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Toxicity Assessment of Produced Water Using Microtox Rapid Bioassay

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Authors' contributions

This work was carried out in collaboration between all authors. Author LOO designed the study and wrote the protocol. Author CAA wrote the first draft of the manuscript, performed the statistical analysis and managed the analyses of the study. Author CCB managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The study aimed at employing the Microtox test procedure in the current biological monitoring protocol as a reliable, rapid and ecologically relevant bioassay tool for toxicity assessment in environmental compliance monitoring of produced water discharges.
Study Design: Inhibition of bioluminescence by *V. fischeri* [median effective concentration (EC_{50})] was employed as the toxicity index.
Place and Duration of Study: Microbiology Department of Halden Laboratories, Port Harcourt, Nigeria / one month.
Methodology: Percent reduction in bioluminescence by *V. fischeri* after 15-min exposure to the PW samples was recorded as median effective concentration (EC_{50}) values.
Results: The 15 min EC_{50} values of the untreated and treated produced water samples for *V. fischeri* was 1.0% and 23.27% respectively. Microtox test indicated the treated and untreated produced water samples were "very toxic" and "extremely toxic" respectively, after 15 min exposure time.

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Conclusion: These findings emphasize the need for adequate treatment of produced water to meet standard discharge limits of regulatory agencies in Nigeria, as both physicochemical analysis and bioassay (Microtox) suggested that the treated PW was toxic to *V. fischeri.* This study thus supports the use of Microtox (bacterial toxicity) system as a sensitive and rapid bioassay tool for biological monitoring protocol in Nigeria's petroleum industry.

Keywords: Acute toxicity; Vibrio fischeri; microtox; produced water; bioassay; median effective concencentration (EC_{50}).

1. INTRODUCTION

Produced water is water that goes along with oil or gas during oil and gas production and it is a mixture of formation water (water in reservoir formation), injection water (water injected into the oil reservoir to enhance maximum oil recovery and to maintain reservoir pressure) and connate water (water trapped in between rocks in the reservoir). It also contains smaller quantities of dissolved organics (including hydrocarbons), traces of heavy metals, dissolved minerals, suspended oil (non-polar), solids (sand, silt) and production chemicals added in the production/separation line [1,2,3]. Produced water is by far the largest volume byproduct of waste stream associated with oil and gas production and its properties and volumes vary considerably depending on the geographical location of the field, the geological formation with which the produce water has been in contact for thousands of years and the type of hydrocarbon product being produced [1]. It has been observed that every aspect of oil operations, in varying degrees, poses significant negative impacts on the environment and that the environmental consequences impose economic effects on the indigenes of that locality [4,5,6]. This is why in the Niger Delta, Nigeria before discharge of produced water in offshore locations, the main regulatory agency [Department of Petroleum Resources (DPR)] requires the constituents of produced water to be within the limits as shown in Table 1, while mandatory sampling, analysis and monitoring are conducted as stipulated intervals. Also, in many countries, for instance, the U.S. Environmental Protection Agency has incorporated various aquatic toxicity tests in the National Pollutant Discharge Elimination System (NPDES) permits since 1984 [7].

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 [8] 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 aquatic organisms exposed. For instance, Chen CY, et al., Chen CM, et al. [9,10] reported that the effluents tested met the Taiwan Environmental Protection Administration's (Taiwan EPA) discharge standards but were found to be toxic to aquatic organisms. Biological monitoring utilizes the responses of living organisms in standardized toxicity tests to assess the potential for toxic effects on inhabitants of surface waters to which complex effluents or wastewaters are discharged. These tests typically use lethality as an endpoint in both acute and chronic tests, and sublethal endpoints, e.g., growth and reproduction in chronic tests. These endpoints are expressed through the median lethal and effective concentrations LC_{50} and EC_{50} respectively [11].

Rapid bioassays (toxicity tests) on the other hand are testing strategies that; (i) have adequate sensitive toxicological endpoint that can be obtained in a short duration (≤ 24 h), (ii) involve minimal test organism maintenance, (iii) is of low technical complexity and, (iv) are cost-effective to conduct relative to conventional standardized tests [12].

Currently, the biological monitoring component of environmental compliance monitoring associated with the discharge of produced water in Nigeria has not been fully developed. Agreement on test species and procedure for biological monitoring is ongoing. These procedures are known to be complex, laborious, time consuming and cost intensive. Thus, this study aimed at employing the Microtox test procedure in the current biological monitoring protocol as a reliable, rapid and ecologically relevant bioassay tool for toxicity assessment in environmental compliance monitoring of produced water discharges.

The Microtox test system is based on measuring changes in the light output of a marine

luminescent bacterium *Vibrio fischeri* following exposure to single chemicals or complex environmental samples. The degree of change in light output relative to a control is directly proportional to the level of toxicity present in test samples [11]. Bioluminescence is an aerobic oxidation process which involves the synthesis of luminescence from a substrate luciferin, catalyzed by the enzyme luciferase and mediated by reduced coenzyme flavin mononucleotides. When the toxicants come in contact with the luminescent bacteria, it results in the inhibition of luminescence synthesis [13,14].

A comprehensive record of comparative results has been generated for the Microtox test and various aquatic organisms by many authors. Stagg et al. [15] recorded a 15 min EC_{50} value for Brent Delta production water to be between 6.2 and 4.3%, [16], recorded values of between 5 and 6% for the same platform. The study by Grigson et al. [17], reported EC_{50} values for 17 produced water samples analysed from 14 different North sea oil platforms ranging from 3.74 – 37.34% and the majority (14) having EC_{50} values between 3 and 10%. Korytar et al. [18] recorded a 15-min EC_{50} of 24.2% for produced formation water from Berge Helene FPSO (floating, production, storage and offloading) facility at Chinguetti oil field, Mauritania.

Table 1. The DPR Standard Limits for Offshore Discharge of Produced Water in Nigeria

Parameters	DPR limit
pH	6.5-8.5
Temperature °C	30.0
Oil/Grease content (mg/l)	40
THC (mg/l)	40
Salinity (psu)	No limit
Total dissolved solid (mg/l)	5000
Total suspended solids (mg/l)	50
Chemical oxygen demand (mg/l)	125
Biochemical oxygen demand (mg/l)	125
Chloride (mg/l)	2000
Chromium (mg/l)	0.5
Zinc (mg/l)	5.0
Turbidity (mg/l)	15

Source: EGASPIN (2002) [19]
[1]Note: EGASPIN: Environmental Guidelines and Standards for Petroleum Industries in Nigeria
Psu: Practical Salinity Units

2. MATERIALS AND METHODS

2.1 Test Samples

Samples of untreated and treated produced water were collected from an offshore operational production facility situated at Warri, Delta State with coordinates 4°54'0'' N and 5°28'60'' E. Samples were treated in the following manner (e.g., filtered, centrifuged, dechlorinated, or pH-adjusted) prior to the initiation of testing with species. All samples were stored at 4°C prior to testing.

2.2 Physicochemical Analysis of Test Samples

The pH, temperature, electrical conductivity, dissolved oxygen (DO), biochemical oxygen demand (BOD), salinity, total dissolved solids (TDS), total suspended solids (TSS), nitrates and phosphate of both produced water (PW) samples were analyzed following standard methods by American public health association [20].

2.3 Detection of Heavy Metals

Lead, chromium, cadmium, arsenic, mercury, nickel, iron and zinc were detected by flame analysis Method 7000B using the Atomic absorption Spectrophotometer Model AA500 (PG instruments) after sample preparation and digestion [20].

2.4 Gas Chromatography of Oils

Total petroleum hydrocarbons (TPH), polycyclic aromatic hydrocarbons (PAH) and monocyclic aromatic hydrocarbons (MAH) were extracted and quantified using Gas chromatograph equipped with single flame ionization detector (GC-FID) Model 6890 (Agilent instruments, USA) according to the method adopted from US Environmental protection agency (USEPA 8015 and 8270c protocol). Benzene, toluene, ethyl benzene and xylene (BTEX) were analysed using the sri8610c purge and trap Gas chromatography according to the method described by (USEPA 5030 protocol) [21].

2.5 Enumeration of Total Culturable Heterotrophic Bacteria

Total culturable heterotrophic bacterial (TCHB) counts were determined using the spread plate method on plate count agar (PCA) described by

Chikere and Ekwuabu [22]. From each sample, One ml was homogenized in 9 ml of 0.85% normal saline using Heindolph vortexing machine. Serial dilutions (10-fold) of the samples were prepared and dilutions (10^{-4}- 10^{-5}) of samples were plated out on agar medium and incubated at 30°C for 24 h. The colony forming units were afterwards enumerated.

2.6 Enumeration of Hydrocarbon Utilizing Bacteria

Hydrocarbon utilizing bacteria (HUB) were enumerated by a method adapted from Hamamura et al. [23] which involved the dilutions of the appropriate sample and plating out on Bushnell-Haas agar (Sigma-Aldrich, USA). Hydrocarbons were supplied through the vapour phase by placing sterile Whatman No.1 filter papers impregnated with 5 ml Bonny light crude oil on the lids of the inverted plates and incubated for 7 days at 30°C.

2.7 Test Organisms

The freeze-dried reagent of the luminescent marine bacterium, *V. fischeri*, was obtained from the manufacturer (MODERN WATER INC, Delaware, USA) and used for conducting the Microtox tests.

2.8 Test Methodology

Microtox acute toxicity test was conducted with the Model 500 Microtox Analyzer (MODERN WATER INC, Delaware, USA) using protocols for the 45% and 81.9% Basic Test protocol [12,24,25]. A standard procedure is detailed in the manufacturer's manual. Each test consisted of blank and serial dilutions of produced water samples. The Inhibition test involved exposure of the reconstituted freeze-dried bacteria to test samples. The reconstituted freeze-dried bacteria were distributed to cuvettes containing cooled (15°C) 2% saline solution (diluent). An initial light output (I_0) from each cuvette was recorded after a 15-min stabilization period (bioluminescence is measured in a temperature-controlled Luminometer). Subsequently, the produced water samples (also precooled to 15°C) were added to appropriate cuvettes and after a 5 to 15-min exposure period, the final light output (I_{15}) was measured relative to a control. The inhibition of the luminescence was correlated with the toxicity of the water samples tested. The test organism was subjected to quality control testing using zinc sulphate in reference tests. Each assay on the PW samples was accompanied by Zinc sulphate as the positive control (reference toxicant). The results of reference toxicant test conducted during the study period fell within the acceptable range for the species and reference material.

2.9 Statistics and Data Analysis

Median effective concentrations (EC_{50}) were calculated using the software that accompanied the Microtox system known as MicrotoxOmni software [26] which uses linear regression analysis. A set of developed guidelines (with categories broadly defining the degree of toxicity) by the manufacturer was used for interpreting the results of the Microtox Inhibition tests for toxicity assessment. The results of the tests were compared against toxicity categories developed as presented in Table 4.

The quality of the data was based on an assessment of the confidence range calculated for each EC_{50} value and the coefficient of determination (R^2), an expression of the quality of the estimating equation from which the EC_{50} is obtained. Ideally, 95% confidence range values for each replicate should not exceed 30% of the EC_{50} value [17].

3. RESULTS AND DISCUSSION

3.1 Physicochemical Properties of Untreated and Treated Produced Water

The Physicochemical properties of produced water (PW) samples are shown in Table 2. Both untreated and treated PW samples had an alkaline pH (8.21 and 8.02 respectively) due to the presence of high levels of carbonates. The total petroleum hydrocarbons (TPH), Total hydrocarbon content (THC) and Oil and Grease levels in the treated produced water were lower than that of untreated produced water. This could be attributed to the treatment process (Hydro cyclone units and Induced gas floatation units) the treated produced water was subjected to. A higher turbidity was observed for Untreated PW compared to Treated PW because of higher levels of TPH, THC, Oil and Grease, total dissolved solids (TDS) and total suspended solids (TSS) in the untreated PW. Monocyclic aromatic hydrocarbons (MAHs) and Benzene, Toluene, Ethyl benzene and Xylene (BTEX) were detected at very low concentrations, while polycyclic aromatic hydrocarbons (PAHs) were not detected in both PW samples. Heavy metals analysed were detected at very low

concentrations. Results from physicochemical analyses reveal that pH, temperature and TSS in treated PW were within recommended discharge limit. Some constituents of the PW samples were above the Department of Petroleum Resources' (DPR) recommended offshore discharge limits even though their concentrations were reduced in the treated PW compared to untreated PW. TPH, THC, Oil and Grease, total dissolved solids (TDS) and chlorides exceeded DPR set limits as presented in Table 1.

Similar findings were recorded by Isehunwa and Onovae, Onojake and Abanum, Onyema et al., Ozulu [27,28,29,30] who established that produced water sourced from some Nearshore and Offshore production and treatment facilities in the Niger Delta, Nigeria, were yet to meet DPR allowable discharge limits. Findings of Darlington and Kenneth [31] were in contrast to findings from this study. They reported that constituents (Oil and Grease, TDS and TSS) of produced water from a certain nearshore produced water treatment facility were reduced far below DPR limit for nearshore discharge limit.

3.2 Relative Population Densities of Microorganisms Found in the Produced Water

The presence of microbial activity in the produced water was determined by the enumeration of total culturable heterotrophic bacteria and total hydrocarbon utilizing bacteria as presented in Table 2. The total culturable heterotrophic bacteria (TCHBC) and hydrocarbon utilizing bacteria (HUB) counts were highest in the untreated produced water with mean values of 4.5×10^5 and 3.2×10^4 cfu/ml respectively, while treated PW had mean values of 4.0×10^5 and 2.5×10^4 cfu/ml for TCHBC and HUB respectively. Okoro, Maggot [3,32] recorded similar findings. They suggested that these low population densities indicate that oil field waters constitute a nutrient limiting environment.

Table 2. Physicochemical and microbiological properties of produced water samples

Parameters	Untreated produced water	Treated produced water
Ph	8.21	8.02
Temperature	27.0	27.0
Electrical conductivity (mS/cm)	22.1	16.0
TDS (mg/l)	12,870	9,040
Salinity (psu)	16.256	11.896
Turbidity, NTU	906	113
DO (mg/l)	1.98	2.77
BOD (mg/l)	22.8	17.0
Nitrate (mg/l)	11.0	0 .80
Phosphate (mg/l)	6.40	1.46
Chlorides (mg/l)	10,562	7,210
TPH (mg/l)	714	48.2
BTEX (mg/l)	0.005	<0.0001
PAHs (mg/l)	-	-
MAHs (mg/l)	0.005	<0.0001
Oil and Grease (mg/l)	852	65.2
THC (mg/l)	801	58.7
Lead (mg/l)	<0.05	<0.05
Chromium (mg/l)	<0.05	<0.05
Cadmium (mg/l)	<0.05	<0.05
Arsenic (mg/l)	<0.001	<0.001
Mercury (mg/l)	<0.001	<0.001
Nickel (mg/l)	<0.05	<0.05
Iron (mg/l)	0.28	<0.05
Zinc	<0.05	<0.05
TCHB (cfu/ml)	4.5×10^5	3.2×10^4
HUB (cfu/ml)	4.0×10^5	2.5×10^4

[2]Note: TCHB: Total culturable heterotrophic bacteria
HUB: Hydrocarbon utilizing bacteria

The untreated PW had TCHBC of 4.5×10^5 cfu/ml and 7.1% of it had the capability to degrade hydrocarbons. Treated PW also had TCHBC of 4.0×10^5 and 6.25% of it had the ability to degrade hydrocarbons. These findings revealed that PW samples had a population of hydrocarbon utilizing bacteria (HUB), suggesting that the components of the PW samples are biodegradable. Okoro, Okoro and Amund, Okoro [3,33,34] reported similar results.

Physicochemical analyses showed that nutrients in the form of Nitrogen and Phosphate in untreated produced water were high as such were considered as not limiting. Hence the support for microbial growth and proliferation as indicated by the population of TCHBC and HUB in untreated PW mentioned above. This was supported by Head et al. [35].

3.3 Microtox Toxicity Tests

The results of the Microtox assay on the produced water samples (untreated and treated PW) are summarized in Table 3. The toxicity of two (2) samples corresponded to changes in reagent light output in the Microtox test. Untreated PW was most toxic with a 15 min EC_{50} value of 1.0%, while the treated had a 15min EC_{50} value of 23.27%.

In this study, the acute toxicity of produced water from an offshore production facility was measured using Microtox (*V. fischeri*). The results showed that untreated and treated were acutely toxic to *V. fischeri* (Microtox) though at varying degrees when compared against toxicity categories developed by manufacturers for interpreting results as presented in Table 4. The 15 min EC_{50} values for Microtox (*V. fischeri*) exposed to untreated and treated PW were 1.0% and 23.27% respectively, suggesting that the untreated PW lies under the “Extremely toxic” category (0-19%) and treated lies under the “Very toxic” category (20-39%). The variance in toxicity could be attributed to the general physicochemical characteristics of the PW samples. As presented in Table 2, the untreated produced water had higher concentrations of these known key compounds of environmental concern: Oil and Grease (852 mg/l), total hydrocarbon content [THC (801 mg/l)] and total petroleum hydrocarbon [TPH (714 mg/l) than that of treated PW Onyema et al., Ozulu [28;29]. Although concentrations of Oil and Grease, TPH and THC in treated PW were reduced compared to the untreated PW, the treated PW was, however, "very toxic" to *V. fischeri* and this could be associated to the fact that concentrations of the compounds mentioned earlier exceeded the Department of Petroleum Resources allowable offshore discharge limits for such compounds, hence the persistence of toxicity.

Stagg et al. [15] recorded a 15 min EC_{50} values of between 6.2 and 4.3%, while [16] also obtained values of between 5 and 6% for produced water from the same platform. Grigson et al. [17], reported EC_{50} values ranging from 3.74 – 37.34% with a majority (14) having EC_{50} values between 3 and 10%. The study by Manfra et al. [36] reported that treated produced samples employed in their study were also toxic to *V. fischeri*. Zinc sulphate was used as the reference toxicant in the microtox assay with a 15 min EC_{50} of 3.75 mg/L. These levels were within the ranges suggested in the manufacturer’s operations manual (15- min EC_{50}: 3 - 10 mg/L), indicating consistency and reproducibility of this assay. Lui et al. [8] also reported similar findings.

It was also observed from this study that the 95% confidence range values exceeded 30% of the EC_{50} for both untreated and treated PW as presented in Table 3. Further, the coefficient of determination (R^2) for both PW samples was >0.91, suggesting the estimating equation for calculating the EC_{50} was of reasonable quality for both samples. This underpins the findings of Grigson et al. [17], who also observed that 95% confidence range for a range of produced water components exceeded 30% of their EC_{50} values and the coefficient of determination (R^2) was >0.91 for all replicates.

Table 3. Toxicity of produced water samples and reference toxicant to microtox (*V. fischeri*)

Effluent type	Microtox 15-min EC_{50}	Toxicity category	Coefficient of determination (R^2)
Untreated produced water	1.00% (0.6752 - 1.493)	Extremely toxic	0.9544
Treated produced water	23.27% (17.40 - 31.12)	Very toxic	0.9862
Zinc sulphate	4.849 mg/l or 0.00048 (2.906 – 8.090)	Extremely toxic	0.9586

[3]*Note: Figures in parentheses indicate 95% confidence range*

Table 4. Result interpretation for Microtox

Microtox EC_{50}	Apparent toxicity level
0-19 %	Extremely toxic
20-39 %	Very toxic
40-59 %	Toxic
60-79 %	Moderately toxic
80-99 %	Slightly toxic
>100 %	Non-toxic

Source: Modern Water Incorporation (2016)

4. CONCLUSION

This study revealed that despite the guidelines and regulations pertaining to the discharge of produced water in Nigerian oil and gas operations, the treated PW analysed in this study is yet to meet the DPR permissible discharge limit as some of its constituents (Oil and Grease, TDS, THC, TPH and chlorides) were above set standard limits, as shown in comparison of Tables 1 and 2. Therefore, emphasizing the need for adequate monitoring and enforcement of disposal guidelines and set limits by regulatory agencies.

Both PW samples tested in this study showed toxicity to Microtox bacterium *V. fischeri*, however, at varying levels. The untreated PW elicited a higher toxicity compared to the treated PW. Also, the sensitivity of Microtox in responding to the reference toxicant throughout the study was very consistent. This is similar to findings by Doherty et al. [11] who conducted reference toxicant tests with *Daphnia* species (*Daphnia* mortality test) and reported that sensitivity during the study period was within acceptable historical range.

Therefore, the sensitivity of the Microtox test can be employed by the petroleum companies in Nigeria as high-throughput bioassay tool particularly when screening a large number of samples. It also proves to be highly reproducible, easy to use, short exposure times, low detection levels and minimal sample size requirements compared to other standard bioassay tools.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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