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RESEARCH ARTICLE

Toxicity response of *Chlorella* microalgae to glyphosate herbicide exposure based on biomass, pigment contents and photosynthetic efficiency

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ABSTRACT
The extensive use of glyphosate (*N*-(phosphonomethyl) glycine) herbicide in agriculture is accompanied by the risk of environmental contamination of aquatic ecosystems. In this study, the effects of glyphosate at different concentrations (50–500 µg ml^{-1}) on three *Chlorella* species including *Chlorella ellipsoidea*, *Chlorella sorokiniana* and *Chlorella vulgaris* especially in relation to the biomass, pigment contents and photosynthetic efficiency were assessed. After treatment for 24 hr, the acute toxicity results showed that *C. vulgaris* (IC_{50} = 449.34 ± 6.20 µg ml^{-1}) was more tolerant to glyphosate than *C. ellipsoidea* (IC_{50} = 288.23 ± 23.53 µg ml^{-1}) and *C. sorokiniana* (IC_{50} = 174.28 ± 0.50 µg ml^{-1}). After a 72-hr chronic toxicity treatment with glyphosate, glyphosate concentrations decreased to 400–500 µg ml^{-1} in *C. ellipsoidea*, 200–300 µg ml^{-1} in *C. sorokiniana* and 200–500 µg ml^{-1} in *C. vulgaris* respectively. During 24-hr acute toxicity exposure to glyphosate, the pigment contents and maximum quantum efficiency of photosystem II (Fv/Fm) decreased as the concentration of glyphosate increased. Overall, the biomass, pigment contents and photosynthetic efficiency presented a high positive correlation. It is worthwhile to mention that our study provides detailed information on the toxicity and sensitivity of these *Chlorella* species to glyphosate.

Introduction

Herbicides are commonly used in agricultural systems on a global scale to control weeds and to increase crop yield and quality. However, due to improper application practices and excessive use, the lack of control and unbalanced usage of herbicides largely impact environment and lead to detrimental effects on human health and ecosystems, especially in aquatic environments (1). Microalgae communities serve as an indicators help to evaluate the effects of both chemical and physical environmental parameters on ecosystems (2). In the case of microalgae, herbicides have the potential to disrupt the balance of the whole ecosystem (3-5). Specifically, herbicides have shown to seriously limit impact the biodiversity and limit the number of organisms in microalga ecological systems (6). In addition, microalgae are primary producers, base link of the aquatic food chain, and respond to environment and chemicals in aquatic ecosystems, thus their sensitivity to herbicides is critical (7, 8). Much research supports that microalgae might be the most promising early-alert indicator of changes in the ecological system caused by chemicals (9-11).

The *Chlorella* genus pertains to a small globular single-celled green algae belonging to the Chlorophyta division, is found in many aquatic systems, and is a representative of microalga in aquatic systems (12). The *Chlorella* species has attracted much interest for its importance in several applications, including agrochemical treatments (13, 14), animal feed (15-17, biofuels (18, 19), biological indicator (20, 21), food supplement (22-25) and wastewater treatment (26-28). As previously mentioned, this species, including *C. kessleri* (29), *C. Protothecoides* (30), *C. pyrenoidosa* (31), *C. sorokiniana* (30) and *C. vulgaris* (20) has also been considered a potential bio-indicator of the ecosystem, such as for chemical contamination in aquatic

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environments. Notably, it is able to respond quickly to toxicity and exhibits high sensitivity towards herbicides (21).

In agricultural systems, glyphosate (*N*-(phosphonomethyl) glycine) is a commonly broad-spectrum herbicide for weed control. Specifically, glyphosate interferes with 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS), which block enzymes and prevents the production of aromatic amino acids, including phenylalanine, tyrosine and tryptophan in plants and microorganisms, through the shikimate pathway (32-34). Previous studies have been demonstrated that toxicity differences to glyphosate exposure in *Chlorella* microalgae, including *C. kessleri* (35, 36), *C. pyrenoidosa* (37-41), *C. saccharophila* (42), *C. sorokiniana* (43) and *C. vulgaris* (20, 42, 44-49). While the toxicity of *Chlorella* microalgae can be evaluated based on their ubiquity and short life cycle (50), there are no reports on the half-maximal inhibition in response to glyphosate exposure at 24 hr acute toxicity.

Since 1979, only 233 reports on the toxicological effects of glyphosate in aquatic environments have been published in the Web of Science database. This scarcity of research indicates the lack of knowledge on the risks of glyphosate exposure and contamination (51). More specifically, studies on the difference in sensitivity responses to the toxicity effect of glyphosate herbicide among various species and respective treatment within a short time are largely unexplored. Therefore, further research is in demand to understand herbicide toxicity and response of microalgae.

In order to assess the toxic stress of herbicides, this study examined the evolution of the toxic impact of glyphosate on the biomass, pigment contents and photosynthetic efficiency in representative of *Chlorella* sp. including *C. ellipsoidea, C. sorokiniana* and *C. vulgaris*. Therefore, the results provide knowledge on the acute and chronic toxicity of glyphosate exposure to advance our current understanding of its effects in aquatic organisms in a short time by employing unicellular *Chlorella* species as a further biological indicator.

Materials and Methods

General chemicals and materials

The reagents and chemicals were purchased as follows: tris base ($H_2NC(CH_2OH)_3$ Tris (hydroxymethyl)-aminomethan; Carlo Eraba, France), NH_4Cl (FLUKA, Switzerland), $MgSO_4·7H_2O$ (Fisher Scientific, UK), $CaCl_2·2H_2O$ (Ajax Finechem, Australia), K_2HPO_4 (Ajax Finechem, Australia), KH_2PO_4 (Merck, Germany), $Na_2EDTA·2H_2O$ (Fisher Scientific, UK), $ZnSO_4·7H_2O$ (Ajax Finechem, Australia), H_3BO_3 (Merck, Germany), $MnCl_2·4H_2O$ (Carlo Eraba, France), $FeSO_4·7H_2O$ (Merck, Germany), $CoCl_2·6H_2O$ (Ajax Finechem, Australia), $CuSO_4·5H_2O$ (Merck, Germany), $(NH_4)6MoO_3$ (Mallinckrodt Chemical, USA), Acetic acid glacial (Carlo Eraba, France), Dimethyl sulfoxide (DMSO; Fisher Scientific, UK) and glyphosate (*N*-(Phosphonomethyl)glycine; HPLC grade, Sigma-Aldrich, Germany). Spectrophotometric determinations were performed using a UV-1800 UV-visible spectrophotometer (Shimadzu, Japan). The effective quantum yield was determined by pulse amplitude modulation (PAM 2500, Walz, Germany).

Herbicide stock preparation

The glyphosate used in this study was analytical grade and prepared in sterile distilled water. Serial dilution was performed to achieve the required range of concentrations from 100–500 µg ml^{-1} for *C. ellipsoidea* and *C. vulgaris* and 50–300 µg ml^{-1} for *C. sorokiniana*. The culture medium was used as diluent.

Strains and culture conditions

C. ellipsoidea (TISTR 8260) and *C. vulgaris* (TISTR 8580*)* were purchased from the Thailand Institute of Science and Technology. *C. sorokiniana* strain KU.B2, which was isolated from an agricultural drainage in Nonthaburi Province, Thailand (July 2018), was obtained from the culture collection of the Department of Botany, Faculty of Science, Kasetsart University. *Chlorella* species was cultured in liquid TAP medium (52) containing the following micronutrients: 2.42 gm tris base, 25 ml TAP-salt (15 gm l^{-1} NH_4Cl, 4 gm l^{-1} $MgSO_4·7H_2O$ and 2 gm l^{-1} $CaCl_2·2H_2O$), 1 ml phosphate solution (288 gm l^{-1} K_2HPO_4 and 144 gm l^{-1} KH_2PO_4), 1 ml trace elements solution (Hutner's trace elements; 50 gm l^{-1} $Na_2EDTA·2H_2O$, 22 gm l^{-1} $ZnSO_4·7H_2O$, 11.4 gm l^{-1} H_3BO_3, 5 gm l^{-1} $MnCl_2·4H_2O$, 5 gm l^{-1} $FeSO_4·7H_2O$, 1.6 gm l^{-1} $CoCl_2·6H_2O$, 1.6 gm l^{-1} $CuSO_4·5H_2O$ and 1.1 gm l^{-1} $(NH_4)6MoO_3$) and 1 ml acetic acid. Then, the medium was adjusted to pH 7.0. *Chlorella* species were cultivated at 30 ± 1 °C, under controlled conditions using white cool fluorescent light lamps (330 µmol photons m^{-2} s^{-1}). The cultures were shaken five times per day during incubation. The concentration of each *Chlorella* mixture was quantified by cell counting with a Neubauer chamber (53). The regression equation for the relationship between cell density (y; $1.0×10^6$ cells ml^{-1}) and absorption (x; wavelength at 750 nm) was calculated as follows: y =33.944x - 0.8972 (R^2 = 0.9998) for *C. ellipsoidea*, y = 44.001x - 0.7281 (R^2 = 0.9999) for *C. sorokiniana* and y = 46.72x - 0.3799 (R^2 = 1) for *C. vulgaris*. The same incubation conditions were used for the determination of IC_{50} and in the glyphosate toxicity treatments.

Toxicity experiments

The biomass was measured as the optical density (OD) at 750 nm, according to the standard method (54). The exponential growth phase of samples was cultured in 150 ml culture medium in an Erlenmeyer flask with initial cell density of $1.0 × 10^6$ cells ml^{-1} for 24, 48 and 96 hr. The biomass yield was calculated at 24, 48 and 72 hr. Inhibition of growth was monitored as an index to determine the glyphosate toxicity and the IC_{50} value for biomass was calculated based on OD_{750}. In order to enable direct comparison of dose responses for IC_{50}, all responses were converted to % of control.

Photosynthetic pigment determination

The pigment contents were determined by spectrophotometry according to a previously

described procedure (55). 5 ml of each treatment was harvested then centrifuged at 3000 gm for 15 min. Subsequently, the supernatant was discarded and 5 ml DMSO was added to the extract of photosynthetic pigments. The samples were sonicated for 1 hr and stored in the dark. After 24 hr, each sample was centrifuged at 3000 gm for 15 min. The pigments of the *Chlorella* microalgae samples in the supernatant were analyzed by using a spectrophotometer at appropriate wavelengths (470, 649 and 665 nm). The resulting absorbance measurements were obtained from Wellburn (56). The equations used to calculate the pigment concentrations are as follows:

Chlorophyll *a* (Chl *a*) = 12.19 A_{665} - 3.45 A_{649}

Chlorophyll *b* (Chl *b*) = 21.99 A_{649} - 5.32 A_{665}

Total Chlorophyll = Chlorophyll *a* + Chlorophyll *b*

Total Carotenoids = (1000 A_{470} - 2.86 Chl *a* - 129.2Chl *b*)/221

The pigment contents are represented as the concentrations in µg ml^{-1}.

Chlorophyll fluorescence

The effect of glyphosate on chlorophyll fluorescence was measured as the effective quantum yield using a pulse amplitude modulation (PAM) fluorometer with a suspension cuvette (KS2500; diameter of 7.5 mm, depth of 9.0 mm). Monitoring of the photoinhibition in microalgae was performed using standard method (57). The effective quantum yield was determined by immersing the probe directly into the culture (measuring light intensity = 9, gain = 4) and one measurement was taken per replicate after 24 hr treatment. The toxic response in each treatment was expressed as a percentage of control values.

Statistical analysis

The experimental treatments had three independent replicates. The results were analyzed using GraphPad Prism 6 Software (San Diego, USA). P-values lower than 0.05 were considered as statistically significant with two-way analysis of Dunnett's test. Pearson's correlation was obtained for all treatments.

Results and Discussion

Growth inhibition test to assess the glyphosate toxicity

Our study investigated the glyphosate toxicity in *Chlorella* microalgae, including *Chlorella ellipsoidea*, *Chlorella sorokiniana* and *Chlorella vulgaris* (Fig. 1) for further use as a biological indicator model. To examine the toxicity effects, *C. ellipsoidea*, *C. sorokiniana* and *C. vulgaris* were treated with 100–500, 50–300 and 100 to 500 µg ml^{-1} glyphosate respectively. The growth inhibition test clearly manifested that glyphosate treatment induced a significant inhibitory effect on the acute toxicity in the three species after 24 hr (Fig. 2). Compared to the control, the biomass of *C. ellipsoidea* and *C. sorokiniana* was significantly inhibited by 50–500 µg ml^{-1} glyphosate and that of *C. vulgaris* was inhibited at 100 µg ml^{-1}. The

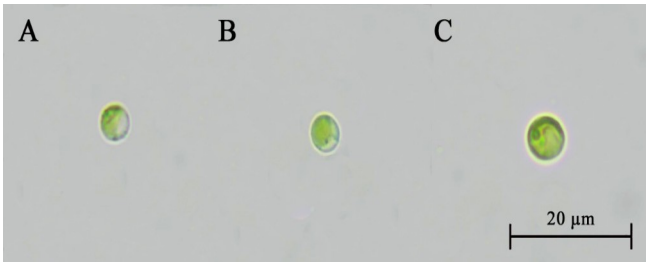


Fig. 1. Microalgae: (A) *Chlorella ellipsoidea*, (B) *Chlorella sorokiniana* and (C) *Chlorella vulgaris*.

growth inhibition in *C. ellipsoidea* and *C. sorokiniana* was not significantly affected by glyphosate concentrations of 300–500 µg ml^{-1} and 200–300 µg ml^{-1} respectively. *C. vulgaris* (IC_{50} = 449.34 ± 6.20 µg ml^{-1}) showed the greatest tolerance to glyphosate compared to *C. ellipsoidea*

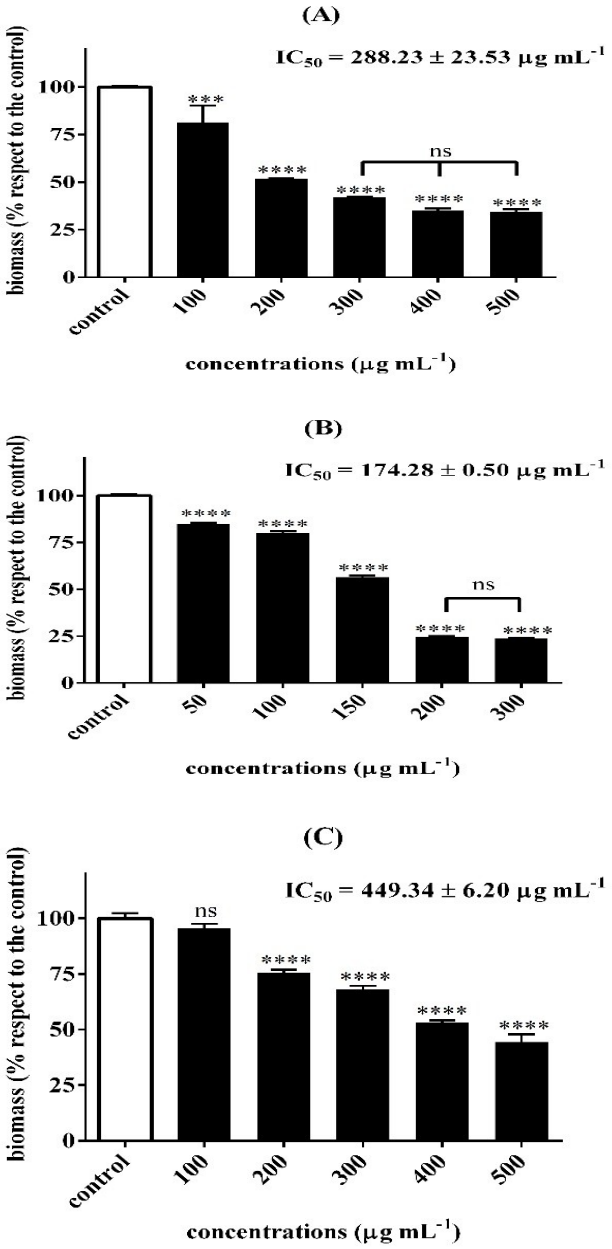


Fig. 2. Biomass of (A) *Chlorella ellipsoidea*, (B) *Chlorella sorokiniana* and (C) *Chlorella vulgaris* as a percentage measured from the optical density (OD) at 750 nm in the control (TAP) after 24 hr exposure to 50–500 µg ml^{-1} glyphosate. The results are presented as the mean ± standard deviation in triplicate (*n* =3).

(IC_{50} = 288.23 ± 23.53 µg ml^{-1}) and *C. sorokiniana* (IC_{50} = 174.28 ± 0.50 µg ml^{-1}). As seen in Fig. 3, glyphosate caused a decrease in biomass of all species as both concentration and time increased, leading to chronic toxicity. Between 24 and 48 hr treatment, the biomass of *C. ellipsoidea* and *C. sorokiniana* did not exceed 300 µg ml^{-1} and 150 µg ml^{-1} respectively. After 72 hr of treatment, the

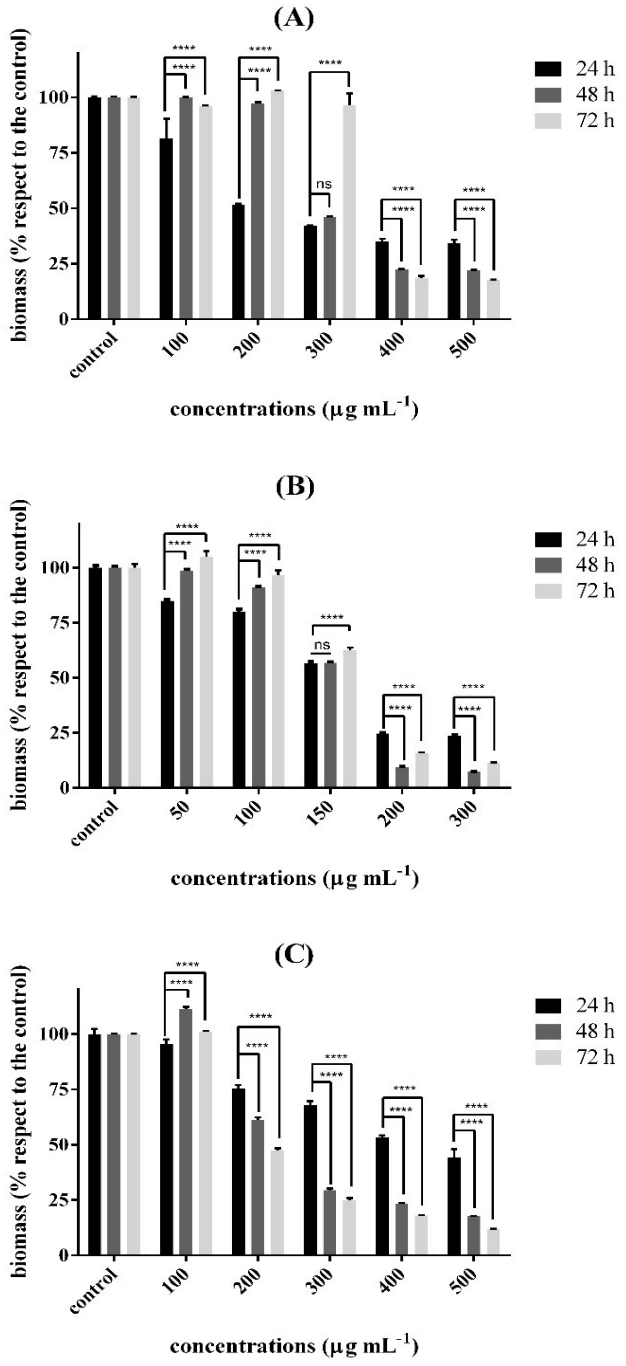


Fig. 3. Biomass of (A) *Chlorella ellipsoidea*, (B) *Chlorella sorokiniana* and (C) *Chlorella vulgaris* measured as the cell viability percentage at optical density (OD) of 750 nm in the control (TAP) after 24, 48 and 72 hr exposure to 50–500 µg ml^{-1} glyphosate. The results are presented as the mean ± standard deviation in triplicate (*n* =3).

glyphosate concentration inhibit growth in the following order: 400–500 µg ml^{-1} for *C. ellipsoidea*, 200–300 µg ml^{-1} for *C. sorokiniana* and 200–500 µg ml^{-1} for *C. vulgaris*.

Many researchers have confirmed that phytoplankton appears to be the most promising early indicator of changes in aquatic system caused by chemicals (9, 10). Within the investigated period and database, there are no reports on IC_{50} that have investigated the toxicological effects of glyphosate in 24 hr. Furthermore, it was reported that *C. sorokiniana* microalgae in South African waters were the most sensitive species to glyphosate herbicide after 24 hr of exposure (30). Previous reports have demonstrated that at low glyphosate concentrations, cell growth of *C. vulgaris* increased within 24–48 hr, which is similar to our results that the cell growth of *Chlorella* species can recover after 24 hr. This is attributed to the phosphorus content in glyphosate, which is essential to microalgae growth (20). Several other studies also suggested that *Chlorella* species, including *C. pyrenoidosa*, *C. saccharophila*, *C. sorokiniana* and *C. vulgaris* are sensitive to glyphosate depending on its concentration and exposure time, which are critical parameters to determine the damage to the balance of the aquatic environment (31, 37, 42, 48, 58).

Pigment contents after glyphosate exposure

After the 24 hr treatment, the concentrations of total chlorophyll and carotenoids were significantly different among *Chlorella* sp. and the control. As indicated in Table 1, increased glyphosate concentration had a negative linear effect on the chlorophyll and carotenoids concentrations. Interestingly, the total carotenoid content was determined to be in the range of 300–500 µg ml^{-1} for *C. ellipsoidea* and 200–300 µg ml^{-1} for *C. sorokiniana*. For both *C. ellipsoidea* and *C. sorokiniana* inhibited growth, the value of total carotenoid content was relate similar to biomass result at the same concentration.

According to a previous report, the total chlorophyll content in *C. kessleri* decreased when exposed to glyphosate in the range 84.54–338.14 µg ml^{-1} within 24–96 hr (59). In another work, *C. pyrenoidosa* treated with increasing glyphosate concentrations (16.9–169.07 µg ml^{-1}) exhibited decreased cell growth, chlorophyll and carotenoid contents (37). Compared with other green microalgae sp. chlorophyll-a content in *Scenedesmus quadricauda* reduced from 2–200 µg ml^{-1} after treated with glyphosate due to the degradation of chlorophyll biosynthesis (60-64). The toxicity of glyphosate may also affect the integrity of thylakoid membranes, preventing absorbed light energy from reaching reaction centers (65). Glyphosate may also indirectly inhibit chlorophyll synthesis by decreasing the Mg content (66). In fact, the Mg incorporation from Mg-chelatase in the porphyrin structure is an important step leading to the chlorophyll synthesis (67). It has been suggested that glyphosate may induce Fe deficiency and, thus, prevent the biosynthesis of δ-aminolevulinic acid (ALA), a significant component of the chlorophyll biosynthetic pathway (68).

Table 1. Pigment contents of chlorophyll-a (chl *a*), chlorophyll-b (chl *b*), and carotenoid in **(A)** *Chlorella ellipsoidea*, **(B)** *Chlorella sorokiniana* and **(C)** *Chlorella vulgaris* after treated with different concentrations of glyphosate (50–500 µg ml^{-1}) for 24 hr. The results are presented as the mean ± standard deviation in triplicate (n = 3).

(A)

Pigment content (µg ml^{-1})	Glyphosate (µg ml^{-1})					
	control	100	200	300	400	500
chl *a*	6.70 ± 0.06	4.81 ± 0.01	4.25 ± 0.01	2.06 ± 0.02	1.70 ± 0.01	1.96 ± 0.01
chl *b*	5.50 ± 0.04	3.99 ± 0.06	3.57 ± 0.16	1.72 ± 0.02	1.26 ± 0.02	1.42 ± 0.02
total chlorophyll	12.20 ± 0.04^{a}	8.80 ± 0.08^{b}	7.82 ± 0.15^{c}	3.78 ± 0.04^{d}	2.96 ± 0.03^{e}	3.37 ±.0.21^{f}
total carotenoid	1.84 ± 0.09^{a}	1.50 ± 0.01^{b}	1.24 ± 0.06^{c}	0.53 ± 0.01^{d}	0.37 ± 0.00^{d}	0.43 ± 0.01^{d}

(B)

Pigment content (µg ml^{-1})	Glyphosate (µg ml^{-1})					
	control	50	100	150	200	300
chl *a*	3.24 ± 0.01	2.36 ± 0.01	0.68 ± 0.01	0.44 ± 0.01	0.12 ± 0.00	0.09 ± 0.00
chl *b*	2.83 ± 0.01	2.08 ± 0.03	0.73 ± 0.00	0.55 ± 0.01	0.22 ± 0.01	0.20 ± 0.01
total chlorophyll	6.08 ± 0.02^{a}	4.44 ± 0.04^{b}	1.41 ± 0.01^{c}	0.99 ±0.02^{d}	0.34 ± 0.01^{e}	0.30 ± 0.01^{f}
total carotenoid	0.82 ±0.01^{a}	0.64 ± 0.00^{b}	0.26 ± 0.00^{c}	0.17 ± 0.01^{d}	0.10 ± 0.00^{e}	0.090 ± 0.00^{e}

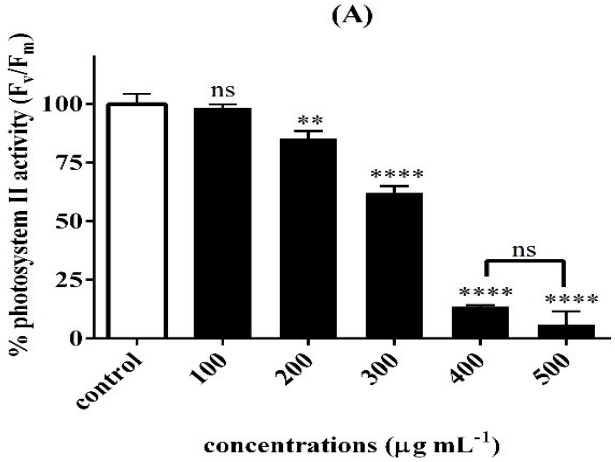
(C)

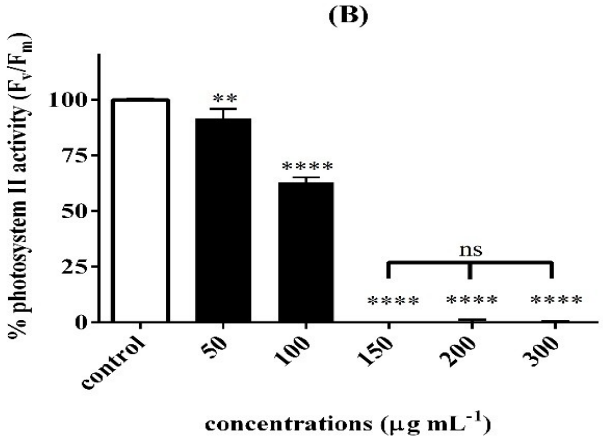
Pigment content (µg ml^{-1})	Glyphosate (µg ml^{-1})					
	control	100	200	300	400	500
chl *a*	5.92 ± 0.19	4.69 ± 0.02	2.80 ± 0.01	1.98 ± 0.00	1.66 ± 0.01	1.54 ± 0.01
chl *b*	2.79 ± 0.11	3.41 ± 0.02	2.20 ± 0.01	1.71 ± 0.01	1.30 ± 0.01	1.16 ± 0.02
total chlorophyll	8.71 ±0.09^{a}	8.10 ± 0.01^{b}	5.00 ± 0.02^{c}	3.69 ± 0.01^{d}	2.96 ± 0.02^{e}	2.70 ± 0.01^{f}
total carotenoid	1.38 ± 0.02^{a}	1.26 ± 0.01^{a}	0.86 ± 0.01^{b}	0.67 ± 0.02^{cd}	0.55 ± 0.00^{d}	0.48 ± 0.00^{de}

Photosynthetic efficiency after exposed to glyphosate

The inhibition of effective quantum yield (F_v/F_m) using pulse amplitude modulation (PAM) fluorometer revealed similar patterns in the biomass and pigment contents of the three *Chlorella* species after 24 hr cumulative glyphosate exposure (Fig. 4). The results demonstrate that 400, 150 and 500 µg ml^{-1} glyphosate had the highest inhibition of quantum yield of *C. ellipsoidea*, *C. sorokiniana* and *C. vulgaris*, respectively. In additional, glyphosate at concentrations of 300 and 400 µg ml^{-1} in *C. vulgaris* presented non-significant photosynthetic effects; thus, the findings indicate that *C. vulgaris* was least influenced by glyphosate.

Glyphosate may affect photosynthesis by indirectly inhibiting the amino acids, fatty acids, carotenoids and chlorophyll biosynthesis. A previous study showed that glyphosate acts as a photosynthetic electron transport inhibitor, specifically inhibiting the activity of photosystem II





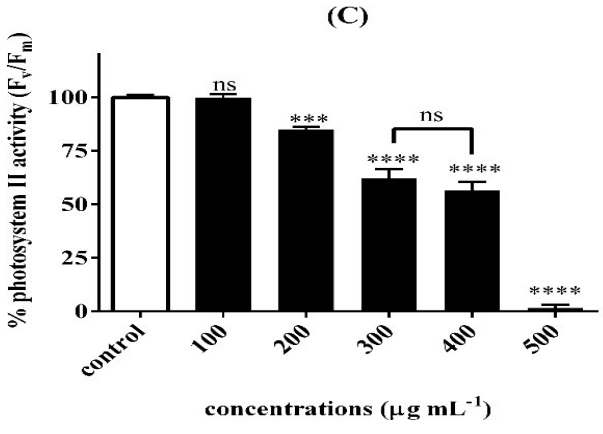


Fig. 4. The maximum quantum efficiency of photosystem II in (A) *Chlorella ellipsoidea*, (B) *Chlorella sorokiniana* and (C) *Chlorella vulgaris* after treated with different concentrations glyphosate (50–500 µg ml^{-1}) for 24 hr. The results are presented as the mean ± standard deviation in triplicate (n = 3).

(PSII) in *C. pyrenoidosa* (37). Later research evaluated the effects of glyphosate on the maximum quantum efficiency of PSII using *in vitro* and *in vivo* treatments (62, 69-71). It was found that the mode of action of glyphosate inhibits the electron transport rate, PSII activity and non-photochemical energy dissipation processes. Moreover, glyphosate can alter the PSI activity (72) and decrease NADH and NADPH pools (69). In another report on the relation between biomass and photosynthesis, electron transport inhibition due to PSII inhibitors at low concentrations decreased growth rates and biomass on two tropical benthic microalgae; *Navicula* sp. (Heterokontophyta) and *Nephroselmis pyriformis* (Chlorophyta) (57).

Correlation analysis

In this study, the obtained Pearson's correlation was utilized as a guide to evaluate the correlation coefficient (r), according to standard method (73). The correlation coefficients of the biomass, pigment contents and photosynthetic performance of three *Chlorella* species indicate that all species present high positive correlation ($r > 0.7$) after 24 hr exposure. Comparing with previous study, the biomass estimates of total green algae were related to pigment contents including chlorophyll *a* and *b* and carotenoids (74). Especially, biomass and pigment contents including chlorophyll *a* and *b*. Our results showed that *Chlorella* species growth as biomass value positive correlated with pigment contents. This results similar to previous study that the highly significant linear regressions were obtained for chlorophyll *a*, total biomass, chlorophyll b green algae and lutein-green algae (74). Moreover, the pigment indices were correlated and revealed importantly community features as biomass and diversity (75). Therefore, all results suggest that the relationship between biomass and pigment contents is directly linked to photosynthesis.

Conclusion

Our experimental results conclude that glyphosate exposure affects biomass, pigment contents and photosynthetic efficiency of *Chlorella* species. After 24-hr acute toxicity glyphosate exposure in terms of biomass, *C. vulgaris* showed the greatest tolerance, while *C. sorokiniana* was the most sensitive. After 72-hr chronic toxicity, the biomass yield of all *Chlorella* species was at a relatively low concentration level, further indicating that the effect of glyphosate is both concentration and time-dependent. Similar to the biomass results, glyphosate exposure lead to reduced pigment contents and photosynthetic efficiency following 24-hr exposure. This indicates that the relationship between the biomass, pigment contents and photosynthetic efficiency is significantly correlated in this study. These results could be beneficial to understanding the impact and potential risk of glyphosate toxicity on microalgae in aquatic environments.

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Authors’ contributions

NS provided conceptualization and resources, review and edited the manuscript. RS investigated experiment, review and edited the manuscript. SK investigated experiment, analyzed data and wrote the first draft of manuscript. All authors have read and approved to the published the manuscript.

Conflict of interests

The authors declare no conflict of interest.

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