Assessment of the potential toxicity of glyphosate-based herbicides on the photosynthesis of *Nitella microcarpa* var. *wrightii* (Charophyceae)

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ABSTRACT: Although macroalgae are considered one of the most important primary producers in streams, to our knowledge there has been no research on the effects of herbicides on these organisms. Such studies are crucial for improving our understanding of the impact of these substances on stream ecology. In this study, we assess the effects of technical-grade glyphosate, Roundup and aminomethylphosphonic acid (AMPA; the main degradation product of glyphosate) on the photosynthetic rate, dark respiration rate and chlorophyll *a* content of *Nitella microcarpa* var. *wrightii*, a green algae found worldwide. Three concentrations of technical-grade glyphosate and Roundup were tested (0.28, 3.5 and 6 mg l^{-1}), while for AMPA only one concentration was evaluated (0.03 mg l^{-1}). Our results indicate that glyphosate has a stronger inhibitory effect on photosynthetic rate when applied in association with a surfactant (Roundup). These effects are related both to the concentration of the active ingredient and to exposure time. On the other hand, treatment with AMPA had a stimulatory effect on the photosynthetic rate, which may be associated with an increased supply of phosphorus available to the algae from the AMPA degradation process. From an ecological perspective, our results show that the ecological distribution of *N. microcarpa* var. *wrightii*, in terms of both spatial and temporal scales, can be affected by glyphosate-based herbicides in streams.

KEY WORDS: AMPA, Glyphosate, Green algae, Herbicides, Photosynthesis, Stream

INTRODUCTION

Herbicides containing glyphosate, *N*-(phosphonomethyl) glycine, are the most widely used herbicides in Brazil, with almost 188,000 tons sold in 2013 (IBAMA 2013). Absorbed by the plant through the leaves, glyphosate acts on aromatic amino acid synthesis by competitive inhibition of the enzyme EPSPs (5-enolpyruvylshikimate-3-phosphate synthase), blocking the metabolic synthesis of phenylalanine, tyrosine and tryptophan (Forlani *et al*. 2008). Some secondary effects have also been reported (Zobiole *et al*. 2012; Serra *et al*. 2013; Gomes *et al*. 2014), such as the assimilation of Ca, Fe and Mn, which can be affected by the glyphosate complexation of these nutrients, inhibiting the translocation processes inside the plant (Senem Su *et al*. 2009; Zobiole *et al*. 2012). The increased oxidative stress caused by the inhibition of aromatic amino acid synthesis has been observed with greater concentrations of ascorbate and inositol (Serra *et al*. 2013; Gomes *et al*. 2014). Glyphosate reduces the formation of microtubules, slowing down cell division, leading to nuclei with a lobed appearance and causing malformations in chloroplasts, which acquire a spiral shape, or disruptions of the chloroplast envelope (Campbell *et al*. 1976; Vaughn & Duke 1986).

The photosynthetic rate is affected by the drainage of carbon from the Calvin cycle for the synthesis of shikimate. After the inhibition of EPSP, arogenate, the allosteric inhibitor of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase, stops being produced, resulting in the consumption of erythrose-4-phosphate in this pathway and the inhibition of the regeneration of ribulose-1,5-bisphosphate in the Calvin cycle (Gomes *et al*. 2014). However, exactly how glyphosate inhibits chlorophyll synthesis is still not clear. Some studies have shown that the main degradation product of glyphosate, aminomethylphosphonic acid (AMPA), is responsible for this effect, although the exact mechanism remains unknown (Reddy *et al*. 2004; Serra *et al*. 2013; Gomes *et al*. 2014).

Several studies from around the world have uncovered cases of glyphosate contamination in streams (Battaglin *et al*. 2005; Peruzzo *et al*. 2008). Samples from 51 streams belonging to nine Midwest states in the United States revealed the presence of glyphosate and AMPA in 36% and 69% of samples, respectively (Battaglin *et al*. 2005). Runoff analysis of natural rainfall uncovered extremely high concentrations of glyphosate (up to 5.2 mg l^{-1}; Edwards *et al*. 1980). Furthermore, high glyphosate concentrations have also been reported in streams from forests in the United States (1.24 mg l^{-1}; Newton *et al*. 1994) and the Argentinean pampas (0.7 mg l^{-1}; Peruzzo *et al*. 2008). In terms of AMPA concentrations in surface water, a review by Villeneuve *et al*. (2011) reported concentrations ranging from 0.04 to 66 µg l^{-1} in Canada, 0.02 to 41 µg l^{-1} in the United States and 2.1 to 48.1 µg l^{-1} in France. In a more recent study involving streams located in agricultural regions of the United States, the average concentration of AMPA ranged from 0.14 to 26 µg l^{-1} (Coupe *et al*. 2012).

The glyphosate concentration of water permitted by law varies depending on the country. In the United States, the limit of glyphosate permitted in drinking water is 0.7 mg l^{-1} (US Environmental Protection Agency 2009); whereas, for the Council of the European Union (1998), the maximum

concentration allowed in drinking water is 0.0001 mg l^{-1}, and the maximum concentration of a pesticide mixture is 0.0005 mg l^{-1}. In Brazil, the limit for glyphosate established by Resolution No. 357/05 of the National Environmental Council (2005) for drinking water is 0.065 mg l^{-1}; whereas, in water for irrigation and animal consumption, it is 0.28 mg l^{-1}.

In terms of freshwater benthic algae, studies investigating the physiological and/or ecological effects of glyphosate-based herbicides on these organisms have been conducted using only microbial communities (Tsui & Chu 2003; Pérez *et al.* 2007; Vera *et al.* 2010, 2012; Pizarro *et al.* 2016). Some have suggested that responses to glyphosate exposure are specific to the species involved and, more generally, among algal phyla (Vera *et al.* 2010, 2012). A reduction in cover due to glyphosate exposure was observed in diatoms (Vera *et al.* 2010); whereas, a significant increase in the growth of filamentous green algae and cyanobacteria was reported in a periphyton community (Vera *et al.* 2012). The green alga *Scenedesmus vacuolatus* Shihira & Krauss was also stimulated when subjected to low concentrations of glyphosate (Daouk *et al.* 2013). This increase in growth rate is probably related to an increase in the concentration of phosphorus generated by the herbicide degradation process (Forlani *et al.* 2008; Daouk *et al.* 2013).

To date, no studies have been published describing the possible effects of glyphosate-based or other herbicides on the macroscopic components of freshwater benthic algae referred to as macroalgae (Sheath & Cole 1992). However, considering the contamination risks reported elsewhere (Edwards *et al.* 1980; Newton *et al.* 1994; Villeneuve *et al.* 2011; Coupe *et al.* 2012) and the fact that macroalgae are considered one of the most important primary producers in streams and an ideal organism for monitoring water quality in lotic habitats (Branco *et al.* 2014), experimental studies evaluating the potential toxic effects of glyphosate-based herbicides on these organisms are vital to improve our understanding of the impact of these substances on stream systems.

Among stream macroalgae, those belonging to the green algal family Characeae play an important ecological role in aquatic ecosystems (Van Donk & Van de Bund 2002). Members of this family, which are considered among the closest relatives of terrestrial plants (Karol *et al.* 2001), have a strong influence on nutrient cycling and, as a consequence, on the richness and abundance of plankton (Van Donk & Van de Bund 2002). In addition, the group is considered an indicator of healthy and clear-water ecosystems (Krause 1981), and their ecological features make them particularly relevant in the management of water and wetlands (Van Nes *et al.* 2002).

Thus, our aim is to assess the effects of technical-grade glyphosate, AMPA, and a commercial glyphosate-based herbicide (Roundup) on the photosynthetic rate, dark respiration rate and chlorophyll *a* content of a tropical stream green macroalga. Considering the phylogenetic proximity of *Nitella microcarpa* var. *wrightii* H. Groves & J. Groves to terrestrial plants, we hypothesised that macroalgae would be sensitive to glyphosate-based herbicides and AMPA, suffering an inhibition of photosynthetic rate and chlorophyll *a* content and an increase in dark respiration rate after exposure to herbicides.

MATERIAL AND METHODS

Considering the relevance of charophycean green algae to aquatic ecosystems, the present study used *N. microcarpa* var. *wrightii* as a test organism. Samples containing specimens of *N. microcarpa* var. *wrightii* were collected in a stream in western São Paulo State (22°38′52″S, and 50°12′14″W), southeastern Brazil. The stretch of the stream where the macroalgal samples were gathered is characterized by continuous rock substrate, low current velocity (0.35 ± 0.02 m s^{-1}, $\bar{X} \pm s$) and sunlight to the bottom of the stream (an 'opened stream', according to the classification by DeNicola *et al.* [1992]). After being collected, the specimens were placed in flasks with stream water and sent to the laboratory *in vivo*, where they were cleaned and weighed.

The cleaning process was performed using a stereoscopic microscope, paintbrush and pressurised distilled water sprays to remove all possible sediments and epiphytes. Each sample had an initial fresh weight of 150 ± 10 mg. After cleaning and weighing, each specimen was transferred to an Erlenmeyer flask containing 100 ml of Bold's basal medium (without EDTA) (Watanabe 2005) and kept for 3 days in a BOD incubator (model 411/FDP355; Nova Ética, São Paulo, Brazil) at a constant temperature (20 ± 0.5°C) and irradiance (140 ± 15 μmol m^{-2} s^{-1}) and a 12:12 light:dark photoperiod. The irradiance was determined using a quantum meter (model LI-189; Li-Cor, Lincoln, Nebraska USA) connected to an LI-193 SA spherical quantum sensor.

After the 3-day acclimation, the specimens were transferred to Erlenmeyer flasks containing new Bold's basal medium with three different concentrations of technical-grade glyphosate (≥ 95% purity, CAS: 1071-83-6) and Roundup and one concentration of AMPA (≥ 99% purity, CAS: 1066-51-9). The treatment concentrations of technical-grade glyphosate and Roundup were chosen because they represent (1) the maximum concentration for irrigation and animal consumption allowed under Brazilian law (0.28 mg l^{-1}) (National Environmental Council 2005), (2) the recommended concentration to control plagues (3.5 mg l^{-1}) (Vera *et al.* 2012), and (3) the highest concentration found in a natural aquatic environment (6 mg l^{-1}) (Edwards *et al.* 1980). For AMPA, we used a treatment concentration of 0.03 mg l^{-1}, which is the median value reported for lotic environments (Villeneuve *et al.* 2011; Coupe *et al.* 2012).

The exposure concentrations for the Roundup treatment were calculated based on acid equivalent (a.e.) value (360 g l^{-1}). For these calculations, we added 0.77 μl of Roundup to 1 litre of Bold's basal medium to obtain an initial concentration of 0.28 mg a.e. l^{-1}, 9.7 μl to an initial concentration of 3.5 mg a.e. l^{-1}) and 16.6 μl to an initial concentration of 6 mg a.e. l^{-1}. For the technical-grade glyphosate and AMPA treatments, the concentrations tested were the nominal concentrations. Thus, to obtain, for instance, a technical-grade glyphosate initial concentration of 0.28 mg l^{-1}, we added 0.28 mg of this herbicide to 1 litre of Bold's basal medium. The same procedure was applied to all

the technical-grade glyphosate and AMPA treatments. Additionally, for all treatments, one control group (Bold's basal medium without herbicide) was simultaneously assessed.

The preparation of the exposure concentrations used in the toxicity tests for glyphosate-based herbicides and AMPA was carried out in accordance with the Organisation for Economic Cooperation and Development (OECD) guidelines for the testing of chemicals 221 (OECD 2011). The exposure procedures described in these guidelines, although proposed for *Lemna* sp., also apply to *N. microcarpa* var. *wrightii*, as both are macroscopic freshwater primary producers. The test itself consists of a semistatic test with response analysis on the first (T1) and seventh day (T7) after herbicide exposure and with the renewal of the experimental media on the third and fifth day to avoid nutrient and active ingredient loss (Nešković *et al.* 1996; OECD 2011).

The effects of each treatment were evaluated by examining the response of the photosynthetic and dark respiration rates and the chlorophyll *a* content. Photosynthetic and dark respiration rates were measured by changes in oxygen concentrations using the light- and dark-bottle technique (Littler & Arnold 1985; Thomas 1988) and following the same procedures described by Vieira & Necchi (2003). This technique is considered one of the most efficient for analysing the effects of glyphosate-based herbicides on photosynthetic and dark respiration rates (Ralph 2000).

In the experiments involving the evaluation of photosynthetic rates, for each temporal analysis (i.e. T1 and T7), five replicates of the samples from each treatment condition (including the control) were transferred to 110-ml glass bottles with 98.5% transparency (light bottles) containing culture medium. The same incubation conditions were maintained during the experiments, with a constant temperature (20 $\pm$ 0.5°C), irradiance (140 $\pm$ 15 µmol m^{-2} s^{-1}) and agitation (100 $\pm$ 2.5 rpm) for a 1-hour period. For the evaluation of dark respiration rates (three replicates), we used the same procedures used for the assessment of photosynthetic rates, but the glass bottles were covered with aluminium foil (dark bottles) and incubated in complete darkness.

We measured the initial (before incubation period) and final (after incubation period) concentrations of dissolved oxygen in all the replicates from both the light and the dark bottles. These data were obtained on T1 and T7 using an oximeter (model 5100; YSI, Yellow Springs, Ohio USA), equipped with a self-stirring probe. The difference between the initial and final dissolved oxygen concentrations in the light and dark bottles was used to calculate the photosynthetic and dark respiration rates, using the formulas proposed by Littler & Arnold (1985).

After the oxygen concentration analysis, the macroalgal samples were dried at 70°C until they reached a constant weight, and their dry weight was measured using an analytical balance (AUW220D; Shimadzu, Kyoto, Japan). The chlorophyll *a* analysis was performed by applying a spectrophotometric technique using a 90% acetone solution as a solvent for extraction and a spectrophotometer (model SP220; Biospectro, Curitiba, Brazil) at wavelengths of 665

and 750 nm. We used a 90% acetone solution as a sample blank (Wetzel & Likens 2000).

The results of the technical-grade glyphosate and Roundup treatments were initially analysed using a Scott–Knott test (Scott & Knott 1974) in Sisvar (Ferreira 2000). The results of the AMPA treatment were analysed using an unpaired *t* test in Minitab v17 (Ryan *et al.* 1985). In addition, the effects of the treatments and exposure time on the photosynthetic rate, dark respiration rate and chlorophyll *a* content were assessed using a multivariate analysis of variance (MANOVA), performed in Minitab. In these analyses, photosynthetic rate, dark respiration rate and chlorophyll *a* content were dependent variables; whereas, the treatments and exposure time were covariates.

RESULTS

Photosynthetic rate

All the treatments with technical-grade glyphosate (–3% to –35.4% in relation to control) and Roundup (–4.3% to –14.3%), except the 0.28 mg a.e. l^{-1} treatment concentration from Roundup (+32.4%), showed a reduction in photosynthetic rate compared to the control group on T1 (Figs 1, 2). However, the results from the AMPA treatment indicated an increase in photosynthetic rate (+14.8%) (Fig. 3). On T7, the technical-grade glyphosate treatments showed values similar to those seen in the control group, varying from slight inhibition, seen in the 0.28 and 3.5 mg l^{-1} treatments (–6.5% and –7.6%, respectively), to slight stimulation, seen in the 6 mg l^{-1} treatment (+3%) (Fig. 1, Table 1).

No statistical differences were found between the technical-grade glyphosate, Roundup and AMPA treatments on T1. On T7, the photosynthetic rates for both Roundup ($F = 29.99$, $P < 0.001$) and AMPA ($t = 3.04$, $P < 0.01$) were significantly affected but in different ways (Figs 2, 3). For Roundup, all treatments showed a clear dose-dependent reduction in photosynthetic rates (Fig. 2). Thus, treatments of 0.28, 3.5 and 6 mg a.e. l^{-1} reduced the photosynthetic rate to 42.1%, 69% and 76% of the control values, respectively. In addition, treatment with 0.03 mg l^{-1} AMPA resulted in a significant increase in photosynthetic rate when compared to controls (+58.8%) (Fig. 3).

The MANOVA of the Roundup treatments showed that the interaction between treatment and exposure time significantly affected the photosynthetic rate ($F = 11.9$, $P < 0.01$) (Table 1). For the technical-grade glyphosate ($F = 8.1$, $P < 0.01$) and AMPA ($F = 4.98$, $P < 0.05$), only exposure time had a significant effect on photosynthetic rate (Table 1).
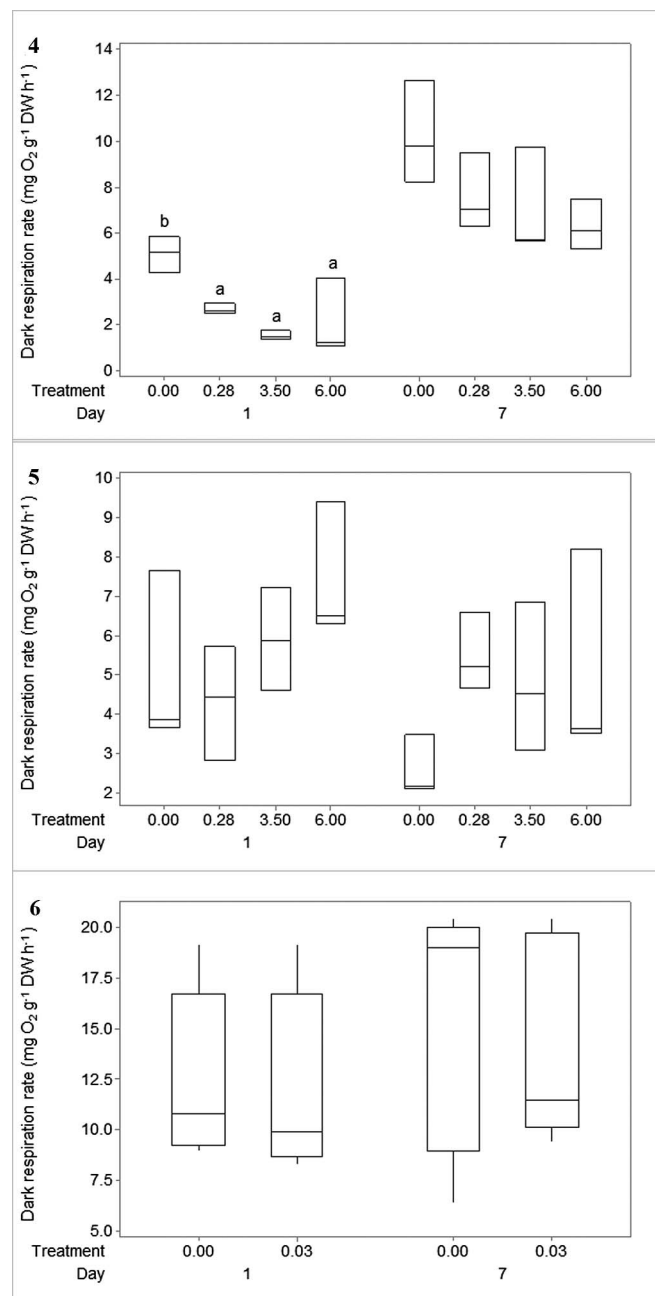
Dark respiration rate

The results on T1 showed a reduction in dark respiration rate in the technical-grade glyphosate treatments (–47.1% to –69.6% in relation to control) and in the 0.28 mg a.e. l^{-1} treatment of Roundup (–14.4%) (Figs 4, 5). An increase in dark respiration rate was observed in the Roundup treatments with 3.5 mg a.e. l^{-1} (+17%) and 6 mg a.e. l^{-1} (+46.5%) and in the AMPA treatment (+21.2%) (Figs 5, 6, Table 1). On T7, all
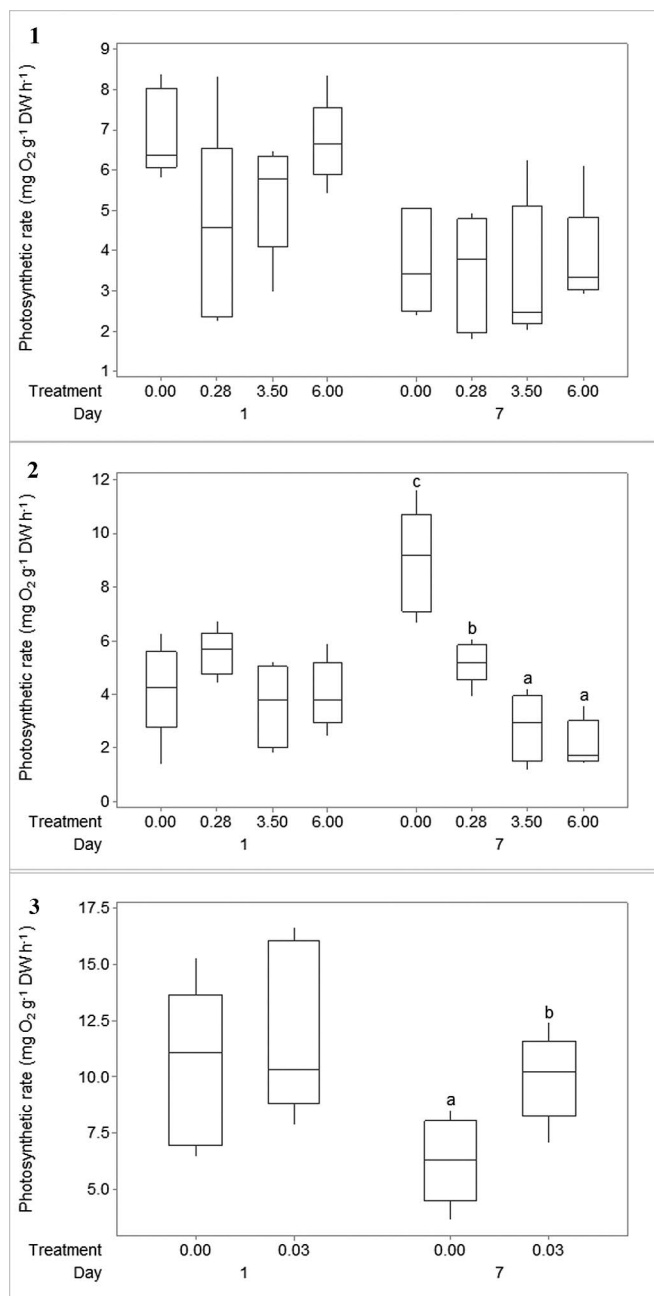
Figs 1–3. Photosynthetic rate ($n = 5$) for *Nitella microcarpa* var. *wrightii* for each treatment on days of exposure. Different letters represent significant statistical differences ($P < 0.05$). Horizontal lines depict medians, boxes represent interquartiles and vertical lines represent the range.

　Fig. 1. Technical-grade glyphosate.
　Fig. 2. Roundup.
　Fig. 3. AMPA.

Figs 4–6. Dark respiration rate ($n = 3$) for *Nitella microcarpa* var. *wrightii* for each treatment on days of exposure. Different letters represent significant statistical differences ($P < 0.05$). Horizontal lines depict medians, boxes represent interquartiles and vertical lines represent the range.

　Fig. 4. Technical-grade glyphosate.
　Fig. 5. Roundup.
　Fig. 6. AMPA.

the technical-grade glyphosate treatment concentrations (reduction varying from –25.4% to –38.4% in relation to the control) and the AMPA treatment (–37.1%) showed a decrease in dark respiration rate. In the Roundup treatments, stimulation of the dark respiration rates was observed, with the gains varying from +86.1% to +111.8% relative to the control.

A statistical difference was found only for technical-grade glyphosate on T1 ($F = 10.49$, $P < 0.01$), with the control showing higher values than all the treatments (Fig. 4, Table 2).

The MANOVA results showed that exposure time ($F = 28.4$, $P < 0.01$) had a significant impact on the variation in the *N. microcarpa* var. *wrightii* dark respiration rates in the technical-grade glyphosate treatments (Table 2). The MAN-

Table 1. MANOVA using the Wilks lambda test, with photosynthetic rate for *Nitella microcarpa* var. *wrightii* as the dependent variable and exposure time and treatment as covariates. Statistically significant differences at $P < 0.05$ were considered.

	Glyphosate			Roundup			AMPA		
	Wilks	*F*	*P*	Wilks	*F*	*P*	Wilks	*F*	*P*
Time	0.81	8.1	< 0.01	0.79	9.06	< 0.01	0.76	4.98	< 0.05
Treatment	0.98	0.61	0.43	0.99	0.07	0.79	0.98	0.29	0.59
Time × treatment	0.99	0.2	0.65	0.75	11.9	< 0.01	0.95	0.66	0.42

OVA showed no significant results for Roundup and AMPA (Table 2).

Chlorophyll *a* content

On T1, the chlorophyll *a* content decreased in the 0.28 mg l^{-1} (–2.6% in relation to control) and 6 mg l^{-1} (–4.5%) technical-grade glyphosate treatments and the 0.03 mg l^{-1} AMPA treatment (–15%); whereas, for the 3.5 mg l^{-1} (+3.3%) technical-grade glyphosate treatment and all the Roundup treatments (+32.3% to +70.5%), we observed an increase in this parameter (Figs 7–9, Table 3). On T7, the AMPA treatment showed lower values of chlorophyll *a* (–1.4%) in comparison to the control group; whereas, the technical-grade glyphosate (+20.5% to +49.2%) and all Roundup treatments (+78.74% to +125.41%) showed higher values than the control group.

Differences in chlorophyll *a* content were significant in the Roundup treatments only on T7 ($F = 7.32$, $P < 0.01$) (Fig. 8, Table 3).

The MANOVA showed that exposure time to technical-grade glyphosate ($F = 5.59$, $P < 0.05$), Roundup ($F = 5.72$, $P < 0.05$) and AMPA ($F = 40.4$, $P < 0.01$) had a significant effect on chlorophyll *a* content (Table 3).

DISCUSSION

Despite the fact that most of the Roundup treatments showed a reduction in photosynthetic rates of *N. microcarpa* var. *wrightii*, a statistically significant difference was observed only after 7 days of exposure, indicating that the amount of time that the organism remained in contact with this herbicide was crucial for determining its negative effects on macroalgal productivity. This long-term effect has also been observed in other studies related to the periphyton community (Vera *et al.* 2012). Additionally, the MANOVA showed that the inhibition of *N. microcarpa* var. *wrightii* photosynthetic rates was affected by an interaction between the concentration of Roundup and

exposure time to the herbicide ($F = 11.9$, $P < 0.01$) (Table 1), with photosynthesis declining only when concentration and exposure time increased. From an ecological perspective, the reduction in productivity in this primary producer may have a negative impact not only on abundance but also on other primary producers that use the macroalgae as a substrate and on the many herbivores that feed from them (Hua & Relyea 2014). A negative impact on biomass/abundance through indirect trophic cascade effects has been reported for other aquatic organisms and systems (Baker *et al.* 2014).
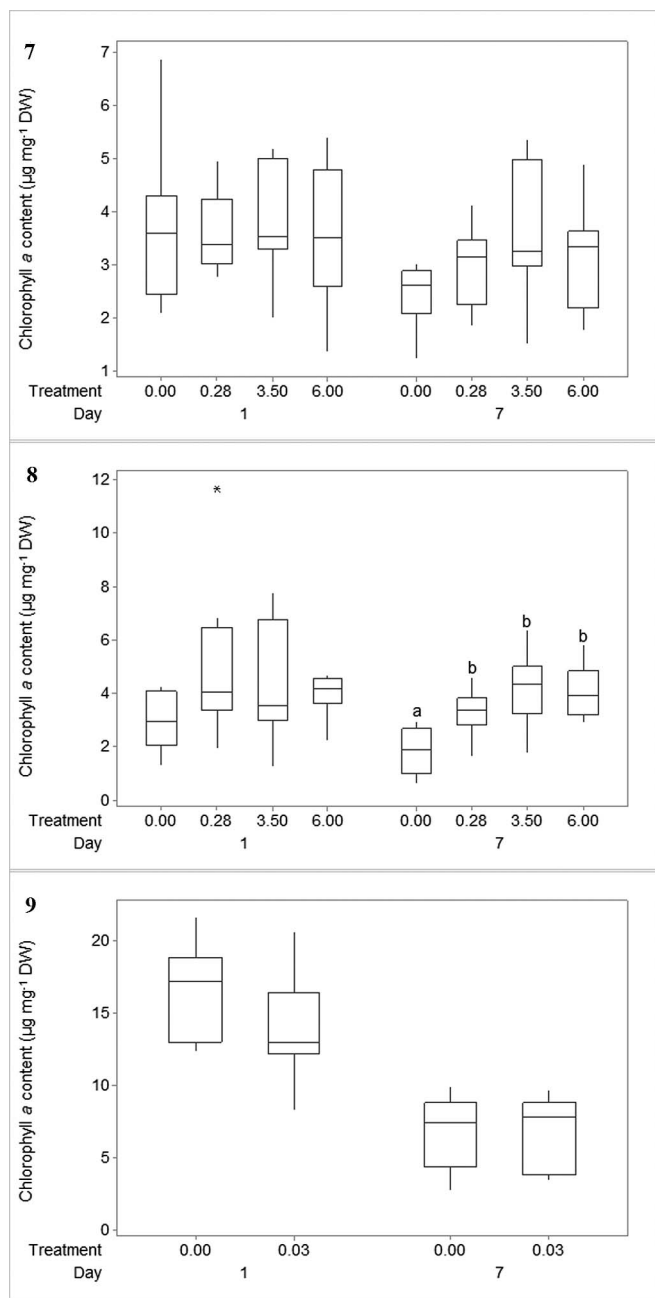
One of the most dramatic findings of this study is that a significant reduction in photosynthetic rate of *N. microcarpa* var. *wrightii* was observed (–42.1%) even at the lowest Roundup concentration tested (0.28 mg l^{-1}). Considering that 0.28 mg l^{-1} is the maximum concentration of this herbicide allowed by Brazilian law in water used for irrigation and animal consumption (National Environmental Council 2005) and the occurrence of glyphosate in the environment (Coupe *et al.* 2012; Majewski *et al.* 2014), this result suggests that even legal concentrations of Roundup in water bodies commonly used for these activities (e.g. low-order streams and small lakes) may present significant environmental risks.

The significant impact of exposure time on the inhibition of photosynthetic rates and chlorophyll *a* content observed in the MANOVA analyses for technical-grade glyphosate and AMPA were related not to the presence of these herbicides but rather to the normal decay of these parameters over time.

No significant differences were found between the control group and the tested concentrations of technical-grade glyphosate on either T1 or T7. Tsui & Chu (2003) pointed out that the combined effects of both the active ingredient and the surfactant present in its formulation [polyoxyethylene amine – (POEA)] causes the toxicity of Roundup and that, depending on the organism considered, POEA could be more toxic than technical-grade glyphosate. Our results confirm this suggestion by Tsui & Chu (2003) in *N. microcarpa* var. *wrightii*, as the significant negative effect on

Table 2. MANOVA using the Wilks lambda test, with dark respiration rate for *Nitella microcarpa* var. *wrightii* as the dependent variable and exposure time and treatment as covariates. Statistically significant differences at $P < 0.05$ were considered.

	Glyphosate			Roundup			AMPA		
	Wilks	*F*	*P*	Wilks	*F*	*P*	Wilks	*F*	*P*
Time	0.41	28.4	< 0.01	0.98	0.34	0.56	0.95	0.79	0.38
Treatment	0.89	2.29	0.14	0.83	3.95	0.06	0.99	0.005	0.94
Time × treatment	0.99	0.15	0.7	0.96	0.65	0.42	0.99	0.02	0.87

photosynthetic rates produced by Roundup on T7 was not observed for technical-grade glyphosate. In addition, the MANOVA revealed that an increase in technical-grade glyphosate concentration causes a decrease in dark respiration rates, indicating a hormesis response in *N. microcarpa* var. *wrightii* (Cedergreen *et al.* 2007).

The AMPA treatment showed a clear tendency towards increasing *N. microcarpa* var. *wrightii* photosynthetic rates on T7. The increase in photosynthetic rates is probably related to AMPA degradation by photolysis and hydrolysis, during which phosphorous is produced (Giesy *et al.* 2000). In addition, our results from the AMPA treatment suggest that, despite the fact that *N. microcarpa* var. *wrightii* can be adversely affected immediately after contact with herbicides (as shown by the negative effects on photosynthetic rates produced by technical-grade glyphosate and Roundup), the productivity of this algae can be stimulated over time (Coupe *et al.* 2012). This stimulation is probably due to the stoichiometric degradation of glyphosate, which results in an increase in AMPA and phosphorus in the water (Vera *et al.* 2012; Daouk *et al.* 2013). Thus, given the fluctuation in the productivity of *N. microcarpa* var. *wrightii* caused by these herbicides, we recommend that future ecological studies on the spatial and temporal distribution of this species of green algae take into account the concentration of glyphosate and AMPA in the water, alongside the other traditionally used parameters, when investigating the ecology of stream macroalgae (e.g. water temperature, pH, dissolved oxygen and nutrients, among others).

In terms of chlorophyll *a* content, the reduction observed in the control group relative to the Roundup treatments on T7, exactly when the highest photosynthetic rate was reported, suggests the occurrence of a physiological reallocation of resources. As such, part of the chlorophyll *a* content of *N. microcarpa* var. *wrightii* was likely degraded to generate additional nitrogen to sustain the increase in the photosynthetic rate. This strategy has been documented in several other producers, including phytoplanktonic algae (Adams & Bugbee 2014).

In the present study, glyphosate-based herbicides showed different effects on the photosynthetic parameters of *N. microcarpa var. wrightii*, suggesting that the form that these herbicides take in the environment (only active ingredient, i.e. technical-grade glyphosate; active ingredient + surfactant, i.e. Roundup; or the degraded form, i.e. AMPA) can be crucial in determining the intensity of the effect on the productivity of aquatic primary producers. These effects suggest that the productivity of aquatic primary producers could show relevant fluctuations based on the agricultural land use of the region in which the aquatic ecosystem is

Figs 7–9. Chlorophyll *a* content (*n* = 8) for *Nitella microcarpa* var. *wrightii* for each treatment (0, 0.03, 0.28, 3.5 and 6 mg l^{-1}) on T1 and T7 days of exposure. Different letters represent significant statistical differences (*P* < 0.05). Vertical lines depict medians, boxes represent interquartiles and horizontal lines represent the range.
    Fig. 7. Technical-grade glyphosate.
    Fig. 8. Roundup.
    Fig. 9. AMPA.

Table 3. MANOVA using the Wilks lambda test, with chlorophyll *a* content for *Nitella microcarpa* var. *wrightii* as the dependent variable and exposure time and treatment as covariates. Statistically significant differences at *P* < 0.05 were considered.

	Glyphosate			Roundup			AMPA		
	Wilks	*F*	*P*	Wilks	*F*	*P*	Wilks	*F*	*P*
Time	0.91	5.59	< 0.05	0.91	5.72	< 0.05	0.4	40.4	< 0.01
Treatment	0.99	0.11	0.73	0.99	0.06	0.79	0.91	2.58	0.11
Time × treatment	0.97	1.25	0.26	0.95	2.74	0.1	0.95	1.23	0.27

located. Consequently, the presence of glyphosate-based herbicides in water must be considered in ecological studies of stream macroalgae, especially when analyzing spatial and temporal distribution.

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REFERENCES

ADAMS C. & BUGBEE B. 2014. Nitrogen retention and partitioning at the initiation of lipid accumulation in nitrogen-deficient algae. *Journal of Phycology* 50: 356–365.

BAKER L.F., MUDGE J.F., HOULAHAN J.E., THOMPSON D.G. & KIDD K.A. 2014. The direct and indirect effects of a glyphosate-based herbicide and nutrients on Chironomidae (Diptera) emerging from small wetlands. *Environmental Toxicology and Chemistry* 33: 2076–2085.

BATTAGLIN W.A., KOLPIN D.W., SCRIBNER E.A., KUIVILA K.M. & SANDSTROM M.W. 2005. Glyphosate, other herbicides, and transformation products in Midwestern streams, 2002. *Journal of American Water Resources Association* 41: 323–332.

BRANCO C.C.Z., BISPO P.C., PERES C.K., TONETTO A.F. & BRANCO L.H. 2014. The roles of environmental conditions and spatial factors in controlling stream macroalgal communities. *Hydrobiologia* 732: 123–132.

CAMPBELL W.F., EVANS J.O. & REED S.C. 1976. Effects of glyphosate on chloroplast ultrastructure of quackgrass mesophyll cells. *Weed Science* 24: 22–25.

CEDERGREEN N., STREIBIG J.C., KUDSK P., MATHIASSEN S.K. & DUKE S.O. 2007. The occurrence of hormesis in plants and algae. *Dose-Response* 5: 150–162.

COUNCIL OF THE EUROPEAN UNION. 1998. Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. *Official Journal of the European Communities* 330: 32–54.

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 Management Science* 68: 16–30.

DAOUK S., COPIN P.J., ROSSI L., CHÈVRE N. & PFEIFER H.R. 2013. Dynamics and environmental risk assessment of the herbicide glyphosate and its metabolite AMPA in a small vineyard river of the Lake Geneva catchment. *Environmental Toxicology and Chemistry* 32: 2035–2044.

DENICOLA D.M., HOGLAND K.D. & ROEMER S.C. 1992. Influence of canopy cover on spectral irradiance and periphyton assemblages in a prairie stream. *Journal of the North American Benthological Society* 11: 391–404.

EDWARDS W.M., TRIPLETT G.B. & KRAMER R.M. 1980. A watershed study of glyphosate transport in runoff. *Journal Environmental Quality* 9: 661–665.

FERREIRA D.F. 2000. Análises estatísticas por meio do Sisvar para Windows versão 4.0. In: *45a Reunião Anual da Região Brasileira da Sociedade Internacional de Biometria*, pp. 255–258. UFSCAR, São Carlos, Brazil.

FORLANI G., PAVAN M., GRAMEK M., KAFARSKI P. & LIPOK J. 2008. Biochemical bases for a widespread tolerance of cyanobacteria to the phosphonate herbicide glyphosate. *Plant and Cell Physiology* 49: 443–456.

GIESY J.P., DOBSON S. & SOLOMON K.R. 2000. Ecotoxicological risk assessment for Roundup® herbicide. *Reviews of Environmental Contamination and Toxicology* 167: 35–120.

GOMES M.P., SMEDBOL E., CHALIFOUR A., HÉNAULT-ETHIER L., LABRECQUE M., LEPAGE L., LUCOTTE M. & JUNEAU P. 2014. Alteration of plant physiology by glyphosate and its by-product aminomethylphosphonic acid: an overview. *Journal of Experimental Botany* 65: 4691–4703.

HUA J. & RELYEA R.A. 2014 Chemical cocktails in aquatic systems: pesticide effects on the response and recovery of > 20 animal taxa. *Environmental Pollution* 189: 18–26.

IBAMA 2013. Relatórios de Comercialização de Agrotóxicos – Boletim Anual de Produção, Importação, Exportação e Vendas de Agrotóxicos no Brasil. Ministério do Meio Ambiente, Brasilia. http://www.ibama.gov.br/areas-tematicas-qa/relatorios-de-comercializacao-de-agrotoxicos/pagina-3; searched on 13 May 2015.

KAROL K.G., MCCOURT R.M., CIMINO M.T. & DELWICHE C.F. 2001. The closest living relatives of land plants. *Science* 294: 2351–2353.

KRAUSE W. 1981. Characeen als bioindikatoren für den gewässerzustand. *Limnologica* 13: 339–418.

LITTLER M.M. & ARNOLD K.E. 1985. Electrodes and chemicals. In: *Handbook of phycological methods. Ecological field methods: Macroalgae* (Ed. by M.M. Little & D.S. Little), pp. 349–375. Cambridge University Press, Cambridge, UK.

MAJEWSKI M.S., COUPE R.H., FOREMAN W.T. & CAPEL P.D. 2014. Pesticides in Mississippi air and rain: a comparison between 1995 and 2007. *Environmental Toxicology and Chemistry* 33: 1283–1293.

NATIONAL ENVIRONMENTAL COUNCIL. 2005. Resolução no 357 de 17 de março de 2005. *Diário Oficial da União* 53: 58–66.

NEŠKOVIĆ N.K., POLEKSIĆ V., ELEZOVIĆ I., KARAN V. & BUDIMIR M. 1996. Biochemical and histopathological effects of glyphosate on carp, *Cyprinus carpio* L. *Bulletin of Environmental Contamination and Toxicology* 56: 295–302.

NEWTON M., HORNER L.H., COWELL J.E., WHITE D.E. & COLE E.C. 1994. Dissipation of glyphosate and aminomethylphosphonic acid in North American forests. *Journal of Agricultural and Food Chemistry* 42: 1795–1802.

ORGANISATION FOR ECONOMIC COOPERATION AND DEVELOPMENT. 2011. *Lemna sp. growth inhibition test*. Guideline 221. OECD, Paris.

PÉREZ G.L., TORREMORELL A., MUGNI H., RODRIGUEZ P., VERA M.S., NASCIMENTO M., ALLENDE L., BUSTINGORRY J., ESCARAY R., FERRARO M., IZAGUIRRE I., PIZARRO H., BONETTO C. & MORRIS D.P. 2007. Effects of the herbicide Roundup on freshwater microbial communities: a mesocosm study. *Ecological Applications* 17: 2310–2322.

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. *Environmental Pollution* 156: 61–66.

PIZARRO H., VERA M.S., VINOCUR A., PÉREZ G., FERRARO M., HELMAN R.M. & DOS SANTOS AFONSO M. 2016. Glyphosate input modifies microbial community structure in clear and turbid freshwater systems. *Environmental Science and Pollution Research* 23: 5143–5153.

RALPH P.J. 2000. Herbicide toxicity of *Halophila ovalis* assessed by chlorophyll a fluorescence. *Aquatic Botany* 66: 141–152.

REDDY K.N., RIMANDO A.M. & DUKE S.O. 2004. Aminomethylphosphonic acid, a metabolite of glyphosate, causes injury in glyphosate-treated, glyphosate-resistant soybean. *Journal of Agricultural and Food Chemistry* 52: 5139–5143.

RYAN B.F., JOINER B.L. & RYAN T.A. 1985. *Minitab handbook*. Duxbury Press, Boston.

SCOTT A.J. & KNOTT M.A. 1974. A cluster analysis method for grouping means in the analysis of variance. *Biometrics* 30: 507–512.

SENEM SU Y., OZTURK L., CAKMAK I. & BUDAK H. 2009. Turfgrass species response exposed to increasing rates of glyphosate application. *European Journal of Agronomy* 31: 120–125.

SERRA A.A., NUTTENS A., LARVOR V., RENAULT D., COUÉE I., SULMON C. & GOUESBET G. 2013. Low environmentally relevant levels of bioactive xenobiotics and associated degradation products cause cryptic perturbations of metabolism and molecular stress responses in *Arabidopsis thaliana*. *Journal of Experimental Botany* 64: 2753–2766.

SHEATH R.G. & COLE K.M. 1992. Biogeography of stream macro-algae in North America. *Journal of Phycology* 28: 448–460.

THOMAS M.L.H. 1988. Photosynthesis and respiration of aquatic macro-flora using the light and dark bottle oxygen method and dissolved oxygen analyser. In: *Experimental phycology: A laboratory manual* (Ed. by C.S. Lobban, D.J. Chapman & B.P. Kremer), pp. 64–77. Cambridge University Press, Cambridge, UK.

TSUI M.T. & CHU L.M. 2003. Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. *Chemosphere* 52: 1189–1197.

US ENVIRONMENTAL PROTECTION AGENCY. 2009. Drinking water contaminants. http://water.epa.gov/drink/contaminants/index.cfm#List; searched on 13 May 2015.

VAN DONK E. & VAN DE BUND W.J. 2002. Impact of submerged macrophytes including charophytes on phyto- and zooplankton communities: allelopathy versus other mechanisms. *Aquatic Botany* 72: 261–274.

VAN NES E.H., SCHEFFER M., VAN DEN BERG M.S. & COOPS H. 2002. Dominance of charophytes in eutrophic shallow lakes – when should we expect it to be an alternative stable state? *Aquatic Botany* 72: 275–296.

VAUGHN K.C. & DUKE S.O. 1986. Ultrastructural effects of glyphosate on *Glycine max* seedlings. *Pesticide Biochemistry and Physiology* 26: 56–65.

VERA M.S., LAGOMARSINO L., SYLVESTER M., PÉREZ G.L., RODRÍGUEZ P., MUGNI H., SINISTRO R., FERRARO M., BONETTO C., ZAGARESE H. & PIZARRO H. 2010. New evidences of Roundup® (glyphosate formulation) impact on the periphyton community and the water quality of freshwater ecosystems. *Ecotoxicology* 19: 710–721.

VERA M.S., DI FIORI E., LAGOMARSINO L., SINISTRO R., ESCARAY R., IUMMATO M.M., JUÁREZ A., MOLINA M.C.R., TELL G. & PIZARRO H. 2012. Direct and indirect effects of the glyphosate formulation Glifosato Atanor® on freshwater microbial communities. *Ecotoxicology* 21: 1805–1816.

VIEIRA J.J.R. & NECCHI O.JR. 2003. Photosynthetic characteristic of charophytes from tropical ecosystems. *Phycological Research* 51: 51–60.

VILLENEUVE A., LARROUDÉ S. & HUMBERT J.F. 2011. Herbicide contamination of freshwater ecosystems: Impact on microbial communities. In: *Pesticides: Formulations, effects, fate* (Ed. by M. Stoytcheva), pp. 285–312. InTech Open Access, Rijeka, Croatia.

WATANABE M.M. 2005. Freshwater culture media. In: *Algal culture techniques* (Ed. by R.A. Andersen), pp. 13–20. Elsevier, New York.

WETZEL R.G. & LIKENS G.E. 2000. *Limnological analyses*. Springer, New York. 429 pp.

ZOBIOLE L.H.S., KREMER R.J., DE OLIVEIRA R.S. & CONSTANTIN J. 2012. Glyphosate effects on photosynthesis, nutrient accumulation, and nodulation in glyphosate-resistant soybean. *Journal of Plant Nutrition and Soil Science* 175: 319–330.