO_2$) per litre was autoclaved at 121°C for 15minutes and reconstituted 30±2°C with 100ml distilled water which in turn was transferred aseptically into 250ml Erlenmeyer flasks, this served as diluents for the effective determinations of subculture on Winogradsky agar.

Toxicity Test Procedure for *Nitrobacter* species

The acute toxicity bioassays were determined for a duration of 24hours as described in the guidelines (APHA, 1992; DPR, 2002 (formally NNPC Inspectorate Division). The test was carried out in separate test tubes containing appropriate volume of filtered waters; FW, BW and MW from the organism's habitat. For each of the experimental set up, a toxicant in percentage (%) concentrations of 50, 25, 12.5, 6.5 and 3.25% were added into tubes later inoculated with test organism and loosely plugged with cotton wool and repeated for the other toxicant (Table 1). Aliquots (0.1ml) of each concentrations of the effluent was

Table 1: Toxicity test set-up using industrial and local refined kerosene on Nitrobacter sp. in Freshwater (FW), Brackish water (BW) and Marine Water (MW)

Industrial Refined Kerosene (IRK)					Local Refined Kerosene (LRK)				
Microcosm Setup Label	Concentration	Volume of Toxicant	Volume of Diluent	Volume of Test Organism	Microcosm Setup Label	Concentration	Volume of Toxicant	Volume of Diluent	Volume of Test Organism
1	Control (0%)	0.0ml IRK	10ml FW	1ml	*19*	Control (0%)	0.0ml LRK	10ml FW	1ml
2	3.25%	0.3ml IRK	9.7ml FW	1ml	*20*	3.25%	0.3ml LRK	9.7ml FW	1ml
3	6.5%	0.7ml IRK	9.3ml FW	1ml	*21*	6.5%	0.7ml LRK	9.3ml FW	1ml
4	12.5%	1.3ml IRK	8.7ml FW	1ml	*22*	12.5%	1.3ml LRK	8.7ml FW	1ml
5	25%	2.5ml IRK	7.5ml FW	1ml	*23*	25%	2.5ml LRK	7.5ml FW	1ml
6	50%	5.0ml IRK	5.0ml FW	1ml	*24*	50%	5.0ml LRK	5.0ml FW	1ml
7	Control (0%)	0.0ml IRK	10ml BW	1ml	*25*	Control (0%)	0.0ml LRK	10ml BW	1ml
8	3.25%	0.3ml IRK	9.7ml BW	1ml	*26*	3.25%	0.3ml LRK	9.7ml BW	1ml
9	6.5%	0.7ml IRK	9.3ml BW	1ml	*27*	6.5%	0.7ml LRK	9.3ml BW	1ml
10	12.5%	1.3ml IRK	8.7ml BW	1ml	*28*	12.5%	1.3ml LRK	8.7ml BW	1ml
11	25%	2.5ml IRK	7.5ml BW	1ml	*29*	25%	2.5ml LRK	7.5ml BW	1ml
12	50%	5.0ml IRK	5.0ml BW	1ml	*30*	50%	5.0ml LRK	5.0ml BW	1ml
13	Control (0%)	0.0ml IRK	10ml MW	1ml	*31*	Control (0%)	0.0ml LRK	10ml MW	1ml
14	3.25%	0.3ml IRK	9.7ml MW	1ml	*32*	3.25%	0.3ml LRK	9.7ml MW	1ml
15	6.5%	0.7ml IRK	9.3ml MW	1ml	*33*	6.5%	0.7ml LRK	9.3ml MW	1ml
16	12.5%	1.3ml IRK	8.7ml MW	1ml	*34*	12.5%	1.3ml LRK	8.7ml MW	1ml
17	25%	2.5ml IRK	7.5ml MW	1ml	*35*	25%	2.5ml LRK	7.5ml MW	1ml
18	50%	5.0ml IRK	5.0ml MW	1ml	*36*	50%	5.0ml LRK	5.0ml MW	1ml

plated out after 4, 8, 12 and 24hours onto Winogradsky agar and incubated for 4days. Plates were then counted as colony forming unit per millilitre (CFU/ml).

The Percentage Log Survival of *Nitrobacter* in Kerosene

The percentage log survival of the bacterial isolates in the kerosene effluent used in the study was calculated using the formula adopted by (Williamson and Johnson 1981; Nrior and Obire, 2015). The percentage log survival of the bacterial isolates in the effluent was calculated by obtaining the log of the count in each toxicant concentrations (*Log C*), divided by the log of the count in the zero toxicant concentration (*Log c)* and multiplying by 100. Thus:

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

The Percentage Log Mortality of *Nitrobacter* in Kerosene

The formula for the calculation of percentage (%) mortality was adopted from (APHA, 1992). The percentage (%) log mortality was done by using the percentage (%) log survival in zero toxicant concentration to subtract the percentage (%) log survival. Thus: percentage (%) log mortality = % log survival in zero toxicant concentration (100) - percentage (%) log survival in test concentrations.

Statistical Analysis

The results from toxicity screening were subjected to statistical analysis using Analysis of Variance (ANOVA) and student t-test at 0.05 confidence limit (Reish and Oshida, 1987) to determine the significant difference between mortality of the test bacterium and toxicants, kerosene. The median lethal concentrations of toxicants with respect to bacterium with respect were calculated using regression analysis.

RESULTS AND DISCUSSION

The Log Mortality of *Nitrobacter* with LRK and IRK at different (%) concentrations; 0, 3.25, 6.5, 12.5, 25 and 50 in triaquatic microcosms ((Freshwater, brackish and marine water) exposed for periods of; 0, 4, 8, 12 and 24h are shown in Figures 1-3. There is a constant decrease in the log survival of the test species, *Nitrobacter* sp. which reflected in the increase in mortality rate from 0-24hours. FW, BW and MW samples were used in the study as a specimen to assess the probable toxic level of LRK and IRK on *Nitrobacter* which is a key environmental pollution monitor in aquatic ecosystem.

The Lethal toxicity of IRK and LRK results also indicated that the increased kerosene concentrations resulted in high mortality rate of *Nitrobacter* which is suggestive of adverse

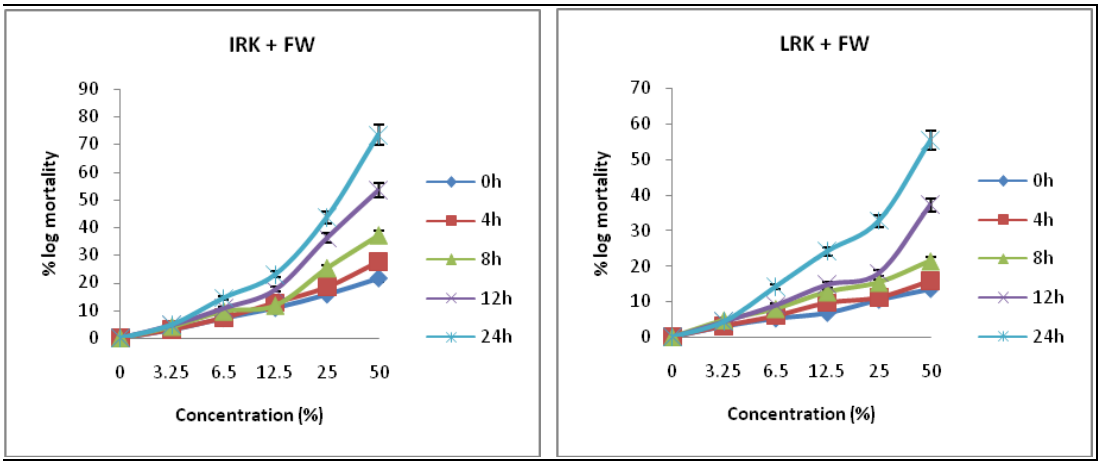

Figure 1: Percentage (%) log mortality of *Nitrobacter* with IRK and LRK in freshwater (FW)

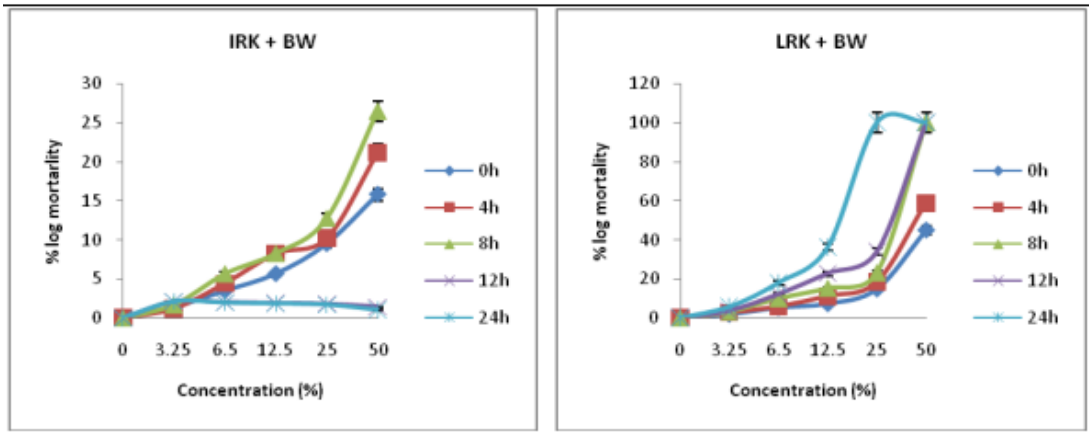

Figure 2: Percentage (%) log mortality of *Nitrobacter* with IRK and LRK in Brackish water (BW)

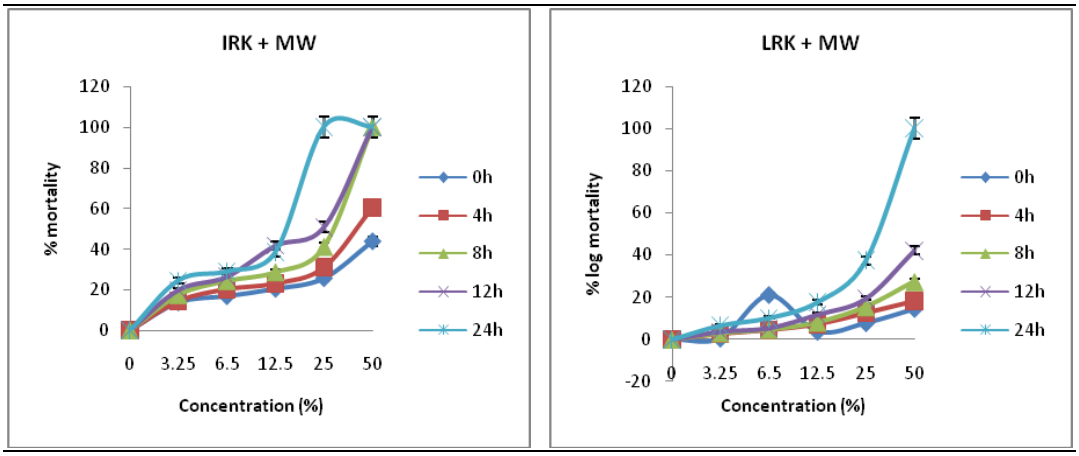

Figure 3: Percentage (%) log mortality of *Nitrobacter* with IRK and LRK in Marine water (MW)

negative effect on growth and survival of the species as earlier reported (Kobeticova et al., 2012). On the other hand, earlier Laboratory investigations have shown that nitrifying bacteria could utilize kerosene and other petroleum products as carbon source which may vary both in rates of utilization and growth profile (Eze et al., 2013a,b). Furthermore, the influence of salinity on the sensitivity of *Nitrobacter* to various microcosms was

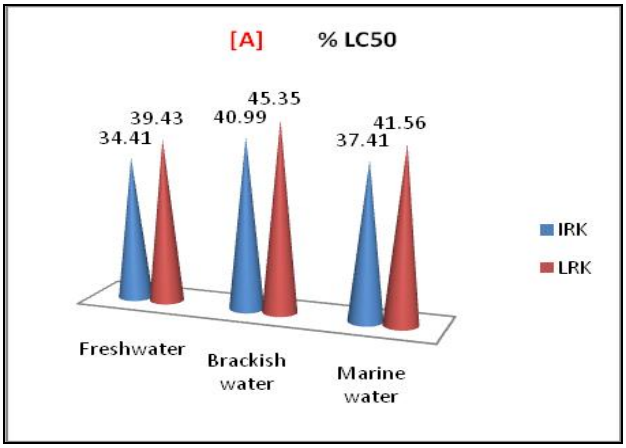

Figure 4A: Percentage Median Lethal Concentration (LC_{50}) of IRK and LRK on *Nitrobacter* in tri-aquatic ecosystem.

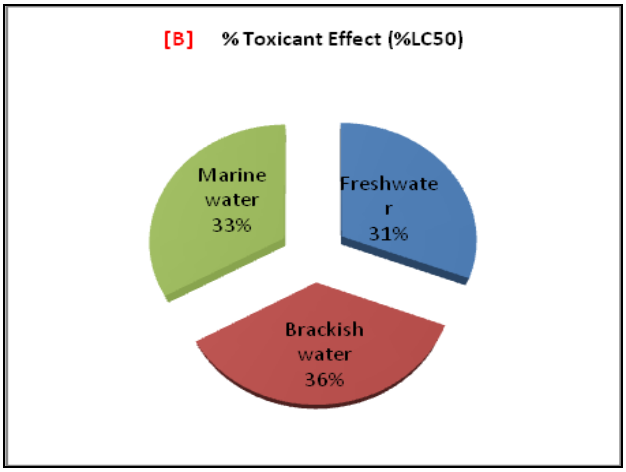

Figure 4B: Percentage Toxicant effect of IRK and LRK on *Nitrobacter* in tri-aquatic ecosystem

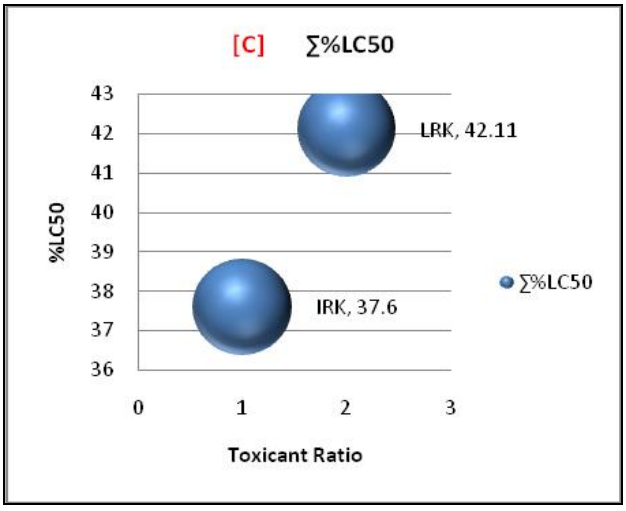

Figure 4C: The Summation of Percentage Median Lethal Concentration ($\sum$%LC_{50}) of IRK and LRK in the three aquatic ecosystem combined

which corroborates earlier work reported (Wemedo and Nrior, 2017).

The percentage median Lethal Concentration (%LC_{50}) of microcosms, toxicant effect and toxicant ratio of *Nitrobacter* exposed for periods of 0, 4, 8, 12 and 24h at different (%) concentrations of; 0, 3.25, 6.5, 12.5, 25 and 50 in LRK and IRK are represented in Figures 4A-C. The results indicated that IRK was more toxic than LRK (Figure 4A). Comparative values of the various microcosms were in the order (noting; the lower the %LC50 the more toxic the test toxicant): *Nitrobacter* with IRK in FW (34.41%) < *Nitrobacter* with IRK with MW (37.89%) <. *Nitrobacter* with LRK in FW (39.43%) < *Nitrobacter* with IRK in BW (40.99%MW (41.56%), *Nitrobacter* with LRK in BW (45.35%) respectively (Figure 4A).

This study also demonstrated that the level of purification and concentrations of un volatilized substances in the Kerosene may be toxic and affect the test bacteria which confirms similar observation on substances in electronics (Hermann and Urbach, 2000). Some advantages observed in the use of bacterial bioassay organism include; low cost, small space, simplicity and rapidity. The use of *Nitrobacter* sp. mortality rate to express as Median Lethal Concentration (LC_{50}) in this study as indices to monitor toxicity has earlier been reported (Odokuma and Nrior, 2015).

Toxicity seems to be affected by the salinity of the medium, as the kerosene shows to be more toxic in freshwater (LC_{50} 31%) > Marine water (33%) and least in Brackish water (36%); noting that the lower the LC_{50} the more toxic the toxicant (Figure 4B). This may be attributed to chemical reactions with the compounds in Kerosene and the salt likely to be present in the waters, particularly in BW. The analytical summation of Percentage LC_{50} in the three aquatic microcosms combined revealed that IRK ($\sum LC_{50}$ 37.6±3.29%) was more toxic to the test bacteria, *Nitrobacter* sp. than in LRK ($\sum LC_{50}$ 42.11±3.0%) (Figure 4C). Comparative evaluation of the toxicity strength gap between the two toxicants shows a significant gap between the toxicants. This also, may be due to combined effect of Kerosene and the salinity in these waters. The marked decrease in the number of *Nitrobacter* sp. as the Kerosene concentration increases, suggest that components present in the kerosene is highly toxic to *Nitrobacter* and may interfere with the nitrogen cycle in the environment (Nrior and Gboto, 2017). Futhermore, the inability of the organism to thrive well at kerosene concentration above 1% (v/v) may be due to enzyme inactivation at increased concentrations. Such adverse effects may also be attributed to sudden exposure of the organism to hostile xenotic microenvironment which obstructed gradual recovery within study period.

Conclusion

This study revealed that different concentrations of kerosene had profound negative effects on growth and

survivability (i.e., high mortality rate) of *Nitrobacter* species. Toxicity results indicated that the sensitivity of *Nitrobacter* species was a function of both the contact time and concentrations, and also reflected lethal effects of the pollutants/toxicants (kerosene). This study also, demonstrated that IRK + FW microcosm was the most toxic (LC_{50}) and least in BW microcosms.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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