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

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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).

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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.