Effect of Glyphosate on Dehydrogenase Activity of Bacterial and Filamentous Fungal Isolates from a Freshwater Sediment

C. O. Aneni* and C. N. Ariole

Department of Microbiology, University of Port Harcourt, P.M.B. 5323, Port Harcourt, Rivers State, Nigeria.

Abstract: The effect of glyphosate on bacteria and filamentous fungi isolated from Yeghe River sediment in Bori LGA, Rivers state, Nigeria was investigated. The isolates were phenotypically identified using standard methods and assayed to determine the Minimum Inhibitory Concentration (MIC) of the glyphosate. The dehydrogenase activity (DHA) of the most sensitive isolates were determined using 2, 3, 5-triphenyltetrazolium chloride (TTC) as the artificial electron acceptor. The triphenylformazan (TPF) produced was extracted in 4 ml of amyl alcohol and determined spectrophotometrically at 500 nm. The DHA was expressed as the mg of TPF formed per dry weight of cell biomass per hour and was plotted against the glyphosate concentration. A total of thirteen (13) bacteria of the *generaAmphibacillus, Brochothrix, Aeromicrobium, Staphylococcus, Sporosarcina, Kurthia, Enterococcus, Acinetobacter, Erysipelothrix, Vagococcus, Alcaligenes, Caryophanon* and *Escherichia*, and nine (9) moulds of the genera *Microsporum, Acrophialophora, Colletotrichum, Histoplasma, Trichophyton, Aspergillus, Cladosporium, Scedosporium* and *Chrysosporium* were isolated. The result of MIC assay showed that 50% of the bacteria and 0% of the mould isolates were susceptible to concentrations of the glyphosate between 0.075 and 5.0 mg/ml while all isolates were susceptible-to concentration of 9.0 mg/ml glyphosate. However, all the microbial isolates used for DHA were found to be sensitive to concentration of 0.075 mg/ml glyphosate, showing that DHA is more sensitive than the MIC assay in toxicity testing. The results have also shown that low concentrations of glyphosate, usually associated with run-off and spray drifts, are sufficient to reduce the dehydrogenase activity of microorganisms and have the capacity to debilitate freshwater systems.

Keywords: Bacteria, dehydrogenase activity, filamentous fungi, freshwater sediment, glyphosate, minimum inhibitory concentration, triphenylformazan, triphenyltetrazolium chloride.

Introduction

Microorganisms play an important role in the macro and micro-environments of aquatic systems, whether at the lotic or benthic levels. These microorganisms function as primary producers, decomposers and nutrient recyclers; specific roles which are necessary for maintaining the biogeochemical balance of the environment. A disruption or change in the population, diversity, biomass or microbial activity of the microorganisms within the environment due to the influence of external factors will lead to a change in the community composition, with probable far reaching implications for the environment (Pepper *et al.*, 2015).

Microbial activity influences the ecosystem stability and thus can be used as an indicator of the environmental condition. Consequently, the enzymatic activities present within a particular environment can also serve as an indicator as they are maintained by microbial processes (Kumar *et al.*, 2013). Enzyme activities in soil (and sediment) are very sensitive to natural and anthropogenic disturbances and show a quick response to these changes (Kumar *et al.*, 2013). Dehydrogenase activity is a parameter of microbial activity which is influenced by many factors and it is used as a common method of estimating potential microbiological activity (Beyer *et al.*, 1992).

*Corresponding author
christopher.aneni@yahoo.com; ***C. O. Aneni***

In the world today, due to ever-increasing anthropogenic activities in the environment, an increasing number of organic and inorganic chemical substances are constantly introduced into the environment. A large percentage of them end up as run-off into aquatic bodies where they settle at the sediment level and contribute to influencing changes in the community compositions within the environments. Of these external factors, pesticides play a major role (Widenfalk, 2005). Pesticides are toxic substances which are intentionally released into the environment to kill living things. They fall into different classes: herbicides (used to kill weeds), rodenticides (for rodents), insecticides (for insects) and fungicides (used to kill fungi) (Widenfalk, 2005).

Glyphosate has been advertised as the most popular herbicide in the world by its manufacturer, Monsanto (*Farmer'sWeekly*,1992).

Glyphosate (N-(phosphonomethyl) glycine) is a broad spectrum herbicide used in over 130 countries – including Nigeria, as at 2010 (Dill *et al.*, 2010). In Nigeria and the study area, the herbicide Glyphosate (Uproot®) is the most commonly used herbicide.

This study aims to contribute information on the effect of Uproot®'s Glyphosate (active ingredient N-(phosphonomethyl) glycine in the form of Isopropylamine salt) on microbiota isolated from the sediment of a freshwater as well as contribute information on the microbial diversity of the Yeghe River in Bori Local Government Area of Rivers State, Nigeria. The assay will also demonstrate a comparison on the efficacy of the Dehydrogenase assay and the

Minimum Inhibitory Concentration Assay used in the assessment of the Glyphosate effect on microorganisms.

Materials and Methods

Sample Collection

The sediment samples were collected along the course of Yeghe River in Bori Local Government area, Rivers state, Nigeria.

Source of Glyphosate

The herbicide (Uproot®) contains 360 g/litre of Glyphosate in the form of 480 g/litre Isopropylamine salt. It was purchased from an agro-chemical shop in Port Harcourt, Rivers State, Nigeria.

Bacterial Isolation and Identification

Twenty five (25) grammes of the sediment was aseptically suspended in 225 ml of sterile physiological saline and serially diluted up to 10^{-5} dilution. Sub samples of 0.1ml of the dilutions were cultured on sterile nutrient agar plates in triplicates using spread plate method. The plates were incubated at 37°C for 24 – 48 hours. Isolates with distinct colony morphology were picked and streaked repeatedly on nutrient agar plates until pure. The purified isolates were identified to generic level based on their morphological and biochemical characteristics (Holt *et al.*, 1994; Garrity *et al.*, 2005; De La Maza *et al.*, 2013).

Fungal Isolation and Identification

Twenty five (25) gramme of the sediment was aseptically suspended in 225 ml of sterile normal saline and serially diluted up to 10^{-5} dilution. To sterile plates of acidified potato dextrose agar, sub samples of 0.1ml of the dilutions were inoculated in triplicates using spread plate method. The plates were incubated at 28°C for 5-7 days. A portion of each fungal colony which developed was picked with a sterile inoculating needle and aseptically subcultured into fresh acidified potato dextrose agar plates for purification. The mould isolates were examined macroscopically and then microscopically (Samson and De Boer, 1995). Through this examination of the isolated moulds, in conjunction with the cultural characteristics as well as the back view of the plate culture, the moulds were identified (Samson and De Boer, 1995; De Hoog *et al.*, 2000; Larone, 2011). Yeast isolates were characterized based on their colonial morphology, cell micromorphology and biochemical characteristics. Identification to generic level was performed using the keys provided by Samson and De Boer (1995).

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) is given as the lowest concentration of an antimicrobial agent that is capable of inhibiting growth of a particular organism. The MIC of glyphosate was prepared for a twofold dilution to give varying concentrations of 5.0, 2.40, 1.20, 0.60, 0.30, 0.15 and 0.075 mg/ml as prescribed by Shehata *et al.* (2012). Two fold dilutions for 9.0, 8.0, 7.0, 6.0 mg/ml were further prepared.

One millilitre of each of the dilutions was added and mixed with 18 ml of molten nutrient agar in McCartney bottles and then poured in pre-sterilized Petri dishes and then allowed to set and dry properly before streaking with overnight broth cultures of isolates. Fungal isolates were picked and dropped in the centre of the plates for the moulds while yeast isolates were streaked. The bacterial plates were labeled accordingly and incubated at 37°C for up to 72 hours. Fungal isolates were incubated at 28°C for up to 96 hours. Examination was done for the presence or absence of growth, with the lowest concentration where growth was absent taken as the MIC of the herbicide for the particular organism.

Dehydrogenase Assay

The dehydrogenase assay method as described by Akujobi *et al.* (2010) and Nweke *et al.* (2007) was adopted for the study. The dehydrogenase activity (DHA) was determined using 2, 3, 5-triphenyltetrazolium chloride (TTC) as the artificial electron acceptor which was reduced to the red coloured triphenylformazan (TPF). The assay was carried out in 4 ml volumes of nutrient broth-glucose-TTC medium supplemented with varying concentrations (0- 5.0 mg/ml) of glyphosate as recommended by Akujobi *et al.* (2010) and further concentrations between 6.0 – 9.0 mg/ml of the glyphosate dilution were also prepared in separate screw-capped test tubes.

Exactly 0.3 ml volume of the bacterial suspension was inoculated into triplicate glass tubes containing 2.5 ml of phosphate-buffered (pH 6.8) nutrient broth-glucose medium supplemented with varying concentrations of the glyphosate. They were incubated in a rotary incubator (150 rpm) at room temperature (28 ± 2°C) for 30 min. Thereafter, 1 ml of 0.4% (w/v) TTC in deionized water was added to each tube to obtain final glyphosate concentrations of 0.075, 0.15, 0.3, 0.6, 1.2, 2.4 and 5.0 mg/ml in different test tubes.

The control consisted of the isolates and the medium without glyphosate. The reaction mixtures were further incubated statically at room temperature (28 ± 2°C) for 16 h. The triphenylformazan (TPF) produced was extracted in 4 ml of amyl alcohol and determined spectrophotometrically at 500 nm. The amount of TPF produced was plotted against the concentration of glyphosate applied to give a response curve for the organism. The Dehydrogenase activity (DHA) was plotted against the glyphosate concentration. The DHA is expressed as the mg of TPF formed per dry weight of cell biomass per hour.

Results

Fourteen bacterial genera (*Amphibacillus*, *Brochothrix*, *Aeromicrobium*, *Staphylococcus*, *Sporosarcina*, *Kurthia*, *Enterococcus*, *Acinetobacter*, *Erysipelothrix*, *Vagococcus*, *Alcaligenes*, *Caryophanon*, *Streptococcus* and *Escherichia*) were isolated from the sediment sample and characterized based on cultural, morphological and biochemical characteristics (Table 1). Nine fungal genera (*Microsporum*, *Acrophialophora*, *Colletotrichum*, *Histoplasma*, *Aspergillus*, *Trichophyton*, *Cladosporium*, *Scedosporium* and *Chrysosporium*) were isolated from the sediment sample and were characterized based on colony morphology and microscopic characteristics (Table 2).

Table 1: Minimum Inhibitory Concentration (MIC) of glyphosate for bacterial isolates

Isolate code	Probable identity	MIC (mg/ml)
BS1	*Brochothrix* sp.	2.4
BS2	*Aeromicrobium* sp.	6.0
BS3	*Streptococcus* sp.	0.6
BS4	*Staphylococcus* sp.	6.0
BS5	*Amphibacillus* sp.	6.0
BS6	*Kurthia*sp.	0.6
BS7	*Sporosarcina* sp.	6.0
BS8	*Acinetobacter* sp.	8.0
BS9	*Enterococcus* sp.	0.6
BS10	*Alcaligenes* sp.	0.6
BS11	*Vagococcus* sp.	0.6
BS12	*Caryophanon* sp.	0.6
BS13	*Erysipelothrix* sp.	0.6
BS14	*Escherichia coli*	6.0

Table 2: Minimum Inhibitory Concentration (MIC) of glyphosate for fungal isolates

Isolate code	Probable identity	MIC (mg/ml)
FS1	*Microsporum* sp.	9.0
FS2	*Acrophialophora* sp.	8.0
FS3	*Colletotrichum*sp.	8.0
FS4	*Histoplasma* sp.	9.0
FS5	*Aspergillus* sp.	9.0
FS6	*Trichophyton* sp.	8.0
FS7	*Aspergillusniger*	8.0
FS8	*Cladosporum* sp.	8.0
FS9	*Trichophyton* sp.	8.0
FS10	*Scedosporium* sp.	8.0
FS11	*Chryosporium* sp.	8.0

Table 3: Minimum Inhibitory Concentration (MIC) of glyphosate for bacterial isolates using dehydrogenase assay

Isolate code	Probable identity	MIC (mg/ml)
BS1	*Brochothrix* sp.	2.4
BS2	*Aeromicrobium* sp.	6.0
BS3	*Streptococcus* sp.	0.6
BS4	*Staphylococcus* sp.	6.0
BS5	*Amphibacillus* sp.	6.0
BS6	*Kurthia*sp.	0.6
BS7	*Sporosarcina* sp.	6.0
BS8	*Acinetobacter* sp.	8.0
BS9	*Enterococcus* sp.	0.6
BS10	*Alcaligenes* sp.	6.0
BS11	*Vagococcus* sp.	0.6
BS12	*Caryophanon* sp.	0.6
BS13	*Erysipelothrix* sp.	0.6
BS14	*Escherichia coli*	6.0

Table 4: Minimum Inhibitory Concentration (MIC) of glyphosate for fungal isolates using dehydrogenase assay

Isolate code	Probable identity	MIC (mg/ml)
FS1	*Microsporum* sp.	8.5
FS2	*Acrophialophora* sp.	8.0
FS3	*Colletotrichum* sp.	8.0
FS4	*Histoplasma* sp.	9.0
FS5	*Aspergillus* sp.	9.0
FS6	*Trichophyton* sp.	8.0
FS7	*Aspergill:usniger*	8.0
FS8	*Cladosporum* sp.	8.0
FS9	*Trichophyton* sp.	8.0
FS10	*Scedosporium* sp.	8.0
FS11	*Chryosporium* sp.	8.0

Table 5: Tripheny lformazan (TPF) (in mg) produced by selected organisms at varying concentrations of glyphosate

Probable identity	Glyphosate concentration (mg/ml)													
	0.075	0.15	0.30	0.60	1.20	2.40	5.0	6.0	6.5	7.0	7.5	8.0	8.5	9.0
Escherichia coli	0.000033	0.00002873	0.0000207	0.0000132	0.0000113	0.0000067	0.000004	0	-	-	-	-	-	-
***Acinetobacter* sp.**	0.000041	0.0002 79	0.0002 12	0.000153	0.0000713	0.000034	0.0000303	0.0000208	0.000014	0	-	-	-	-
***Aspergillus* sp.**	0.000547	0.000517	0.00049	0.000465	0.000465	0.000419	0.0002	0.000186	0.00016	0.000129	0.00008294	0.00005736	0.00003643	0

- = No TPF (in mg) produced

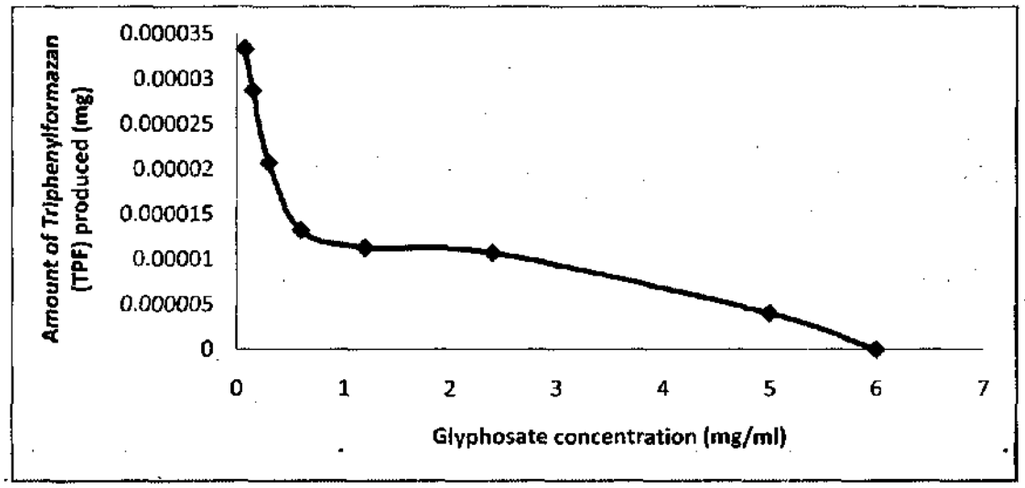

Fig. 1: Triphenylformazan produced by *Escherichia coli* against glyphosate concentrations

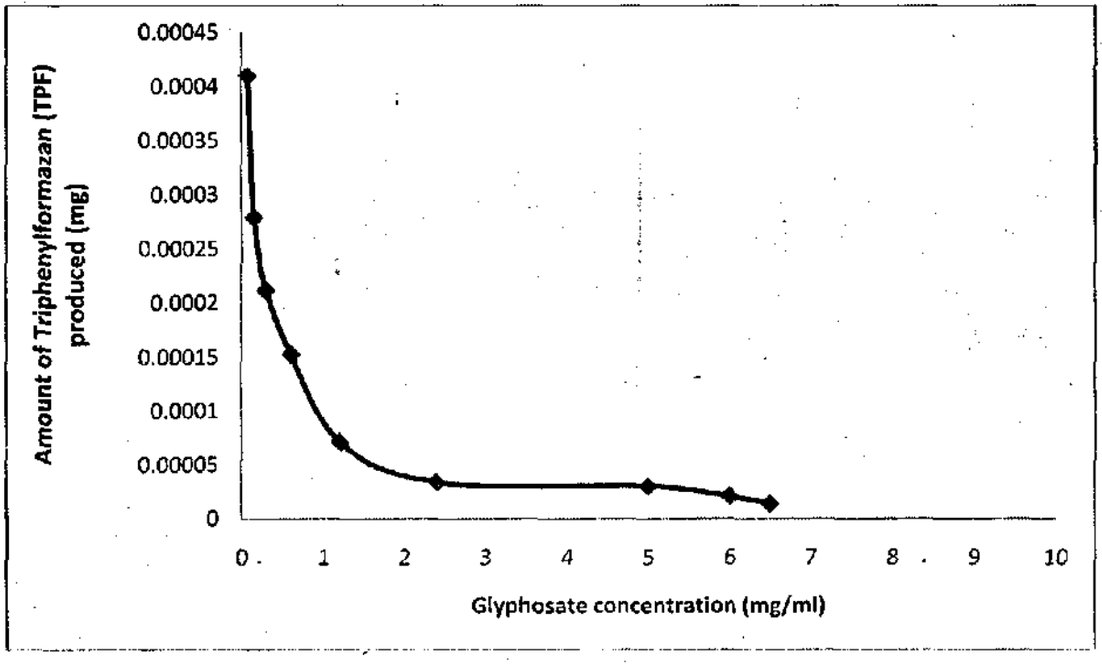

Fig. 2: Triphenylformazan produced by *Acinetobacter* sp. against glyphosate concentrations

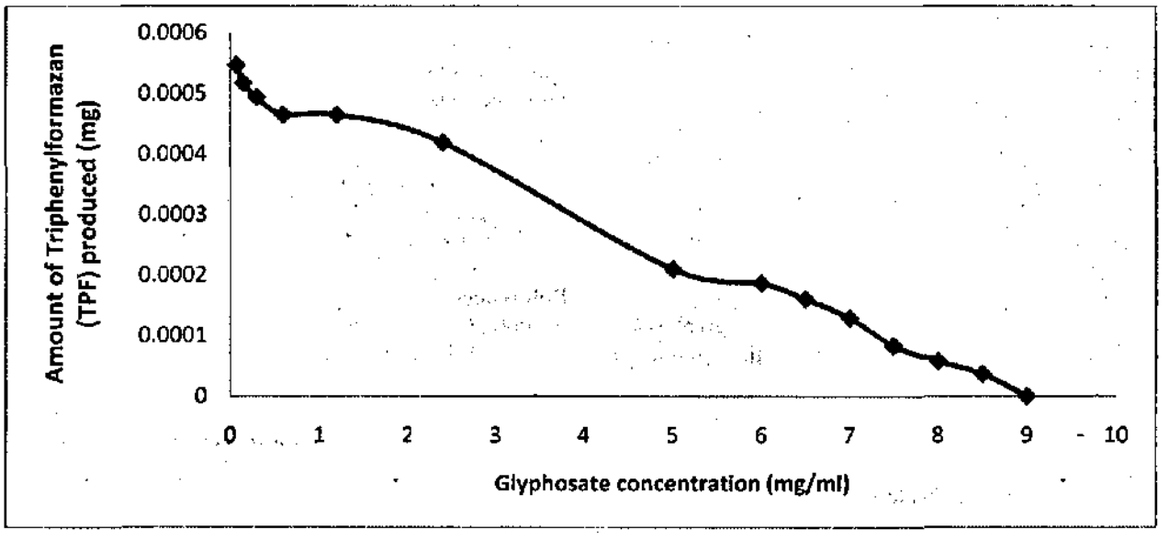

Fig. 3: Triphenylformazan produced by *Aspergillus* sp. against glyphosate concentrations

Discussion

This study was carried out to determine the effect of glyphosate on microorganisms isolated from the sediment of a freshwater river in order to show the effect of this notorious compound on these non-target organisms as well as to provide a comparison on the different techniques employed for the study (Minimum Inhibitory Concentration and Dehydrogenase Assay) and provide information on the microbial diversity of the Yeghe River sediment. Glyphosate is the active ingredient of many herbicides and it functions by inhibiting the actions of the EPSP (5-enolpyruvylshikimate-3-phosphate) synthase – an enzyme which functions in the production of amino acids for growth and the production of hormones of fungi and bacteria.

The Uproot® brand was chosen for this study because based on market survey, it is the most popular herbicide being used in Nigeria. The sample site at Yeghe River was chosen because it is located in Bori Local Government Area of Rivers state, Nigeria, home to numerous farmlands which operate both subsistence and specialized mechanized farming techniques. An unspecified number of these farmlands utilize glyphosate based herbicides like Uproot® as part of their farming techniques.

Aquatic dwelling microorganisms play vital roles in the environment; as producers to bring nutrients into the nutrient cycle and also as decomposers by breaking down dead organic matter to reintroduce nutrients to the cycle. These vital roles in the ecosystem ensure the biogeochemical cycles remain stable. Fourteen bacterial genera were isolated from the sediment sample (Table 1). Of the fungi, nine genera were gotten from the sediment sample, all of which were moulds in nature (Table 2).

Some of the microorganisms isolated in this study play important roles in the ecosystem and biogeochemical cycles, examples include: *Escherichia coli*, *Erysipelothrix* sp., *Aspergillus* sp. and *Staphylococcus* sp. which cause various human pathogenic diseases and are important indicators in the environment when water quality is considered. The fungal genera, *Aspergillus,* which is used to produce citric acid and *Colletotrichum* which is also important as an endophytic plant pathogen, all play important roles in the environment which necessitates their preservation (Pepper *et al.*, 2015).

According to Akujobi *et al.* (2010) and Shehata *et al.* (2012), the dilutions prescribed for this study fall within the range of 0.075 mg/ml and 5.0 mg/ml of the active ingredient, and in previous studies this has proven sufficient in determining the MIC of glyphosate for the various isolates used for those studies. In this study, however, most of the tested isolates were found to be highly resistant to these concentrations of glyphosate (Tables 1, 2, 3 and 4). As a result of this, the concentrations of glyphosate prescribed for the study were modified to include the following concentrations: 6.0 mg/ml, 7.0 mg/ml, 8.0 mg/ml and 9.0 mg/ml for the Minimum Inhibitory Concentration assay and 6.0 mg/ml, 6.5 mg/ml, 7.0 mg/ml, 7.5 mg/ml, 8.0 mg/ml, 8.5 mg/ml and 9.0 mg/ml for the dehydrogenase assay and 50% of the bacteria and 100% of the fungi had MIC values outside of the literature specified range.

This implies that the organisms have developed some level of resistance to the glyphosate, and this can be due to a number of reasons, such as long term exposure to the herbicide contaminant glyphosate in form of spray drifts, run-offs and other forms of dispersal from the surrounding farmlands resulting in the development of resistance within the impacted communities. While glyphosate has been associated with antibiotic resistance for some time, it also stands to reason that the continued indiscriminate use of

antimicrobials has resulted in an inadvertent increase in glyphosate resistance (Kurenbach *et al.*, 2015). Another reason could be a single amino acid substitution in the EPSP-synthase – which is the enzyme targeted by glyphosate thus leading to the inhibition of the action of the herbicide compound (Stalker *et al.*, 1985).

The bacterial isolates were the more susceptible group of microorganisms with only *Acinetobacter* sp. showing strong resistance to the glyphosate active ingredient. The fungal isolates showed characteristic resistance to the glyphosate, however in percentages exceeding the minimum inhibitory concentrations prescribed by literature.

In the dehydrogenase assay, at low concentrations of the glyphosate active ingredient, the amount of triphenylformazan produced reduced significantly. This indicates that the dehydrogenase assay is a more sensitive assay for determining toxicity levels than the Minimum Inhibitory Concentration assay (Figs. 1 – 3). A reduction in the Dehydrogenase Activity (DHA) of the microorganisms occurred with increased concentrations of the glyphosate active ingredient (Figs. 1 – 3). This corresponds with the findings of Nweke *et al.* (2007) and Ariole and Aneni (2015) who were able to detect changes in DHA with minute concentrations of test chemicals. Therefore, the MIC assay is insufficient to assess the effect of glyphosate on test microorganisms as the DHA assay is more effective.

The government should restrict farmlands which engage in mechanized agriculture or use herbicides and pesticides from coming within a certain radius of freshwater bodies in order to reduce the impact of these herbicides – which can occur in the form of run-offs, spray drifts or leaching, on the growth and proliferation of indigenous microbiota and other members of the aquatic ecosystem. Irrigation systems channeled from rivers and lakes to farmlands should be set with valves and pumps that ensure the herbicides and pesticides used in the farm do not flow back towards the river. Further research is needed to determine the contributions of the microorganisms isolated from Yeghe river sediment to the micro-community and what results the deleterious effects of the herbicide would have in the long term. It is also important to ascertain if the river sediment actually contains glyphosate, and if it does to determine if it is as a result of the nearness of farmlands using pesticides to rivers and of what concentration. This will aid in determining if the herbicide is the actual cause of the resistance to the concentrations used in this study and to establish safety protocols to prevent that from reoccurring.

Conclusion

The effect of glyphosate on microorganisms isolated from Yeghe River sediment was determined. The results have confirmed that low concentrations of glyphosate are sufficient to reduce the dehydrogenase activity of the microorganisms. This indicates that low concentrations of glyphosate such as are released from farmlands via run-offs, leaching and spray drifts have the capacity to impact freshwater sediments with debilitating effects. Furthermore, the work shows that as a tool for toxicity testing, dehydrogenase activity assays provide a more sensitive method than the minimum inhibitory concentration assay.

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