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Acute Toxicity of Two Oil Spill Dispersants Used In Nigerian Petroleum Industries to Nitrobacter and Thiobacillus.

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***Abstract*:** *The acute toxicity of oil spill dispersants -GoldCrew and Biosolve was monitored in seawater obtained from Bonny Estuary in Rivers state, Nigeria. Toxicity of dispersants was assessed using Nitrobacter and Thiobacillus as test organisms using mortality as index in a 96-hour exposure period. There was an increase in percentage mortality with increasing concentration of the toxicants and increasing contact time. The toxicity of the dispersants was in decreasing order of GoldCrew>Biosolve. The sensitivity trend for the test organisms showed a decreasing order; Thiobacillus>Nitrobacter sp. The 96hour–LC_{50}GoldCrew on these test organisms were 0.05 and 0.0006ppt respectively while that of Biosolve were 0.07 and 0.001ppt respectively.Nitrobacter is responsible for the oxidation of nitrites to nitratres in the nitrogen cycle while Thiobacillus is responsible for the oxidation of reduced sulphur compounds to sulphur and sulphates. The study revealed that these dispersants are toxic to the test organisms. Growth inhibition of these microorganisms can upset the balance of biogeochemical cycles where they are key players. This can trigger inadequate waste management and affect other environmental activities. Proper monitoring of the use of dispersants should be ensured.*

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I. Introduction

An oil spill is the leakage or discharge of liquid petroleum hydrocarbons into the environment. It is a form of pollution and may happen due to release of crude oil from tankers, offshore platforms, drilling rigs, wells, etc. The aquatic ecosystem that may be affected by spilled oil consists of shoreline, sea surface, water column and sediments. Natural events cause the spilled oil or its components to be moved from one compartment of the environment to another (IPIECA-IOGP, 2015).

Dispersants are a class of chemical compounds used in the control of oil spilled in aquatic environment. In Nigeria, the use of dispersants is permitted only in the offshore environment (EGASPIN, 2002). They weakens interfacial tension between oil and water in order to enhance the natural process of dispersion by generating larger numbers of small droplets of oil that are entrained within the water column (Fingas*et al*., 1995). The aim of dispersion is to expel the oil from the surface where it is at its most potentially toxic concentration, dilute it into water column to low concentration and facilitate its biodegradation. Environmental factors, including temperature, salinity and conditions at sea influence the adequacy of dispersants.

Toxicity is a relative property of a chemical, which refers to its potential to have deleterious effects on living organism. It is the inherent ability of a substance to cause harm. Acute toxicity is a property of a substance that has toxic effects on an organism, when the organism is exposed to a lethal dose of a substance once. Also, chronic toxicity is a property of a substance that has toxic effects on a living organism, when the organism is ceaselessly or repeatedly exposed to that substance (Klaassen and Eaton, 1991).

The destructive impacts of oil spill on the environment are numerous. Expansive release of oil is most likely to produce physical and habitat effects. Anthropogenic PAHs affect living organisms through their toxicity (Neff, 1985). Oil on water surface meddles with gaseous interchange at the sea surface and dissolved oxygen levels will thereby be lowered. This diminishes the life expectancy of marine organisms (Nwilo and Badejo, 2001).

In Earth science, a biogeochemical cycleis a pathway by which a chemical substance moves through biotic (biosphere) and abiotic (lithosphere, atmosphere, and hydrosphere) compartments of Earth. The ecosystems have many biogeochemical cycles operating as a part of the system; however, Sulphur and nitrogen occupy very strategic positions in the earth biogeochemical cycle. The sulfur cycle is the collection of processes by which sulfur moves to and from minerals (including the waterways) and living systems. Sulphur cycles are also important for life because sulfur is an essential element, being a constituent of many proteins and cofactors. Oxidation of sulphide to elemental sulphur is mainly en sued by sulphur oxidizing bacteria. Nitrogen is present in the environment in a wide variety of chemical forms including organic nitrogen,ammonium(NH^+_4), nitrite

(NO^{-}_{2}), nitrate (NO^{-}_{3}), nitrous oxide (N_2O), nitric oxide (NO) or inorganic nitrogen gas (N_2). The conversion of nitrogen can be carried out through both biological and physical processes. Important processes in the nitrogen cycle include fixation, ammonification, nitrification, and denitrification. *Nitrobacter* are however responsible for the oxidation of the nitrites (NO^{-}_{2}) into nitrates (NO^{-}_{3}). The aim and objective of this study is to determine the possible toxic effects of Biosolve and GoldCrew on *Nitrobacter* and *Thiobacillus*.

II. Materials And Methods

Source and types of oil spill dispersants

Biosolve, and Goldcrew were obtained from different licensed oil field chemical stores in Port Harcourt, Rivers State.

Source of microorganisms for toxicity test

The test organisms consisting of *Nitrobacter* and *Thiobacillus* were isolated from the sea water sample collected off the coast of Bonny in Rivers State, Nigeria.

Isolation of toxicity test organisms

(i) Nitrobacter

The method used for isolation of *Nitrobacter*was adopted from Colwell and Zambruski (1972). *Nitrobacter*was isolated using Winogradsky medium phase 2 (KNO_2, 0.05g; Na_2CO_3, 1.0g; NaCl, 0.5g; $FeSO_4.7H_2O$, 0.4g; agar 15.0g; distilled water 1000ml). The medium was sterilized by autoclaving and aseptically dispersed into sterile Petri dishes after cooling to about 45^0C. The plates were then inoculated and incubated aerobically for 4 days at room temperature (28 ± 2^0C). Colonies that developed on the Winogradsky agar media for nitrification phases 2 after incubation period were aseptically sub-cultured on freshly prepared Winogradsky agar media phase 2 for further identification and confirmation. Pure isolates were sub-cultured to Winogradsky agar slants and stored for further use (Odokuma and Akponah, 2008).

(ii) Thiobacillus

The method used for isolation of sulfur oxidizing bacteria was adopted from Behera*et al.*, (2014). Sulfur-oxidizer medium (0.1g NH_4Cl, 3.0g KH_2PO_4, 0.1g MgCl, 0.35g CaCl, 5.0g $Na_2S_2O_3.5H_2O$, 1000ml of distilled water and 20g of agar) was utilized. Direct plating method was used. 0.1ml of the aliquot of the serially diluted seawater were spread onto the medium plates and incubated at 30^0C for 24 to 72hours. The well-defined isolated colonies that appeared on the plates were picked up using sterile wire loop and streaked on the fresh medium agar plate for purification and confirmation.

Identification and characterization of isolates

Pure isolates from the corresponding agar slants were characterized and identified using morphological (colonial morphology, motility, and gram reaction), biochemical and physiological attributes (Holt *et al.*, 1994; Cheesbrough, 2004).

Chemical Composition of the Source of Microorganism

The seawater sample was analyzed for its physiochemical properties using pH, total dissolved solids, electric conductivity, salinity, dissolve oxygen, total suspended solid,total organic carbon, biochemical oxygen demand, chemical oxygen demand, nitrate, phosphate and sulphate.

Chemical Composition of Dispersants

The chemical composition ofBiosolve and Goldcrew was carried out using parameters such as pH, Dissolved oxygen (DO), Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), total hydrocarbon, total petroleum hydrocarbon, nitrate, sulfate, phosphate and chloride.

Toxicity of dispersants

Preparation of test medium

The different concentrations (1000ppt, 100ppt, 10ppt, 1ppt, 0.1ppt and 0.01ppt) of the different toxicants (Biosolve, and GoldCrew) were prepared and dispensed into different and appropriate medium.

Preparation of test organisms

Loopful of the test organisms (sulphur oxidizing bacteria and *Nitrobacter* sp.) were transferred into 10ml sterile appropriate broth and incubated for 2-4days at appropriate temperature. One millilitre of aliquot of each culture were transferred into fresh sterile broth and incubated for 24 hours to ensure that actively growing organisms were used for toxicity test.

Toxicity test procedure

Tubes containing 9ml of appropriate broth for each test organisms and the different concentrations (0.01ppt, 0.1ppt, 1ppt, 10ppt, 100ppt, and 1000ppt) of each toxicant (oil spill dispersants – Biosolve, and GoldCrew) were set up. However, the 1000ppt tubes contained no broth and the control tubes contained no toxicants. One millilitre of the test organisms were added to their respective labeled tubes and 0.1millilitre of

each were plated out immediately on appropriate agar plates. This was to determine the 0hour count. Aliquot (0.1ml) of each concentration of the toxicants were then plated out in triplicates at 24 hours, 48 hours, 72 hours and 96 hours on appropriate agar plates. The plates were incubated for appropriate period and at appropriate temperature. The colonies were then counted and average colony taken.

The percentage log survival of isolates in the toxicants

The percentage log survival of the isolates in the toxicant used in the study was calculated using the formula adopted from Williamson and Johnson (1981). This was calculated by obtaining the log of the count in each toxicant concentration and dividing the count in the zero toxicant concentration and then multiplying the product by 100.

Therefore %log survival = $\frac{LogC}{Logc} \times 100$

Where $LogC$ = log count in each toxicant concentration,

$Logc$ = log count in the zero toxicant concentration.

Determination of LC_{50}

The lethal concentration of the toxicant that will kill 50% of the test organism (LC_{50}) was determined byProbit analysis

III. Results

The source of the test microorganisms was analysed for its physicochemical properties. The Seawater has pH 7.50, total dissolved solids 21550mg/L, electric conductivity 43100µs/cm, salinity 25880mg/L. Dissolved oxygen was measured to be 3.41mg/L, total suspended solids 8.00mg/L, total organic carbon 0.16%, biochemical oxygen demand 11.59mg/L, chemical oxygen demand 19.19mg/L. The level of some nutrients was also measured – nitrate 1.10mg/L, phosphate 3.80mg/L and sulphate 14.00mg/L.The morphological and biochemical characteristics of *Nitrobacter* and *Thiobacillus* are shown in table 1.

GoldCrew and Biosolveused for the study were analyzed. GoldCrew appeared golden yellow with pH of 8.49, total dissolved solid – 5225mg/L,salinity-5880mg/L, total suspended solid- 47mg/L, total petroleum hydrocarbon- 3673.03mg/L. nitrate, phosphate and sulphate were 8mg/L, 15mg/L and 20mg/L respectively. Biosolve appeared pink with pH of 8.12, total dissolved solid – 2459mg/L,salinity-2620mg/L, total suspended solid- 110mg/L, total petroleum hydrocarbon- 2484.42mg/L. nitrate, phosphate and sulphate were 58mg/L, 11mg/L and 240mg/L respectively.

The two dispersants GoldCrew and Biosolve that served as the toxicants expressed different level of toxicity on the test organisms. However, a general pattern was recorded for all the dispersants in which the percentage of organisms that died with increasing concentrations and exposure time was more. The percentage mortality was used as an index in measuring toxicity, which implies that the higher the mortality recorded the more toxic the dispersant was to the test organisms. The lethal concentration of the dispersant able to kill fifty percent of the test organisms in 96hours is calculated as the 96hours – LC_{50} and is used to determine which of the dispersant was non toxic or least toxic to the organisms.

Figure 1 to 4 represents theresponse of the test organisms to GoldCrew and Biosolve dispersants.

In view of the concentration of the toxicant able to kill fifty percent of the test organism, the LC_{50} of the dispersants on *Nitrobacter*and *Thibacillus* was determined using Probit software.

Table 1: Morphological and biochemical characteristics of *Nitrobacter* sp. and *Thiobacillus* sp.

Tests	Isolate A	Isolate B
Colour of colony	Greyish/Whitish	Whitish
Shape of colony	Round	Round
Size of colony	Tiny	Tiny
Cell shape	Pear shaped short rod	Rod shaped
Gram Staining	-	-
Catalase	+	+
Motility	-	+
Urease	-	-
Indole	-	-
Coagulase	-	-
Oxidase	-	+
Citrate Utilization	-	+
Nitrate reduction	-	+
Nitrate oxidation	+	-
Sulphur oxidation	-	+

Methyl Red	-	+
VougesProskauer	-	-
Glucose	-	+
Mannitol	-	+
H_2S production	-	+
Starch hydrolysis	-	+
Suspected Organism	*Nitrobacter*sp.	*Thiobacillus*sp

Key: + = positive reaction and - = negative reaction.

Table 2: Lethal cancentration of toxicants on test organisms

Organism	Biosolve 96hour – LC_{50} (ppt)	Goldcrew 96hour – LC_{50} (ppt)
*Nitrobacter*sp	0.07	0.05
*Thiobacillus*sp	0.001	0.0006

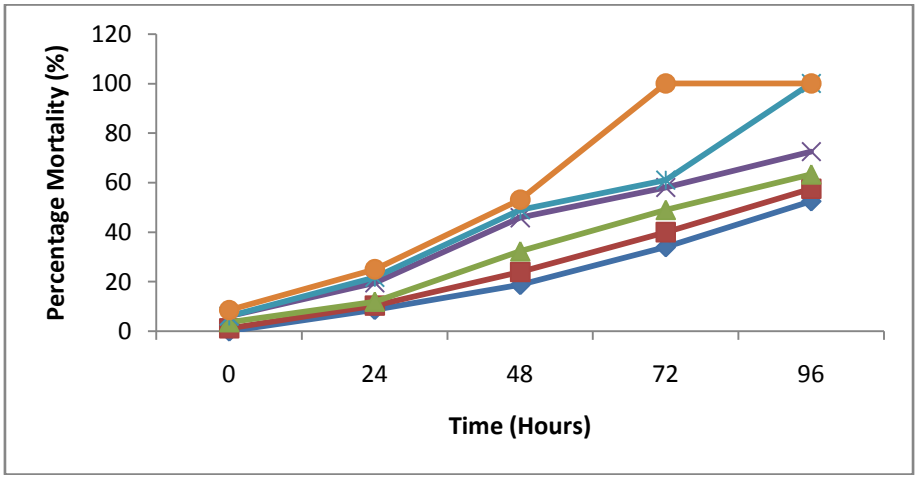

Figure 1: Response of *Nitrobacter* sp. to GoldCrew

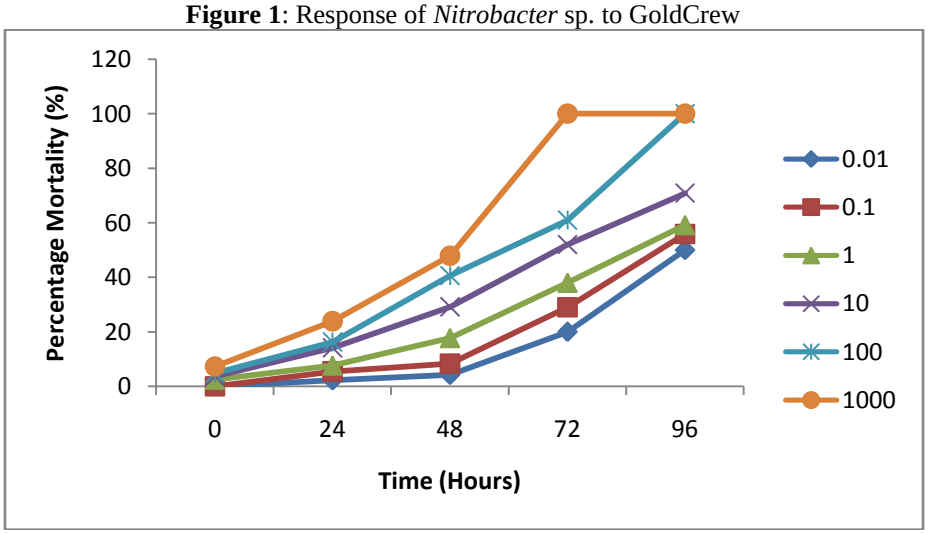

Figure 2: Response of *Nitrobacter* sp. to Biosolve

GoldCrew on *Nitrobacter* sp. (Fig. 1) for the first 24hourrs, concentrations of 0.01 and 0.1ppt exhibited some level of stimulatory effects on *Nitrobacter* after which it became inhibiting. However, concentrations 1, 10, 100, and 1000ppt were all inhibitory to the test organism. At 1000ppt concentration for 72hours exposure period, all the test organisms were completely inhibited by the toxicant, recording 100% mortality rate.Inspite of the high sensitivity of *Nitrobacter*sp to the toxicants, concentrations 0.01 and 0.1ppt of Biosolve (Fig. 2) did not

induce any form of mortality to the test organisms. However at 96hrs exposure period, the percentage mortality for both concentrations was reported as 50% and 55.83% respectively.

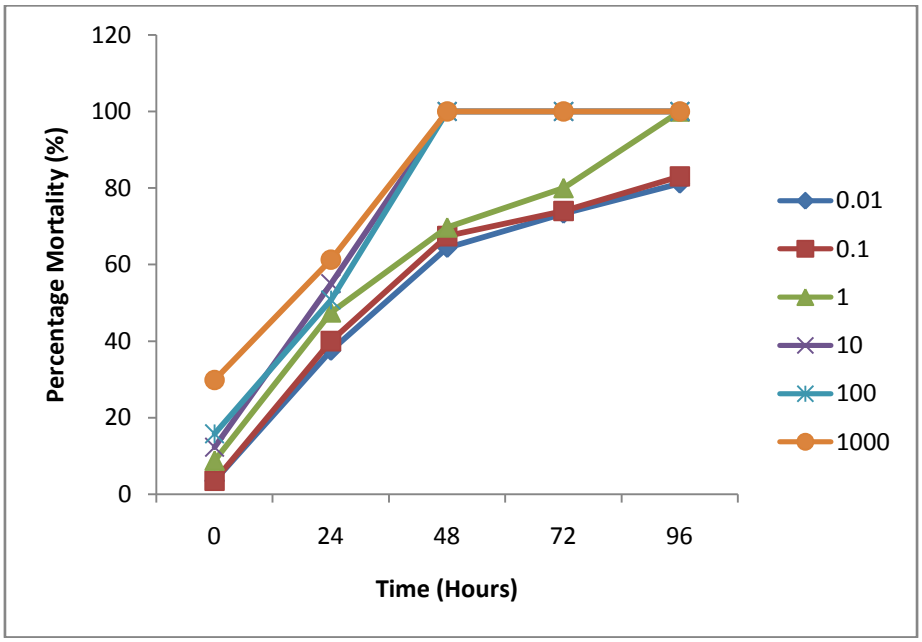

Figure 3: Response of *Thiobacillus* sp. to GoldCrew

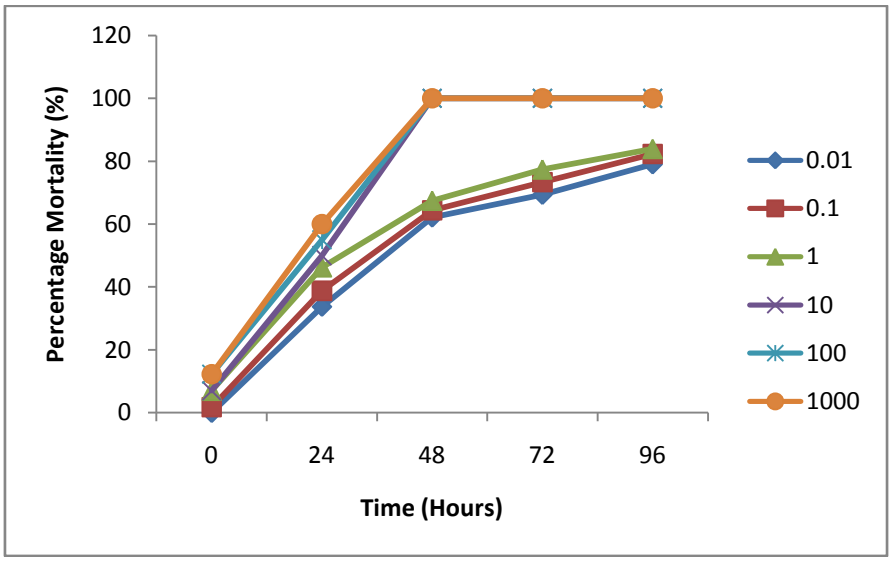

Figure 4: Response of *Thiobacillus* sp. to Biosolve

Goldcrew (Fig 3) which was used as a toxicant in the study at concentrations of 0.01, 0.1, 1, 10, 100, and 1000ppt, expressed a general increase in percentage mortality of*Thiobacillus* sp. with increase in exposure time. The highest toxic effect and mortality rates were recorded at concentration of 1000ppt, while the least mortality was recorded 0.01ppt concentration. However, the Goldcrew dispersant did not express any stimulatory effect on the test organisms; rather a decline in the number of organisms as compared to the control was recorded. Higher concentrations of Biosolve (Fig. 4) expressed very toxic effects on the test organism, expressed by the high rate of mortality at these concentrations. After 24 hours, the percentage mortality recorded for these concentrations were 100% respectively. Although lower concentrations of 0.01, 0.1, 1 and 10ppt did have up to 100% mortality, they however expressed varying degree of mortality in which the general pattern observed was an increasing mortality with increasing concentration and increasing exposure time.

IV. Discussion

The physicochemical results of the source of the test microorganism suggest that there had been no previous exposure to increased industrial activity or anthropogenic disturbances. *Nitrobacter* sp. and *Thiobacillus* sp.were isolated.The toxicity of the two test dispersants on two groups of test organisms were tested. However, it has been described by literature that the toxicity of complex mixtures such as surfactants cannot be unequivocally attributed to one or few compounds (Nrior and Odokuma, 2015).

The organisms that make up the two groups of test organisms in this study were the nitrifying bacteria and sulphur oxidizing bacteria made up of *Nitrobacter* sp. and *Thiobacillus*sp.

The result from this study clearly demonstrates the response of the test organisms to the dispersants. From the result, the test organism responded in varying degrees to dispersant. However a general trend was observed for all test organism. There was an increase in percentage mortality with increasing concentration of dispersants and increasing contact time. This result is in alliance with reports of Odokuma and Okpokwasili (1994), Odokuma and Akpponah (2008), Luke and Odokuma (2017). The controls showed an increase in the population of viable cells (apparently absence of mortality) with increase in exposure time.

Mortality was used as the index of toxicity of the dispersants on the test organisms (Odokuma and Akponah, 2008). The responses of *Nitrobacter* sp. to the toxicants with increasing concentrations are recorded in figures 1 and 2. Studies have confirmed that the target of toxicant activity on bacterial system include cell wall, cytoplasmic membrane, enzymes mediated activities and genetic machinery (Kobeticora*et al*., 2012). All results obtained followed a general pattern of increasing mortality with increasing concentration and time. For all test dispersants, it was recorded that at 1000ppt concentration and 96hours exposure period, all the test organisms were reportedly killed as 100% mortality was recorded.

The lethal effect of the toxicant on *Nitrobacter* sp. was used as an index of measuring the toxicity of the test dispersants on *Nitrobacter*. However, lower concentrations of 0.01ppt and 0.1ppt were stimulatory to *Nitrobacter* at the initial 24 hours exposure, after which it became inhibitory.

As compared with the *Nitrobacter* sp., the sulphur oxidizing bacteria, *Thiobacilus* sp was more susceptible to the toxicant than *Nitrobacter* sp.. Concentrations of 10ppt of all toxicant samples were shown to express 100% mortality on *Thiobacillus* at 48 hours exposure period. The trend observed for all the toxicants samples were increasing percentage mortality with increasing exposure period and increasing concentration. Similar observations have been made by Okpokwasili and Odokuma (1997) who observed an increase in percentage log mortality with increasing contact time and concentration when *Nitrobacter* was exposed to three oil spill dispersants and five domestic detergents. Okpokwasili and Odokuma (1997) suggested that the toxicity of dispersants of microbial population might have resulted from dispersant effect on any of the target sites of the organisms. The site of action of a toxicant is a function of the nature, concentration and contact time of the toxicant (Odokuma and Okpokwasili, 2003). The present study revealed that the dispersants caused cell mortality leading to a reduction in the viable cell count. All two microbial species exhibited this effect.

The main index for determining the toxicity of the dispersants on the test microbial species *Nitrobacter* and *Thiobacillus* was the determination of the 96 hours – LC_{50} of the toxicants on the test species. This index has been used by Odokuma and Akponah (2008), Okpokwasili and Odokuma (1997), Nrior and Odokuma (2016), Luke and Odokuma (2017). The methods express the lethal dose or concentration of the toxicant sample able to kill fifty percent of the test organism in 96 hours. The values are representatives of the concentrationof the toxicant able to kill 50% of the organism. The method for calculation of the 96 hours – LC_{50} of the toxicants was Probit method for determination of LC_{50} as recommended by DPR in EGASPIN (2002). This method have been previously used by Odokuma and Akponah (2008); Nrior and Odokuma (2016), Luke and Odokuma (2017). The 96 hours – LC_{50} for GoldCrew on *Nitrobacter* and *Thiobacillus* were revealed to be 0.05 and 0.0006 ppt. With *Thiobacillus* being the most susceptible to the toxic effect of GoldCrew, the trend in increasing order was *Thiobacillus*>*Nitrobacter*.

Biosolve however expressed a lesser toxic effect on the test organisms when compared with GoldCrew. 96 hours – LC_{50} of Biosolve on *Nitrobacter* and *Thiobacillus* were 0.07 and 0.001ppt respectively. The order was also similar to those reported for GoldCrew.

V. Conclusion

At high concentration and exposure time, the growth and activities of the organisms were influenced. From the results of 96 hours – LC_{50} of the two dispersants (GoldCrew and Biosolve) on the independent test organisms, GoldCrewhad the lowest LC_{50} value by Biosolve. The trend observed for *Nitrobacter* was GoldCrew>Biosolve and the trend also for *Thiobacillus* was GoldCrew>Biosolve.Generally it was revealed that GoldCrew was more toxic than Biosolve. The toxicity of these dispersants inhibits the growth of these microorganisms and this can upset the balance of biogeochemical cycles where they are key players.

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