1 **Freshwater dissolved oxygen dynamics: changes due to glyphosate, 2,4-D and**

2 **their mixture, both under clear and turbid-organic conditions.**

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18

19 **Abstract**

20 We performed two independent outdoor mesocosm experiments where we measured the

21 variation of DO saturation (DO%) in freshwater after a single input of Roundup Max® (G)

22 (glyphosate-based formulation), AsiMax 50® (2,4-D) (2,4-D-based formulation) and their

23 mixture (M). Two concentration levels were tested; 0.3 mg/L G and 0.135 mg/L 2,4-D

24 (Low; L) and 3 mg/L G and 1.35 mg/L 2,4-D (High; H). We assayed consolidated microbial

25 communities coming from a system in organic turbid eutrophic status and a system in

26 clear mesotrophic status during 21 and 23 days, respectively. A sample of phytoplankton

27 (micro+nano, pico-eukaryotes, pico-cyanobacteria), mixotrophic algae and heterotrophic

28   bacteria was collected to determine abundances at each of four sampling dates. The clear

29   and turbid systems showed similar, but not synchronized, patterns of daily DO% changes

30   in relation to the controls (DO%$_v$), after exposure to both single and combined

31   formulations. Under glyphosate scenarios (GL, GH, ML and MH), the two types of systems

32   showed similar DO%$_v$ but different microbial abundances, being associated to an increase

33   in the micro+nano and pico-eukaryotic phytoplankton fractions for the clear system. In

34   contrast, in the turbid system changes were associated with increased pico-cyanobacteria

35   and decreased mixotrophic algae. Effects of 2,4-D were only observed in the turbid

36   system, leading to decreased micro+nano phytoplankton abundances. Under the turbid

37   scenario, the herbicide mixture at high concentration had a synergistic effect on DO%$_v$

38   and recovery was not detected by the end of the experiment. Our results revealed that

39   herbicides inputs induced changes in phytoplankton abundances that leads to measurable

40   DO variations.

41   **Keywords:** dissolved oxygen, glyphosate, 2,4-D, herbicide mixtures, microbial

42   communities, freshwater.

43

44   **1. Introduction**

45   In aquatic systems, dissolved oxygen (DO) integrates the dynamics of physical,

46   chemical and biological variables, and its concentration results from the balance of several

47   processes. With regard to biological processes, DO results from the balance between

48   photosynthesis and respiration. Photosynthesis by autotrophic freshwater communities

49   (eg., phytoplankton, attached microbial communities and macrophytes) is a major source

50   of oxygen for all living organisms, while oxygen is consumed by the respiration of free and

51   attached bacteria, phyto and zooplankton, attached auto- and heterotrophic communities,

52 macroinvertebrates and vertebrates. Moreover, DO concentration in freshwater systems

53 may be affected by biogeochemical processes such as mineralization of allochthonous

54 organic matter and nitrification. For these reasons, DO has been fully considered as an

55 ecological indicator.

56 Therefore, DO monitoring in natural freshwater systems is a useful tool for different

57 applications, including management, conservation or restoration.

58 The techniques for measuring DO have evolved from chemical reactions (e.g.,

59 Winkler method) to the use of microelectrodes and optical sensors. The latter are small,

60 cheap and have long-term stability (Klimant et al., 1995). Currently, monitoring of DO in

61 aquatic systems, both *in situ* and under laboratory conditions, is easy providing instant

62 results (Warkentin et al., 2007).

63 In Argentina, about 100,000 shallow lakes are located in the Pampa plain, one of the

64 largest and most productive agroecosystems worldwide (Dukatz et al., 2006). Most of

65 these shallow lakes alternate between two contrasting limnological states: the clear state,

66 where the primary productivity is mainly driven by submerged macrophytes; and the turbid

67 state, which shows prevalence of phytoplanktonic productivity (Allende et al., 2009). Some

68 studies have provided evidence that shallow lakes remain turbid due to agrochemical

69 contamination, caused by fertilizers (Quirós et al., 2002) and herbicides such as

70 glyphosate (Vera et al., 2010, Castro Berman et al., 2018).

71 There was an increase in agrochemical-based agriculture between 1995 and 2015

72 worldwide, which was accompanied by an increase in the use of insecticides and

73 herbicides of about 3.5 and 4.4 times, respectively (FAOSTAT, 2017). Scientific results

74 show that agrochemicals exert direct and indirect toxic effects on freshwater organisms

75 living in agricultural landscapes (Lampert et al., 1989; Fleeger et al., 2003). The

76 ecosystem approach is broadly accepted as the most realistic framework to investigate

77 the impact of anthropogenic disturbances on freshwater organisms, while DO has

78 historically been used as a reliable indicator of water quality. In this regard, King et al.

79 (2015) pointed out that DO is particularly sensitive to herbicide contamination in water.

80 In Argentina, glyphosate (N-phosphonomethylglycine) and 2,4-D (2,4-

81 dichlorophenoxyacetic acid) are the most widely used herbicides. They show different

82 modes of action. Glyphosate is a reversible inhibitor of EPSP (5-enolpyruvylshikimate-3-

83 phosphate) synthase, a key enzyme in the synthesis of aromatic amino acids in plants,

84 algae, bacteria and fungi (Pollegioni et al., 2011). On the side, 2,4-D is an auxin-type

85 herbicide, which causes the death of dicotyledonous by inducing overgrowth of vascular

86 cambium (Song, 2014). The appearance of glyphosate-resistant weeds due to its

87 continued extensive use prompted farmers to apply mixtures as a control option. An

88 experimental laboratory study reported that the mixture of these herbicides has a

89 synergistic effect on freshwater cyanobacteria (Lozano et al., 2018). However, the

90 environmental implications of their combined application have been poorly investigated.

91 The aim of the present research was to study the daily changes in dissolved oxygen

92 saturation percentage (DO%) resulting from the impact of Roundup Max®, (a glyphosate-

93 based herbicide formulation) and AsiMax 50® (a 2,4-D-based herbicide formulation), alone

94 and in mixture, on two freshwater systems of different turbidity. We carried out two outdoor

95 mesocosm experiments to analyze the daily DO% variation with respect to control (DO%v)

96 in water, and the difference in DO%$_v$ between a single dose of Roundup Max®, AsiMax

97 50® or their mixture with respect to the controls, under different turbidity conditions, i.e.

98 clear and organic turbid. High and low concentrations of each herbicide and their

99 respective mixtures were assayed to assess a hypothetical dose-dependence effect. A

100   sample was obtained on each of four occasions during the experiment to determine the

101   abundance of the following communities: micro+nano phytoplankton (>2 µm, M+N);

102   photosynthetic picoplankton (PPP, 0.2-2 µm), which includes prokaryotic

103   picocyanobacteria (Pcy) and eukaryotic phototrophs (PEuk); and heterotrophic

104   bacterioplankton (HB).

105   Algae and cyanobacteria are possibly sensitive to herbicide contamination because

106   they share several metabolic pathways with vascular plants (Ferrari et al., 2018). In

107   addition, we may assume that glyphosate and 2,4-D have different mode of action but

108   similar toxicological endpoint. Then, it is reasonable to predict that their combination will

109   show additive effects, as postulated by Faust et al. (2001). On the other hand,

110   photosynthetic microbial communities are more abundant in organic turbid than in clear

111   systems. Based on these considerations, we tested the following hypotheses:

112   1. Daily $DO\%_v$ pattern are similar for the organic turbid system and for the clear

113   one after exposure to Roundup Max[®] and AsiMax 50[®], alone and in mixture.

114   2. The impact of Roundup Max[®] and AsiMax 50[®] on the daily pattern of $DO\%_v$ are

115   similar.

116   3. The effect of the mixture of Roundup Max[®] and AsiMax 50[®] on the daily pattern

117   of $DO\%_v$ is similar to the sum of the effects of the herbicides acting

118   independently (additive effect).

119   4. The impact of the herbicides on the dynamics of daily $DO\%_v$ is higher at high

120   doses than at low doses.

121   5. $DO\%_v$ pattern resulting from the impact of Roundup Max[®], AsiMax 50[®] and their

122   mixture may be explained by phytoplanktonic abundance variations.

123

2. Material and methods

2.1. Experimental design

124

125

126 Natural freshwater systems in the Pampean plain of Argentina are exposed to an

127 intensive herbicide spraying and the possibility of contamination cannot be ruled out.

128 Therefore, to obtain microbial communities without previous exposition, we established

129 freshwater systems through colonization in outdoor tanks of 3,000 L, allowing them to

130 evolve for two years. This decision was taken for avoiding the use of natural lake water

131 where possible previous contamination is impossible to be guaranteed. We performed two

132 outdoor mesocosm experiments using these consolidated microbial communities coming

133 from systems developed in two tanks: (a) a system in organic, turbid eutrophic status

134 (hereafter referred to as turbid) with chlorophyll a concentration = 31.7 μg/L and turbidity

135 = 12 NTU; and; (b) a system in clear mesotrophic status (hereafter referred to as clear)

136 with chlorophyll a concentration = 8.7 μg/L and turbidity = 2 NTU. The turbid experiment

137 was carried out 21 d in late spring, while the clear one was performed 23 d in late summer,

138 using a similar experimental design. Experimental units (EUs) were 40-45-L plastic bags

139 incubated in three 3,000-L outdoor tanks located in the experimental campus at the

140 University of Buenos Aires. The EUs of the first experiment were filled with turbid water

141 and the EUs of the second one with clear water, both of which were taken from the natural

142 colonized systems. Prior to the assays, the EUs were subjected to a 3-day stabilization

143 period and randomly assigned to one of the following treatments: Control (C); a single

144 dose of Roundup Max® (glyphosate-based formulation, active ingredient, a.i.: G) at low

145 (0.3 mg/L G) and high (3 mg/L G) concentrations; a single dose of AsiMax 50® (2,4-D-

146 based formulation, a.i.: 2,4-D) at low (0.135 mg/L 2,4-D) and high (1.35 mg/L 2,4-D)

147 concentrations; and a herbicide mixture at two concentrations with the same ratio of active

148 ingredients (i.e. 1 G : 0.45 2,4-D), namely a low mixture concentration of 0.3 mg/L G +

149 0.135 mg/L 2,4-D and a high mixture concentration of 3 mg/L G + 1.35 mg/L 2,4-D).

150 Hereafter, these treatments will be referred to as Control, GL, GH, 2,4-DL, 2,4-DH, ML

151 and MH, respectively. Treatments were realized by simple overthrow andperformed in

152 triplicate, except for Control, which was replicated six times (two per tank). The summary

153 of the experimental design is shown in Fig. 1.

Fig. 1: Scheme of the experimental design of clear and turbid assays. Treatments are listed on the left and variables on the right. GL: glyphosate low concentration, GH: glyphosate high concentration, 2,4-DL: 2,4-D low concentration, 2,4-DH: 2,4-D high concentration, ML: mixture low concentration, MH: mixture high concentration, M+N: micro+nano phytoplankton, PPP: photosynthetic picoplankton, PEuk: eukaryotic phototrophs, Pcy: prokaryotic picocyanobacteria, Mixo: mixotrophic micro+nano plankton, HB: heterotrophic bacteria. Black circle: mesocosm.

2.2.   Variables measured in clear and turbid experiments:

2.2.1. Environmental variables

164 Daily maximum and minimum data of air temperature were determined from the

165 National Weather Service of Argentina (Servicio Metereológico Nacional, SMN), using

166 available data from a station at less than 2 km away from the experimental campus.

167 Values were used to calculate daily mean air temperature (T°C) defined as ($T_{maximum}$ +

168   $T_{minimum}$)/2. An *ad-hoc* device of the same shape and dimensions as those of an EU was

169   placed near the tanks during the whole study periods to measure rainfall volume (in mL).

170   *2.2.2. Chemical variables*

171   The concentration of DO and DO% was monitored daily at 8:30 AM with an optical

172   dissolved oxygen meter (YSI® ProODO™; error = 0.01 mg/L) by immersing the probe to

173   a depth of 10 cm in the center of each EU. Water temperature was continuously monitored

174   using thermo buttons (Akribis®) immersed in each EU. For herbicides analysis, 50 mL of

175   water was taken from the center of each EU with plastic flasks and kept at -20°C.

176   Glyphosate was determined by ELISA Microtiter Plate test (Abraxis®) and 2,4-D by HPLC-

177   UV (APHA, 2005; U.S.EPA, 1980) on days 0, 1, 7 and 21 in the turbid experiment and on

178   days 0, 1, 7 and 23 in the clear experiment. Half-lives were calculated considering that

179   glyphosate and 2,4-D residue adjusted to a logarithmic function assuming a first-order

180   kinetic.

181

182   *2.2.3. Biological variables*

183   *2.2.3.1. M+N phytoplankton abundances*

184   Total abundances of live micro (>20 μm) and nano   (2–20   μm)

185   phytoplankton (M+N) and mixotrophic micro + nanophytoplankton (Mixo) fractions were

186   analyzed with inverted microscope. For this purpose, 200 mL of water was sampled from

187   each EU at T0 (immediately after treatment application), T1 (1 d), T2 (7 d) and TF (21 and

188   23 d). Samples were fixed with 1% acidified Lugol´s iodine solution for algal and

189   cyanobacteria quantification (> 2 μm) following the technique of Utermöhl (1958). In

190   general, Bourrelly's systematic scheme (1970) was followed for taxonomic identification.

191 *Peridinium* sp. and *Ochromonas* sp. were considered to be mixotrophic organisms

192 (Andersson et al., 1989, Palsson & Granéli, 2004)

193

194 *2.2.3.2. PPP and HB abundances*

195 Total abundances of photosynthetic picoplankton (PPP) (0.2–2 µm), which includes

196 picocyanobacteria (Pcy) and eukaryotic phototrophs (PEuk), and heterotrophic bacteria

197 (HB), were obtained by flow cytometry. A FACSAria II (Becton Dickinson®) flow cytometer

198 equipped with a standard 15 mW blue argon-ion (488 nm emission) laser and a red laser

199 diode (635 nm) was used. Samples (3.6 mL) were taken on the same sampling dates as

200 the M+N and Mixo samples. They were fixed with 10% cold glutaraldehyde (1% final

201 concentration), left in the dark for 10 min at room temperature and then stored at -80°C.

202 A known volume of beads (1-µm diameter, Fluospheres®) was added to all unfrozen

203 samples. PPP were identified in plots of SSC (Side Scatter) versus blue laser-dependent

204 red fluorescence (PerCP or FL3, 670 nm), orange fluorescence (PE or FL2 585/42 nm)

205 versus FL3, and red laser-dependent far-red fluorescence (APC or FL4, 661 nm) versus

206 FL3. For the HB determination, 400 µL-samples were previously stained with SYBRGreen

207 I (Sigma-Aldrich®) diluted in DMSO to a final concentration of 1X, left for about 10 min in

208 the dark to complete the nucleic-acid staining and run in the flow cytometer. Abundances

209 were calculated by their signature in plots of side scatter light (SSC) versus green

210 fluorescence of nucleic acid-bound stains FITC (FL1 530 nm) and SSC (side scatter) axes

211 (Gasol et al. 1999).

212

213 *2.3. Statistical and numerical analysis*

214   A *t*-test was used to compare between the real values of G and 2,4-D for each

215   treatment at T0.

216   Given that DO depends on temperature and that both experiments were carried out

217   at different times of the year, we evaluated the effect of herbicides on the daily DO

218   percentage of saturation variation ($DO\%_v$), calculated as $DO\%_v = DO\%_x - DO\%_{control}$,

219   where $DO\%_x$ is the value obtained from each treatment with herbicide (x). Then, $DO\%_v$

220   can attain positive or negative values. Differences in $DO\%_v$ among the seven treatments

221   over time were analyzed with one-way RM-ANOVA with *treatment* as the factor with 7

222   levels (GL, 2,4-DL, ML, GH, 2,4-DH, MH and C) and 21 (turbid experiment) and 23 (clear

223   experiment) measurement days. Statistical significance was set at $p<0.05$, considering

224   periods of at least 3 days showing significant differences compared with the controls. The

225   three tanks served as replicates.

226   A one-way RM-ANOVA was performed to test possible additive effects on $DO\%_v$,

227   with *mixture* as the factor with 2 levels: observed and expected and 21 (turbid experiment)

228   and 23 (clear experiment) measurement days, for both clear and turbid experiments.

229   Expected values were obtained by summing the values of $DO\%_v$ measured for each

230   herbicide separately. When the difference between the observed and expected $DO\%_v$ was

231   significant, the interaction was synergistic if the observed mean $DO\%v$ concentration was

232   higher than the expected mean $DO\%_v$ concentration; otherwise, the interaction was

233   antagonistic (Lozano et al., 2018).

234   A one-way RM-ANOVA was conducted for M+N, Mixo, as well as for PPP and HB

235   abundances with *treatment* as the factor with 7 levels (GL, 2,4-DL, ML, GH, 2,4-DH, MH

236   and C) and 21 (turbid experiment) and 23 (clear experiment) sampling days. All data were

237 previously analyzed for normality and homocedasticity using the Shapiro-Wilks and

238 Levene tests, respectively, and log-transformed if appropriate.

239 Pearson correlation matrix was calculated among the abundance values of the

240 autotrophic, mixotrophic and heterotrophic fractions and DO%, for both experiments. With

241 these correlation values, graphical network was built in Cytoscape® v3.7.1 (Shannon et

242 al. 2007). Thickness of lines was set proportionally to the r values obtained while the size

243 of the circles was set proportionally to the mean values of abundance of the different

244 biological fractions from the controls (ind/mL) in each system at the initial time.

245 Statistical analyses were performed using SigmaPlot® 11.0 and InfoStat® software

246 version 2015.

247 3. **Results**

248 *3.1. Environmental variables*

249 As expected for independent experiments made in different moments, there were

250 differences in climatic conditions throughout the study periods, which are shown in Fig. 2.

251 Daily mean air temperature was 21.9°C (min. 15.0 and max. 24.1°C) for the clear

252 experiment and 17.9°C (min. 13.8 and max. 23.2°C) for the turbid one. Rainfall volume in

253 each EU was 7,840 mL (6 rain episodes) for the entire clear experiment and 7,275 mL (6

254 rain episodes) for the turbid one.

255
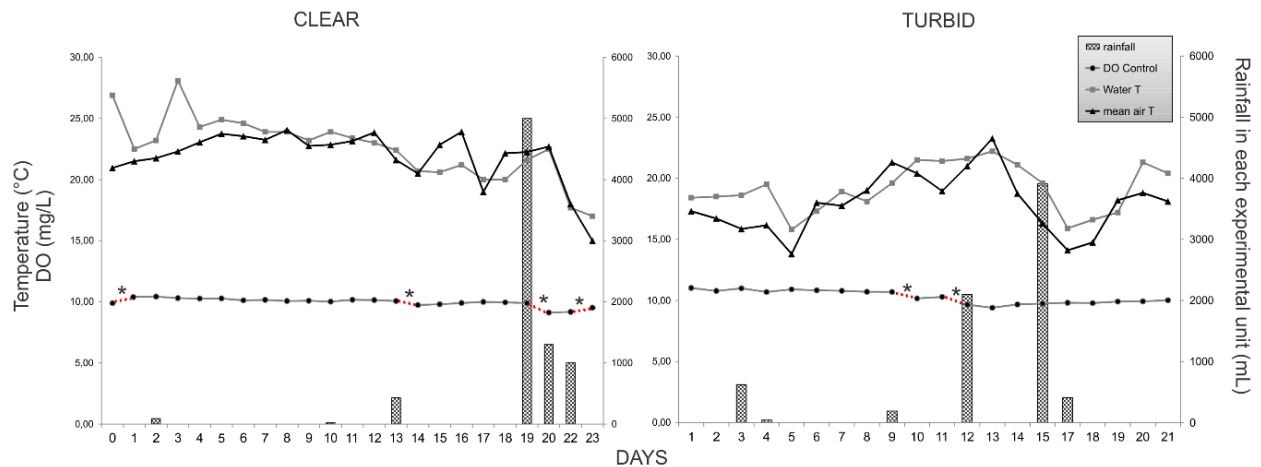
Fig. 2: Mean daily DO concentration calculated from control EUs (n=6) and mean air and water temperature (°C) values and rainfall (mL) accumulated in each EU for the clear and turbid experiments along the study period. Significant differences in DO values (RM-ANOVA, p<0.05) are shown in dotted red lines with asterisk (*).

3.2. Physical and chemical variables

Initial herbicide concentrations were similar to the nominal concentration applied (Table 1); no differences in herbicide concentrations were found between clear and turbid experiments for all treatments at initial time (*t*-test, p>0.1 in all cases). The half-lives of herbicides where 16 and 41 days for glyphosate and 99 and 69 for 2,4-D in the clear and turbid experiments respectively. Water temperature measured in EUs using thermos-buttons showed a mean daily variation between maximum and minimum values of 2.32 ± 0.68 °C in each EU for the clear experiment, and 2.55 ± 0.92 °C for the turbid experiment.

	Nominal (mg/L)		Real (mg/L)			
			CLEAR		**TURBID**	
	G	**2,4-D**	**G**	**2,4-D**	**G**	**2,4-D**
C	0	0	< LOD	< LOD	< LOD	< LOD
GL	0.30	0	0.28 ± 0.06	< LOD	0.36 ± 0.08	< LOD
2,4-DL	0	0.135	< LOD	0.11 ± 0.01	< LOD	0.14 ± 0.01
ML	0.30	0.135	0.25 ± 0.05	0.12 ± 0.01	0.33 ± 0.05	0.11 ± 0.01
GH	3	0	3.09 ± 0.08	< LOD	3.14 ± 0.09	< LOD
2,4-DH	0	1.35	< LOD	1.02 ± 0.07	< LOD	1.28 ± 0.02
MH	3	1.35	2.93 ± 0.33	1.05 ± 0.13	2.82 ± 0.43	1.26 ± 0.08
Half-lives (days)			16	99	41	69

Table 1: Comparison between nominal and mean real values (± 1 SD) of herbicide concentrations and half-lives for both herbicides during the two experiments. LOD: Limit of detection.

Maximum and minimum absolute values of DO concentrations (mg/L) in EUs treated with herbicides ranged between 8.15 mg/L (MH, day 20) and 11.00 mg/L (GH, day 8) for the clear system, and between 8.52 mg/L (MH, day 13) and 12.09 mg/L (GH, day 6) for the turbid one. The percentage of oxygen saturation always exceeded 94% in both water systems. In regard to daily DO concentrations in the controls, the clear system appeared to be more affected by rainfall periods than the turbid one (Fig. 2). The maximum DO%$_v$ was observed in the clear system with a mean increase of +10.30% under the GH treatment, while the minimum percentage was detected in the turbid system with a mean decrease of -9.17% under the MH treatment. For G, the turbid system showed a slight but significant increase in mean DO%$_v$ of +2.60% (±0.44%) at 19, 20 and 21 d under the GL treatment ($p=0.012$, $p=0.002$ and $p<0.001$, respectively), while no increase was observed for the clear system (Fig. 3a and 4a). In the GH treatment under turbid experiment, DO%$_v$ increased between days 3 and 9 with a mean of +6.56% (±1.35%), and between 14 and 21 d with a mean of +3.59% (±0.69%) ($p<0.001$ in all cases, Fig. 4b); a similar increase of +6.33% (±3,10%), was recorded a few days later, between days 7 and 12, under clear

293 conditions (Fig. 3b). The maximum percentage of DO%$_v$ was reached under the GH

294 treatment, with an average increase of +7.92% at day 6 (p<0.001) for the turbid and

295 +10.30% at day 8 (p<0.001) for the clear experiments. Although DO%$_v$ increased similarly

296 in both systems, the impact started earlier and lasted longer under the turbid condition.

297 The addition of 2,4-D to the water did not modify DO%$_v$ in all treatments, except for

298 a decrease observed under the 2,4-DH treatment in the turbid system (p<0.001 for all

299 days), observed between days 2 and 9 with a mean of -5.14% (±0.98%) (Fig. 4d).

300 The mixture of herbicides at low and high doses impacted DO%, under both clear

301 and turbid conditions. Under turbid conditions, DO%$_v$ increased in the ML treatment 3, 4

302 and 5 days after application with a mean of +2.44% (±0.46%) (p<0.001, p=0.02 and

303 p=0.004, respectively) and between days 9 and 15 with a mean value of +3.39 (±0.56%)

304 (p<0.001 for all days except day 12 with p=0.002) (Fig. 4e). Under clear conditions, ML

305 treatment reduced DO in the period of 14 to 20 days (Fig. 3e). In the clear system, MH

306 increased DO%$_v$ by +6.19% (±1.60%) between days 7 and 12 (p<0.001 for all days, except

307 day 12 with p=0.005) (Fig. 3f). In the turbid system, MH increased DO%$_v$ by +2.83%

308 (±0.45%) at days 4-6 (p<0.001) and 7 (p=0.005), followed by a decrease of -5.22%

309 (±2.75%) between days 9 and 14. Thereafter, DO%$_v$ incresd by +4.38% (±1.35%) from

310 day 18 onwards (p<0.001 for all days) (Fig. 4f).

311 At the end of the experiment, the herbicide treatments under the clear condition

312 had DO% values similar to those of the controls, suggesting a recovery of the system. A

313 contrasting result was obtained for the turbid system, where the treatments with

314 glyphosate (GL, GH and MH) showed a net significant increase in DO%$_v$ (Fig. 4a,b,f).

315 Compared with the other glyphosate-related treatments, the MH treatment in turbid

316   experiment trended toward no recovery to control levels of DO$\%_v$ at the end of the study

317   (Fig. 4e).

318   In general, the combined exposure to G and 2,4-D formulations caused additive

319   effects on the examined variables, except for MH in the turbid system, which exhibited

320   significant synergism ($p < 0.05$) between days 9 and 14 (Fig. 4f).
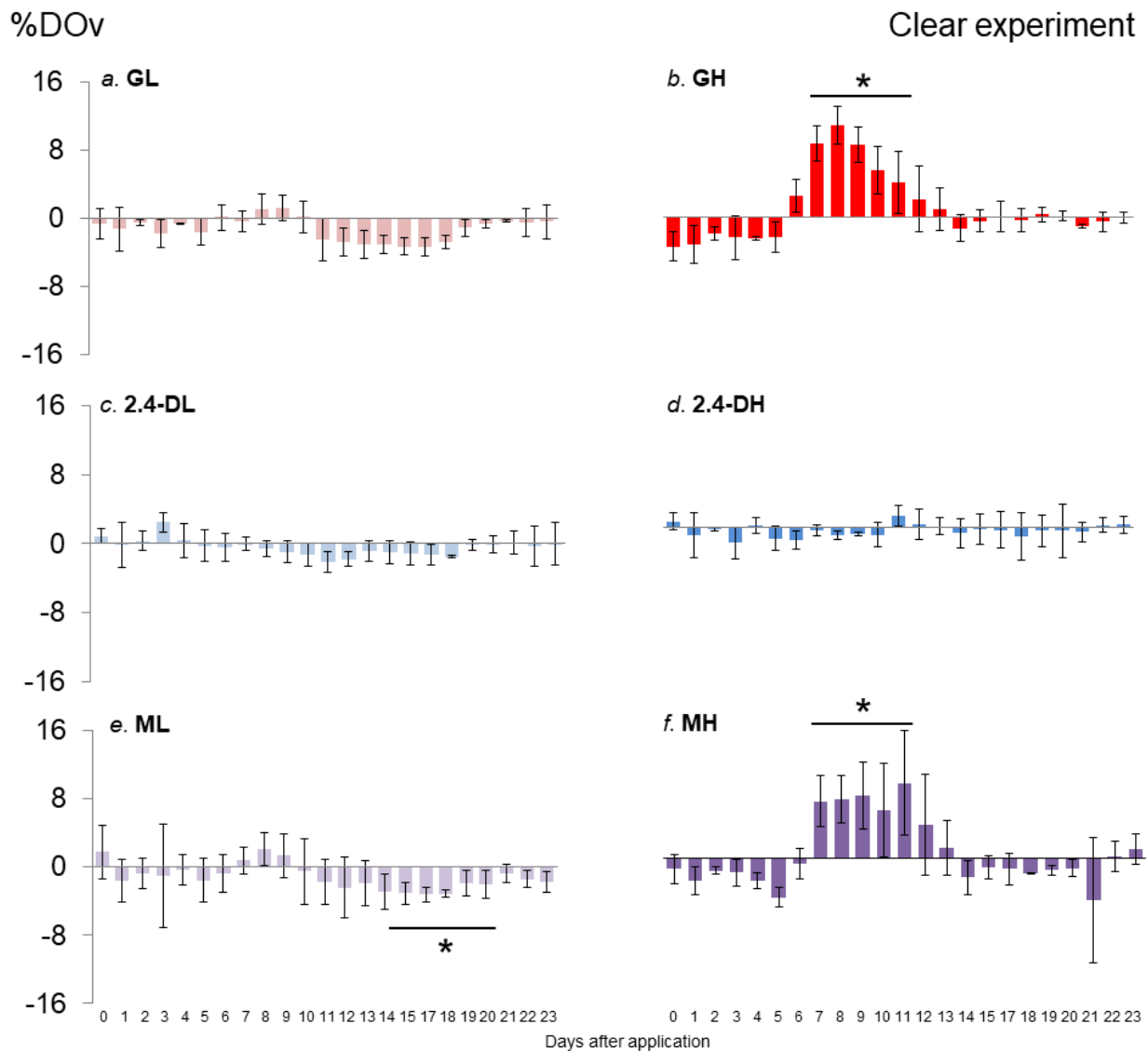
DO means (mg/L)

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	17	18	19	20	21	22	23
CONTROL	9.93	10.41	10.42	10.30	10.26	10.27	10.12	10.16	10.06	10.09	10.02	10.16	10.14	10.06	9.74	9.80	9.90	9.99	9.95	9.89	9.12	9.18	9.51
GL	9.89	10.45	10.38	10.18	10.23	10.12	10.09	10.12	10.13	10.17	10.06	9.95	9.89	9.78	9.45	9.47	9.57	9.70	9.83	9.82	9.08	9.10	9.46
2,4-DL	10.00	10.52	10.44	10.41	10.32	10.23	10.07	10.13	10.03	10.03	9.97	9.94	10.03	9.98	9.64	9.65	9.79	9.84	9.93	9.87	9.12	9.13	9.50
ML	10.07	10.38	10.36	10.19	10.23	10.10	10.03	10.20	10.24	10.19	10.02	9.95	9.94	9.88	**9.48**	**9.48**	**9.58**	**9.68**	**9.76**	**9.70**	9.05	9.01	9.32
GH	9.72	10.28	10.28	10.14	10.08	10.06	10.29	**10.84**	**10.91**	**10.74**	**10.49**	**10.48**	10.31	10.11	9.62	9.73	9.87	9.94	9.96	9.89	9.03	9.11	9.49
2,4-DH	10.07	10.43	10.42	10.18	10.25	10.13	9.98	10.10	9.98	10.00	10.00	10.22	10.14	10.07	9.66	9.73	9.86	9.87	9.92	9.85	9.07	9.17	9.51
MH	9.80	10.29	10.31	10.12	10.01	9.88	10.02	**10.67**	**10.61**	**10.67**	**10.51**	**10.79**	10.45	10.15	9.54	9.67	9.76	9.80	9.81	9.77	8.70	9.16	9.58

321

322 **Fig. 3:** Daily percentage of DO saturation in relation to the controls (DO%$_v$) in the clear experiment after
323 herbicide application (G: Roundup Max®; 2,4-D: AsiMax 50®; M: Roundup Max® + AsiMax 50®; L: low
324 concentration; H: high concentration). Asterisks indicate significant differences with controls (RM-ANOVA,
325 $p < 0.05$). Below: Daily DO mean values (mg/L) for each treatment; in grey and bold: values corresponding
326 to significant differences showed in the graphs below (RM-ANOVA, $p < 0.05$).
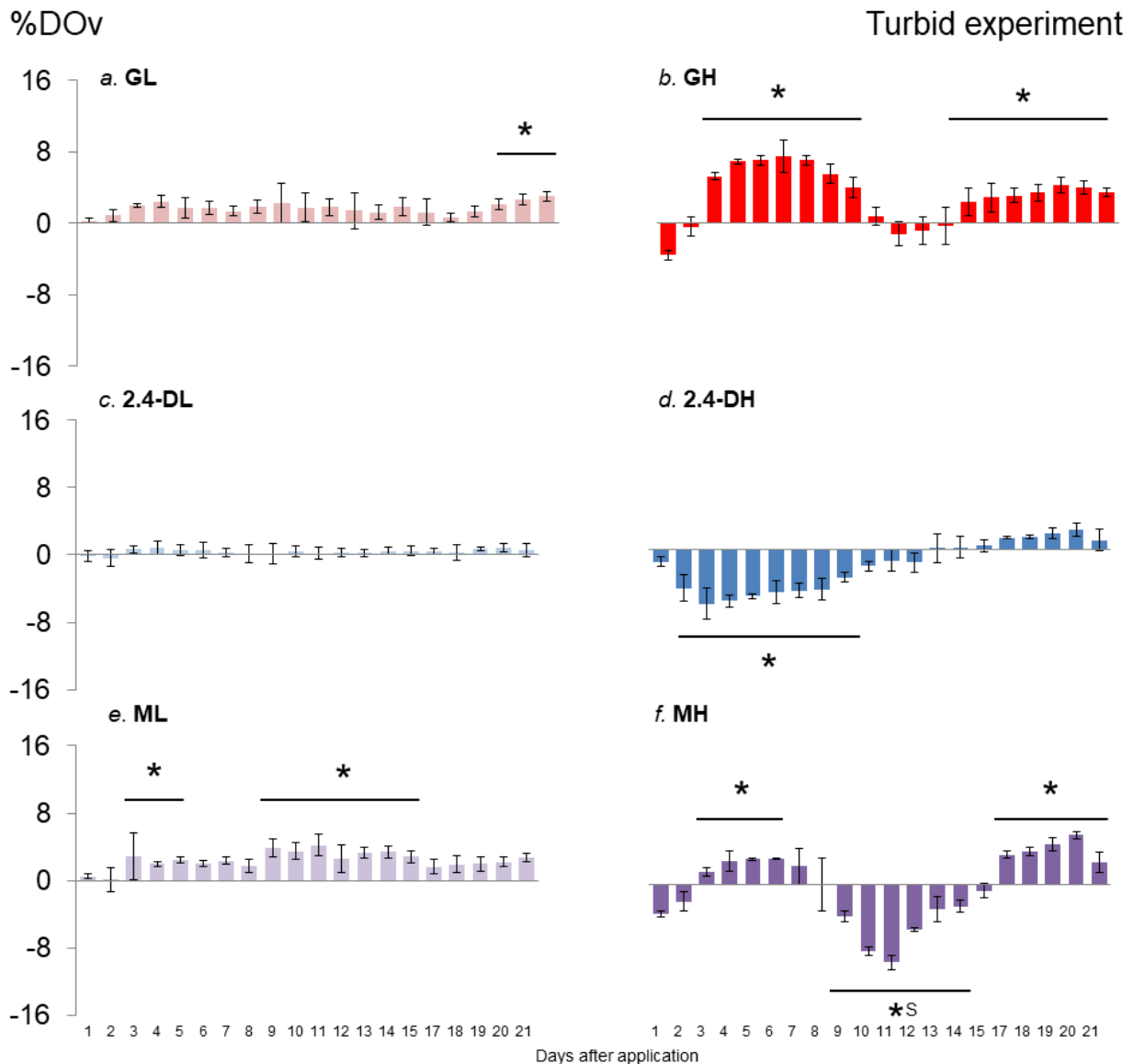
DO means (mg/L)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	17	18	19	20	21
CONTROL	11.02	10.83	10.99	10.69	10.91	10.83	10.79	10.71	10.70	10.17	10.29	9.65	9.40	9.67	9.75	9.81	9.78	9.91	9.93	10.01
GL	11.03	10.87	11.13	10.92	11.03	10.90	10.88	10.84	10.88	10.29	10.41	9.78	9.51	9.87	9.88	9.91	9.85	**10.09**	**10.11**	**10.28**
2,4-DL	10.98	10.74	10.96	10.75	10.88	10.73	10.74	10.64	10.68	10.18	10.26	9.67	9.41	9.76	9.81	9.85	9.75	9.94	9.95	10.05
ML	10.97	10.77	**11.02**	**10.83**	**11.02**	10.82	10.89	10.73	**11.01**	**10.43**	**10.60**	**9.89**	**9.63**	**9.99**	**10.02**	9.96	9.84	10.02	10.04	10.23
GH	10.63	10.73	**11.51**	**11.70**	**11.58**	**11.45**	**11.41**	**11.21**	**11.05**	10.22	10.13	9.56	9.36	**9.93**	**10.04**	**10.16**	**10.10**	**10.31**	**10.25**	**10.33**
2,4-DH	10.82	**10.34**	**10.26**	**10.10**	**10.25**	**10.16**	**10.22**	**10.12**	**10.35**	9.95	10.13	9.51	9.37	9.72	9.80	9.94	9.85	10.03	10.06	10.09
MH	10.56	10.75	10.98	**10.89**	**11.06**	**10.91**	**10.90**	10.58	10.33	**9.43**	**9.48**	**9.19**	**9.09**	**9.45**	9.71	**10.16**	**10.01**	**10.25**	**10.38**	**10.21**

327

328 **Fig. 4:** Daily percentage of DO saturation in relation to the controls (DO%v) in the turbid experiment after

329 herbicide application (G: Roundup Max®; 2,4-D: AsiMax 50®; M: Roundup Max® + AsiMax 50®; L: low

330 concentration; H: high concentration). Asterisks indicate significant differences with controls (RM-ANOVA,

331 p<0.05) and (S): synergism. DO means values (mg/L) are listed below, in grey and bold: values

332 corresponding to significant differences showed in the graphs below ((RM-ANOVA, p<0.05).

3.3.   Biological variables

In the clear experiment, the mean abundance of M+N ranged from a minimum of $3.4 \times 10^2 \pm 3.1 \times 10^2$ ind/mL (GL, TF) to a maximum of $1.8 \times 10^4 \pm 6.8 \times 10^3$ ind/mL (GH, T2). This microbial fraction showed behavioral differences among treatments over the study period. In general, there was a trend toward a significant increase in abundance under treatments GL, ML, GH and MH. On the contrary, M+N showed a trend toward decreased abundance under treatments Control, 2,4-DL and 2,4-DH, probably as a consequence of an enclosure effect. The abundance of PEuk ranged from a minimum of $4.2 \times 10^2 \pm 3.4 \times 10^2$ ind/mL (2,4-DL, TF) to a maximum of $1.5 \times 10^4 \pm 7.8 \times 10^3$ ind/mL (GH, T2). For all treatments, this microbial fraction showed similar abundance values between TF and T0, with intermediate patterns. The abundance of Pcy ranged from a minimum of $4.6 \times 10^4 \pm 1.3 \times 10^4$ ind/mL (GH, T0) to a maximum of $2.4 \times 10^5 \pm 1.7 \times 10^5$ ind/mL (GH, TF). Likewise, the abundance values of Pcy were similar between Tf and T0 for all treatments. The abundance of Mixo ranged from a minimum of $1.4 \times 10^1 \pm 2.3 \times 10^1$ ind/mL (GH, TF) to a maximum of $7.1 \times 10^3 \pm 3.4 \times 10^3$ ind/mL (2,4-DL, T0). The abundances of this algal fraction showed a decreasing trend toward TF for all treatments, except for ML and MH where abundance values were similar between T0 and TF. The abundance of heterotrophic bacteria ranged between a minimum of $6.1 \times 10^6 \pm 1.3 \times 10^6$ ind/mL (Control, T1) and a maximum of $2.6 \times 10^7 \pm 3.6 \times 10^6$ ind/mL (Control, T2) (Supplementary Material 1)

When compared with controls, significant treatment-related effects on M+N were observed at T2, for both GH with a mean of +769% (ANOVA, p<0.001) and MH with a mean of +267% (ANOVA, p=0.01) (Fig. 4a). These increases persisted until

357   TF, with +125% for MH (ANOVA, p<0.001) and +55% for GH (ANOVA; p=0.004). At

358   TF, GL showed a decrease of -83% in relation to the controls (ANOVA, p=0.001).

359   For PEuk, abundance increased at T2 in GH treatment with a mean value of 414%

360   and in MH with an increase of 204%. No treatment-related effects were observed for

361   Pcy and Mixo fractions. HB fraction had consistent variations in abundance across

362   treatments at various sampling times: a mean increase of +52% in GH (p<0.001) at

363   T0; +60, +49 and +136% (p<0.001 in all cases) in 2,4-DH, GH and MH, respectively

364   at T1. In contrast, some treatments showed lower abundances than controls at T2,

365   with -26% (p=0.011), -27% (p=0.008) and -27% (p=0.009) for GH, 2-4-DH and MH,

366   respectively (Fig. 5e).

		GL		2,4-DL		ML		GH		2,4-DH		MH	
		%	SD	%	SD	%	SD	%	SD	%	SD	%	SD
a. M+N	T0	-4	12	8	13	-25	25	18	26	7	25	26	7
	T1	1	42	-4	26	-23	9	-16	29	-18	9	-32	19
	T2	20	33	-24	18	-27	19	**769** *	322	-21	19	**267** *	93
	T3	**-83** *	15	-44	27	47	64	**55** *	60	-23	64	**125** *	72
b. Mixo	T0	52	60	116	104	-58	27	-21	38	-33	27	-24	51
	T1	-6	30	-17	43	-41	25	-28	26	2	25	-54	4
	T2	-24	43	-29	31	-73	34	-63	25	-30	34	-46	16
	T3	586	1000	468	309	993	228	-76	41	111	228	206	451
c. PEuk	T0	66	89	20	65	20	50	94	44	-17	50	89	118
	T1	8	12	11	16	20	9	-6	11	11	9	2	20
	T2	6	7	0	21	-13	36	**414** *	277	-40	36	**204** *	37
	T3	82	149	-49	41	45	13	211	227	-19	13	165	178
d. Pcy	T0	2	32	-11	33	10	17	-37	18	-27	17	-22	26
	T1	6	8	16	25	17	17	21	10	0	17	39	7
	T2	17	98	53	77	-48	11	7	43	-55	11	6	41
	T3	43	76	5	45	-28	82	204	213	45	82	34	91
e. HB	T0	-6	6	-21	9	-15	10	**52** *	21	-19	10	-9	24
	T1	4	9	77	117	21	38	**49** *	24	**60** *	38	**136** *	52
	T2	-17	6	-15	2	-18	4	**-26** *	3	**-27** *	4	**-27** *	4
	T3	6	6	5	3	11	7	29	7	28	7	21	15

Fig. 5: Percentages (%) and standard deviation (SD) of the abundance of microbial communities for each treatment in the clear system. M+N: micro+nano phytoplankton; PEuk: picoeukaryotes; Pcy: picocyanobacteria; Mixo: mixotrophic algae and HB: heterotrophic bacteria. (G: Roundup Max®; 2,4-D: AsiMax 50®; M: Roundup Max® + AsiMax 50®; L: low concentration; H: high concentration). Bars show the mean % (n=3) around controls (central line). Asterisks indicate significant differences with respect to controls for each microbial fraction (RM-ANOVA, p<0.05).

372   In the turbid experiment the mean abundances of M+N ranged from a minimum

373   of $1.1 \times 10^4 \pm 3.5 \times 10^3$ ind/mL (2,4-DH, TF) to a maximum of $1.6 \times 10^5 \pm 4.9 \times 10^4$ ind/mL

374   (GL, T2). There was a trend toward decreased abundance under treatments Control,

375   GL, ML and 2,4-DH, where values at TF were lower than those at T0. The abundance of

376   PEuk varied from a minimum of $3.3 \times 10^4 \pm 5.0 \times 10^3$ ind/mL (GL, TF) to a maximum of

377    $2.3 \times 10^5 \pm 7.2 \times 10^3$ ind/mL (2,4-DH, T3). The abundance of the Pcy fraction varied from

378   a minimum of $2.0 \times 10^5 \pm 3.9 \times 10^4$ ind/mL (GL, TF) to a maximum of $8.2 \times 10^5 \pm 2.7 \times$

379   10^5 ind/mL (MH, T3). Mixo abundance ranged from a minimum of $3.0 \times 10^3 \pm 3.2 \times 10^3$

380   ind/mL (MH, TF) to a maximum of $5.5 \times 10^4 \pm 2.2 \times 10^4$ ind/mL (Control, T3). The

381   abundance of heterotrophic bacteria ranged between a minimum of $2.5 \times 10^6 \pm 6.4 \times 10^5$

382   ind/mL (Control, T0) and a maximum of $1.4 \times 10^7 \pm 2.6 \times 10^6$ ind/mL (ML, T3)

383   (Supplementary Material 2).

384   In relation to controls, M+N showed significant decreases in 2,4-DH both at T2

385   and TF, with mean values of -67% (ANOVA, p<0.001) and -67% (ANOVA, p=0.003),

386   respectively (Fig. 6a). The abundance of Mixo showed significant decreases with respect

387   to control at T2 in GH (-73%), MH (-72%), 2,4-DH (-64%), ML (-50%) and 2,4-DL (-49%)

388   (ANOVA, p<0.001 in all cases). Only MH showed a sustained decrease in abundance of

389   -88% toward TF (ANOVA, p=0.006) (Fig. 6b). PEuk abundance values showed

390   significant treatment-related effects for GH at T1, with a mean variation of -42% (ANOVA,

391   p=0.007), and for GL, 2,4-DH and MH at TF, with mean variations of -60% (ANOVA, p

392   <0.001), +58% (ANOVA, p=0.004) and +53% (ANOVA, p=0.004) respectively (Fig. 6c).

393   Pcy abundance in ML and GH increased slightly with respect to Control, with means of

394   +33 and +34% (ANOVA, p=0.005 and 0.007, respectively) at T1. There were significant

395   increases in Pcy abundance in GH and MH from T2 to TF, with means of +58 and +104%

396   for GH, and +89 and +98% for MH (ANOVA, p<0.001 in all cases). In addition, ML

397   showed an increase in the abundance of the Pcy fraction at T1 and TF, with a mean of

398   +33 and +55% respectively (ANOVA, both p<0.001) (Fig. 6d). HB showed rapid

399   variations with respect to control at T0 and, except for GL, all treatments showed

400   increases until TF: 2,4-DL +67%, ML +90%, GH +157%, 2,4-DH +94% and MH 250%

401   (ANOVA, p<0.001 in all cases) (Fig. 6e).

402

403

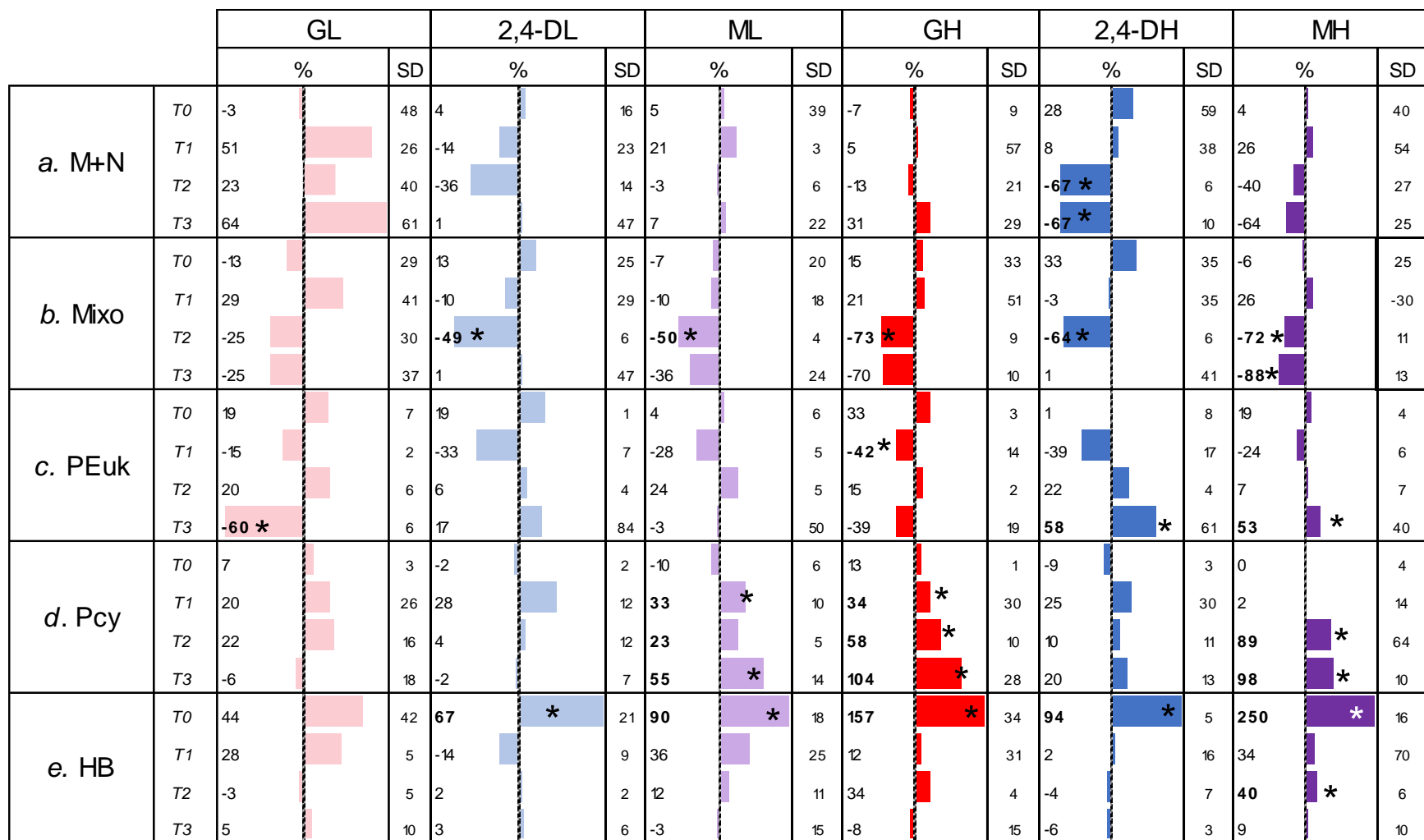|		GL %	GL SD	2,4-DL %	2,4-DL SD	ML %	ML SD	GH %	GH SD	2,4-DH %	2,4-DH SD	MH %	MH SD
a. M+N	T0	-3	48	4	16	5	39	-7	9	28	59	4	40
	T1	51	26	-14	23	21	3	5	57	8	38	26	54
	T2	23	40	-36	14	-3	6	-13	21	-67 *	6	-40	27
	T3	64	61	1	47	7	22	31	29	-67 *	10	-64	25
b. Mixo	T0	-13	29	13	25	-7	20	15	33	33	35	-6	25
	T1	29	41	-10	29	-10	18	21	51	-3	35	26	-30
	T2	-25	30	-49 *	6	-50 *	4	-73 *	9	-64 *	6	-72 *	11
	T3	-25	37	1	47	-36	24	-70	10	1	41	-88 *	13
c. PEuk	T0	19	7	19	1	4	6	33	3	1	8	19	4
	T1	-15	2	-33	7	-28	5	-42 *	14	-39	17	-24	6
	T2	20	6	6	4	24	5	15	2	22	4	7	7
	T3	-60 *	6	17	84	-3	50	-39	19	58 *	61	53 *	40
d. Pcy	T0	7	3	-2	2	-10	6	13	1	-9	3	0	4
	T1	20	26	28	12	33 *	10	34 *	30	25	30	2	14
	T2	22	16	4	12	23	5	58 *	10	10	11	89 *	64
	T3	-6	18	-2	7	55 *	14	104 *	28	20	13	98 *	10
e. HB	T0	44	42	67 *	21	90 *	18	157 *	34	94 *	5	250 *	16
	T1	28	5	-14	9	36	25	12	31	2	16	34	70
	T2	-3	5	2	2	12	11	34	4	-4	7	40 *	6
	T3	5	10	3	6	-3	15	-8	15	-6	3	9	10

Fig. 6: Percentages (%) and standard deviation (SD) of the abundance of microbial communities for each treatment in the clear system. M+N: micro+nano phytoplankton; PEuk: picoeukaryotes; Pcy: picocyanobacteria; Mixo: mixotrophic algae and HB: heterotrophic bacteria. (G: Roundup Max®; 2,4-D: AsiMax 50®; M: Roundup Max® + AsiMax 50®; L: low concentration; H: high concentration). Bars show the mean % (n=3) around controls (central line). Asterisks indicate significant differences with respect to controls for each microbial fraction (RM-ANOVA, $p < 0.05$).

409   To analyze the relationship between DO% and variations in the abundance of

410   all the fractions of autotrophic and bacterial communities, a correlation network was

411   built with these variables at T0, T1, T2 and TF by means of Cytoscape® using

412   Pearson's r values (Fig. 7). Under clear conditions, DO% correlates positively with

413   M+N phytoplankton abundances (r=0.53, p<0.0001), which, in turn correlates

414   positively with PEuk abundances (r=0.54, p=0.0001). On the other hand, HB

415   correlates negatively with DO% (r=-0.24, p=0.0408). Under turbid conditions,, DO%

416   correlates positively with Pcy abundances (r=0.38, p=0.0044) and negatively with

417   mixotrophic algae (r=-0.36, p=0.008). In regard to relationships between abundance

418   variations, Pcy correlates negatively with Mixo (r=-0.52, p<0.0001), which also

419   correlates negatively with M+N phytoplankton (r=-0.5, p=0.0001). Finally,

420   mixotrophic algae correlates positively with M+N phytoplankton (r=0.43, p= 0.0001).
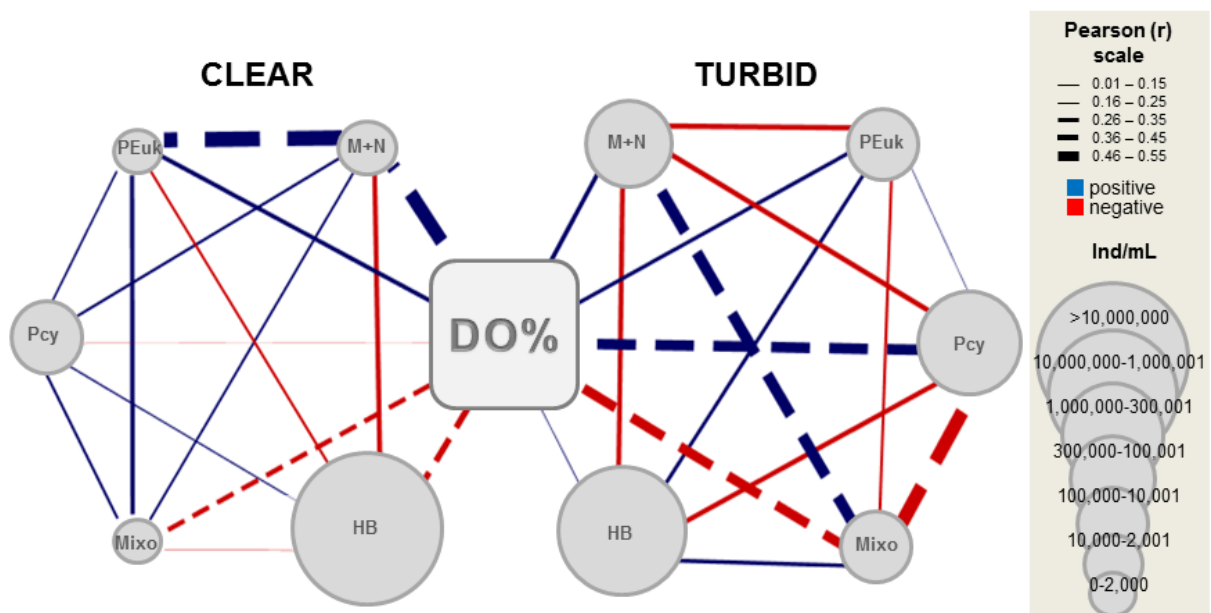

421

Fig. 7: Correlation network among DO%, and micro + nanophytoplankton (M+N), picoeukaryotes (PEuk), picocyanobacteria (Pcy), mixotrophic algae (Mixo) and heterotrophic bacteria (HB) abundances under clear and turbid conditions at all times (T0, T1, T2 and TF). Circle size: mean

425   abundance value (ind/mL) of each microbial community for the controls at T0. Dotted line: p<0.05. n

426   = 72. Networks were built with Cytoscape® v3.7.1 software.

427

428   **4. Discussion**

429   Our results strongly suggest that DO is sensitive for monitoring ecological

430   changes driven by single and mixed herbicide formulations on freshwater systems.

431   Despite the different modes of action of G and 2,4-D, their toxicity appeared to be

432   primarily targeted at the algal and cyanobacteria fractions, probably because they

433   share common pathways with plants (Couderchet & Vernet, 2003). Both herbicides,

434   alone and/or in combination, can directly affect the autotrophic fraction of plankton

435   in different ways, as already observed in microcosms (Lozano et al., 2018), leading

436   to changes in DO dynamics in mesocosms.

437   Changes in DO levels in water may result not only from physical and chemical

438   processes, but also from other environmental drivers of change acting on the biota,

439   from individual to community scales. Contaminants such as herbicides may cause

440   organisms to die or survive (Peck, 2011). The responses of the survivors depend on

441   the rate of change and vary from biochemical buffering through gene expression and

442   physiological flexibility at the individual scale, to changes in gene frequency, genetic

443   drift and selection at the population scale. The latter are likely to occur relatively

444   rapidly in microbial communities. Moreover, contaminants may induce stress

445   responses in microbial communities, modifying their structure and function. These

446   responses involve changes in respiration and photosynthesis rates, which affect the

447   primary and secondary production of the ecosystem. Therefore, it is expected that

448   herbicide inputs will increase the abundance of decomposers, thus decreasing DO

449   concentration. An increased mortality of some autotrophic species may also cause

450   a drop in DO concentration, as it contributes to additional decomposable matter and

451   leads to a reduction in the photosynthetic rate. In contrast, physiological stimulation

452   would increase the abundance of some autotrophic species, with the concomitant

453   rise in photosynthetic rate and in DO concentration.

454   It is important to highlight that there are other aquatic microbial communities

455   involved in the scenario of DO concentration changes driven by herbicides. Vera et

456   al. (2010) found a strong impact of Roundup® on periphyton, which is composed of

457   autotrophic and heterotrophic fractions. On the other hand, Pizarro et al. (2015b)

458   documented an increase in metaphyton (macroscopic mats of microscopic

459   filamentous algae) biomass, following glyphosate addition. The impact of herbicides

460   on freshwater systems may also include indirect effects on autotrophs. For example,

461   Vera et al. (2012) reported that zooplankton preying on phytoplankton is affected by

462   Glifosato Atanor®, a commercial glyphosate-based formulation commonly used in

463   Argentina. Thus, variations in DO concentration reflect changes in the food web and

464   in community structural and functional variables. Even if the overall picture of

465   biological changes is incomplete, DO is a useful integrative variable that summarizes

466   processes working in different directions.

467   The increase in DO at the higher concentration of G under the clear condition

468   may be explained by the rise in the abundances of M+N phytoplankton and PEuk.

469   In this sense, Pizarro et al. (2015a) showed a significant increase in PEuk in

470   response to the input of Glifosato Atanor®. In the present work, the fact that no

471   increase in PEuk abundance was induced at the low concentration of Roundup Max®

472   suggests a dose-response behavior. In contrast, in the turbid system, a similar

473   pattern of increased DO would be related to an increase in Pcy abundance. Indeed,

474   Pérez et al. (2007) observed that the addition of Roundup® to earthen mesocosms

475   triggered an increase in primary production related to a 40-fold rise in pico-

476   cyanobacteria abundance. Pcy are autotrophic prokaryotes, with some species

477   being tolerant to glyphosate (Pérez et al., 2007), regardless of water turbidity

478   (Pizarro et al., 2015a), and capable of phosphonate degradation because they use

479   it as phosphorus source (Ilikchyan et al. 2009, 2010).

480   There is very little information on the impact of AsiMax 50® on freshwaters

481   (Lozano et al., 2019) and only a few records concerning the effects of its active

482   ingredient, 2,4-D, are available. The impact of 2,4-D on freshwater in general and on

483   algal photosynthesis in particular is currently controversial, with some authors

484   reporting stimulation (Kobraei & White, 1996; Boyle, 1980; Relyea, 2009) and others

485   inhibition (Singh & Shrivastava, 2016; Zhu et al., 2016). In our study, the DO

486   decrease in the turbid system at the high dose would be the result of the combination

487   of a negative impact on one or more autotrophic populations and/or a positive impact

488   on heterotrophic populations. The significant decline in M+N phytoplankton

489   abundance observed at T2, that could have started before, and the increase in HB

490   abundance at T0, were in accordance with the decrease in DO%. In contrast, neither

491   DO% decrease nor variations in the phytoplankton fractions were observed in the

492   clear system. After the addition of herbicides, changes in microbial communities

493   occur relatively quickly as reported in previous studies (Pizarro et al., 2015a). As

494   microbial communities respond quickly to impact factors (Peck 2011) they can

495   recover quickly, depending on their own characteristics that can provide greater

496   resilience (Oliver et al. 2015).

497   Our first hypothesis was related to the response of the two contrasting

498   systems in terms of DO% dynamics under a similar contamination scenario while

499   our second hypothesis was related to the similarity between the impact of Roundup

500   Max® and AsiMax 50® on the daily DO%v dynamic. Glyphosate appeared to have

501   an impact under both scenarios while 2,4-D had a stronger effect on DO dynamics

502   only in the turbid system, hence, the first and the second hypothesis are rejected.

503   Our results reveal that glyphosate, the most used herbicide worldwide, has

504   significantly modified the dynamics of oxygen in water regardless of the type of

505   system. This fact was already pointed out by Lozano et al. (2018), who showed that

506   glyphosate had a higher effect than 2,4-D, either alone or in mixture, on the structure

507   of phytoplankton and periphyton communities. Notwithstanding this, in our study G

508   induced higher variations in the percentages of DO saturation and in the abundance

509   of the phytoplankton in the clear system than in the turbid one. For example, in

510   regard to abundance variation, the M+N phytoplankton showed a maximum mean

511   value of 902% in the clear system, while the Pcy fraction reached a maximum of

512   58% in the turbid one under the GH treatment at T2. Although changes in microbial

513   abundances due to glyphosate addition were higher in the clear than in the turbid

514   system, values of DO%$_v$ were not recovered at the end of the experimental period in

515   the latter system. Changes in microbial abundances were lower in magnitude in the

516   turbid than in the clear system, but the variation in the Pcy fraction in the last one

517   would have had a higher influence, accounting for the DO% variations observed at

518   TF with respect to control. The preference for phosphonates by the Pcy fraction has

519   been already documented in different experimental approaches that were used to

28

520   test glyphosate-based formulations in both clear and turbid systems (Pérez et al.,

521   2007; Pizarro et al., 2015).

522   The network correlation revealed that the turbid system showed more

523   correlations among the abundance of microbial fractions and DO%, suggesting that

524   it is less resilient than the clear system for all herbicide treatments. A greater stability

525   of the clear system under herbicide stress was also found by Lozano et al. (2019),

526   who analyzed the impact of AsiMax 50® on clear and turbid systems using a

527   laboratory microcosms approach. The fact that in the clear system DO% was highly

528   correlated with M+N phytoplankton abundances, while in the turbid one it was

529   correlated with Pcy abundances may reflect different community responses to the

530   herbicide (Cottingham et al., 1998) and supports the fifth hypothesis of this work.

531   These community-related responses may depend on their structure and on the ability

532   of each component to take advantage of the additional phosphorus provided by G.

533   In terms of competition, it is probable that the M+N phytoplankton fraction was

534   favored by the supply of nutrients under clear conditions, while the Pcy fraction was

535   favored by light and nutrients under turbid conditions. The latter statement should be

536   taken with caution considering that there are many other factors that are involved in

537   the DO dynamics, such as the water chemistry and the fate of each contaminant in

538   the environment.

539   The third hypothesis postulated that the DO%$_v$ pattern followed an additive

540   model in the presence of both herbicides. It was supported by all the results

541   presented here, except for the synergistic effect observed at the high mixture

542   concentration in the turbid system during the course of the experiment. A synergistic

543   effect of a mixture of G and 2,4-D on phytoplankton structure has also been

544   described in the context of freshwater communities by Lozano et al. (2018).

545   The fourth hypothesis that the impact of herbicides is stronger at high

546   herbicide doses was supported by our results because the greatest variations in both

547   DO% and abundance were observed in the treatments at high concentration. A

548   dose-dependent impact of these herbicides on structural features of microbial

549   communities has also been found previously (Lozano et al., 2018, 2019).

550   In our outdoor study, all the units in each experiment were subjected to the

551   same climatic conditions (i.e., temperature, atmospheric pressure, rainfall and wind).

552   Therefore, it can be assumed that DO% variations in water were exclusively

553   determined by the impact of herbicide formulations on the structure of microbial

554   communities and functional properties of the systems. To assess the effect of active

555   principles and adjuvants present in commercial formulations on DO% variations, the

556   present work focused on autotrophic microbial fractions and heterotrophic bacteria.

557   However, other microbial fractions such as colorless flagellates, protozoa and

558   rotifers should also be considered to draw more accurate conclusions (Vera et al.,

559   2012). Certainly, biological interactions (e.g. competition, grazing) are known to play

560   a relevant indirect role in DO dynamics.

561   Taking into account that most studies assessing the effect of binary mixtures

562   of herbicides have dealt only with monospecific experiments (Backhaus et al., 2004),

563   our work using two contrasting freshwater conditions at the ecosystem level is

564   expected to provide more realistic results. The synergistic decrease of dissolved

565   oxygen in the water due to the input of a mixture of herbicides raises concerns about

566   environmental risks.

567   DO fluctuations in aquatic ecosystems may have several ecological

568   consequences, including alterations in food web structure. Changes in DO have

569   been assumed to affect fish behavior (difference of 1 mg/L) (Davis, 1975) and

570   microbial stratification (Yu et al., 2014). On this basis, the magnitude of DO

571   fluctuations measured in our study, with maximum values of 2.55 mg/L and 3.57

572   mg/L for clear and turbid scenarios, respectively, are expected to have relevant

573   effects on for freshwater ecosystems.

574   Knauer and Hommen (2012) discussed the importance of functional vs.

575   structural variables in assessing the collateral damage of herbicides in mesocosm

576   studies. They concluded that functional variables generally show less experimental

577   variability between replicates and provide a better integration of ecosystem function

578   than do structural ones. Our results suggest that a simple metrics of DO may be

579   sufficiently flexible to monitor the effects of herbicide mixtures on aquatic

580   ecosystems regardless of their trophic status. This is especially true for outdoor

581   mesocosm studies with all EUs having almost identical physical properties (Caquet

582   et al., 2001). It is important to underline that a daily follow-up of DO concentration

583   (e.g., with submersible data loggers) provides additional useful information. In

584   freshwaters, DO monitoring is an effective tool to prevent environmental

585   contamination, to evaluate the recovery of heavily polluted ecosystems and to

586   analyze the potential impact of herbicides on the source-sink balance (Seguin et al.,

587   2002). An assessment of the impact of agrochemical residues on water bodies is

588   crucial for environmental protection, thus preserving the goods and services they

589   provide to people. In addition, DO measures can give early and rapid warning of

590 changes in the structure and abundance of communities from perturbed lentic

591 freshwater systems.

592 Even though the variations in DO recorded as consequence of herbicide

593 inputs may seem small, we believe that they are strong evidences of what would be

594 happening in natural systems. Shallow lakes and rivers immersed in an agricultural

595 matrix, such as that of the Pampa plain in Argentina, which are continually subjected

596 to the arrival of herbicides, would have their DO dynamics also affected by these

597 agents. Under a global warming scenario of increasing average temperatures and

598 more frequent and longer droughts, water pollution by herbicides may contribute to

599 anoxia, thus threatening water quality and aquatic life.

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608 **References**

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