International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304, ISSN(Online): 2455-9563
Vol.9, No.6, pp 446-454, 2016

Bioresponsce of microalgae *Oscillatoria limnetica* to organophosphorous pesticide Glyphosate

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Abstract: The microalga species, *Oscillatoria limnetica*, isolated from the artificial canal around University of Babylon in Al-Hilla city was cultured in the laboratory using BG-11 growth medium for biomass production and to test the effect of organophosphorous pesticide glyphosate on gowth rate, doubling time and photosynthesis pigments (chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, phycoerythrin, phycocyanin, allophycocyanin and phycobilliproteins).

The presence of glyphosate caused an inhibitory effect on the growth rate of *O.limnetica* and increase doubling time. Comparisons with a control, which supported 0.0361 growth rate and 8.337days doubling time, showed that the highest reduction of the growth rate was 0.0285 and the top rise of the doubling time was 10.561 days at 15mg/l of glyphosate.

In addition to, glyphosate caused inhibitory effects on photosynthetic pigments of the isolated algae.Maximum reduction of chlorophyll a, chlorophyll b, total chlorophyll and carotenoids was 0.6224, 0.1138, 0.736 and 0.143 mg/1, respectively in the presence of 15mg/1 of glyphosate. Moreover, maximum reduction of phycocyanin, allophycocynin, phycoerythrin and phycobiliproteins was 0.00207, 0.00361, 0.00165 and 0.0072mg/1 respectively, in 20mg/1 glyphosate.

Keywords: Cyanophytae, bioresponce, photosynthesis pigments, orgnophosphorous pesticide, glyphosate.

Introduction

Over the years, pesticides have resulted in problems caused by their interactions with the biological systems in the environment and have harmful effects on algae, especially nitrogen fixing cyanophyta by influencing growth, photosynthesis, nitrogen fixation, biochemical and molecular composition, and metabolic activities[1].

Glyphosate-based herbicides are the world's leading post-emergent, broad spectrum and non-selective herbicides for the control of annual and perennial weeds in agricultural lands, ornamental and residential gardens and in aquatic systems[2]. While the physicochemical and acute toxicological properties of glyphosate are well known[3], with numerous studies focusing on aquatic animals (invertebrates and fish), limited information is available on the responses of photosynthetic microorganisms to the herbicide[4-5].

Glyphosate is a competitive inhibitor of the enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS), which is involved in the pathway of synthesis of shikimic acid[6 - 7]. It, will also affect photosynthesis [8] by forming complexes with cations which are co-factors of important photosynthetic components: magnesium as a co-factor in chlorophyll synthesis[9] , manganese and calcium as co-factors of the Mn-cluster[10] and iron as an essential component of the ferredoxin protein[11].

In general, glyphosate (roundup) reduced micro- and nano- phytoplankton, while the abundance of picocyanophyta increased by a factor of about 40 [12]. Commercial formulations (e.g. Ron-do® and Roundup®) of glyphosate were more toxic than glyphosate alone. For example, Tsui and Chu [4]observed a 7 folds higher toxicity of Roundup® than the IPA salt of glyphosate in the green algae *Selenastrum capricornutum*. Alike results were reported for *Selenastrum capricornutum* and the macrophyte *Lemma minor*, showing 4 folds higher toxicity of Roundup® than glyphosate[13]. Lower differences in toxicity were registered by Sáenz *et al* [14] and Sobrero *et al*[15], reporting between 1.2 to 1.8 folds higher toxicity of commercial formulations than active ingredient. POEA itself contributed to Roundup® toxicity with values ranged from about 45% for *Skeletonema costatum* to 85 % for *Selenastrum capricornutum*[4].[15] also reported that *Microcystis aeruginosa* based EC50 was 18 X more sensitive compared to the green algae *Chlorella vulgaris*, when exposed to the glyphosate-based herbicide Roundup®. According to the same authors, this higher sensitivity was attributable to isopropylamine found in the glyphosate based herbicides, rather than to the pure glyphosate molecule. [16] found that glyphosate could influence the composition of algae community in summer. Three algal genera (Asterionella, Cyclotella and Oocystis) disappeared after glyphosate exposure, while pennate diatoms (Gomphonema, Navicula and Nitzschia Scenedesmus) remained relatively stable and homogeneous.

Materials and Methods

Sample collection and identification

Samples of freshwater algae were collected from artificial canal around University of Babylon in Al-Hilla city by using phytoplankton net[17]. Experimental cultures were incubated in a sterile BG-11 medium[18-19] at 26± 1°C under cool white fluorescent lamps (200) µE/m²/s with a light–dark cycle of 16/8 h.[20]. Furthermore the cultures were mildly shaken by hand on alternate days[21].

Pesticide

The organophosphorus pesticide used in this study is the formulation of the herbicide glyphosate commercially available as Roundup® (containing 480 g active ingredient/L of glyphosate).

Experimental Design

The isolated alga was batch-cultured in 500 ml Erlenmeyer flasks. Into each flask 200 ml of liquid culture media, BG11 medium for *O.limnetica* ,was added. Glyphosate was added to the culture medium to the final concentrations 5, 10, 15, 20 mg/l. The flasks were cultivated under the conditions described above. Response of glyphosate toxicity on *O. limnetica* (at the stationary phase) was investigated by determination of growth rate (k), doubling time (G), chlorophyll a, chlorophyll b, total chlorophyll, carotenoid, Phycocyanin, Allophycocyanin, Phycoerythrin and phycobiliprotein (All analysis done in three replicates).

Determination of Growth rate and Doubling time.

O.limnetica cell density was estimated with a UV-Vis spectrophotometer by converting the OD at 750nm into cell density (cells ml-1) based on a linear relationship between these two parameters. Optical density was measured daily to follow the evolution of the biomass, identify growth phases and calculate growth rates [22].

The specific growth rate, ì (day-1), and doubling time were calculated during the exponential growth phase, according to the following equation [23]:

$K = 3.322 * (\log OD_t - \log OD_0) / t$
$G = 0.301 / K$
G: growth rate
K: doubling time
t: time
OD0: optical density at the beginning of the experiment (zero time).
ODt: optical density after (t) day.

Estimation of Chlorophyll

The estimation of chlorophyll was done by the method of Arnonn[24]. *O.limnetica* cells were collected and resuspended in 1 ml of 80% acetone. After centrifugation, the chlorophyll content of the supernatant was measured according to optical absorbance at 663nm and 645 nm by using a UV-VIS spectrophotometer. The chlorophyll content was determined by the following Equations (1) – (3)[25]:

Chlorophyll a (mg/l) = (12.7 × A663) − (2.698 × A645) (1)
Chlorophyll b (mg/l) = (22.9 × A645) − (4.68 × A663) (2)
Total chlorophyll (ìg/ml) = chlorophyll a + chlorophyll b = (20.2 × A645) + (8.02 × A663) (3)

Estimation of Carotenoid

An aliquot (5 ml) of *O. limnetica* cell suspension was taken and subjected to centrifugation (4000 r\min for 10 mins.). Discarded the supernatant and washed the pellet 2-3 times with distilled water to remove traces of adhering salts. To the pellet, added 2-3 ml of acetone (80%) and vortex mixed until a white precipitate appeared (which, generally required 1min). The cell membrane gets ruptured because of organic solvent (acetone). Acetone extract separated from cell debris by centrifugation it at 3000 r\min for 10min, After centrifugation, the carotenoid content of the supernatant was measured according to the equation reported by Lichtenthaler and Wellburn [26] as follows:

Total carotenoids (mg/l) = 1000 A470 – 2.860 Ca – 129.2 Cb /245
(Ca = chlorophyll a, Cb = chlorophyll b)

Estimation of Phycobiliproteins

The estimation of phycobiliproteins was done by the method of Bennet and Bogorad [27]. An aliquot of 10ml of the sample was centrifuged at the rate of 4500 r/min for 20min, and then the supernatant was decanted. The pellets were washed with distilled water, suspended in 10ml phosphate buffer (0.05 M, pH 7.0) and homogenized, then the contents were freeze thawed, repeated and centrifuged at 4500 r/min for 10min. The absorbance of the supernatant was determined at the wavelengths of 652, 615, and 562nm, using phosphate buffer as a blank. The concentrations of phycocyanin (PC), allophycocyanin (APC), and phycoerythrin (PE) were calculated according to (4) to (7), respectively [28].

Phycocyanin (PC) = OD615-0.474(OD652) / 5.34 (4)
Allophycocyanin (APC) = OD652-0.208 (OD615) / 5.09 (5)
Phycoerythrin (PE) = OD562-2.41(PC)-0.849(APC) / 9.62 (6)
Phycobiliproteins= PC + APC + PE (7)

Statistical Analysis

General Treatment Structure was used as an experimental design. Data were analyzed to study the bioresponce ability of *O. limnetica* to glyphosate and Least significant difference (LSD) was used to compare the significant difference between means at $p<0.05$.

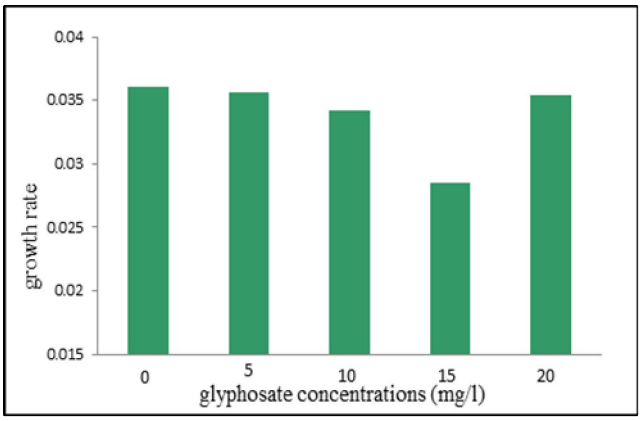
Results And Discussion

Effect of Glyphosate Concentrations on Growth rate and doubling time of *O. limnetica*.

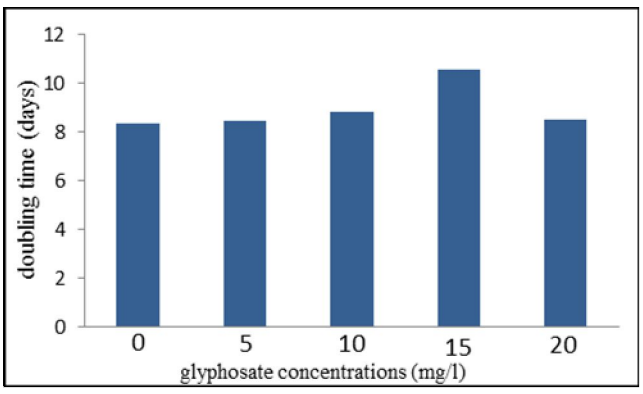
Growth was the endpoint that constantly detected inhibitory effects and significant differences between control and treatments. The mechanism for the growth reduction by glyphosate was perhaps inhibition of the chlorophyll synthesis, most likely mediated by the effects of the herbicide of its precursor ALA (5-aminolevulinic acid) [29-30-31] and the decreasing of growth rate led to slow of cell division so doubling of the cells took more time [32].

The different concentrations of glyphosate caused different growth rate and doubling time for isolated alga. The growth rate value was decreased and the doubling time was increased when glyphosate concentration increased. The highest growth rate inhibition 21.052% was recorded at 15mg/l and the lower growth rate

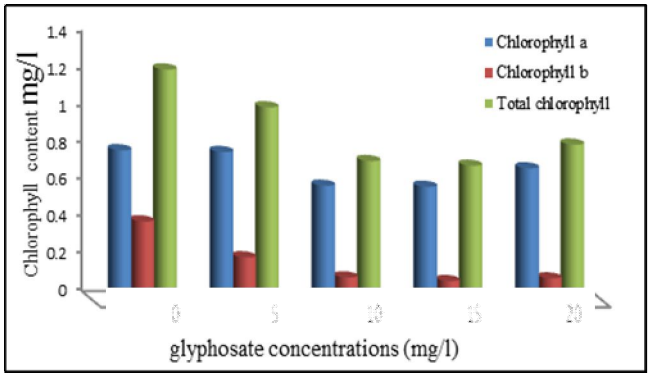
inhibition 1.385% was recorded at 5mg/l. Significant differences were recorded in K value and G between control and all treatments except treatment 5mg/l and 20mg\l (figure 1 and figure 2). The findings of this study agreed with Vendrell *et al* [33] who reported that the acute toxicity of glyphosate herbicide on the four species of freshwater phytoplankton, *Scenedesmus acutus*, *Scenedesmus subspicatus*, *Chlorella vulgaris* and *Chlorella saccharophila* was caused 50% growth reduction over 72 h when glyphosate ranged from 24.5 to 41.7 mg/l, while 10% growth inhibition is achieved by herbicide concentrations ranging from 1.6 to 3.0 mg/l. Similar results were stated by Issa [34] who found that glyphosate at two concentrations (5 and 20 mmol/L) inhibited growth rate of *Oscillatoria angustissima* and *Calothrix parietina*. Another study by Christy *et al* [35] examined the growth rate reduction of *Chlorella sorokiniana* which was cultured in various concentrations of the herbicide glyphosate. Growth rates, in terms of cell doublings per day, were determined for cells inoculated into medium containing from $5.91x10^{-6}$M to $591x10^{-6}$M glyphosate. Comparisons with a control, which supported 10.4 doublings/day, showed growth only slightly reduced at $5.91x10^{-6}$M and $11.8x10^{-6}$M, with averages of 9.7 and 9.5 doublings/day; reduced by more than half at $17.7x10^{-6}$M with an average of 4.4 doublings/day; and completely preventing growth at $23.7x10^{-6}$M and all higher concentrations. Moreover, Lipok *et al* [2] found that market available formulation of Roundup® was inhibited the growth of studied strains (eight aquatic microphotoautotrophs; seven cyanobacterial strains representing either saline or freshwater communities, and common eukaryotic algae *Chlorella vulgaris* Beijerinck) in a dose-dependent manner. The physiological and biochemical responses of *Microcystis aeruginosa* to glyphosate and its formulation in the common herbicide, Roundup®, were studied by Qiu *et al* [36]. However, they found that Roundup® showed low-dose (below 1 mg/l) stimulation and high-dose (above 1 mg/l) inhibition on *Microcystis aeruginosa* cell density and chlorophyll content. Furthermore, Ruan and Murray [37] investigated the effects of glyphosate exposure (0.15, 0.30, 0.45 and 0.6 mmol/L Gly acid) on colony size, dry biomass accumulation, chlorophyll a fluorescence (Fv/Fm) and chlorophyll a biosynthesis in *Nostoc sphaeroides*. They found all parameters were significantly inhibited in a concentration used and time dependent way.



*($p<0.05$)
Significant differences between control and all treatments expect 5mg/l and 20mg/l.
Figure 1: Growth rate of *O.limnetica* at different glyphosate concentrations (mg/l).

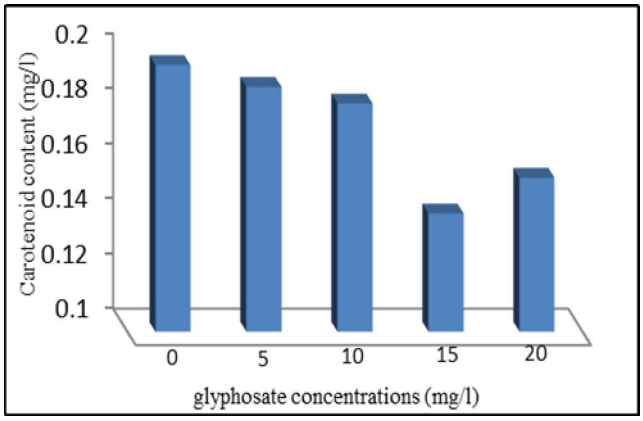


*($p<0.05$)
Significant differences between control and all treatments expect 5mg/l and 20mg/l.
Figure 2: Doubling time of *O.limnetica* at different glyphosate concentrations (mg/l).



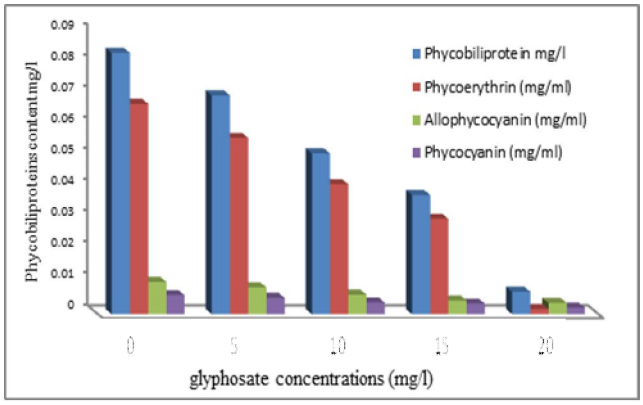
*(p<0. 05)
Significant differences between control and all treatments expect 5mg/l in chlorophyll a and total chlorophyll content.

Figure 3: Chlorophyll a, chlorophyll b and total chlorophyll content of *O.limnetica* at different glyphosate concentrations (mg/l).



*(p<0.05)
Significant differences between control and all treatments expect 5mg/l and 10mg/l.

Figure 4: Carotenoid content of *O.limnetica* at different glyphosate concentrations (mg/l).



*(p<0.05)
Significant differences between control and all treatments expect 5mg/l in phycocyanin and phycobiliprotein content.

Figure 5: Phycocyanin, Allophycocyanin, Phycoerythrin and total phycobiliproteins content of *O.limnetica* at different glyphosate concentrations (mg/l).

Effect of Glyphosate on photosynthesis pigments of *O.limnetica*

Results show that glyphosate inhibited all photosynthetic pigments like chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, phycoerythrin, phycocyanin, allophycocyanin and phycobilliproteins (figure 3, figure 4 and figure 5). These results imply that glyphosate-based herbicides might interfere, not only via the shikimic acid pathway, but also affected photosynthesis indirectly by inhibiting the biosynthesis of carotenoids, chlorophylls, fatty acids, or amino acids [38].

Experimental results indicated that the effect of glyphosate on chlorophyll may be indirect through photobleaching and/or peroxidation of chlorophyll however, it is not well known how exactly glyphosate might interfere with chlorophyll content, either by a perturbation of chlorophyll synthesis or by the formation of reactive oxygen species degrading the photosynthetic pigments [39] and loss of carotenoids pigment might be another reason to protect against ROS generation and inhibition of photosynthetic electron transfer[40] .

Although glyphosate did not directly target photosystemII electron transport, but it indirectly affected photosynthetic activity by interfering with protein and pigment biosynthesis like phycobiliprotein-pigment complexes which could constitute about 50% of the total cellular protein in cyanophyta[31].

In agreement with these results [41]reported that glyphosate caused reduction in the content of pigments (chlorophyll, carotenoids, phycobiliprotein) in *Chlorella vulgaris*, *Chlamydomonas reihardii*, *Anabaena cylindrica*, and *Chroococcus turgidus*. Alike results were reported by [42]who found that glyphosate [N-(phosphonomethyl) - glycine] in concentrations ranging from 0.1mM to 1mM, decreased photosynthetic pigment content in *Chlorella pyrenoidosa* Pringsheim and had two different effects upon photosynthetic pigments: inhibition of chlorophyll synthesis and a decrease in carotenoids. Wong [43]too stated that the presence of 2 mg/l of glyphosate was significantly inhibited the photosynthesis and chlorophyll content, whereas the presence of 20mg/l or more of glyphosate completely inhibited photosynthesis and chlorophyll synthesis in *Scenedesmus quadricauda*. In the study of Inderjit and Kaushik[44], glyphosate suppressed chlorophyll a concentrations of *Anabaena fertilissima* at doses above 10 mg/l, but glyphosate showed no inhibitory effect at low doses (0 to 1.5mg/l).

In accordance with these results, a laboratory study on the effects of commonly field sprayed herbicide, glyphosate (Roundup) on the nitrogen fixing biofertilizer cyanophyta, *Mastigocladus laminosus* Cohn. indicated that this herbicide caused significant decrease in chlorophyll and phycobilin pigments even at 10mM concentrations within 24 hours leading to bleaching of cultures. It is suggested that the greater amino acid pools of the cells could have been possible by the break down of cellular proteins, including the major pigment, phycobiliprotein[31]. Also, biosynthesis of carotenoids and phycobiliproteins could be inhibited in *Anabaena doliolum*[45]. In general, data obtained from Peterson *et al* [46] who found that the significant inhibitory effects on the algal growth, photosynthesis and chlorophyll a synthesis were only observed in the presence of expected environmental concentration (EEC) of glyphosate and paraquat. Romero *et al*[47] also suggested that the surfactant alone or combined with high doses of the glyphosate formulation could inhibit carotenoid synthesis. A similar observation has been reported by Allen *et al* [48] and Wanner *et al* [49]who stated that glyphosate induced loss of pigments in both wild type and mutant strains was found to be higher under Pi-starved than under the Pi-supplemented condition and this could be due to alternations in the phyisological state of organisms such as nitrogen and phosphate metabolism.

Conclusion

Glyphosate treatments affected the activities in the cyanophyta species by reducing the growth rate, Chlorophyll a, chlorophyll b, total chlorophyll, carotenoid, phycocyanin, allophycocyanin, phycoerythrin, phycobiliproteins as well as increased doubling time.

Acknowledgment

This work was supported by department of Biology,College of Science and Environmental Research Center,University of Babylon .

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