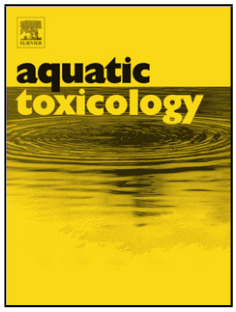

New findings on the effect of glyphosate on autotrophic and heterotrophic picoplankton structure: a microcosm approach

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New findings on the effect of glyphosate on autotrophic and heterotrophic picoplankton structure: a microcosm approach

Running page head: Glyphosate alters freshwater picoplankton structure

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Highlights

- Glyphosate exposure decreases PC-rich Pcy cytometric population's abundances.
- Glyphosate changes HNA/LNA bacteria ratio from a clear system.
- The structure of dominant bacterial OTUs is altered by glyphosate.

ABSTRACT

Massive use of glyphosate-based herbicides in agricultural activities has led to the appearance of this herbicide in freshwater systems, which represents a potential threat to these systems and their communities. These herbicides can affect autotrophic and heterotrophic picoplankton abundance. However, little is known about glyphosate impact on the whole structure of these assemblages. Herein, we used an 8-day long microcosm approach under indoor controlled conditions to analyze changes in the structure of picoplankton exposed to a single pulse of glyphosate. The analyzed picoplankton correspond to two outdoor ponds with contrasting states: "clear" (chlorophyll-a= 3.48 µg L^{-1} ± 1.15; nephelometric turbidity, NTU= 1) and "turbid" (chlorophyll-a= 105.96 µg L^{-1} ± 15.3; NTU= 48). We evaluated herbicide impact on different picoplankton cytometric populations and further explored changes in bacterial dominant

operational taxonomic units (OTUs) fingerprinting. We observed that glyphosate induced a drastic decrease in the abundance of phycocyanin-rich picocyanobacteria. Particularly, in the turbid system this effect resulted in an 85 % decrease in the abundance of the whole autotrophic picoplankton. Glyphosate also changed the structure of the heterotrophic fraction by means of changing bacterial dominant OTUs fingerprinting patterns in both systems and by shifting the relative abundances of cytometric groups in the clear scenario. These results demonstrate that upon glyphosate exposure picoplanktonic fractions face not only the already reported changes in abundance, but also alterations in the composition of cytometric groups and of bacterial dominant operational taxonomic units. This research provides suitable and still little explored tools to analyze agrochemical effects on picoplanktonic communities.

Keywords: Glyphosate - Picoplankton - Microcosms - Flow cytometry - DGGE - Freshwater ecosystems.

1. INTRODUCTION

Glyphosate-based herbicides are the most massively applied agrochemicals in agriculture worldwide. Nowadays, the use of these herbicides continues growing and only in 2014, 826 million kilograms of glyphosate-based herbicide were applied (Benbrook 2016). Glyphosate [N-(phosphonomethyl) glycine] success has been favored by the belief that it rapidly dissipates through microbial degradation and that it is highly specific towards organisms carrying the target enzyme 5-enolpyruvyl-shikimate-3-phospate synthase (Duke and Powles, 2008). However, glyphosate half-life ranges between 2 and 215 days in soils and 2 and 91 days in water (Battaglin et al., 2014). Moreover, it has harmful effects on non-target organisms including vertebrates; furthermore, it has been declared as potentially carcinogenic for humans (Myers et al., 2016). An increasing bibliography supports that glyphosate-based herbicides reach water bodies (e.g. Peruzzo et al., 2008; Battaglin et al., 2014; Ronco et al., 2016; Berman et al., 2018) and negatively affect aquatic organisms (Annett et al., 2014, and references therein). Through an outdoor mesocosm experiment, Vera et al. (2010) demonstrated that Roundup® increases the phosphorous (P) content, thus favoring the eutrophication of freshwater systems. Other similar studies in outdoor and indoor devices have reported that these herbicides alter the composition and abundance of phytoplankton, periphyton and zooplankton assemblages (Pérez et al., 2007; Pesce et al., 2009; Vera et al., 2012, 2010; De Stefano et al., 2018; Lozano et al., 2018).

Picoplanktonic communities play a key role in nutrient and carbon recycling in ecological food webs (Azam et al. 1983, Callieri 2007, Reed and Hicks 2011). Therefore, changes in their structure can affect the whole ecosystem function.

To date, however, a few studies performed in experimental ecosystems have included the impact of glyphosate-based herbicides on the structure of picoplankton assemblages and only analyzed changes in the abundances of picoeukaryotes, picocyanobacteria (Pcy) or the whole bacteria/heterotrophic picoplankton fraction (HPP) through epifluorescence microscope technique. In these assays, which use concentrations of herbicides active ingredient ranging between 2.4 and 12 mg L^{-1}, Pcy increased when exposed to Roundup® (Pérez et al., 2007), Glyphosate Atanor® (Vera et al., 2012) or the isopropilamine salt of glyphosate (Pizarro et al., 2015). On the other hand, HPP abundance increased (Vera et al., 2012) or was unaffected (Pérez et al., 2007; Pesce et al., 2009) under similar conditions. Moreover, the exposure to 2.4 mg L^{-1} of isopropilamine salt of glyphosate evoked opposite responses in bacterial communities from a clear or a turbid organic system (Pizarro et al. 2015). Altogether, these findings highlight the complexity inside picoplanktonic communities.

Through molecular and biochemical approaches, several groups have identified, at a single species level, different bacteria and cyanobacteria resistant/tolerant to glyphosate (Ilikchyan et al. 2009; Hove-Jensen et al. 2014; Forlani et al. 2015), as well as bacterium strains that can degrade this molecule (Zhan et al. 2018). However, there is a gap between information regarding glyphosate-based herbicides effect on single species and on assemblages. This may be partially due to the lack of simple and inexpensive techniques to analyze these highly diverse communities, which, furthermore, are mostly uncultivable.

Flow cytometry provides a suitable tool to deepen in the analysis of picoplankton community structure and its changes. This approach allows the

clustering of different picoplankton communities based on different characteristics. The autotrophic picoplankton (APP) can be clustered according to size/intracellular complexity and expression of pigments, whereas the HPP is clustered based on size/intracellular complexity and nucleic acid content. For instance, this technique allows to discriminate between phycocyanin rich (PC-rich) and phycoerythrin rich (PE-rich) Pcy, which are likely present in environments of high or low turbidity, respectively (e.g. Stomp et al., 2007, Schiaffino et al. 2013). In addition, it discriminates heterotrophic groups with high nucleic acid content (HNA) and groups with low content (LNA), which are suggested to dominate in environments with high and low nutrient load, respectively (Harry et al. 2016, Pradeep Ram et al. 2016).

The aim of this research was to further explore isopropilamine salt of glyphosate (from now on IPA) effect on the abundance and composition of cytometric populations by studying picoplankton assemblages from 2 freshwater systems with contrasting conditions of turbidity, nutrients and chlorophyll-*a* concentration. We analyzed the response of cytometric populations from APP and HPP upon an exposure to 4 mg L^{-1} of IPA for 8 days, under indoor controlled conditions. In addition, considering the relevance of bacteria in glyphosate degradation, we complement the analysis by studying changes in the relative abundance of bacterial dominant operational taxonomic units (OTUs). For this purpose, we performed denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments in samples from both aquatic systems. Considering previous reports on glyphosate-based herbicides effects over Pcy, we hypothesize that upon IPA exposure 1) the relative abundances of autotrophic picoplankton cytometric populations, and particularly Pcy groups,

change. Besides, glyphosate molecule incorporates phosphorous to the system, producing an increment in nutrient load that would be more pronounce in a scenario with lower nutrient concentration. Considering that nutrient load was positively correlated with HNA cytometric populations, we also hypothesize that upon IPA addition 2) HNA/LNA ratio increases particularly in the clear microcosms. On the other hand, several glyphosate-resistance mechanisms have been described in bacteria; therefore, we expect that in IPA treated scenarios 3) dominant OTUs fingerprinting changes. Finally, 4) some of the OTUs that are favored by the exposure, taxonomically would affiliate with groups previously described as resistant/tolerant to glyphosate in single species studies.

2. MATERIALS & METHODS

2.1 Experimental design

A microcosm assay was performed by using autoclaved flasks (500 mL) incubated with gentle shaking at 25 ± 1°C under a 12:12 h light-dark photoperiod provided by cool fluorescent white light of 9 µmol photon m^{-2} s^{-1}. The water used in the assays belonged to two outdoor artificial ponds, which had been filled with tap water and where microbial assemblages naturally evolved for a year. The initial conditions of the outdoor ponds, which markedly differed in their turbidity values, chlorophyll-*a* and nutrient concentrations, are described in Table 1. Their limnological attributes represented the general conditions of clear and organic turbid natural freshwater systems (hereafter “clear” and “turbid”, respectively). Both ponds are set in an experimental campus of the Buenos Aires University, where the assays were performed.

Water samples were collected in 5 L recipients and immediately transferred to laboratory microcosms.

Two sets of 20 flasks were filled with 450 mL of water from either the clear pond or the turbid pond. After a 48-h stabilization period in the incubator, 4 mg L^{-1} of isopropilamine salt of glyphosate solution (40 wt. % in H_2O, CAS Number: 38641-94-0, SIGMA Aldrich) was added to 10 flasks from each system (G), whereas the remaining 10 flasks of each system were assigned to control conditions (C). Therefore, 4 different groups were established as follows: 1) C of clear system; 2) G of clear system; 3) C of turbid system and 4) G of turbid system. Three flasks of each treatment were removed from the incubator 20-25 min (0.4 h) and 24 h after IPA incorporation; the remaining microcosms (in quadruplicates for each treatment) were removed 192 h later.

IPA was chosen to evaluate only the effect of the active ingredient of glyphosate-based herbicides considering that there are a variety of these herbicides that differ in the composition of its adjuvants and the form of its active ingredient, and that the adjuvants might exert effects over aquatic ecosystems *per se*. The selected form is the most frequently used in commercial formulations to date. Four mg L^{-1} of active ingredient was applied because this concentration remains below the values expected in worst-case scenarios in the environment (Wagner et al., 2013). Other reason to select this concentration was that it allows us to make comparisons with previous manipulative experiments in which the concentrations of glyphosate applied were within this range (Pérez et al., 2007; Vera et al., 2012; Pizarro et al., 2015). A water sample was obtained from each flask 20 min after IPA addition to evaluate chemical concentrations. In G flasks, the concentration of

glyphosate measured was 4.28 ± 0.45 mg L^{-1}, whereas in C flask glyphosate it was undetectable.

The selected 8-day period allows the assessment of the acute response of picoplankton assemblages to glyphosate exposure, because these communities have short generation times and rapid responses (Peck, 2011). A similar time-scale in other microcosm's assays was applied to evaluate the response of different microbial groups to diverse pesticides (e.g., Flores Vargas et al., 2019; Lozano et al., 2018; Muturi et al., 2017; Stachowski-Haberkorn et al., 2008).

2.2 Determination of glyphosate concentration

Water samples (50 mL) taken 0.4 h after IPA addition were conserved at –20 °C until use. The glyphosate concentration was assessed as follows. The samples were thawed and then homogenized to subsequently perform a chloromethane extraction protocol with 3 mL to 10 mL of water. Then the extracted water was centrifuged for 10 min at 17,000 g to exclude solid residues. The obtained liquid phase was analyzed in an ultra-performance liquid chromatography (UPLC) Water Acquity with single quadrupole mass detector set up in the ESI negative mode to determine glyphosate concentration. The chromatographic separation was set up with 1% acetic acid in water: MeOH, at the following gradients: (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. A Hypercarb 2.1 × 100 mm 5 µm column was used and quantification was performed using the ion monitoring (SIM) mode (168 m/z and ion 150 m/z). The calibration curves were prepared with water with a range between 5.00 µg L^{-1} and 15.00 mg L^{-1}.

2.3 Measurements of physical and chemical variables and chlorophyll-*a* concentrations

Physical and chemical variables as well as chlorophyll-*a* concentration of the outdoor ponds were assessed before collecting the water samples for the microcosm assay. In addition, chemical variables and chlorophyll-*a* were measured again after a period of stabilization of the microcosms to evaluate the enclosure effect.

Temperature, pH and conductivity were measured with a Hach® portable multiparameter sensor; nephelometric turbidity and dissolved oxygen were assessed with a 2100P Hach® portable turbidimeter and an HI 9146 Hanna® portable meter, respectively.

The chlorophyll-*a* determination was assessed by filtering samples through a Whatman® GF/F filter. Pigments were extracted from the filters after an incubation in 90% acetone at 4 °C overnight and by a subsequent centrifugation for 10 min at 4500 g. The obtained pigments were measured by spectrophotometry (lectures at 665 and 750 nm) before and after acidification with HCl 1 N. Chlorophyll-*a* concentrations were determined following Marker et al.'s equation (1980). Soluble reactive phosphorous and total phosphorous were determined from filtered and unfiltered water, respectively. Total phosphorous was transformed to soluble reactive phosphorous through acid digestion with potassium persulfate. Soluble reactive phosphorous and total phosphorous were determined as molybdate reactive phosphorous following standard procedures described in APHA (2005).

2.4 Picoplankton analyses

Flow cytometry analyses of the structure of picoplankton communities were performed both in the original ponds and in the stabilized control microcosms to evaluate the enclosure effect. Subsequently, the impact of glyphosate on the cytometric populations was analyzed at 0.4, 24 and 192 h. The effect of glyphosate on the HPP structure was further evaluated through DGGE at 0.4 and 192 h.

Cytometric analyses of APP and HPP fractions. Samples (3.6 mL) were filtered through an 18 µm mesh to avoid cytometer clogging issues; subsequently, glyTE 10X (50 % glycerol and 10X TE) was added to a final concentration of 1X, left 10 min at room temperature in the dark and preserved at –80 °C until needed. Each sample was thawed and divided in 2 subsamples to analyze APP and HPP in a FACSAria II flow cytometer equipped with blue (488 nm) and red (633 nm) lasers. The obtained data were analyzed with FlowJo® software. The settings used to process samples in the cytometer and the subsequent analysis were based on the protocols of Gasol & Moran (2015). Briefly, autotrophic picoplankton cytometric populations were clustered by cell complexity/size and pigments expression. Complexity/size was determined using side scattered light, whereas the presence of chlorophyll-*a*, PE and PC were assessed by detecting the fluorescence emitted by the pigments after laser excitation. By contrast, the HPP fraction was treated with SYBR® green (Sigma-Aldrich) dye for 10 min to stain nucleic acids before running in the flow cytometer. Therefore, the HPP cytometric groups were clustered using side scatter light and SYBR® green signals. The changes in HNA/LNA ratio were assessed by measuring the mean intensity of fluorescence of nucleic acids of the whole HPP fraction, since an increase of HNA/LNA ratio increases the

mean intensity of fluorescence of nucleic acids and the opposite effect would result from a decrease of this ratio.

HPP analysis by DGGE. Samples (200 mL) from 0.4 and 192 h microcosms were pre-filtered through an 18-µm mesh, then filtered through a 0.2 µm filter (polycarbonate membrane filters, 47 mm, Isopore™) and the filters were preserved at –80 °C until DNA extraction. The extractions were performed using CTAB buffer and washed with chloroform/isoamyl alcohol as described by Fernández Zenoff et al. (2006). Subsequently, the variable V3–V5 region within the 16S rRNA gene fragment was PCR amplified with the primers described by Muyzer et al. (1993). DGGE of the amplified products was performed in 6% polyacrylamide gels with 40-80% denaturant gradient agent run in a DGGE-2000 system (CBS Scientific Company) according to Schiaffino et al. (2011). DGGE gels were analyzed using TotalLab® TL120 v software to obtain matrices of presence-absence of bands as well as matrices of the relative intensity of bands. The relative intensities within each lane were calculated as the contribution of each band to the sum of signals of all bands in the lane. Each band position was considered, at least, one dominant operational taxonomic unit (OTU).

Band sequencing. Some of the more conspicuous bands from each gel were excised, PCR re-amplified and sequenced in Macrogen Sequencing Service (Macrogen, Republic of Korea). The selection included bands with different patterns between treatments: i.e. bands that increased/appeared, decreased/disappeared or maintained similar intensity. The obtained sequences (c.500 bp) were screened for chimeras using the Ribosomal Database Project

(Maidak et al., 2001) and then compared with public DNA databases using SILVA and BLAST (Altschul et al., 1997).

2.5 Statistical analyses

The differences between both conditions (clear and turbid), regarding autotrophic and heterotrophic picoplankton total abundances and the chemical parameters, were assessed through two-sample t-tests. On the other hand, the enclosure effect (i) on the proportion of picoplanktonic cytometric populations (both, APP and HPP fractions) and (ii) on chemical parameters were evaluated by one-way frequency analyses and two-sample t-tests, respectively.

The IPA exposure and incubation time effects on the population abundances were assessed by generalized linear models. Variance was modeled with a negative binomial distribution. Tukey´s contrasts ($p< 0.05$) was used for *post-hoc* multiple comparisons of means. All analyses were performed using Infostat® (version 2018. FCA, UNC, Argentina) and R statistical software (version 3.5.1; R Development Core Team 2014).

A non-metric multidimensional scale (NMDS) analysis using Bray-Curtis similarity measurement was used to analyze OTU matrices of relative intensities and the significant differences were further evaluated ($p< 0.05$) with a one-way analysis of similarity (ANOSIM) of pairwise comparisons given by Bonferroni-corrected p-value. These analyses were performed with the Past version 2.17c Software. Differences on OTU richness were analyzed using the non-parametric test Mann-Whitney ($\alpha= 0.05$) with Infostat® software.

3. RESULTS

3.1 Characterization of outdoor ponds and stabilized microcosms

For microcosm assays, we used samples from a clear and a turbid outdoor pond that differed in the structure of their cytometric populations (see Supplementary Figs. 1a.i-b and 2). Both outdoor scenarios had similar abundances of total HPP (mean ± SD clear *vs.* turbid: $1.6x10^6 \pm 7.8x10^5$ *vs.* $2.4x10^6 \pm 6.1x10^5$ cells mL^{-1}; $p > 0.05$). The clear pond, however, presented a markedly higher abundance of LNA than HNA, whereas the turbid system displayed similar proportions of both HPP cytometric groups. The abundance of APP fraction was lower, although not significantly, in the clear pond (mean ± SD clear *vs.* turbid: $1.1x10^3 \pm 0.1x10^2$ *vs.* $2.7x10^4 \pm 4.6x10^3$ cells mL^{-1}; $p = 0.058$). Both systems contained a picoeukaryote group and different Pcy cytometric groups. In the clear system, PE-rich Pcy dominated the APP fraction. In the turbid scenario, by contrast, the PC-rich Pcy group, which comprised two subgroups (PC-rich Pcy1 and PC-rich Pcy2) prevailed by far over PE-rich Pcy. No significant changes ($p > 0.05$) in total abundances of APP or HPP fractions or in HNA/LNA proportion were detected in the stabilized clear and turbid microcosms in comparison to their respective clear and turbid outdoor ponds. However, picoeukaryote abundance increased and Pcy relative abundances changed in both microcosm conditions ($p < 0.001$, in both systems). In addition, the whole APP abundance in the clear microcosms was significantly lower than in the turbid microcosms ($p = 0.01208$). This observation confirms the tendency observed in the original ponds. Despite these variations in the APP fraction, PC-rich Pcy and PE-rich Pcy cytometric groups maintained their predominance within Pcy groups in the clear and turbid systems, respectively, at the microcosm scale (see Supplementary Figs. 1a.ii and 2).

The concentrations of chlorophyll-*a* and total phosphorous in the clear and

turbid microcosms (Table 1) showed no significant differences ($p > 0.05$) with respect to the outdoor ponds. Only soluble reactive phosphorous significantly increased in the turbid microcosms (mean ± SD for turbid outdoor ponds *vs.* microcosms 0.126 ±0.04 *vs.* 0.24 ±0.04 mg L^{-1}, $p < 0.05$).

3.2 Glyphosate effect on picoplanktonic communities

IPA had different impact on APP and HPP fractions in the clear and turbid microcosms (Table 2). The abundance of the APP remained unaltered between treatments in the clear microcosms throughout the assay. In the turbid scenario, however, this fraction abundance was affected by treatment-time interaction ($p < 0.001$). Notably, APP diminished in the turbid microcosms exposed to IPA for 24 h (post-test C vs. G at 24 h, $p < 0.05$) and further decreased by the end of the assay (post-test C vs. G at 192 h, $p < 0.05$ and 24 h vs. 192 h for G, $p < 0.05$) to finally represent 15% of its control counterpart.

The HPP abundance was affected by treatment-time interaction in both scenarios. In fact, this fraction increased after 24 h of incubation (post-test 0.4 vs. 24 h for C and G in both systems, $p < 0.05$) to later decrease at 192 h (post-test 24 vs. 192 h for C and G in both systems, $p<0.05$), in both conditions. In addition, in the clear system, HPP abundance was lower at 24 h in the G microcosms (post-test C vs. G at 24 h, $p < 0.05$). Despite these effects, no differences in HPP abundances were detected between treatments at the end of the assay (post-test C vs. G at 192 h, $p > 0.05$, for both conditions).

Glyphosate impact on APP cytometric groups

We subsequently analyzed the effect of IPA on the abundance of cytometric populations within the APP fraction (Fig. 1a and Supplementary Figs. 3-4). In

the clear condition, picoeukaryotes were affected by treatment-time interaction ($p< 0.0001$). The abundance of this population decreased in G microcosms at 24 h in comparison to C microcosms at the same time-point (post-test C vs. G at 24 h, $p< 0.05$). By contrast, only time ($p< 0.0001$) altered picoeukaryotes abundance in the turbid system. In both scenarios, picoeukaryotes decreased in time (0.4 h vs. 192 h in the respective comparisons for both scenarios $p< 0.05$), regardless of glyphosate exposure.

PE-rich Pcy abundance varied through time (clear: $p= 0.0002$; turbid: $p= 0.0329$) in both systems. However, whereas the abundance of this population progressively increased throughout the evaluated times in the clear condition, in the turbid system it decreased by the end of the assay.

Remarkably, the abundance of PC-rich Pcy cytometric populations was markedly lower in G than in C microcosms at the end of the assay (Fig. 1 and Supplementary Figs. 3 and 4) and was affected by treatment-time interaction, in both scenarios (clear: $p= 0.048$; turbid: $p< 0.0001$). Particularly, PC-rich Pcy decreased in clear G microcosms at 192 h compared to its control counterpart (post-test C vs. G at 192 h, $p< 0.05$), whereas PC-rich Pcy2 decreased in the turbid G microcosms compared to its control counterpart at 24 h (post-test C vs. G at 24 h, $p< 0.05$), with a further decreased at 192 h (post-test C vs. G at 192 h, $p< 0.05$; 24 vs. 192 h for G, $p< 0.05$). The exception was PC-rich Pcy1 that also drastically decreased in the G turbid microcosms but was affected only by IPA exposure ($p< 0.05$) and not by time. The severe decline of PC-rich Pcy groups in the turbid system explains the drop of the initial abundance in APP fraction to 15% (Table 2).

Glyphosate effect on HPP structure

HNA/LNA ratio shifted in clear microcosms after 24 h of IPA exposure compared to 24 h control counterpart. Moreover, by the end of the assay, at 192 h, the relative abundance of HNA and LNA were approximately 3.5-fold higher and 4-fold lower, respectively, than in the non-treated systems at the same time-point (Fig. 2a). Accordingly, the mean intensity of fluorescence of nucleic acids in the clear scenario was affected by time-treatment interaction ($p < 0.0001$) with a rise of this parameter in IPA-exposed microcosms at 24 h and 192 h compared to its respective control counterparts (post-test C vs. G at 24 and 192 h, $p < 0.05$) (Fig. 2b.i). In the turbid system, HNA/LNA remained unaltered between C and G microcosms and only time accounted for differences of nucleic acids signal ($p=0.0145$) (Figs. 2a, 2b.ii and Supplementary Fig. 5). Despite the effects over HNA/LNA ratio in the clear scenario, no changes in HPP total abundances were detected between C and G microcosms at 192 h (Table 2) in either scenario.

Notably, the cytograms of the IPA-exposed clear microcosms displayed an evident leftward displacement of the whole HPP compared to C microcosms, at 192 h of exposure (Fig. 2b). An analysis of mean intensity of fluorescence of side scatter light revealed that time-treatment interaction affected this parameter in both scenarios ($p= 0.0018$ and $p < 0.0001$ for clear and turbid microcosms, respectively). Whereas C and G microcosms from the clear system differed at 24 and 192 h (post-test C vs. G at 24 and 192 h, $p < 0.05$), the turbid system only displayed significant differences at 192 h (post-test C vs. G at 192 h $p < 0.05$). Notably, side scatter light signal increased in the clear microcosms exposed to IPA, whereas it decreased in the turbid scenarios under the same condition.

A further characterization of IPA effect on HPP fraction structure was performed by separately running DGGE for samples of both systems (Fig. 3).

A total of 200 bands in 44 different migration positions were present in the DGGE of samples from the clear microcosms (sum of bands from C and G at 0 and 194 h) (Fig. 3a). After 192 h (lanes 7-14), almost all OTUs were somehow affected by IPA exposure. For example, the relative intensity of some bands from IPA-exposed microcosms diminished or disappeared (positions 7 to 9; in lanes 7-10 vs. 11-14), whereas new OTUs became detectable (e.g. band position 62 in lanes 12-14). Particularly, 5 bands disappeared from all the replicates of G microcosms at 192 h, whereas 14 new bands appeared in at least one of these replicates.

In the DGGE of the turbid samples, 174 bands in 24 migration positions were detectable (Fig. 3b). In this case, the relative intensity of some OTUs from G microcosms decreased (e.g. band position 4 in lanes 8-11 vs. 12-15), whereas others increased (e.g. band positions 6 and 7 in lanes 8-11 vs. 12-15) or remained unaltered (e.g. band positions 3 and 9 in lanes 8-15).

The mean richness of dominant OTUs at the end of the assay showed no significant differences between treatments (C vs G at 192 h, $p > 0.05$) in the clear (C= 13.25 ±1.56; G= 16.25 ±1.56) or turbid microcosms (C= 10.25 ±1.03; G= 10.5 ±1.03). Surprisingly, at the beginning of the assay richness of G turbid microcosms was higher than that of its control (C= 11 ±0.89; G= 16 ±0.89; C vs. G $p < 0.05$); by contrast, no differences were observed between treatments in clear microcosms (C = 12.67 ±3.05, G= 12.67 ±3.21, $p > 0.05$).

The NMDS analyses showed a clear grouping of samples from same time and treatment (stress value: 0.1053 for clear and 0.108 for turbid microcosms).

Furthermore, samples exposed to glyphosate for 192 h were further separated from the rest of the groups in both conditions (Fig. 3b). A statistical analysis with ANOSIM confirmed the observed clustering and showed significant differences between samples incubated with glyphosate for 192 h and all the rest of the treatments (clear ANOSIM R value= 0.87 and turbid ANOSIM R value= 0.91, both $p< 0.05$). No differences were detected between C and G at 0.4 h. However, a significant effect was also evident between controls at these 2 evaluated times.

From the total number of sequenced bands, only 10 bands from the samples of the clear system and 4 of the turbid scenario were successfully sequenced (Table 3). The obtained sequences affiliated mostly to Bacteroidetes and Proteobacteria in both systems. Remarkably, 4 OTUs phylogenetically affiliated to Alphaproteobacteria and 3 from the clear and 1 from the turbid microcosms increased after 192 h in the glyphosate treatment. In the clear system, an OTU that affiliated to Gammaproteobacteria showed the same trend, whereas a band that affiliated to Betaproteobacteria decreased 192 h post glyphosate application. The bands that affiliated to Bacteroidetes showed dissimilar trends in both scenarios.

4. DISCUSSION

Even though picoplankton is a key component of aquatic ecosystems (Azam & Malfatti 2007, Callieri 2007), the effect of glyphosate on the structure of this size fraction has been scarcely studied from an ecological perspective. Herein, through a relatively simple approach, we have demonstrated that a single pulse

of glyphosate can change not only the abundance of picoplanktonic communities, but also their whole structure.

We studied glyphosate impact on APP cytometric population under the hypothesis that the herbicide changes the relative abundances of Pcy cytometric populations. We expected to observe an increase of some Pcy cytometric groups considering previous studies in which this effect was reported using comparable concentrations of the active ingredient of glyphosate-based herbicides (Pérez et al., 2007; Pizarro et al., 2015; Vera et al., 2012). Surprisingly, and despite we corroborated that IPA alters the abundance of Pcy cytometric populations, we found an unexpected drastic decrease of Pcy groups.

Our results evidence marked dissimilarities in the effect of glyphosate on Pcy in comparison to the systems analyzed in each of the cited works. Indeed, whereas in our study glyphosate exposure evoked an 85% reduction of APP fraction because of PC-rich Pcy decrease in the turbid system, in Pérez et al.'s study (2007), by contrast, this fraction showed a 40-fold increase. The authors assumed that Pcy rise was mainly due to mechanisms of tolerance/resistance to glyphosate in cyanobacteria, which has been previously described in single species studies. Cyanobacteria displayed diverse sensitivity to glyphosate, which is associated to overexpression of 5-enolpyruvyl-shikimate-3-phospate synthase or to the expression of an alternative form of the glyphosate target enzyme (Forlani et al., 2015, 2008, Powell et al., 1992, 1991). Also, Ilikchyan et al. (2009) have reported a phosponate transporter gene that can metabolize glyphosate in *Synechococcus* spp and *Prochlorococcus* spp. In view of this diversity observed in culture assays, we cannot rule out the existence of

sensitive Pcy groups in the study presented here. Noticeable, the system analyzed by Perez et al. (2007) was set near agriculture fields where glyphosate is frequently applied for weed control. Therefore, the communities that grow in those mesocosms may have evolved to assemblages with a predominance of glyphosate-resistant/tolerant Pcy populations. On the other hand, the ponds in our study were set in an experimental campus with no agricultural field nearby and therefore glyphosate sensitive populations could establish.

In other manipulative experiments in which previous exposure of aquatic communities to glyphosate was unlikely (Vera et al. 2012, Pizarro et al. 2015), Pcy were also favored by glyphosate; however, the magnitude of the effect was lower. It should be noted that turbidity (in NTU) in those studies was up to 3 folds lower than that of the turbid system assayed herein. Considering that turbidity favors PC/PE-rich Pcy ratio (e.g. Schiaffino et al. 2013, Stomp et al. 2007), those studies may have evaluated systems with lower relative abundances of PC-rich Pcy cytometric populations. The turbid system assessed in the present study, in which, by contrast, PC-rich Pcy largely prevailed, showed the strongest variations upon glyphosate exposure. Since the studies mentioned above used epifluorescence microscope technique to quantify Pcy and did not distinguish Pcy groups, a potential negative effect of the herbicide over some less represented PC-rich Pcy groups may have been overlooked. Overall, this finding highlights the importance of analyzing communities from different environments rather than assessing single species in standard cultures. Moreover, this result puts into debate the general assumption that Pcy are favored by glyphosate exposure. This fact opens the question regarding

which Pcy groups are favored and which are negatively affected by glyphosate-based herbicides in communities from different freshwater systems. It would be important to address this question in future research by analyzing Pcy taxonomic groups to complement the cytometric data presented herein. Additional factors related to differences with our experimental design, such as indoor condition and limited water volume can neither be discarded. Nevertheless, we did not detect major effects on the Pcy cytometric groups due to enclosure (i.e. outdoor ponds vs. microcosms).

Finally, the observed changes in Pcy may be ascribed to other indirect effects of IPA, besides the possible differences in Pcy groups sensitivities to glyphosate discussed above. Indeed, IPA can impact other biological, physical or chemical components of the system affecting Pcy groups. For instance, we have recently observed that glyphosate alters the dynamics of dissolved oxygen in clear and turbid outdoor mesocosms (Lozano et al, unpublished data).

We also hypothesized that glyphosate increases HNA/LNA ratio because of an increment in total phosphorous given by the agrochemical, particularly in the clear scenario, which originally had a lower concentration of this nutrient. We based this hypothesis on previous reports in which nutrient concentration correlated with HNA bacterial rise (Harry et al. 2016, Pradeep Ram et al. 2016). Indeed, in our study glyphosate increased HNA relative abundance in the clear system, which was originally dominated by LNA cytometric groups. The turbid scenario received the same amount of phosphorous, but originally had a higher concentration of this nutrient and higher relative abundances of HNA than the clear system. Therefore, glyphosate contribution to nutrient further enrichment may not have been decisive to affect the bacterial ratio in the turbid condition.

Another not exclusive interpretation for HNA/LNA ratio changes is that glyphosate could have affected bacterial predators in the clear scenario, thus causing a top down effect that resulted in an HNA/LNA shift. Indeed, ciliates and flagellates selectively graze over HNA or LNA group and in turn control their ratio (Sintes and del Giorgio, 2014; Shafi et al., 2017). Although the analysis of other aquatic food web components is beyond the scope of this study, we cannot exclude indirect effects of glyphosate as previously described for other microbial communities (Vera et al. 2012).

A third possibility is that glyphosate may have a direct and differential impact on heterotrophic cytometric populations. Lebaron et al. (2002) interpreted that the HNA cytometric groups were the active components of microbial communities, whereas LNA were the less active, or dormant, communities. Later on, these populations were explained by differences in genome sizes of the different phylogenetic groups (Pradeep Ram et al., 2016; Schattenhofer et al., 2011). Other authors, on the other hand, proposed an intermediate scenario in which both interpretations coexist (Bouvier et al. 2007).

Even though we cannot establish if the observed shift in HNA/LNA ratio after IPA exposure in the clear condition is due to metabolic activation of dormant cells or to changes in the proportions of phylogenetic groups, our DGGE analysis confirmed that the herbicide changed, at least, dominant OTU composition in both scenarios. Furthermore, the herbicide also altered the cell morphology established by flow cytometry in both systems. Altogether, this finding suggests structural changes of the heterotrophic fraction in both cases. In our assay, glyphosate did not alter the richness of the dominant OTUs, in accordance with similar results observed with Illumina technology in a soil

bacterial community exposed to Roundup® for 60 days (Dennis et al., 2018). Unexpectedly, we found that bacterial community richness was higher in G than in C turbid microcosms at the beginning of the assay (0.4 h); however, this difference was not observed between clear microcosms. It is possible that during the stabilization period prior to the assay, turbid replicates evolved differently resulting in the differences observed. Despite this, the effect of IPA on bacterial community structure on both scenarios after a 192 h exposure was clear, as evidence by NMDS and ANOSIM analyses.

Most of the successful OTU sequencing reactions belonged to the clear microcosms. A high percentage of bands from samples of the turbid system were superposed and therefore they were discarded from the analyses. Most of the OTUs that were favored by glyphosate exposure affiliated to Alphaproteobacteria. Indeed, Alpha-, after Gammaproteobacteria, is the main group containing species with identified strategies of tolerance or resistance to this agrochemical (Hove-Jensen et al. 2014) and even some Alphaproteobacteria are able to use glyphosate as a phosphorous source (Hove-Jensen et al. 2014, Zhan et al. 2018). Another sequenced OTU that increased in the presence of glyphosate affiliated to *Arthrobacter* sp. (Actinobacteria), a group with a strain that was reported to be capable of degrading glyphosate (Pipke and Amrhein 1988). Band sequencing results from DGGE should be taken with caution, because some methodological biases in affiliation procedures have been described (Pesce et al., 2008). Despite this, the results presented herein supports our hypothesis that glyphosate alters heterotrophic picoplankton structure and that bacterial groups found to be resistant/tolerant in single species assays are favored when analyzed in

assemblages. Moreover, these findings suggest that glyphosate should exert a direct effect on bacterial composition. This do not exclude potential indirect effects previously discussed. In any case, glyphosate induced changes in picoplankton structure that may have consequences in other components of the food webs.

The approach used in this study involved the analysis of glyphosate effects on natural picoplanktonic communities from outdoor ponds through an 8-day indoor microcosm assay controlling most abiotic and biotic factors. Although the confinement and the reduced water volume can change the picoplankton structure and function (Calvo-Díaz et al. 2011), herein the main structure of the cytometric groups found in outdoor ponds remained unaltered in the microcosms. Furthermore, and as previously mentioned, the differences in relative abundances of the cytometric populations between the analyzed clear and turbid systems were consistent with those observed in natural scenarios (Stomp et al., 2007; Navarro et al., 2009; Harry et al., 2016; Pradeep Ram et al., 2016; Schiaffino et al., 2013). From this initial characterization changes in the structure of cytometric groups because of IPA exposure were evidenced. We also analyzed glyphosate consequences over the structure of bacterial dominant OTUs through DGGE. Today, new generation sequence technology gives more powerful tools to analyze picoplanktonic communities, because it includes less abundant or rare microorganisms. Nevertheless, a recent study of biogeographical patterns of microbial eukaryote communities from different lakes reported similar tendencies for richness comparisons and for lake clustering based on the dominant OTUs obtained by DGGE or the more

abundant OTUs obtained by Illumina (Schiaffino et al. 2016). This finding suggests that DGGE is useful to evaluate changes in more abundant OTUs and that it might be sufficient for general evaluations of changes in the structure of bacterial communities.

In summary, in this work we used a microcosms approach and applied relatively simple and inexpensive techniques to evaluate changes in the structure of picoplanktonic communities upon IPA exposure. We corroborated our hypotheses regarding 1 -changes in the relative abundances of different cytometric populations from both autotrophic and heterotrophic picoplankton fractions, 2- differences in IPA impact on the picoplankton from turbid and clear systems and 3- the presence of bacteria potentially tolerant/resistant to glyphosate in a community analysis. Furthermore, to our knowledge, we reported for the first time a drastic negative effect of glyphosate on two PC-rich Pcy cytometric populations and an increase of HNA/LNA ratio, as well as changes in size/morphology of cytometric heterotrophic groups.

The results presented herein provided new information about glyphosate impact on the picoplanktonic fraction of freshwater ecosystems and the techniques applied can be used in major scale experiments to further explore glyphosate impact through studies like risk assessment, monitoring or early alert systems.

Author statement

All authors listed in the manuscript contributed to this paper. Sabio y García C. conceived and designed the experiments, performed the experiments, analyzed and interpreted the results and prepared the manuscript. Pizarro H. conceived and designed the experiments, analyzed and interpreted the data; contributed reagents, materials, analysis tools or data and prepared the manuscript; Izaguirre I. collaborated in analysis and interpretation of molecular and cytometric results and manuscript preparation; Schiaffino R. participated in molecular procedures, analysis and interpretation of the results and manuscript preparation; Lozano V. collaborated in microcosm preparation and sample collection; Ferraro M. collaborated in

molecular procedures and Vera S. collaborated in statistical analysis and manuscript preparation.

Declarations of interest: none.

Conflict of interestings statement

All authors listed in the manuscript certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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FIGURES

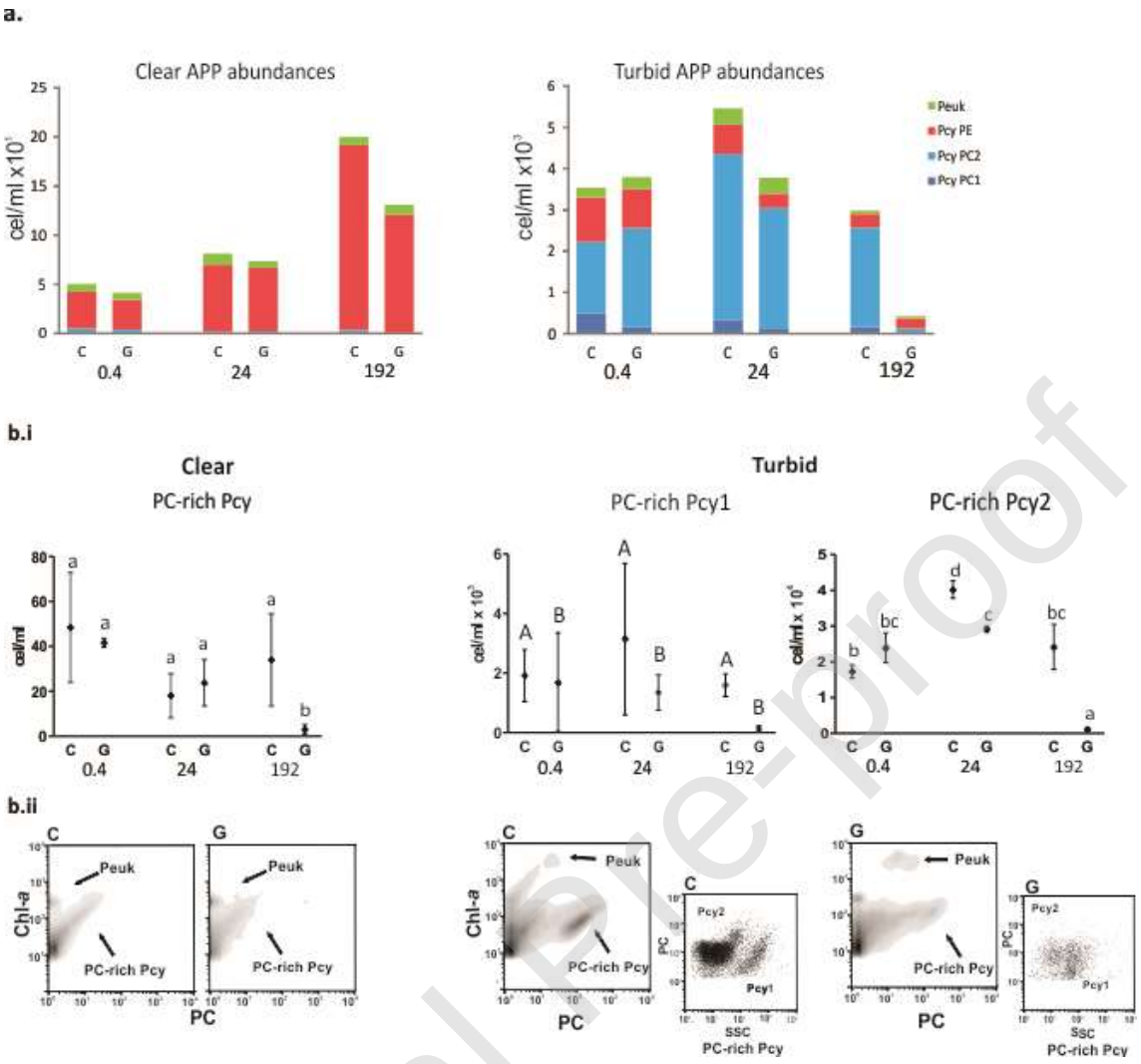

Figure 1. Glyphosate effects on autotrophic cytometric populations from control (C) and IPA (G) treated clear and turbid microcosms. **a**) Mean relative abundances of autotrophic picoplankton (APP) cytometric groups after 0.4 h, 24 h and 192 h of incubation. **b.i)** Scatter plots of mean (±SD) abundance of phycocyanin (PC)-rich Picocyanobacteria (Pcy) cytometric groups after 0.4 h, 24 h and 192 h of incubation; and **b.ii)** representative density cytograms of PC-rich Pcy populations of C and G microcosms at 192 h of incubation, from clear and turbid scenarios (left and right, respectively). Dot plots obtained from a gating of PC-rich Pcy to visualize PC-rich Pcy1 and PC-rich Pcy2 separately

were included for turbid microcosms. Lowercase letters indicate significant differences ($p < 0.05$) in Tukey´s contrasts for *post-hoc* multiple comparisons of means when treatment-time interaction effect was significant ($p < 0.05$); whereas uppercase letters indicate significant differences ($p < 0.05$) between C and G because of IPA simple effect in PC-rich Pcy1 (no interaction effect). Chl-*a*: Chlorophyll *a*.

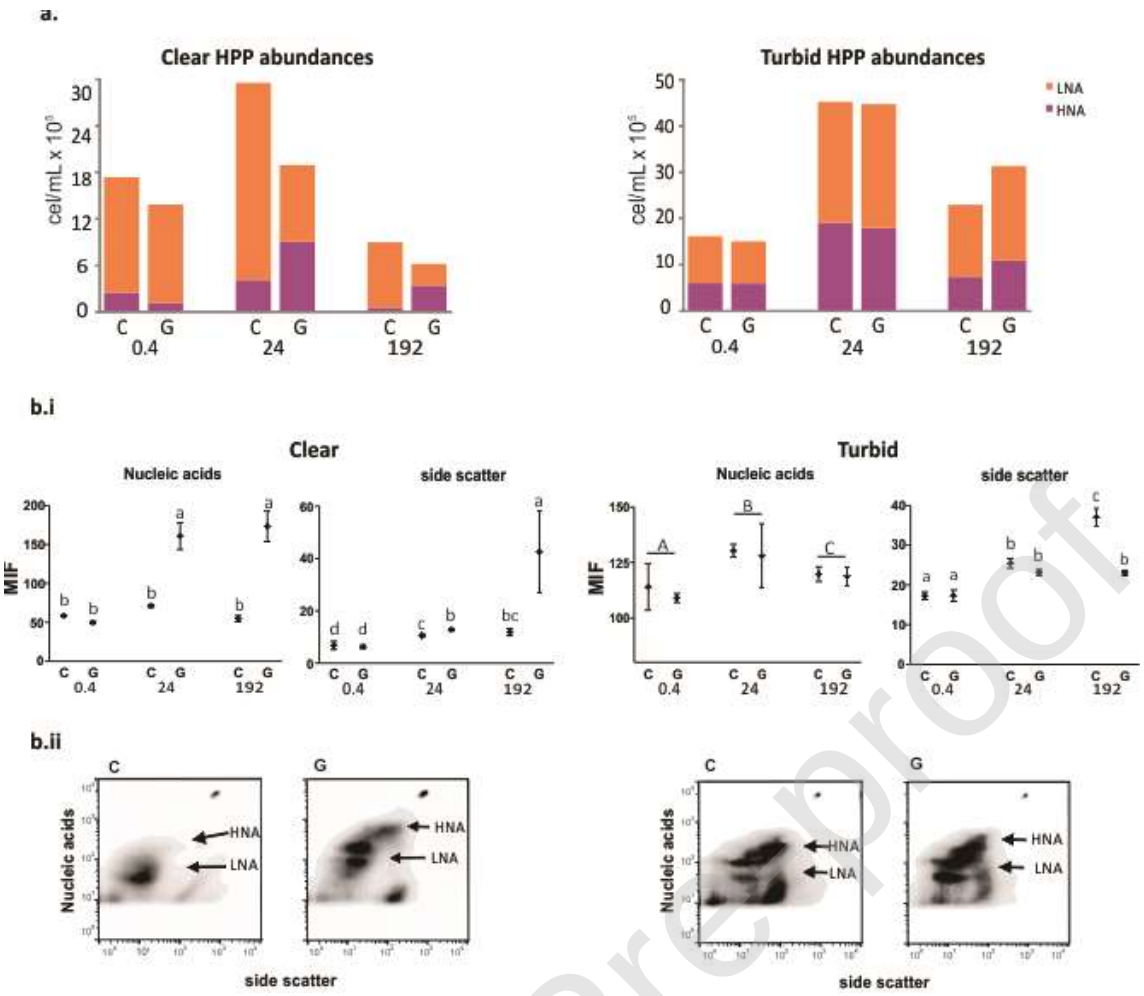

Figure 2. Glyphosate effects on HPP cytometric populations from control (C) and IPA (G) treated clear and turbid microcosms. **a)** Relative abundances of heterotrophic picoplankton (HPP) cytometric groups after 0.4 h, 24 h and 192 h of incubation. **b.i)** Scatter plots of mean intensity of fluorescence (MIF) (± SD) of nucleic acids SYBR®green stained (nucleic acids), and side scatter signal of HPP after 0.4 h, 24 h and 192 h of incubation. **b.ii)** Representative density cytograms showing high and low nucleic acid content cytometric groups (HNA and LNA, respectively) from C and G clear and turbid (left and right, respectively) microcosms at 192 h of incubation. Lowercase letters indicate significant differences ($p < 0.05$) in Tukey´s contrasts for *post-hoc* multiple comparisons of means when treatment-time interaction effect was significant

(p< 0.05); whereas uppercase letters indicate significant differences (p< 0.05) between times simple effect (no interaction effect).

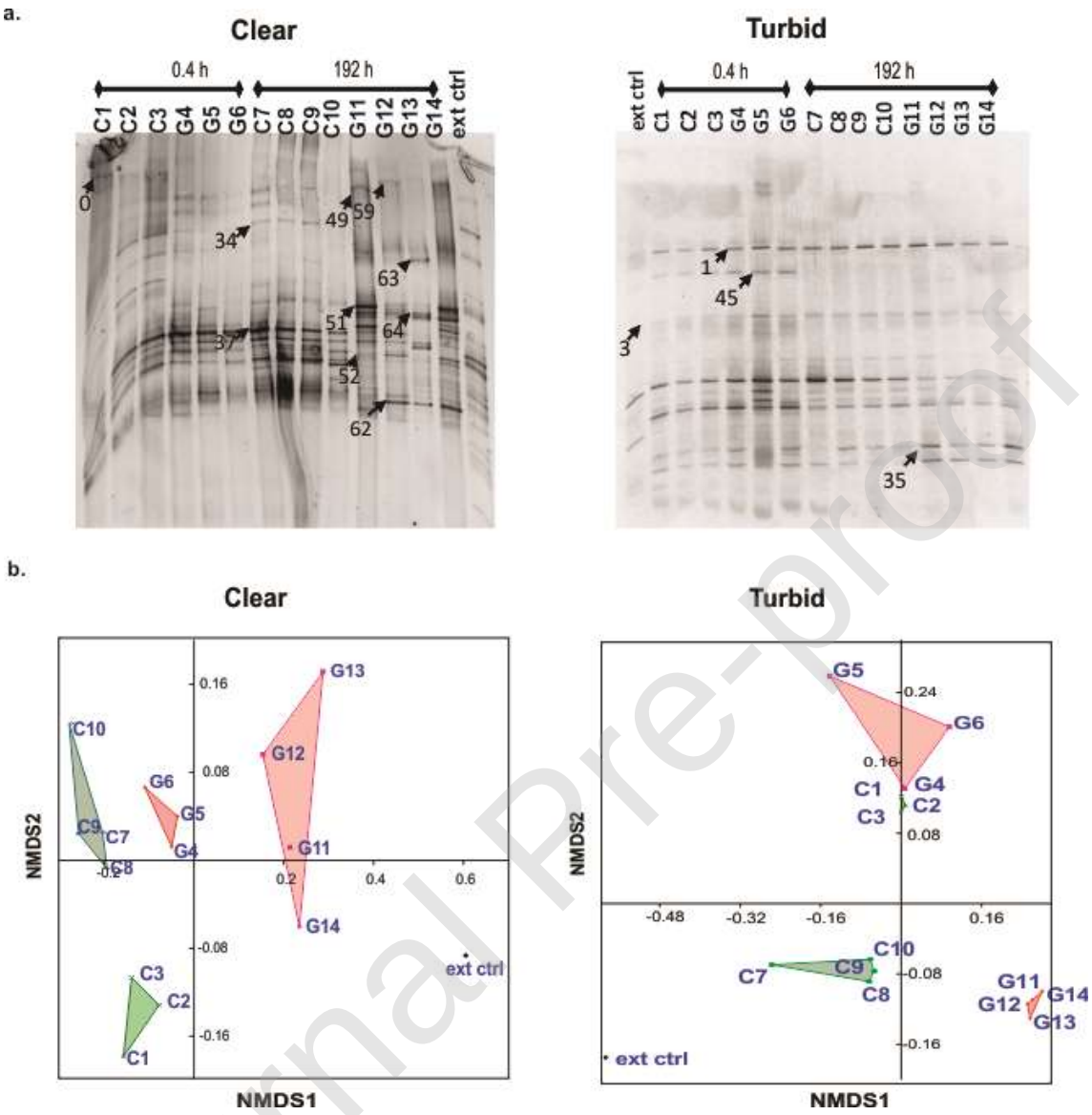

Figure 3. Glyphosate effects on dominant Operational Taxonomic Units (OTUs) from clear and turbid microcosms**. a)** DGGE of 16S rDNA band profiles at 0.4 and 192 h in control (C) and glyphosate exposed (G) microcosms. C1 to C3 and G4 to G6: C and G samples replicates at 0.4 h; C7 to C10 and G11 to G14: C and G samples replicates at 192 h; ext ctrl: non-related sample used as external controls. **b**) NMDS analyses performed with the DGGE band relative intensity profiles using Bray-Curtis similarity index. Green and red colors

correspond to control and glyphosate treated samples, respectively. Stress value: 0.1053 for clear and 0.108 for turbid microcosms.

TABLES

Table 1. **Physical and chemical variables of the outdoor freshwater artificial ponds**.

System Typology	Turbidity (NTU)	$Kd_{15/9}$ (m^{-1})	Conductivity (µS cm^{-1})	DO (mg L^{-1})	pH	Chl-*a* (µg L^{-1})	SRP (mg L^{-1})	TP (mg L^{-1})
CLEAR	1	0.516	106.5	9.6	9.0	3.48 (±1.15)	0.04 (± 0.01)	0.22 (± 0.13)
TURBID	48	3.919	59.8	10.1	9.4	105.96 (±15.3) *	0.126 (± 0.04) *	0.52 (± 0.06) *

Soluble reactive phosphorus (SRP), total phosphorus (TP) and chlorophyll-*a* (Chl-*a*) concentrations were measured in triplicate. Values are expressed as mean (±SD) and significant differences between both systems are expressed with an *($p<0.05$). NTU: nephelometric turbidity; DO: dissolved oxygen.

Table 2. Mean values (± SD) of autotrophic picoplankton (APP) and heterotrophic picoplankton (HPP) abundances in microcosms of clear and turbid systems

a. Clear Microcosms

	APP			HPP	
hours	**C**	G	hours	**C**	**G**
0.4	$0.99x10^{3} \pm 0.39x10^{3}$ a	$1.13x10^{3} \pm 0.04x10^{3}$ a	**0.4**	$1.8x10^{6} \pm 0.40x10^{6}$ cd	$1.49x10^{6} \pm 0.55x10^{6}$ bc
24	$1.36x10^{3} \pm 0.10x10^{3}$ a	$0.8x10^{3} \pm 0.35x10^{3}$ a	**24**	$3.26x10^{6} \pm 0.16x10^{6}$ e	$2.26x10^{6} \pm 0.17x10^{6}$ d
192	$2.1x10^{3} \pm 0.7x10^{3}$ a	$1.35x10^{3} \pm 0.5x10^{3}$ a	**192**	$1.04x10^{6} \pm 0.15x10^{6}$ ab	$0.86x10^{6} \pm 0.22x10^{6}$ a

b. Turbid Microcosms

	APP			HPP	
hours	**C**	**G**	hours	**C**	**G**
0.4	$3.13x10^{4} \pm 0.58x10^{4}$ b	$3.85x10^{4} \pm 0.43x10^{4}$ ab	**0.4**	$1.75x10^{6} \pm 0.12x10^{6}$ a	$1.55x10^{6} \pm 0.06x10^{6}$ a
24	$5.56x10^{4} \pm 1.25x10^{4}$ a	$3.84x10^{4} \pm 0.42x10^{4}$ c	**24**	$4.94x10^{6} \pm 0.46x10^{6}$ c	$4.77x10^{6} \pm 0.20x10^{6}$ c
192	$3.03x10^{4} \pm 0.62x10^{4}$ b	$0.46x10^{4} \pm 0.17x10^{4}$ a	**192**	$2.37x10^{6} \pm 0.22x10^{6}$ ab	$3.32x10^{6} \pm 0.80x10^{6}$ b

Autotrophic and heterotrophic picoplankton abundances (cells mL^{-1}) in clear (a) and turbid (b) microcosms at 0.4 h, 24 h and 192 h after IPA application for control (C) and glyphosate (G) treatments. Significant differences ($p< 0.05$) in Tukey´s contrasts for *post-hoc* multiple comparisons of means of HPP or APP abundances within each system are expressed by different lowercase letters.

Table 3. Closest sequence affiliation of 16S rRNA gene fragment from bands excised from the DGGE

clone	sample	closest sequence affiliation	Taxon	% similarity	Glyphosate effect on relative abundance
0	clear	*Flavobacterium*	Bacteroidetes (Flavobacteria)	98	decreased 0.4 h post application
60	clear	Sphingomonadaceae	Proteobacteria (Alphaproteobacteria)	99	increased 192 h post application
64	clear	Sphingomonadaceae	Proteobacteria (Alphaproteobacteria)	99	increased 192 h post application
49	clear	*Flavobacterium*	Bacteroidetes (Flavobacteria)	98	no effect
59	clear	*Flavobacterium*	Bacteroidetes (Flavobacteria)	97	no effect
52	clear	Sphingomonadaceae	Proteobacteria (Alphaproteobacteria)	99	increased 192 h post application
51	clear	*Legionella*	Proteobacteria (Gammaproteobacteria)	95	increased 192 h post application (in 2 of 4 microcosms)
63	clear	*Chryseobacterium*	Bacteroidetes (Flavobacteria)	99	increased 192 h post application (in 2 of 4 microcosms)
62	clear	*Arthrobacter*	Actinobacteria	99	increased 192 h post application
37	clear	*Polynucleobacter*	Proteobacteria (Betaproteobacteria)	99	decreased 192 h post application
34	clear	*Sediminibacterium*	Bacteroidetes (Sphingobacteriia)	96	decreased 192 h post application
45	turbid	Chitinophagaceae	Bacteroidetes (Sphingobacteriia)	93	Increased 0.4 h post application
35	turbid	Rhodobacteraceae	Proteobacteria (Alphaproteobacteria)	92	increased 192 h post application
1	turbid	*Sediminibacterium* sp	Bacteroidetes (Sphingobacteriia)	97	no effect
3	turbid	*Sediminibacterium*	Bacteroidetes (Sphingobacteriia)	97	no effect