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Impact of diuron and S-metolachlor on the freshwater diatom *Gomphonema gracile*: complementarity between fatty acid profiles and different kinds of ecotoxicological impact-endpoints.

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Abstract

Fatty acids (FA) are crucial for the maintenance of membrane fluidity and play a central role in metabolic energy storage. Polyunsaturated fatty acids play an essential ecological role since they are key parameters in the nutritional value of algae. Pesticide impacts on fatty acid profiles have been documented in marine microalgae, but remain understudied in freshwater diatoms. The aims of this study were to: 1) investigate the impact of diuron and S-metolachlor on "classical descriptors" (photosynthesis, growth rate, pigment contents, and on the expression levels of target genes in freshwater diatoms), 2) examine the impact of these pesticides on diatom fatty acid profiles and finally, 3) compare fatty acid profiles and "classical descriptor" responses in order to evaluate their complementarity and ecological role. To address this issue, the model freshwater diatom *Gomphonema gracile* was exposed during seven days to diuron and S-metolachlor at 10 $\mu g.L^{-1}$.

G. gracile was mostly composed of the following fatty acids: 20:5n3; 16:1; 16:0; 16:3n4; 14:0 and 20:4n6 and highly unsaturated fatty acids were overall the best represented fatty acid class. S-metolachlor decreased the growth rate and chlorophyll *a* content of *G. gracile* and induced the expression of *cox1*, *nad5*, *d1* and *cat* genes, while no significant impacts were observed on photosynthesis and carotenoid content. In a more global way, S-metolachlor did not impact the fatty acid profiles of *G. gracile*. Diuron inhibited photosynthesis, growth rate, chlorophyll *a* content and induced *cat* and *d1* gene expressions but no significant effect was observed on carotenoid content. Diuron decreased the percentage of highly unsaturated fatty acids but increased the percentage of monounsaturated fatty acids. These results demonstrated that fatty acids responded to diuron conversely to pigment content, suggesting that fatty acids can inform on energy content variation in diatoms subjected to herbicide stress.

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Keywords

Microbial ecotoxicology; Microalgae; Bacillariophycae; Fatty acid; Herbicides; Toxicity

1. Introduction

The intensive use of pesticides for crop protection causes many environmental problems, making pesticides major pollutants of aquatic ecosystems (Aydinalp and Porca 2004, Vidal et al. 2012). Indeed, whereas the use of these pesticides takes place in the terrestrial environment, by aerial spraying, leaching, runoff or accidental spills, the final receptacles of these chemicals are aquatic environments (Vidal et al. 2012). In France, many different herbicides are present in rivers. For example, the tributaries of Arcachon Bay are contaminated by diverse mobile and persistent herbicides, such as diuron and S-metolachlor (Fauvelle et al. 2012).

The herbicide diuron 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea, is a compound of the substituted urea family and is resistant to photolysis and hydrolysis (Okamura 2002). This herbicide is an inhibitor of photosystem II (PSII) which inhibits photosynthesis by preventing the electron transport chain (Rutherford and Krieger-Liszkay 2001) and stopping oxygen formation (Moisset et al. 2015). Furthermore, diuron is known to generate reactive oxygen species (ROS) which induce a gap in the balance between prooxidative and antioxidative reactions (Geoffroy et al. 2002). Moreover, it is also demonstrated that diuron inhibits polyunsaturated fatty acid synthesis by inhibiting the desaturation process (Troton et al. 1986).

S-metolachlor is a compound of the chloroacetamides family and is a molecule that is potentially dangerous to the environment (Liu et al. 2006). This herbicide was developed in order to control grass weeds following pre-emergence application (Neves et al. 2015). It was shown that this pesticide inhibits synthesis of chlorophyll, proteins, fatty acids, lipids and

finally, growth of the organism (Fuerst 1987, Rivard 2003). In fact, S-metolachlor inhibits elongases involved in the elongation of highly unsaturated fatty acid (HUFA) and especially very long-chain fatty acids (VLCFA) (Trenkamp et al. 2004). Furthermore, its toxicity is linked with the oxidation of components of cells increasing the production of reactive oxygen species (ROS) and starting lipid peroxidation (Maronić et al. 2018).

Many microorganisms co-occur in watercourses, especially periphytic communities and particularly diatoms which, at certain times of the year, represent the bulk of periphyton (Moisset et al. 2015). Among primary producers in watercourses, freshwater diatoms are the dominant phototrophic organisms and play an important role in ecological processes such as primary production (Potapova and Charles 2002, Rimet 2012). Diatoms are relevant bioindicators because of their adaptation capacities and their tolerance of environmental factors and aquatic pollution (Potapova and Charles 2002).

Several monitoring tools based on diatom community composition (such as the Biological Diatom Index, BDI; Coste et al., 2009) have been developed to assess the ecological quality of watercourses, but unfortunately these indices are not suitable to detect the impacts of water pollution by pesticides on diatoms (Lenoir 1996, Prygiel and Coste 1996, Dorigo et al. 2004).

It was shown in the literature that the growth and physiology of diatoms can be impacted by herbicide pollution (DeLorenzo et al. 2001, Debenest et al. 2010). Differential gene expression proved to be an early and sensitive biomarker of toxicant exposure (Kim Tiam et al. 2012, Moisset et al. 2015, Kim Tiam et al. 2018), compared to more global endpoints.

Chemical pollution may impact organisms at the biomolecular and biochemical levels being sensitive and quick-responding bioindicators (Adams and Greeley 2000). Alteration in FA profiles is a sensitive early bioindicator of environmental and chemical stress in zooplankton, fish, crustaceans and diatoms, as demonstrated by recent studies (Maazouzi et al. 2008,

Gonçalves et al. 2012, Ramírez et al. 2013, Sánchez-Muros et al. 2013, Gonçalves et al. 2018).

Fatty acids contribute to the fluidity, production and permeability of cell membranes, and they are the main components of lipids that play multiple roles in the cell. Moreover, saturated and monounsaturated fatty acids (SFA and MUFA) are the principal forms of stored energy (Olofsson et al. 2012), whereas polyunsaturated fatty acids (PUFA with > 1 double bonds) and highly unsaturated fatty acids (HUFA) (with ≥ 20 carbons and ≥ 3 double bonds) play a significant role in the organism, being major constituents of cellular membranes (Gonçalves et al. 2017). PUFA and HUFA take part in the regulation of membrane fluidity and serve as precursors of many hormones (Brett and Müller-Navarra 1997). PUFA and HUFA are only synthesized by photosynthetic organisms and are thus the most important molecules to be transferred in the trophic chain (Dalsgaard et al. 2003, Allan et al. 2010).

HUFA such as eicosapentaenoic acid (20:5n3, EPA) and docosahexaenoic acid (22:6n3, DHA) are key nutritional compounds of zooplankton diet because of their energetic efficiency (Perhar and Arhonditsis 2012). They are used as fuel in all metabolic systems for all organisms, having an important role on physiological response (Arts et al. 2009).

Some studies have demonstrated the impact of pesticides on fatty acid profiles of marine microalgae (Robert et al. 2007, Filimonova et al. 2016, Filimonova et al. 2018). For example, Filimonova et al. (2016) observed that exposure to the Primextra® herbicide decreased the percentage of PUFA and particularly of HUFA but increased the percentage of SFA in the marine diatom *Thalassiosira weissflogii*. Likewise, Robert et al. (2007) demonstrated that the herbicide metolachlor decreased the percentage of HUFA in the microalga *Melosira moniliformis*.

Moreover, few studies have demonstrated the effect of herbicides on freshwater microalgae, such as green algae (Weisshaar et al. 1988, El-Sheekh et al. 1994, Böger et al. 2000, Chalifour

et al. 2014). For instance, an increase of MUFA and a decrease of PUFA and HUFA content were found on the green algae *Scenedesmus* and *Chlamydomonas reinhardtii* after respectively chloroacetamide treatment; norflurazon and fluridone exposure (Böger et al. 2000, Chalifour et al. 2014).

To our knowledge, no study has yet focused on the impact of pesticides on fatty acid profiles of freshwater diatoms, and on the variable impacts of distinctly-acting toxic compounds such as diuron and S-metolachlor. We therefore tested the effects environmentally realistic concentrations of both pesticides (10 $\mu g.L^{-1}$ each) (Coquillé et al. 2015, Moisset et al. 2015) on FA profiles and classical endpoints in the freshwater diatom *G. gracile* at 20.5°C for seven days. The aims of our study were: 1) to evaluate the impact of these herbicides on "classical" global descriptors such as growth rate, photosynthesis, pigment contents and to investigate the subsequent expression levels of the genes involved in mitochondrial metabolism (*cox1* (cytochrome C oxidase subunit I), *12S* (mitochondrial ribosomal RNA 12S) and *nad5* (NADH dehydrogenase subunit 5)), photosynthetic activity (*d1 (D1 protein)* and *psA* (PsaA protein)), oxidative stress response (*cat* (catalase) and *gpx* (glutathione peroxidase)) and frustule synthesis (*sil1* (silaffin-1 protein)) known to be putatively differentially expressed during pesticide exposure; 2) to explore the impact of two herbicides with contrasting cellular targets (diuron and S-metolachlor) on fatty acid profiles of freshwater diatoms, hypothesizing that diatom fatty acid responses would be similar to those of green algae; and finally, 3) to compare fatty acid profiles and "classical descriptors" responses in order to show their complementarity based on physiological and ecological responses.

2. Materials and methods

2.1. Diatom culture: *Gomphonema gracile*

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147 *G. gracile* Ehrenberg (1838) (Bacillariophyceae) is a benthic diatom found preferentially in
148 biofilms from slightly acidic waters (Coste et al. 2009). It is a pennate diatom and has a
149 bidirectional movement, as most raphid species (Cohn 2001). According to the Specific
150 Pollution Sensitivity Index (Cemagref 1982), this diatom is considered to be sensitive to
151 macropollutants.
152 The freshwater diatom culture was isolated in December 2013 from a field sample (collected
153 from an upstream section of the Leyre river, which is the main tributary of Arcachon Bay
154 (South West France)) by micromanipulation under an inverted microscope (Coquillé et al.
155 2015). The diatom culture was maintained non axenic in the laboratory and was cultured in
156 sterile Dauta medium (Dauta 1982) at 20.5°C in a thermostatic chamber 610 XAP (LMS
157 LTD®, UK) at 45 µmol photons.m^{-2}.s^{-1} with a dark: light cycle of 8:16h, in 1-L erlenmeyers.
158 Every week, the culture was renewed with new sterile Dauta medium for the purpose of
159 keeping diatoms in an exponential growth phase.
160

161 ## 2.2. Experimental design

162 The experiment was carried out in round, 100 mL borosilicate sterile glass flasks previously
163 heated to 550°C for 4h. One week before pesticide contamination, glass flasks were
164 inoculated with 40 mL of stock culture in exponential growth phase and 40 mL of Dauta
165 culture medium. This step allowed a one-week adaptation of the culture to the glass flasks.
166 The contamination experiment lasted 7 days. For the first day of contamination, glass flasks
167 were inoculated with 300,000 cell.mL^{-1} using adapted cultures in exponential growth phase
168 and Dauta medium (total volume = 80 mL). Three treatments were run: a control treatment
169 (no pesticide) and two pesticide treatments: diuron and S-metolachlor at 10 µg.L^{-1}. Controls
170 and herbicide-exposed cultures were carried out in quadruplicate.

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Chemical (nutrients and pesticides) analyses were carried out at the beginning (t0) and at the end (t7) of the experiment, following the protocols described in Supplementary Material (SI 1 and 2). Detailed environmental conditions in the cultures are provided in Table SI 1.

2.3.Biological parameters

2.3.1. Photosystem II effective quantum yield – Photosynthesis

The analysis of inhibition of photosynthesis was carried out using a PHYTO-PAM (Pulse-Amplitude Modulated (PAM)) (Heinz Walz, GmbH, Germany) every day of the experiment. PHYTO-PAM is a device used to evaluate the physiological state of algae. It is based on the quantitative relationship between the efficiency of photosynthetic energy conversion and chlorophyll fluorescence (Corcoll et al. 2012) and its use for determining pesticide effects on photosynthesis (Dorigo and Leboulanger 2001).

The PSII effective quantum yield (Φ'_M) was determined using PHYTO-PAM with an emitter-detector unit (PHYTO-ED), in the "brown algae" mode. Measurements were performed in 1cm-wide quartz cuvettes using 2 mL of diatom culture. Five Φ'_M measurements were performed for each condition. Φ'_M is described by the following equation (Genty et al. 1989):

$$\Phi'_M = (F'_M - F) / F'_M$$

With: - F being the minimum fluorescence determined after exposure to a weak far red modulated light

- F'_M being the maximum level of fluorescence measured during a saturating white light pulse

2.3.2. Growth rate

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Cellular concentrations of diatoms were quantified every day of the experiment from 1mL of each sample fixed in Lugol (4%) and stored at 4°C until counting. Cell density was determined by counting using a Nageotte counting chamber (Marienfield, Germany) and following the protocol below. Each fixed sample was homogenized with a vortex. After homogenization, 125 µL of sample was dropped into the counting chamber. The number of cells was identified, under light microscopy, in ten columns of the counting area (1.25 µL each, 0.5 mm depth). The distinction between live and dead organisms was estimated by the observation of the color of the chloroplasts and by the observation of their turgescence (Morin et al. 2010).

Cellular concentration was noted in number of individuals per milliliter, and then average growth rates were calculated to optimally fit the daily counting over the exponential growth phase, as the slope of the regression line of Ln (cell.mL^{-1}) over time (days).

2.3.3. Chlorophyll a and carotenoid contents

Chlorophyll *a* and carotenoid content analyses were performed at t0 and t7 of the experiment. An aliquot of 20 mL (t0) and another of approximately 10 mL (t7) of each sample were filtered on Whatman® GF/C filters and frozen at -20°C until the time of analysis. Samples were then defrosted at room temperature before adding 10 mL of 80% acetone (v/v). Each tube was wrapped with aluminum (to induce darkness) and shaken. The tubes were placed in a beaker containing water (to ensure stability) and ultrasonicated for 20 min before being left to settle over a 10 min period, in the dark. Each sample was filtered on Whatman® GF/C filters and the filters were recovered in the filter cone. The tubes were rinsed with 80% acetone and the filters were pressed. The filtrates were collected in the initial tube and the final volume was noted (graduated tube).

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Chlorophyll *a* content was determined with a Shimadzu UV-1800 spectrophotometer (Shimadzu Inc., Kyoto, Japan). Absorbance measurements were made at the following wavelengths: 470 nm, 663.2 nm and 648.8 nm. The chlorophyll *a* and carotenoid contents were calculated with Lichtenthaler equations (Lichtenthaler and Wellburn 1983, Iummato et al. 2017) and expressed as milligrams per liter ($mg.L^{-1}$).

2.3.4. Genetic analyses

Four milliliters, with a cell concentration of 576400 ± 140224 $cells.mL^{-1}$, were sampled at t7 and were centrifuged at 1.500g during 5 min in order to the pellet cells and thus remove culture medium. To avoid degradation of RNA, 200 µL of RNA later buffer were added to the pellet. Samples were stored one night at 4°C and then at -80°C until the genetic analysis.

The protocols of RNA extraction, Reverse Transcription of RNA and Real-Time q-PCR were based on the protocol of Kim Tiam et al. (2012) and carried out at the end of the experiment.

Ten genes were studied; three involved in mitochondrial metabolism (*cox1* (cytochrome C oxidase subunit I), *12S* (mitochondrial ribosomal RNA 12S) and *nad5* (NADH dehydrogenase subunit 5)), two involved in photosynthetic activity (*d1 (D1 protein)* and *psA* (PsaA protein)), two involved in oxidative stress (*cat* (catalase) and *gpx* (glutathione peroxidase)) and one involved in frustule synthesis (*sil1* (silaffin-1 protein)).

The level of expression of each gene was determined in relation to that of the reference gene (*β-actin*) whose expression was stable and did not show any variation depending on the experimental treatments applied. Indeed, the reference gene *rpl7* was not stable and so was not retained.

The detailed primers for each gene are provided in Table SI 3.

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2.3.5. Fatty acid profiles

Twenty milliliters were sampled for each condition at t0 and t7. In order to remove medium culture samples were centrifuged at 1,500 *g* for 5 min and then 1 mL of methanol containing 2.5% H_2SO_4 (*v/v*) was added to the pellet. The fatty acid methyl esters were obtained according to the protocol described by Le Guédard et al. (2008).

Separation of FAMEs was carried out using gas chromatography (Agilent 7890A) on a DB-WAX column (15 m x 1 µm x 0.53 mm, Agilent) and with flame ionization detection. The oven temperature was 150°C for 1 min, followed by a 5°C.min^{-1} ramp to 230°C. This last temperature was maintained for a further 8min (total run time = 25 min). The limit of detection was 0.25µg of fatty acid.

Identification of fatty acids was performed by comparing their retention times with PUFA No3 standard (Sigma, Aldrich). For each fatty acids or class of fatty acids (SFA, MUFA, PUFA, HUFA) results were expressed in percentage of total fatty acids.

2.4. Statistical analyses

To detect significant differences between the beginning and the end of the experiment (FA profiles), and the control conditions and herbicide-exposed cultures (all descriptors), a non-parametric Wilcoxon-Mann-Whitney test was performed because of the non-normal distribution of the data (with a p-value <0.05 considered as statistically significant). All statistical analyses were carried out with the R studio software (RStudioTeam 2016).

Principal Component Analyses (PCA) were performed for the purpose of highlighting correlations between different variables such as photosynthesis activity, growth rate, contents of chlorophyll *a* and carotenoid, the percentage of the four classes of fatty acids (SFA, MUFA, PUFA and HUFA), the percentage of each fatty acid (14:0, 16:0, 16:1 etc.), the repression or induction factor of each gene of interest, nutrient concentrations and treatment

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with pesticides. In order to show significant difference between groups of individuals a PERMANOVA test was performed (with a p-value <0.05 considered as statistically significant) and three confidence ellipses were represented on the PCA plot.

3. Results

3.1."Classical descriptors"

3.1.1. Cellular concentration, growth rate, photosynthesis and pigment contents

Control cultures grew during the seven days of the experiment with an average growth rate of 0.11 div.day^{-1}. The cellular concentration increased between t1 and t7 from 373,000 cells.mL^{-1} to 576,400 cells.mL^{-1}. However, the chlorophyll a and carotenoid contents were stable over the time. Chlorophyll a content was in average of 0.84 ± 0.36 mg.L^{-1} at t0 and 1.49 ± 0.61 mg.L^{-1} at t7 and carotenoid content was 0.19 ± 0.05 mg.L^{-1} at t0 and 0.42 ± 0.17 mg.L^{-1} at t7. Moreover, the photosynthetic activity was stable over time, with an average effective quantum yield of 0.54 ± 0.01.

S-metolachlor and diuron significantly decreased the growth of *G. gracile* (pvalue<0.05) with a decrease in growth rate from 0.11 div.day^{-1} to 0.07 div.day^{-1} and to 0.02 div.day^{-1}, respectively. The cellular concentration of *G. gracile* was significantly lower in both S-metolachlor and diuron-treated cultures than in the controls (pvalue<0.05), being 427,600 ± 26,368 cells.mL^{-1} and 308,600 ± 57,189 cells.mL^{-1}, respectively.

No significant difference in photosynthesis was observed between control and S-metolachlor conditions, whereas diuron decreased photosynthesis with an average effective quantum yield of 0.40 ± 0.02 (pvalue<0.05 for all dates). However, it was interesting to note that photosynthesis at t7 under diuron treatment was significantly higher than t1, t2, t5 and t6 with values of 0.37 ± 0.01, 0.37 ± 0.00, 0.36 ± 0.01 and 0.35 ± 0.02, respectively.

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Chlorophyll *a* concentration significantly decreased with the two pesticide treatments from 1.49 ± 0.61 mg.L^{-1} to 0.80 ± 0.17 mg.L^{-1} for diuron (pvalue<0.05) and from 1.49 ±0.61 mg.L^{-1} to 0.78 ± 0.19 mg.L^{-1} for S-metolachlor (pvalue<0.05), while carotenoid contents were not impacted by either herbicide.

3.1.2. Genetic analyses

The expression of the photosynthesis gene *psaA* and the mitochondrial metabolism gene *12s* were identical with that of the control after seven days of exposure to both pesticides (Table 1). The results of the oxidative stress gene *gpx* and the frustule gene *sil1* were not exploitable in this study.

Conversely, the oxidative stress gene *cat* and photosynthetic gene *d1* were induced by 18.58 and 2.83 respectively with diuron treatment, while S-metolachlor induced the photosynthetic gene *d1*, the mitochondrial metabolism genes *cox1* and *nad5* and the oxidative stress gene *cat* respectively by 6.31, 4.99, 5.08 and 86.57 (Table 1).

3.2.Fatty acid profiles

3.2.1. Variability of fatty acid profiles with time

During the seven days of culture without herbicides, changes in the fatty acid composition of diatoms were observed. At the beginning of the experiment (t0), *G. gracile* was composed mainly of seven fatty acids: EPA (26.17 ± 0.08%); 16:1 (18.63 ± 0.18%); 16:0 (17.57 ± 0.22%); 16:3n4 (9.45 ± 0.06%); 14:0 (7.14 ± 0.12%); 18:1n9 (5.71 ± 0.12%) and 20:4n6 (5.63 ± 0.01%). At the end of the experiment (t7), the six major fatty acids were 20.5n3 (28.49 ± 0.53%); 16:1 (18.21 ± 1.99%); 16:0 (16.57 ± 0.50%), 16:3n4 (11.16 ± 0.52%); 14:0 (7.01 ± 0.33%) and 20:4n6 (5.67 ± 0.27%). The rest of fatty acids that were not described previously had content lower than 5%.

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The percentage in 16:0 and 18:1n9 significantly decreased (pvalue<0.05) respectively from 17.57 ± 0.22% to 16.57 ± 0.50% and from 5.71 ± 0.12% to 3.11 ± 1.78% between the beginning and the end of the experiment.

SFA were affected by the seven days of culture with a significant decrease (pvalue<0.05) in their percentage between t0 and t7 from 24.71 ± 0.32% to 23.58 ± 0.48%. In the same way, the percentage in MUFA significantly decreased (pvalue<0.05) between t0 and t7 from 24.34 ± 0.25% to 21.32 ± 1.21%. The percentage in polyunsaturated fatty acids 16:3n4 and 18:4n3 significantly increased (pvalue<0.05) respectively from 9.45 ± 0.06% to 11.16 ± 0.52% and from 0 ± 0% to 0.56 ± 0.02% and significantly decreased (pvalue<0.05) for 18:2n6 from 1.64 ± 0.02% to 1.44 ± 0.05% between t0 and t7. Overall, the percentage in PUFA significantly increased (pvalue<0.05) from 16.42 ± 0.12% to 18.78 ± 0.94%. The percentage in highly unsaturated fatty acid EPA significantly increased (pvalue<0.05) from 26.17 ± 0.08% to 28.49 ± 0.53% and the percentage in 22:5n3 significantly decreased (pvalue<0.05) from 2.42 ± 0.04% to 1.85 ± 0.20%. The overall percentage in HUFA significantly increased (pvalue<0.05) from 34.51 ± 0.63% to 36.31 ± 1.13%.

3.2.2. Variability of fatty acid profiles with herbicide exposure

Under diuron treatment, the major fatty acid classes were HUFA (31.12%); MUFA (28.84%) (Figure 1) and the major fatty acids were EPA (24.88%); 16:1 (20.64%); 16:0 (14.97%); 16:3n4 (12.73%); 18:1n9 (8.20%) and 14:0 (6.41%) (Figure 2). Diuron significantly increased the percentage in MUFA from 21.32% to 28.84% and significantly decreased SFA and HUFA percentage from 23.58% to 21.38% and from 36.31% to 31.12%, respectively (Figure 2). Furthermore, a significant increase in the percentage of 18:1n9, 16:2n4 and 16:3n4 was noted between control and diuron conditions from 3.11% to 8.20%, from 3.51% to 3.86% and from 11.16% to 12.73%, respectively. A significant decrease in the percentage of 14:0, 16:0,

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18:2n6, 18:4n3 and EPA was observed from 7.01% to 6.41%, from 16.57% to 14.97%, from 1.44% to 0.29%, from 0.56% to 0% and from 28.49% to 24.88%, respectively. Moreover, a non-significant decrease in the percentage of 22:5n3 and 22:6n3 was noted between control and diuron treatment from 1.85% to 0.50% and from 0.30% to 0%, respectively. Also, the percentage of 16:1 increased in a non-significant way from 18.21% to 20.64%.

Under S-metolachlor contamination, no significant impact was observed on fatty acid classes. However, a significant increase in the percentage of 16:0 from 16.57% to 17.25% and a significant decrease in the percentage of 18:2n6, 18:4n3 and 20:4n6 from 1.44% to 1.28%, from 0.56% to 0.52% and from 5.67% to 5.12%, respectively, were observed (Figure 3).

3.3. Correlations between « classical descriptors » and fatty acid profiles

The results obtained at the end of the experiment (t7) for "classical descriptors" and fatty acid profiles were correlated using a Principal Component Analysis (Figure 4). Percentages of saturated (SFA) and highly unsaturated fatty acids (HUFA), photosynthetic activity (Photo_t7) and growth rate were positively correlated with the axis 1 while monounsaturated fatty acid (MUFA) was negatively correlated with this axis. Control and S-metolachlor treatments were correlated with the axis 1 while diuron treatment was negatively correlated with this axis.

These results permitted to conclude that : 1) SFA, MUFA HUFA, photosynthetic activity and growth rate was impacted by herbicide pollutions conversely to pigment contents; 2) Diuron seemed more impacted on "classical descriptors" previously mentioned, conversely to S-metolachlor; 3) Diuron seemed more toxic than S-metolachlor in this study.

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4. Discussion

4.1. "Classical descriptors"

With chemical contamination such as pesticides, reactive centers of diatom photosystems are saturated faster, and light is used less efficiently by diatoms. Therefore, potential damages and the physiological state of the organism can be reflected by the effective quantum yield (Laviale 2008, Bonnineau et al. 2012, Coquillé et al. 2015, Moisset et al. 2015). *G. gracile* cultures exposed to environmental concentration of diuron showed that this herbicide targets photosynthesis and thus cell development. Larras et al. (2013) observed the sensitivity of benthic diatoms to diuron with an establishment of an EC50 value of 9.50 $\mu g.L^{-1}$ based on the 96h growth rate of the population. Diuron acts by blocking electron transport between PSII and PSI and as a consequence, it is considered to be one of the most potent photosynthetic inhibitors (Hall Jr et al. 1999, Thomas et al. 2003).

With S-metolachlor contamination, no impact was observed on photosynthetic activity of *G. gracile* culture exposed to environmental concentrations. Coquillé et al. (2015) demonstrated the same result on photosynthetic activity of *G. gracile* with this pesticide at 1, 10 and 100 $\mu g.L^{-1}$.

However, environmental concentration of S-metolachlor impacted growth rate of *G. gracile*. Previous findings were obtained at 100 $\mu g.L^{-1}$ of S-metolachlor (Coquillé et al. 2015) and 5 and 30 $\mu g.L^{-1}$ (Debenest et al. 2009) of this pesticide. These observations can be explained by the target of S-metolachlor: Carder and Hoagland (1998) noted that chloroacetamides disrupt fatty acid synthesis and finally inhibit cell division. The inhibition of diatom cell division can result in a decrease of chlorophyll content (Debenest et al. 2009).

In diatoms, pigment content is also an indicator of cell physiology: in order to estimate the influence of contaminants, many studies use pigment content as a biomarker (Teisseire and Vernet 2001, Geoffroy et al. 2002). Silkina et al. (2009) showed a decrease in pigment

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contents in the diatom *Staurosirella pinnata* under diuron at 1mg.L^{-1}. The results of the present study showed the same observations for chlorophyll a content conversely to carotenoid content. The applied concentration, more environmentally realistic, was 100-fold lower than the above-mentioned one and could explain this lack of carotenoid content decrease.

As found here, Coquillé et al. (2015) demonstrated a decrease in chlorophyll a content under S-metolachlor at 100µg.L^{-1}.

It is also well-known that pesticides can provoke oxidative stress resulting in DNA breakage, mitochondrial and/or photosynthetic metabolism dysfunction and apoptotic mechanisms induction, leading to the death of the cell (Rutherford and Krieger-Liszkay 2001, Slaninova et al. 2009, Fukuyama et al. 2010).

ROS are one of the earliest responses to chemical stress. When present in low quantities, ROS are rapidly converted to a less reactive form (Lauritano et al. 2015). In order to maintain healthy equilibrium of ROS, effective enzymatic mechanisms such as catalase (CAT) were developed by diatoms (Gonçalves et al. 2018, Gonçalves et al. 2018). However, at abnormally high quantities, they may provoke an alteration of the normal metabolism of the cell impacting nucleic acids, proteins and photosynthetic pigments resulting in an inactivation of enzymes, membrane damage and lipid peroxidation and affecting diverse physiological processes such as viability of cells (Arora et al. 2002, Jaganjac et al. 2016). This could explain differences in results observed between diuron and s-metolachlor used at the same environmental concentration.

Under S-metolachlor contamination, photosynthetic defense mechanism (gene *d1*) seemed efficient since no impact was noted on photosynthetic activity: D1 protein activity was activated increasing PSII reaction centers and allowed countering toxicant effects of this pesticide. The induction of the genes *cox1*, *nad5* suggests an increase of ATP production to

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counter the effects of a toxicant (Kim Tiam et al. 2012), since these genes are involved in the functioning of the respiratory chain and PS II. Moreover, the increase in ATP demand linked to the significant decrease of growth rate in *G. gracile* suggests that energy in the cell was reallocated to detoxification mechanisms to fight against the molecular effects of S-metolachlor (Kim Tiam et al. 2018).

However, *G. gracile* culture exposed to diuron showed a lower level of *d1* and *cat* than those exposed at the same environmental concentration to S-metolachlor. The antioxidative defense mechanisms of *G. gracile* exposed to diuron seem to be not sufficiently effective to protect cells against ROS since, unlike the response with S-metolachlor, a decrease in photosynthesis was observed.

4.2. Fatty acid profiles

4.2.1.1. Variability of fatty acid profiles with time

Culture parameters are important to consider when analyzing biochemical composition of microalgae. It has been demonstrated in many studies that the percentage of fatty acid and particularly EPA content of algal strains is not only associated with heredity, but also influenced by different growing conditions (Teshima et al. 1983, Thompson et al. 1990, Renaud et al. 1991, Yongmanitchai and Ward 1991, Alonso et al. 1992, Reitan et al. 1994, Renaud et al. 1995, Tan and Johns 1996) and by the growth stage of the algae (Chu and Dupuy 1980, Fernández-Reiriz et al. 1989, Brown et al. 1996). In the present study, the fatty acid profile of *G. gracile* was modified with the sampling time, especially with a decrease of SFA and MUFA and an increase of PUFA and HUFA.

Li et al. (2014) noted this variability with the accumulation of neutral lipids rich in SFA (14:0, 16:0) and MUFA (16:1) during a stationary growth phase. These types of lipids are used to store excess carbon accumulated during photosynthesis. Since structural components are

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necessary for the growth, the surplus of neutral lipid accumulation was reduced in diatoms at the exponential growth phase. Finally the proportion of PUFA and HUFA, which are fatty acids of membrane lipids and structural components, are increased in diatoms during the exponential growth phase (Kates and Volcani 1966).

Concerning microalgae, it was noted that *Cylindrotheca* strains accumulated a high proportion of PUFA and HUFA at the exponential phase (29.5%–42.9%), particularly EPA and 20:4n6 (Ying et al. 2002). A study carried out on the diatom *Thalassiosira pseudonana* highlighted that the proportion of total lipid content was higher in the late stationary phase of the culture, whereas PUFA and HUFA decreased during this later phase or in the early stationary phase. The proportion of PUFA and HUFA was higher in the exponential phase (Brown et al. 1996).

4.2.1.2. Variability of fatty acid profiles with herbicide exposure

Numerous studies demonstrated that marine and freshwater microalgae confronted to acute exposition to pesticides (not only herbicides) responded by a modification of their fatty acid profiles with a decrease of PUFA and HUFA content, particularly EPA and PUFA with 18-C, such as linoleic acid (18:2), linolenic acid (18:3); but also, with an increase of MUFA content with palmitoleic acid (16:1) and oleic acid (18:1) (Sicko-Goad et al. 1989, Sicko-Goad et al. 1989, Robert et al. 2007).

4.2.1.2.1. MUFA

The increase in the percentage of MUFA showed under diuron treatment can be explained by the inhibition of photosynthetic activity by this pesticide. Without stress factors and under normal growth conditions, ATP and NADPH, which are products of photosynthesis, are consumed to generate biomass. Various studies have highlighted that when photosystem II is altered and proliferation is finally inhibited because of stress factors such as a lack of

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nutrients, photoinhibition, or other sorts of damages, the major electron acceptor for photosynthesis can become depleted and NADPH is consumed in the biosynthesis of fatty acids. The cells deposit fatty acids in triacylglycerol (TAG), which are reserve lipids and contained majority of MUFA, because there is no need to produce new membrane compounds (Thompson Jr 1996, Hu et al. 2008, Converti et al. 2009). Finally, higher neutral lipid content in algae can be a biological reaction to physiological stress and results observed in the present study are likely the result of a modification in lipid metabolism from the synthesis of membrane lipids to storage of neutral lipids (Hu et al. 2008). To confirm this explanation, it could be relevant in future studies to carry out lipid measurement in diatoms using TAG dosage (Lupette et al. 2019) or fluorescent Nile red and BODIPY, for example (Rumin et al. 2015).

4.2.1.2.2. PUFA and HUFA

With herbicide contamination, it was suggested that the process of fatty acid desaturation is inhibited by the direct inhibition of the desaturase enzymes involved in PUFA and HUFA synthesis (Filimonova et al. 2016). For example, it was shown in the literature that the substituted pyridazinone herbicide inhibited the desaturation process and especially the synthesis of omega-3 fatty acids, decreasing the 18:3n3 content in the glycolipids of algae. In the same way, the herbicide norflurazon inhibited desaturation system and particularly the omega 6 fatty acids (Cohen et al. 1993). Filimonova et al. (2016) observed a decrease of HUFA content of the marine diatom *T. weissfloggi* from 16.48% to 12.32% under Primextra® herbicide treatment.

Under diuron treatment, the decrease of HUFA in *G. gracile* can thus be explained by the inhibition of the desaturation process. Troton et al. (1986) demonstrated identical results on the species *Euglena gracilis* with the decrease of PUFA induced by 5.8 $mg.L^{-1}$ of diuron. The

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authors explained this result by the inhibition of desaturation and the activation of elongation in the process of fatty acid synthesis with diuron contamination. In future studies, it could be relevant to explore genetic responses of desaturase genes concurrent with fatty acid profiles in order to compare their results.

Surprisingly, no effect was observed on fatty acid classes under S-metolachlor treatment. However, a significant decrease of 18:2n6, 18:4n3 and 20:4n6 was observed (pvalue<0.05). It has been shown that the chloroacetamide herbicide metolachlor modifies the synthesis of very long chain fatty acids by inhibiting the action of the enzyme involved in the condensation of malonyl-CoA and acyl-CoA to produce CO_2 and 3-ketoacyl-CoA and impacts fatty acid elongase (FAE) in *Melosira* cf. *moniliformis* with the decrease of the fatty acid content of 18:0, 18:1n9, 18:4 and 20:5 (Thakkar et al. 2013). The marine diatom *Melosira* cf. *moniliformis* contains a FAE-type that converts 16:0-ACP into 18:0-ACP (ACP: Acyl Carrier Protein is an important compound in the process of fatty acid synthesis (Berg et al. 2008)), and 18:1 acid is then produced by n9-desaturation. Moreover, the inhibition of n3 and n6-desaturation induces a decrease of linoleic and linolenic acids content in diatoms (Filimonova et al. 2016). In order to confirm these hypotheses, it could be relevant to contrast genetic responses of desaturase and elongase genes at the same time.

4.3.Fatty acid relevance as biomarker of water contamination

4.3.1. Complementarity between “classical descriptors” and fatty acid profiles

The induction of the oxidative stress gene *cat* under diuron treatment suggests that alteration of HUFA content synthesis may be related to ROS production. To respond to oxidative stress, cells can activate enzymatic antioxidants including catalase (CAT), but when antioxidative defenses fail, HUFA are attacked by the free radicals on their bisallylic site resulting in lipid

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519 peroxidation (Maronić et al. 2018). Conversely, with S-metolachlor the gene *cat* was induced
520 much more importantly than diuron and as a consequence HUFA are less impacted, even not
521 impacted. Thus, this response could be explained by the efficiency of the antioxidant
522 mechanism.
523 Studies on higher plants showed that fatty acids impacted by the organic and inorganic
524 contaminants were the most unsaturated fatty acids and were those present in the chloroplast
525 lipids and necessary for photosynthetic activity. Reductions of these lipids resulted in the
526 dismantling of the photosynthetic membranes and prevented PSI and PSII activity (Kobayashi
527 2016). It was shown in the literature that the fatty acids 18:4 and 20:5 were mainly linked
528 with MGDG (monogalactosyldiacylglycerol) and DGDG (digalactosyldiacylglycerols), which
529 are mostly chloroplastic lipids (80%) (Nakamura and Li-Beisson 2016, Zulu et al. 2018),
530 which can explain the decline of these fatty acids along with photosynthesis in this study.
531 Finally, a decrease in photosynthesis leads to a reduction of the growth rate, that it is
532 consistent with the ACP results in this study. The decrease of photosynthesis activity despite
533 the induction of *d1* gene under diuron treatment could be explained previous by the decrease
534 of HUFA content. Photosynthetic yield was not impacted by S-metolachlor and this might be
535 the result of the efficiency of the *d1* gene against contaminant exposure but also due to very
536 low effect observed on HUFA.
537 The growth rate was damaged under S-metolachlor treatment but in lower proportion than
538 under diuron stress; it could be explained by the induction of mitochondrial metabolism genes
539 and, at the same time, by the very low effect on HUFA which are membrane structure
540 compounds needful for diatom growth.
541 Finally, S-metolachlor contamination seemed to be less toxic than diuron at the same
542 environmental concentration. Several hypotheses can be formulated: 1) contrary to other
543 studies, the herbicide was applied alone and without adjuvant, making the toxicity of

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compounds lower in the present case. Filimonova et al. (2016) studied the impact on the marine diatom *Thalassiosira weissflogii* of the commercial compound Primextra® Gold TZ, which is produced by Syngenta AG and is made-up of two main active ingredients: 17.75% (w/w) of terbuthylazine (TBA) and 30.2% (w/w) of S-metolachlor; 2) the applied concentration was 10 $\mu g.L^{-1}$, a much lower concentration compared to other studies enabling the defense mechanisms to be efficient. For example, Robert et al. (2007) studied the impact of metolachlor but the concentrations applied were 5.7; 7.09 and 8.5 $mg.L^{-1}$; 3) the high concentrations of nutrients provided by the Dauta medium may have stimulated diatoms metabolism and masked, at least partially, pesticide toxicity (Guasch et al. 1998, Guasch et al. 2004); 4) the species *G. gracile* is less sensitive than those used in the previous studies. Wood et al. (2016) showed that the mode of action of herbicide did not change the most sensitive benthic diatom response used in their study, except for *G. gracile*. In fact, *G. gracile* was the third most sensitive to the PSII inhibitors but was ranked as less sensitive to herbicide with another mode of action. Larras et al. (2012) reported the same results: PSII inhibitors are more phytotoxic to diatoms than herbicides with other modes of action. In the current study, the finding of diuron being more toxic than S-metolachlor could so be an artefact of the test organism *G. gracile.*

Finally and according to the results of the PCA analysis, it was shown that fatty acid profiles provide complementary information of herbicide pollution compared to pigment content responses where no impacts were found. Conversely, fatty acid profile responses were correlated with photosynthesis and growth rate responses. However, fatty acid profiles give supplementary information of mode of action of herbicide with especially induction of ROS species, conversely to growth rate and photosynthesis descriptors.

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4.3.2. Ecological relevance of fatty acid profiles

Fatty acid profiles analyses were often used to identify relationships in trophic chain (El-Sabaawi et al. 2009, Van den Meersche et al. 2009, Allan et al. 2010, Kelly and Scheibling 2012). HUFA and especially EPA are necessary in the health and function of all animals at all trophic levels, including plankton invertebrates, fish and humans. Animals are able to convert one form of HUFA to another using elongation and desaturation process but very few can synthesize HUFA *de novo* (Brett and Müller-Navarra 1997). Moreover, it was shown in the literature that bacillariophyceae as diatoms had high contents of EPA and thus were excellent quality food resources for zooplankton (Taipale et al. 2013).

However, lipid components and fatty acid profiles are very sensitive to stressors and environmental modifications (Arts et al. 2009). Filimonova et al. (2016) noted modifications in the FA profiles of the primary producer *T. weissflogii* and primary consumers *A. tonsa* and *A. franciscana* under Primextra® herbicide treatment with the increase of SFA and decrease of PUFA and especially HUFA contents. Thus, it was suggested that pesticide could affect the nutritive value of primary producers and primary consumers impacting the entire trophic chain.

Conversely to "classical descriptors", FA profiles could provide information of nutritional quality of diatom. In the way where diuron decreased HUFA and especially EPA content of *G. gracile*, it could be decreased the nutritional quality of *G. gracile* and consequently the trophic chain could be impacted, conversely to S-metolachlor where no impact where found on EPA. In this context, it could be pertinent in future studies to explore the impact of diuron and S-metolachlor at the same concentration on fatty acid profiles of a consumer of *G. gracile* contaminated by trophic and direct ways.

In summary, while pigment content did not capture pesticide effects at environmentally realistic concentrations, a combination of classical endpoints such as photosynthesis or

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growth rate reduction and FA profiles is recommended for future studies. The latter endpoints were able to capture pesticide effects on diatoms, with FA profiles yielding additional information on the underlying mode of action (e.g. ROS formation) and the ecological relevance (e.g. trophic impact) of pesticide pollution.

5. Conclusions

The impact of diuron and S-metolachlor at 10 µg.L^{-1} was investigated on the freshwater diatom *G. gracile*. At the same concentration, these two pesticides had different effects on "classical descriptors" of *G. gracile*. Fatty acid profiles of *G. gracile* were also affected by these two pesticides, with a considerably stronger response to diuron, suggesting that this pesticide was more toxic to *G. gracile* in this study than S-metolachlor. Both pesticides seemed to induce ROS formation but according to impacted fatty acids, it could be possible that diuron also inactivated desaturation enzymes. The low but environmentally realistic concentrations tested in the present study did not allow to show a high effect of S-metolachlor on fatty acid profiles and it is difficult to conclude on its mode of action of *G. gracile*'s fatty acid profiles. However, considering the target specificity of herbicides, the fatty acids profiles used to assess chemical toxicity might provide additional information on the mode of action and as consequence on the type of pesticide studied.

Moreover, this study proves the complementarity between the fatty acid profiles and the "classical descriptors" since FA indicate herbicide effects conversely to pigment contents and provide additional nutritive values involving ecological information particularly on the trophic chain.

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