Original Research Article

TOXICITY ASSESSMENT OF PRODUCED WATER USING *Nitrosomonas Sp.*

ABSTRACT

Aim: Most studies on the effect of produced water are centred on the physicochemical parameters of the recipient environment, but not much has been conducted on biogeochemical processes such as the nitrogen cycle, hence this study 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).

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

Conclusion:Effects of toxicity for 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..

Keywords: Produced water, Nitrosomonas species, Chronic toxicity.

UNDER PEER REVIEW

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 (Lee and Neff *et al.,* 2011). Following this, Lee *et al.,* (2005a) 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 (2002) as cited in Neff *et al*. (2011) 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 (Neff *et al.,* 2011).

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.

UNDER PEER REVIEW

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 (Lui *et al*., 2001) 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. MATERIAL 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 with GPS coordinate 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 were used to 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 coordinate 05'35.002N 005'50.984'E, Army Estate, Effurun, Delta State, Nigeria.

2.1 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)(Tudararo-Aherobo and Egieya, 2023).

2.2. Laboratory Analysis of Untreated and treated Produced wastewater

UNDER PEER REVIEW

These 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 THCof the samples was analysed using ASTM D3921 method. (Tudararo-Aherobo and Egieya, 2023).

UNDER PEER REVIEW

2.3. Isolation of nitrifying bacteria (*Nitrosomonas* sp)

The method used for isolation of *Nitrosomonas* was adopted from Odokuma and Akponah (2008). *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).

One gram of the soil sample was measured and aseptically transferred into 9mL of distilled water contained in a test tube plugged with cotton wool. 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°C). 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 (Olutiola *et al*., 1991; Holt *et al*., 1994; Cheesbrough, 2005).

2.4.Molecular identification of bacteria isolate

2.4.1. DNA extraction (boiling Method)

Five milliliters of an overnight broth culture of the bacterial isolate in Luria Bertani (LB) were spun at 14000rpm for 3 min. The cells were re-suspended in 500ul of normal saline and heated at 95^{0}C for 20 min. The heated bacterial suspension was cooled on ice and spun for 3 min at 14000rpm. The supernatant containing the DNA was transferred to a 1.5ml microcentrifuge tube and stored at -20°C for other downstream reactions.

2.4.2. DNA quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was lunched by double clicking on the Nanodrop icon. The equipment was initialized with 2 µl of sterile distilled water and blanked using normal saline. Two microliter of the extracted DNA was loaded onto

UNDER PEER REVIEW

the lower pedestal; the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the “measure” button.

2.4.3. 16S rRNA Amplification

The 16s rRNA region of the rRNA gene of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3’ primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microliters for 35 cycles. The PCR mix included: The X2 Dream Tag Master mix supplied by Inqaba, South Africa (tag polymerase, DNTPs, MgCl), the primers at a concentration of 0.5uM and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 30 minutes and visualized on a blue light trans illuminator.

2.4.4. Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10ul, the components included 0.25 ul BigDye® terminator v1.1/v3.1, 2.25ul of 5 x BigDye sequencing buffer, 10uM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing condition were as follows 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min.

2.4.5. Phylogenetic Analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using MAFFT. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor 1969).

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 4ºC. The slant cultures served as stock cultures. The standard inocula

UNDER PEER REVIEW

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 II broth for *Nitrosomonas*) contained in a 250ml conical flask. These were incubated at 37°C 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°C 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^{0}C 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.

UNDER PEER REVIEW

3. RESULTS AND DISCUSSION

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)	1.018894±0.0000	1.017778±0.0000	0.99704	Universal
pH	8.10±0.03	8.27±0.01	6.5 – 8.5	WHO, 2011
Electrical Conductivity, µS/cm	35700±11	41600±17	1400	WHO, 2011
Total Dissolved Solids, mg/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), mg/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(2023)

Results from Table 1, showed that, besides turbidity, six (6) key physicochemical parameters has 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 (Tudararo-Aherobo and Egieya, 2023).

3.2 Isolation, Identification and Characterization of Microorganisms for Acute toxicity (Bioassay)

The result for identification and characterization of microorganisms for toxicity testing is presented below.

UNDER PEER REVIEW

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)

Nitrosomonas species was tentatively identified from the conventional or non-molecular characterization and identification. *Nitrosomonas sp* showed positive results to Catalase and Voges Proskauer tests, its morphological properties indicated that it is motile and rod shaped.

3.3 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. The mean % mortality of *Nitrosomonas sp.* after 48-hours exposure to the untreated Produced wastewater samples ranged from 37%(0.01mg/l) to 90%(100mg/l) (Fig 1), while mean % mortality of the test organisms ranged from 26.65%(0.01mg/l) to 84.45%(100mg/l) (Fig 2) for the treated produced wastewater. The % 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 (Neff *et al.,* 2011).

UNDER PEER REVIEW

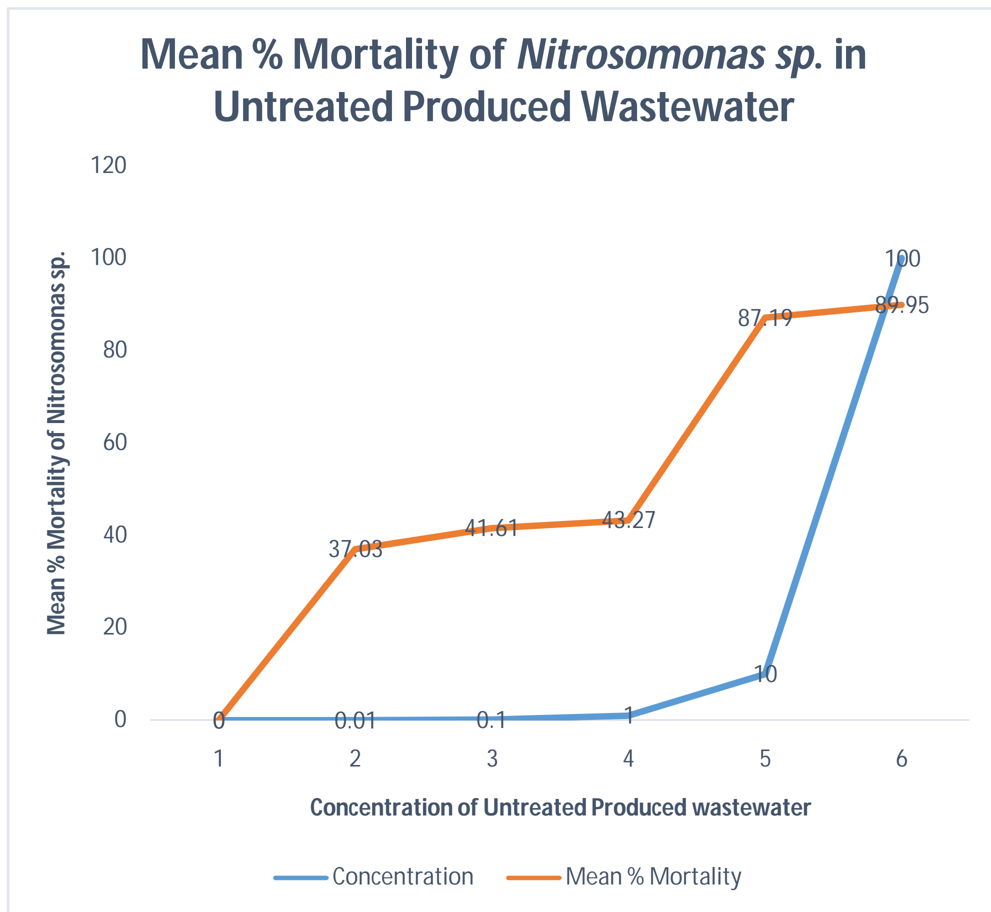

Fig 1: Mean % Mortality of *Nitrosomonas sp.* exposed to Untreated Produced wastewater for 48 hours.

UNDER PEER REVIEW

.

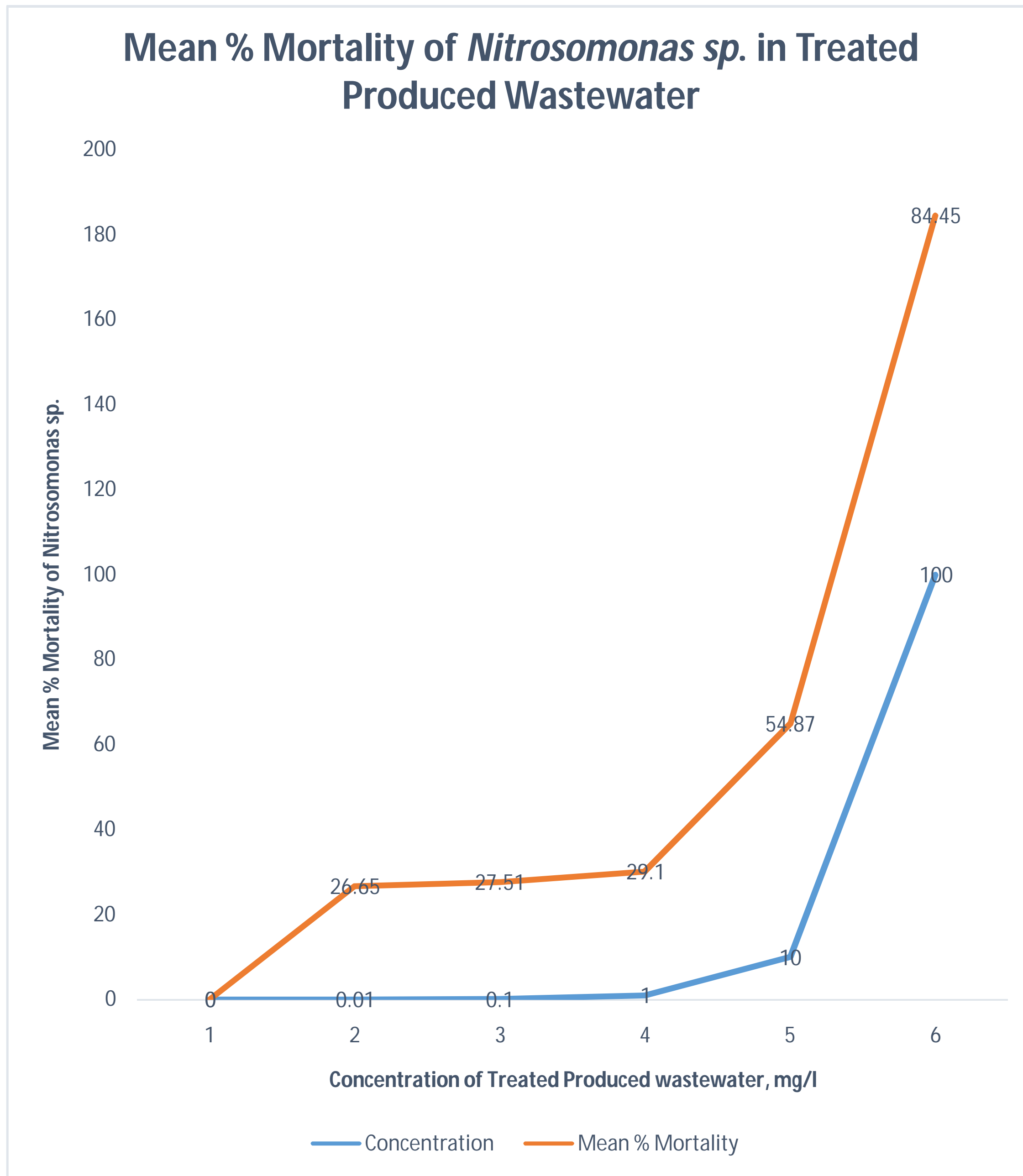

Fig 2: Mean % mortality of *Nitrosomonas sp.* exposed to treated produced wastewater for 48 hours

UNDER PEER REVIEW

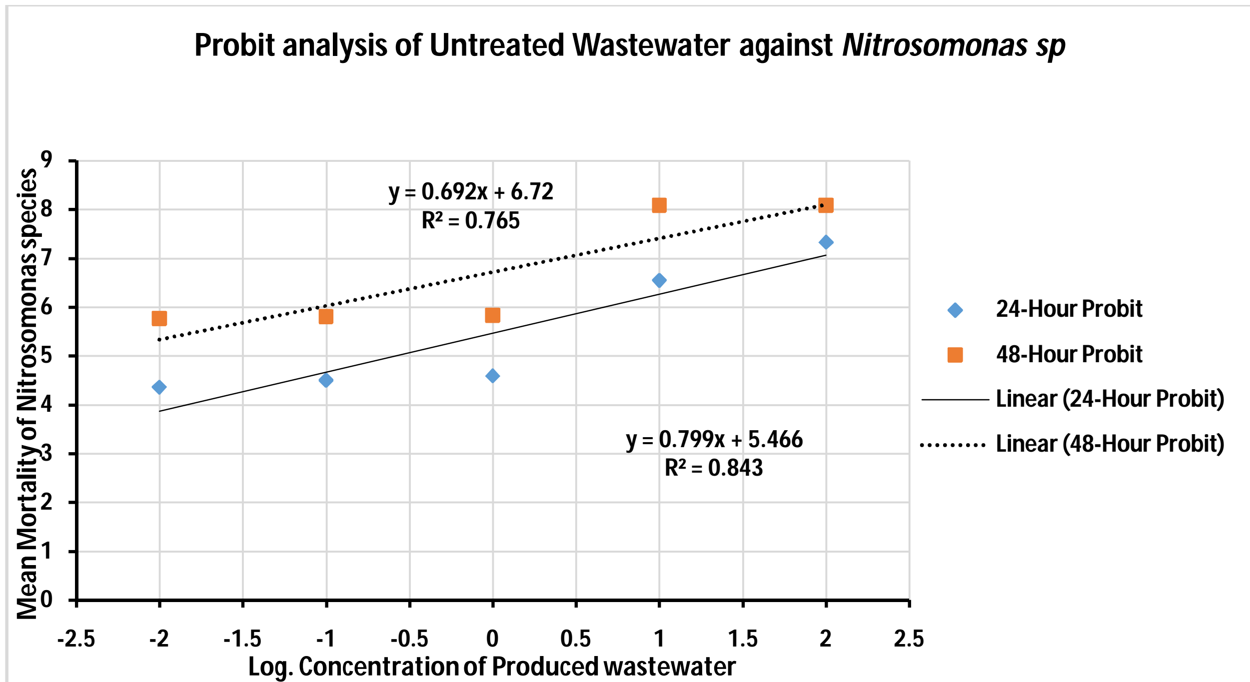

Fig.3: Probit Analysis of Untreated Wastewater against *Nitrosomonas sp* for 24-and 48 Hour Period

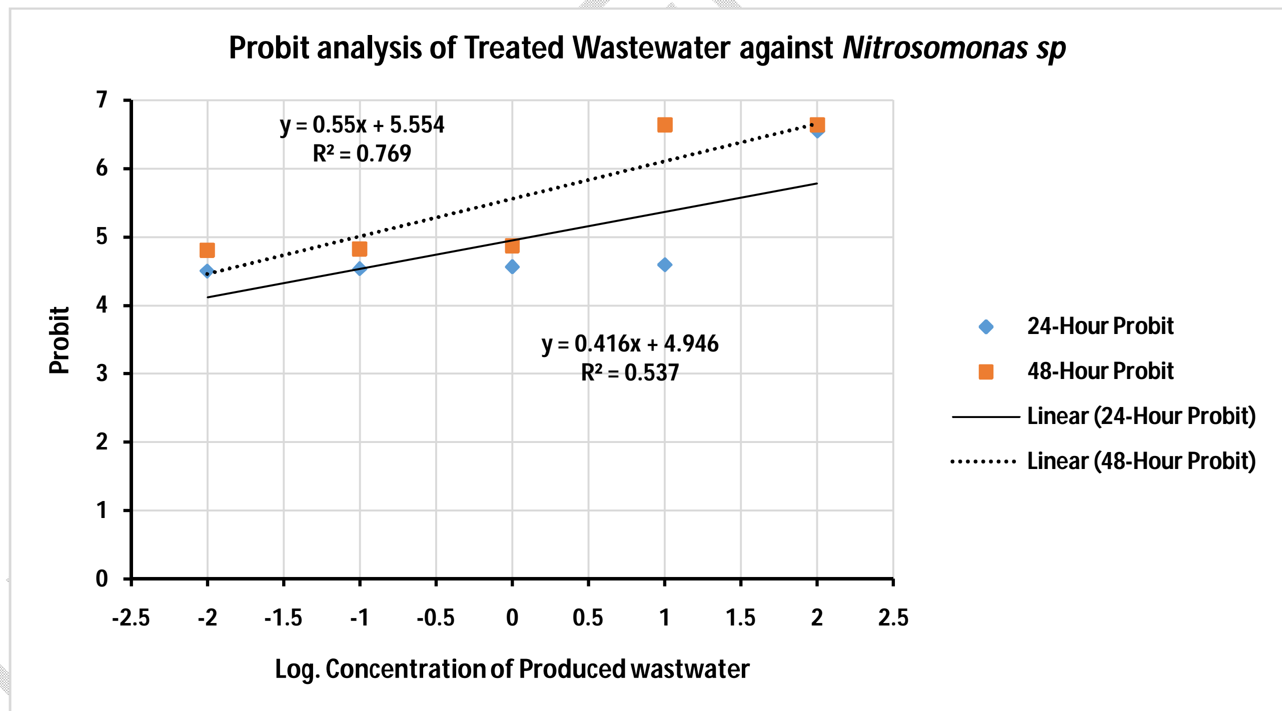

Fig.4: Probit analysis of Treated Wastewater against *Nitrosomonas sp* for 24- and 48 Hour Period

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 (2023)

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, Biochemical Oxygen Demand, TPH,Oil & grease(Table 1).

The test of significant difference between treated and untreated wastewater was found to be significant at 5% level with values in 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. Statistical analysis also shows strong correlation between toxicity values for untreated and treated wastewater against all organisms.

REFERENCES

American Public Health Association (APHA). (2018). Standard Methods for the Examination of water and Wastewater (23rd Edition). Edited by Rice, E.W., Baird, R.B., Eaton, A.D., Clesceri, L.S., America Public

UNDER PEER REVIEW

Health Association (APHA), America Water Works Association (AWWA) and Water Environmental Federation (WEF), Washington D.C., USA. 10200.

Cheesbrough, M. (2005). Discrete Laboratory Practice in Tropical Countries Part 1, Cambridge Second Editions. Cambridge University press. New York.

Environmental Guidelines and Standards for the Petroleum Industry in Nigeria (EGASPIN). (2018). Nigeria Upstream Petroleum Regulatory Commission.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.

Holdway, D. A. (2002). The acute and chronic effects of wastes associated with offshore oil and gas production on temperate and tropical marine ecological processes. Marine Pollution Bulletin 44:185-203.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., and Williams, S. T. (1994). Genus Acetobacter and Gluconobacter. Bergey's Manual of Determinative Bacteriology, 19:71, 84

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Stanley, J. F., and Williams, S. T. (1994). Bergey's Manual of Determinative 19th edition. Williams and Wilkins's, Baltimore, Maryland, USA.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Stanley, J. T., and Williams, S. T. (eds). (1994). *Bergey's Manual of Determinative Bacteriology*. The Williams and Wilkins Company, Baltimore, USA: 566 – 568

Jukes, T. H., and Cantor, C. R. (1969). Evolution of protein molecules. In Munro HN, editor, Mammalian Protein Metabolism, pp. 21-132.

Lee, K., Azetsu-Scott, K., Cobanli, S. E., Dalziel, J., Niven, S., Wohlgeschaffen, G., and Yeats, P. (2005a). Overview of potential impacts of produced water discharges in Atlantic Canada. Pages 319-342 In: S.L. Armworthy, P.J. Cranford, and K. Lee (Eds.). Offshore Oil and Gas Environmental Effects Monitoring: Approaches and Technologies. Battelle Press, Columbus, OH. 631

Lee, K., Neff, J. (2011). Produced water: environmental risks and advances in mitigation technologies. DOI:

- 10.1007/978-1-4614-0046-2

Lui, M. C., Chen, C. M., Cheng, H. Y., Chen, H. Y., Su, Y. C., and Hung, T. Y. (2008). Toxicity evaluation of industrial and land base sources in a river basin. *Desalination.* 226: 1–3, , Pages 348-356.

Lui, M. C., Chen, C. M., Cheng, H. Y., Chen, H. Y., Su, Y. C., and Hung, T. Y. (2002).Toxicity of different industrial effluents in Taiwan: A comparison of the sensitivity of *Daphnia similis* and Microtox. Environ Toxicol. 17:93-97.

Neff, J. M. (2002). Bioaccumulation in Marine Organisms. Effects of Contaminants from Oil Well Produced Water. Elsevier Science Publishers, Amsterdam. 452 pp.

Neff, J. M, Kenneth. L and Deblois, E (2011).Produced Water: Overview of Composition, Fates, and Effects In: Produced Water: Environmental Risks and Advances in Mitigation Technologies. Elsevier Science Publishers, Amsterdam. 452 pp.

Odokuma, L and Akponah, E (2008).Inhibition of nitrification and carbon dioxide evolution as rapid tools for ecotoxicological assessment of drilling fluids. *African Journal of Applied Zoology and Environmental Biology*. 6(1). DOI: 10.4314/ajazeb.v6i1.41168

UNDER PEER REVIEW

Odokuma, L. O., and Ogbu, H. I. (2002). Tolerance of Bacteria and Crustaceans to oil spill dispersants. Afri J Appl Zool Environ Biol. 4:50-55.

Olutiola, P. O., Famurewa, O., and Sontang, H. G. (1991). An introduction to general microbiology: a practical approach. Heidelberger Verlagsanstalt und Druckerei GmbH, Heidelberg, 267.

Tudararo-Aherobo and Egieya(2023*)*.Physicochemical and Microbial Characterization of Treated and Untreated Produced Water *J. Adv. Microbiol., 2: 4, pp. 38-49*

UNDER PEER REVIEW