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Ecotoxicity of Gammalin 20 on *Nitrobacter vulgaris* and *Nitrosomonas halophila* in Different Aquatic Environments

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ABSTRACT

Aquatic ecosystems have been shown to be adversely affected by pesticides such as Gammalin 20, which can have an adverse effect on primary and secondary producers. This study investigated the ecotoxicity of gammalin 20 on *Nitrobacter vulgaris* and *Nitrosomonas halophila* in Freshwater, Marine water and Estuarine water samples collected from Adoni Local Government Area, Rivers State. The test isolates were isolated from roots of aquatic plants on Winogadsky agar using standard microbiological method. Various concentrations (0, 5, 15, 25, 50 and 75%) of the toxicant were prepared and transferred into conical flasks containing different measurements of the various water samples. The toxicity test and determination of Lethal concentration (LC_{50}) of the toxicant on the isolates was conducted at 4 hours intervals for 24 hours. The mean percentage survival of *Nitrobacter vulgaris* in different aquatic environments exposed to different concentrations of gammalin 20 after 24 hours in marine, estuarine and freshwater were 5.013±11.21 to 100±0.0, 24.84±25.75 to 100±0.0 and 14.52±13.26 to 100±0.0%, respectively. There was a significant difference ($P<0.05$) in the percentage survival of test organisms in all the aquatic environments. The mean percentage survival *of Nitrosomonas halophila* in known aquatic environments mixed with known concentrations of gammalin 20 after 24 hours in marine water, estuarine water and freshwater ranged from 4.86±10.87 to 100±0.0, 0.00±000 to 100±0.0 and 6.85 ±15.32 to 100±0.0%, respectively. The *Nitrosomonas* load of the control in all the aquatic environments was significantly ($P < 0.05$) higher than other concentrations with the 75% concentrations having the lowest survival counts. The LC_{50} for *Nitrosomonas halophila* ranged from 13.40 to 16.89 while that of *Nitrobacter vulgaris* was 13.10 to 27.88. Thus, gammalin had low LC_{50} on both isolates in freshwater than in other aquatic environments. Therefore use of Gammalin 20 in aquatic environment is not recommended.

Keywords: Ecotoxicity, Gammalin 20, *Nitrobacter vulgaris*, *Nitrosomonas halophila*, LC_{50}, aquatic environments.

Introduction

Gammalin 20 (Lindane), also known as gamma-hexachlorocyclohexane (gamma HHC or BCH), is an organochlorine pesticide that was once extensively used for controlling various agricultural pests and parasites (Oni *et al.*, 2020). It belongs to the group of chlorinated hydrocarbons and has been employed in agriculture, forestry, and public health applications (Lawson *et al.*, 2011).

The active ingredient in Gammalin-20 is lindane which is used extensively in the treatment of seed, and making of shampoo and lotion cream for the control lice and mites in humans and other domestic animals (Lawson *et al.*, 2011). However, Lindane has been reported to be highly toxic to bees, fish, and other aquatic organisms.

It is being used to control a wide range of crops to aphididae larvae of coleopteran and diptera. Gammalin 20 have been implicated in illegal fishing and some of its ecotoxicological signs and symptoms among several others includes; difficulty in breathing, irritability, convulsion, staggering and death (Dede and Dogara, 2005; Oni *et al.*, 2020).

The compound has been classified as persistent organic pollutant (POP) and it is subject to international restrictions under agreements and Stockholm Conventions on "Persistent Organic Pollutants" (Ezemonye and Ogbomida, 2010; Radhaiah *et al*., 1987), although there was specific exemption which allowed its use as second line treatment for lice and scabies in the United State (Nolan *et al*., 2012).

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The unregulated release of agrochemicals, particularly pesticides herbicide into the environment may pollute adjoining water bodies including groundwater and could pose a significant threat to the health of aquatic ecosystems including the integrity of groundwater (Odokuma and Nrior, 2015; Ahmad *et al*., 2024). Pesticides are designed to control or eliminate pests in agricultural settings, but when they enter water bodies through runoff or direct application, they can have detrimental effects on all levels of organisms in that inhabit (Ezemonye and Ogbomida, 2010).

The loss of biodiversity in aquatic ecosystems can have far-reaching consequences (Ezemonye and Ogbomida, 2010). One course of 19 part per trillion (ppt) topical lindane would contaminate an estimated 8 million gallon of water (Nolan *et al*., 2012). It is important to note that while Lindane has been effective in pest control, it is also classified as an organochlorine pesticide, and its use has raised environmental and health concern (Jayaraj *et al*., 2016).

The aquatic ecosystems are intricate and vital components of the Earth, harboring an abundance of life at the microscopic level. Microorganisms, including bacteria, fungi, and algae, thrive in these aquatic environments, participate in fundamental ecological processes that maintain the health and balance of the ecosystems (Ankit *et al*., 2017; Oni *et al.*, 2020).

Introduction of chemical substances could alter the biodiversity of aquatic environment which could result to the imbalance in nutrient cycle and other biogeochemical processes. *Nitrosomonas* sp and *Nitrobacter* sp are implicated in the conversion of atmospheric nitrogen into a useable form for plants both in aquatic and terrestrial ecosystem, by extension they are key players in nitrogen cycling.

This study investigated the ecotoxicity of gammalin 20 on *Nitrobacter vulgaris* and *Nitrosomonas halophila* in different aquatic environment, to monitor the effect of the selected toxicant on the test isolate and its implication on the nitrogen cycle.

Material and Methods

Study Area

The study area was in Andoni Local Government Area in Rivers State, South-South, Nigeria. Andoni Local Government Area is among the 23 Local Government Areas in Rivers State. Andoni Territory extend from the Andoni River (7.21 E) to the Qua Iboe River (8.00 E). The area is bounded by Bonny and Kalabari to the West, Okirika and Ogoni to the north, Ibibio to the north-east, Ibeno to the east and the Atlantic Ocean to the south (Ejituwu, 1991). The rivers and creeks of Andoni Local Government Area of Rivers State remain the home of large splendor of biodiversity ranging from microbes, wildlife, fishes, brackish mangroves and other exotic aquatic life which has been the source of revenue for the people apart from the oil exploration in the area. The people of Andoni (Obolo) are predominately fisher men and women and treaders. Thus, their livelihood depends on the surrounding aquatic ecosystem in the Local Government Area.

Collection of Water Sample

Freshwater sample was obtained from Mudim Ejitorong Asarama in Andoni Local Government Area with coordinates latitude: 4.5170, longitude: 7.4640; the stream runs through the lengths of Asarama, Ataijong and Dimmama Asarama and empty into the river that separates Asarama from Nkoro in Opobo/Nkoro Local Government Area. Estuarine water was collected from the Okwan Odung-ama river separating Asarama and Ebukuma (Asarama Ebukuma Bridge), in Andoni Local Government Area with coordinates latitude 4.5260, longitude 7.4520. The Marine water sample was collected from Ibegi the section of Atlantic Ocean with coordinates latitude; 4.5010, longitude; 7.4680, bordering Ikuru Town and adjoining communities in Andoni Local Government Area. The Map of Study Area is presented in Figure 1.

Collection of Toxicant

The toxicant used for this study was Gammalin 20 and it was obtained from a local fumigator at 10 Station Road, Port Harcourt, Rivers State.

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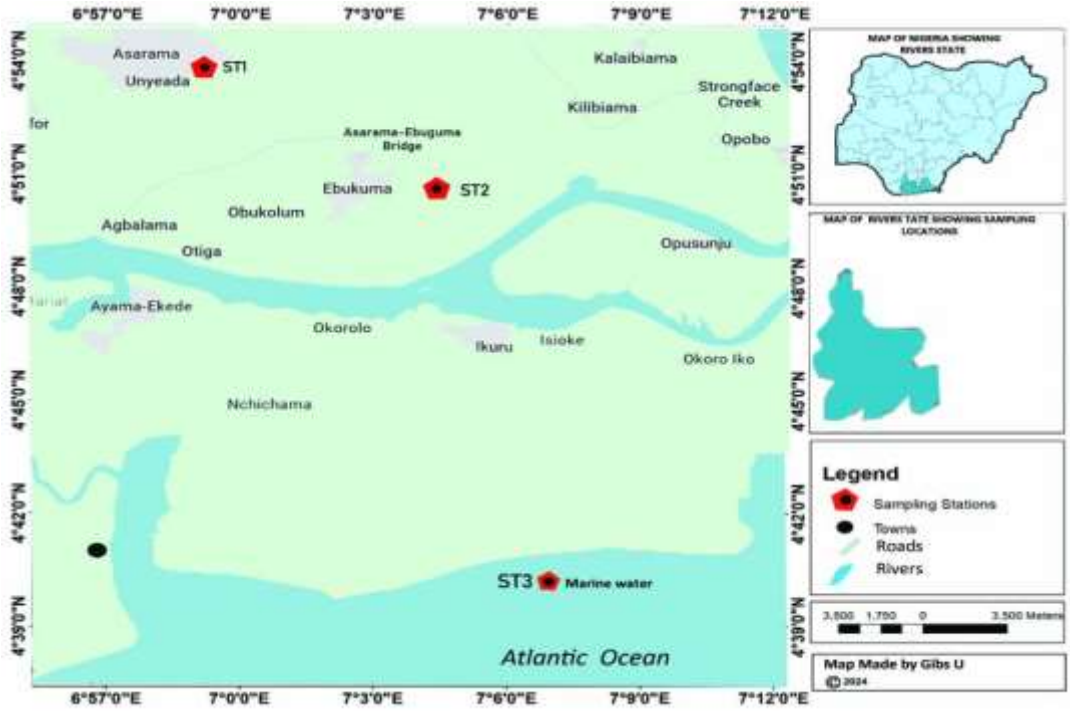

Fig. 1: Map of study area showing locations of sampled aquatic environments

Microbiological Analysis

Isolation of *Nitrosomonas halophila* and *Nitrobacter vulgaris*

The microbiology technique used in the isolation of *Nitrosomonas halophila* and *Nitrobacter vulgaris* (test organisms) using Winogradsky agar was adopted from (Odokuma and Nrior, 2015)

The composition of Winogradsky medium for isolation of *Nitrobacter* sp; K_2HPO_4 - 0.5g, Na_2CO_3 – 1 g, $NaNO_2$ - 0.05 g, NaCl – 0.3 g, $ZnCl_2$ - 0.03 g, $MnSO_4$. H_20 - 0.02 g, $FeSO_4.7H_20$ - 0.02g, Agar - 15g, Distilled water – 1,000 ml. while

The composition of the medium for isolation of *Nitrosomonas* sp; K_2HPO_4 - 0.5g, $(NH_4)_2SO_4$ – 2 g, NaCl – 2 g, $FeSO_4.7H_20$ - 0.4 g, $CaCO_3$ – 0.01g, Agar - 15g, $MgSO_4$. $7H_20$ - 0.5g, Distilled water – 1,000 ml.

Isolation and Characterization of Test Bacteria

The test organisms *Nitrobacter vulgaris* and *Nitrosomonas halophila* used in this study were isolated from water sample collected close the root of mangrove (aquatic plants) in the sampling station using sterile container.

An aliquot (0.1 ml) of water sample was aseptically transferred onto well dried Winogradsky medium and generously spread with a flamed bent glass rod evenly on plate and incubated for 48 hours at 37^0C.

Distinct colonies on the agar plate for *Nitrosomonas* sp and *Nitrobacter* sp were subcultured onto different nutrient agar freshly prepared using streak method. The pure culture was aseptically transferred into 10% (v/v) glycerol suspension and store at – 4^0C which serve as stock cultures (Douglas *et al.*, 2018).

The pure isolates were further subjected to standard biochemical tests for identification in the laboratory (Douglas *et al*., 2018) the samples were further subjected to molecular identification.

Preparation of Bacterial Inoculum

The discrete colonies of pure culture *Nitrosomonas halophila* and *Nitrobacter vulgaris* obtained were inoculated separately into freshly prepared 100 ml peptone water broth in 100 ml conical flask and incubated for 24 hours. This served as bacterial inoculum for the toxicity test.

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Toxicity Test for Bacteria

The toxicity was done using a setup of fifteen (15) 100 ml conical flasks capped with cotton wool wrapped in foil paper containing sterile water from the three aquatic environment. Five (5) conical flask was use for each aquatic environment, four (4) contains the different concentration (5%, 25% 50% 75%) of toxicant concentration in 100 ml flask and the fifth conical flask was the control, containing no toxicant.

Aliquot of 1.0 ml of test organism inoculum was aseptically transferred into various concentrations (75 %, 50 %, 25 % and 5 %) of prepared toxicant concentration respectively. Aliquot (0.1 ml) from each of the concentration from the setup containing test organism was plated out on well dried Winogradsky agar immediately after addition and spread evenly using bent grass rod, this was then incubated for 48 to 72 hours at 37 ± 2 ^{0}C this was recorded for zero hour and was repeated after 4, 8, 12, and 24 hours respectively and colonies of test organism on the plates were counted and counts express as colony forming unit (CFU/ml) then converted logarithm based 10 ($\log_{10}$) (Koops *et al*., 2004; Nrior and Gboto, 2017; Douglas *et al*., 2018; Kpormon and Douglas, 2018)

Percentage (%) Log Survival of the Bacteria in the Different Toxicant

Percentage log survival of the test isolate was obtained by the formula adopted from Nrior and Odokuma, (2017); Williamson and Johnson (1981). The Percentage log survival of the bacterial isolate is obtained by dividing the log of counts in each toxicant concentration, by the log of the count of control, multiplied by 100. Thus:

$$\% \text{ Survival} = \frac{\text{Log Count of toxicant Concentration}}{\text{Log Count of control}} \text{ X } 100. \qquad Eqn.\ 1$$

Percentage Mortality of the Isolates

The calculation of percentage mortality of the test organism was adopted from the formular from (Douglas *et al.*, 2018; Nrior and Obire, 2015). The percentage mortality was obtained from the formular

$$\% \text{ Mortality} = 100 - \% \log survival \qquad Eqn.\ 2$$

Median lethal concentration (LC_{50}) Determination

The lethal concentration of gammalin 20 in the tri-aquatic environment were determined by the formular (Douglas *et al.*, 2018)

$$\text{Lethal Conc.}\ (LC_{50}) = \frac{\text{Log } \sum \text{Con. Diff} \times \text{Mean \% Mortality}}{\text{\% control}} \text{ x } 100. \quad Eqn.\ 3$$

LC_{50} = Lethal concentration
LC_{100} = Value of the highest concentration value used
Σ conc. Diff = summation of concentration difference
mean % mortality.

Statistical Analysis

All data in this study were expressed as mean ± standard deviation. Statistical analysis was carried out by two-way analysis of variance (ANOVA) to compute statistically significant differences at $p < 0.05$. Duncan multiple range test was used to separate the means. All graphics were constructed using Microsoft Excel V21 while the statistical tool used was SPSS (v27).

Results

The colonial, morphology and biochemical characteristics of the isolates are presented in Table 1. The results showed that the isolates were in accordance with the *Nitrobacter* and *Nitrosomonas* sp.

The molecular characteristics confirmed the isolates were within the database of *Nitrobacter vulgaris* and *Nitrosomonas halophila* and showed 100% similarity index thereby confirming the identity of the isolates.

The Phylogenetic tree of the evolutionary relationship between the bacterial Isolates is presented in Fig. 1. Results showed that isolate NIT.B1 had 100% similarity index with *Nitrobacter vulgaris* NZ_MWPQ01000085.1, isolate NIT.S2 had 97.78% similarity index with *Nitrosomonas europaea* AF353160.1.

On the other hand, isolate NIT.S1 had 100% similarity index with *Nitrosomonas halophila* AF272413.1. *Nitrobacter vulgaris* and *Nitrosomonas halophila* were used as microbial test organisms throughout of this research.

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Table 1: Colonial, Morphological and Biochemical Characterization and Identification of Bacterial Isolates

Isolates	Cultural	Gram rxn	Catalase	Oxidase	Coagulase	Urease	Indole	Citrate	Nitrate reduction	Ammonium reduction	Glucose	Lactose	Probable ID
NIT.B1	Greyish, mucoid, flat colonies	-ve rod	+	-	-	-	-	-	-	-	A	-	*Nitrobacter* sp
NIT.S1	Whitish small round mucoid wet	-ve rod	+	-	-	-	-	+	-	+	A	-	*Nitrosomonas* sp

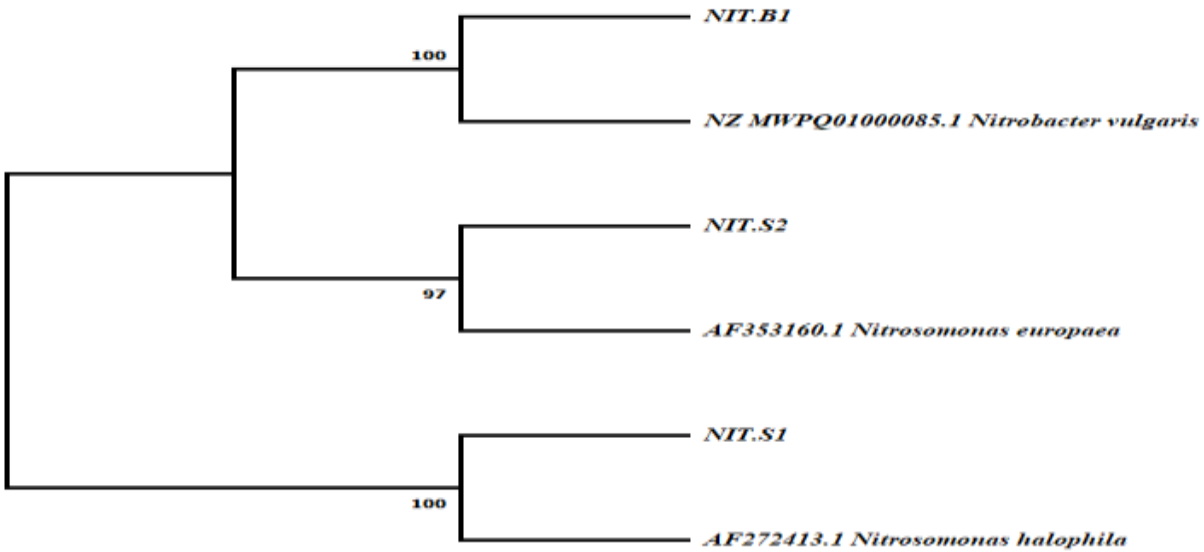

Fig. 1: Phylogenetic Tree of the evolutionary Relationship of the bacterial Isolates

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Toxicity Testing Results

Results of the mean percentage survival of *Nitrobacter vulgaris*. in different aquatic environments exposed to various concentrations of Gammalin 20 (G20) after 24 hours (Fig. 2) showed that the mean percentage survival of the isolate in marine, estuarine and freshwater were 5.013±11.21 to 100±0.0, 24.84±25.75 to 100±0.0 and 14.52±13.26 to 100±0.0%, respectively. There was a significant difference ($P<0.05$) in the percentage survival of test organisms in all the aquatic environments.

The result of the mean percentage survival of *Nitrosomonas halophila* in different aquatic environments mixed with different concentrations of Gammalin 20 after 24 hours is presented in Figure 3. It shows that the mean percentage survival of the isolate in marine water, estuarine water and freshwater ranged from 4.86±10.87 to 100±0.0, 0.00±000 to 100±0.0 and 6.85 ±15.32 to 100±0.0%, respectively. The *Nitrosomonas* load of the control in all the aquatic environments treated with the toxicant was significantly ($P < 0.05$) higher than the microcosm treated with various concentrations of gammalin 20 with the 75% concentrations having the lowest survival counts.

The lethal concentration of 50 (LC_{50}) for *Nitrobacter vulgaris* and *Nitrosomonas halophila* in the toxicant is presented Table 2.

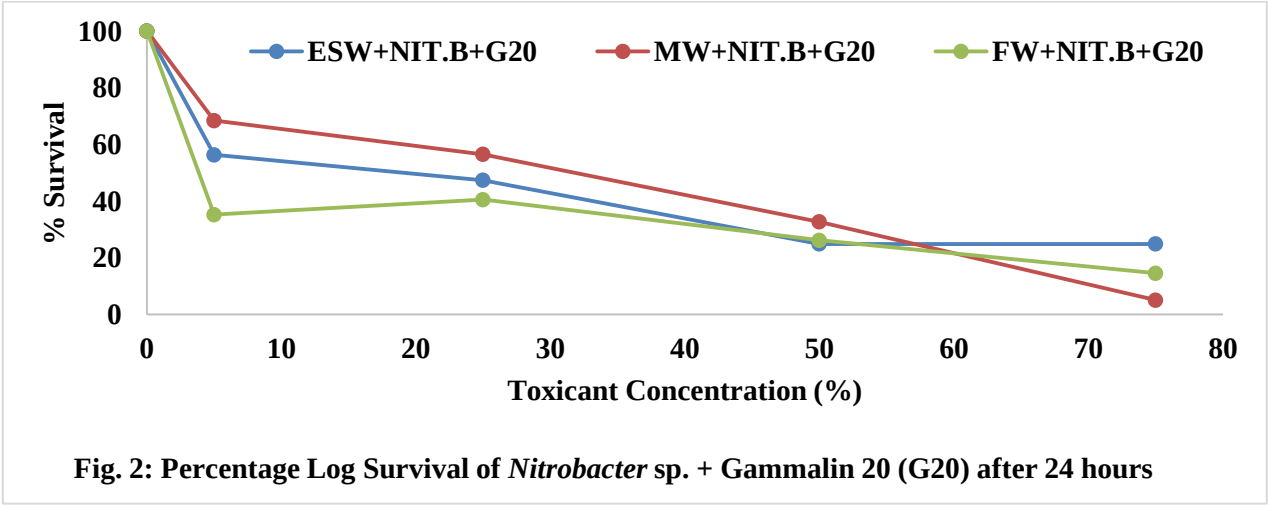

Fig. 2: Percentage Log Survival of *Nitrobacter* sp. + Gammalin 20 (G20) after 24 hours

*** Keys:** NIT.B = Nitrobacter, ESW = Estuarine Water, MW = Marine Water, FW = Freshwater, G20 Gammalin 20

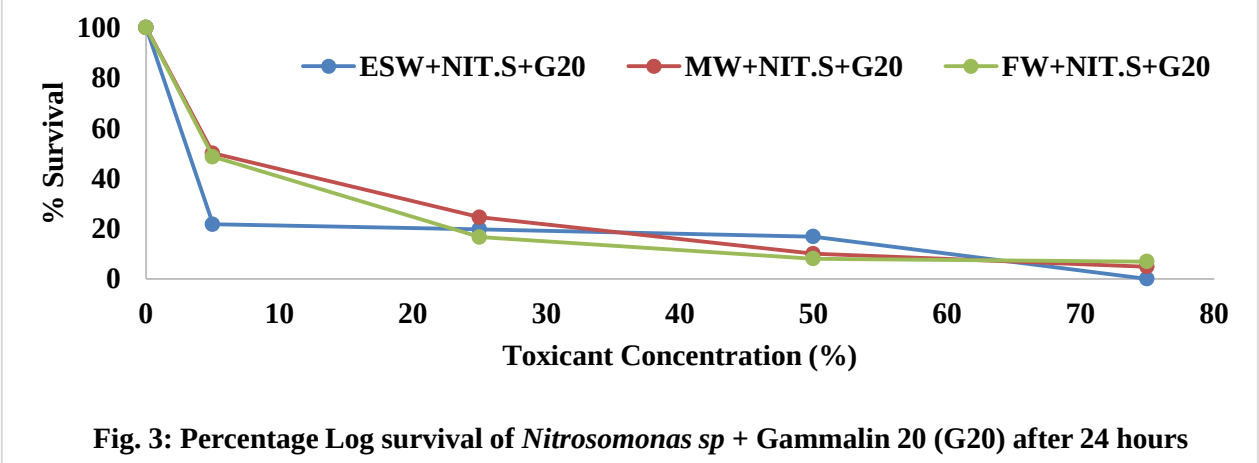

Fig. 3: Percentage Log survival of *Nitrosomonas sp* + Gammalin 20 (G20) after 24 hours

Keys: NIT.S = Nitrosomonas, ESW = Estuarine Water, MW = Marine Water, FW = Freshwater, G20 Gammalin 20

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Table 2: **Lethal Concentration 50 (LC_{50}) of *Nitrobacter* and *Nitrosomonas* in Various Toxicant**

Toxicant	Organisms	Marine Water	Estuarine Water	Freshwater
Gammalin 20	*Nitrosomonas halophila*	16.89	14.71	13.40
	Nitrobacter vulgaris	17.88	17.60	13.31

Discussion

Gammalin 20 have enormous ecotoxicity effect on both the bacteria and higher organism in an aquatic ecosystem where it is used (Teklit and Semere, 2016). Gammalin 20 is enormously toxic as its ecotoxicity potential was tested against *Nitrobacter vulgaris* and *Nitrosomonas halophila* in trio-aquatic ecosystems. The LC_{50} of *Nitrobacter vulgaris* in various aquatic ecosystem are 13.31, 17.60, 17.88 in freshwater, estuarine water and marine water respectively. While that for *Nitrosomonas halophila* was observed to be 13.40, 14.71, 16.89 in freshwater, estuarine water and marine water respectively. Findings showed that *Nitrosomonas halophila* is more sensitive to gammalin 20 compared to *Nitrobacter vulgaris* especially in freshwater. However, *Nitrobacter vulgaris* is slightly more tolerance to gammalin 20, especially in marine ecosystem compared to *Nitrosomonas halophila.* The observed variation in the LC_{50} between the test organism may have resulted in their different pathways of metabolizing and biotransformation of gammalin 20 among the organisms (Johnson and Toledo, 1993; Lawson *et al*., 2011). From this observation the continued use of gammalin 20 in aquatic environment for purpose of illegal fishing can alter or inhibit the rate nitrification and by extension the aquatic life as evidence in its toxic nature to *Nitrosomonas halophila* and *Nitrobacter vulgaris* involve in nitrification. The observation from this study is in agreement with the report of Nrior and Odokuma, (2017) that suggested that the high mortality recorded on the exposure *Nitrobacter* to the degreaser (Aquabreak) after 24 hours may be due to the inhibition of nitrification process or direct cell death resulting from exposure to toxicant. It could also be due to the inhibition or alteration of steps in the metabolic activity of the organism. In a previous study, shock-related stress caused by the introduction of a toxic substance have been reported to cause damage and death of cells (Ihechu *et al.*, 2023).

In conclusion, this study has showed that, the effect of gammalin 20 on *Nitrosomonas halophila* and *Nitrobacter vulgaris* in the tri-aquatic environment - Marine water, Estuarine water and Freshwater varied; implying that the physicochemical parameters of the water could have influenced the activity of the chemical on the organisms. More so, despite gammalin 20 being a potent chemical on the isolates, it had high toxicity on both isolates in freshwater than in marine and estuarine environment. The use of this chemical should be disregarded in fishing and other agricultural activities.

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