Interspecific differences in the response of autotrophic microorganisms to atrazine and S-metolachlor exposure

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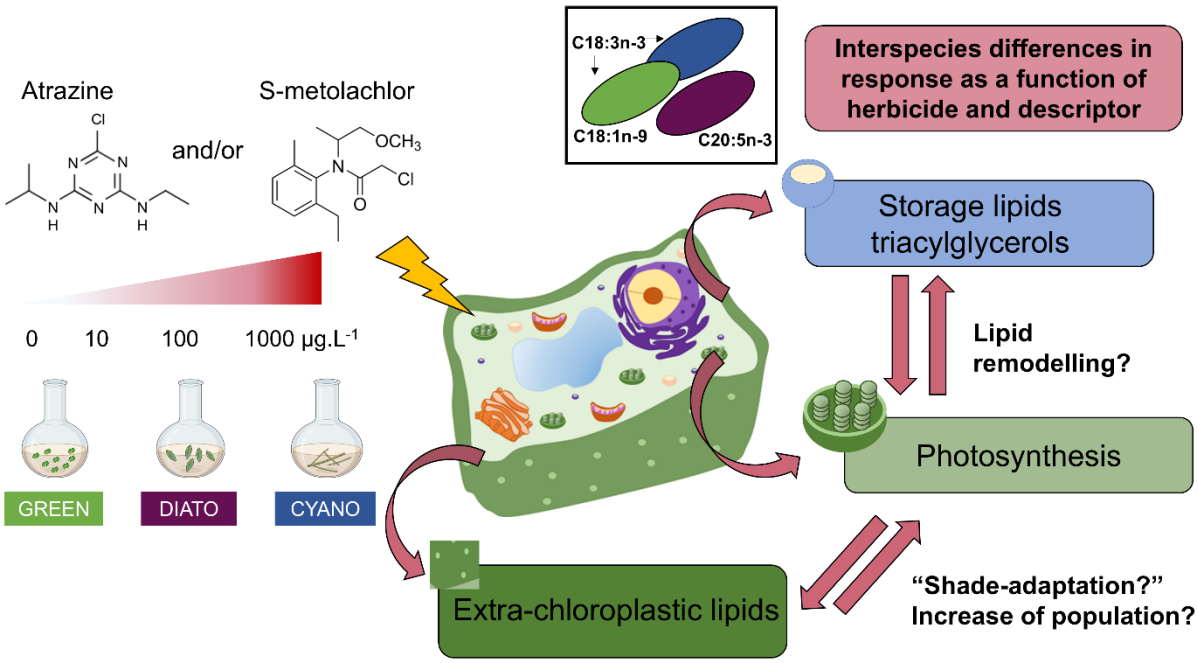
Highlights:

- **Atrazine and S-metolachlor significantly impacted photosynthesis parameters**
- **Atrazine and S-metolachlor significantly impacted lipids and fatty acids profiles**
- **Environmental concentration of S-metolachlor decreased C18:3n-3 content in green algae**
- **Mixture effects were driven by atrazine and S-metolachlor according to their mode of action**
- **Ecotoxicological studies should consider the response of multiple species to various descriptors**

Abstract:

Atrazine and S-metolachlor are herbicides widely used on corn and soybean crops where they are sometimes found in concentrations of concern, in nearby aquatic ecosystems, potentially affecting autotrophic organisms. The aim of this study was to investigate the response of the green alga *Enallax costatus*, the diatom *Gomphonema parvulum* and a culture of the cyanobacteria *Phormidium* sp. and *Microcystis aeruginosa*, to atrazine and S-metolachlor alone and in mixture (0, 10, 100 and 1000 $\mu g.L^{-1}$, for 7 days). For each culture, chlorophyll fluorescence and effective quantum yield of photosynthesis were determined and compared with lipid and methyl-ester fatty acid profiles. In general, the green algae was most strongly affected by atrazine and S-metolachlor. In particular, atrazine led to a total inhibition of photosynthesis and a sharp decrease in triacylglycerols (TAGs), while S-metolachlor caused a partial decrease in photosynthesis in the green algae and a sharp increase in reserve lipids in the diatom when the herbicide was in mixture. The effect of the mixture of compounds depended on the descriptor considered. Indeed, atrazine seemed to explain the toxicity of the mixture for photosynthetic parameters, while certain lipid classes showed intermediate responses between compounds. The results suggest mechanisms of shade adaptation, algal population increase and lipid remodeling in response to compound exposure. The results reveal differences in sensitivity between species after 7 days exposure to the two compounds alone and in mixture. These results support the value of using the study of lipid and fatty acid profiles as complementary information to traditional descriptors for the assessment of pesticide exposure on photoautotrophic organisms.

Graphical abstract:



Keywords:

Herbicide, mixture, microalgae, cyanobacteria, photosynthesis, lipids

1. Introduction

The extensive use of herbicides in agriculture results in global terrestrial and aquatic ecosystems contamination where they can potentially impact non-target organisms (Gao et al., 2019; Ou-Yang et al., 2022). Atrazine and S-metolachlor are two herbicides commonly used on corn and soybean crops around the world. After application, they may remain in soils for several months and may be transported to nearby aquatic systems by surface runoff (Steffens et al., 2022). Both herbicides are widely detected in freshwaters systems, with concentrations reaching hundreds $\mu g.L^{-1}$ in the United States of America (Ghirardelli et al., 2021; Hansen et al., 2019; Kapsi et al., 2019).

Atrazine [IUPAC name: 6-chloro-4-*N*-ethyl-2-*N*-propan-2-yl-1,3,5-triazine-2,4-diamine] is one of the most widely used herbicides in the world (Religia et al., 2019). It is a photosystem II inhibitor binding with the D1 protein, and thus it interrupts photosynthesis (Vallotton et al., 2008). Certain studies have shown that atrazine has adverse effects on photosynthesis (Gao et al., 2019) and community structure (Pannard et al., 2009) of non-target photosynthetic organisms, and that it represents a potential risk to amphibians, fish, and aquatic invertebrates in highly contaminated areas (USEPA, 2016). The threat atrazine poses to terrestrial and aquatic organisms (de Albuquerque et al., 2020) led to its restriction in Europe since 2003-2004 (European Commission, 2004). S-metolachlor (IUPAC name: 2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-[(2S)-1-methoxypropan-2-yl]acetamide) is a chloroacetanilide composed of 20% R-isomers and 80% of S-isomers, the latter having higher herbicidal activity. It binds to FAE1-synthase to inhibit the elongation of C16 and C18 to C20 fatty acids, limiting the formation of very long chain fatty acids (VLCFAs), and thus disrupting cells membrane stability (Vallotton et al., 2008) leading to impaired growth and development (Copin et al., 2016; Demailly et al., 2019; Liu and Xiong, 2009). Although it does not appear to induce lethal effects on animals such as daphnia or fish at environmental concentrations, S-metolachlor has been classified as a potential endocrine disruptor (Ou-Yang et al., 2022). These two herbicides and many others are found concomitantly in aquatic ecosystems, resulting in a complex mixture of compounds (Cedergreen et al., 2007) that could interact together. Co-exposure to multiple herbicides could lead to additive, synergic or antagonist effects on organisms as compared to individual exposure. It should be noted that some compounds, which not be inherently toxic on their own, can increase the toxicity of one or more other compounds when mixed together. This process is known as potentiation. Risk assessment based on one chemical substance alone, as

routinely conducted in exposure experiments, may thus underestimate the toxicity to organisms. Additional studies that examine the complexity of exposure (compound alone vs. reconstituted or environmental mixtures) must therefore be conducted to improve biological risk assessment protocols (Gardia-Parège et al., 2022).

Microorganisms such as green algae, diatoms and cyanobacteria, are phototrophic organisms widely found in aquatic ecosystems where they may be affected by pesticides. Because they are involved in several environmental processes and that they occupy a pivotal position at the basis of the food web, aquatic phototrophic organisms are relevant as experimental models. Several studies showed differences in sensitivity between phototrophs, and even within distinct taxonomic groups or between genera within a group (Vonk and Kraak, 2020). Sensitivity to a chemical also depends on the duration of the experiment, the compound itself, its concentration and the endpoints studied (Morin and Artigas, 2023; Weiner et al., 2004).

Endpoints may exhibit differences in the degree of precocity and specificity of their response, particularly in relation to the mode of action of the compound being tested. Thus, it seems appropriate to adopt a multi-descriptor approach including several "classical descriptors" such as cellular density and photosynthesis, in combination to complementary descriptors such as lipids and fatty acids profiles (Demailly et al., 2019). In recent years, -omics technologies have been developed and used in ecotoxicology to study the effects of toxic substances at different levels of organisation: individuals (i.e. molecular, cellular, tissue), populations, and communities (Zhang et al., 2018). Among them, lipidomic focuses on changes in lipid profiles that may be used as biomarkers of herbicide exposure in phototrophs (Shanta et al., 2021). Lipids are important constituents of cells; phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are phospholipids mostly found in extra-chloroplastic membranes (e.g., cellular membrane), while chloroplastic membranes (i.e. thylakoids) are characterized by high proportion of glycolipids such as monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG) and the phospholipid phosphatidylglycerol (PG). MGDG and DGDG contribute to the stability and integrity of photosynthetic membranes (Dörmann and Benning, 2002). In addition, triacylglycerols (TAG) are energy-storage compounds (Nakamura and Li-Beisson, 2016).

Lipids are composed of fatty acids (FAs) that are important for the structure and the functioning of cells, and for the nutrition of consumers. In plant cells, saturated fatty acids (SFAs) and mono-unsaturated fatty acids (MUFAs) are mostly dedicated to energy storage,

while poly-unsaturated fatty acids (PUFAs) are involved in maintaining membrane structure and integrity as well as in cellular signalling (Huggins et al., 2004). Certain fatty acids such as eicosapentaenoic acid (EPA, 20:5n-3) or α-linolenic acid (ALA, 18:3n-3) are respectively present in notable proportion in diatoms and green algae (Drerup and Vis, 2016), while odd-chain SFAs (13:0-19:0) and branched-chain FAs highlight the presence of bacteria. Several studies have shown that herbicides can affect the lipid and fatty acid composition of photosynthetic aquatic organisms (Gonçalves et al., 2021; Malbezin et al., 2024; Špoljarić Maronić et al., 2018). In particular, lipid peroxidation from contaminant exposure and the modification of community structure in favor of microorganisms that are poorer in PUFAs (e.g., cyanobacteria) can result in a reduction in the abundance of PUFAs, which are essential FAs for the development of organisms at higher trophic levels (Brett and Müller-Navarra, 1997).

Pesticides may directly (e.g., photosynthesis) and indirectly (e.g., nutritional composition) affect the basis of aquatic food webs which may lead to cascading effects on higher trophic levels (Fleeger et al., 2003). The study of these lipidomic descriptors, in combination to classical descriptors such as photosynthetic parameters, is of great interest for assessing the effects of herbicides on photosynthetic aquatic microorganisms and their potential resulting consequences on aquatic and terrestrial trophic chains. This laboratory study aimed to mimic a more realistic exposure condition than traditional ecotoxicology studies by investigating the response of microalgae and cyanobacteria to herbicide exposure alone and in mixture. Furthermore, the incorporation of multiple descriptors at varying levels of biological organization is intended to provide a comprehensive understanding of herbicide toxicity. The main objectives of this study were (1) to determine the effects of two widely used herbicides, atrazine and S-metolachlor, individually and in mixture, on photosynthesis, lipid content and fatty acid composition of different cultures of phototrophic organisms, (2) to evaluate if there are differences in sensitivity between organisms and (3) to explore potential interactive effects of the two tested herbicides.

2. Materials and methods

2.1. Tested organisms and culture conditions

The green alga *Enallax costatus* (Schmidle) Pascher 1943 TCC744 (formerly *Scenedesmus costatus*) and the diatom *Gomphonema parvulum* (Kützing) Kützing 1849 TCC612 were purchased from INRAE, Thonon-les-Bains, France, while *Phormidium* sp., PMC847 and *Microcystis aeruginosa* Kützing 1846 PMC679 composing the cyanobacteria culture were obtained from the Muséum National d'Histoire Naturelle (Paris, France). All cultures were non-axenically grown in Dauta medium (Dauta, 1982) at pH 7.5. They were maintained in an incubator (Cooled Incubator, LMS) at 20°C, under an average light of 66.7 ± 14.7 µmol photons.m^{-2}.s^{-1} with a 16:8 light:dark cycle (Mazzella et al., 2023a). Prior to exposure, 40 mL of culture were inoculated into 40 mL of Dauta medium in 100 mL sterile borosilicate flasks for at least two growing cycles of 7 days before the beginning of the experiment. Prior and during the experiment, all flasks were gently agitated by hand once a day for 4 seconds to ensure the uniformity of the culture and that no clumps are formed. Hirschmann™ Silicosen silicon caps were used to seal the flasks while allowing for gaseous exchanges between the cultures and the environment. Flasks were moved around the shelves to ensure homogeneous illumination among all flasks.

2.2. Experimental setup

For herbicides exposure, stock solutions of 10 mg.L^{-1} atrazine (>99.4 %) and S-metolachlor (97.9 %) (certified reference materials, Dr. Ehrenstorfer™) were used to prepare 20, 200 and 2000 µg.L^{-1} solutions of each compound alone and in mixture in Dauta medium and stored at 4°C in the dark between each experiment (approximately four months in total). The two herbicides and the mixture were tested on each phototrophic organism in independent exposure experiments. The three experiments lasted 7 days and were conducted sequentially, with experiment 1 involving the green alga *Enallax costatus* (later referred to as GREEN), experiment 2 involving the diatom *Gomphonema parvulum* (DIATO), and experiment 3 involving the cyanobacteria *Phormidium* sp. and *Microcystis aeruginosa* (CYANO).

On the first day of each experiment, the phototrophic organisms under study were diluted with fresh Dauta medium where 40 mL of Dauta were added to 42 mL of culture to reach a total culture volume of 82 mL and herbicides nominal concentrations of 0, 10, 100 and

1000 µg.L^{-1} for each herbicide alone and in mixture. The nominal concentrations of 10 and 100 µg.L^{-1} were selected as they correspond to previously measured concentrations around the world, such as in Europe and in North America (Battaglin et al., 2000; Giroux, 2022; Hansen et al., 2019). The concentration of 1000 µg.L^{-1} is a relatively high concentration in comparison to those typically measured in the environment and was used to determine the potential maximal impacts that can be expected in the cultures. At d0 (d0, after 2-3 hours of exposure) and d7, 40 mL of all biotic conditions were collected in 50 mL Falcon® cryotubes, quenched in liquid nitrogen and stored at -80°C before being freeze-dried using a VirTis BenchTop Pro (SP Scientific, United Kingdom) for subsequent lipidomic analyses. On day 0, herbicide concentrations were verified collecting six flasks of biotic treatments (initial control replicates, medium + culture) and one for abiotic conditions (only medium). On day 7, the four biotic control replicates and the abiotic condition were sampled. Herbicide concentrations were determined by filtering 2 mL of each replicate (0.45 µm polyethersulfone filter) and stored at -20°C pending analysis by high performance liquid chromatography (Dionex Ultimate 3000 HPLC; Thermo Fisher scientific, Illkirch-Graffenstaden, France) coupled to a tandem mass spectrometer (API 2000 triple quadrupole, Sciex, Les Ulis, France) (see Lissalde et al., 2011 for detailed methods). For nutrient analysis (anions and cations), 5 mL of each replicate of the same condition were filtered (0.45 µm PTFE filter) and combined to obtain a 20 mL composite water sample per condition and were stored at 4°C in the dark (24-48 hours) until analysis by ionic chromatography (Metrohm 881 Compact Ionic Chromatograph pro; Metrohm) (supplementary material Table A.1). Quality controls and analytical blanks were regularly added to the analysis sequences. Finally, the fluorescence of chlorophyll a and the effective photosynthetic quantum yield were monitored throughout the experiment, but only data from day 0 and day 7 are shown. For photosynthesis analyses, samples were acclimatised at room light intensity then 2.5 mL of each sample were transferred in quartz cuvettes for measurement with a Pulse Amplitude Modulated fluorimeter (Phyto-PAM-ED; Heinz Walz GmbH, Germany).

2.3.Determination of cellular density for the green algae experiment

The same 2.5 mL of samples used for Phyto-PAM were fixed at 4% with basic Lugol solution and stored at 4°C and in the dark until counting. For each replicate, the sample was homogenized with a vortex, diluted with spring water, and then 150 µL were placed on a

Nageotte counting chamber and left for 10 minutes at room temperature to allow for sedimentation. The number of algal cells was recorded in ten bands of 1.25 µL each (0.5mm depth) with an optical microscope at 400X magnification (Carl Zeiss Ltd Axio Imager 2) to obtain cellular density. Cell density could only be determined for the green algae experiment due to a problem with laboratory logistics.

2.4.Photosynthetic parameters

Each measurement for effective quantum yield and chlorophyll a fluorescence was performed three times for each sample so that the values presented are averages. Chlorophyll a fluorescence was measured and expressed as µg chlorophyll a.L^{-1}. For each culture, a specific calibration was performed based on spectral fingerprints of the culture established prior to exposure (supplementary material Table A.2).

2.5.Lipid and fatty acid analyses

2.5.1. Lipids extraction

Lipids extraction was based on the protocol of Mazzella et al., (2023b). During the extraction process, freeze-dried samples were kept at a cool temperature. For each sample, 10 mg of dry weight (dw) were added to 150 mg of glass beads (0.5 mm diameter, BioSpec Products) and 1 mL of a Methyl Tert-Butyl Ether (MTBE):Methanol (MeOH) (3:1) solution was added. This solution contained also butylated hydroxytoluene (BHT, 0.01% w/v) to avoid lipid peroxidation. This step was followed by 15 s of homogenisation at 6.5 nm using a FastPrep-24™5G (mpbio) after which samples were cooled at -80 °C for 3 min. This step was conducted twice. Then, 650 µL of H_20:MeOH (3:1) were added, followed by another homogenisation cycle and a cooling phase at -80°C. After centrifugation for 5 min at 4°C and 12,000 rpm, 500 µL of the upper phase were transferred to vials. The pellet was saved for another extraction using the same procedure as described above but with 700 µL for MTBE:MeOH and 455 µL of H_20:MeOH. The upper phase of this second extraction was added to the vial containing the first extraction. Samples were kept at -80°C until injection and fractionation. Lipid extracts were analyzed by high performance liquid chromatography (Dionex Ultimate 3000 HPLC; Thermo Fisher scientific, Illkirch-Graffenstaden, France) coupled to a tandem mass spectrometer (API 2000 triple quadrupole, Sciex, Les Ulis, France). A Luna® NH2 HILIC column (100 x 2 mm, 3 µm) from Phenomenex was used for the chromatographic separation of both phospholipids and glycolipids. A mixture of acetonitrile and 40 mM ammonium acetate buffer was used as the mobile phase and the flow rate was set at 400 $\mu L.min^{-1}$. For the analysis of the triglyceride class, a KINETEX® C8 column (100 x 2.1 mm, 2.6 µm) from Phenomenex was used as the stationary phase, and the mobile phase was a mix of a solution of acetonitrile/ultra-pure water/40 mM ammonium acetate buffer (600/390/10, v/v/v) and a solution of isopropanol/acetonitrile/1 M ammonium acetate buffer (900/90/10, v/v/v). The flow rate was set at 300 $\mu L.min^{-1}$. More details are available in (Mazzella et al., 2023a).

2.5.2. Lipids fractionation

The lipid fractionation step allows for the separation of fatty acids from neutral lipids (storage lipids) and polar lipids (chloroplastic and non-chloroplastic membranes) by solid phase

extraction. The protocol was adapted from the Hamilton and Comai, (1988) method. To summarise, 5 mL of MeOH (HPLC grade) were used to dehydrate the Sep-Pak cartridge (1 g of silica, Waters), and then 5 mL of MTBE (HPLC grade) were used to condition it. For each sample, 150 µL of previously obtained lipid extracts were poured into a SPE-conditioned cartridge, then 10 mL of MTBE (HPLC grade) were added to elute glycerides in a 15 mL tube. A clean tube was used to collect glycolipids after the addition of 10 mL of acetone, and phospholipids were collected after addition of 10 mL of MeOH. Both fractions were evaporated under a nitrogen flow and 300 µL of MeOH were added. After homogenisation with a vortex, samples were placed in clear vials and stored at -80°C before derivatisation.

2.5.3. Fatty acid derivatisation

For the two lipid fractions, 150 µL of each sample were transferred in 10 mL crimp vials and evaporated. Then, 6 µL of standard C17:0 at 1 $g.L^{-1}$ in isopropanol were added, followed by 2 mL of MeOH at 2.5% of H_2SO_4 and 5% of ultra-pure water. Vials were crimped, vortexed for 30 s and heated for 1 hour at 80°C with a stirring speed of 150 rpm. Vials were left to cool for 10 min at room temperature and then the content was transferred to 15 mL tubes. 6 µL of C19:0 in hexane, 3 mL of ultra-pure water and 300 µL of hexane were added and tubes were vortexed for 30 s. This was followed by a centrifugation for 5 min at 1500 g to obtain two phases. A maximum of 300 µL of the supernatant (i.e. hexane) containing the fatty acid methyl esters (FAMEs) were recovered and transferred to 1-mL glass vials which were stored at -80°C pending GC-MS analysis (GC Thermo Scientific Trace 1310 coupled to a Thermo Scientific ISQ mass spectrometer single quadrupole detector, Agilent J&W GC column DB-FastFAME column (30 m length, 0.250 mm inner diameter, 0.25 µm film thickness)).

2.6.Data analyses

All analyses were performed in RStudio 2024.04.2 Build 764 (R version 4.2.2). Normality and homogeneity of variances were verified using residual plots as well as with Shapiro-Wilk and Levene's tests. For all parameters, statistical tests were performed on raw data. Differences in controls between initial day (d0) and final day (d7) were investigated by Student's tests or Wilcoxon-Mann-Whitney tests depending on whether assumptions were met or not. One-way ANOVA with Dunnett's test or Kruskal-Wallis with Dunn's test (Holm adjustment) were used to assess the effect of exposure concentration. For visualization, figures were performed on chlorophyll fluorescence and PSII quantum yield data at day 7 (normalized to the control). Day 7 averages represented a 100% reference point for recalculating percentages for all conditions. Figures were created using *drc* (Ritz et al., 2015, R version: 3.0-1) and *ggplot2* (Wickham, 2016, R version: 3.5.1) with *drm* method to perform a four-parameter log-logistic model because lower limit was not null except for effective PSII quantum yield of the GREEN experiment. FAMEs data were corrected using blank averages, recalculated as percentages. FAMEs with a relative abundance greater than 5% were included in a multivariate analysis using non-metric multidimensional scaling (nMDS) on a dissimilarity matrix (method="gower") with the *vegan* package (Oksanen et al., 2022, R version 2.6-4). PERMANOVAs were performed with the "adonis2" (method="gower") from *vegan* package and "pairwise.adonis2" functions from *pairwiseAdonis* package (Arbizu, 2017, R version: 0.2) to identify differences in fatty acid profiles between conditions. The model employed was as follows: distance matrix ~ Organism * Nominal Concentration + Herbicide + Nominal Concentration. A Principal Component Analysis (PCA) on total fatty acids was performed on FAMEs with a relative abundance greater than 5% and filtered by cos^2>0.10, using *FactoMineR* (Lê et al., 2008, R version: 2.10) and *factoextra* (Kassambara and Mundt, 2020, R version: 1.0.7). Details of statistical tests are presented as supplementary material (Table A.3, Table A.4, Table A.5, Table A.6, Table A.7, Table A.8, Table A.11). Raw data are available at https://doi.org/10.57745/JYZDDE.

3. Results

For all experiments, control conditions showed no presence of atrazine or S-metolachlor. Both herbicides remained relatively stable under biotic and abiotic conditions. However, atrazine at nominal concentration of 10 $\mu g.L^{-1}$ increased from the first experiment (GREEN culture, Table 1), to the second experiment (DIATO culture, Table 2) and to the third experiment (CYANO culture, Table 3). This difference in atrazine concentration between experiments (for the same stock solution) may be due to incomplete solubilisation of atrazine powder during the GREEN experiment, followed by progressive dissolution over time despite storage at 4°C, thus increasing the effective concentration of the contaminant in the solution. Measured S-metolachlor concentrations were close to nominal concentrations for the DIATO experiment, while it decreased between day 0 and day 7 under biotic conditions for the GREEN and the CYANO experiments. Despite targeted concentrations deviating from nominal values, a concentration gradient was still observed in all experiments for both herbicides, individually and in mixture.

333 Table 1: Herbicides concentration (mean µg.L^{-1}±standard deviation) measured at initial and final days of experiment for GREEN (*Enallax costatus*).

Initial day	**Nominal concentration** (µg.L^{-1})	**Atrazine concentration at day 0**		**S-metolachlor concentration at day 0**	
Medium + culture	**Atrazine**	Mean±standard deviation	%nominal	Mean±standard deviation	%nominal
	10	11.8±1.6	118		
	100	113±19	113		
	1000	941±114	94		
	S-metolachlor				
	10			9.3±0.9	93
	100			77±6	77
	1000			749±77	75
	Mixture				
	10/10	9.6±1.0	96.4	10.5±1.1	105
	100/100	119±8	119.2	87±8	87
	1000/1000	869±154	86.9	667±111	67

Final day		**Atrazine concentration at day 7**		**S-metolachlor concentration at day 7**	
Medium + culture	**Atrazine**	Mean±standard deviation	%nominal	Mean±standard deviation	%nominal
	10	9.1±0.4	91		
	100	119±6	119		
	1000	1046±194	105		
	S-metolachlor				
	10			1.9±0.1	19
	100			11±3	11
	1000			66±12	7
	Mixture				
	10/10	7.7±0.5	77	2.2±0.2	22
	100/100	115±8	115	12.7±1.6	13
	1000/1000	1090±98	109	242±34	24

Medium	**Atrazine**	Value (one replicate)	%nominal	Value (one replicate)	%nominal
	10	14.4	144		
	100	105	105		
	1000	1025	103		
	S-metolachlor				
	10			9.6	96
	100			61	61
	1000			685	69
	Mixture				
	10/10	10.0	100	8.9	89
	100/100	130	130	91	91
	1000/1000	970	97	835	84

334

335 Table 2: Herbicides concentration (mean µg.L^{-1}±standard deviation) measured at initial and final days of experiment for DIATO (*Gomphonema parvulum*).

Initial day	**Nominal concentration** (µg.L^{-1})	**Atrazine concentration at day 0**		**S-metolachlor concentration at day 0**	
Medium + culture	**Atrazine**	Mean±standard deviation	%nominal	Mean±standard deviation	%nominal
	10	29.2±1.0	292		
	100	86±3	86		
	1000	1002±12	100		
	S-metolachlor				
	10			8.4±0.5	84
	100			80±7	80
	1000			793±100	79
	Mixture				
	10/10	12.9±0.7	129	6.8±0.7	68
	100/100	86±0.5	86	65±12	65
	1000/1000	964±142	96	754±56	75
		Atrazine concentration at day 0		**S-metolachlor concentration at day 0**	
Medium	**Atrazine**	Value (one replicate)	%nominal	Value (one replicate)	%nominal
	10	28.4	284		
	100	80	80		
	1000	981	98		
	S-metolachlor				
	10			8.8	88
	100			90	90
	1000			750	75
	Mixture				
	10/10	12.4	124	6.9	69
	100/100	85	85	73	73

	1000/1000	1033	103	795	80
Final day		**Atrazine concentration at day 7**		**S-metolachlor concentration at day 7**	
Medium + culture	**Atrazine**	Mean±standard deviation	%nominal	Mean±standard deviation	%nominal
	10	30.0±0.6	300		
	100	85±2	85		
	1000	1047±52	105		
	S-metolachlor				
	10			8.9±0.8	89
	100			85±6	85
	1000			939±122	94
	Mixture				
	10/10	13.0±0.3	131	8.9±1.9	89
	100/100	90±2	90	74±5	74
	1000/1000	1101±38	110	831±79	83
		Atrazine concentration at day 7		**S-metolachlor concentration at day 7**	
Medium	**Atrazine**	Value (one replicate)	%nominal	Value (one replicate)	%nominal
	10	29.8	298		
	100	81	81		
	1000	1037	104		
	S-metolachlor				
	10			9.5	95
	100			84	84
	1000			815	82
	Mixture				
	10/10	12.7	127	7.8	78
	100/100	90	90	63.5	64

	1000/1000	1137	114	955	96

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337 Table 3: Herbicides concentration (mean µg.L^{-1}±standard deviation) measured at initial and final days of experiment for CYANO (*Phormidium* sp. and *Microcystis*
338 *aeruginosa*).

Initial day	**Nominal concentration** (µg.L^{-1})	**Atrazine concentration at day 0**		**S-metolachlor concentration at day 0**	
Medium + culture	**Atrazine**	Mean±standard deviation	%nominal	Mean±standard deviation	%nominal
	10	75±7	750		
	100	79±14	79		
	1000	969±67	97		
	S-metolachlor				
	10			7.7±1.1	77
	100			69±4	69
	1000			725±103	73
	Mixture				
	10/10	41±10	408	6.6±0.6	66
	100/100	87±3	87	57±5	57
	1000/1000	928±100	93	629±96	63
		Atrazine concentration at day 0		**S-metolachlor concentration at day 0**	
Medium	**Atrazine**	Value (one replicate)	%nominal	Value (one replicate)	%nominal
	10	80	796		
	100	81	81		
	1000	900	90		
	S-metolachlor				
	10			7.6	76
	100			72	72
	1000			735	74
	Mixture				

	10/10	31	311	5.4	54
	100/100	84	84	83	83
	1000/1000	825	83	600	60
Final day		**Atrazine concentration at day 7**		**S-metolachlor concentration at day 7**	
Medium + culture	**Atrazine**	Mean±standard deviation	%nominal	Mean±standard deviation	%nominal
	10	69±4	692		
	100	86±7	86		
	1000	835±57	84		
	S-metolachlor				
	10			3.5±0.4	35
	100			8.6±0.9	9
	1000			417±57	42
	Mixture				
	10/10	57±6	568	3.3±0.4	33
	100/100	74±29	74	28±4	28
	1000/1000	950±99	95	416±59	42
		Atrazine concentration at day 7		**S-metolachlor concentration at day 7**	
Medium	**Atrazine**	Value (one replicate)	%nominal	Value (one replicate)	%nominal
	10	62	620		
	100	78	78		
	1000	1000	100		
	S-metolachlor				
	10			7.1	71
	100			68	68
	1000			720	72

Mixture					
10/10	13	127	7.8	78
100/100	90	90	64	64
1000/1000	1137	114	955	96

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3.1. Classical descriptors

3.1.1. Cellular density for the GREEN experiment

The cell density of the green alga *Enallax costatus* increased from $6.15x10^6 \pm 0.97x10^6$ cells.mL^{-1} to $2.19x10^7 \pm 0.45x10^7$ cells.mL^{-1} in controls after 7 days (p-value<0.01) (Figure 1, supplementary material Table A.3). Exposure to atrazine resulted in a significant decrease in cell density at the 100 µg.L^{-1} treatment compared to control ($1.27x10^7 \pm 0.33x10^7$ cells.mL^{-1} ; p-value<0.01). Cell density at the highest atrazine concentration was also significantly lower than control ($0.98x10^7 \pm 0.03x10^7$ cells.mL^{-1}; p-value<0.001). The higher concentration of S-metolachlor, as well as the mixture of the two herbicides, also led to a significant decrease in cell density compared to control, respectively $0.99x10^7 \pm 0.22x10^7$ cells.mL^{-1} (p-value<0.001) and $0.81x10^7 \pm 0.14x10^7$ cells.mL^{-1} (p-value<0.001). Chlorophyll a concentration per cell (estimated by fluorescence) slightly decreased in controls (from $0.95x10^{-7} \pm 0.11x10^{-7}$ to $0.65x10^{-7} \pm 0.17x10^{-7}$ µgchla.cell^{-1} between d0 and d7), while atrazine exposure resulted in an increase reaching up to $2.81x10^{-7} \pm 0.15x10^{-7}$ µgchla.cell^{-1} for the 1000 µg.L^{-1} concentration (p-value<0.01). The mixture led to a significant increase, reaching up to $2.91x10^{-7} \pm 0.63x10^{-7}$ µgchla.cell^{-1} (p-value<0.05), while S-metolachlor alone had no effect on chlorophyll concentration per cell.

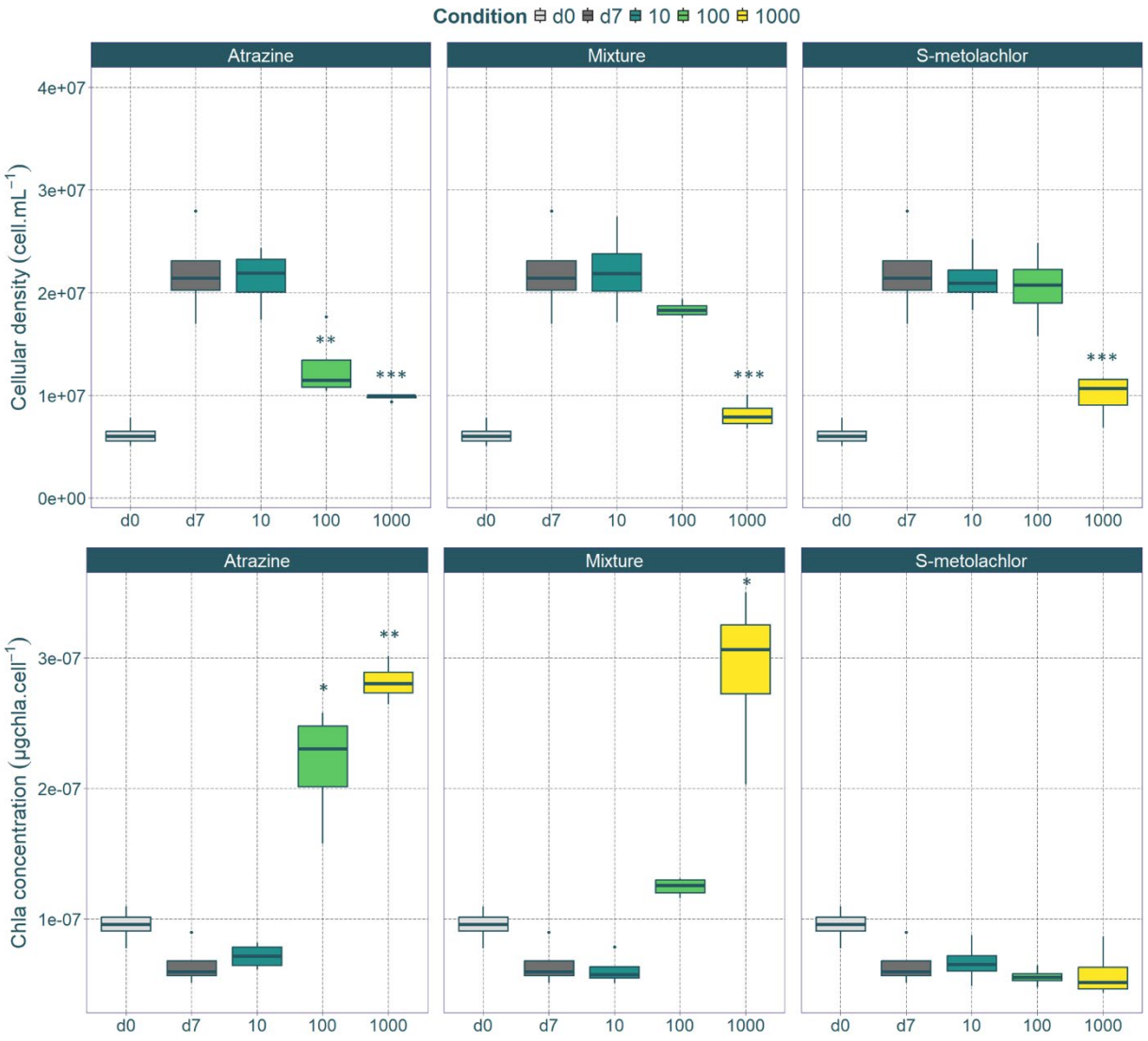
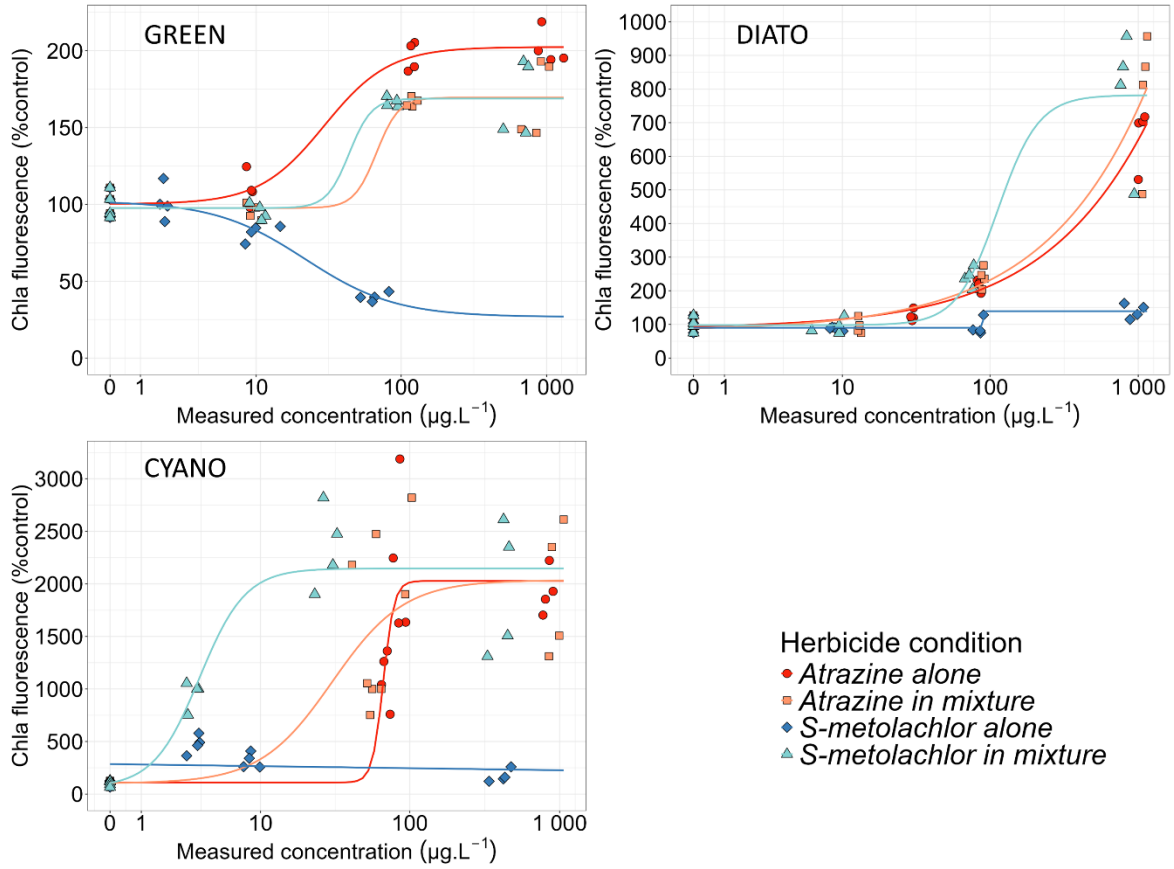


Figure 1: Cellular density (cells.mL^{-1}) and chlorophyll fluorescence normalized by cell number (µg chla.cell^{-1}) for the GREEN experiment at the different exposure treatments (nominal concentrations). d0= initial control, d7= final control (* p-value<0.05, ** p-value <0.01 and *** p-value<0.001 for difference with final control (d7)).

3.1.2. Effect of herbicides on photosynthetic parameters

Chlorophyll fluorescence increased in controls between the beginning and the end of the GREEN and DIATO experiments (from 577 ± 51 µgchla.L^{-1} to 1369 ± 111 µgchla.L^{-1} and from 5 ± 1 µgchla.L^{-1} to 8 ± 2 µgchla.L^{-1}, respectively, p-values<0.05) (Figure 2, supplementary material Table A.4). Pigment fluorescence markedly increased compared to controls under atrazine exposure, especially for the DIATO and CYANO, with respective fluorescence values of 663 ± 80% and 1927 ± 198% at 1000 µg.L^{-1} compared to controls (p-value<0.01) (Figure 2). Significant differences in chlorophyll fluorescence between atrazine exposure and controls were observed at 10 µg.L^{-1} for the CYANO experiment (p-value<0.05) and at 100 µg.L^{-1} of atrazine and 100 µg.L^{-1} of the mixture for both DIATO and GREEN experiments (all p-values<0.001). In GREEN, S-metolachlor led to a lower fluorescence at 100 µg.L^{-1} (p-value<0.05) and 1000 µg.L^{-1} (p-value<0.001), while in CYANO it increased the fluorescence at 10 µg.L^{-1} (p-value<0.001) and 100 µg.L^{-1} (p-value<0.01), but not at 1000 µg.L^{-1}. The increase in chlorophyll a fluorescence under mixture conditions showed similar trends as for exposure to atrazine alone.



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380 Figure 2: Chlorophyll a fluorescence as a function of the control (% control at day 7) after 1-week exposure to
381 atrazine and S-metolachlor alone and in mixture for all experiments, with GREEN for the green algae, DIATO for
382 the diatom and CYANO for the cyanobacteria. Red circles: atrazine alone; orange squares: atrazine in mixture;
383 blue diamonds: S-metolachlor alone; cyan triangles: S-metolachlor in mixture.

At the beginning of the experiment (day 0), the effective PSII quantum yield in the controls was 0.30 ± 0.03 for GREEN, 0.42 ± 0.07 for DIATO and 0.27 ± 0.05 for CYANO. This parameter slightly increased to 0.41 ± 0.04 for GREEN and decreased to 0.29 ± 0.03 for DIATO after 7 days (both p-value<0.01), while no changes were observed for CYANO.

After 7 days of herbicide exposure, all cultures showed an inhibition of effective PSII quantum yield compared to controls, however, not all were affected to the same extent (Figure 3, supplementary material Table A.5). Atrazine at the highest concentration led to the total inhibition of photosynthesis for GREEN (p-value<0.01) and to a partial inhibition for CYANO (p-value<0.01) and for DIATO (p-value<0.001), leading to respective responses of 16.2 ± 23.7% and 51.7 ± 5.4% compared to controls at day 7. DIATO was the only culture showing a significant inhibition at 10 $\mu g.L^{-1}$ (p-value<0.05) and 100 $\mu g.L^{-1}$ of atrazine (p-value<0.001). The highest concentration of S-metolachlor significantly reduced the effective quantum yield of GREEN (47.2 ± 4.8% of the control, p-value<0.001), while the yield of CYANO was slightly impacted at 10 $\mu g.L^{-1}$ (p-value<0.05). The effective quantum yield of DIATO was not impacted by S-metolachlor exposure at any tested concentration.

In general, the dose-response patterns observed under exposure to the mixture of atrazine and S-metolachlor were similar to those of atrazine alone. The mixture affected all cultures similarly under the same concentrations of atrazine. Moreover, the inhibition of the effective quantum yield under S-metolachlor alone was in general low, for a given concentration, compared to atrazine at the same exposure concentration.

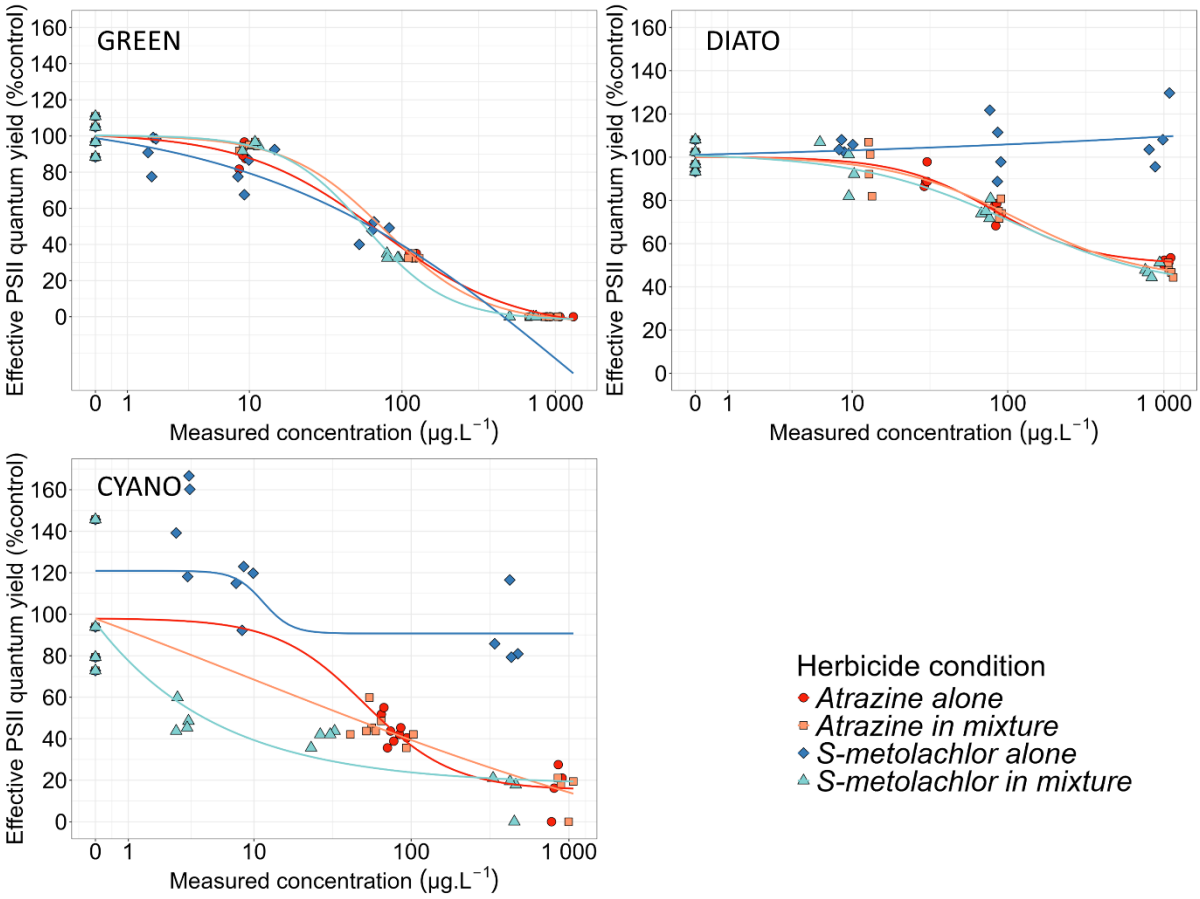

Figure 3: Effective PSII quantum yield as a function of the control (%control at day 7) after 1-week exposure to atrazine and S-metolachlor alone and in mixture for all experiments, with GREEN for the green algae, DIATO for the diatom and CYANO for the cyanobacteria. Red circles: atrazine alone, orange squares: atrazine in mixture, blue diamonds: S-metolachlor alone and cyan triangles: S-metolachlor in mixture.

3.2.Lipidomic descriptors

3.2.1. Effect of herbicides on lipid content

3.2.1.1. Control condition over time

Proportions of each lipid class are shown for the GREEN, DIATO and CYANO experiments (Table 4, Table 5 and Table 6, respectively). On day 0, higher polar lipid content was detected in GREEN (47.3 ± 5.8 nmol.mg^{-1} dw) and CYANO (16.1 ± 2.4 nmol.mg^{-1} dw) compared to DIATO where it reached 8.0 ± 2.4 nmol.mg^{-1} dw. Polar lipids were mostly represented by MGDG followed by PG, PE, DGDG, SQDG and PC. Triacylglycerols (TAGs), i.e. non-polar lipids, were particularly abundant in DIATO (18.6 ± 10.0 nmol.mg^{-1} dw) and in GREEN (18.0 ± 4.0 nmol.mg^{-1} dw), whereas they were not detected in CYANO.

Between the beginning and the end of the experiment, lipid content slightly changed in the controls. In GREEN, glycolipids, phospholipids and total polar lipids decreased (p-values<0.05, Table 1). In DIATO, phospholipids such as PE and PC increased, while DGDG and SQDG increased (all p-values<0.05) without changing total glycolipids content. Lipid content remained stable in CYANO, with only a slight increase of PE.

3.2.1.2. Effect of contaminants on lipid profiles

Both herbicides, individually and in mixture, affected lipid content at different concentrations and induced different responses depending on the phototroph culture. After 7 days of exposure, atrazine appeared to have a concentration-dependent effect in CYANO, with a significant increase in total polar lipids from 16.4 ± 3.2 nmol.mg^{-1} dw to 24.0 ± 2.3 nmol.mg^{-1} dw for the 10 µg.L^{-1} treatment (p-value<0.01) (Table 3). At this condition, glycolipids as SQDG and phospholipids as PG increased (all p-value <0.05). On the contrary, the highest concentration of 1000 µg.L^{-1} led to a small decrease of total polar lipids (p-value<0.05), explained by a decrease in the glycolipids MGDG and DGDG. In DIATO (Table 2), polar lipids increased from 10.0 ± 2.5 nmol.mg^{-1} dw to 20.9 ± 6.8 nmol.mg^{-1} dw at the highest exposure concentration of atrazine (p-value<0.01). This increase is mainly explained by the increase in MGDG in this culture, from 3.55 ± 0.89 nmol.mg^{-1} dw in controls to 12.0 ± 3.82 nmol.mg^{-1} dw at 1000 µg.L^{-1} (p-value<0.05). Atrazine exposure also resulted in a significant decrease in TAGs content, only in GREEN (Table 1), between the control condition (22.9 ± 4.1 nmol.mg^{-1}

dw) and the 100 µg.L^{-1} condition (12.6 ± 2.9 nmol.mg^{-1} dw; p-value<0.05) as well as the 1000 µg.L^{-1} condition (4.6 ± 1.0 nmol.mg^{-1} dw; p-value<0.001).

Exposure to S-metolachlor appeared to have a limited effect on total polar lipid levels in all cultures. However, it led to the increase of total phospholipids in both GREEN and DIATO (all p-value<0.05), increasing from 14.5 ± 1.7 to 20.4 ± 4.4 nmol.mg^{-1} dw at 100 µg.L^{-1} and to 24.7 ± 2.4 nmol.mg^{-1} dw at 1000 µg.L^{-1} for GREEN and from 4.78 ± 1.74 to 7.92 ± 1.60 nmol.mg^{-1} dw at 1000 µg.L^{-1} for DIATO. The highest concentration of S-metolachlor led to small increases in PE of GREEN, DIATO and CYANO, to the increase of PG only in GREEN while the lowest concentration (10 µg.L^{-1}) led to an increase in PC of DIATO (all p-value<0.05). Finally, concentrations of 10 and 100 µg.L^{-1} led to a decrease in DGDG in CYANO, while the highest concentration led to a decrease in GREEN.

Herbicide mixture led to significant effects on GREEN polar lipids, whereas this was not the case with the compounds alone. For example, the 100 µg.L^{-1} mixture led to an increase in polar lipids from 36.6 ± 3.9 nmol.mg^{-1} dw to 50.3 ± 3.2 nmol.mg^{-1} dw (p-value<0.05). This increase is mainly due to the increase of glycolipids MGDG and DGDG as well as phospholipid PG. In addition, TAGs content increased from 22.9 ± 4.1 nmol.mg^{-1} dw (control) to 67.9 ± 15.4 nmol.mg^{-1} dw at 100 µg.L^{-1} (p-value<0.001), but returned to a level comparable of the controls at 1000 µg.L^{-1}. In DIATO, TAGs content also increased from 21.2 ± 3.1 to 34.1 ± 3.6 nmol.mg^{-1} dw only at the lowest concentration.

Table 4: Evolution of lipid contents (nmol.mg^{-1} dry weight) for the GREEN experiment (* p-value<0.05, ** p-value<0.01 and *** p-value<0.001; for controls: significant differences between initial and final control, for contaminated conditions: differences between herbicide concentration and final control d7). Statistics for Wilcoxon-Mann-Whitney test (W) or Student's test (t) for initial and final control comparison: MGDG t=4.1, DGDG t=3.9, ∑Glycolipids t=3.4, ∑Phospholipids t=2.4. Statistics for ANOVA (F) or Kruskal-Wallis (Chi-squared): MGDG F=9.8 for atrazine and F=7.3 for mixture, DGDG F=22.7 for atrazine and F=10.5 for mixture, SQDG F=19.3, PG F=10.5 for mixture and F=18.1 for S-metolachlor, PE Chi-squared=9.6, ∑Glycolipids F=5.3 for mixture and F=4.7 for S-metolachlor, ∑Phospholipids F=7.3 for mixture and F=18.4 for S-metolachlor, ∑Polar lipids F=4.6 for atrazine and F=7.2 for mixture, TAG F=19.6 for atrazine and F=11.1 for mixture (with degree of freedom in the numerator, i.e DFn=3, and the degree of freedom in the denominator, i.e DFd=12).

GREEN	Initial control (d0)	Final control (d7)	Atrazine			Mixture			S-metolachlor		
Nominal concentration (µg.L^{-1}) / Contamination levels	0	0	10	100	1000	10	100	1000	10	100	1000
MGDG	23.2 ± 2.7	**16.6 ± 2.1****	15.1 ± 2.2	**22.4 ± 2.1***	**24.6 ± 4.5****	17.0 ± 4.3	**24.5 ± 2.4***	**24.0 ± 3.4***	15.5 ± 0.6	17.9 ± 4.1	11.4 ± 3.5
DGDG	1.86 ± 0.09	**1.47 ± 0.23****	1.46 ± 0.09	1.12 ± 0.17	**0.41 ± 0.26*****	1.62 ± 0.24	1.09 ± 0.34	**0.72 ± 0.16****	1.69 ± 0.25	1.74 ± 0.40	1.67 ± 0.26
SQDG	3.67 ± 0.45	4.03 ± 0.45	3.95 ± 0.72	5.07 ± 1.18	3.65 ± 1.05	4.06 ± 0.59	4.47 ± 0.17	4.30 ± 0.79	3.99 ± 0.66	3.53 ± 0.11	**1.99 ± 0.34*****
PG	13.2 ± 2.4	10.2 ± 1.2	8.5 ± 2.0	11.3 ± 1.3	11.2 ± 1.4	9.1 ± 2.3	**15.7 ± 1.1****	**15.0 ± 3.0***	8.3 ± 1.2	**14.8 ± 2.2***	**16.9 ± 2.6****
PE	4.82 ± 1.28	3.65 ± 1.21	2.53 ± 1.94	4.19 ± 0.99	5.12 ± 1.27	4.23 ± 1.24	4.19 ± 0.80	5.59 ± 1.70	2.66 ± 0.28	4.91 ± 2.21	**6.72 ± 0.38***
PC	0.57 ± 0.20	0.85 ± 0.49	0.41 ± 0.32	0.76 ± 0.54	0.54 ± 0.22	0.49 ± 0.17	0.38 ± 0.11	0.54 ± 0.18	1.11 ± 0.67	0.91 ± 0.57	1.09 ± 0.38
∑Glycolipids	28.8 ± 3.2	**22.1 ± 2.7****	20.1 ± 3.0	28.6 ± 3.2	28.7 ± 5.7	22.7 ± 4.4	**30.0 ± 2.6***	**29.0 ± 4.4***	21.2 ± 1.25	23.1 ± 4.6	**15.0 ± 4.0***
∑Phospholipids	18.6 ± 3.1	**14.5 ± 1.7***	11.5 ± 3.3	16.0 ± 2.5	16.9 ± 2.7	13.8 ± 2.9	**20.3 ± 0.7***	**21.0 ± 4.4***	12.1 ± 0.9	**20.4 ± 4.4***	**24.7 ± 2.4*****
∑Polar lipids	47.3 ± 5.8	**36.6 ± 3.9***	31.6 ± 6.1	44.6 ± 5.7	45.5 ± 8.4	36.5 ± 6.4	**50.3 ± 3.2***	**50.0 ± 8.4***	33.3 ± 2.1	43.5 ± 8.6	39.8 ± 6.0
Neutral lipids (TAG)	18.0 ± 4.0	22.9 ± 4.1	22.1 ± 5.9	**12.6 ± 2.9***	**4.6 ± 1.0*****	**58.0 ± 24.3***	**67.9 ± 15.4****	16.4 ± 9.8	47.5 ± 43.0	44.7 ± 7.4	26.6 ± 14.3

469 Table 5: Lipids content (nmol.mg^{-1} dry weight) of the DIATO experiment (* p-value<0.05, ** p-value<0.01 and *** p-value<0.001; for controls: significant differences between
470 initial and final control, for contaminated conditions: differences between herbicide concentration and final control d7). Statistic of Wilcoxon-Mann-Whitney test (W) or
471 Student's test (t) for initial and final control comparison: DGDG t=-2.6, SQDG t=-3.0, PE W=0, PC t=-2.6, ∑Phospholipids t=-2.7. Statistic of ANOVA (F) or Kruskal-Wallis
472 (Chi-squared): MGDG F=10.4 for atrazine and F=4.5 for mixture, PE F=15.7, ∑Glycolipids F=8.3, ∑Phospholipids F=6.2, ∑Polar lipids F=5.0, TAG=5.3 (with degree of
473 freedom in the numerator, i.e DFn=3, and the degree of freedom in the denominator, i.e DFd=12).

DIATO	Initial control (d0)	Final control (d7)	Atrazine			Mixture			S-metolachlor		
Nominal concentration (µg.L^{-1}) / Contamination levels	0	0	10	100	1000	10	100	1000	10	100	1000
MGDG	4.28 ± 1.92	3.55 ± 0.89	5.68 ± 2.01	5.89 ± 0.89	**12.0 ± 3.82***	4.95 ± 1.11	**5.57 ± 1.27***	**6.05 ± 0.72***	4.85 ± 1.34	3.86 ± 0.83	2.82 ± 0.62
DGDG	0.47 ± 0.18	**0.72 ± 0.09****	0.75 ± 0.25	0.48 ± 0.14	0.56 ± 0.25	0.82 ± 0.30	1.07 ± 0.16	0.66 ± 0.08	0.32 ± 0.20	0.63 ± 0.24	0.67 ± 0.31
SQDG	0.52 ± 0.16	**0.94 ± 0.28***	1.14 ± 0.20	1.06 ± 0.11	1.43 ± 0.46	0.94 ± 0.18	1.10 ± 0.17	0.73 ± 0.11	0.73 ± 0.15	0.78 ± 0.15	0.78 ± 0.13
PG	1.36 ± 0.49	2.21 ± 1.01	2.20 ± 0.78	2.07 ± 0.51	3.36 ± 1.16	1.87 ± 0.61	1.49 ± 0.26	1.30 ± 0.06	2.54 ± 0.48	1.92 ± 0.51	3.08 ± 1.12
PE	0.96 ± 0.17	**1.83 ± 0.60***	1.78 ± 0.75	1.60 ± 0.50	2.40 ± 1.14	1.99 ± 0.51	1.46 ± 0.22	2.19 ± 0.40	1.85 ± 0.24	1.68 ± 0.21	**4.14 ± 0.97*****
PC	0.40 ± 0.20	**0.74 ± 0.21****	1.36 ± 0.47	1.16 ± 0.28	1.20 ± 0.10	0.92 ± 0.40	0.94 ± 0.15	0.58 ± 0.05	1.17 ± 0.27	0.93 ± 0.48	0.70 ± 0.26
∑Glycolipids	5.27 ± 2.12	5.21 ± 0.97	7.57 ± 2.42	7.43 ± 0.88	**14.0 ± 4.46****	6.71 ± 1.45	7.74 ± 1.57	7.44 ± 0.85	5.90 ± 1.54	5.27 ± 0.96	4.26 ± 0.38
∑Phospholipids	2.71 ± 0.61	**4.78 ± 1.74***	5.33 ± 1.93	4.83 ± 0.95	6.96 ± 2.32	4.78 ± 1.37	3.89 ± 0.40	4.07 ± 0.43	5.56 ± 0.60	4.53 ± 0.48	**7.92 ± 1.60****
∑Polar lipids	8.0 ± 2.4	10.0 ± 2.5	12.9 ± 4.2	12.3 ± 1.8	**20.9 ± 6.8***	11.5 ± 2.7	11.6 ± 1.9	11.5 ± 0.8	11.5 ± 2.1	9.8 ± 0.7	12.2 ± 1.9
Neutral lipids (TAG)	18.6 ± 10.0	21.2 ± 3.1	15.9 ± 8.1	18.6 ± 4.8	15.1 ± 4.7	**34.1 ± 3.6***	23.2 ± 10.4	16.6 ± 5.9	23.7 ± 11.3	34.8 ± 7.4	22.9 ± 7.3

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Table 6: Lipids content (nmol.mg^{-1} dry weight) of the CYANO experiment (* p-value<0.05, ** p-value<0.01 and *** p-value<0.001; for controls: significant differences between initial and final control, for contaminated conditions: differences between herbicide concentration and final control d7). Statistic of Wilcoxon-Mann-Whitney test (W) or Student's test (t) for initial and final control comparison: PE t=-8.1. Statistic of ANOVA (F) or Kruskal-Wallis (Chi-squared): MGDG F=17.9 for atrazine and F=28.2 for mixture, DGDG F=7.9 for atrazine and F=6.8 for S-metolachlor, SQDG F=20.7, PG F=6.4, PE=30.9 for atrazine, F=90.0 for mixture and F=14.6 for S-metolachlor, PC F=3.6, ∑Glycolipids F=26.9 for atrazine and F=4.8 for mixture, ∑Phospholipids= 5.1, ∑Polar lipids F=21.0. (with degree of freedom in the numerator, i.e DFn=3, and the degree of freedom in the denominator, i.e DFd=12 for ∑Polar lipids). No TAGs were detected for this experiment.

CYANO	Initial control (d0)	Final control (d7)	Atrazine			Mixture			S-metolachlor		
Nominal concentration (µg.L^{-1}) / Contamination levels	0	0	10	100	1000	10	100	1000	10	100	1000
MGDG	5.52 ± 0.83	4.92 ± 0.30	4.67 ± 1.08	**3.73 ± 0.4***	**2.14 ± 0.11****	5.66 ± 0.87	**3.71 ± 0.64****	**2.12 ± 0.30*****	4.39 ± 0.85	4.51 ± 0.70	4.99 ± 0.74
DGDG	4.40 ± 0.65	4.16 ± 0.60	4.64 ± 0.38	4.23 ± 0.49	**3.02 ± 0.50***	3.77 ± 0.92	3.52 ± 0.23	2.96 ± 0.27	**2.68 ± 0.42****	**2.81 ± 0.51****	3.28 ± 0.50
SQDG	3.58 ± 0.80	4.07 ± 1.54	**9.95 ± 1.93*****	6.44 ± 0.9	2.83 ± 0.86	5.01 ± 1.14	5.49 ± 1.00	4.02 ± 1.08	2.98 ± 1.14	2.86 ± 0.19	2.98 ± 1.22
PG	2.59 ± 0.47	3.04 ± 0.92	**4.54 ± 0.41***	4.05 ± 0.54	2.84 ± 0.57	3.39 ± 0.42	3.73 ± 0.39	3.18 ± 0.25	3.05 ± 0.49	2.55 ± 0.32	2.99 ± 0.42
PE	0.04 ± 0.02	**0.14 ± 0.01*****	0.21 ± 0.07	**0.34 ± 0.13***	**0.67 ± 0.08*****	0.13 ± 0.06	0.19 ± 0.02	**0.63 ± 0.07*****	0.16 ± 0.04	0.16 ± 0.05	**0.33 ± 0.06*****
PC	0.02 ± 0.01	0.02 ± 0.00	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.00	**0.03 ± 0.01***	0.03 ± 0.01	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
∑Glycolipids	13.5 ± 2.0	13.2 ± 2.3	**19.3 ± 1.9****	14.4 ± 1.4	**8.0 ± 1.4****	14.4 ± 2.7	12.7 ± 1.8	**9.1 ± 1.4**	10.1 ± 2.0	10.2 ± 1.0	11.3 ± 2.4
∑Phospholipids	2.64 ± 0.47	3.20 ± 0.92	**4.77 ± 0.40**	4.42 ± 0.65	3.53 ± 0.56	3.55 ± 0.44	3.95 ± 0.40	3.83 ± 0.23	3.23 ± 0.49	2.73 ± 0.32	3.34 ± 0.46
∑Polar lipids	16.2 ± 2.4	16.4 ± 3.2	**24.0 ± 2.3****	18.8 ± 1.5	**11.5 ± 1.8***	18.0 ± 2.9	16.7 ± 2.1	12.9 ± 1.5	13.3 ± 2.5	12.9 ± 0.7	14.6 ± 2.8

The ratio of chloroplastic/extra-chloroplastic lipids differed between cultures and was in general much higher in CYANO, followed by GREEN and DIATO (supplementary material Table A.6). Between the beginning and the end of the experiment, this ratio remained stable in GREEN controls, whereas it decreased significantly in DIATO (from 4.9 ± 0.7 to 2.9 ± 0.4; p-value<0.01) and CYANO (300.6 ± 116.0 to 104.0 ± 23.9; p-value<0.05) (Figure 4, top). Exposure to the highest concentration of atrazine led to an increase in this ratio (2.9 ± 0.4 to 4.9 ± 0.7; p-value<0.01) for DIATO. The highest concentration of S-metolachlor led to a decrease in the chloroplastic/extra-chloroplastic lipid ratio from 2.9 ± 0.4 to 1.6 ± 0.4 for DIATO (p-value<0.05) and from 104 ± 23.9 to 40.8 ± 5.9 for CYANO (p-value<0.01). The diatom response to the mixture exposure was intermediate between the responses observed under atrazine and S-metolachlor alone. The mixture resulted in a significant effect only for CYANO at the highest concentration (p-value<0.05).

The ratio of MGDG and DGDG lipid contents also differed between cultures. On day 7, CYANO showed the lowest ratio at 1.1 ± 0.1 followed by DIATO (5.0 ± 1.8) and GREEN (11.3 ± 1.0) (Figure 4, bottom; supplementary material Table A.7). At the highest concentration, atrazine led to a significant decrease in the MGDG/DGDG ratio down to 0.7 ± 0.1 (p-value<0.01) in CYANO, but to an increase up to 22.6 ± 6.4 in DIATO (p-value<0.001) and up to 79.8 ± 48.6 (p-value<0.01) in GREEN. S-metolachlor decreased the MGDG/DGDG ratio in GREEN from 11.0 ± 1.0 to 6.7 ± 1.3 at the highest concentration (p-value<0.001). The mixture of compounds followed the same trends as for atrazine alone, with an increase in the ratio for the two highest concentrations for GREEN (p-value<0.01) and a decrease in the ratio for CYANO (p-value<0.05).

Figure 4: Chloroplastic/extra-chloroplastic lipid ratios (top) and MGDG/DGDG ratios (bottom) at the different exposure treatments (nominal concentrations) for the GREEN, DIATO and CYANO experiments. (* p-value<0.05, ** p-value<0.01 and *** p-value<0.005; difference with final control (d7)).

3.3.Fatty acids composition

3.3.1. Interspecific differences in fatty acid composition

A total of 41 FAMEs were quantified in both TAGs (supplementary material Table A.9) and polar lipids (supplementary material Table A.10), where 28 FA showed a relative proportion >5%. As previously stated in the lipid result section, CYANO does not possess TAGs, so FAMEs were absent for this fraction in CYANO. In GREEN, the TAGs fraction in the control condition was mainly composed of MUFAs (41%; in particular C18:1n-9), followed by PUFAs (30%; in particular C18:3n-3 and C18:2n-6) and SFAs (28%; in particular C16:0). In DIATO, MUFAs were the most abundant (63%; in particular C16:1n-7), followed by SFAs (32%; in particular C16:0) and PUFAs (5%; in particular C20:5n-3). In the polar lipid fraction, CYANO had more SFAs (24%) and less MUFAs (5%) than GREEN and DIATO. All cultures had a majority of PUFAs in their polar lipid fraction, from 61% for DIATO, to 71% for CYANO and 77% for GREEN. However, DIATO had a higher proportion of VLCFAs (C≥20) (61%) than GREEN (1%) and CYANO (0%) (Table 5).

Differences in FAMEs composition were notable between fractions (TAGs or polar lipids) and cultures (GREEN, DIATO or CYANO) (see Tables 7 and 8). A species-specific difference in FAMEs composition is shown in Figure 5. On this nMDS, GREEN and CYANO had similar FAMEs compositions, which differed from DIATO, regardless of herbicide and exposure concentration. This pattern was similar for both lipid fractions (Supplementary material, TAGs: Figure A.1, polar lipids: Figure A.2). The PCA (Figure 6) showed a clear separation of DIATO on the left-hand side, while GREEN and CYANO were better represented on the right-hand side. DIATO's fatty acid profiles were mainly driven by MUFAs, in particular C16:1n-7, but also by HUFAs such as C20:5n-3 present only in this culture. On the contrary, this culture seemed to be anti-correlated with C18:3n-3 fatty acid, which appeared to be correlated with GREEN and CYANO on the right side of the ordination. In addition, C18:1n-9 was quantified almost exclusively in GREEN, while C18:2n-6 was present in GREEN and CYANO but not in DIATO. Overall, PUFAs were mainly correlated with the right-hand side of the PCA. TAGs were slightly correlated with DIATO, while they were anti-correlated with CYANO, as implied by the lipid content results (cos2 of variables associate with the two first dimensions of PCA are presented in supplementary material, Table A.12). PERMANOVAs (supplementary material Table A.11) suggested that these differences in FAME profiles were largely explained by phototrophic group (p-value<0.05). However, nominal herbicide

543 concentration also modulated FAMEs profiles in all cultures, with a significant interaction
544 between species and nominal concentrations.

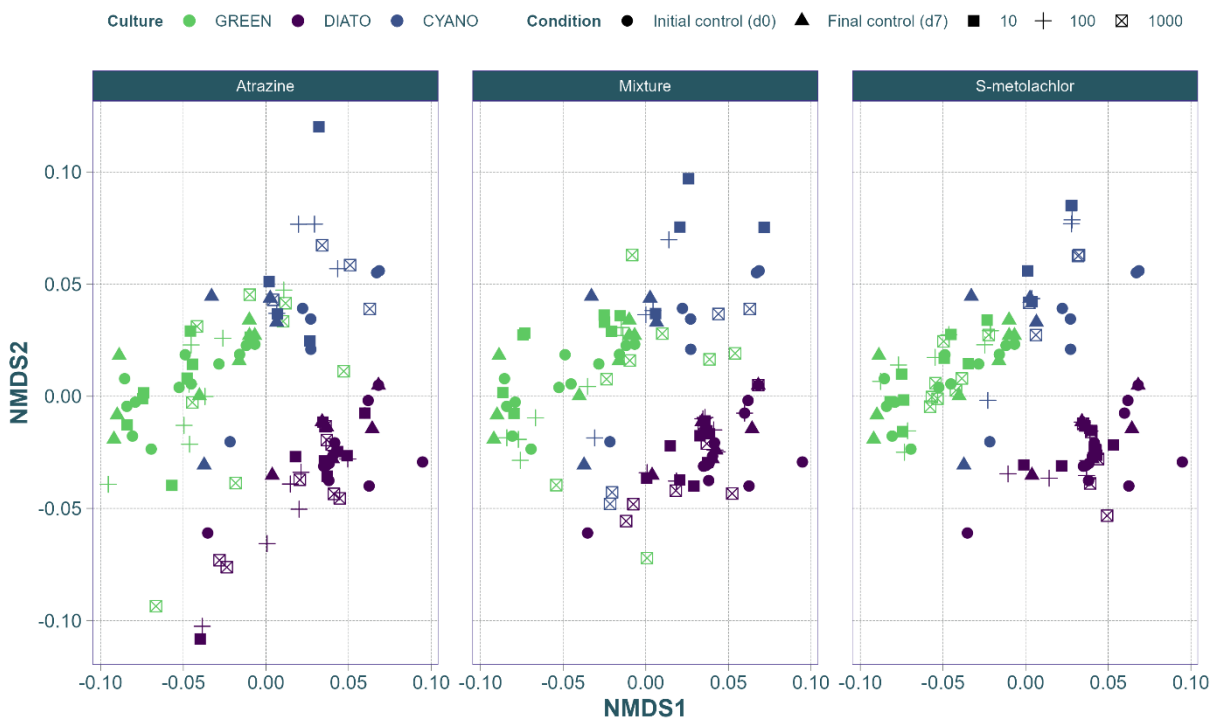

Figure 5: Non-metric Multi Dimensional Scaling (distance matrix) performed on FAMEs relative proportion of all fatty acids from both TAG and polar lipids (Stress<0.18).

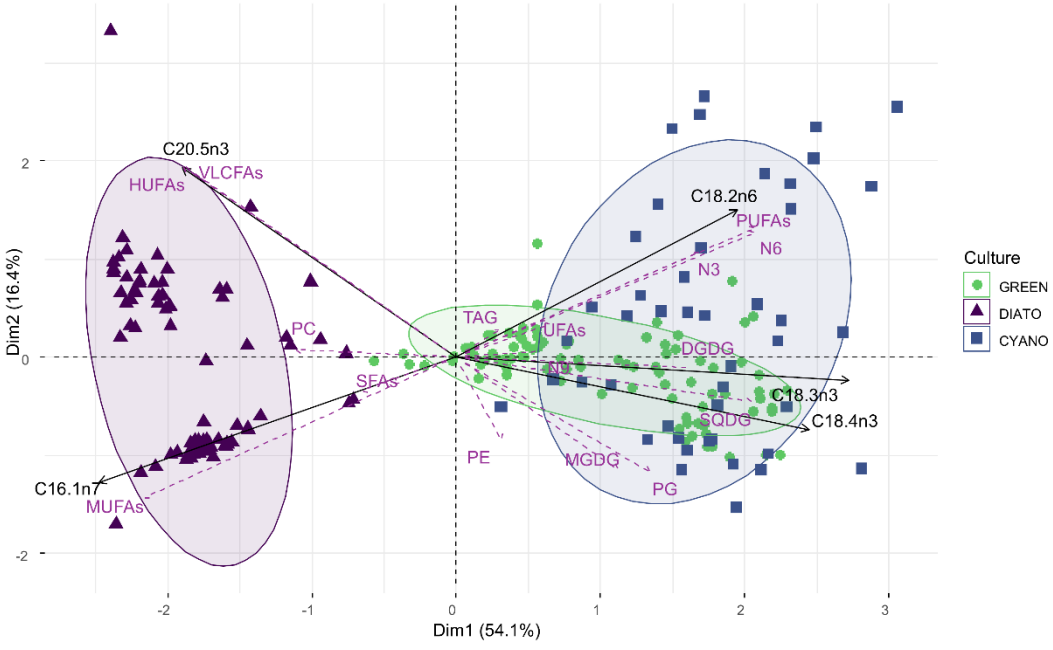

Figure 6: Principal Component Analysis on total fatty acids (from both TAG and polar lipids; proportion >5%) filtered by cos2>0.10. Lipids and FAMEs groups were added as supplementary quantitative variables and are represented in purple color.

Table 7: Relative proportion of FAMEs (%) in TAGs[1] for GREEN and DIATO experiments at different exposure conditions (* p-value<0.05, ** p-value<0.01 and *** p-value<0.001 for significant differences between initial and final control and differences between contaminated and final control d7). For GREEN: Statistic of Wilcoxon-Mann-Whitney test (W) or Student's test (t) for initial and final control comparison: C18:1n-9 t=4.3, C18:2n-6 t=-4.5, MUFAs t=4.8. Statistic of ANOVA (F) or Kruskal-Wallis (Chi-squared): C18:1n-9 F=27.1 for atrazine and 10.5 for S-metolachlor, C18:2n-6 F=17.0 for atrazine and F=7.6 for mixture, C18:4n-3 F=14.1, SFAs F=4.5, MUFAs F=29.7 for atrazine and F=5.0 for S-metolachlor, UFAs F=4.7 and VLCFAs F=6.7. For DIATO: Statistic of Wilcoxon-Mann-Whitney test (W) or Student's test (t) for initial and final control comparison: C20:5n-3 t=2.3. Statistic of ANOVA (F) or Kruskal-Wallis (Chi-squared): C20:5n-3 F=3.9, PUFAs F=5.4 and VLCFAs F=3.9. (degree of freedom in the numerator, i.e DFn=3, and the degree of freedom in the denominator, i.e DFd=12).

GREEN	Initial control (d0)	Final control (d7)	Atrazine			Mixture			S-metolachlor		
Nominal concentration ($\mu g.L^{-1}$) / Contamination levels	0	0	10	100	1000	10	100	1000	10	100	1000
C16:1n-7	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
C18:1n-9	51.6 ± 3.2	**41.6 ± 4.3****	42.7 ± 5.4	38.0 ± 9.9	**5.1 ± 6.7*****	45.9 ± 1.6	46.6 ± 3.7	10.0 ± 12.5	43.8 ± 1.2	48.1 ± 3.1	**48.6 ± 1.4***
C18:2n-6	3.03 ± 0.61	**5.74 ± 1.29****	5.50 ± 1.82	**3.21 ± 0.83****	**0.30 ± 0.60*****	5.82 ± 0.73	5.53 ± 3.16	**0.60 ± 1.21*****	6.89 ± 0.59	4.30 ± 0.98	5.04 ± 0.56
C18:3n-3	15.3 ± 2.2	17.4 ± 4.9	19.0 ± 6.4	13.3 ± 4.5	30.9 ± 25.2	18.9 ± 3.5	11.8 ± 1.6	45.8 ± 38.1	17.2 ± 2.5	14.5 ± 2.4	16.3 ± 0.7
C18:4n-3	2.78 ± 0.68	5.09 ± 1.77	5.75 ± 2.33	4.54 ± 1.57	2.54 ± 5.08	6.14 ± 1.39	3.42 ± 0.77	0.53 ± 1.07	5.08 ± 1.06	4.13 ± 0.89	**4.55 ± 0.46*****
C20:5n-3	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
SFAs	23.2 ± 3.5	25.8 ± 7.3	23.4 ± 2.0	38.3 ± 13.3	**56.0 ± 23.8***	17.8 ± 4.2	27.0 ± 4.1	38.5 ± 22.5	21.7 ± 3.9	23.6 ± 4.9	22.8 ± 1.8
MUFAs	54.3 ± 2.9	**43.4 ± 4.4*****	44.3 ± 6.3	38.5 ± 9.2	**5.1 ± 6.7*****	49.7 ± 1.5	49.8 ± 3.9	10.0 ± 12.5	46.9 ± 1.4	**51.23 ± 3.4****	48.6 ± 1.4
PUFAs	22.4 ± 1.9	30.6 ± 4.4	32.3 ± 5.4	22.1 ± 5.2	38.3 ± 24.4	32.5 ± 4.2	22.6 ± 3.0	49.2 ± 34.3	31.2 ± 3.1	24.7 ± 1.8	28.6 ± 1.0
UFAs	76.7 ± 3.5	74.0 ± 7.2	76.6 ± 2.0	60.5 ± 13.1	**43.4 ± 23.7***	82.1 ± 4.2	72.4 ± 4.3	59.2 ± 24.6	78.1 ± 3.9	75.9 ± 5.0	77.1 ± 1.8
VLCFAs	2.33 ± 1.43	2.80 ± 1.87	1.75 ± 1.31	0.91 ± 1.83	2.26 ± 4.52	3.90 ± 0.41	3.90 ± 0.70	0.41 ± 0.82	3.26 ± 0.44	3.51 ± 1.48	**0.11 ± 0.14***
DIATO	Initial control (d0)	Final control (d7)	Atrazine			Mixture			S-metolachlor		

Nominal concentration ($\mu g.L^{-1}$) / Contamination levels	0	0	10	100	1000	10	100	1000	10	100	1000
C16:1n7	62.98 ± 4.26	62.54 ± 4.48	62.65 ± 2.27	59.47 ± 9.35	68.93 ± 7.64	60.19 ± 4.02	62.5 ± 4.51	60.23 ± 19.19	61.98 ± 4.98	58.67 ± 5.97	64.31 ± 3.3
C18:1n-9	0 ± 0	0.17 ± 0.34	1.29 ± 2.59	2.47 ± 3.20	0 ± 0	0.75 ± 0.59	0.45 ± 0.76	4.21 ± 4.88	1.06 ± 2.11	1.51 ± 2.56	0 ± 0
C18:2n-6	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.14 ± 0.29	0 ± 0
C18:3n-3	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.00 ± 0.00	0 ± 0
C18:4n-3	0 ± 0	0.16 ± 0.32	0 ± 0	0 ± 0	0 ± 0	0.36 ± 0.42	0.15 ± 0.31	0 ± 0	0.17 ± 0.33	0.14 ± 0.29	0 ± 0
C20:5n-3	6.31 ± 1.68	**4.22 ± 0.76***	4.57 ± 0.93	3.91 ± 1.31	**6.06 ± 0.75***	3.42 ± 0.32	3.71 ± 0.62	4.67 ± 1.68	3.78 ± 0.57	3.97 ± 1.13	4.53 ± 1.61
SFAs	30.5 ± 3.6	32.5 ± 3.9	31.4 ± 3.6	33.5 ± 6.0	24.1 ± 7.3	34.5 ± 2.7	32.9 ± 3.9	28.1 ± 13.5	33.0 ± 3.1	34.9 ± 3.2	31.0 ± 3.7
MUFAs	63.0 ± 4.3	62.7 ± 4.2	64.0 ± 3.8	61.9 ± 6.2	68.9 ± 7.6	61.0 ± 3.5	63.0 ± 3.9	64.4 ± 14.7	63.0 ± 3.1	60.2 ± 3.6	64.3 ± 3.3
PUFAs	6.31 ± 1.68	4.78 ± 1.00	4.57 ± 0.93	3.91 ± 1.31	6.06 ± 0.75	4.33 ± 1.16	4.16 ± 1.05	**7.46 ± 1.90***	3.95 ± 0.59	4.69 ± 1.43	4.53 ± 1.61
UFAs	69.3 ± 3.7	67.5 ± 3.9	68.5 ± 3.8	65.9 ± 7.1	75.0 ± 8.2	65.3 ± 3.1	67.1 ± 3.9	71.9 ± 13.5	67.0 ± 3.2	64.9 ± 3.4	68.8 ± 4.0
VLCFAs	6.31 ± 1.68	4.22 ± 0.76	4.57 ± 0.93	3.91 ± 1.31	**6.06 ± 0.75***	3.42 ± 0.32	3.71 ± 0.62	4.67 ± 1.68	3.78 ± 0.57	3.97 ± 1.13	4.53 ± 1.61

[1] The FAMEs presented here are among those best represented on the PCA in Figure 6.

Table 8: Relative proportion of FAMEs (%) in polar lipids[1] for GREEN, DIATO and CYANO experiments at different exposure conditions (* p-value<0.05, ** p-value<0.01 and *** p-value<0.001 for significant differences between initial and final control and differences between contaminated and final control d7). For GREEN: Statistic of Wilcoxon-Mann-Whitney test (W) or Student's test (t) for initial and final control comparison: C16:1n-7 W=2, C18:4n-3 t=-2.8. Statistic of ANOVA (F) or Kruskal-Wallis (Chi-squared): C18:1n-9 Chi-squared=12.4, C18:3n-3 F=9.2, SFAs F=3.8, MUFAs Chi-squared=10.9, PUFAs F=6.9, UFAs F=3.6 and VLCFAs F=9.6 for mixture and F=7.2 for S-metolachlor. For CYANO: Statistic of Wilcoxon-Mann-Whitney test (W) or Student's test (t) for initial and final control comparison: C16:1n-7 F=7.1, C18:3n-3 t=2.6, C18:3n-4 W=0. Statistic of ANOVA (F) or Kruskal-Wallis (Chi-squared): C18:4n-3 F=3.7, SFAs F=4.6 and UFAs F=4.6. (degree of freedom in the numerator, i.e DFn=3, and the degree of freedom in the denominator, i.e DFd=12). No statistical differences were observed for DIATO.

GREEN	Initial control (d0)	Final control (d7)	Atrazine			Mixture			S-metolachlor		
Nominal concentration ($\mu g.L^{-1}$) / Contamination levels	0	0	10	100	1000	10	100	1000	10	100	1000
C16:1n7	3.9 ± 3.1	**6.74 ± 0.5***	2.89 ± 3.42	3.96 ± 5.01	3.59 ± 4.16	4.24 ± 2.83	7.67 ± 6.19	3.5 ± 2.7	2.71 ± 3.14	3.35 ± 3.87	4.79 ± 4.36
C18:1n-9	10.2 ± 5.6	4.1 ± 3.4	14.6 ± 11.5	11.6 ± 8.4	6.8 ± 9.4	8.8 ± 2.8	9.3 ± 7.4	3.5 ± 3.0	11.2 ± 2.3	14.7 ± 2.3	**25.2 ± 16.3*****
C18:2n-6	4.06 ± 2.73	7.19 ± 0.60	11.26 ± 4.44	9.11 ± 6.36	2.79 ± 2.91	8.41 ± 0.48	3.47 ± 2.87	0.57 ± 1.14	9.64 ± 0.50	8.32 ± 4.44	6.83 ± 2.55
C18:3n-3	53.9 ± 11.7	58.1 ± 6.3	36.4 ± 22.6	48.6 ± 16.9	55.8 ± 14.5	53.9 ± 4.4	39.4 ± 28.0	65.6 ± 9.3	**42.8 ± 6.4***	**39.4 ± 7.6***	**30.5 ± 9.6*****
C18:4n-3	9.1 ± 1.9	**12.3 ± 1.6***	8.6 ± 5.6	10.2 ± 3.3	11.4 ± 3.4	12.4 ± 1.5	7.5 ± 5.5	11.6 ± 1.8	9.6 ± 2.4	10.9 ± 1.5	9.4 ± 4.1
C20:5n-3	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
SFAs	18.1 ± 7.6	10.8 ± 2.9	23.3 ± 14.4	15.5 ± 11.1	18.1 ± 7.7	10.6 ± 4.1	7.5 ± 9.0	13.9 ± 7.4	21.0 ± 6.4	21.1 ± 6.1	**22.2 ± 5.9***
MUFAs	14.7 ± 3.5	11.3 ± 4.7	18.9 ± 9.9	15.6 ± 4.0	11.0 ± 6.8	14.5 ± 2.7	16.9 ± 11.5	7.8 ± 2.5	15.4 ± 1.7	19.4 ± 2.3	**30.7 ± 19.7***
PUFAs	67.2 ± 11.0	77.6 ± 7.8	57.5 ± 24.3	68.8 ± 14.8	70.3 ± 13.9	74.7 ± 5.7	50.4 ± 34.9	77.8 ± 9.8	63.5 ± 5.5	**59.4 ± 5.9***	**47.0 ± 15.6****
UFAs	81.9 ± 7.6	88.9 ± 3.1	76.5 ± 14.6	84.4 ± 11.2	81.2 ± 7.5	89.3 ± 4.1	67.3 ± 45.6	85.6 ± 7.6	78.9 ± 6.4	78.8 ± 6.1	**77.6 ± 6.0***

VLCFAs	0.74 ± 1.25	0.08 ± 0.15	1.24 ± 0.93	0.87 ± 0.70	0.42 ± 0.50	**0.63 ± 0.22****	0.12 ± 0.25	0 ± 0	**1.76 ± 0.56****	**1.34 ± 0.61***	0.74 ± 0.69
DIATO	Initial control (d0)	Final control (d7)	Atrazine			Mixture			S-metolachlor		
Nominal concentration ($\mu g.L^{-1}$) / Contamination levels	0	0	10	100	1000	10	100	1000	10	100	1000
C16:1n7	26.62 ± 40.7	34.06 ± 23.29	33.79 ± 12.35	24.59 ± 4.49	31.06 ± 15.6	45.41 ± 10.93	45.63 ± 5.57	34.64 ± 23.28	42.86 ± 8.61	44.06 ± 3.49	43.04 ± 10.7
C18:1n-9	3.05 ± 7.47	0 ± 0	0 ± 0	3.82 ± 4.63	1.29 ± 2.59	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
C18:2n-6	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.11 ± 0.23	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
C18:3n-3	1.20 ± 2.93	0 ± 0	0 ± 0	0.41 ± 0.83	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
C18:4n-3	0 ± 0	0 ± 0	0.59 ± 1.17	1.74 ± 2.29	0.74 ± 0.90	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
C20:5n-3	38.2 ± 36.4	61.3 ± 26.5	34.3 ± 24.7	23.7 ± 15.5	29.6 ± 14.7	41.9 ± 4.1	48.4 ± 4.9	53.2 ± 31.5	39.9 ± 6.0	46.0 ± 2.4	29.4 ± 10.7
SFAs	29.5 ± 33.7	4.7 ± 4.2	25.2 ± 26.5	38.3 ± 14.1	27.1 ± 16.2	12.7 ± 8.1	6.0 ± 8.7	8.5 ± 9.4	17.3 ± 6.4	9.9 ± 4.6	26.9 ± 18.7
MUFAs	29.7 ± 39.9	34.1 ± 23.3	34.8 ± 11.0	30.4 ± 5.7	34.4 ± 11.6	45.4 ± 10.9	45.6 ± 5.6	34.6 ± 23.3	42.9 ± 8.6	44.1 ± 3.5	43.0 ± 10.7
PUFAs	40.6 ± 34.4	61.3 ± 26.5	39.8 ± 16.1	30.5 ± 11.2	37.5 ± 6.5	41.9 ± 4.1	48.4 ± 4.9	56.9 ± 29.8	39.9 ± 6.0	46.0 ± 2.4	29.4 ± 10.7
UFAs	70.3 ± 33.9	95.3 ± 4.2	74.7 ± 26.7	60.8 ± 14.4	71.9 ± 17.7	87.3 ± 8.1	94.0 ± 8.7	91.5 ± 9.4	82.7 ± 6.4	90.1 ± 4.6	72.4 ± 19.9
VLCFAs	38.2 ± 36.4	61.3 ± 26.5	40.4 ± 15.2	29.5 ± 4.8	31.1 ± 13.2	41.9 ± 4.1	48.4 ± 4.9	53.2 ± 31.5	39.9 ± 6.0	46.0 ± 2.4	29.4 ± 10.7
CYANO	Initial control (d0)	Final control (d7)	Atrazine			Mixture			S-metolachlor		

Nominal concentration ($\mu g.L^{-1}$) / Contamination levels	0	0	10	100	1000	10	100	1000	10	100	1000
C16:1n7	0.32 ± 0.78	1.52 ± 1.75	0 ± 0	3.49 ± 4.1	8.91 ± 10.29	0 ± 0	2.85 ± 3.83	8.73 ± 6.02	0 ± 0	2.78 ± 3.21	**8.96 ± 4.65***
C18:1n-9	0.21 ± 0.53	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.79 ± 1.58	0.43 ± 0.86	0 ± 0	2.64 ± 5.28	0 ± 0
C18:2n-6	8.9 ± 7.9	11.9 ± 1.3	21.2 ± 11.7	10.7 ± 13.3	16.7 ± 19.6	3.8 ± 7.5	21.1 ± 10.5	25.5 ± 17.0	4.6 ± 7.2	3.2 ± 3.7	5.6 ± 6.8
C18:3n-3	70.8 ± 16.4	**44.7 ± 13.7***	35.6 ± 26.6	53.4 ± 14.2	57.2 ± 11.1	59.8 ± 7.4	41.8 ± 14.7	29.6 ± 20.2	52.9 ± 7.2	46.5 ± 14.4	52.8 ± 16.9
C18:4n-3	0 ± 0	**7.3 ± 3.7****	6.6 ± 5.2	8.6 ± 7.5	6.3 ± 9.8	9.7 ± 6.8	7.4 ± 4.1	**0 ± 0****	14.5 ± 6.6	11.2 ± 4.6	11.9 ± 5.0
C20:5n-3	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
SFAs	10.0 ± 12.3	24.4 ± 8.2	12.1 ± 10.0	7.6 ± 12.6	**1.3 ± 2.5****	4.8 ± 8.8	17.4 ± 16.4	22.0 ± 15.1	8.5 ± 10.3	16.6 ± 21.2	11.8 ± 14.2
MUFAs	8.0 ± 11.7	4.5 ± 5.2	6.0 ± 7.0	8.5 ± 9.8	18.5 ± 21.5	8.9 ± 10.4	8.3 ± 10.5	21.7 ± 12.9	9.0 ± 10.5	12.5 ± 9.4	14.4 ± 10.3
PUFAs	81.9 ± 11.7	71.1 ± 13.2	81.9 ± 6.6	83.9 ± 9.2	80.2 ± 20.2	86.3 ± 9.2	73.9 ± 17.2	56.0 ± 3.9	82.5 ± 3.5	70.0 ± 19.1	73.8 ± 6.7
UFAs	90.0 ± 12.3	75.6 ± 8.2	88.0 ± 10.0	92.4 ± 12.6	**98.8 ± 2.5****	95.2 ± 8.8	82.1 ± 16.7	77.8 ± 15.2	91.5 ± 10.3	82.5 ± 22.8	88.2 ± 14.2
VLCFAs	0 ± 0	0 ± 0	0 ± 0	3.21 ± 6.41	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

1 The FAMEs presented here are among those best represented on the PCA in Figure 6.

3.3.2. Effect of herbicide exposure on fatty acid composition

Herbicide exposure to atrazine and S-metolachlor alone and in mixture showed the strongest effect on FA composition in the GREEN experiment. For the TAGs fraction of GREEN (Table 4), atrazine alone led to an increase in SFAs and to a decrease in unsaturated fatty acids (UFAs) (p-value<0.05). MUFAs decreased drastically at the highest atrazine concentration (43.4 ± 4.4% to 5.1 ± 6.6%; p-value<0.001). This decrease may be explained by the significant reduction in C18:1n-9 (p-value<0.001). In contrast, S-metolachlor at 100 $\mu g.L^{-1}$ led to an increase in MUFAs (43.4 ± 4.4 to 51.3 ± 3.4%; p-value<0.05) and to a decrease in PUFAs (30.6 ± 4.4% to 24.7 ± 1.8%; p-value<0.05).

In general, only S-metolachlor affected the polar lipids of the cultures (Table 5). In the GREEN experiment, the 1000 $\mu g.L^{-1}$ condition of S-metolachlor alone led to an increase in MUFAs (11.3 ± 4.7% to 30.7 ± 19.7%; p-value<0.05) and to a decrease in PUFAs including C8:3n-3 (77.6 ± 7.8% to 47.0 ± 15.6%; p-value<0.01). The C18:3n-3 decreased at 10 $\mu g.L^{-1}$ of S-metolachlor from 58.1 ± 6.3% to 30.5 ± 9.6% (p-value<0.001). Atrazine had a significant effect only on CYANO. The 1000 $\mu g.L^{-1}$ treatment led to a drop in SFAs from 24.4 ±8.2% to 1.3 ± 2.5% (p-value<0.01) and to an increasing in UFAs from 75.6 ± 8.2% to 98.8 ± 2.5% (p-value<0.01). The mixture of compounds showed little effect on CYANO apart from the disappearance of C18:4n-3 at the highest concentration (p-value<0.01).

4. Discussion

Atrazine and S-metolachlor affected photosynthesis as well as lipid and fatty acid profiles of the diatoms, green algae, and cyanobacteria. In general, the green alga was the most sensitive phototroph tested, presenting a 100% inhibition of its photosynthesis yield and a marked change in lipid and fatty acid compositions at high concentrations of the two herbicides. Atrazine mostly affected photosynthesis parameters, while S-metolachlor had the greatest impact on the fatty acid composition of polar lipids. The choice of the biological descriptor (photosynthesis versus lipidomic) is a key factor to consider as it may highlight compound-specific effects in line with their mode of action. Photosynthetic parameters indicated that atrazine was the main driver of toxicity in the mixture, while the effect was subtle for lipids. Mixture effect on TAGs seemed driven by S-metolachlor at low concentrations only, while other results suggested intermediate effects between single herbicide responses.

4.1. Interspecific differences under control conditions

Photosynthetic and lipidomic results showed differences between cultures. For example, the green algae had twice the polar lipids content of diatoms and cyanobacteria. Through their evolutionary history, algae acquired chloroplastic membranes that are similar to the membrane lipide structure of cyanobacteria. However, the quantitative and qualitative composition of lipids vary considerably between different taxonomic groups, but also between species and strains within the same group (Kalisch et al., 2016). Unlike unicellular algae, which possess neutral lipids mainly in the form of TAGs, only a handful of cyanobacteria are known to accumulate these TAGs (Alvarez, 2016; Hu et al., 2008), which explains the absence of these lipids in the cyanobacterial culture used in the present study.

The content of different lipid classes can change depending on the age and growth phase of the culture during experiments (Nakamura and Li-Beisson, 2016). It has been observed that there is generally a strong increase in TAGs between the logarithmic phase and the stationary phase of algae (Hu et al., 2008). In the present study, although some classes were slightly modified between the beginning and the end of the three experiments, TAGs levels remained rather stable over time, suggesting that the cultures effectively stayed in the growth phase for the duration of the herbicide exposures.

FAMEs results highlighted different profiles depending on the culture, partly explained by certain fatty acids being specific to each phototroph group. The diatom culture was characterized by a high proportion of eicosapentaenoic fatty acid (EPA; C20:5n-3) and a low proportion of α-linolenic acid (ALA; C18:3n-3). C18:1n-9 and C18:3n-3 were almost exclusively present in the green algae culture. These two FAs have already been identified as good biomarkers of green algae such as *Chlorella pyrenoidosa* (Shen et al., 2016). The results from the present study are in line with the work of Taipale et al., (2013) who highlighted that C16:1n-7 and C20:5n-3 effectively contributed to the similarity of species in the Bacillariophyceae group (diatoms), while C18:1n-9 and C18:3n-3 contributed to the similarity of the Chlorophyceae group (green algae). Thus, lipids and fatty acid profiles may be relevant markers of microbial community composition (Maltsev and Maltseva, 2021; Mazzella et al., 2023c) as well as markers of exposure to contaminants.

4.2. Response of phototrophic organisms to atrazine exposure

Atrazine exposure affected photosynthetic quantum yield of all tested cultures. The findings are in accordance with (Chalifour and Juneau, 2011) who showed a reduction in the effective PSII quantum yield of the species *Scenedesmus obliquus* (green alga), *Navicula pelliculosa* (diatom) and two strains of *Microcystis aeruginosa* (cyanobacteria) at 21.6 $\mu g.L^{-1}$ of atrazine after 72 hours of exposure. Atrazine disrupts photosynthesis by competing with plastoquinone for the quinone binding site on the D1 protein (QB site) in PSII leading to the interruption of the electron flow from plastoquinone QA to QB (Rea et al., 2009), and thus the reduction of effective PSII quantum yield. The interruption of electron flow may lead to the re-emission of excitation energy as fluorescence (Muller et al., 2008), which was measured as increased pigment fluorescence. In addition, certain photosynthetic organisms may demonstrate a "shade-adaptation" strategy resulting in an increase in pigment concentration per cell (Fernández-Naveira et al., 2016; Pannard et al., 2009). This was indeed suggested by the significant increase in pigment fluorescence per cell (μg chla.$cell^{-1}$) observed in the atrazine exposure treatments (alone and in mixture) for the green algae experiment. Moreover, the experiment revealed that atrazine resulted in a reduction in cell density. This finding is consistent with the observation that atrazine may result in higher nutrient concentrations in the medium (lower uptake) (supplementary material Table A.1).

Exposure to atrazine led to an increase in the ratio of chloroplastic lipids and extra-chloroplastic lipids in the diatom culture. This suggests an increase in lipids associated with photosynthetic functions and supports the "shade-adaptation" hypothesis as a preferred strategy against the mode of action of atrazine. Among chloroplastic lipids, MGDG is a glycolipid and the main constituent of thylakoid membranes. It is notably involved in chloroplast development and in the maintenance of photosynthetic complexes (Nakamura and Li-Beisson, 2016). Thus, the increase in MGDG suggests an increase in thylakoid membranes and, therefore in chloroplasts. This response aims at counteracting the negative effect of atrazine on photosynthesis. The increase in the ratio chlorophyll a fluorescence/MGDG (Supplementary material, Figure A.3) may suggest an increase in pigment concentration in the collecting antennae of PSII to compensate for the inhibition of photosynthesis as proposed by Chin et al., (2019). It should be noted, however, that the evolution of chlorophyll fluorescence may also be explained by changes in other chloroplastic lipids such as DGDG and SQDG (Supplementary material, Figure A.3, Figure A.4, Figure A.5, Table A.8).

The results of the present study showed an increase in the MGDG/DGDG ratio in the green algae and in the diatom exposed to atrazine. MGDG is a precursor for the biosynthesis of DGDG, another major galactolipid present in chloroplasts. DGDG is also involved in the stability and integrity of photosynthetic membranes (Dörmann and Benning, 2002), hence the importance of maintaining a balanced MGDG/DGDG ratio. Under stress conditions, a change in the MGDG/DGDG ratio may represent an adaptation of photosynthetic membranes through a remodeling of glycolipid metabolism to ensure photosynthetic function (Moellering and Benning, 2011). It is worth noting that, unlike the green algae and diatom cultures, the cyanobacteria culture showed a decrease in the MGDG/DGDG ratio under exposure to atrazine. Hypotheses behind these interspecific differences will be discussed further in section 4.5.

Some algae may be able to use TAGs as substrates for the biosynthesis of polar lipids such as MGDGs (for example *Porphyridium cruentum*, Khozin-Goldberg et al., 2000). Exposure to atrazine led to a decrease in TAGs content in the green algae, while several studies suggest that stress conditions lead to an increase in TAGs content (Du and Benning, 2016). The decrease in TAGs observed in the present study may reflect a rapid adaptive reorganization of membranes (Guschina and Harwood, 2009) to maintain cellular functions. The composition of TAGs themselves can be altered under conditions of stress. TAGs are generally composed of high proportions of SFAs and MUFAs, whereas structural lipids (such as polar lipids) are generally richer in PUFAs (Olofsson et al., 2012). In this study, FAMEs from TAGs showed

an increase in saturation (i.e. increased SFAs) and a significant decrease in UFAs, including MUFAs. Finally, MGDG is also thought to play a role in promoting the xanthophyll cycle (Garab et al., 2016). These pigments are involved in cellular photoprotection and in the management of oxidative stress, as in the context of pesticide exposure, and thus participate in the maintenance of membrane stability (Chalifour and Juneau, 2011).

4.3. Response of phototrophic organisms to S-metolachlor exposure

Unlike atrazine, S-metolachlor had a limited effect on the cultures studied. Demailly et al., (2019) and Coquillé et al., (2015) showed that the exposure of S-metolachlor (respectively, 10 $\mu g.L^{-1}$ for 7 days and 1, 10 and 100 $\mu g.L^{-1}$ for 7 days) did not inhibit the effective quantum yield of the diatom *Gomphonema gracile,* which is consistent with the results from this study where no effects were observed for the diatom *Gomphonema parvulum*. However, S-metolachlor led to a decrease in the chloroplastic/extra-chloroplastic lipid ratio of the cyanobacteria and diatom cultures. This decrease may be explained by an increase in PE, one of the components of extra-chloroplastic membrane lipids contributing to the structural integrity and permeability of cell membranes.

In contrast with observations for the diatom culture, S-metolachlor led to the reduction of chlorophyll fluorescence and effective quantum yield of the green algae at the highest concentration. Machado and Soares, (2020) exposed the green algae *Pseudokirchneriella subcapitata* to different concentrations of metolachlor (40, 45, 115 and 235 $\mu g.L^{-1}$) for 72 hours. Their results showed that exposure at 115 $\mu g.L^{-1}$ affected the photosynthetic performance, possibly associated with an electron transport disorder in the photosynthetic chain. Špoljarić Maronić et al., (2018) suggested that the decrease in chlorophyll concentration in response to S-metolachlor exposure may be an indicator of inhibition of pigment synthesis or degradation by S-metolachlor. Indeed, S-metolachlor may generate reactive oxygen species (ROS) which may then interact with biological components of the cell. In their study, 72 h exposure of the green algae *Parachlorella kessleri* to 100 and 200 $\mu g.L^{-1}$ S-metolachlor led to a significant increase in ROS levels. ROS generated by S-metolachlor exposure may target the double bond sites of PUFAs, leading to lipid peroxidation and to their depletion in the cells. Indeed, in the present study, S-metolachlor exposure led to a decrease in PUFAs and to an increase in MUFAs for the green algae. For example, C18:3n-3 was strongly decreased under S-metolachlor exposure with a relative abundance dropping from 58.1 ± 6.3% to 30.5 ± 9.6% at the highest

concentration. Similarly, Roux et al., (2024) observed that the exposure of freshwater biofilm to the biocide dodecylbenzyldimethylammonium chloride led to the decline in C18:3 content. This decrease was accompanied by an increase in hydroxyoctadecadienoic acids (HODEs) which are produced by the oxidation of C18:3 by ROS. Finally, as previously stated, certain fatty acids such as C18:3n-3 have already been reported as taxonomic group "markers". However, the decrease in this fatty acid, which is present in high proportions in green algae, suggests that such descriptors may also represent a physiological response to stress, and that their use as taxonomic markers should also consider the results from this study to ensure proper interpretation.

4.4. Herbicide mixture showed varying effects depending on the descriptor

In general, the toxicity of the atrazine + S-metolachlor mixture on photosynthetic parameters was mainly attributed to the effects of atrazine. Atrazine is a photosynthesis inhibitor that is considered more toxic than S-metolachlor at equivalent concentrations. In fact, atrazine typically exhibits lower EC_{50} values than S-metolachlor. Leboulanger et al., (2001) determined an EC_{50} values of 42 $\mu g.L^{-1}$ for the green algae *Chlorella vulgaris*, 52 $\mu g.L^{-1}$ for the cyanobacteria *Oscillatoria limnetica* and of 104 $\mu g.L^{-1}$ for the diatom *Navicula accomoda*. For comparison, EC_{50} values of 68 $\mu g.L^{-1}$ for the green algae *Chlorella pyrenoidosa* (Liu and Xiong, 2009) and 341 - 697 $\mu g.L^{-1}$ for the green algae *Scenedesmus vacuolatus* were determined for S-metolachlor (Vallotton et al., 2008).

The effect of the mixture on the TAGs of the green algae and the diatom appeared to be contingent upon the toxicity of S-metolachlor. Interestingly, in the green algae experiment, the intermediate concentration (100 $\mu g.L^{-1}$) resulted in an increase in TAGs, whereas the highest concentration (1000 $\mu g.L^{-1}$) exhibited a level comparable to the control. Consequently, the response to the herbicide mixture tested does not follow a monotonic concentration-dependent relationship. Unlike polar lipids, TAGs have no structural or functional role in photosynthesis, but serve as a form of carbon and energy storage. As already mentioned, the accumulation of neutral lipids in the form of TAGs seems to be a common response in algae and could represent a biological response, i.e., shifting lipid metabolism from membrane lipid synthesis to neutral reserve lipids (Du and Benning, 2016). Furthermore, *de novo* synthesis of TAGs may act as a sink for excess electrons that accumulate in the electron transport chain upon disruption of

photosynthesis (Hu et al., 2008), serving as a protective mechanism against oxidative stress induced by exposure to S-metolachlor.

Certain lipid results indicate that the observed effects in mixture condition may be attributed to S-metolachlor, rather than atrazine. For example, in green algae, exposure to the highest concentrations of S-metolachlor alone and in mixture resulted in an increase in phospholipids, whereas atrazine had no effect on these lipids. However, for green algal glycolipids, atrazine individually had no effect; high concentration of S-metolachlor led to a significant decrease in glycolipid content, while the mixture of the two compounds led to a significant increase in glycolipid content at the highest concentrations. There are still relatively few studies on co-exposure to pesticides/herbicides of aquatic and marine organisms. Wang et al., (2022) observed that co-exposure of the diatom *Phaeodactylum tricornutum* to three triazine herbicides (atrazine, prometryn and terbutryn) led to a synergistic effect leading to strong growth inhibition compared to the compounds alone. However, S-metolachlor and atrazine are two herbicides with different modes of action. Few studies have looked at the co-exposure of such different compounds, even though they are often found together in aquatic environments. Carder and Hoagland, (1998) demonstrated a rather additive interaction between atrazine and alachlor (herbicide of the chloroacetanilide family) on the biovolume of an algal community, while Kotrikla et al., (1999) observed an antagonistic interaction between atrazine and metolachlor (racemic) in a mixture on the growth rate of the green alga *Chlorella fusca var-fusca*. This antagonistic interaction may be due to a reduction in the uptake rate of one herbicide by the other (Barbieri et al., 2022) or to an increase in detoxification mechanisms. Interactions between herbicides therefore seem to depend on the species tested, the exposure time (effect at different time scales between herbicides) and the mode of action of the compounds.

In addition to pesticides, agricultural practices contribute to the enrichment in nitrogen and phosphorus in aquatic ecosystems. This process of eutrophication can modify aquatic communities, particularly the biomass of phytoplankton and periphyton, as well as their composition (Morin et al., 2009; Zhang and Mei, 2013). Nutrients may then interact with pesticides (Pannard et al., 2009). For example, Vijayaraj et al., (2022) demonstrated a synergistic interaction between nitrates and the pesticides terbuthylazine (herbicide), pirimicarb (insecticide) and tebuconazole (fungicide) on autotrophic communities. This interaction resulted in a change in the proportion of primary producers, with a reduction in the abundance of macrophytes in favour of phytoplankton and periphyton. This change was explained by the presence of nitrate facilitating the growth of periphyton, but also by a negative effect of the

788 pesticides on *Daphnia magna*, a phytoplankton and periphyton grazer. Therefore, it seems
789 important to increase the number of studies investigating a combination of factors, in particular
790 pesticides and nutrients, and to include climate change (increased temperature as an additional
791 stress). A better understanding of these complex interactions is fundamental for predicting the
792 effects of the many stressors in this changing world.

4.5. Inter-specific differences in herbicide toxicity

Results from photosynthesis and lipids/fatty acid profiles showed that the green algae, the cyanobacteria, and the diatom were affected by atrazine and S-metolachlor exposure, but not to the same extent. At the two highest atrazine concentrations, the green algae was the most affected followed by cyanobacteria and the diatom. Chlorophytes have previously been reported as one of the most sensitive groups among freshwater phototrophs (Schmitt-Jansen and Altenburger, 2007; Weiner et al., 2004), which is in line with the results obtained in this study.

Cyanobacteria are photosynthetic organisms which, because of their physiological similarities to higher plants and algae, can be affected by herbicides (Benegas et al., 2023). Chalifour et al., (2016) investigated the sensitivity to atrazine of monocultures of the green algae *Scenedesmus obliquus*, two strains (toxic and non-toxic) of the cyanobacteria *Microcystis aeruginosa* and a mixed culture before, during and after a period of acclimatization to 0.1 µM atrazine (21.6 $\mu g.L^{-1}$). The results showed that *M. aeruginosa* exhibited lower photosynthetic efficiency, but higher growth rates. Thus, despite reduced photosynthesis, cyanobacteria may be more efficient than green algae at maintaining cell division. This difference in sensitivity may be due to different photosynthetic and metabolic processes. This is also supported by chlorophyll a fluorescence and effective PSII quantum yield values that were lower in cyanobacteria than in green algae, suggesting different structure and function of the algal photosynthetic apparatus (Campbell et al., 1998). In a plant cell, ATP is produced via electron transfer along the photosynthetic chain, but also via auxiliary pathways (Peltier et al., 2010). These pathways may be more efficient in cyanobacteria than in green algae, enabling photosynthetic processes to function more effectively even under stress conditions. Cyanobacteria tolerance to herbicides may also be related to their capacity of using accessory pigment composition (Koenig, 1990), their alternative carbon fixation pathways (Egorova and Bukhov, 2006) or their specific enzymatic and/or non-enzymatic systems acting in response to the oxidative effects of herbicides (Pileggi et al., 2020).

In contrast to the green alga, the lipid and fatty acid profiles of the cyanobacteria culture remained less affected by exposure to atrazine and S-metolachlor. This difference in response may be attributed to the utilization of distinct galactolipids biosynthesis pathways in cyanobacteria (Kalisch et al., 2016). Whereas atrazine resulted in an increase in the ratio of chloroplastic lipids to extra-chloroplastic lipids in the diatom, this ratio decreased in cyanobacteria. Cyanobacteria may exhibit other adaptation strategies for maintaining

membrane lipid structure and the growth of the population instead of maintaining optimal photosynthetic function. Membrane remodeling was also suggested by the decrease in the MGDG/DGDG ratio with atrazine exposure. The biosynthetic pathway may differ between cyanobacteria and microalgae. Awai et al., (2014) even suggest that MGDG and DGDG may not be essential for photosynthesis in certain cyanobacteria like *Synechocystis* sp.

Photosynthetic parameters for the diatom culture were the least affected by both herbicides in the present study. Diatoms are composed of a frustule made of hydrated silica involved in light harvesting, nutrient uptake, and the protection of the cytoplasm from the environment (Townley, 2011). Diatoms are also known to secrete extracellular polymeric substances (EPS) composed of a wide variety of polysaccharides, proteins, lipids and nucleic acids which may play a role in chemical defence (Gonçalves et al., 2018) and preventing herbicide internalization into cells (Melo et al., 2022). In addition, small cells with a larger surface/volume ratio may show higher contaminant uptake, making them more sensitive (Magnusson et al., 2008; Weiner et al., 2004). Microscopic observations did not reveal any differences in biovolume between the green algae, the diatom and the cyanobacteria cultures at the start of the experiments. However, data showed considerable variability within the same condition (data not shown). Other mechanisms may explain the tolerance of diatoms, such as specific pigment composition, higher PSII cycling of electrons and non-photochemical quenching protecting against photooxidation (Wilhelm et al., 2006), or the use of alternative energy sources (Tuchman et al., 2006).

5. Conclusions

This study highlights the potential of atrazine, S-metolachlor and their mixture to affect various biological descriptors of a diatom, a green alga, and two cyanobacteria. The impact of atrazine on the three cultures studied was more pronounced on photosynthetic parameters, while the impact of S-metolachlor was mainly observed on fatty acid profiles. These results align with the respective mode of action of the compounds. However, atrazine also had a significant effect on lipid profiles, which may be attributed to lipid remodeling in response to the stress caused by atrazine exposure. In general, atrazine and S-metolachlor affected organisms at concentrations of 100 $\mu g.L^{-1}$ and 1000 $\mu g.L^{-1}$. However, significant effects of S-metolachlor at 10 $\mu g.L^{-1}$, concentration that can be measured in agricultural areas, were found on some lipid classes and on the proportion of C18:3n-3 in the green algae. Thus, environmental concentrations of these two compounds, either alone or in mixtures, may impact non-target aquatic organisms *in situ*. Atrazine appears to be the main contributor to the observed toxicity in the mixture, as evidenced by the photosynthetic parameters. However, this observation was more subtle looking at lipid content. Mixture effect on TAGs seems driven by S-metolachlor at low concentrations only. Other descriptors suggest that the response to the mixture is intermediate between the response to atrazine and to S-metolachlor alone.

Pesticides can affect non-target organisms present in aquatic ecosystems, as illustrated in this study for phototrophs exposed to the herbicides atrazine and S-metolachlor. The direct effects of these compounds on autotrophs or the modification of their fatty acid profiles and, therefore, their nutritional quality may lead to effects on higher trophic levels. Changes in lipid and fatty acid composition under environmental stress may represent an ecologically relevant response for the study of toxic exposure such as herbicide contamination in agricultural streams. Indeed, these biological descriptors provide complementary information to traditional descriptors such as chlorophyll fluorescence or cell density. Because the response of FAMEs differed depending on the lipid fraction considered (TAGs or polar lipids), it is worth considering studying both fractions instead of just the whole/total lipid composition as it is often the case. Herbicide exposure did not affect the three cultures to the same extent and the sensitivity of the different phototrophs depended on the descriptor measured. The higher tolerance of the diatom and cyanobacteria compared to the green algae may be attributed to differences in pigment composition and/or to more efficient photosynthesis and protective mechanisms against oxidative stress. However, species-specific sensitivity to contamination

879 differed between endpoints. Results from this study support the relevant use of lipidomic
880 descriptors in combination with classical fluorescence descriptors to assess the effects of
881 herbicide mixtures on phototrophic aquatic organisms and their potential effects on higher
882 trophic levels.

Acknowledgments

The authors would like to thank Sylvia Moreira for her help in setting up experiments. Thank you to Débora Millan Navarro for her help on nutrients and herbicides analysis and to Aurélie Moreira for her help on nutrients, herbicides analysis as well as the fractioning and derivation of FAMEs extracts. We want to thank Mélissa Eon and Nicolas Creusot for their help and involvement in the lipid extraction protocol. The experiments were performed at the Pole Végétation Aquatique from the Collective Scientific Infrastructure XPO (DOI: 10.17180/brey-mr38). This work was also supported by the Bordeaux Metabolome Facility, the MetaboHUB (ANR11-INBS-0010) and the PHENOME (ANR-11-INBS-0012) projects.

Author contributions

All authors made substantial contributions to this paper. L.M was in charge of conceptualization, methodology, investigation, formal analysis and writing-original draft. N.M was involved in investigation, writing-review & editing. S.B was involved in formal analysis and writing-review & editing. I.L was in charge of conceptualization, funding acquisition, writing-review & editing. S.M was in charge of project administration, conceptualization, funding acquisition, writing-review & editing.

Funding

We would like to thank the Fonds de recherche du Québec (FRQNT) for a grant to I. Lavoie (FRQNT Relève professorale; 2021-NC-285440), as well as the FRQNT and the Centre de recherche en écotoxicologie du Québec (EcotoQ) for their mobility funding (Programme de stages internationaux du FRQNT) and the LabEx COTE (Laboratories of Excellence, evolution, adaption et gouvernance des écosystèmes continentaux et côtiers) for their mobility grant to Laura Malbezin.

Research data for this article

Data are available at https://doi.org/10.57745/JYZDDE.

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