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The Effect of Glyphosate on Microorganisms Isolated From a Tropical River Water

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ABSTRACT The effect of glyphosate on bacteria, fungi and cyanobacteria isolated from surface water collected from Yeghe River in Bori Local Government Area of Rivers state, Nigeria was evaluated. The organisms were isolated from the sample using serial dilution and spread plate technique and were phenotypically identified using standard methods. The isolates were assayed to determine the Minimum Inhibitory Concentration (MIC) of the glyphosate and the most sensitive assayed to determine changes in Dehydrogenase activity. Diverse microorganisms were isolated from the composite water sample. A total of twelve (12) bacterial genera - *Aquaspirillum*, *Sporosarcina*, *Gemella*, *Micrococcus*, *Kurthia*, *Erysipelothrix*, *Enterococcus*, *Escherichia*, *Vagococcus*, *Staphylococcus*, *Amphibacillus* and *Bacillus*, eleven (11) fungal genera - *Trichophyton*, *Phaeoacremonium*, *Aspergillus*, *Paecilomyces*, *Microsporum*, *Cylindrocarpon*, *Coccidioides*, *Mucor Colletotrichum*, *Malassezia* and *Candida* and two (2) cyanobacterial genera - *Chroococcus* and *Microcystis* were identified. The result of Minimum Inhibitory Concentration (MIC) assay showed that 15.4% of the bacteria, 0% of the fungi and 0% of the cyanobacterial isolates were susceptible to concentrations of the glyphosate between 0.075 mg/ml and 5.0 mg/ml while 100%) of the bacteria, 100% of the fungi and 100% of the cyanobacterial isolates were susceptible to concentration of 9.0 mg/ml glyphosate. The bacterial isolates were found to be more susceptible to the glyphosate active ingredient than the fungal and cyanobacterial isolates. However, all the microbial isolates used for dehydrogenase assay were found to exhibit 100% sensitivity to concentration of 0.075 mg/ml glyphosate with a reduction of their dehydrogenase activity. This implies that dehydrogenase assay is more sensitive than the Minimum Inhibitory Concentration assay in toxicity testing. Therefore, low concentrations of glyphosate such as are found in runoffs are limiting to dehydrogenase activity which is a necessary metabolic process for the survival of microorganisms in aquatic ecosystem.

Keywords: Glyphosate, bacteria, fungi, cyanobacteria, surface water, dehydrogenase activity

Introduction

Microorganisms are important inhabitants of aquatic ecosystems where they fulfill critical roles as primary producers, nutrient recyclers and decomposers. Aquatic ecosystems are made up of communities of organisms living together in dependence within a body of water such as streams, rivers, oceans and lakes. Microorganisms found in aquatic ecosystems include bacteria, yeasts, moulds, cyanobacteria and algae. Other microorganisms found in the aquatic environment include the phytoplankton, protozoa and periphyton/biofilms assemblages. These organisms have been utilized in the medical, food, pharmaceutical and environmental sectors of economy as quality indicators, brewers, bioactive substance producers and in bioremediation. Aquatic microorganisms can be pathogenic however studies have shown that the beneficial microorganisms outnumber the pathogenic or harmful variety (Willey *et al.*, 2011). Humans interact with the aquatic environment and this interaction between the aquatic environment and Man subjects the ecosystem to increasing pressure from the anthropogenic activities which include contamination by a variety of organic and mineral pollutants. Most of these pollutants are herbicides which are not only used in mechanized agriculture but also for many other purposes which ranged from domestic use in gardens to maintenance of railway tracks (Perez *et al.,* 2007). These herbicides enter aquatic ecosystems as a result of terrestrial runoff, and to a lesser extent aerial spraying and direct application. The microbial communities in freshwater ecosystems are not targeted but nevertheless, they are exposed to herbicides and can be directly or indirectly affected by these compounds. According to Ayansina and Mohammed (2014), absolute specificity of pesticides has not been achieved and non-target organisms may be at risk.

In Nigeria, the herbicide glyphosate (active ingredient, N-(phosphonomethyl) glycine), is presently the most commonly used weed pesticide especially with the recent focus on mechanized and large scale farming which has deterred most farm workers from the more arduous traditional methods of weeding**.** Information on impact of glyphosate (Uproot®) on the dehydrogenase activity of microbes in freshwater ecosystem is scarce. Therefore, the aim of this present work is to contribute information on the microbial diversity of Yeghe River surface water as well as demonstrate a comparison in the efficacy of the dehydrogenase and Minimum Inhibitory Concentration assays in the assessment of glyphosate effect on microorganisms.

Materials and Methods

Sample Collection

The water 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) millilitre of the composite water sample was aseptically introduced into 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^{o}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; Garrrity *et al*., 2005; De La Maza *et al*., 2013).

Fungal Isolation and Identification

To sterile plates of acidified potato dextrose agar, sub samples of 0.1ml of 10 -fold serial dilutions of the composite water sample were inoculated in triplicates using spread plate method. The plates were incubated at 28^{o}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; 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).

Cyanobacterial Isolation and Identification

Isolation of cyanobacteria followed the methods prescribed by Cetinkaya-Donmez *et al.* (1999). One thousand millilitres of the water sample was filtered using sterilized filter paper and the filtrate was discarded. The residue was collected and rinsed in 1000 ml of sterile water. One millilitre of the resulting mixture was collected and serially diluted in normal saline. Dilutions were plated in duplicate on plates of BG-11 media supplemented with 1.5% agar agar and Nystatin and Streptomycin to prevent growth of bacteria and fungi respectively and left under room temperature in continuous light. After 2 weeks the plates were observed for growth and distinct colonies were collected using a Pasteur micropipette and introduced gently into liquid BG-11 media in conical flasks and left under room temperature in continuous light. After 7 days, 1ml was taken from each of the flasks and diluted in 9 ml of sterile water. Then 0.1 ml was taken from the dilution and plated on BG-11 media overnight. The plates were then observed under a light microscope and single cells picked and transferred to liquid BG-11 media as stock culture. Cyanobacterial isolates were identified using macroscopic and microscopic techniques (Lawton *et al.,* 1999).

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/L as prescribed by Shehata *et al.* (2012). Dilutions for 9.0, 8.0, 7.0, 6.0 mg/L 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^{o}C for up to 72 hours. Fungal isolates were incubated at 28^{o}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 col-

oured triphenylformazan (TPF). The assay was carried out in 4 ml volumes of nutrient broth-glucose-TTC medium supplemented with varying concentrations (0- 5000 µg/ml) of glyphosate as recommended by Akujobi *et al.* (2010) and further concentrations between 6000 - 9000 µg/ml of the glyphosate dilution were also prepared in separate screw-capped test tubes.

About 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^{o}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 75, 150, 300, 600, 1200, 2400 and 5000 µg/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^{o}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

Twelve genera of bacteria (*Aquaspirillum, Sporosarcina*, *Gemella*, *Kurthia*, *Erysipelothrix*, *Enterococcus*, *Escherichia*, *Micrococcus, Vagococcus*, *Staphylococcus*, *Amplibacillus* and *Bacillus*) were isolated from the composite water sample (Table 1). Eleven fungal genera were isolated from the water. Nine of these fungal genera were moulds (*Trichophyton*, *Phaeoacremonium*, *Aspergillus*, *Paecilomyces*, *Microsporum*, *Cylindrocarpon*, *Coccidioides*, *Mucor* and *Colletotrichum*) and two of them were yeasts (*Malassezia* and *Candida*) (Table 2). Two cyanobacterial isolates (*Chroococcus* sp. and *Microcystis* sp.) were identified (Table 3). Bacterial isolates were mostly resistant between concentrations of 0.075 mg/ml to 5.0 mg/ml of the glyphosate active ingredient, with the average Minimum Inhibitory Concentration (MIC) at 6.0 mg/ml. However, isolates like *Kurthia* sp. and *Bacillus* sp. were susceptible at 5.0 mg/ml and 2.4 mg/ml respectively (Table 1). *Gemella* sp. was also highly resistant with MIC of 7.0 mg/ml (Table 1). Fungal isolates had dispersed results. Many of the fungi recorded 9.0 mg/ml as the MIC of glyphosate, while some recorded 8.0 mg/ml. The yeasts, *Candida* sp. and *Malassezia* sp., were in the more susceptible range than the other fungi with MIC of 7.0 mg/ml (Table 2). The two cyanobacteria isolated from the water were also resistant between the concentrations of 0.075 mg/ml and 6.0 mg/ml with MIC at 7.0 mg/ml (Table 3).

Selected microorganisms (bacteria and fungi) were used for the dehydrogenase test. The results of the dehydrogenase test for the bacterial isolates (Table 4) were similar to those of the Minimum Inhibitory Concentration (MIC) assay (Table 1). However, *Gemella* sp. exhibited a lower MIC than with the MIC assay. Some fungi isolates such as *Candida* sp., *Trichophyton* sp. and *Colletotrichum* sp. exhibited slightly lower MIC in dehydrogenase test (Table 5) than with the MIC assay (Table 2). The amount of triphenylformazan formed (in milligrams) by *Bacillus* sp., *Gemella* sp. and *Candida* sp. when subjected to varying concentrations of glyphosate in the dehydrogenase assay are shown in Table 6 and Figures 1 – 3. The dehydrogenase activity was derived as a function of the amount of triphenylformazan produced by the isolate in mg per the dry weight of cell biomass in mg per hour. The Dehydrogenase activities of the isolates before exposure to varied concentrations of glyphosate were 0.000072 mg/cell biomass weight/hour, 0.000432 mg/cell biomass weight/hour and 0.000552 mg/cell biomass weight/hour for *Gemella* sp., *Candida* sp. and *Bacillus* sp. respectively. Upon exposure to glyphosate these values decreased with increase in glyphosate concentrations (Figures 4 – 6).

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

Isolate code	Probable identity	MIC (mg/ml)
BW1	*Aquaspirillum* sp.	6.0
BW2	*Vagococcus* sp.	6.0
BW3	*Enterococcus* sp.	6.0
BW4	*Kurthia* sp.	5.0
BW5	*Erysipelothrix* sp.	6.0
BW6	*Gemella* sp.	7.0
BW7	*Escherichia coli*	6.0
BW8	*Micrococcus* sp.	6.0
BW9	*Sporosarcina* sp.	6.0
BW10	*Staphylococcus* sp.	6.0
BW11	*Bacillus* sp.	2.4
BW12	*Staphylococcus aureus*	6.0
BW13	*Amphibacillus* sp.	6.0

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

Isolate code	Probable identity	MIC (mg/ml)
FW1	*Trichophyton* sp.	7.0
FW2	*Phaeoacremonium* sp.	9.0
FW3	*Aspergillus flavus*	9.0
FW4	*Paecilomyces* sp.	9.0
FW5	*Trichophyton* sp.	9.0
FW6	*Microsporum* sp.	9.0
FW7	*Cylindrocarpon* sp.	9.0
FW8	*Trichophyton* sp.	9.0
FW9	*Coccidioides* sp.	8.0
FW10	*Mucor* sp.	8.0
FW11	*Colletotrichum* sp.	8.0
FW12	*Microsporum* sp.	8.0
FW13	*Malassezia* sp.	7.0
FW14	*Candida* sp.	7.0

Table 3: Minimum Inhibitory Concentration (MIC) of glyphosate for cyanobacterial isolates

Isolate code	Probable identity	MIC (mg/ml)
CW1	*Chroococcus* sp	7.0
CW2	*Microcystis* sp.	7.0

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

Isolate code	Probable identity	MIC (mg/ml)
BW1	*Aquaspirillum* sp.	6.0
BW2	*Vagococcus* sp.	6.0
BW3	*Enterococcus* sp.	6.0
BW4	*Kurthia* sp.	5.0
BW5	*Erysipelothrix* sp.	6.0
BW6	*Gemella* sp.	6.5
BW7	*Escherichia coli*	6.0
BW8	*Micrococcus* sp.	6.0
BW9	*Sporosarcina* sp.	6.0
BW10	*Staphylococcus* sp.	6.0
BW11	*Bacillus* sp.	2.4
BW12	*Staphylococcus aureus*	6.0
BW13	*Amphibacillus* sp.	6.0

Table 5: Minimum Inhibitory Concentration (MIC) of glyphosate for fungal isolates using dehydrogenase assay

Isolate code	Probable identity	MIC (mg/ml)
FW1	*Trichophyton* sp.	7.0
FW2	*Phaeoacremonium* sp.	9.0
FW3	*Aspergillus flavus*	9.0
FW4	*Paecilomyces* sp.	9.0
FW5	*Trichophyton* sp.	9.0
FW6	*Microsporum* sp.	8.5
FW7	*Cylindrocarpon* sp.	9.0
FW8	*Trichophyton* sp.	8.5
FW9	*Coccidioides* sp.	8.0
FW10	*Mucor* sp.	7.0
FW11	*Colletotrichum* sp.	6.5
FW12	*Microsporum* sp.	8.0
FW13	*Malassezia* sp.	7.0
FW14	*Candida* sp.	6.5

Table 6: Triphenylformazan (TPF) (in mg) produced by selected organisms at varying concentrations of glyphosate

Table 6:	Triphenylformazan (TPF) (in mg) produced by selected organisms at varying concentrations of glyphosate																	
Probable identity	Glyphosate concentration (mg/ml)																	
	0.07	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				
	5																	
Gamella sp.																		
	0.00	0.000	0.000	0.0000	0.000	0.0000	0.000	0.0000										
	0.067	0.517	0.458	358	0.238	117	01	0175										
Bacillus sp.	0.00	0.000	0.000	0.0002	0.000	-	-	-	-	-	-	-	-	-				
	047	36	33	9	235													
Candida sp.	0.00	0.000	0.000	0.0001	0.000	0.0000	0.000	0.0000	0.000	-	-	-	-	-				
	0.262	231	214	74	157	885	08	314	0171									

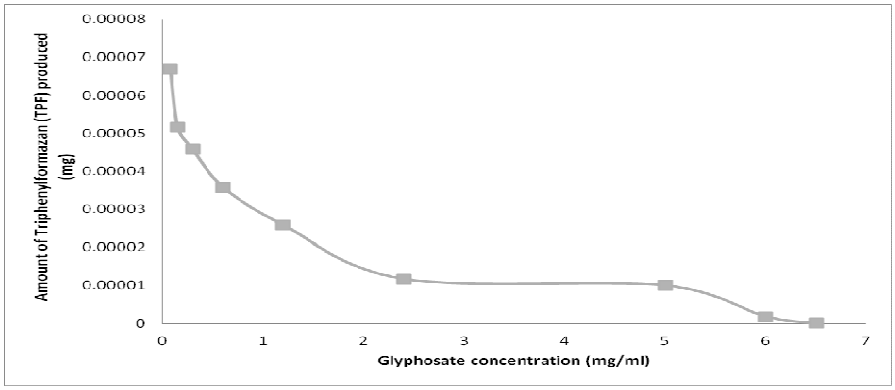

Fig. 1: Triphenylformazan produced by Gemella sp. against glyphosate concentrations

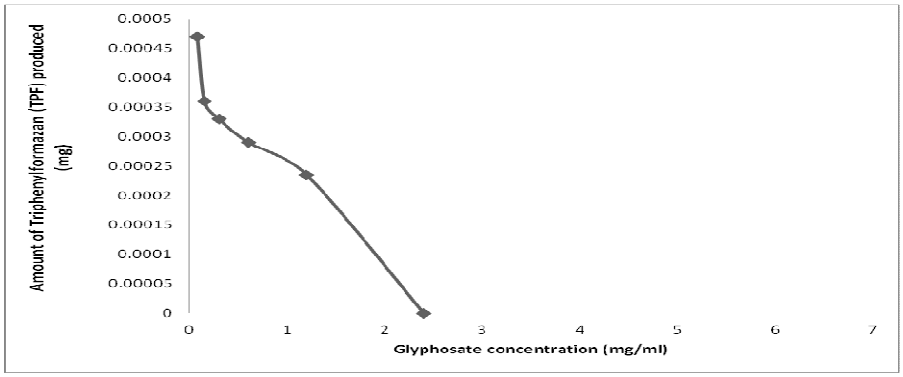

Fig. 2: Triphenylformazan produced by Bacillus sp. against glyphosate concentrations

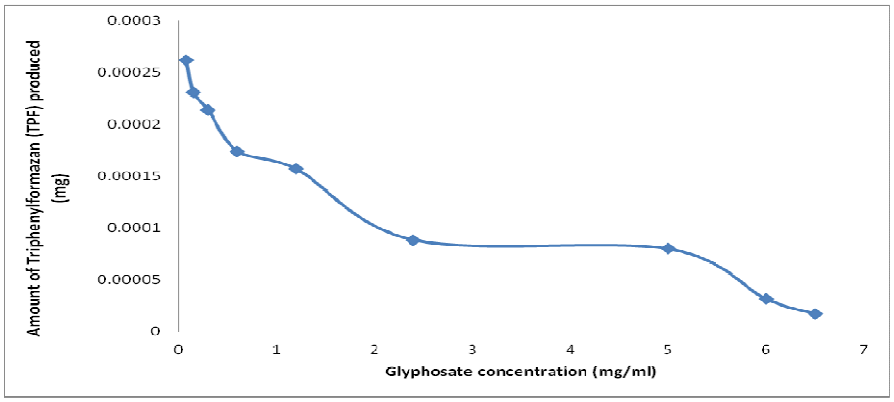

Fig. 3: Triphenylformazan produced by Candida sp. against glyphosate concentrations

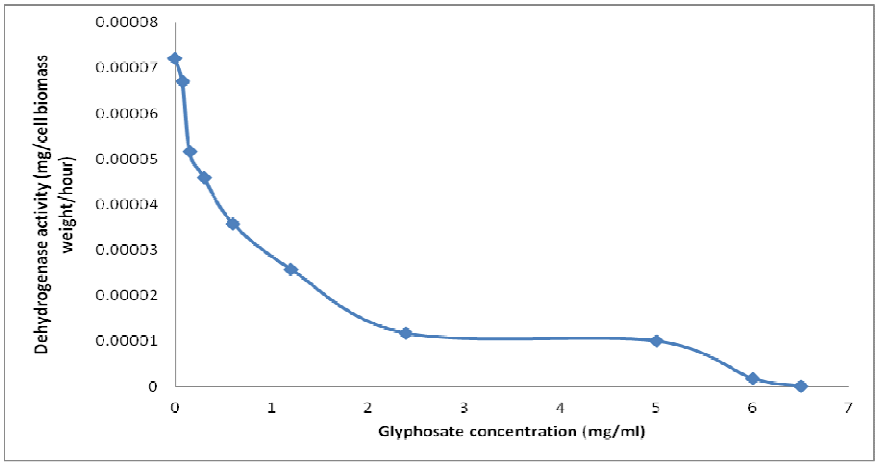

Fig. 4: Dehydrogenase activity of Gemella sp. against glyphosate concentrations

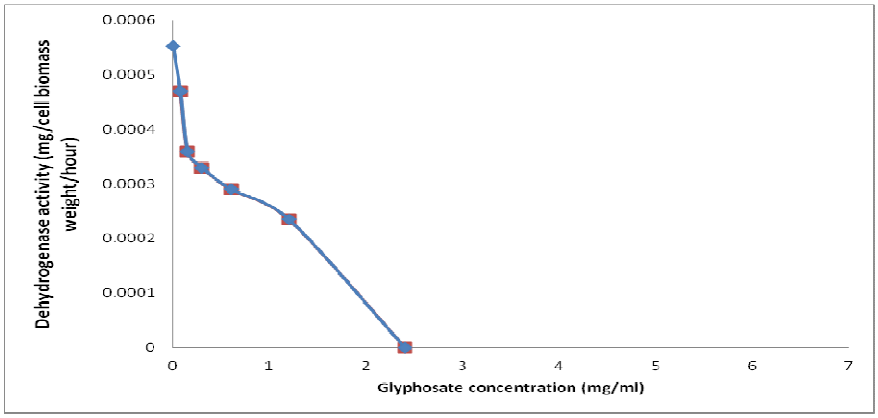

Fig. 5: Dehydrogenase activity of Bacillus sp. against glyphosate concentrations

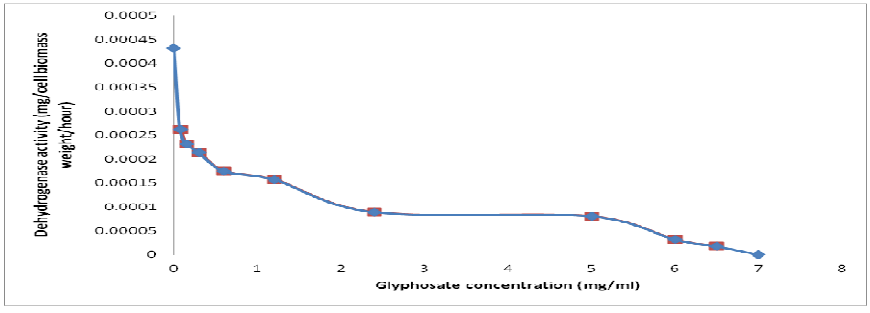

Fig. 6: Dehydrogenase activity of Candida sp. against glyphosate concentrations

Discussion

This study was carried out to determine the effect of glyphosate on microorganisms isolated from river water. Glyphosate is the active ingredient of many herbicides and it functions by inhibiting the actions of the Enolpyruvylshikimate-3-phosphate (EPSP) synthase – an enzyme which functions in the production of amino acids and which is also present in many fungi, algae and bacteria.The Uproot® brand was chosen for this study because it is currently the most popular herbicide being used in Nigeria based on market survey. The sample site at Yeghe River was also chosen for practical reasons. The Yeghe River is located in Bori Local Government Area of Rivers state, Nigeria which is home to numerous farmlands, both of subsistence and industrial quality. An unspecified number of these farmlands utilize glyphosate based herbicides as part of their farming techniques.

Aquatic dwelling microorganisms play vital roles in the ecosystem to ensure the biogeochemical cycles remain stable. Some of the microorganisms isolated in this study play important roles in the ecosystem and biogeochemical cycles, examples include: the cyanobacteria, *Chroococcus* sp., which functions by utilizing a large quantity of carbon dioxide for photosynthesis, thus freeing up large amounts of oxygen into the atmosphere. It is also one of the first genera to use water to access electrons and hydrogen which it uses for photosynthesis thus freeing up oxygen in both processes of hydrolysis and photosynthesis (Hong *et al.,* 2005). *Microsystis* sp., which is another cyanobacterium isolated in this study is commonly recognized for the ability to cause algal blooms (Qiu, 2013). Other nefarious microorganisms isolated in this study include *Candida* sp., *Malessezia* sp., *Escherichia coli*, *Erysipelothrix* sp., *Aspergillus flavus*, and *Staphylococcus* sp. are known to have species which cause various human pathogenic diseases (Willey *et al.*, 2011). The fungal genera; *Aspergillus,* which is used to produce citric acid (Ramesh and Kalaiselvam, 2011), *Paecilomyces* which is used as a nematocide (Mukhtar *et al*., 2013) and *Colletotrichum* which is also important as an endophytic plant pathogen, all play important roles in the environment which necessitates their preservation.

According to Akujobi *et al.* (2010) and Shehata *et al.* (2012), the dilutions prescribed for this type of study fall within the range of 0.075 mg/ml and 5.0 mg/ml of the active ingredient. However, in this study, most of the tested isolates including bacteria, fungi and cyanobacteria were found to be highly resistant to these concentrations of glyphosate (Tables 1, 2 and 3). Up to 84.6% of the bacteria, 100% of the fungi and 100% of the cyanobacteria had MIC values outside of this literature specified range (0.075 - 5.0 mg/ml). 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 glyphosate in form of spray drifts, run-offs and other forms of

dispersal from the surrounding farmlands. Furthermore, glyphosate has for a long time been associated with antibiotic resistance and the application of glyphosate in the environment has induced antibiotic resistance in organisms (Kurenbach *et al.,* 2015). The converse can also be the case with the widespread application and use of antimicrobials leading to a reduction in glyphosate susceptibility. It has also been reported that a single amino acid substitution in EPSP-synthase (an enzyme targeted by glyphosate that leads to the inhibition of growth of organism) can confer resistance to glyphosate (Stalker *et al.,* 1985). This may account for the resistance of the cyanobacterial isolates to the glyphosate.

The dehydrogenase assay results (Table 6 and Figures 1 – 3) show that even at lower concentrations of glyphosate, the amount of triphenylformazan produced by the microorganisms had already begun to reduce indicating an effect of the glyphosate at even low concentrations. The dehydrogenase activities of the isolates decreased with increase in glyphosate concentrations (Figures 4 – 6). This corresponds with the results of Nweke *et al.,* (2007) who were also able to detect changes in dehydrogenase activity at minute concentrations of the test chemicals. Therefore, MIC assay is insufficient to be used in assessing the effect of the herbicidal agent on the microorganisms as it gives a null result for the smaller concentrations. This implies that dehydrogenase assay is more sensitive, as it shows the effect of smaller concentrations, than the MIC assay.

Conclusion

The effect of glyphosate on microorganisms isolated from Yeghe River water was determined. The results have shown that low concentrations of glyphosate are sufficient to reduce the dehydrogenase activity of the microorganisms. These low concentrations are usually associated with run-offs and spray drifts. This indicates that low concentrations of glyphosate released from farmlands via run-offs, leaching and spray drifts have the capacity to impact freshwater ecosystems with debilitating effects.

Recommendations

Further research is needed to determine the contributions of the microorganisms to the ecosystem health and what results the deleterious effects of the herbicide would have in the long term. It is also important to ascertain if the river water actually contains glyphosate as a result of the nearness of farmlands using pesticides to rivers and of what concentration to determine 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 occurring.

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