Influence of light intensity on the toxicity of herbicides (alone or in mixture) to freshwater phytoplankton

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Highlights

- The sensitivity to herbicides (alone or in mixture) differs among freshwater algae
- Two cyanobacteria adapted to high light have distinct sensitivity to herbicides
- High light adaptation decreased the toxicity of single herbicide and binary mixtures
- Light intensity changes the interaction types of herbicide binary mixtures

Abstract:

Some mixtures of photosynthetic inhibitor herbicides have synergistic effects on the inhibition of the photosynthetic electron transport. Light-sensitive photoprotective ability is involved in the tolerance to single pesticides. Phytoplankton are likely to be simultaneously stressed by light intensity fluctuations and pesticide mixtures in aquatic ecosystems. However, the effect of light intensity on the toxicity of mixed pesticides is very limited. We assessed the influence of light adaptation (40-VLL, 100-LL, and 400-ML μmol photons m^{-2} s^{-1}) on the toxicity of atrazine and simazine, singly and in combination for three freshwater phytoplankton (*Chlorella vulgaris*-CPCC90, *Microcystis aeruginosa*-toxic-CPCC299 and non-toxic-CPCC632). Toxicity of atrazine and simazine on photosynthesis was greater for the three species grown under the LL condition than under the VLL condition. However, ML-adapted *C. vulgaris* and non-toxic *M. aeruginosa* were less sensitive to atrazine and simazine compared to VLL and LL conditions. A mixture of atrazine and simazine produced synergistic (for *C. vulgaris*), additive (for toxic *M. aeruginosa*), and antagonistic (for non-toxic *M. aeruginosa*) effects on the photosynthetic toxicity of the three species at 40 μmol photons m^{-2} s^{-1}. The interaction in both *M. aeruginosa* strains shifted to synergism when light intensity increased (VLL-LL, VLL-ML). Under LL and ML conditions, photoprotective ability (NPQ) was extremely sensitive to the inhibitory effects of atrazine and simazine alone, as well as their mixtures. Our results demonstrate that environmental factors (non-chemical) in freshwater habitats can enhance the toxicity of

mixed herbicides with the same mode of action on photosynthesis, indicating that light intensity cannot be ignored when assessing the toxic impact of single and binary herbicides on phytoplankton in aquatic ecosystems.

Keywords:

Freshwater phytoplankton, atrazine, simazine, mixture toxicity, light, photosynthesis

Abbreviation:

VLL: very low light intensity 40 μmol photons m^{-2} s^{-1}; LL: low light intensity 100 μmol photons m^{-2} s^{-1}; ML: medium light intensity 400 μmol photons m^{-2} s^{-1}; HL: high light intensity 1100 μmol photons m^{-2} s^{-1}; Photosystem I and II: PSI, II, CEF: cyclic electron flow; PQ: plastoquinone; Q_B: plastoquinone B.

1. Introduction

An important anthropogenic impact on freshwater ecosystems is the rise in pesticide usage in regions of intensive agriculture (Melero-Jimenez et al. 2021). Due to the long half-life of most herbicides, causing their high persistence in water bodies, these chemicals can exert toxic effects on numerous non-target organisms, such as primary producers: phytoplankton and cyanobacteria (Arts and Hanson 2018, Melero-Jimenez et al. 2021, Smedbol et al. 2018). Atrazine and simazine are frequently detected in aquatic ecosystems as they are widely used in agriculture owing to their low cost and high efficiency (Giroux 2015, 2019). Atrazine concentrations currently observed in some water bodies, reaching up to 30 µg/L (Sullivan et al. 2009) and exceeding the aquatic life protection standards of 1.8 µg/L in Canada and 1.5 µg/L in the USA (MDDEP 2008, US EPA 2004). The maximum residue for simazine in drinking water is limited to 4 µg/L based on the EPA directive (Callahan 1980) and it has been detected in surface waters with peak concentrations up to 1.2 µg/L (Li et al. 2018). Atrazine and simazine are well-known photosynthetic inhibitor herbicides that can bind to the plastoquinone B (Q_B) site of the D1 protein on PSII to block the photosynthetic electron transport chain between photosystem II (PSII) and photosystem I (PSI). This blockage results in a decrease of ATP and NADPH required for carbon fixation, which affects the growth of algae and higher plants (Bai et al. 2015, DeLorenzo 2001, Gomes and Juneau 2017). Furthermore, this inhibition of the photosynthetic electron transport chain caused by photosynthesis inhibitor herbicide also induces Reactive Oxygen Species (ROS) generation. The constant accumulation of ROS induces cellular

oxidative damage, leading to the degradation of lipids, proteins, and pigments connected to the photosynthetic apparatus (Singh et al. 2016, Wang et al. 2020). Consequently, herbicides can reduce the primary productivity of phytoplankton and have significant impacts on aquatic ecosystems due to their toxic effects (Melero-Jimenez et al. 2021, Zhao et al. 2020).

In aquatic ecosystems impacted by human activities, phytoplankton as primary producers, are often exposed to a mixture of chemicals rather than to any single one. Thus, studying the toxicity of a single chemical is insufficient to evaluate the environmental risk given that interactions between substances can occur (Gonzalez-Pleiter et al. 2013, Magdaleno et al. 2015). The impact of the binary combination of pesticides on phytoplankton has been previously studied, demonstrating that these chemicals cause greater toxicity than the sum of each one alone (called synergistic effect) (Bighiu et al. 2020, Dupraz et al. 2019, Liu et al. 2013). Pesticides and antifouling biocides in binary mixtures were reported to exhibit synergistic effects in approximately 7% and 26% of cases (reviewed by Cedergreen 2014). Further to synergistic effects, binary mixtures of pesticides may also have additive effects or antagonistic effects (lower effects than additive) on algal physiology (Crain et al. 2008). The type of interaction effects observed depends on the mode of action of the chemicals and the sensitivity of the physiological and protective mechanisms (non-photochemical quenching and antioxidant enzyme activity) of the affected organisms (Korkaric et al. 2015).

In aquatic environments, light intensity is one of the main environmental factors that can alter phytoplankton photosynthesis (Virtanen et al. 2021). Therefore, in contaminated waters, the effects caused by herbicides affecting photosynthesis can be modulated by light intensity, resulting in a different response than the one expected when these stressors are present alone (Fischer et al. 2010). Previous studies have clearly showed the importance of considering the mechanisms involved in the interaction between light and herbicides in aquatic ecosystems (Deblois et al. 2013, Gomes and Juneau 2017). However, most studies on phytoplankton to date have focused only on the interactions between an environmental factor and a single pesticide. Moreover, light variation and mixture of pesticides can occur simultaneously in freshwater habitats. To our knowledge, there has been no investigation of how light intensity affects the toxicity of herbicide mixtures. In this study, we thus investigated the combined effects of light intensities with atrazine and simazine (single and in binary mixtures) on the growth and photosynthetic processes of three freshwater phytoplankton taxa, including a green microalga and two strains (toxic and non-toxic) of a cyanobacteria.

2. Materials and methods

2.1 Phytoplankton

The cyanobacteria species *Microcystis aeruginosa* CPCC632 (non-toxic strain) and CPCC299 (toxic strain) and the green alga *Chlorella vulgaris* CPCC90, were obtained from the Canadian Phycological Culture Centre (Waterloo, ON, Canada). Each species was cultivated in 250 mL flasks with a total volume of 100 mL BG11

growth medium (Devgoswami et al. 2011). Cultures were grown (for a minimum of eight generations) under three different light intensities of very low light (VLL), low light (LL) and medium light (ML): VLL = 40, LL = 100, and ML = 400 µmol photons m^{-2} s^{-1}. Cultures were all kept at 24 °C with a light:dark (14:10 h) illumination cycle and gently mixed every day. To keep cells in their exponential growth phase they were transferred regularly (every 3 days) into fresh growth medium. Sub-samples were collected every day to quantify biovolume and cell density by using Multisizer 3 Coulter Counter particle analyzer (Beckman Coulter Inc., USA). The following formula was used to evaluate the growth rate (µ): $\mu = (\ln N_3) - (\ln N_0)/(t_3 - t_0)$, where N_3 is the number of cells at day 3 (t_3) and N_0 is the number at time 0 (t_0).

2.2 Herbicide and high light exposures

Atrazine and simazine were obtained from Sigma-Aldrich (PESTANAL®, analytical standard, Canada). Pure acetone (≥ 99%) was used as the solvent for dissolving pesticide stock solutions. Cultures were harvested while in their exponential growth phase and placed into sterile 24-well transparent polystyrene microplates with an initial cell density of 2.5×10^6 cells/mL and exposed to atrazine or simazine for 72 h under the three different light intensities mentioned in section 2.1. The initial concentrations of atrazine and simazine were measured as described in Du et al 2023. Measured initial concentrations were 4-10% of the nominal concentrations. For atrazine the initial concentrations were 0, 5.5, 26.9, 48.0, 108.4 and 246.8 µg/L and for simazine they were 0, 4.9, 24.8, 52.7, 103.9 and 252.9 µg/L. In the figures, these initial concentrations are noted as 0, 5, 25, 50, 100 and 250 µg/L. For each herbicide,

concentration-response tests were carried out to determine the 72 h-EC_{50}. The final percentage of acetone used in the microplate was 0.01%. Each sample had four replicates.

High light intensity treatments (HL; 1100 µmol photons m^{-2} s^{-1} for 60 min) were obtained by using a halogen lamp (250 W; Winchester, UK) following the 72-h exposure to growth light intensities (with or without herbicides, single or in mixtures). At the end of the experiment, cell density and cell biovolume were measured by using the Multisizer 3 Coulter Counter particle analyzer (Beckman Coulter Inc., USA).

2.3 Mixture toxicity tests

The EC_{50} of the operational PSII quantum yield (Φ'_M) from the single herbicide toxicity test was used to determine the herbicide concentration in the binary mixture experiments. Concentration-response tests were conducted on the single herbicide (considered as a mixture ratio of 0:100% and 100:0%) and mixtures at two effective concentration ratios of 75:25%, and 25:75% (atrazine:simazine), using four concentrations for two mixture ratios in four replicates. The isobole model was used to analyze the interactive effect on the herbicide mixtures. More information about this model is described in (Dupraz et al. 2018).

2.4 Chlorophyll fluorescence measurements

Pulse Amplitude Modulated fluorometer (Maxi-Imaging PAM, Heinz Walz GmbH, Effeltrich, Germany) was used to determine the light curves with a series of 60 s light exposures to 12 levels of irradiance (1, 21, 56, 83, 111, 186, 281, 336, 396, 461, 531 and 611 µmol photons m^{-2} s^{-1}) according to (Du et al. 2023), after being exposed

to various herbicide concentrations/ratios for 72 h. The Φ_M (maximum PSII quantum yield), Φ'_M (operational PSII quantum yield) and NPQ (non-photochemical quenching) were then evaluated from the obtained light curves according to (Bilger and Björkman 1990, Du et al. 2023, Genty et al. 1989).

2.5 Statistical analyses

We used R opensource software 4.2.1 to estimate the isobole model curve with the 'drc' package for analyzing the concentration-response curves. More details are provided in (Dupraz et al. 2018). The EC_{50} was obtained from the nonlinear least-square fits by using the regression curve inversely (described in Van der Heever and Grobbelaar 1996).

3. Results

3.1 Effects of growth light intensity

The growth of three phytoplankton taxa (non-toxic *Microcystis aeruginosa* CPCC632, toxic *Microcystis aeruginosa* CPCC299 and *Chlorella vulgaris*-CPCC90) was significantly enhanced with increasing growth light intensity (VLL-LL-ML, VLL-40, LL-100, ML-400) except for non-toxic cyanobacteria CPCC632 at ML compared to LL (Fig. 1, Tukey's HSD, $P < 0.05$). The maximal PSII quantum yield (Φ_M) of this non-toxic strain (CPCC632) without atrazine and simazine treatment significantly increased with increasing growth light intensity (VLL-LL-ML, Tukey's HSD, $P < 0.05$). However, the operational PSII quantum yield (Φ'_M) of CPCC632 decreased remarkably with increasing growth light intensity (Fig. 2, Tukey's HSD, $P < 0.05$).

For the toxic cyanobacterial strain (CPCC299), Φ_M and Φ'_M decreased with increasing growth light intensity (VLL-LL, Fig. 2). Interestingly, CPCC299 grown under the ML condition had the same growth rate as VLL and LL conditions, but the values of Φ'_M and Φ'_M were almost zero. Therefore, the results of CPCC299 grown under ML condition are no longer considered in the following discussion as there was no photosynthetic activity.

For the green alga (CPCC90), Φ_M and Φ'_M also declined significantly under ML compared to VLL and LL conditions and the decline amplitude of Φ'_M was greater than Φ_M (Tukey's HSD, $P < 0.05$). On the other hand, while CPCC299 did not show any non-photochemical quenching (NPQ) when grown under ML condition, the NPQ of CPCC632 and CPCC90 grown under ML condition were five times higher than under the LL condition (Table 1). While no NPQ was observed for the cyanobacteria strains (CPCC632 and CPCC299) under the VLL condition, the green alga CPCC90 demonstrated similar NPQ levels under both VLL and LL conditions (Table 1).

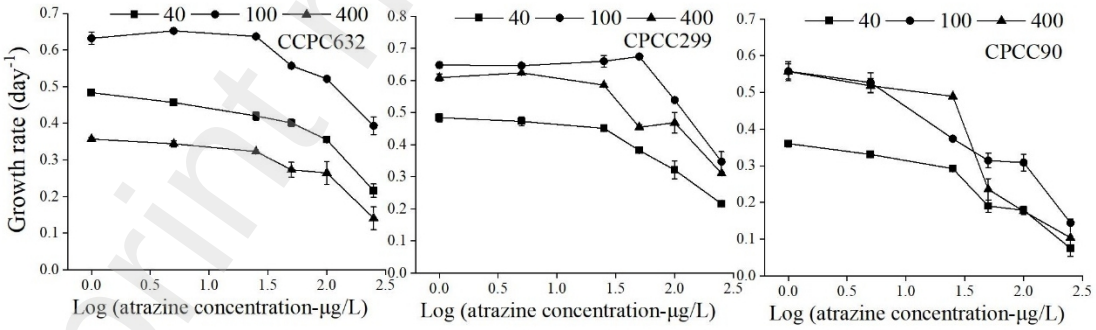

Figure 1. The effect of light intensity (VLL-40 μmol photons m^{-2} s^{-1}: square; LL-100 μmol photons m^{-2} s^{-1}: circle; ML-400 μmol photons m^{-2} s^{-1}: triangle) on the growth rate of three phytoplankton taxa (non-toxic *M. aeruginosa*-CPCC632, toxic *M. aeruginosa*-

CPCC299, and *C. vulgaris*-CCPC90) after exposure to various atrazine concentrations for 72 h. Data presented as means ± SD (n = 4-8).

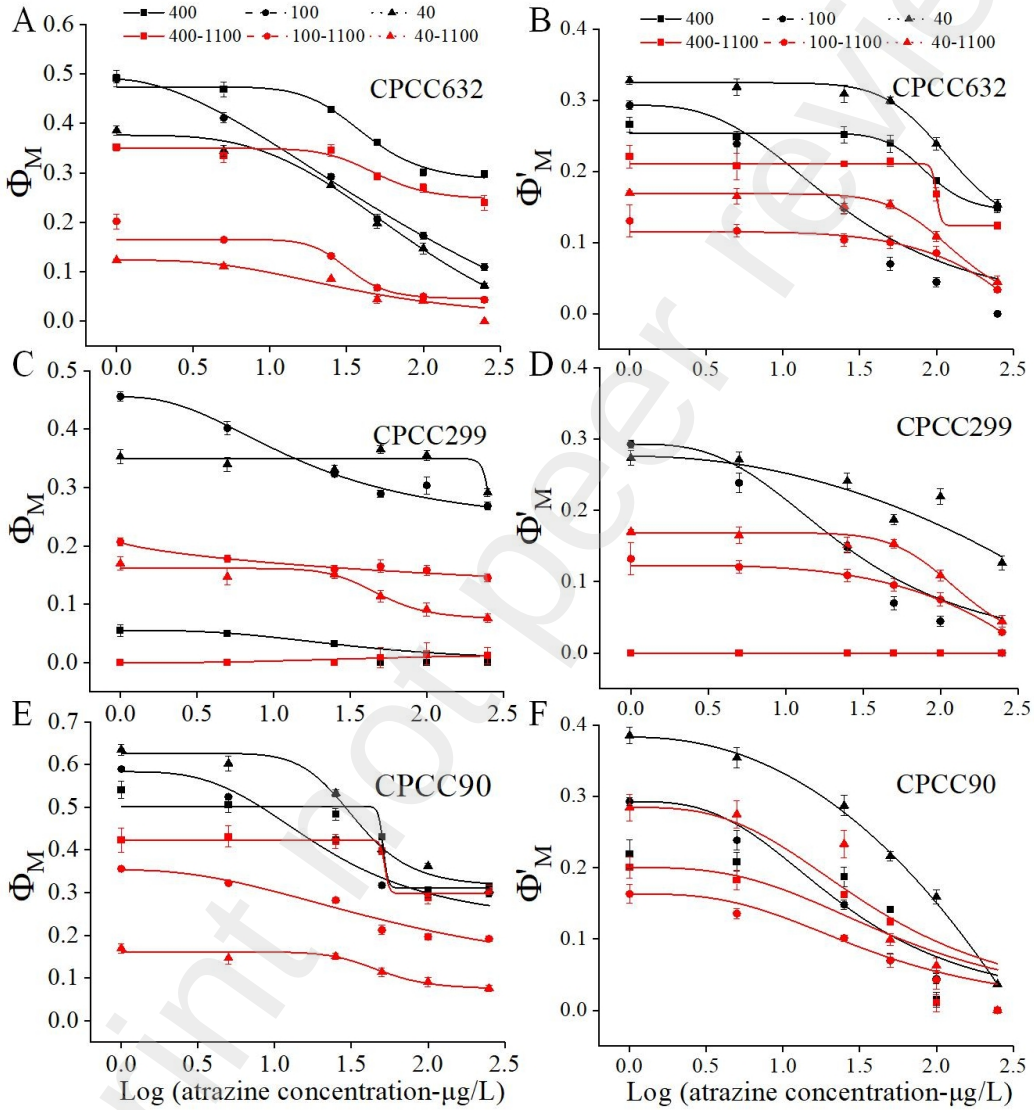

Figure 2. The effect of light intensity (VLL-40 μmol photons m^{-2} s^{-1}: square; LL-100 μmol photons m^{-2} s^{-1}: circle; ML-400 μmol photons m^{-2} s^{-1}: triangle) on the maximum (Φ_M) and operational (Φ'_M) PSII quantum yields of three phytoplankton taxa (non-toxic *M. aeruginosa*-CPCC632, toxic *M. aeruginosa*-CPCC299, and *C. vulgaris*-CCPC90)

after being exposed to various atrazine concentrations for 72 h, and subsequently shifted to high light condition for 60 min (red color 400-1100 (ML-HL): straight line; 100-1100 (LL-HL): dashed line; 40-1100 (VLL-HL): dotted line). Data presented as means ± SD (n = 4-8).

Table 1. NPQ and EC_{50} values of the growth rate and operational PSII quantum yield (Φ'_M) for three phytoplankton (non-toxic *M. aeruginosa*-CPCC632, toxic *M. aeruginosa*-CPCC299, and *C. vulgaris*-CPCC90) under three different light intensities (VLL-40 µmol photons m^{-2} s^{-1}, LL-100 µmol photons m^{-2} s^{-1}, ML-400 µmol photons m^{-2} s^{-1}) after being exposed to various concentrations of atrazine and simazine for 72 h and Φ'_M-EC_{50} after being shift to HL-1100 µmol photons m^{-2} s^{-1} for 60 min. Data expressed as means ± SD (n = 4).

Species	Growth light	NPQ	µ-EC_{50} (µg/L)		Φ'_M-EC_{50} (µg/L)		Treat light-Φ'_M-EC_{50} (µg/L)	
			Atrazine	Simazine	Atrazine	Simazine	Atrazine	Simazine
CPCC632	40	0.00±0.01[a]	217.1±12.7[a]	256.3±13.1[a]	66.4±8.5[a]	296.4±21.1[a]	20.6±11.4[a]	142.8±12.6[a]
	100	0.13±0.01[b]	608.4±39.6[b]	702.7±46.3[b]	22.3±1.4[b]	154.1±7.3[b]	29.2±0.5[a]	142.7±23.1[a]
	400	0.54±0.14[c]	99.4±5.6[c]	155.5±21.3[ab]	54.9±4.0[a]	224.0±9.0[ab]	64.5±5.8[b]	291.9±22.7[b]
CPCC299	40	0.00±0.01[a]	196.9±14.5[a]	245.8±36.3	69.1±9.5[a]	235.8±14.8[a]	21.1±2.4[a]	92.6±17.5[a]
	100	0.09±0.06[b]	346.5±12.4[b]	378.6±38.9	16.1±3.1[b]	131.2±4.1[b]	16.6±8.7[a]	108.6±10.9[a]
	400	0.12±0.01[b]	426.2±34.6[b]	497.4±53.7	N.D	N.D	N.D	N.D

CPCC90	40	0.08±0.01[a]	38.2±2.1[a]	59.3±9.7[a]	75.8±1.1[a]	539.1±49.7[a]	43.2±2.1[a]	477.6±126[a]
	100	0.10±0.02[a]	116.4±9.5[b]	163.8±21.3[b]	30.8±1.1[b]	323.5±15.5[b]	44.9±12.5[a]	339.6±77.1[a]
	400	0.49±0.03[b]	68.2±3.6[a]	73.8±12.3[a]	57.9±2.1[a]	213.8±24.5[b]	55.2±4.0[a]	438.9±76.1[a]

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238 3.2 Effects of light intensity on atrazine and simazine toxicity

239 Growth rates were inhibited to varying degrees by taxon with atrazine treatment
240 for 72 h under three different light intensities (VLL, LL, and ML) (Fig. 1). The three
241 phytoplankton species grown under LL condition exhibited a lower inhibitory effect on
242 growth than cells grown under VLL. Under ML condition, the growth of all taxa was
243 more inhibited in the presence of high concentrations of atrazine (≥ 5 μg/L) relative to
244 the other two light intensities (VLL and LL) as also shown by the μ-EC_{50} (Table 1).
245 Simazine effects on growth rates demonstrated a similar trend in all microalgal species
246 (Fig. S1). Under three different light intensities (VLL, LL, and ML), Φ_M and Φ'_M
247 showed taxon-specific declines with increasing atrazine and simazine concentrations
248 (Fig. 2 and 3). Declines in Φ_M and Φ'_M were greater in the presence of atrazine and
249 simazine under the LL condition compared to the VLL condition. However, Φ_M and
250 Φ'_M of the green CPCC90 and non-toxic CPCC632 taxa declined less when grown
251 under ML compared to VLL and LL conditions, except at high herbicide concentrations
252 of 100 and 250 μg/L. The NPQ of the three studied species under all light conditions
253 also showed a significant downward trend with increasing concentrations of each
254 herbicide (Fig. 4, Tukey's HSD, $P < 0.05$), with the decline in NPQ occurring more

255 strongly under LL than ML conditions. Finally, NPQ completely disappeared at the
256 highest concentrations of the herbicides (≥ 50 µg/L) (Fig. 4).
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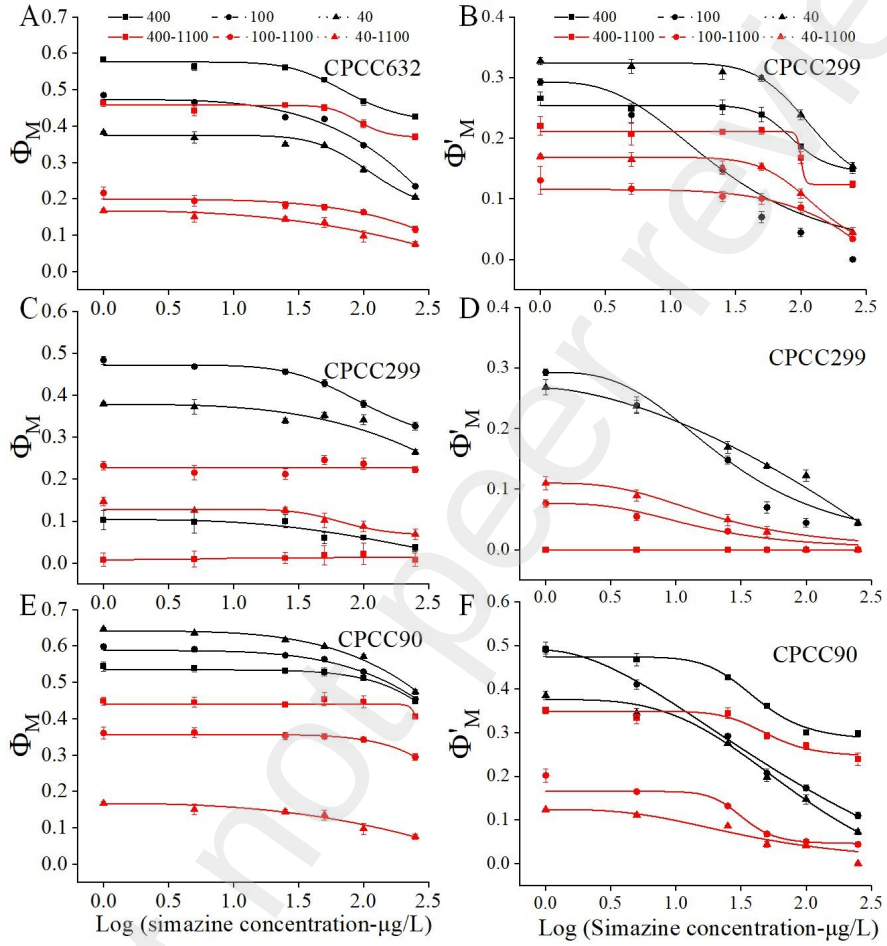

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260 Figure 3. The effect of light intensities (VLL-40 µmol photons m^{-2} s^{-1}: square; LL-100
261 µmol photons m^{-2} s^{-1}: circle; ML-400 µmol photons m^{-2} s^{-1}: triangle) on the maximum
262 (Φ_M) and operational (Φ'_M) PSII quantum yields of three phytoplankton species (non-
263 toxic *M. aeruginosa*-CPCC632, toxic *M. aeruginosa*-CPCC299, and *C. vulgaris*-
264 CCPC90) after being exposed to various simazine concentrations for 72 h, and
265 subsequently shifted to high light condition for 60 min (red color 400-1100 (ML-HL):

straight line; 100-1100 (LL-HL): dashed line; 40-1100 (VLL-HL): dotted line). Data presented as means ± SD (n = 4-8).

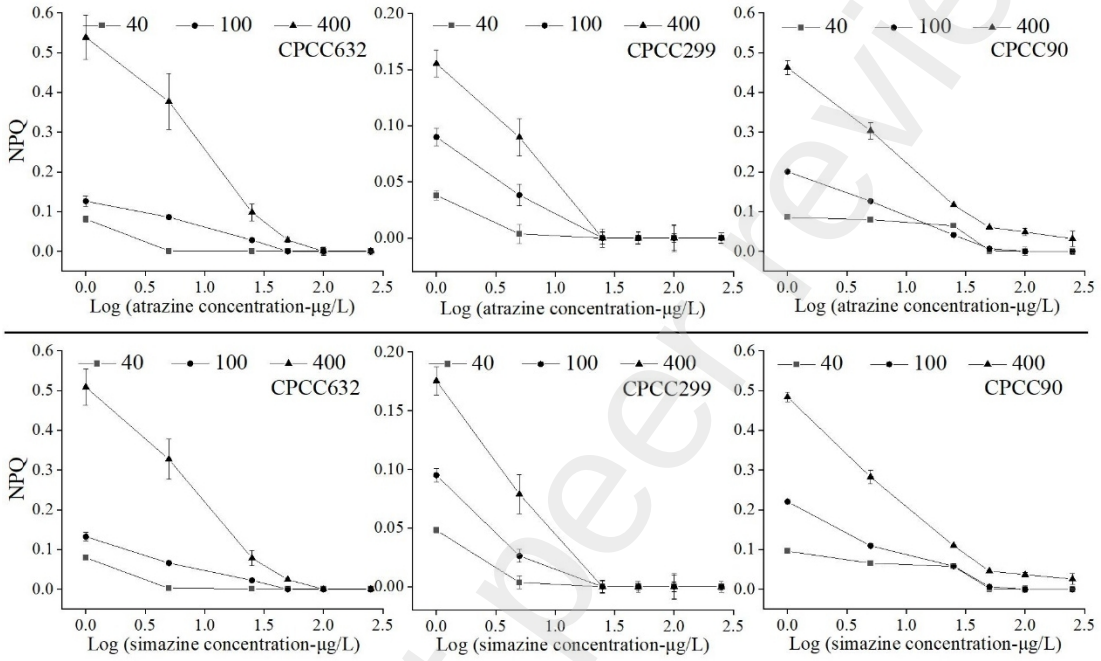

Figure 4. The effects of light intensities (VLL-40 μmol photons m^{-2} s^{-1}: square; LL-100 μmol photons m^{-2} s^{-1}: circle; ML-400 μmol photons m^{-2} s^{-1}: triangle) on the non-photochemical quenching (NPQ) of three species (non-toxic *M. aeruginosa*-CPCC632, toxic *M. aeruginosa*-CPCC299, and *C. vulgaris*-CCPC90) after being exposed to various atrazine and simazine concentrations for 72 h. Data presented as means ± SD (n = 4-8).

3.3 Effects of high light intensity exposure

When the three phytoplankton taxa grown without herbicides under each light conditions (VLL, LL, and HL) were then transferred to high light intensity (HL: 1100 µmol photons m^{-2} s^{-1}) for 60 min (VLL-HL, LL-HL, and ML-HL), their Φ_M and Φ'_M significantly decreased (Tukey's HSD, $P < 0.05$, Fig. 2 and 3- atrazine and simazine concentration = 0 µg/L). The observed decline amplitude of Φ'_M was greater than Φ_M for all species under all growth light conditions. NPQ of all taxa also significantly decreased when cells were transferred to HL for 60 min, *C. vulgaris*-CCPC90 and non-toxic *M. aeruginosa*-CPCC632 decreased 1.9 and 3.2 times respectively from ML to HL (Tukey's HSD, $P < 0.05$, Fig. S2).

3.4 Effects of high light intensity exposure on atrazine and simazine toxicity

After 72-h of atrazine and simazine treatment under the three growth light intensities and subsequent HL exposure for 60 min (VLL-HL, LL-HL, and ML-HL), a significant decrease in Φ_M and Φ'_M of all taxa were observed with increasing atrazine and simazine concentrations (Fig. 2 and 3). The previously observed decline in Φ_M and Φ'_M of CPCC90 and CPCC632 grown under ML and shifted to HL was less than in cells shifted to HL after grown under VLL and LL in the presence of herbicides (Fig. 2A-B, E-F and 3). Declines in the presence of atrazine were also observed in the EC_{50}-Φ'_M (Table 1), EC_{50}-Φ'_M of the non-toxic CPCC632 with the shift from ML to HL leading to effects that were 3 and 2 times greater than those observed for the LL to HL and VLL to HL shifts respectively. In the presence of atrazine, EC_{50}-Φ'_M of the green alga CPCC90 at ML to HL was only higher than 1.3 times than those occurring when

shifts from VLL and LL to HL occurred. With simazine treatment, EC_{50}-Φ'_M for CPCC90 and CPCC632 showed a similar trend to atrazine, but with EC_{50}-Φ'_M values in the presence of simazine was always higher than those of atrazine under any treatment conditions. Similar trend was also observed for the toxic CPCC299 strain with LL grown cells (Fig. 2C-D and 3C-D). For all studied taxa, NPQ was absent in presence of atrazine or simazine when exposed to the HL treatment for 60 min (data not shown).

3.5 Effects of the atrazine-simazine mixture

The mixture toxicity of atrazine and simazine on the green alga CPCC90 induced a synergistic effect at the three light intensities (40-VLL, 100-LL, and 400-ML; Fig. 5). Moreover, the synergistic effect was enhanced at increasing light intensities (VLL-LL, LL-ML). Antagonism was observed for the non-toxic cyanobacterial strain CPCC632 under VLL condition, but the interaction shifted to synergism under LL and ML intensities. This synergistic effect was boosted with increasing light intensity (LL to ML). A slight, but significant additive effect of the two studied herbicides was found for the toxic CPCC299 under the VLL condition, but the interaction became synergistic under LL intensity. Declines in Φ_M for the green CPCC90 and non-toxic CPCC632 under ML condition after binary herbicide mixtures 72 h exposure were less extensive relative to those observed under VLL and LL conditions, while Φ_M of the toxic CPCC299 did not significantly change under the same situation (Table 2, Tukey's HSD, $P < 0.05$). For Φ'_M, CPCC299 under the LL condition decreased less relative to the VLL

condition, but CPCC90 and CPCC632 did not significantly change (Table 2, Tukey's HSD, $P < 0.05$). Furthermore, Φ_M and Φ'_M in CPCC90 and CPCC299 under ML condition after HL treatment 60 min decreased more than under VLL and LL conditions, and Φ'_M in CPCC632 did not significantly change under the same condition (Table 2, Tukey's HSD, $P < 0.05$).

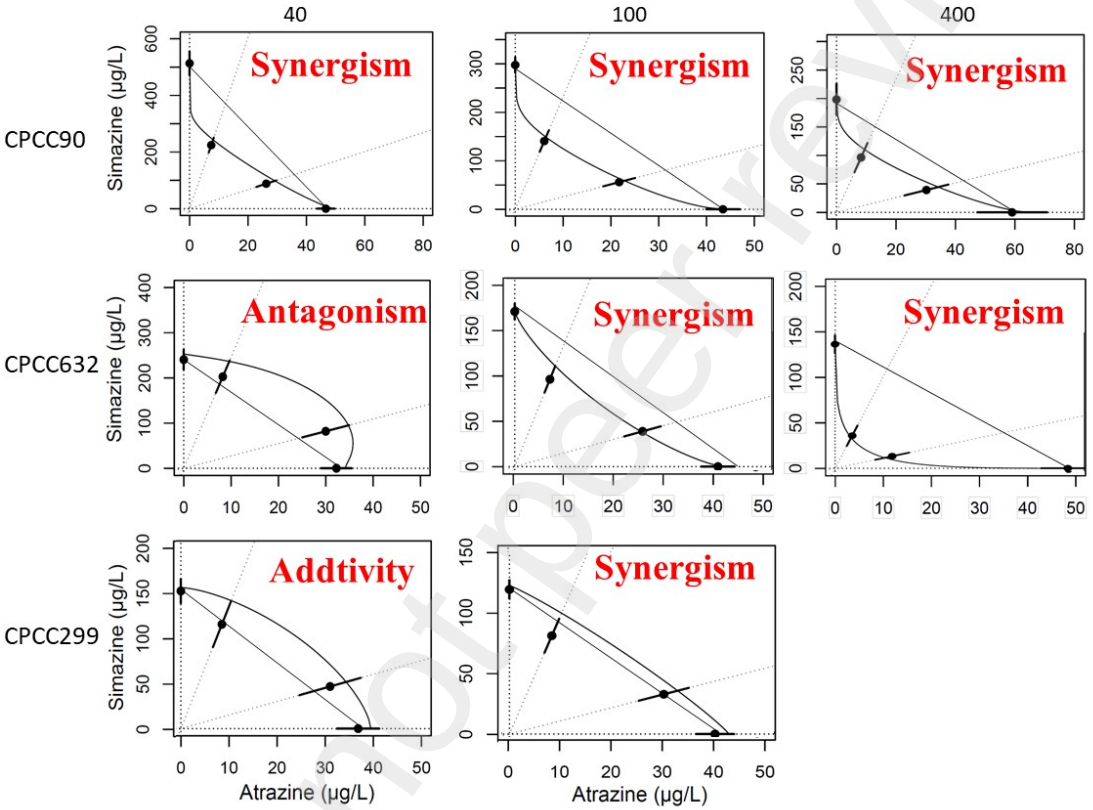

Fig. 5. Isobolograms of binary herbicide mixtures with the same mode of action under different light intensities for three species (non-toxic *M. aeruginosa*-CPCC632, toxic *M. aeruginosa*-CPCC299, and *C. vulgaris*-CCPC90). The dots show the EC_{50}± standard error. The solid line indicates the CA isobole.

Table 2. The effect of light intensities (40 µmol photons m^{-2} s^{-1},100 µmol photons m^{-2} s^{-1}, 400 µmol photons m^{-2} s^{-1}) on the maximum (Φ_M) and operational (Φ'_M) PSII

quantum yields of three phytoplankton species (non-toxic *M. aeruginosa*-CPCC632, toxic *M. aeruginosa*-CPCC299, and *C. vulgaris*-CCPC90) after being exposed to mixture herbicides (atrazine*simazine=EC25:EC25) for 72 h, and subsequently shifted to high light condition (1100 μmol photons m^{-2} s^{-1}) for 60 min. The numbers in parentheses are the decreased percentages relative to the control. N.D. = not determined. Different superscript letters (a-b) indicate significant differences between the percentages (Tukey's HSD, $P < 0.05$). Data presented as means ± SD (n = 4-8).

	Growth			Treatment light	
Species	light	Φ_M	Φ'_M	Φ_M	Φ'_M
CPCC632	40	0.393±0.02 (100)^{a}	0.332±0.01 (100)^{a}	0.137±0.02 (100)^{a}	0.113±0.02 (100)^{a}
Control	100	0.521±0.01 (100)^{a}	0.294±0.01 (100)^{a}	0.280±0.01 (100)^{a}	0.164±0.01 (100)^{a}
	400	0.536±0.02 (100)^{b}	0.199±0.01 (100)^{a}	0.469±0.02 (100)^{b}	0.186±0.01 (100)^{a}
	40	0.239±0.01 (39)^{a}	0.174±0.01 (48)^{a}	0.087±0.01 (36)^{a}	0.050±0.01 (56)^{a}
EC_{25}*EC_{25}	100	0.306±0.02 (41)^{a}	0.142±0.01 (52)^{a}	0.198±0.01 (29)^{ab}	0.080±0.01 (51)^{a}
	400	0.417±0.00 (22)^{b}	0.088±0.01 (56)^{a}	0.394±0.01 (16)^{b}	0.089±0.01 (52)^{a}
CPCC299	40	0.283±0.01 (100)^{a}	0.222±0.01 (100)^{a}	0.081±0.02 (100)^{a}	0.051±0.01 (100)^{a}
Control	100	0.500±0.00 (100)^{a}	0.383±0.01 (100)^{a}	0.177±0.02 (100)^{a}	0.105±0.01 (100)^{a}
	400	N.D	N.D	N.D	N.D
	40	0.200±0.01 (29)^{a}	0.092±0.01 (59)^{a}	0.042±0.01 (48)^{a}	0.000±0.01 (100)^{a}
EC_{25}*EC_{25}	100	0.366±0.01 (27)^{a}	0.231±0.01 (40)^{b}	0.087±0.01 (51)^{a}	0.029±0.00 (72)^{b}
	400	N.D	N.D	N.D	N.D

CPCC90	40	0.603±0.02 (100)[a]	0.372±0.01 (100)[a]	0.302±0.01 (100)[a]	0.198±0.01 (100)[a]
Control	100	0.530±0.01 (100)[a]	0.289±0.01 (100)[a]	0.301±0.01 (100)[ab]	0.145±0.01 (100)[a]
	400	0.526±0.01 (100)[b]	0.215±0.01 (100)[a]	0.453±0.01 (100)[b]	0.193±0.01 (100)[a]
	40	0.448±0.00 (36)[a]	0.248±0.01 (44)[a]	0.284±0.01 (6)[a]	0.134±0.01 (32)[a]
EC_{25}*EC_{25}	100	0.414±0.01 (22)[b]	0.175±0.01 (40)[a]	0.247±0.01 (28)[b]	0.072±0.02 (50)[b]
	400	0.427±0.01 (19)[b]	0.105±0.01 (51)[a]	0.377±0.03 (27)[b]	0.100±0.03 (48)[b]

4. Discussion

4.1 Effects of single herbicide on phytoplankton grown under different light conditions

As reported by Graymore and collaborators (Graymore et al. 2001), extremely high atrazine concentrations of up to 1000 μg/L has been found in groundwater near agricultural fields and attributable to its long half-life. Furthermore, concentrations as high as 80 μg/L of atrazine have been found in drinking water reservoirs in USA. The concentrations of herbicides we studied here are thus within the range of concentrations that can be found in the environment. Both the cyanobacteria *M. aeruginosa* (non-toxic-CPCC632 and toxic-CPCC299 strains) and the green algae *C. vulgaris* exhibited different susceptibilities to atrazine and simazine for growth and photosynthesis under three different light intensities (VLL, LL, and ML). Both cyanobacteria strains were more sensitive to atrazine and simazine than was *C. vulgaris* according to their photosynthesis-EC_{50} (Table 1). In contrast, μ-EC_{50} indicates that *C. vulgaris* was the most sensitive to herbicides, agreeing with a previous study showing that green algae had higher sensitivity to atrazine than cyanobacteria (Lockert et al. 2006). These results indicated that despite a lower or equal photosynthesis sensitivity to both herbicides, *M. aeruginosa* (CPCC299 and CPCC632) can support a faster or similar cell division rate as *C. vulgaris*, depending on the strain. It is well-known that photosynthesis and respiration share the same electron transport chain in cyanobacteria enabling the respiratory chain to supply electrons by NADPH hydrogenase to the plastoquinone (PQ) pool associated with the photosynthetic chain (Lea-Smith et al. 2016). Atrazine or simazine can bind to the Q_B site of PSII thereby influencing the photosynthetic electron

transport of the adjacent PQ pool. Under these conditions, electrons from the respiratory chain may compensate by providing electrons to the PQ pool, helping to maintain the production of ATP and NADPH (Chalifour et al. 2016). Furthermore, cyanobacteria are known to exhibit high levels of cyclic electron flow relative to green algae (Peltier et al. 2010), and this process is strongly induced by DCMU, an inhibitor of photosynthesis (You et al. 2015). The induction of this alternative electron flow in presence of atrazine or simazine may explain the higher growth of both *M. aeruginosa* compared to *C. vulgaris* in the presence of herbicides affecting PSII. This might also explain why *M. aeruginosa* CPCC299 grew better when exposed to high concentrations of atrazine and simazine than *C. vulgaris* under ML and HL conditions, as cyclic electron flow may also be induced by higher light conditions (Du et al. 2019).

On the other hand, atrazine and simazine significantly decreased the photosynthetic efficiency (Φ_M and Φ'_M) of both *M. aeruginosa* and *C. vulgaris* and this inhibitory effect was enhanced under LL (VLL-LL, Fig. 2 and 3). However, following ML adaption non-toxic cyanobacteria CPCC632 and the green alga CPCC90 were less affected by atrazine and simazine. Indeed, they were less affected by herbicides compared to the VLL and LL conditions following ML adaption and subsequent exposure to HL for 60 min. Both situations can be mainly attributed to the highly activated non-photochemical quenching (NPQ) under ML condition (Table 1). NPQ can dissipate the excess light energy generated by the blockage of the photosynthetic electron transport chain to reduce the overexcitation pressure of PSII (Goss and Lepetit 2015, Müller et al. 2001). Interestingly, the toxic *M. aeruginosa* strain CPCC299

showed high growth rate but relatively low photosynthetic activity under ML conditions, while the non-toxic strain (CPCC632) showed low growth rates and high photosynthetic activity. This difference between the toxic and the non-toxic strains could be linked to the yet unknown mechanistic role of microcystin when *M. aeruginosa* is grown under high light (Xu et al. 2013), but further investigation is needed. Another reason may be related to the difference in the photoprotective ability (NPQ) between toxic and non-toxic strains as they deal with photodamage under LL and ML conditions. In addition, while atrazine and simazine share the same mode of action, we showed that the toxicity to photosynthesis of atrazine was 4-10 times that of simazine for the studied taxa. For growth, atrazine was approximately 1.5 times more toxic than simazine. These results suggest that the degree of toxicity of the herbicides depends on the evaluated parameters, and thus choosing which parameter to assess for mixed pesticides (with known mode of action) interactions is essential for future research on mixtures (Moreira et al. 2020).

4.2 Effects of herbicide mixtures on phytoplankton grown under different light conditions

The assessment of the ecological risk of chemicals using single substances in the lab may underestimate impacts in aquatic ecosystems owing to the interactions occurring among various chemicals in the natural environment (Bighiu et al. 2020). The concentration addition (CA) model theory is the basis for the isobole method, and it is mostly used to qualitatively analyze the effects of combined chemicals regardless of whether they exhibit synergism or antagonism (Chen et al. 2014). The basic assumption

410 of the CA model is that mixed chemicals have the same mode of action as the individual
411 chemical and that one chemical in the mixture can be replaced by the other and be
412 considered as dilution of each other (Crain et al. 2008). Atrazine and simazine are the
413 most well-known photosynthesis inhibitor herbicides, which mainly affect the
414 photosynthetic electron transport chain (Bai et al. 2015). Different results were obtained
415 when various parameters were used to assess the type of interaction on the pesticide
416 mixtures by the same model (Moreira et al. 2020). Atrazine and simazine have the same
417 mode of action on the photosynthetic electron transport chain, so the parameter Φ'_M,
418 reflecting the efficiency of the entire chain of photosynthesis electron transport, was
419 chosen rather than growth rate or another photosynthetic parameter to improve the
420 accuracy of the interaction assessment. As we expected, Φ'_M was the most sensitive
421 indicator in this study. We observed a stronger reduction in Φ'_M for the mixture of
422 atrazine and simazine over single additions for all the taxa and light conditions
423 investigated (except for both *M. aeruginosa* strains under VLL conditions) at all tested
424 concentrations, showing that the herbicide mixtures produced a synergistic effect (Fig.
425 5). Considering that atrazine and simazine act similarly on PSII, the observed synergism
426 for all phytoplankton might be caused by a combined toxicity effect on the
427 photosynthetic apparatus. Regarding the photosynthetic toxicity of herbicide mixtures,
428 it appears that the three species showed different sensitivities under different light
429 conditions (VLL, LL, ML). However, the green alga (CPCC90) was more sensitive to
430 the herbicide mixtures than either cyanobacteria taxon (CPCC632 and CPCC299) under
431 VLL and LL conditions, while the opposite response occurred with single herbicides.

This result indicates that natural phytoplankton community compositional shifts may occur in contaminated areas depending on the light conditions in the waterbody and given that different phytoplankton taxa often occur at different depths. Moreover, mixtures of pesticides also modified the sequence of taxon sensitivity levels to herbicides even without considering the light intensity factor. Thus, phytoplankton community compositional changes should also be sensitive to differences in herbicide mixture toxicity as has been previously advanced (Gregorio et al. 2012).

4.3 Combination of light and binary herbicide mixtures

It is generally recognized that pesticides can interact with each other in the aquatic environment and that light intensity is an environmental factor that may also become stressful. Thus, pesticides and light are part of a combination of multiple factors that may contribute to changes in phytoplankton community composition (Fischer et al. 2010, Laetz et al. 2014). Our study has demonstrated that moderate increases in ambient light augmented the synergistic phototoxicity for the green alga CPCC90 and the non-toxic cyanobacteria CPCC632 at low or high herbicide concentration ranges. Furthermore, the magnitude of the synergism observed for herbicide mixtures also depended on the taxon: strong for CPCC632, moderate for CPCC90, and close to additivity for the toxic strain CCPCC299. The effect of light as an environmental factor on the toxicity of single pesticide in numerous aquatic species has been studied (Baxter et al. 2016). However, studies to date have only assessed the interaction between light and a single chemical (first-order interactions), with increasing light intensity ordinarily

453 enhancing the toxicity (Deblois et al. 2013, Wood et al. 2016). A few studies have
454 reported that high light intensity can increase the uptake of pollutants, such as cadmium,
455 zinc, and phosphorus (Du et al. 2019, Sforza et al. 2018, Xu and Juneau 2016). We also
456 shown recently that the removal of atrazine from the growth medium by marine
457 phytoplankton (green algae and diatoms) was enhanced at higher light intensity (Du et
458 al. 2023). Our current evaluation of the interaction of light and binary herbicide
459 mixtures showed reduced effects on photosynthetic efficiency (Φ'_M) for the three
460 species grown under VLL condition (additive/antagonistic effect) compared to LL
461 (synergistic effect). This may be attributed to the impact of light intensity on the uptake
462 of herbicide, since light intensity alone did not affect photosynthetic efficiency (all taxa
463 having the same Φ'_M under VLL and LL conditions). Another reason behind the
464 increased toxicity of binary herbicide mixtures in the LL condition may be related to
465 the muted induction of the photoprotective ability (NPQ) in all taxa. However, the
466 inhibitory effect of binary herbicide mixtures under ML condition was further enhanced
467 even though NPQ was highly induced. This result suggests that other protective
468 measures, such as the antioxidant enzyme system, may play a major role against the
469 photosynthetic damage caused by mixtures of herbicides (Du et al. 2023, Lozano et al.
470 2014, Mofeed and Mosleh 2013). Therefore, further mechanistic studies are needed to
471 determine how each of these processes contributes to the overall response. Medium
472 light adaptation (ML) decreased the toxicity of binary herbicide mixtures compared to
473 the low light adaptation (VLL and LL) (Table 2), similar to the results obtained for the
474 single herbicide treatments (atrazine or simazine). Moreover, this adaptation process

was enough to cope against the dual effects of the high light (HL) treatment and a single herbicide, but it was not sufficient to protect against damage caused by the ~~combined impacts~~ presence of more than one pesticide and high light. Therefore, studies considering only single herbicide and environmental factor (such as light, temperature, and nutrients) effects may underestimate the toxic effects of herbicides on algal communities since herbicide and environmental stressor interaction can occur in water bodies.

5. Conclusions

We investigated the combined effect of light levels with single and binary herbicide mixtures on photosynthetic parameters of three freshwater phytoplankton strains. We found that both toxic and non-toxic cyanobacteria (*Microcystis aeruginosa*) strains were more sensitive than a green alga taxon (*Chlorella vulgaris*) to single photosynthetic herbicide application. On the other hand, the opposite was observed for binary herbicide mixtures, indicating that care should be taken when extrapolating the impact of single herbicide on phytoplankton communities in aquatic ecosystems. Furthermore, light intensity should be considered in assessments of herbicide risks. Indeed, we have shown that high light intensity stress increased the toxicity of single and binary herbicide mixtures to three freshwater phytoplankton taxa. However, adaptation to high light was shown to protect against exposure to low concentrations of single and binary herbicide mixtures. Our results underline the necessity to understand

the interactions between contaminants in relation to the impact that variable environmental factors may have on their toxicity.

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