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Toxicity Assessment of Produced Water Using *Nitrosomonas sp.*

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Authors' contributions

This work was carried out in collaboration between both authors. Author LETA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author AJE managed the analyses of the study and the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Aim: Most studies on the effect of produced water are centred on the effects on the physicochemical parameters of the recipient environment, but not much has been conducted on biogeochemical processes such as the nitrogen cycle. This study aims to determine the median lethal concentration (LC50) of the produced water that would cause mortality of 50% of the exposed population of *Nitrosomonas sp*. (test organism) which is involved in the ammonification step of the Nitrogen cycle.

Study Design: For the purpose of this study, acute toxicity testing was done for 48 hours to determine the effect of the untreated and treated produced wastewater on the test organism. The LC50 is the median lethal concentration that would cause mortality of 50% of the exposed population of the test organisms.

Methodology: Toxicity effects of treated and untreated produced wastewater were determined following standard procedures against the test organism (*Nitrosomonas sp*). The resultant output of the probit analysis was generated from the SPSS statistical programme.

Results: Results showed that the mean LC50 for the untreated produced wastewater at the 48-hour test period of bioassay was 3.27mg/L, while the mean LC50 for the treated wastewater was

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found to be 6.69mg/L. The mean percentage mortality of *Nitrosomonas sp*. for the untreated produced waster water ranged from 37%(0.01mg/l) to 90%(100mg/l), while for the treated, percentage mortality ranged from 27%(0.01mg/l) to 84%(100mg/l) at the end of the 48 hour test duration.

Conclusion: Toxicity of treated and untreated wastewater against *Nitrosomonas sp* increased with time and decreased considerably with treatment, corresponding to positive correlation as observed statistically between toxicants with respect to concentration and time of exposure for untreated and treated wastewater against *Nitrosomonas sp*. These results indicate a likey inteference of the nitrogen cycle by contamination of untreated produced water in the environment and by extension impairment of the productivity of the environment.

Keywords: Produced water; Nitrosomonas species; toxicity.

1. INTRODUCTION

The effects of produced water discharged in the environment are usually in accordance with its concentration and relative toxicity of the chemicals and dispersion and biodegradation/ transformation rates of the receiving water, which may be a potential marker for acute toxicity beyond the immediate surroundings of the produced water discharge into offshore waters [1]. Following this, Lee et al*.,* [2] supported the hypothesis by sensitive biotests- primarily regulatory acute toxicity assays, and the rapid dispersion and degradation of the produced water plume in receiving waters. It was also proposed by Holdway [3] as cited in Neff et al. [4] that the chronic impact associated with long-term exposures must be quantified to fully explore the potential long-term ecological impacts of the produced water discharges. Furthermore, frequent exposure of the environment may cause sub-lethal changes in populations and communities, including decreased growth and fecundity, respiratory problems, behavioral and physiological disorder, decreased developmental success and endocrine disruption [5].

Nitrifying bacteria are a small group of aerobic bacteria (family Nitrobacteraceae) that use inorganic chemicals as an energy source. They are microorganisms that are important in the nitrogen cycle as converters of soil ammonia to nitrates, compounds usable by plants. The nitrification process requires the mediation of two distinct groups: bacteria that convert ammonia to nitrites (*Nitrosomonas, Nitrosospira, Nitrosococcus,* and *Nitrosolobus*) and bacteria that convert nitrites (toxic to plants) to nitrates (*Nitrobacter, Nitrospira* and *Nitrococcus*). In agriculture, irrigation with dilute solutions of ammonia results in an increase in soil nitrates through the action of nitrifying bacteria. *Nitrosomonas* is a genus of ammonia-oxidizing proteobacteria. They are important players in wastewater treatment plants, where they get rid of excess ammonia by converting it to nitrite. One species, *Nitrosomonas europaea*, is especially interesting because of its unique metabolism.

Nitrosomonas are rod-shaped chemolithoautothrophs with an aerobic metabolism. While they do not grow by photosynthesis, their unusual metabolic behavior involves burning ammonia with oxygen. Long, thin membranes inside the bacteria's cell use electrons from ammonia's nitrogen atom to produce energy. In order to complete cell division, *Nitrosomonas* must consume vast amounts of ammonia, making the division process last for several days. The cells grow either in pairs or short chains. In nitrification, *Nitrosomonas* plays the role of oxidizing ammonia to nitrite, which is then converted to nitrate by other bacteria.

However, in Nigeria, the petroleum industry depends majorly on the physicochemical analysis of produced water to monitor and regulate produced water discharge. This strategy has proved inappropriate and inadequate to protect aquatic organisms [6] because it only gives information on the constituents and concentrations of the individual components in the produced water rather than their potential ecological risks/effect (biological interpretations) on microorganisms exposed.

Hence, this study was carried out to determine the median lethal concentration ($LC_{50)}$ of the untreated and treated produced water that would cause mortality of 50% of the exposed population of the test organisms (*Nitrosomonas sp),* which plays a role in the first step of the biogeochemical process of nitrogen cycle in both the aquatic and terrestial environments. This method could be applied in current biological monitoring protocol as a reliable, rapid and ecologically relevant bioassay tool for toxicity assessment in environmental compliance monitoring of produced water discharges.

2. MATERIALS AND METHODS

2.1 Sources of Test Samples Produced Water Effluent (Wastewater)

Samples of produced water (untreated and treated) were collected from an offshore operational facility situated in Akwa Ibom State, Nigeria with GPS coordinates of 03'51.141N; 006'58.794'E.

A 10 -liter sampling can was used for sample collection prior to the initiation of testing with test organisms. Sample for BOD was collected in amber bottles. Sterile plastic bottles were used to sample for microbial analysis of the test sample. All samples were stored at 4^0C prior to testing. One (1) litre glass bottle was used to collect the sample for total petroleum hydrocarbon and was preserved with 1:1 sulphuric acid (H_2SO_4).

Nitrifying bacteria (*Nitrosomonas* sp) was cultured from soil collected from a cucumber farm, with GPS coordinates of 05'35.002N 005'50.984'E, Army Estate, Effurun, Delta State, Nigeria.

2.2 *In situ* Parameters Analysis of Untreated and treated Produced wastewater

These *in situ* parameters were assessed on site using the following procedures; pH (APHA 4500-H+ B using Hanna pH electronic meter), temperature (APHA 2550 - B laboratory and field methods), electrical conductivity and total dissolved solids (APHA 2510-B using Hanna desktop conductivity meter) and dissolved oxygen (DO) (APHA 4500-O C by azide modification method) [7].

2.3 Laboratory Analysis of Untreated and Treated Produced Wastewater

The physicochemical parameters were assessed using the following procedures; Salinity (Mohr Argentometric Method, 4500 B-Cl-), biochemical oxygen demand (BOD) (APHA 5210B, by 5-Day test method), Total suspended solids (APHA 2540D), nitrates (APHA 4500-NO3- B) and phosphate (APHA 4500-PE). Oil and grease and THC of the samples was analysed using ASTM D3921 method [8].

2.4 Isolation of Nitrifying Bacteria (*Nitrosomonas* sp)

The method used for isolation of *Nitrosomonas* was adopted from Odokuma and Akponah [8]. *Nitrosomonas* was isolated using Winogradsky medium phase I (($(NH_4)_2SO_4$, 2.0g; K_2HPO_4,1.0g; $MgSO_4.7H_2O$, O.5g; NaCl, 2.0g; $FeSO_4.7H_2O$, 0.4 g; CaCO 0.01, agar 15.0 g; distilled water 1000 mL) from a soil sample.

One gram of the soil sample was measured and aseptically transferred into 9mL of sterilized distilled water contained in a test tube. This was properly shaken and serial dilution was done up to the third dilution factor (10^3). Then 0.1mL each of these suspensions were withdrawn and aseptically inoculated into Winogradsky phase I media using the pour plate method for the isolation of *Nitrosomonas*. The Petri dishes were then incubated aerobically for 4 days at room temperature (28 ± 2^oC). The colonies that developed were sub-cultured on freshly prepared Winogradsky agar media phase I for further identification and confirmation. Pure isolates were sub-cultured to agar slants and stored for further use. The broth media used for isolation of the test organisms also served as diluents for producing the various toxicant concentrations.

Pure isolates from corresponding agar slants were characterized and identified using morphological (colonial morphology, motility, and gram reaction), biochemical and physiological attributes. The biochemical characteristics include: Gram staining, motility test, catalase test, oxidase test, coagulase test, urease test, indole test, citrate utilization test, sugar fermentation test, nitrate reduction [9-11].

2.5 Preparation of Standard Inoculum of the Isolates

Discrete colonies from each of the different culture media were sub-cultured unto fresh media. These were transferred unto slants and stored at 4^oC. The slant cultures served as stock cultures. The standard inocula were prepared from the stock cultures. Each of the isolates were picked from the respective stock cultures using a sterile inoculating needle and inoculated aseptically into 100mL of appropriate broth (Winogradsky phase I broth for *Nitrosomonas*) contained in a 250mL conical flask. These were incubated at 37^oC for 48-hr at room temperature after which 1ml each was separately withdrawn from the respective flask and ten-fold serial dilution was done up to 10^{-3}. Further, 0.1ml of the various dilutions was inoculated into appropriate agar plates using the pour plate technique. Incubation under appropriate conditions followed immediately (28 ± 2^oC for 4 days). The dilution factor that resulted in 30-300 colonies at the end of incubation was selected as standard inoculum for the toxicity test.

2.6 Acute Toxicity Test Methodology (*Nitrosomonas* sp. Toxicity Test)

The set up includes the toxicant (untreated and treated produced water), Winogradsky phase I broth used as diluents for *Nitrosomonas* and broth culture (standard inoculum) of the bacteria. The concentrations (100, 10, 1.0, 0.1 and 0.01 mg/L) were obtained from stock solution of the produced water. The standard inoculum (0.1mL) *Nitrosomonas sp* was added to each of the set toxicant concentrations contained in 250 mL Erlenmeyer flask. Control contained Winodgrasky phase I broth and the organism without toxicant. The mixtures were incubated at room temperature of 28^0C at the various exposure time (0, 6, 12, 18, 24, 30, 36, 40 and 48 hours) respectively.

2.7 Statistical Analysis

ANOVA was used to conduct the test of significant difference between the treated and untreated produced water physicochemical parameters.

3. RESULTS AND DISCUSSION

Results from Table 1, showed that, besides turbidity, six (6) key physicochemical parameters have their values decreased considerably after treatment.These include, temperature, BOD, phosphate, nitrate, oil & grease and THC. The microbial load was also reduced in the treated produced wastewater. This could be due to efficiency of the treatment method [7]. pH, electrical conductivity, TDS and salinity increased slightly after treatment (Table 1). This could be due to leaching of some of the treatment chemicals used in the treatment of the produced water.

3.1 Isolation, Identification and Characterization of Microorganisms for Acute Toxicity (Bioassay)

The result for identification and characterization of the *microorganism* used for the toxicity testing is presented in Table 2.

Nitrosomonas species was identified with the conventional or non-molecular characterization method, using morphological and biochemical tests. *Nitrosomonas sp* showed positive results to Catalase and Voges Proskauer tests, its morphological properties indicated that it is motile and rod shaped (Table 2). Identification was confirmed using the Bergey’s manual of determinative bacteriology [12].

3.2 Toxicity Effects of Treated and Untreated Produced Water

Toxicity effects of treated and untreated produced waste water on nitrifying bacteria (*Nitrosomonas* sp) as test organisms are presented in the section. The probit analysis which showed the relationship between the dose of the sample and the quantal response, as well as the percentage mortality of organisms at each dose was recorded and analyzed using the probit model [13-15]. The mean percentage mortality of *Nitrosomonas sp.* after 48-hours exposure to the untreated produced wastewater samples ranged from 37% (0.01 mg/L) to 90% (100 mg/L) (Fig. 1), while mean percentage mortality of the test organisms ranged from 26.65% (0.01 mg/L) to 84.45% (100 mg/L) (Fig. 2) for the treated produced wastewater. The percentage mortality of the test organism increased with an increase in the toxicant concentration as well as the time of exposure. This could be attributed to the toxic nature of the physicochemical constituents of the produced wastewater [4].

Results from the Probit analysis in Fig. 3 shows that at 24 hours, the untreated produced waster water (toxicant) exhibited a LC50 of 6.74mg/L, while at 48hours a LC50 of 3.27mg/L was recorded. The mean LC_{50} for treated wastewater was found to be 7.10mg/L at 24 hours and 6.69mg/L at 48 hours (Fig. 4). Effects of toxicity for treated and untreated wastewater against *Nitrosomonas sp* increased with time and concentration, while it decreased considerably with reduced concentration of treatment. The LC50 of the treated toxicant was however lower than that of the untreated, indicating a reduction of the toxicant effect as a result of the treatment. The observed higher LC50 of the untreated produced wastewater could be attributed to the higher concentration of some key parameters such as, BOD, TPH, oil & grease (Table 1) [16-18].

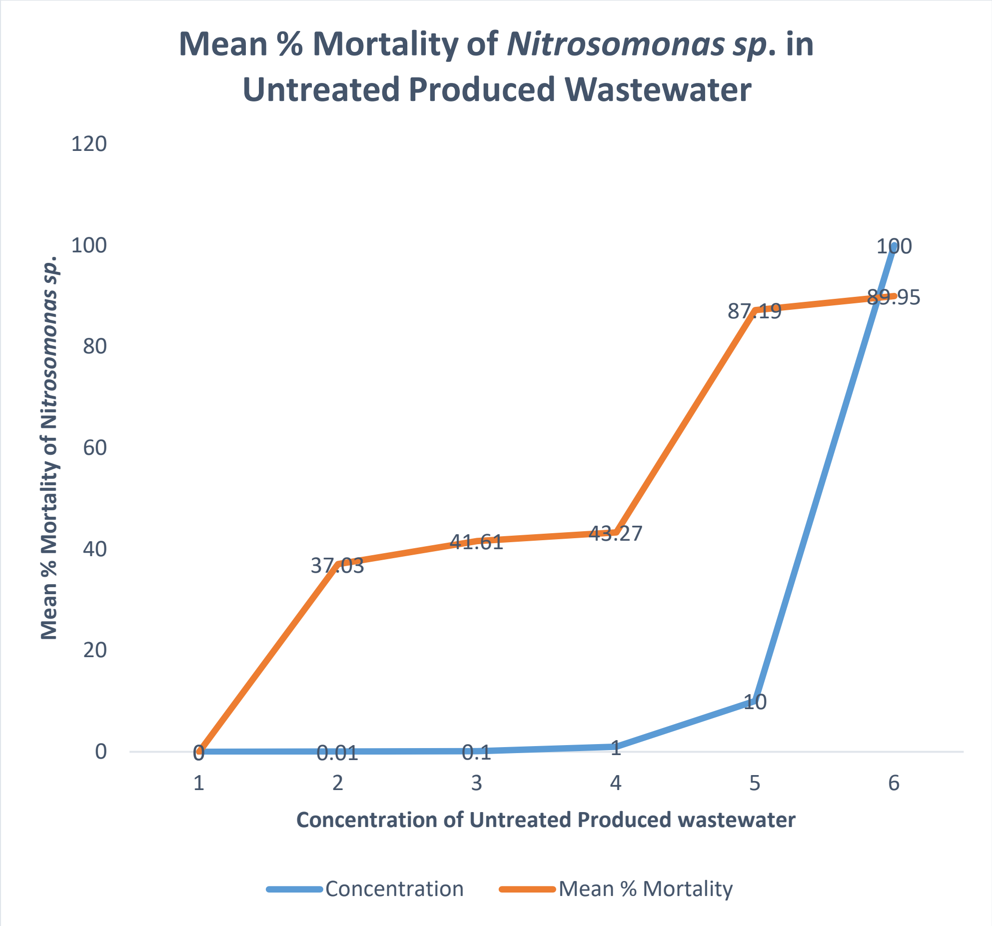

Fig. 1. Mean percentage mortality of *Nitrosomonas sp.* exposed to untreated produced wastewater for 48 hours

Table 1. Mean concentrations of physicochemical properties of treated and untreated produced (Waste Water) water samples

Parameter	Produced Water (Untreated)	Produced water (Treated)	Inland Permissible Limit	
			Limit	Source
Density g/ml (@ 25°C)	1.018±0.0000	1.018±0.0000	0.9970	Universal
pH	8.10±0.03	8.27±0.01	6.5 – 8.5	WHO, 2011
Electrical Conductivity, mS/cm	35700±11	41600±17	1400	WHO, 2011
Total Dissolved Solids, g/L	22848±14	26629±9	2000	EGASPIN, 2018
Temperature, ^{0}C	28.30±0.00	27.60±0.00	Ambient ± 2	EGASPIN, 2018
DO, mg/L	2.05±0.01	4.23±0.03	-	-
BOD, mg/L	28.90±0.7	18.4±0.1	10	EGASPIN, 2018
Phosphate, mg/L	7.56±0.06	3.29±0.09	-	-
Salinity (Chloride), g/L	9529±13	11104.87±7.58	600	EGASPIN, 2018
Turbidity, NTU	31.24±0.27	34.33±0.35	10	EGASPIN, 2018
Nitrate, mg/L	54.82±1.9	50.21±0.9	50	WHO, 2022
Oil & Grease, mg/L	125.00±1.5	47.00±1.1	-	-
THC, mg/L	118.00±0.00	34.00±0.00	10	EGASPIN, 2018
THB, Cfu/mL	4.1×10^4	2.8×10^4	100	WHO, 2011
HUB, Cfu/mL	2.44×10^3	1.58×10^3	-	-

Source: Tudararo-Aherobo and Egieya [7]

Table 2. Morphological and biochemical identification of test microorganism

Test	Result
Gram staining	-
Cell morphology	rod
Motility	+
Catalase	+
Oxidase	-
Indole	-
Citrate utilization	-
Methyl red	-
Voges Proskauer	+
Hydrogen sulfide	-
Sugar fermentation	-
• Glucose (acid)	-
• Glucose (gas)	-

Key: += presence/positive; - = absence/negative (or no visible reaction)

Table 3. Analysis of variance (ANOVA) of physicochemical parameters of wastewater

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	3.53E+09	14	2.52E+08	3.323029	0.002818	2.03742
Within Groups	2.28E+09	30	75957021			
Total	5.81E+09	44				

Source: Tudararo-Aherobo and Egieya [7]

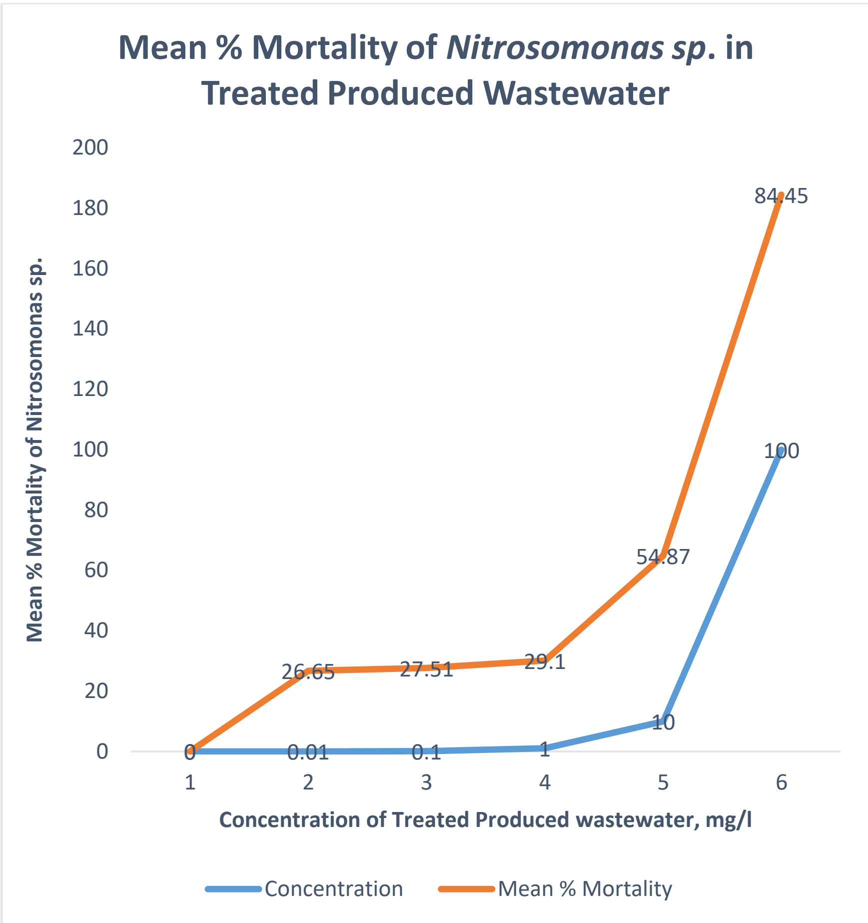

Fig. 2. Mean percentage mortality of *Nitrosomonas sp.* exposed to treated produced wastewater for 48 hours

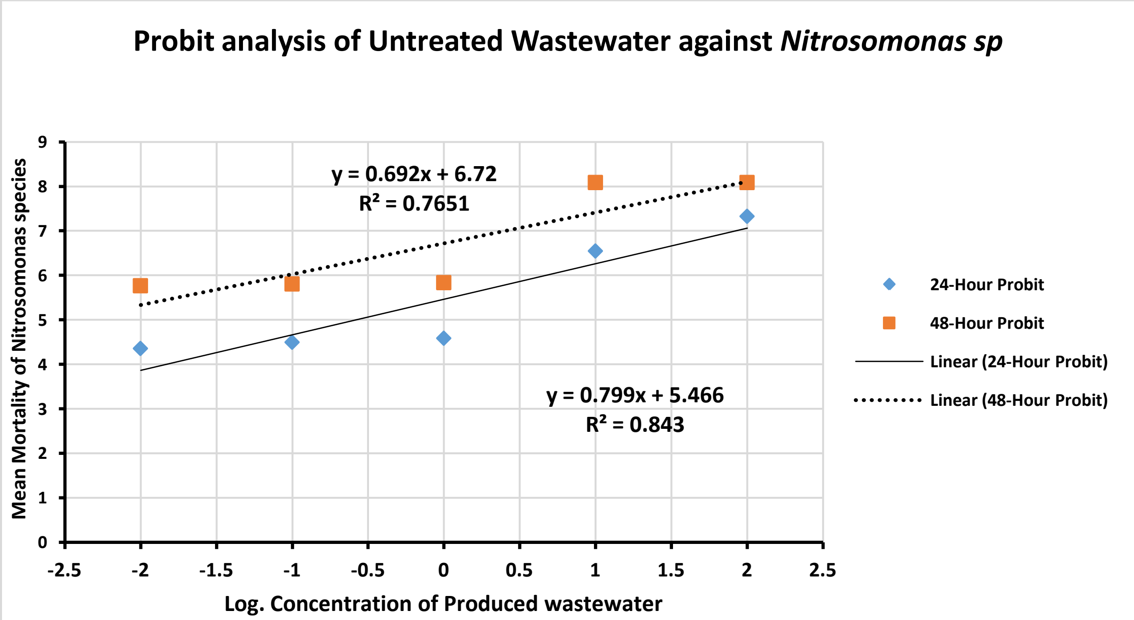

Fig. 3. Probit analysis of untreated wastewater against *Nitrosomonas sp.* for 24-and 48 hour period

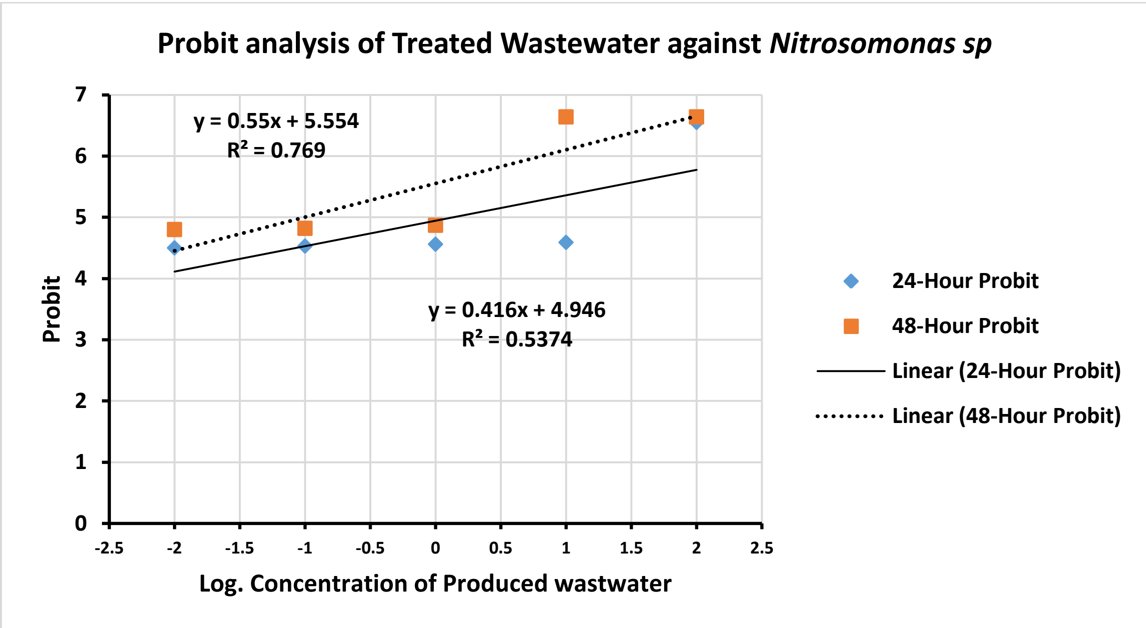

Fig. 4. Probit analysis of treated wastewater against *Nitrosomonas sp* for 24- and 48 hour period

Results from the Probit analysis in Fig. 3 shows that at 24 hours, the untreated produced waster water (toxicant) exhibited a LC50 of 6.74mg/L, while at 48hours a LC50 of 3.27mg/L was recorded. The mean LC_{50} for treated wastewater was found to be 7.10mg/L at 24 hours and 6.69mg/L at 48 hours (Fig. 4). Effects of toxicity for treated and untreated wastewater against *Nitrosomonas sp* increased with time and concentration, while it decreased considerably with reduced concentration of treatment. The LC50 of the treated toxicant was however lower than that of the untreated, indicating a reduction of the toxicant effect as a result of the treatment. The observed higher LC50 of the untreated produced wastewater could be attributed to the higher concentration of some key parameters such as, BOD, TPH, oil & grease (Table 1) [16-18].

Test of significant difference between treated and untreated wastewater was found to be significant at 5% level (Table 3).

4. CONCLUSION

A number of biological variables are directly related to the toxicological condition of wastewater and sediments, while others reflect the overall biological integrity. Monitoring of wastewater is usually aimed at revealing general trends in water quality and sometimes detect the effects of local discharges or other human activities. Overall, the toxicity results of the produced wastewater show linear proportionality with concentration of wastewater and exposure time. Results obtained for the LC50 and mean percentage mortality showed that the untreated produced water had a higher toxic effect on *Nitrosomonas sp* tested, inferring the likely interference of untreated produced water on the nitrogen cycle in the environment and by extension the productivity of the environment. Statistical analysis also shows strong correlation between toxicity values (Table 3) for untreated and treated wastewater against the test organism.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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