Physiological Effects of the Herbicide Glyphosate on the Cyanobacterium Microcystis aeruginosa

Article *in* Aquatic toxicology (Amsterdam, Netherlands) · July 2016

DOI: 10.1016/j.aquatox.2016.07.010

7 authors, including:

Jing Ye
Shanghai Institute of Technology
27 PUBLICATIONS **422** CITATIONS

SEE PROFILE

Aquatic Toxicology 178 (2016) 72–79

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Physiological effects of the herbicide glyphosate on the cyanobacterium *Microcystis aeruginosa*

Liang Wu [a], Zhihao Qiu [a], Ya Zhou [a,b], Yuping Du [a], Chaonan Liu [a], Jing Ye [a,*], Xiaojun Hu [a,*]

[a] School of Chemical and Environmental Engineering, Shanghai Institute of Technology, Shanghai 201418, China
[b] Key Laboratory of Geo-information Science of the Ministry of Education, College of Geographic Science, East China Normal University, Shanghai 200241, China

ARTICLE INFO

Article history:
Received 28 March 2016
Received in revised form 21 June 2016
Accepted 18 July 2016
Available online 21 July 2016

Keywords:
Glyphosate
Oxidative stress
Toxicity
Microcystis aeruginosa

ABSTRACT

Glyphosate has been used extensively for weed control in agriculture in many countries. However, glyphosate can be transported into the aquatic environment and might cause adverse effects on aquatic life. This study investigated the physiological characteristics of cyanobacteria *Microcystis aeruginosa* (*M. aeruginosa*) after exposure to glyphosate, and the results showed that changes in cell density production, chlorophyll *a* and protein content are consistent. In *M. aeruginosa*, oxidative stress caused by glyphosate indicated that 48 h of exposure increased the concentration of malondialdehyde (MDA) and enhanced the activities of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD). To further investigate the toxicity of glyphosate on *M. aeruginosa*, the viability of treated cells was monitored and the toxin release was determined. The results indicated that glyphosate induced apoptosis of and triggered toxin release in *M. aeruginosa*. These results are helpful for understanding the toxic effects of glyphosate on cyanobacteria, which is important for environmental assessment and protection. These results are also useful for guidance on the application of this type of herbicide in agricultural settings.

1. Introduction

Glyphosate (*N*-(phosphonomethyl)glycine) is a post-emergence, non-selective broad-spectrum herbicide that was commercially introduced by the Monsanto Company in 1974 and are applied to many food and non-food field crops, and it is also used for other types of vegetation management. Glyphosate has been used extensively and is the best-selling herbicide in the history of agriculture (Baylis, 2000). In the 1990s, approximately 50,000–80,000 square kilometers were treated with 8.5 million kilogram of glyphosate annually in the United States (RED, 1994). In recent years, glyphosate has been used widely in glyphosate-resistant crops.

It is generally accepted that glyphosate is an environmentally friendly herbicide. However, many studies have reported that glyphosate could have extensive unintended effects on disease severity (Harper, 2007; Larson et al., 2006) and nutrient efficiency (Gordon, 2007; Reichenberger, 2007). Glyphosate can enter the aquatic environment through direct spraying, surface runoff and infiltration (Tsui and Chu, 2008), and recently, glyphosate has been detected in surface water frequently (Coupe et al., 2012; Kolpin et al., 2006; Struger et al., 2008). According to Peruzzo et al. (2008), 0.7 mg/L glyphosate was found in a surface water system in Argentina. Furthermore, certain studies have reported that glyphosate can have physiological effects on aquatic life. Pesce et al. (2009) found that the composition of the algae community can be affected by glyphosate. Wong (2000) observed that the growth of *Scenedesmus quadricauda* could be stimulated by glyphosate at low concentrations. However, studies of the impacts on cyanobacteria caused by glyphosate are insufficient.

Algal blooms caused by cyanobacteria have occurred frequently worldwide (Otten et al., 2012), and serious environmental problems such as hypoxia, unpleasant odors and fish kills are currently attracting increasing concern (Graham et al., 2010). Many known environmental factors that cause these blooms have been investigated, and a number of studies have focused on nutrients, light intensity, pH and temperature (Jiang et al., 2008; Xu et al., 2011). Recently, several studies found that many industrial and agricultural contaminants and their residues, such as antibiotics and herbicides, can affect the growth of cyanobacteria (Perron and

Abbreviations: G, glyphosate; EPSP, 5-enolpyruvylshikimate-3-phosphate; SOD, superoxide dismutase; CAT, catalase; POD, peroxidase; MDA, malondialdehyde; PS, phosphatidylserin.

* Corresponding authors.
E-mail addresses: yejinganna@163.com (J. Ye), hu-xj@mail.tsinghua.edu.cn (X. Hu).

http://dx.doi.org/10.1016/j.aquatox.2016.07.010
0166-445X/

Juneau, 2011; Phlips et al., 1992) and further induce oxidative damage and trigger toxin release. It may affect the entire ecosystem by affecting the food chain. Causative cyanobacteria can produce microcystins (MCs) or other cyclic hepatotoxins which are toxic to domestic livestock and wildlife around the world. They also pose a serious health hazard to humans exposed to them when they use contaminated water for drinking, cooking, or recreation. However, studies on the adverse effects of environmental pollutants on cyanobacteria are still limited.

This study investigated the physiological effects of glyphosate on the cyanobacteria *Microcystis aeruginosa*. Over the past few years in China, *M. aeruginosa* became one of the predominant species involved in water blooms around the entire country (Zhang et al., 2012). The growth curves, chlorophyll *a* and protein content of *M. aeruginosa* were determined after exposure to glyphosate at a range of concentrations. Glyphosate has been proposed to induce oxidative stress in *M. aeruginosa* cells. Therefore, the activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and the concentration of the lipid peroxidation indicator malondialdehyde (MDA) were determined. Cell apoptosis and toxin release were examined to further explore the toxicity of the herbicide glyphosate on *M. aeruginosa*. Understanding the physiological effects caused by glyphosate on cyanobacteria is helpful for risk assessment for this type of herbicide and for protection of the aquatic environment and human health.

2. Materials and methods

2.1. Chemical and cyanobacteria

Glyphosate (≥97% purity) was obtained from JiangShan Co., Ltd. (Nantong, China). The cyanobacteria *M. aeruginosa* was obtained from the Institute of Hydrobiology of Chinese Academy of Sciences (Wuhan, China). The unialgal inoculant (axenic strain) was cultured in sterile BG11 medium under an irradiance of 40 μmol/m^2 s with a photoperiod of 12 h light/12 h dark at 28 ± 1 °C.

2.2. Growth conditions

The algal growth-inhibition tests were carried out according to the OECD guidelines 201-Freshwater Alga and Cyanobacteria (OECD, 2011). Based on a series of pre-experiments, the growth tests were conducted under different nominal concentrations (0, 1, 2, 5 and 10 mg/L) of glyphosate. Three replicates of each concentration were prepared in Erlenmeyer flasks (100 mL) containing 5 mL of algal inoculant and 45 mL of culture medium. The Erlenmeyer flasks were maintained at 28 ± 1 °C and a humidity of 60% in a culture chamber with alternating periods of light and dark (12 h/12 h). Irradiance with a wavelength range from 400 to 750 nm was held constant at 40 μmol/m^2·s. The linear equation relating the cell number and optical density of algal culture was established using a UV/vis spectrometer at 680 nm (TU-1810, China). The initial algal density in every flask was (1.94–2.18) × 10^6 cells/mL. Algal densities were measured every 24 h to obtain the growth curves under different exposure conditions.

2.3. Determination of chlorophyll a content

The chlorophyll *a* content was determined according to the Arnon method (Arnon, 1949). One milliliter of *M. aeruginosa* culture sample was removed from each treatment every three days (day 0, 3, 6, and 9) and extracted by 4 mL of acetone solution at 4 °C in the dark for 24 h. The solutions were centrifuged at 8,000 × *g* (TGL20 M, Hunan Xiangli Scientific Instrument Co., Ltd., China) for 10 min. The supernatant was reserved, and the chlorophyll *a* content was calculated after determination at 663 nm and 645 nm. A solution of 80% (volume percentage) acetone was used as blank correction.

2.4. Determination of protein content

The protein content was determined according to the Bradford method (Bradford, 1976) using the principle of protein-dye binding. One milliliter of *M. aeruginosa* culture sample was removed from each treatment every three days (day 3, 6, and 9). The cell walls were disrupted by ultrasound (JY-96IIN, Ningbo Xinzhi Biotechnology Co., Ltd., China) for 13 min (output power of 6 W) in an ice water bath at 4 °C and centrifuged at 4,000 × *g*. After disruption, 0.5 mL of the disrupted cell suspension was transferred to a test tube, and the optical density was measured at 595 nm.

2.5. Analysis of exposure concentrations

After the chemicals were added into the culture medium, triplicate culture samples were filtered through a 0.45-μm filter and analyzed via HPLC. The analyses were performed on an Agilent 1100 Series HPLC (Agilent, USA) with a G1354A quaternary pump and a G1315B DAD UV/vis detector. The mobile phase was prepared by adding 150 mL of methanol (HPLC grade, Tedia, USA) to the solution, which was mixed by dissolving 13.6 g of potassium dihydrogen phosphate (Shanghai Zhanyun Chemical Co., Ltd.) in 850 mL of purified water (A.S. Watson Group Hong Kong Ltd.). The solution was adjusted to pH 2.0 with phosphoric acid and ultrasonically oscillated for 10 min. The operation conditions consisted of the following: Agilent ZORBAX SAX column (250 mm × 4.6 mm, 5 μm), flow rate of 1.5 mL/min, detection wavelength of 195 nm, and injection volume of 20 μL at room temperature. Preparation and determination followed the GB12686-2004 standard.

2.6. Analysis of antioxidant responses

The SOD activity assay was conducted at 550 nm according to the method of Beauchamp and Fridovich (1971). The CAT activity assay was conducted at 405 nm according to the method of Góth (1991). The POD activity was determined according to Kwak et al. (1995) at 420 nm. The determination of MDA using the thiobarbituric acid (TBA) reactive substances method was performed as described by Hagege et al. (1990) at 532 nm. After 24 h and 48 h of treatment, the activities of SOD, CAT and POD and the MDA concentrations were determined. The SOD, CAT, POD and MDA assay kits were purchased from Jiancheng Bioengineering Institute (Nanjing, China), and the measurements were conducted according to the manufacturer's instructions.

2.7. Determination of cell apoptosis

The viability of *M. aeruginosa* cells after exposure to different concentrations of glyphosate was measured using flow cytometry-based assays. After stabilization of flow, 5000 cells of each sample were counted. The Apopxin PS sensor in the kit binds with PS and emits intense green fluorescence. Each plot is divided into four quadrants, and each individual cell populates in one of the four quadrants. The normal cells of *M. aeruginosa* emit intense red fluorescence such that this cell population populates in the "Live cells" quadrant of the plot. However, cells that are stained with the Apopxin PS sensor are early apoptotic cells, and these cells are populated in the "Apoptotic cells" quadrant of the plot. The Cell Meter™ Phosphatidylserine Apoptosis Assay Kit (Green Fluorescence) was purchased from AAT Bioquest, Inc., and the measurements were conducted according to the manufacturer's

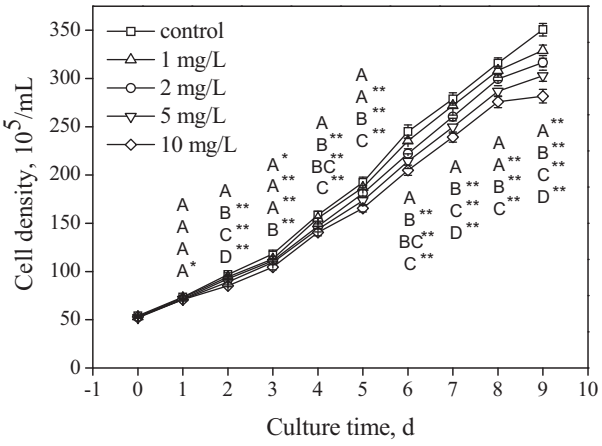

Fig. 1. Growth curves for *M. aeruginosa* exposed to 0, 1, 2, 5 and 10 mg/L of glyphosate for 0–9 days. Results are presented as mean ± SD of three independent assays (*indicates $p < 0.05$, and **indicates $p < 0.01$ relative to the control by ANOVA). Different capitalized letters indicate significant differences ($p < 0.05$) among different exposure concentrations, while the same letter indicates no significant difference (LSD).

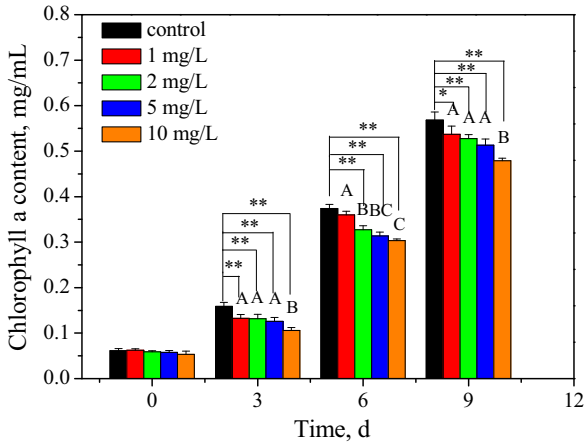

Fig. 2. Chlorophyll *a* content of *M. aeruginosa* cells after treatment with 0, 1, 2, 5 and 10 mg/L of glyphosate for 0, 3, 6 and 9 days. Results are presented as mean ± SD of three independent assays (*indicates $p < 0.05$, and **indicates $p < 0.01$ relative to the control by ANOVA). Different capitalized letters indicate significant differences ($p < 0.05$) among different exposure concentrations, while the same letter indicates no significant difference (LSD).

instructions using flow cytometry (Millipore Guava EasyCyte 5, USA).

2.8. Extraction of MC-LR

The microcystin-LR ELISA Kit was purchased from Beijing Puhuashi Technology Development Co., Ltd. Determination of MC-LR was conducted according to the manufacturer's instructions using a microplate reader (BioTek-BOX-990, USA).

2.9. Data analysis

Statistical analysis was performed using Origin 8.0 (Microcal Software, Northampt, MA, USA) and SPSS 16.0 (SPSS, USA) to determine the significance among the treatments. One-way analysis of variance (ANOVA) was used to determine the differences between control and treatment groups, and $p < 0.05$ was considered statistically significant. Multiple comparisons between the groups were performed using the post-hoc test with the LSD method.

3. Results

3.1. Exposure concentrations of the chemical

For nominal concentrations of 1, 2, 5 and 10 mg/L, the actual exposure concentrations of glyphosate (as measured by HPLC UV/vis) were 0.98 ± 0.054, 1.99 ± 0.101, 4.96 ± 0.235 and 9.71 ± 0.608 mg/L, respectively.

3.2. Growth curves of M. aeruginosa

Growth curves of *M. aeruginosa* exposed to different concentrations of glyphosate are illustrated in Fig. 1. The results showed that on day 1, no significant differences were noted between concentrations. From day 2 to 9, an increase in production of cell density from 1 to 10 mg/L was observed. On day 4, glyphosate at 1 mg/L slightly inhibited the cell density production compared with the control. Glyphosate concentrations of 2, 5 and 10 mg/L significantly ($p < 0.05$) inhibited the cell density production, and the percent inhibitions were 2.19%, 7.39%, 9.43% and 11.45%, respectively. From day 5 to 8, the percent inhibitions at all concentrations were gradually increased and were related to the concentrations of glyphosate. Significant inhibition effects ($p < 0.05$) were observed on day 9 with percent inhibitions of 6.13%, 9.73%, 13.61% and 19.65% for 1, 2, 5 and 10 mg/L, respectively. On day 9, the difference in cell density between the exposure concentrations of 5 mg/L and 10 mg/L was significant ($p < 0.05$).

3.3. Chlorophyll a content of M. aeruginosa

Cyanobacteria contain only chlorophyll *a*. To investigate the effects of glyphosate on the growth of *M. aeruginosa*, the chlorophyll *a* content was determined, and the results are shown in Fig. 2. For every exposure concentration (including control), the content of chlorophyll *a* increased significantly with exposure time. On day 0, the chlorophyll *a* contents were nearly the same at all exposure concentrations. On day 3, the differences in chlorophyll *a* content at 1, 2 and 5 mg/L were not significantly different, but at 10 mg/L, the chlorophyll *a* content was significantly different ($p < 0.05$) compared with the control. However, on day 6, at 1 mg/L, chlorophyll *a* content was slightly lower than in the absence of glyphosate, and at 2, 5 and 10 mg/L, chlorophyll *a* was much lower. The most significant reductions were observed on day 9, highly consistent with the cell density results.

3.4. Protein content of M. aeruginosa

To explore whether protein synthesis of the treated cells was inhibited by glyphosate, the protein content of *M. aeruginosa* cells after exposure to glyphosate are shown in Fig. 3. Similar to chlorophyll *a*, for every exposure concentration (including control), the protein content increased significantly with exposure time. On day 3, the differences in protein content for 1 and 2 mg/L were not significantly different, but significant differences ($p < 0.05$) were observed at 5 and 10 mg/L compared with the control. Similar results were observed on day 9, and different results were observed on day 6. The differences in protein content for 2 and 5 mg/L were not significant. The protein content results at day 3, 6 and 9 were highly consistent with the cell density results and the chlorophyll *a* results.

3.5. SOD, CAT, POD activities and MDA concentration in M. aeruginosa cells

SOD is the first defense against ROS among antioxidant systems (Alscher et al., 2002). The activity of the antioxidant enzyme SOD

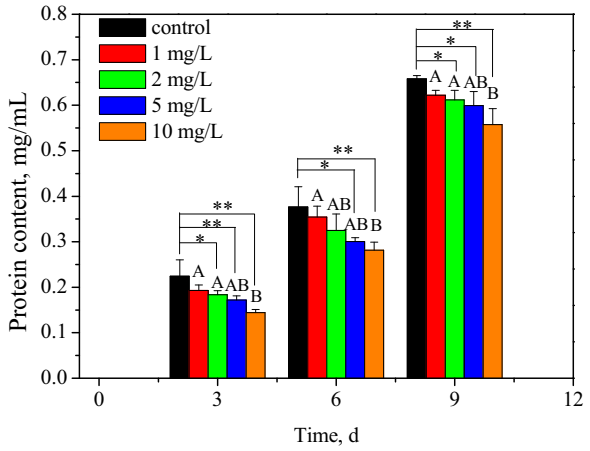

Fig. 3. Protein content of *M. aeruginosa* cells after treatment with 0, 1, 2, 5 and 10 mg/L of glyphosate for 3, 6 and 9 days. Results are presented as mean ± SD of three independent assays (* indicates $p < 0.05$, and ** indicates $p < 0.01$ relative to the control by ANOVA). Different capitalized letters indicate significant differences ($p < 0.05$) among different exposure concentrations, while the same letter indicates no significant difference (LSD).

was examined to determine whether glyphosate affects the antioxidant system. The results are shown in Fig. 4A. After 24 h of exposure, the SOD activity in cells treated with 1 and 2 mg/L glyphosate was decreased, and the SOD activity at 2 mg/L was higher than that at 1 mg/L; however, the differences between them were not significant. After 48 h of exposure, SOD activity at both 1 and 2 mg/L was increased by glyphosate by 1.17-fold and 1.23-fold, respectively.

CAT and POD are the second defense against ROS. The superoxide radicals are converted to hydrogen peroxide by SOD, and they can be further eliminated by CAT and POD (Cho and Seo, 2005). The activities of CAT and POD were examined to further determine whether glyphosate has impacts on the antioxidant system. As shown in Fig. 4B, after 24 h of exposure, the CAT activities were increased 1.47-fold and 2.46-fold by glyphosate at 1 mg/L and 2 mg/L, respectively. After 48 h of exposure, the activities of CAT were greatly increased to 2.03-fold and 4.14-fold at 1 mg/L and 2 mg/L, respectively. As shown in Fig. 4C, after 24 h of exposure, the POD activities at 1 mg/L and 2 mg/L were increased, which were similar to the results obtained from CAT activity. After 48 h of exposure, the tendency of POD activity was the same as after 24 h of exposure; however, the differences between 1 mg/L and 2 mg/L were significantly different ($p < 0.05$) compared with the control, and glyphosate increased the POD activity by 1.26-fold and 1.41-fold, respectively.

MDA is a byproduct of lipid peroxidation, and the concentration of MDA was quantified to ascertain the involvement of lipid peroxidation in the toxicity of glyphosate. As shown in Fig. 4D, after 24 h of exposure, 1 mg/L and 2 mg/L glyphosate induced 1.24-fold and 1.12-fold increases in MDA compared with the control, respectively. After 48 h of exposure, the MDA concentration increased with the increased concentration of glyphosate. Glyphosate induced 1.45-fold and 1.72-fold increases in MDA compared with the control, respectively, and the differences between 1 mg/L and 2 mg/L were significantly different ($p < 0.05$).

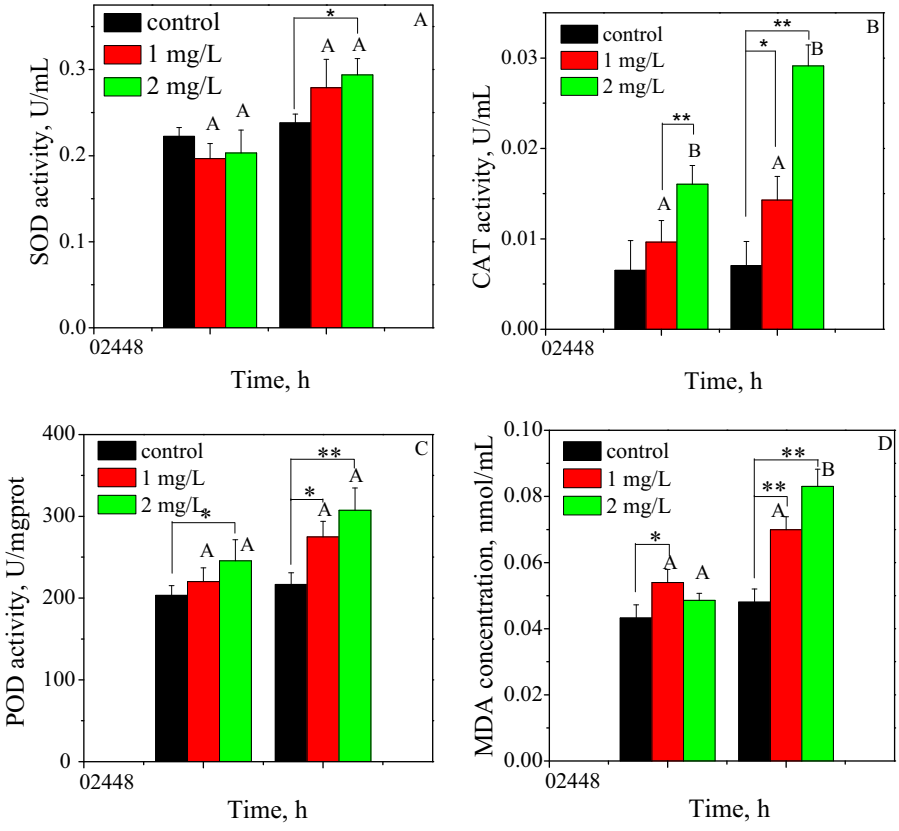

Fig. 4. Activities of SOD (A), CAT (B), POD (C), and MDA (D) concentration in *M. aeruginosa* cells exposed to 0, 1 and 2 mg/L of glyphosate after 24 h and 48 h. Results are presented as mean ± SD of three independent assays (* indicates $p < 0.05$, and ** indicates $p < 0.01$ relative to the control by ANOVA). Different capitalized letters indicate significant differences ($p < 0.05$) among different exposure concentrations, while the same letter indicates no significant difference (LSD).

3.6. Cell apoptosis

Cell viability is one of the most important indicators for determining whether cells are healthy. To further investigate whether the oxidative stress can induce apoptosis, the viability of treated cells was monitored. In the control sample (Fig. 5A), the numbers of cells in the "Live cells" quadrant and "Apoptotic cells" quadrant are 3713 and 161, respectively. As shown in Fig. 5B–E, the numbers of cells in the "Live cells" quadrants are 3453, 3348, 3151 and 3121, respectively, and the number of cells in the "Apoptotic cells" quadrants are 274, 342, 410 and 431, respectively.

3.7. MC-LR analysis of M. aeruginosa

As shown in Fig. 6A, after 24 h of exposure, the intracellular MC-LR concentration increased with increased concentration of glyphosate. In contrast, after 48 h of exposure, the intracellular MC-LR concentration decreased with increased concentration of glyphosate. As shown in Fig. 6B, after 24 h and 48 h of exposure, the extracellular MC-LR concentrations increased with increased concentration of glyphosate.

4. Discussion

4.1. Physiological effects of glyphosate on M. aeruginosa

Many studies have investigated growth inhibition by herbicides on different types of microalgae, such as diclofop (Ye et al., 2013) and sulfonylurea (Nyström et al., 1999); however, the inhibitory effects of glyphosate on cyanobacteria are poorly understood. Qiu et al. (2013) studied glyphosate as the sole P source for growth of *M. aeruginosa* and found that glyphosate can stimulate the growth of *M. aeruginosa* at concentrations ranging from 0.01 to 5 mg/L. However, the results of the current study showed an inhibitory effect, perhaps because the *M. aeruginosa* used in this study was not P-starved, and *M. aeruginosa* did not use glyphosate as the sole P resource. The results showed that as the concentration of glyphosate increased, the cell density production of *M. aeruginosa* was inhibited more significantly, indicating that glyphosate inhibits to the growth of *M. aeruginosa*.

Chlorophyll plays an important role in energy capture and transfer during photosynthesis. Once the biosynthesis of chlorophyll has been inhibited, the growth of the plant is inhibited as well. Many previous studies have suggested that certain pollutants, such as antibiotics, can inhibit the activity of chlorophyll synthase and induce a decrease in chlorophyll content (Bradel et al., 2000). The impacts on the growth of *M. aeruginosa* can be revealed by the decreased chlorophyll *a* content (Kreitlow et al., 1999). Liu et al. (2014) reported that the growth of *M. aeruginosa* was consistent with the changes in chlorophyll *a* content. On the same day (day 3, 6 or 9), the chlorophyll *a* content was decreased and the concentrations of glyphosate increased. The results showed that glyphosate can inhibit the chlorophyll *a* content, and with increased concentrations of glyphosate, the inhibitory effects of chlorophyll *a* content became more apparent.

Protein is indispensable for cell growth, and it has been commonly treated as an important indicator for evaluating the growth condition of cells. Similar to the results of chlorophyll *a* content, protein content was apparently decreased by glyphosate; as the concentration of glyphosate increased, the protein content of *M. aeruginosa* decreased.

The herbicidal action of glyphosate is chelation with Mn, a cofactor for the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase enzyme in the shikimate pathway, and glyphosate inhibits this metabolic pathway in plants and many microorganisms (Cerdeira and Duke, 2006; Jaworski, 1972). Both chlorophyll *a* and protein content reflect the growth condition of *M. aeruginosa*. From the growth curves, it is obvious that glyphosate inhibits the growth of *M. aeruginosa*, and as the concentration of glyphosate increases, the inhibition increases. It can be inferred that glyphosate inhibits the EPSP synthase enzyme of *M. aeruginosa*. Thus, the chlorophyll *a* content was decreased, as was also the protein content. To further investigate the toxicity mechanism of glyphosate and the manner in which it works on *M. aeruginosa*, the antioxidant responses of treated cells were determined.

4.2. Antioxidant responses of M. aeruginosa

Previous studies have revealed that many pollutants can generate intracellular reactive oxygen species (ROS) (Bagchi et al., 1995). The increased ROS can trigger oxidative damage to proteins and lipids, which finally leads to damage of different cellular organelles. The increased ROS also trigger different antioxidant responses that prevent damage to proteins and lipids. Certain responses are increases in the activities of the antioxidant enzymes superoxide dismutase, catalase and peroxidase (Chaufan et al., 2006; Lei et al., 2006).

After 24 h of exposure to glyphosate, the SOD activity was decreased at 1 mg/L and slightly increased at 2 mg/L, and at the same time, the CAT and POD activities were increased with the increased concentration of glyphosate. Morelli and Scarano (2004) found that the SOD and CAT activities in the marine diatom were increased in response to copper treatment. Liu and Pang (2010) found that SOD and POD activities are stimulated to protect two red algae. Decreased MDA concentration was followed by increased POD and CAT activities at concentrations from 1 to 2 mg/L. The results indicated that glyphosate-induced oxidative damage occurred in *M. aeruginosa* after 24 h of exposure at 1 mg/L; however, at 2 mg/L, the oxidative damage caused by glyphosate could be moderated by the increased activities of CAT and POD. After 48 h of exposure, the activities of SOD, CAT and POD showed an increasing tendency from the control to 2 mg/L, and the MDA concentration was also increased with the increased glyphosate concentration. Romero et al. (2011) reported that a significant increase in the MDA concentration of *C. kessleri* exposed to different concentrations of glyphosate indicates the occurrence of damage to the lipid membranes. Similar to previous results, our current findings also showed that glyphosate triggered oxidative damage in the lipids of *M. aeruginosa*. When oxidative damage exceeds a threshold, it might induce apoptosis of survival cells.

4.3. Analysis of cell apoptosis

Apoptosis is the process of programmed cell death that can occur in organisms. However, if apoptosis occurs in a time that is much shorter than the regular lifecycle of cells, it might result from toxicity caused by pollutants. Phosphatidylserine (PS) is located at the inner side of the plasma membrane and can only be transferred to the outer leaflet of the plasma membrane during apoptosis. The appearance of PS on the cell surface is a universal indicator of the initial stage of cell apoptosis and can be detected before morphological changes can be observed. In this study, it was clearly observed that the cells in the "Apoptotic cells" quadrant increased, and the cells in the "Live cells" quadrant decreased. As the concentrations of glyphosate increased, the number of live cells decreased, and the number of apoptotic cells increased. Obviously, the oxidative stress caused by glyphosate can induce apoptosis of *M. aeruginosa*.

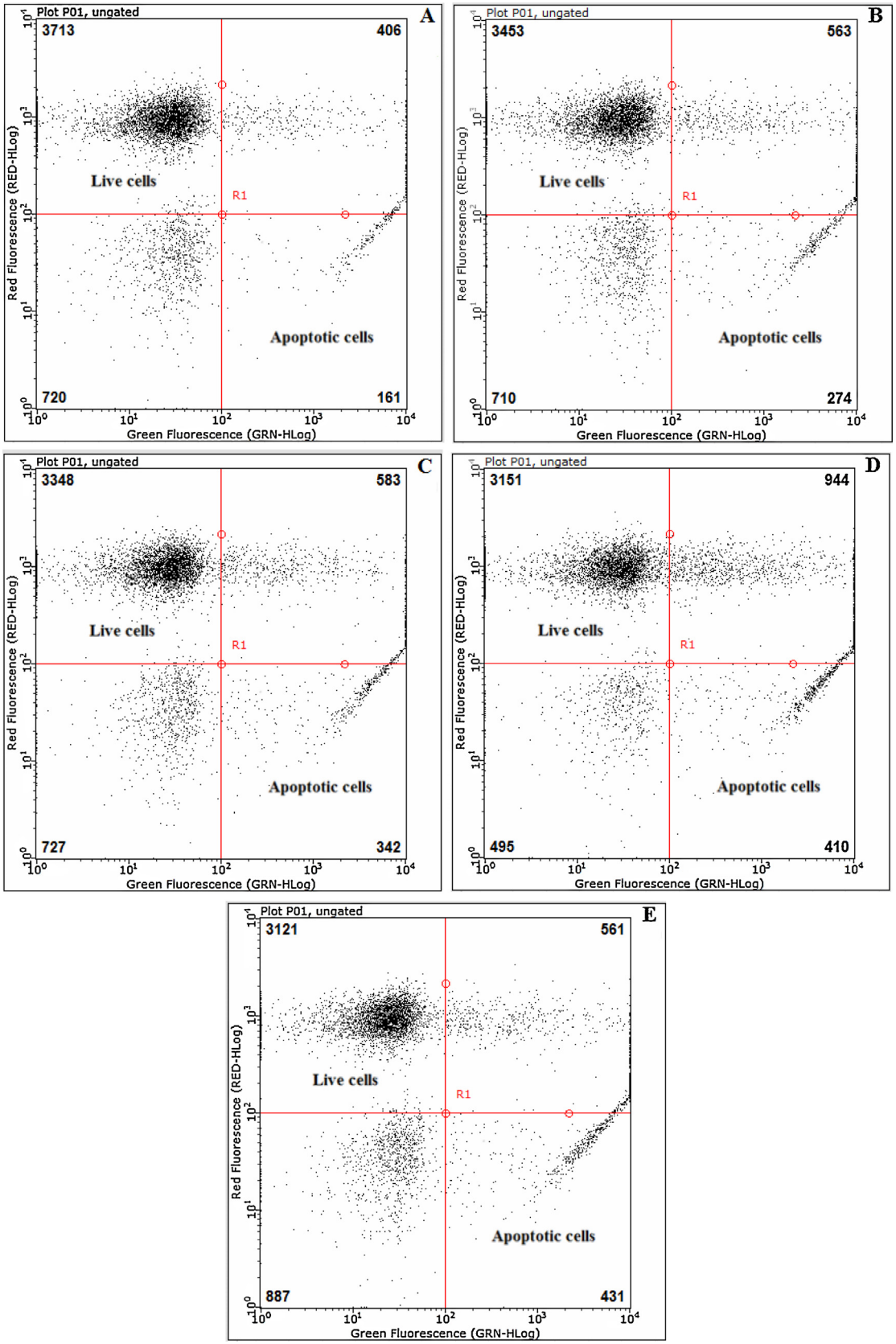

Fig. 5. Apoptosis of *M. aeruginosa* cells in samples after 24-h inoculation with 0, 1, 2, 5 and 10 mg/L (A–E) of glyphosate.

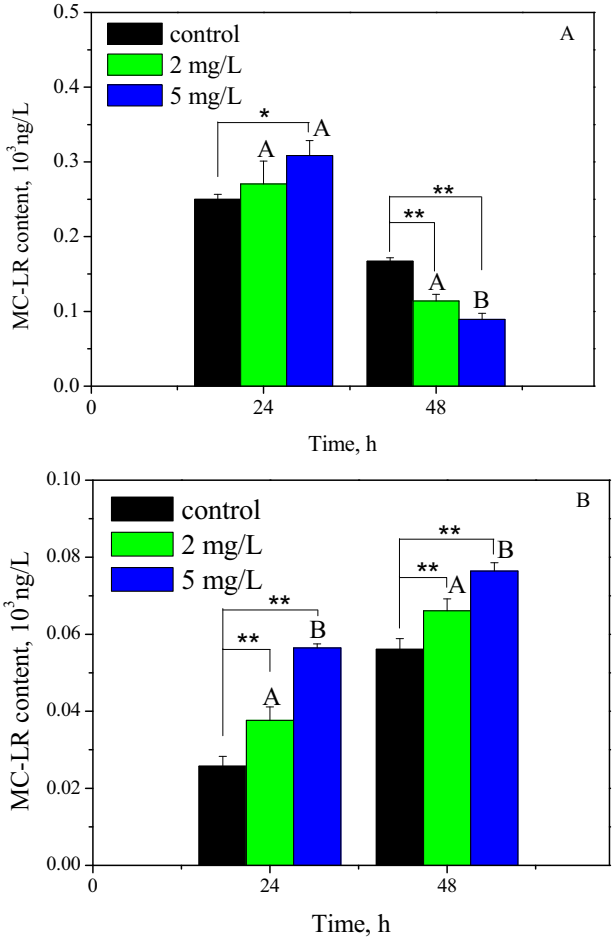

Fig. 6. Intracellular (A) and extracellular (B) MC-LR concentrations in *M. aeruginosa* cells exposed to 2 and 5 mg/L glyphosate after 24 h and 48 h. Results are presented as mean ± SD of three independent assays (* indicates $p < 0.05$, and ** indicates $p < 0.01$ relative to the control by ANOVA). Different capitalized letters indicate significant differences ($p < 0.05$) among different exposure concentrations, while the same letter indicates no significant difference (LSD).

4.4. Toxin release in response to cell damage

Cyanotoxins are retained within the cell structure by many cyanobacteria, and the substance can only be released into surrounding water upon cell lysis (White et al., 2005). Increased intracellular toxins are an indicator of the early stage of a toxic bloom. As the bloom ages, the concentration of extracellular toxins increases (Lahti et al., 1997). The increase in the amount of released toxin has a severely deleterious effect on the water column and can pose adverse effects to aquatic life. Furthermore, the contaminated water can eventually threaten human health. In this study, after 48 h of exposure, the extracellular MC-LR concentration was consistent with the MDA concentration. The oxidative lipid peroxidation and the destruction of the cell membrane promote transfer of the MC-LR from the intracellular to the extracellular space. As the apoptosis of *M. aeruginosa* continues, the treated cells eventually die and release their intracellular contents to the surrounding water, and the microtoxins within the cell structure are also released. The results indicated that toxin release from cultures of *M. aeruginosa* is stimulated by glyphosate.

In conclusion, when *M. aeruginosa* was exposed to the herbicide glyphosate, the chlorophyll *a* content was significantly decreased. At the same time, the protein content was apparently also decreased; both indicate inhibition of the growth of *M. aeruginosa*. The results demonstrated that (1) glyphosate causes adverse effects on the growth of *M. aeruginosa*. (2) The activity of the enzymatic antioxidant SOD slightly decreased, but those of CAT and POD increased in an effort to minimize the potentially destructive conditions, which demonstrated that the treated cells were under oxidative stress. The MDA concentration increased significantly, suggesting that glyphosate induced oxidative damage to *M. aeruginosa*. (3) Cell apoptosis occurred within a short time, and as the concentration of glyphosate increased, the number of apoptotic cells increased, which revealed that glyphosate induced apoptosis in *M. aeruginosa* cells. (4) MC-LR release was significantly increased in cells treated with glyphosate. The results demonstrated that glyphosate initiates destruction of the cell membrane through lipid peroxidation and induces apoptosis in treated cells, which eventually release microtoxins together with the intracellular contents. The results indicate that the glyphosate can pose physiological effects on *M. aeruginosa*. Additionally, the results

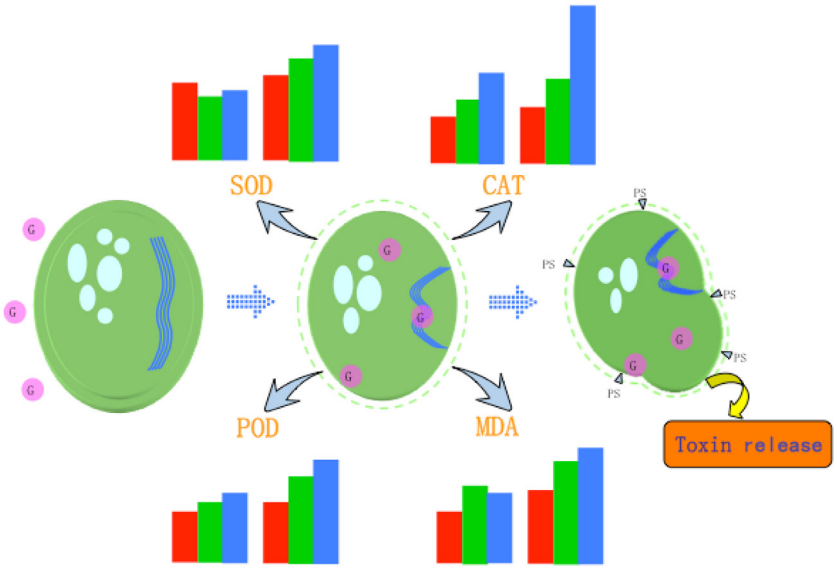

Fig. 7. Schematic diagram showing the morphologic change of *M. aeruginosa*, induction of oxidative damage and subsequent release of toxins from *M. aeruginosa* cells exposed to different concentrations of glyphosate.

suggested that mechanism in *M. aeruginosa* involves inhibition of protein biosynthesis, consistent with the herbicidal action; the inhibition of protein biosynthesis is the specific mode of action of glyphosate (Fig. 7).

Conflict of interest

The authors declare no competing financial interest.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (21307082, 20977062), the project of the Science and Technology Commission of Shanghai Municipality, China (13ZR1421700), the Innovation Program of Shanghai Municipal Education Commission (13YZ116), and the Cultivation Fund of the Key Scientific and Technical Innovation Project, Ministry of Education of China (708052).

References

Alscher, R.G., Erturk, N., Heath, L.S., 2002. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. J. Exp. Bot. 53, 1331–1341.
Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts: polyphenoloxidase in beta vulgaris. Plant Physiol. 24, 1.
Bagchi, D., Bagchi, M., Hassoun, E.A., Stohs, S.J., 1995. In vitro and in vivo generation of reactive oxygen species, DNA damage and lactate dehydrogenase leakage by selected pesticides. Toxicology 104, 129–140.
Baylis, A.D., 2000. Why glyphosate is a global herbicide: strengths, weaknesses and prospects. Pest Manage. Sci. 56, 299–308.
Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 44, 276–287.
Bradel, B.G., Preil, W., Jeske, H., 2000. Remission of the free-branching pattern of Euphorbia pulcherrima by tetracycline treatment. J. Phytopathol. 148, 587–590.
Bradford, M.M., 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Cerdeira, A.L., Duke, S.O., 2006. The current status and environmental impacts of glyphosate-resistant crops: a review. J. Environ. Qual. 35, 1633–1658.
Chaufan, G., Juarez, A., Basack, S., Ithuralde, E., Sabatini, S.E., Genovese, G., Oneto, M.L., Kesten, E., Molina, M.d.C.R.d., 2006. Toxicity of hexachlorobenzene and itstransference from microalgae (Chlorella kessleri) to crabs (Chasmagnatus granulatus). Toxicology 227, 262–270.
Cho, U.H., Seo, N.H., 2005. Oxidative stress in Arabidopsis thaliana exposed to cadmium is due to hydrogen peroxide accumulation. Plant Sci. 168, 113–120.
Coupe, R.H., Kalkhoff, S.J., Capel, P.D., Gregoire, C., 2012. Fate and transport of glyphosate and aminomethylphosphonic acid in surface waters of agricultural basins. Pest Manage. Sci. 68, 16–30.
Góth, L., 1991. A simple method for determination of serum catalase activity and revision of reference range. Clin. Chim. Acta 196, 143–151.
Gordon, B., 2007. Manganese nutrition of glyphosate-resistant and conventional soybeans. Better Crops Plant Food 91, 12–13.
Graham, J.L., Loetin, K.A., Meyer, M.T., Ziegler, A.C., 2010. Cyanotoxin mixtures and taste-and-odor compounds in cyanobacteria blooms from the Midwestern United States. Environ. Sci. Technol. 44, 7361–7368.
Hagege, D., Nouvelot, A., Boucaud, J., Gasper, T., 1990. Malondialdehyde titration with thiobarbiturate in plant extracts: avoidance of pigment interference. Phytochem. Anal. 1, 86–89.
Harper, M., 2007. The Review of the Moratorium on GM Canola, Australia.
Jaworski, E.G., 1972. Mode of action of *N*-phosphonomethyl-glycine: inhibition of aromatic amino acid biosynthesis. J. Agric. Food Chem. 20, 1195–1198.
Jiang, Y., Ji, B., Wong, R.N.S., Wong, M.H., 2008. Statistical study on the effects of environmental factors on the growth and microcystins production of bloom-forming cyanobacterium-Microcystis aeruginosa. Harmful Algae 7, 127–136.
Kolpin, D.W., Thurman, E.M., Lee, E.A., Meyer, M.T., Furlong, E.T., Glassmeyer, S.T., 2006. Urban contribution of glyphosate and its degradate AMPA to streams in the United States. Sci. Total Environ. 354, 191–197.
Kreitlow, S., Mundt, S., Lindequist, U., 1999. Cyanobacteria-a potential source of new biologically active substances. J. Biotechnol. 70, 61–63.
Kwak, S.S., Kim, S.K., Lee, M.S., Jung, K.H., Park, I.H., Liu, J.R., 1995. Acidic peroxidases from suspension-cultures of sweet potato. Phytochemistry 39, 981–984.
Lahti, K., Rapala, J., Fardig, M., Niemela, M., Sivonen, K., 1997. Persistance of cyanobacterial hepatotoxin, Microcystin-LR, in particulate material and dis-solved in lake water. Water Res. 31, 1005–1012.
Larson, R.L., Hill, A.L., Fenwick, A., Kniss, A.R., Hanson, L.E., Miller, S.D., 2006. Influence of glyphosate on Rhizoctonia and Fusarium root rot in sugar beet. Pest Manage. Sci. 62, 1182–1192.
Lei, A., Hu, Z., Wong, Y., Tam, N.F., 2006. Antioxidant responses of microalgal species to pyrene. J. Appl. Phycol. 18, 67–78.
Liu, F., Pang, S.J., 2010. Stress tolerance and antioxidant enzymatic activities in the metabolisms of the reactive oxygen species in two intertidal red algae Grateloupia turuturu and Palmaria palmata. J. Exp. Mar. Biol. Ecol. 382, 82–87.
Liu, Y., Zhang, J., Gao, B., Feng, S., 2014. Combined effects of two antibiotic contaminants on microcystis aeruginosa. J. Hazard. Mater. 279, 148–155.
Morelli, E., Scarano, G., 2004. Copper-induced changes of non-protein thiols and antioxidant enzymes in the marine microalga Phaeodactylum tricornutum. Plant Sci. 167, 289–296.
Nyström, B., Björnsäter, B., Blanck, H., 1999. Effects of sulfonylurea herbicides on non-target aquatic micro-organisms: growth inhibition of micro-algae and short-term inhibition of adenine and thymidine incorporation in periphyton communities. Aquat. Toxicol. 47, 9–22.
OECD, 2011. Freshwater Alga and Cyanobacteria, Growth Inhibition Test (201). OECD Guidelines for the Testing of Chemicals.
Otten, T.G., Xu, H., Qin, B., Zhu, G., Paerl, H.W., 2012. Spatiotemporal patterns and ecophysiology of toxigenic Microcystis blooms in Lake Taihu, China: implications for water quality. Environ. Sci. Technol. 46, 3480–3488.
Perron, M.C., Juneau, P., 2011. Effect of endocrine disrupters on photosystem II energy fluxes of green algae and cyanobacteria. Environ. Res. 111, 520–529.
Peruzzo, P.J., Porta, A.A., Ronco, A.E., 2008. Levels of glyphosate in surface waters, sediments and soils associated with direct sowing soybean cultivation in north pampasic region of Argentina. Environ. Pollut. 156, 61–66.
Pesce, S., Batisson, I., Bardot, C., Fajon, C., Portelli, C., Montuelle, B., Bohatier, J., 2009. Response of spring and summer riverine microbial communities following glyphosate exposure. Ecotoxicol. Environ. Saf. 72, 1905–1912.
Phlips, E.J., Hansen, P., Velardi, T., 1992. Effect of the herbicide diquat on the growth of microalgae and cyanobacteria. Bull. Environ. Contam. Toxicol. 49, 750–756.
Qiu, H., Geng, J., Ren, H., Xia, X., Wang, X., Yu, Y., 2013. Physiological and biochemical responses of Microcystis aeruginosa to glyphosate and its Roundup formulation. J. Hazard. Mater. 248–249, 172–176.
RED, 1994. Facts Glyphosate. United States Environmental Protection Agency, Washington, DC.
Reichenberger, L., 2007. Missing micronutrients: using glyphosate is complicating the uptake of some minor nutrients. Furrow, 22–23.
Romero, D.M., Molina, M.C.R.d., Juarez, A.B., 2011. Oxidative stress induced by a commercial glyphosate formulation in a tolerant strain of Chlorella kessleri. Ecotoxicol. Environ. Saf. 74, 741–747.
Struger, J., Thompson, D., Staznik, B., Martin, P., McDaniel, T., Marvin, C., 2008. Ocurrence of glyphosate in surface waters of Southern Ontario. Bull. Environ. Contam. Toxicol. 80, 378–384.
Tsui, M.T.K., Chu, L.M., 2008. Environmental fate and non-target impact of glyphosate-based herbicide (Roundup) in a subtropical wetland. Chemosphere 71, 439–446.
White, S.H., Duivenvoorden, L.J., Fabbro, L.D., 2005. A decision-making framework for ecological impacts associated with the accumulation of cyanotoxins (cylin-drospermopsin and microcystin). Lake Reserv. Manage. 10, 25–37.
Wong, P.K., 2000. Effect of 2, 4-D, glyphosate and paraquat on growth, photosynthesis and cholorophyll-*a* synthesis of Scenedesmus quadricauda Berb 614. Chemosphere 41, 177–182.
Xu, Y., Yang, F., Liu, Y., Wang, Z., Wang, J., Wang, G., Li, R., 2011. Genetic diversity of Microcystis populations in a bloom and its relationship to the environmental factors in Qinhuai River, China. Microbiol. Res. 167, 20–26.
Ye, J., Wang, L., Zhang, Z., Liu, W., 2013. Enantioselective physiological effects of the herbicide diclofop on cyanobacterium microcystis aeruginosa. Environ. Sci. Technol. 47, 3893–3901.
Zhang, P., Zhai, C., Chen, R., Liu, C., Xue, Y., Jiang, J., 2012. The dynamics of the water bloom-forming Microcystis aeruginosa and its relationship with biotic and abiotic factors in Lake Taihu, China. Ecol. Eng. 47, 274–277.