Ecotoxicology and Environmental Safety 148 (2018) 1010–1019

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Effects of glyphosate and 2,4-D mixture on freshwater phytoplankton and periphyton communities: a microcosms approach

V.L. Lozano[a,b,*], A. Vinocur[a,b,c], C.A. Sabio y García[a,b], L. Allende[d], D.S. Cristos[e], D. Rojas[e], M. Wolansky[f,g], H. Pizarro[a,b]

[a] Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Depto. Ecología, Genética y Evolución, Buenos Aires, Argentina
[b] CONICET – Universidad de Buenos Aires. Instituto de Ecología, Genética y Evolución de Buenos Aires (IEGEBA), Buenos Aires, Argentina
[c] Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Departamento de Biodiversidad y Biología Experimental, FCEyN-UBA, Argentina
[d] Instituto del Conurbano, Universidad Nacional de General Sarmiento, Buenos Aires, Argentina
[e] Instituto Nacional de Tecnología Agropecuaria, Instituto de Tecnología de Alimentos, Argentina
[f] Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Departamento de Química Biológica, Buenos Aires, Argentina
[g] CONICET – Universidad de Buenos Aires. Instituto de Química Biológica de la Facultad de Ciencias Exactas y Naturales (IQUIBICEN), Buenos Aires, Argentina

ARTICLE INFO

Keywords:
Glyphosate
2,4-D
Mixture
Phytoplankton
Periphyton
Freshwater

ABSTRACT

Glyphosate (G) and 2,4-D herbicides are massively applied in agriculture worldwide and the use of their mixture is currently a very common practice. We carried out two experiments using microcosms under laboratory conditions for 7 days each. In the first experiment, we analyzed changes in species composition, abundance and chlorophyll *a* of phytoplankton due to 10 treatments: control; low, medium and high concentrations of G and 2,4-D; and mixtures at low, medium and high concentrations at a G:2,4-D ratio of 1:0.45. In the second experiment we studied changes on the composition of the autotrophic fraction and abundance, chlorophyll *a*, dry weight (DW), ash free dry weight (AFDW) and autotrophic index of periphyton developed in artificial substrata under 7 treatments considering the lowest doses that showed an effect in the previous phytoplankton experiment: control; pure G and Glifosato Atanor® (glyphosate-based formulation); pure 2,4-D and Asi Max 50® (2,4-D-based formulation); mixtures of the a.i at a G:2,4-D ratio of 1:0.45, and mixture of Glifosato Atanor® + Asi Max®. Results showed that G was more toxic than 2,4-D to the algal fraction, decreasing chlorophyll *a*, turbidity and algal abundances in the phytoplankton experiment. The effects of the mixture on phytoplankton were mainly additive, except for total and *Staurastrum* sp. live abundances where an antagonistic effect between herbicides was recorded. Periphyton showed more resistance to the herbicides as it was less affected than phytoplankton by the active ingredients and commercial formulations. The high development of *Leptolyngbya* sp. due to the impact of the herbicide mixture on periphyton might represent the beginning of a more conspicuous community to prevent the impact of contaminants. The study of the impacts of herbicide mixtures on freshwater systems requires the analysis of several variables to better assess the responses of key microbial communities and to predict more realistic scenarios.

1. Introduction

Glyphosate-based herbicides are the most used agrochemicals worldwide (Annett et al., 2014) and different collateral impacts have been reported after 20 years of intensive use in Argentina. For example, an increase in the positive selection of glyphosate (G)-resistant weeds has now become a major problem for farmers (Bonny, 2016). As an alternative, the use of herbicide mixtures is being strongly recommended for a more efficient weed control. In addition, the development of novel transgenic crops that are tolerant to multiple herbicides supports a weed control strategy based on mixtures of different herbicides (Green, 2016).

Glyphosate (N-phosphonomethylglycine) is the most commonly used herbicide in Argentina. It represents 75% of all agrochemicals, with more than 137 million kilograms being applied to croplands per year (Pórfido et al., 2014). The mode of action (MOA) of this non-selective, broad-spectrum herbicide primarily consists of the reversible inhibition of the enzyme EPSP (5-enolpyruvylshikimate-3-phosphate) synthase, and the consequent decrease in the synthesis of aromatic amino acids in plants, algae, bacteria and fungi (Pollegioni et al., 2011).

* Corresponding author at: Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Depto. Ecología, Genética y Evolución, Buenos Aires, Argentina.
E-mail address: vlozano@ege.fcen.uba.ar (V.L. Lozano).

https://doi.org/10.1016/j.ecoenv.2017.12.006
Received 5 September 2017; Received in revised form 28 November 2017; Accepted 1 December 2017

The herbicide 2,4-D (2,4-Dichlorophenoxyacetic acid) is also extensively used in Argentina. Between 2013 and 2015, 2,4-D was the third most imported agrochemical in the country (SENASA, 2017). It is an auxin-type selective herbicide that induces overgrowth of vascular cambium in dicotyledonous plants, ultimately leading to death (Song, 2014). Taking advantage of MOA-based strategies for weed control, 2,4-D is increasingly used by farmers in combination with G, as reported by Pérez et al. (2017), who detected residues of both herbicides in a stream located near croplands in Argentina. In general, these herbicides are commercialized as formulations containing the active ingredient (i.e. G or 2,4-D), adjuvants and water. Today, binary mixtures of formulations of G and 2,4-D at different ratios (e.g. Mestizo® 1:0.45; EnList Duo® 1:0.95; Landmaster II® 1:0.83) are available in the market.

Agrochemicals in general and herbicides in particular affect ecosystems in different ways. Herbicides may build up in aquatic systems directly or indirectly through run-off, air drift or groundwater (Pérez et al., 2007; Aparicio et al., 2015). Many exposure studies performed under laboratory and outdoor mesocosm conditions have shown that glyphosate-based herbicides affect freshwater systems by modifying phytoplankton, zooplankton and other microbial communities (Pérez et al., 2007; Lipok et al., 2010; Vera et al., 2012; Geyer et al., 2016). Although less information is available for 2,4-D, different effects have been demonstrated on microbial freshwater communities, involving algae from monoculture (Wong, 2000) and from phytoplankton community bioassays under laboratory conditions (Kobraei and White, 1996; Boyle, 1980). Moreover, there are limited data on the susceptibility of freshwater communities to the simultaneous or sequential use of G and 2,4-D products consisting of active and non-active ingredients.

Contaminants may act in additive, synergic or antagonistic ways when entering the environment simultaneously (Piggott et al., 2015). There is an emerging debate about the possible synergic effects of multiple herbicides on the environment under realistic scenarios of weed control (US EPA, 2017).

The impact of herbicides on natural shallow lakes has been more studied in phytoplankton than in periphyton communities (165 articles vs 57, respectively) (PubMed, consulted 6–22–2017). Phytoplankton is a free-floating microbial autotrophic community, while periphyton is a sessile microbial community comprising not only autotrophic (i.e. algae and cyanobacteria) but also heterotrophic (i.e. bacteria, fungi, protozoa and animals) components, as well as organic and inorganic detritus (Wetzel, 1983), all of which are embedded in a mucilaginous matrix attached to different types of submerged substrata. We are interested in elucidating how a mixture of G and 2,4-D might impact on these microbial communities playing such an important role in freshwater trophic webs. The fact that these herbicides have a different MOA suggests that they interact with different molecular target sites but that they still trigger a common toxicological endpoint for each organism of the community. Under this assumption, the effects of G and 2,4-D in the mixture are assumed to be independent from each other (Faust et al., 2001; Relyea, 2009).

The objective of this work was to study the joint action of G and 2,4-D as single active ingredients, Glifosato Atanor® (glyphosate-based formulation) and Asi Max® (2,4-D-based formulation), on some structural properties of the phytoplankton and periphyton communities. We performed two 7-day successive experiments using microcosms under laboratory conditions. In the first one, we determined the phytoplankton composition and chlorophyll *a* concentration after exposure to 3 concentrations of G and 2,4-D to obtain a dose-response relationship. Then, we conducted a second experiment to compare the action of the single compound and commercial formulations of these herbicides on the periphyton structure. In the latter approach we tested the resistance of periphyton using the minimum herbicide concentrations that had shown an effect on phytoplankton.

We propose the following hypotheses to be tested: 1. the toxicity of G and 2,4-D on phytoplankton is dose-dependent; 2. phytoplankton and periphyton have different susceptibility to the herbicides of interest because of their distinct biological and ecological nature; 3. the binary mixture of these herbicides has an additive effect on the studied communities based on their different MOAs and independent toxicogenic pathways; 4. the impact of herbicide formulations is different from that of the single active ingredient at the species level; and 5. resistance to the studied herbicides is higher for periphyton than for phytoplankton.

2. Methods

We carried out two experiments in microcosms under laboratory conditions, one using phytoplankton obtained from an organic-turbid system and the other using periphyton developed on artificial substrata placed in a clear-vegetated system. The phytoplankton experiment consisted in the analysis of 3 scenarios of concentrations of each herbicide and 3 scenarios of concentrations of mixtures to explore possible dose-response effects on the community. In the second experiment, we used the lowest dose of both active ingredients and herbicide-based formulations that had an effect on the previous phytoplankton experiment, to test their impact on periphyton.

2.1. Phytoplankton experiment

We used water with algal-turbid eutrophic status (chlorophyll *a* = 71.5 µg/L, nephelometric turbidity = 9 NTU, P-PO_3 = 0.08 mg/L, N-NO_2 + NO_3 = 0.03 mg/L) from an outdoor tank to fill 34 microcosms (experimental units, 500-mL Erlenmeyers). Microcosms were incubated in a shaker under a 12:12 photoperiod at 25 °C with continuous agitation. After 4 days of stabilization 3 samples were processed totally to determine initial time (Ti) conditions. The following treatments were applied by triplicate to the microcosms: G concentrations of 0.3, 3 and 6 mg/L (GL, GM and GH, respectively); 2,4-D concentrations of 0.135, 1.35 and 2.7 mg/L (2,4-DL, 2,4-DM, 2,4-DH, respectively); low mixture concentration of 0.3 mg G/L + 0.135 mg 2,4-D/L (ML); medium mixture concentration of 3 mg G/L + 1.35 mg 2,4-D/L (MM) and high mixture concentration of 6 mg G/L + 2.7 mg 2,4-D/L) (MH). Active ingredients (a.i) were used in all cases: glyphosate monoisopropylamine salt Sigma–Aldrich cat. 338,109 and 2,4-D dimethylamine salt Supelco cat. N-10612-1G. Glyphosate monoisopropylamine salt ($C_6H_{17}N_2O_5P$) has a molecular weight of 228.18 and a water solubility of 786 g/L while the 2,4-D dimethylamine salt ($C_{10}H_{13}Cl_2NO_3$) has a molecular weight of 266.19 and a water solubility of 750 g/L. Final time (Tf) was on day 7 after treatment application. The exposure levels were selected based on the 1:0.45 ratio of a commercial herbicide formulation increasingly used in Argentina (Mestizo®, from Atanor®, Argentina), which was taken as a reference case. The selected exposure scheme closely follows the agronomic recommendations for the use of these herbicide compounds (Metzler et al., 2011).

Turbidity was measured with a Hach® 2100 P portable turbidimeter. At Tf, water samples (200 mL) were filtered with Whatman® GF/F filters and stored at − 20 °C until chlorophyll *a* quantification. Pigment extraction was performed with acetone, and the extract was preserved// incubated overnight at 4 °C in darkness and then centrifuged for 10 min at 3000 rpm. Absorbance was determined at 665 and 750 nm before and after acidification with HCl 1 N. The final concentration was estimated following Lilchtenthaler and Wellburn (1983).

Another 200-mL sample from each experimental unit was fixed with 1% acidified Lugol's iodine solution for algal and cyanobacteria quantification (> 2 µm) following Utermöhl's (1958) technique, at both Ti and Tf. Counts were made to the lowest possible taxonomic level, distinguishing between live and dead organisms. Individuals with organized cell structure (undamaged chloroplasts and cell wall such as frustules for diatoms) were considered to be alive. Counting errors were estimated according to Venrick (1978), accepting a maximum of 20% for the most abundant taxa.

2.2. Periphyton experiment

Polycarbonate strips were used as artificial substrata; these were suspended by means of an ad hoc device in an outdoor tank during a 60-day colonization period. At the beginning of the colonization, the outdoor tank was in a clear- nephelometric turbidity = 1 NTU, P-PO_3 = 0.08 mg/L, N-NO_2 + NO_3 = 0.01 mg/L. Mean water temperature was 19.5 °C throughout the colonization period. Prior to the exposure experiment, the water from the tank was filtered through an 18-μm pore mesh and sterilized to prevent further biological interaction of periphyton with organisms in the water of the microcosms. Initial nutrient concentrations were 0.13 mg/L PO_3^- and 0.9 mg/L NO_3^- (i.e. non-limiting conditions for algal growth). Microcosms (experimental units) were 250-mL beakers filled with the autoclaved water, where three colonized substrata were immersed vertically by means of ad hoc devices. At Ti, 3 randomly chosen experimental units were processed to determine pre-incubation conditions. The other microcosms were randomly assigned to one of the following 7 treatments: No herbicide added (Control); 3 mg/L active ingredient (a.i) of glyphosate mono-isopropylamine salt (Sigma – Aldrich cat. 338,109) (G a.i.); 3 mg/L a.i of Glifosato Atanor® (43.8% of N-(phosphonomethyl) glycine mono-potassium salt (CAS: 39600-42-5) and 56.2% of inert ingredients and adjuvants) (Glyphosate Atanor®); 1.35 mg/L a.i of 2,4-D dimethylamine salt (Supelco cat. N-10612-1G) (2,4-D a.i.); 1.35 mg/L a.i of 2,4-D Asi Max 50® (60,2 g 2,4-D dimethylamine salt and 100 mL inerts, lot. 26,792) (Asi Max®); the mixture of the active ingredients (G a.i. + 2,4-D a.i), and the mixture of the commercial formulations (Glifosato Atanor® + Asi Max®) in proportions of 3 mg/L G a.i + 1.35 mg/L 2,4-D a.i. For the phytoplankton experiment, we selected the 1G: 0.45 2,4-D ratio present in Mestizo®. These concentrations correspond to the lowest doses that showed an effect in the phytoplankton assay and to the commercial formulations commonly used in Argentina. Experimental units were randomly arranged in a culture chamber at 25 °C and under a 12:12 L:D photoperiod for 7 days.

At Tf, periphyton was scraped from the substrata with a fine brush and diluted in distilled water. Samples from each substrate were divided into three aliquots. One aliquot was filtered through pre-combusted (550 °C) GVS® GF/C filters for ash-free dry weight (AFDW) determinations. AFDW was determined as the difference in mass after calcination (550 °C for 3 h) of dry samples (60 °C on stove) (APHA, 2005). The second aliquot was filtered through Whatman® GF/F filters and chlorophyll *a* determined as described for phytoplankton but related to the surface of the scrapped substrate and expressed as μg chlorophyll *a* per cm^2. Autotrophic index (AI) was calculated as AFDW/chlorophyll *a*; an AI value higher than 200 indicates a high proportion of heterotrophic, non-chlorophyllous organisms or organic detritus (APHA, 2005; Lowe and Pan, 1996). The third aliquot of the sample was fixed with 1% acidified Lugol's iodine solution for algal quantification using the Utermöhl's (1958) technique with a counting error < 20% (Venrick, 1978). Live and dead individuals were counted as mentioned above. The presence of a filamentous cyanobacterium species was confirmed by epifluorescence microscopy. All variables were expressed on an area basis.

2.3. Herbicide quantification

For each experiment, water samples from all microcosms were collected immediately after the administration of herbicides at Ti and stored at − 20 °C until herbicide residue analysis. Samples were thawed and homogenized and a chloromethane extraction protocol was performed with 3–10 mL of water. Next, 10 mL of extracted water samples were transferred to a 15 mL-Falcon tube and centrifuged to remove solid residues (17,000 *g* × 10 min). The liquid phase was transferred to a polypropylene UPLC (Ultra Performance Liquid Chromatography) vials. Water sample concentrations of G and 2,4-D were determined by UPLC Waters Acquity with SQD detector (single quadrupole mass detector) using ESI negative mode. Chromatographic separation was set with 1% acetic acid in water: MeOH at the following gradient: (95:5)–(95:5) 0–2 min, (95:5)–(0:100) 2–5 min, (100:0)–(95:5) 5–6 min, (95:5) 6–10 min, as the mobile phase. The columns used were Hypercarb 2.1 × 100 mm 5 μm column (to analyze G) and ODS Hypersil 2.1 × 150 3 μm column (to analyze 2,4-D). Selected ion monitoring (SIM) mode was used for quantitative analysis (G: ion 168 *m/z* and ion 150 *m/z*; 2,4-D: 220 *m/z* and 161 *m/z*). Calibration curves were constructed in water to cover a range from 5.00 μg/L to 15.00 mg/L, with a detection limit of 1.00 μg/L.

2.4. Statistical analysis

For the phytoplankton experiment, the homogeneity of the experimental units was verified in terms of water turbidity just before the addition of herbicides by a Kruskal–Wallis non-parametric ANOVA by ranks test (KW). For the second experiment, the percentage of variation of the mean periphytic DW at Ti was considered as a measure of homogeneity. The measured value was 6.85%, which appears reasonable in view of the fact that periphyton is a very heterogeneous community (Azim and Asaeda, 2005). Such value is lower than those obtained for periphyton developed on artificial substrata (e.g. Pizarro et. al, 2015).

In the phytoplankton experiment, the Pearson's correlation coefficient was used to analyze trends in turbidity, total live and dead abundances and chlorophyll *a* concentration. The impact of herbicides on algae and cyanobacteria was only assessed for the most abundant species by comparing live and dead abundances in herbicide-treated and control microcosms. At Tf, species were considered rare if they were present in less than 5 samples for all replicates of a treatment or in 1 or 2 replicates of the same treatment, and excluded from the analysis. For both experiments, a KW was used to compare differences between treated and control groups for each variable considered. To test the default hypothesis 3, the expected theoretical additive effect of the mixture was estimated for turbidity, chlorophyll *a* concentration, total live and dead phytoplankton abundances of and for the most abundant species as follows: [(herbicide 1 – control) + (herbicide 2 – control)] (Piggott et al., 2015). If the observed combined impact of herbicides exceeded their expected additive effect, then the interaction was defined as being synergistic. In contrast, if the observed impact was lower than the additive effect, then the interaction was denoted as antagonistic. We used a t-Student test ($p < 0.001$) to compare between the observed and calculated additive responses, after testing for normality and homoscedasticity with the Kolmogorov-Smirnov and Levene's tests, respectively. For graphical representation, confidence intervals (CI95) for the expected and observed values were calculated and relativized to − 1.1 with respect to expected values. The analyses were performed using the InfoStat® program 2015 version.

3. Results

3.1. Herbicides

For both experiments, the herbicide concentrations in microcosms (n = 3) at Ti are shown in Table 1.

3.2. Phytoplankton

At Ti, mean water turbidity was 8.71 ± 0.59 NTU and non-significant differences ($p = 0.08$) were detected between the experimental units. At Tf, values of turbidity ranged from a minimum of 9 NTU (GM) to a maximum of 16 NTU (ML), with a significant decrease ($p = 0.0042$) in GM, GH and the corresponding mixtures (MM and MH) (Fig. 1A). The same trend was observed for Chlorophyll *a* concentrations, with a significant decrease ($p = 0.0061$) in the GM, GH, MM and MH treatments, varying from a minimum of 41 μg/L (MM) to a

Table 1
Initial herbicide concentrations. Values are expressed as mean ± SD.

	Glyphosate (mg/L)	2,4-D (mg/L)
Phytoplankton experiment		
Control	< LOD	< LOD
GL	0.34 ± 0.11	< LOD
GM	3.21 ± 0.22	< LOD
GH	5.45 ± 0.11	< LOD
2,4-D L	< LOD	0.20 ± 0.07
2,4-D M	< LOD	1.76 ± 0.29
2,4-D H	< LOD	2.35 ± 0.85
ML	0.36 ± 0.13	0.18 ± 0.03
MM	2.89 ± 0.30	1.57 ± 0.39
MH	4.51 ± 0.77	2.45 ± 1.31
Periphyton experiment		
Control	< LOD	< LOD
G a.i.	2.39 ± 0.47	< LOD
Glifosato Atanor®	3.65 ± 0.85	< LOD
2,4-D a.i.	< LOD	0.86 ± 0.14
Asi Max®	< LOD	1.18 ± 0.56
G a.i. + 2,4-D a.i.	3.16 ± 1,61	1.24 ± 0.47
Glifosato Atanor® + Asi Max®	2.96 ± 0.64	1.03 ± 0.12

G: glyphosate; L: low concentration; M: medium concentration; H: high concentration; M: mixture. LOD: limit of detection.

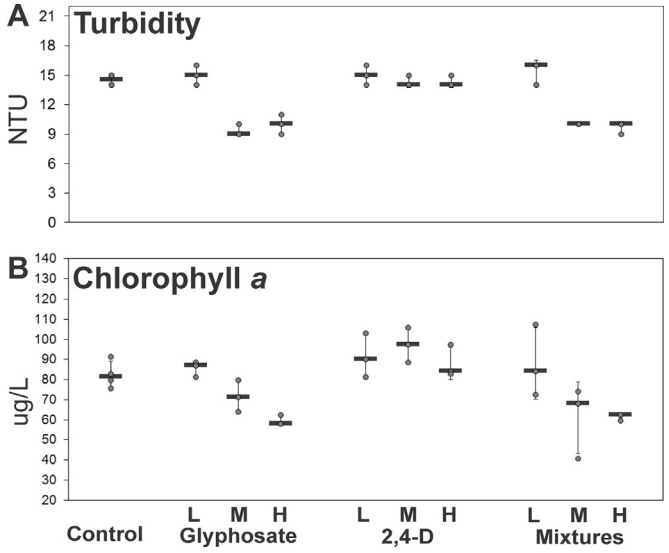

Fig. 1. Turbidity **(A)** and Chlorophyll *a* concentration **(B)** after a 7-day exposure to glyphosate, 2,4-D and mixtures of both herbicides as active ingredients. L: low concentration; M: medium concentration; H: high concentration. Dots show individual measurements. Horizontal bars indicate median values (figures made from templates provided by Weissgerber et al., 2015).

maximum of 107 µg/L (ML) (Fig. 1B). The same treatments showed significant differences in phytoplankton total abundance, with a decrease in live and an increase in dead individuals (Fig. 2A). Maximum mortality was recorded for MH and GM (60% and 53% of dead individuals, respectively), while a minimum of 10% of dead organisms was found for the GL treatment. The following Pearson's correlations were statistically significant: turbidity vs total live abundance ($r = 0.58$, $p = 0.0006$); turbidity vs total dead abundance ($r = -0.61$, $p = 0.0003$); Chlorophyll *a* vs total live abundance ($r = 0.50$, $p = 0.0045$), Chlorophyll *a* vs total dead abundance ($r = -0.39$, $p = 0.03$).

We found a total of 11 species in all microcosms. Chlorophyta was the most abundant group (87.8%) followed by Chrysophyceae (10.5%), Cyanobacteria (0.9%), Dinophyceae (0.78%) and Bacillariophyceae (0.02%) (Table 2).

Staurastrum sp. was the dominant species, with ≈ 83% of the total abundance in all treatments and a mean live abundance of (± SD) of 280,839 ± 75,499 ind/mL, which represented 90.7% of the total live abundance (309,711 ± 77,011 ind/mL). This species showed a significant increase in dead individuals in the GM (74% of total organisms), GH and MM (67%) and MH (67%) treatments (Fig. 2B.1). An increase of 9.95 times of *Ochromonas* sp. was found in GM with respect to control and the same trend was recorded for the MM treatment (5.1 times) (Fig. 2B.2). *Chlamydomonas* sp. showed high mortality in GM while it was absent in the GH treatment (Fig. 2B.3). *Tetraedron minimum* increased in the GL (5.7 times) and MH (8.1 times) treatments (Fig. 2B.4), while the filamentous cyanobacteria *Leptolyngbya* sp. decreased in the GH and MH treatments (Fig. 2B.5). 2,4-D had no effect on any of the phytoplankton variables analyzed for any of the concentrations tested.

Although the most frequently observed response was additive for all variables and concentrations, the mixture of herbicides at high concentration (MH) resulted in a lower abundance of live organisms of *Staurastrum* sp. than that expected for an additive effect. In consequence, this dose-response was also observed for total live abundance (Fig. 3).

3.3. Periphyton

Mean DW was 65.90 ± 4.51 µg/cm^2 at Ti, ranging from a minimum value of 34.62 (MF) to a maximum value of 54.43 (GF) ug/cm^2 at Tf. At Tf, AFDW values ranged from a minimum of 18 µg/cm^2 (M) to a maximum of 55 µg/cm^2 (Control) (Fig. 4A), chlorophyll *a* concentration varied from 0.03 µg/cm^2 (MF) to 0.10 µg/cm^2 (2,4-D) (Fig. 4B) and AI varied from 218 (2,4-D) to 1350 (G) (Fig. 4C). No significant differences were found in mass variables and AI among treatments.

We found a total of 25 species, with Chlorophyta being the most abundant group (81.8%) followed by Bacillariophyceae (13.6%), Dinophyceae (3.0%), Cyanobacteria (1.6%) and Chrysophyceae (0.02%) (Table 2). No significant differences in live percentages were found among treatments (Control 64.1%, G 54.1%, GF 53.5%, 2,4-D 55.9%, 2,4-DF 51.2%, M 43.2%, MF 64.6%) (Fig. 5A). At Tf, two species showed variations in abundance with respect to the Control: the diatom *Achnanthidium* sp. decreased more than 3 times (− 3.26) with respect to the Control at the concentration of 3 mg/L G ($p = 0.03$) and in the treatment of the a.i. mixture (Fig. 5B.1). On the other hand, the filamentous cyanobacteria *Leptolyngbya* sp. appeared significantly only in the treatment of the a.i. mixture ($p = 0.02$) (Fig. 5B.2.1 and B.2.2). There was no significant effect on any of the variables measured for 2,4-D alone.

4. Discussion

Phytoplankton and periphyton communities appeared to be sensitive to G but not to 2,4-D, as no significant effect was detected at the concentrations tested in both experiments. The mixture of herbicides mainly had an additive impact on these communities, especially on phytoplankton. The G and herbicide mixture treatments yielded similar results, suggesting that G is the primary driver factor in the interaction between the two herbicides in the mixture. On one hand, G is a broad spectrum herbicide which inhibits aromatic amino acid synthesis in plants via the shikimate pathway. Because this route is also present in algae (Richards et al., 2006; Tohge et al., 2013), it is expected a significant impact on these organisms, as previously reported by Saxton et al. (2011) and Mensah et al. (2013). On the other hand, 2,4-D is more selective since its auxin-type MOA affects the vascular cambium in dicots (Song, 2014), a tissue absent in algae and cyanobacteria. Therefore, considering that G has a broader spectrum of action, our results support the relevant role of G in the impact exerted by the mixtures with 2,4-D on freshwater algae and cyanobacteria, in both phytoplankton and periphyton communities.

A dose-dependent behavior was confirmed by the decrease in total phytoplankton abundance recorded at medium and high concentrations of G, which is consistent with the results previously reported by

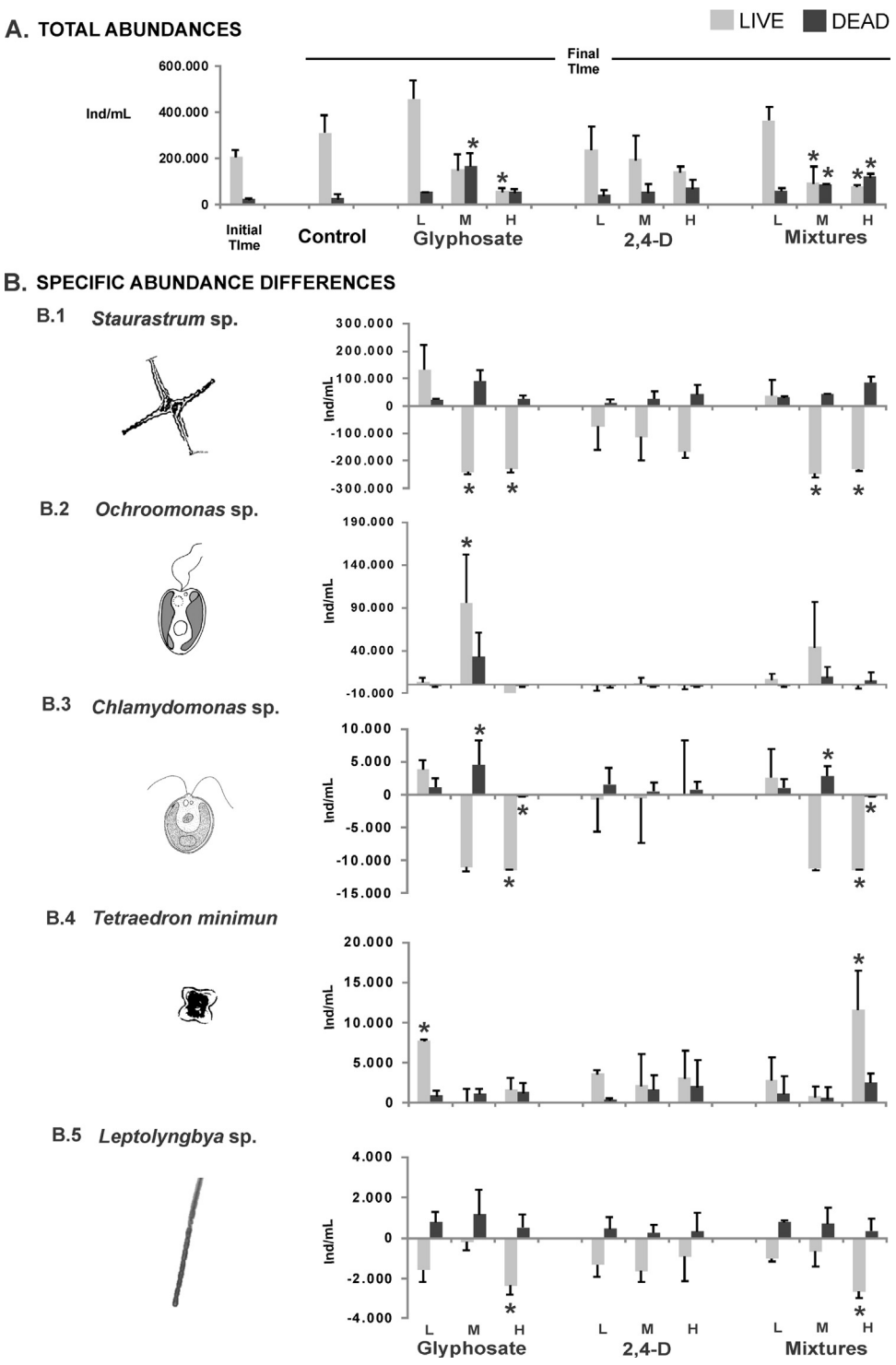

Fig. 2. Phytoplankton abundances after a 7-day exposure to glyphosate, 2,4-D and mixtures of both herbicides as active ingredients. **A.** Mean abundances (ind/mL) of live and dead organisms (n = 3) at Ti and Tf. **B.** Mean differences of live and dead abundances at Tf with respect to Ti for the main phytoplanktonic species by treatment. L: low concentration; M: medium concentration; H: high concentration. Algal images are not at scale. Error bars: 1 SD. (*) significant differences with respect to control (KW, $p < 0.05$; n = 3).

different authors. Pizarro et al. (2015) found a significant decline in the abundance of micro- and nano- phytoplankton in outdoor mesocosms due to the action of Glifosato Atanor® at 3 mg/L of a.i., while Pérez et al. (2007) found a similar decline in these communities after applying Roundup® at 6 and 12 mg/L of a.i. The decrease in phytoplankton abundance with the GM and GH treatments was reflected in a drop in turbidity and chlorophyll *a* concentration. This was also pointed out by Sura et al. (2012) for phytoplankton in outdoor mesocosms, with consequent impact on water quality. It has been observed that G does not completely eliminate some algae and cyanobacteria having the shikimic acid pathway and that they show different reactions to the herbicide (Forlani et al., 2008; Lipok et al., 2010; Saxton et al., 2011; Wang et al., 2016). In this sense, concentrations of 3 and 6 mg/L of G produced a decrease in the abundance of *Staurastrum* sp., which in turn was more sensitive than *Chlamydomonas* sp. and *Leptolyngbya* sp., as these were only affected by the highest G concentration. In contrast, the growth of *Tetraedron minimum* was stimulated by the lowest concentration of G, with no effect on its abundance at higher concentrations. Although the concentrations applied in our experiment were in the order of magnitude of the highest levels reported in natural water (Peruzzo et al., 2008), they were not enough to eliminate populations, but instead elicited different responses in algae and cyanobacteria.

Table 2
List of species found in phytoplankton and periphyton communities from both experiments.

Group	Phytoplankton	Periphyton
Cyanobacteria	*Leptolyngbya* sp.	*Aphanothece nidulans*
		Chroococcus sp.
		Leptolyngbya sp.
Chlorophyta	*Staurastrum* sp.	*Ankistrodesmus spiralis*
	Lagerheimia sp.	*Botryococcus braunii*
	Pandorina sp.	*Fusola viridis*
	Tetraedron mínimum	*Monoraphidium minutum*
	Chlamydomonas sp.	*Nephrocytium* sp.
	Botryococcus braunii	*Oocystis* sp.
		Pediastrum sp.
		Scenedesmus sp.
		Scenedesmus obliquus
		Sphaerocystis sp.
		Tetraedron minimum
		Palmeloid (not identified)
		Cosmarium sp.
		Staurastrum sp.
		Oedogonium sp.1
		Oedogonium sp. 2
		Bulbochaete sp.
Bacillariophyceae	*Achnanthes* sp.	*Achnanthidium* sp.
	Nitzschia sp.	*Fragilaria ulna*
		Gomphonema sp.
		Rophalodia gibba
Dinophyceae	*Peridinium* sp.	*Peridinium* sp.
Chrysophyceae	*Ochromonas* sp.	*Salpingoeca* sp.

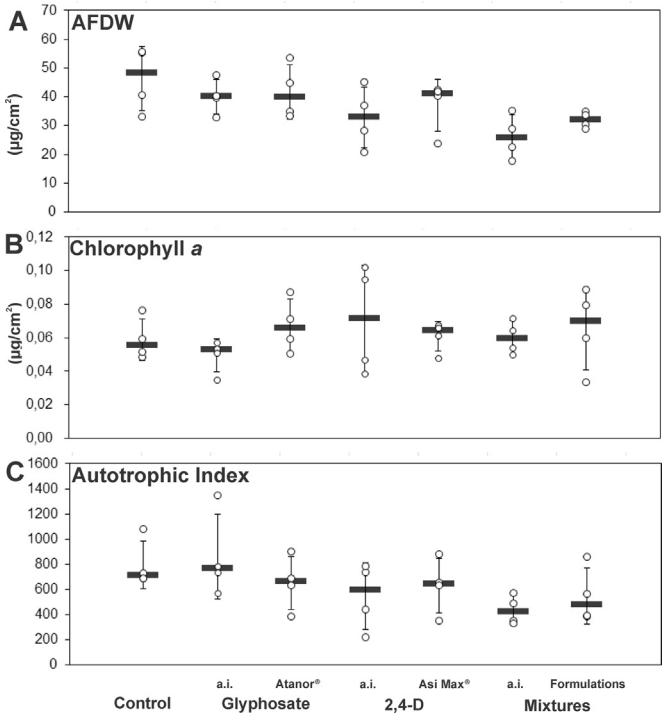

Fig. 4. Periphytic mass variables after a 7-day exposure by treatment. Circles show individual measurements. Horizontal bars indicate median values (figures made from templates provided by Weissgerber et al., 2015).

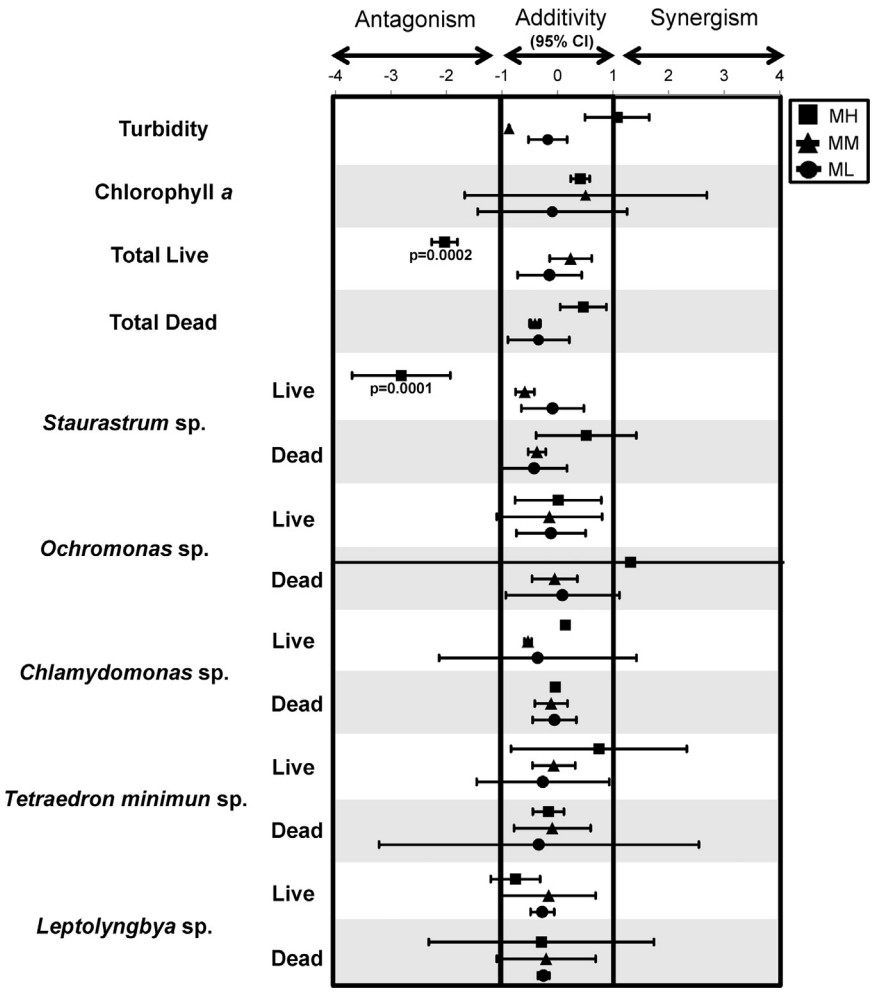

Fig. 3. Mean value and confidence intervals for each variable in the mixture treatments at high (ML), medium (MM) and low (ML) concentrations; values were relativized for comparison across variables; 95% confidence intervals for additivity (center), antagonism (left), and synergism (right). (T-Student $p < 0.05$; $n = 3$).

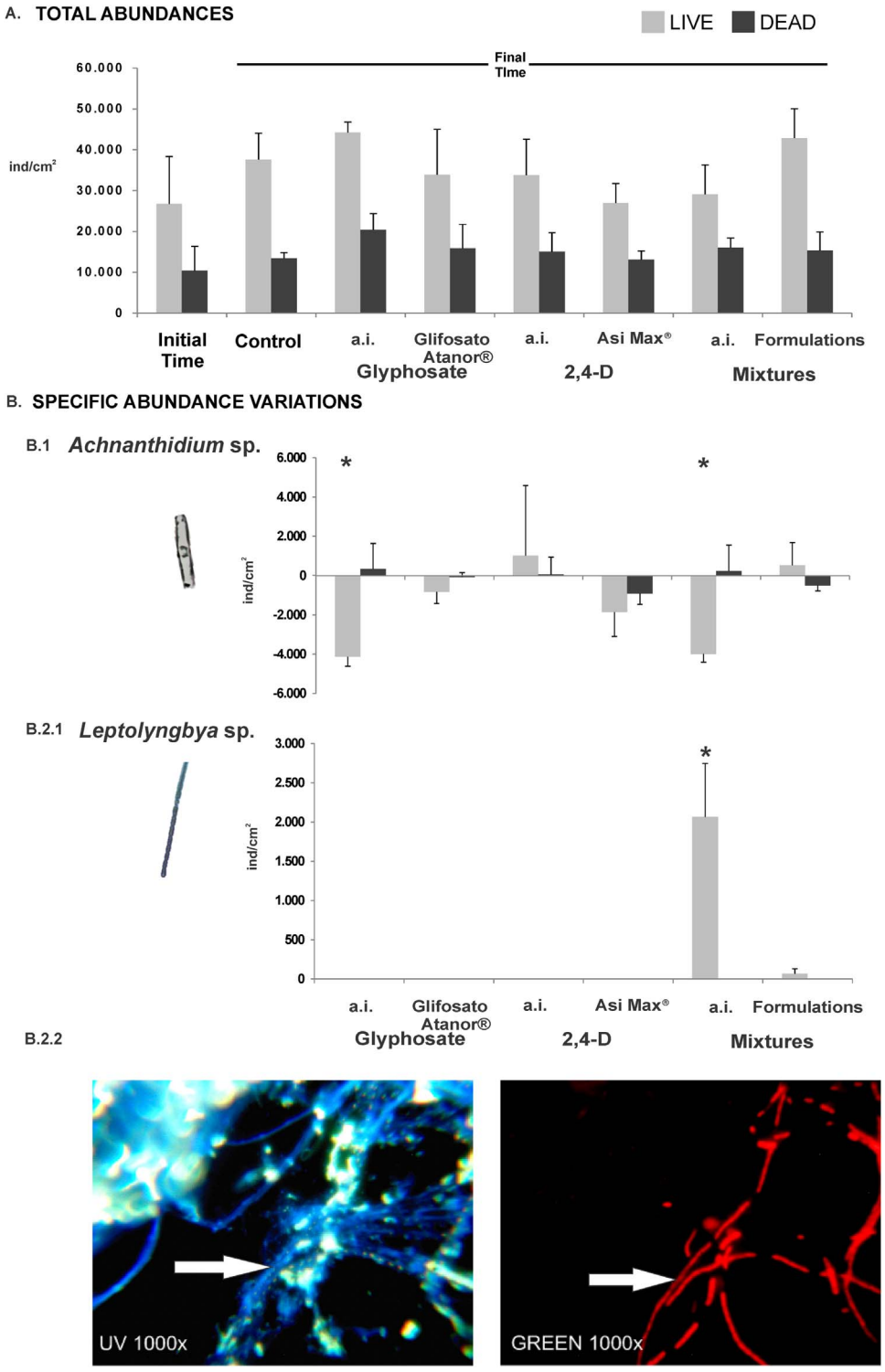

Fig. 5. Periphytic algae and cyanobacteria abundances after a 7-day exposure to herbicide treatments. **A.** Total Abundances (ind/cm^2) at initial time (control) and final time (all treatments). **B.** Mean values of differences in live and dead abundances with respect to the control at Tf for: **1.** *Achnanthidium* sp. **2.1** *Leptolyngbya* sp. **2.2.** Epifluorescence images of *Leptolyngbya* sp. (arrows) from periphyton M-treated samples. Algal images are schemes and are not at scale. Error bars: ± 1 SD. (*) significant differences with respect to control (KW, $p < 0.05$; $n = 3$).

In regard to the impact of 2,4-D on algae, different responses were recorded. Phytoplankton growth was stimulated at low concentrations (0.02–2 mg/L), while it was inhibited at higher ones (between 10 to more than 20 mg/L) (Kobraei and White, 1996; Boyle, 1980; Relyea, 2009). More recently, declines in abundance were reported for *Scenedesmus* sp. at 1.1 mg/L (Singh and Shrivastava 2016) and for some species of diatoms at 0.5 mg/L (Wood et al., 2016). Likewise, 2 mg/L of 2,4-D did not stimulate *Scenedesmus obliquus*' growth and decreased the formation of anti-grazer colonies (Zhu et al., 2016). In contrast, we found that 2,4-D had no significant impacts on phytoplankton abundances, and only a non-significant decreasing trend was seen in live cells of *Staurastrum* sp.

Our results pointed to G as the major driving factor of phytoplankton changes under mixture scenarios. Accordingly, Sura et al. (2012) also found that G has the highest potential to inhibit phytoplankton and periphytic algae in mixtures with auxin-type herbicides. It is important to highlight that the results obtained were conditioned by the experimental design, such as the concentrations of herbicides used and a probable difference in environmental fate (Seiber, 2002). Thus, we applied a ratio of 1:0.45, which is one of the most recommended in

agriculture, but many other ratios could be used instead. On the other hand, we experimentally assessed the impact of the herbicide mixture on the communities for 7 days, which is lower than the half-life in freshwater of G (≈ 33 days) (Pizarro et al., 2015) and 2,4-D (≈ 15 days) (US EPA, 2005). Indeed, a change in the duration of the experiment could have influenced the final results. Therefore, one should be cautious in making generalizations about the results of this study.

Although additivity was the main response for all variables, the herbicide mixture at high concentrations seemed to mitigate the effect of each herbicide separately on *Staurastrum* sp. This response may be explained by differential species sensitivity to herbicides and by ecological interactions at the community level, such as competition among populations (Rohr et al., 2006). For example, the presence of 2,4-D would decrease the abundance of other algae or cyanobacteria or impair their nutrient uptake capability. The competition between a 2,4-D-sensitive species and a G-sensitive species (i.e. *Staurastrum* sp.) could lead to an antagonistic response because the latter species would be directly and negatively affected by G and indirectly and positively affected by 2,4-D, through a lower pressure exerted by the 2,4-D-sensitive competitor.

The concentrations of G and 2,4-D selected for the periphyton experiment did not impact significantly on the variables measured. In fact, no significant differences in mass variables -which included both the autotrophic and heterotrophic fractions of the community- were detected between the active ingredient forms and commercial formulations. This is contrary to, what is expected, given that the co-adjuvants used in commercial formulations may be more toxic than the active ingredients for periphyton (Vera et al., 2014). In an experiment using 70 L-outdoor mesocosms, Vera et al. (2012) recorded a significant decrease in mass variables and AI for periphyton developed in similar substrata, as a consequence of the addition of 3 mg/L of Glifosato Atanor®. These substantial variations in periphyton response are most likely due to differences between their experimental design and ours, mainly determined by the characteristics of the experimental units (i.e. shape and volume) and the experimental conditions (i.e. outdoors vs indoors). Nevertheless, at the specific level *Achnanthidium* sp. was significantly affected by G as active ingredient, both alone and mixed with 2,4-D as active ingredient, indicating once more that responses vary at different levels of analysis. Moreover, the development of the filamentous cyanobacterium *Leptolyngbya* sp. was only observed in the presence of both herbicides as a.i., which could be considered as an unexpected synergic result. Its remarkable development in periphyton after 7 days might represent the beginning of a more conspicuous community to prevent the impact of contaminants. Filamentous organisms play a key role in the tridimensional structure of periphyton by enhancing the cohesion of the microbiota embedded in the mucilaginous matrix. Filamentous cyanobacteria such as *Leptolyngbya* sp. are known to form algal mats (Vincent et al., 1993).

The results of both experiments suggested that periphyton would be more resistant to herbicides than phytoplankton. An ecological community deals with disturbances, either by increasing resistance and/or improving the process of recovery; the ability of communities to use either or both strategies is known as resilience (Oliver et al., 2015). In this context, periphyton would be more resistant to stressing factors than phytoplankton by having a very complex structure and a self-produced matrix of hydrated extracellular polymeric substances (Flemming and Wingender, 2010). The matrix may limit the contact between organisms and contaminants, resulting in a reduced exposure; besides, nutrient recycling takes place within the matrix, providing a degree of independence from the water column. In our experiments we used a periphytic community that had developed over 60 days, forming a matrix consolidated enough to confer resistance to herbicides. Resistance would have also been reinforced by lack of turnover in algal composition between initial and final times. A relative high resilience of the periphyton community has already been observed for the herbicide paraquat by Bonilla et al. (1998).

It is worthwhile to stress the importance of adopting a community ecology approach in studies of herbicide impacts, considering that different results would be obtained from monospecific bioassays. In microbial communities such as phytoplankton and periphyton, different populations may react differently to a toxicant because biological interactions play a significant role in the final response of the community as a whole (Seguin et al., 2001). Likewise, many variables must be included in the analyses when assessing the mechanisms by which microbial communities respond to a mixture of herbicides. In this regard, Magbanua et al. (2013) recorded significant interactions of G with sediments, revealing synergistic effects for some variables and antagonistic effects for others.

There is some evidence that freshwater systems are being affected by land-use changes and the expansion of the industrial agricultural model (Tilman et al., 2001; Quirós et al., 2006; Scanlon et al., 2007; Pérez et al., 2017). In particular for Argentina, the Pampean Plain has undergone a strong agricultural intensification in the last decades (Sánchez et al., 2015). The Pampean Plain represents an agricultural region with thousands of polimitic shallow lakes, giving rise to more than 200,000 permanent and sporadic ones (Dangavs, 2005). Shallow lakes in this region are classified in three states according to their turbidity: clear-vegetated, algal-turbid (Scheffer et al., 1993), and inorganic-turbid (Allende et al., 2009). Although the clear-vegetated state probably was the pristine condition of the Pampean shallow lakes (Quirós et al., 2006), most of them are currently eutrophic/hyper-eutrophic (Quirós and Drago, 1999), present an algal-turbid status and are located in areas of high anthropogenic impact (Quirós et al., 2002). There are evidences that the large amount of algal-turbid shallow lakes is related to the impact of agricultural activities using agrochemicals during many decades (Quirós et al., 2006). Therefore, it cannot be ruled out that the input of agrochemicals such as fertilizers and herbicides in freshwater by different means (accidental spills, wind-drift, surface runoff, etc.) could strongly affect the structural and functional characteristics of freshwater systems in the region.

In this work we performed the experiments by means of laboratory designs similar to those carried out by the governmental agencies responsible for characterizing the use of herbicides in the field. The ecological consequences of the communities responses are difficult to predict under natural conditions. In a realistic scenario, G at a concentration of 3 mg/L or higher, alone or mixed with 2,4-D, would reduce chlorophyll *a* and turbidity due to a decrease in phytoplankton, as supported by the correlation analyses. This would increase light penetration in the water column in algal-turbid shallow lakes as those found in the Pampean Plain, leading to enhanced periphyton growth, which appears to be less affected by herbicides. This could ultimately result in a change in the structure and function of the entire system. Nevertheless, care must be taken when extrapolating to real complex systems.

Despite the difficulty in predicting the co-occurrence of most contaminants, we are able to simulate scenarios of herbicide contamination by studying agronomic recipes through laboratory assays. The effect of herbicide mixtures on the environment is a challenging ecotoxicology problem that needs a deeper study to prevent ecological surprises.

5. Conclusions

We studied the effect of the mixture of G and 2,4-D on freshwater phytoplankton and periphyton at agronomic proportions, using a microcosms approach to test 5 hypotheses. The results indicated that: 1. G and 2,4-D affected some structural variables in different ways, with the effect of G being dose-dependent. 2. Phytoplankton and periphyton showed different susceptibility to G and 2,4-D. 3. The impact of the mixture of both herbicides was additive at low and medium concentrations, while some antagonistic and synergic effects were observed at a higher concentration. In general, the strongest impact of the mixture on algal abundances was related to G, which appeared as the

driving force in the interaction. On the other hand, 2-4-D did not seem to contribute significantly to the impact on the microbial community, either alone or in the mixture. 4. The impacts of G and 2,4-D formulations differed from those of the single active ingredients, and the former did not enhance toxicity to periphyton. 5. Periphyton appeared to be more resistant than phytoplankton to G, 2,4-D and their mixture. The use of several variables is essential to predict more realistic scenarios as well as to analyze changes driven by agrochemical mixtures on key freshwater communities, which could not be detected otherwise.

Acknowledgments

This work was supported by PICT 2014.1586, UBACyT (20020130100248BA) and CONICET PIP11220130100399. We thank Lic. Miranda Cecilia and Garzarón Ignacio (P.E.) for editing assistance. The authors declare that they have no conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors. Informed consent was obtained from all individual participants included in the study.

References

Allende, L., Tell, G., Zagarese, H., Torremorell, A., Pérez, G., Bustingorry, J., Escaray, R., Izaguirre, I., 2009. Phytoplankton and primary production in clear-vegetated, inorganic-turbid, and algal-turbid shallow lakes from the pampa plain (Argentina). Hydrobiologia 624 (1), 45–60.

American Public Health Association, 2005. Standard methods for the examination of water and wastewaters. 21st edn. APHA, American Water Works Association, Water Environmental Federation, Washington, DC.

Annett, R., Habibi, H.R., Hontela, A., 2014. Impact of glyphosate and glyphosate-based herbicides on the freshwater environment. J. Appl. Toxicol. 34, 458–479.

Aparicio, V., De Gerónimo, E., Guijarro, K.H., Pérez, D., Portocarrero, R., Vidal, C., 2015. Los plaguicidas agregados al suelo y su destino en el ambiente. INTA Ediciones Argent. 1–74.

Azim, M.E., Asaeda, T., 2005. Periphyton structure, diversity and colonization. In: Azim, M.E. (Ed.), Periphyton: Ecology, Exploitation and Management. CABI Publ. Oxfordshire, Cambridge, pp. 15–34.

Bonilla, S., Conde, D., Blanck, H., 1998. The photosynthetic responses of marine phytoplankton, periphyton and epipsammon to the herbicides paraquat and simazine. Ecotoxicology 7 (2), 99–105.

Bonny, S., 2016. Genetically modified herbicide-tolerant crops, weeds, and herbicides: overview and impact. Environ. Manag. 57 (1), 31–48.

Boyle, T.P., 1980. Effects of the aquatic herbicide 2,4-D DMA on the ecology of experimental ponds. Environ. Pollut. Ser. A Ecol. Biol. 21 (1), 35–49.

Dangavs, N.V., 2005. Los ambientes acuáticos de la provincia de Buenos Aires. Relatorio del XVI Congreso Geológico Argentino.

Faust, M., Altenburger, R., Backhaus, T., Blanck, H., Boedeker, W., Gramatica, P., Hamer, V., Scholze, M., Vighi, M., Grimme, L.H., 2001. Predicting the joint algal toxicity of multi-component s-triazine mixtures at low-effect concentrations of individual toxicants. Aquat. Toxicol. 56 (1), 13–32.

Flemming, H.C., Wingender, J., 2010. The biofilm matrix. Nat. Rev. Microbiol. 8 (9), 623–633.

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 Cell Physiol. 49 (3), 443–456.

Geyer, R.L., Smith, G.R., Retting, J.E., 2016. Effects of Roundup formulations, nutrient addition, and Western mosquitofish (*Gambusia affinis*) on aquatic communities. Environ. Sci. Pollut. Res. Int. 23 (12), 11729.

Green, J.M., 2016. The rise and future of glyphosate and glyphosate-resistant crops. Pest Manag. Sci. 68 (10), 1323–1331.

Kobraei, M.E., White, D.S., 1996. Effects of 2,4-dichlorophenoxyacetic acid on Kentucky algae: simultaneous laboratory and field toxicity testings. Arch. Environ. Contam. Toxicol. 31 (4), 571–580.

Lilchtenthaler, H.K., Wellburn, A.R., 1983. Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochem. Soc. Trans. 11 (5), 591–592.

Lipok, J., Studnik, H., Guyaert, S., 2010. The toxicity of Roundup® 360 SL formulation and its main constituents: glyphosate and isopropylamine towards non-target water photoautrotrophs. Ecotoxicol. Environ. Saf. 73, 1681–1688.

Lowe, R., Pan, Y., 1996. Benthic algal communities as biological monitors. Algal Ecol.: Freshwater Benthic Ecosyst. 705–739.

Magbanua, F.S., Townsend, C.R., Hageman, K.J., Lange, K., Lear, G., Lewis, G.D., Matthaei, C.D., 2013. Understanding the combined influence of fine sediment and glyphosate herbicide on stream periphyton communities. Water Res. 47 (14), 5110–5120.

Mensah, P.K., Palmer, C.G., Muller, W.J., 2013. Derivation of South African water quality guidelines for Roundup® using species sensitivity distribution. Ecotoxicol. Environ. Saf. 96, 24–31.

Metzler, M.J., Puricelli, E., Peltzer, H.F., 2011. Control de *Conyza* spp. (rama negra) en barbecho de soja con glifosato en mezcla con herbicidas residuales y de contacto. In: Acta del Quinto Congreso de la Soja del Mercosur. Primer Foro de la Soja Asia-Mercosur, pp. 138–140.

Oliver, T.H., Heard, M.S., Isaac, N.J., Roy, D.B., Procter, D., Eigenbrod, F., Freckleton, R., Hector, A., Orme, D.L., Petchey, O.L., Proença, V., Raffaelli, D., 2015. Biodiversity and resilience of ecosystem functions. Trends Ecol. Evol. 30 (11), 673–684.

Pérez, D.J., Okada, E., De Gerónimo, E., Menone, M.L., Aparicio, V.C., Costa, J.L., 2017. Spatial and temporal trends and flow dynamics of glyphosate and other pesticides within an agricultural watershed in Argentina. Environ. Toxicol. Chem. 9999 (9999), 1–11.

Pérez, G.L., Torremorell, A., Mugni, H., Rodríguez, P., Vera, M.S., Do Nascimento, M., Allende, L., Bustingorry, J., Escaray, R., Ferraro, M., Izaguirre, I., Pizarro, H., Bonetto, C., Morris, D.P., Zagarese, H., 2007. Effect of the herbicide roundup on freshwater microbial communities: a mesocosm study. Ecol. Appl. 17 (8), 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. Environ. Pollut. 156 (1), 61–66.

Piggott, J.J., Townsend, C.R., Matthaei, C.D., 2015. Reconceptualizing synergism and antagonism among multiple stressors. Ecol. Evol. 5 (7), 1538–1547.

Pizarro, H., Vera, M.S., Vinocur, A., Pérez, G., Ferraro, M., Helman, R.M., dos Santos Afonso, M., 2015. Glyphosate input modifies microbial community structure in clear and turbid freshwater systems. Environ. Sci. Pollut. Res. 23 (6), 5143–5153.

Pollegioni, L., Schonbrunn, E., Siehl, D., 2011. Molecular basis of glyphosate resistance–different approaches through protein engineering. FEBS J. 278 (16), 2753–2766.

Pórfido, O.D., Butler, E., de Titto, E., Issaly, P., Benítez, R., 2014. Los plaguicidas en la República Argentina. 14. Ministerio de Salud, Departamento de Salud Ambiental, Serie Temas de Salud Ambiental, pp. 193.

Quirós, R., Drago, E., 1999. The environmental state of Argentinean lakes: an overview. Lakes Reserv.: Res. Manag. 4, 55–64.

Quirós, R., Renella, A.M., Boveri, M.B., Rosso, J., Sosnovsky, A., 2002. Factores que afectan la estructura y el funcionamiento de las lagunas pampeanas. Ecol. Austral 12, 175–185.

Quirós, R., Boveri, M.B., Petracchi, C.A., Rennella, A.M., Rosso, J.J., Sosnovsky, A., Von Bernard, H., 2006. The effects of the pampa wetlands agriculturization on shallow lakes eutrophication. Eutrofizaçâon a América do Sul: Causas, conseqüências e tecnologias de gerenciamento e controle. Inst. Int. Ecol. 1–16.

Relyea, R.A., 2009. A cocktail of contaminants: how mixtures of pesticides at low concentrations affect aquatic communities. Oecologia 159 (2), 363–376.

Richards, T.A., Dacks, J.B., Campbell, S.A., Blanchard, J.L., Foster, P.G., McLeod, R., Roberts, C.W., 2006. Evolutionary origins of the eukaryotic shikimate pathway: gene fusions, horizontal gene transfer, and endosymbiotic replacements. Eukaryot. Cell. 5 (9), 1517–1531.

Rohr, J.R., Kerby, J.L., Sih, A., 2006. Community ecology as a framework for predicting contaminant effects. Trends Ecol. Evol. 21 (11), 606–613.

Sánchez, M.L., Lagomarsino, L., Allende, L., Izaguirre, I., 2015. Changes in the phytoplankton structure in a Pampean shallow lake in the transition from a clear to a turbid regime. Hydrobiologia 752, 65–76.

Saxton, M.A., Morrow, E.A., Bourbonniere, R.A., Wilhelm, S.W., 2011. Glyphosate influence on phytoplankton community structure in Lake Erie. J. Gt. Lakes Res. 37 (4), 683–690.

Scanlon, B.R., Jolly, I., Sophocleous, M., Zhang, L., 2007. Global impacts of conversions from natural to agricultural ecosystems on water resources: quantity versus quality. Water Resour. Res. 43 (3), W03437.

Scheffer, M., Hosper, S.H., Meijer, M.L., Moss, B., Jeppesen, E., 1993. Alternative equilibria in shallow lakes. Trends Ecol. Evol. 8, 275–279.

Seguin, F., Leboulanger, C., Rimet, F., Druart, J.C., Bérard, A., 2001. Effects of atrazine and nicosulfuron on phytoplankton in systems of increasing complexity. Arch. Environ. Contam. Toxicol. 40 (2), 198–208.

Seiber, J.N., 2002. Environmental Fate of Pesticides. Marcel Dekker, New York, pp. 127–161.

SENASA, 2017. Importación de Fitoterapicos 2013, 2014, 2015, data on website.

Singh, P.K., Shrivastava, A.K., 2016. Role of initial cell density of algal bioassay of toxic chemicals. J. Basic Microbiol. 56 (7), 812–819.

Song, Y., 2014. Insight into the mode of action of 2,4-dichlorophenoxyacetic acid (2,4-D) as an herbicide. J. Integr. Plant Biol. 56 (2), 106–113.

Sura, S., Waiser, M., Tumber, V., Lawrence, J.R., Cessna, A.J., Glozier, N., 2012. Effects of glyphosate and two herbicide mixtures on microbial communities in prairie wetland ecosystems: a mesocosm approach. J. Environ. Qual. 41 (3), 732–743.

Tilman, D., Fargione, J., Wolff, B., D'Antonio, C., Dobson, A., Howarth, R., Schindler, D., Schlesinger, W.H., Simberloff, D., Swackhamer, D., 2001. Forecasting agriculturally driven global environmental change. Science 292 (5515), 281–284.

Tohge, T., Watanabe, M., Hoefgen, R., Fernie, A.R., 2013. Shikimate and phenylalanine biosynthesis in the green lineage. Front. Plant Sci. 4, 62.

US EPA, 2005. Reregistration Eligibility Decision (RED) 2,4-D (EPA 738-R-05-002). Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances, Office of Pesticide Programs, U.S. Government Printing Office, Washington, DC.

US EPA, 2017. Registration of Enlist Duo|US EPA. [online] Available at: 〈https://www.epa.gov/ingredients-used-pesticide-products/registration-enlist-duo〉, (Accessed 14 July 2017).

Utermöhl, H., 1958. Zurvervollkommung der quantitativen phytoplankton Methodik. Int. Assoc. Theor. Appl. Limnol. 9, 1–38.

Venrick, E.L., 1978. Systematic sampling in a planktonic ecosystem. Fish. Bull. 76 (3), 617–627.

Vera, M.S., Di Fiori, E., Lagomarsino, L., Sinistro, R., Escaray, R., Iummato, M.M., Juárez,

A., Ríos de Molina, M.C., Tell, G., Pizarro, H., 2012. Direct and indirect effects of the glyphosate formulation Glifosato Atanor® on freshwater microbial communities. Ecotoxicology 21, 1805–1816.
Vera, M.S., Juárez, Á.B., Pizarro, H.N., 2014. Comparative effects of technical-grade and a commercial formulation of glyphosate on the pigment content of periphytic algae. Bull. Environ. Contam. Toxicol. 93 (4), 399–404.
Vincent, W.F., Castenholz, R.W., Downes, M.T., Howard-Williams, C., 1993. Antartic cyanobacteria: light, nutrients, and photosynthesis in the microbial mat environment. J. Phycol. 29 (6), 745–755.
Wang, C., Lin, X., Li, L., Lin, S., 2016. Differential growth responses of marine phytoplankton to herbicide glyphosate. PLoS One 11 (3), e0151633.
Weissgerber, T.L., Milic, N.M., Winham, S.J., Garovic, V.D., 2015. Beyond bar and line graphs: time for a new data presentation paradigm. PLoS Biol. 13 (4), e1002128.
Wetzel, R.G., 1983. Opening Remarks. Periphyton of Freshwater Ecosystems. Dr. W. Junk Publishers, The Hage, Netherlands, pp. 3–4.
Wong, P.K., 2000. Effects of 2,4-D, glyphosate and paraquat on growth, photosynthesis and chlorophyll-a synthesis of *Scenedesmus quadricauta* Berb 614. Chemosphere 41 (1–2), 177–182.
Wood, R.J., Mitrovic, S.M., Lim, R.P., Kefford, B.J., 2016. How benthic diatoms within natural communities respond to eight common herbicides with different modes of action. Sci. Total Environ. 557, 636–643.
Zhu, X., Sun, Y., Zhang, X., Heng, H., Nan, H., Zhang, L., Huang, Y., Yang, Z., 2016. Herbicides interfere with antigrazer defenses in *Scenedesmus obliquus*. Chemosphere 162, 243–251.