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

4
5 Floriane Demailly^{a*}, Imane Elfeky^a, Laura Malbezin^b, Marina Le Guédard^c, Mélissa Eon^a,
6 Jean-Jacques Bessoule^d, Agnès Feurtet-Mazel^b, François Delmas^a, Nicolas Mazzella^a, Patrice
7 Gonzalez^b, Soizic Morin^a

8
9 ^a Irstea, UR EABX, 50 avenue de Verdun, 33612 Cestas cedex – France.

10 ^b Univ. Bordeaux, EPOC, UMR CNRS 5805, Station Marine d'Arcachon, Place du Docteur
11 Bertrand Peyneau, 33120 Arcachon Cedex – France

12 ^c LEB Aquitaine Transfert, ADERA, Bâtiment A3, INRA Bordeaux Aquitaine, 71 avenue
13 Edouard Bourlaux, CS 20032, 33140 Villenave d'Ornon – France

14 ^d CNRS – Univ. Bordeaux, Laboratoire de Biogenèse Membranaire, UMR 5200, Bâtiment
15 A3, INRA Bordeaux Aquitaine, 71 avenue Edouard Bourlaux, CS 20032, 33140 Villenave
16 d'Ornon – France

17
18 *Corresponding author.

19 *E-mail address:* floriane.demailly@irstea.fr (F. Demailly)

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22

23 **Abstract**

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

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

46

47

48 **Keywords**

49 Microbial ecotoxicology; Microalgae; Bacillariophyceae; Fatty acid; Herbicides; Toxicity

50

51 **1. Introduction**

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

60 The herbicide diuron 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea, is a compound of the
61 substituted urea family and is resistant to photolysis and hydrolysis (Okamura 2002). This
62 herbicide is an inhibitor of photosystem II (PSII) which inhibits photosynthesis by preventing
63 the electron transport chain (Rutherford and Krieger-Liszkay 2001) and stopping oxygen
64 formation (Moisset et al. 2015). Furthermore, diuron is known to generate reactive oxygen
65 species (ROS) which induce a gap in the balance between prooxidative and antioxidative
66 reactions (Geoffroy et al. 2002). Moreover, it is also demonstrated that diuron inhibits
67 polyunsaturated fatty acid synthesis by inhibiting the desaturation process (Troton et al.
68 1986).

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

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

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

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

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

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

97 Gonçalves et al. 2012, Ramírez et al. 2013, Sánchez-Muros et al. 2013, Gonçalves et al.
98 2018).

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

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

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

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

122 et al. 2014). For instance, an increase of MUFA and a decrease of PUFA and HUFA content
123 were found on the green algae *Scenedesmus* and *Chlamydomonas reinhardtii* after
124 respectively chloroacetamide treatment; norflurazon and fluridone exposure (Böger et al.
125 2000, Chalifour et al. 2014).

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

143

144

145 **2. Materials and methods**

146 **2.1. Diatom culture: *Gomphonema gracile***

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 $\mu\text{mol photons.m}^{-2}.\text{s}^{-1}$ 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 $\mu\text{g.L}^{-1}$. Controls
170 and herbicide-exposed cultures were carried out in quadruplicate.

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

174

175 **2.3. Biological parameters**

176 ***2.3.1. Photosystem II effective quantum yield – Photosynthesis***

177 The analysis of inhibition of photosynthesis was carried out using a PHYTO-PAM (Pulse-
178 Amplitude Modulated (PAM)) (Heinz Walz, GmbH, Germany) every day of the experiment.

179 PHYTO-PAM is a device used to evaluate the physiological state of algae. It is based on the
180 quantitative relationship between the efficiency of photosynthetic energy conversion and
181 chlorophyll fluorescence (Corcoll et al. 2012) and its use for determining pesticide effects on
182 photosynthesis (Dorigo and Leboulanger 2001).

183 The PSII effective quantum yield (Φ'_M) was determined using PHYTO-PAM with an emitter-
184 detector unit (PHYTO-ED), in the “brown algae” mode. Measurements were performed in
185 1cm-wide quartz cuvettes using 2 mL of diatom culture. Five Φ'_M measurements were
186 performed for each condition. Φ'_M is described by the following equation (Genty et al. 1989):

$$187 \quad \Phi'_M = (F'_M - F) / F'_M$$

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

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

192

193

194 ***2.3.2. Growth rate***

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

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

207

208 ***2.3.3. Chlorophyll a and carotenoid contents***

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

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

224

225 **2.3.4. Genetic analyses**

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

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

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

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

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

242

243

244 **2.3.5. Fatty acid profiles**

245 Twenty milliliters were sampled for each condition at t0 and t7. In order to remove medium
246 culture samples were centrifuged at 1,500 g for 5 min and then 1 mL of methanol containing
247 2.5% H₂SO₄ (v/v) was added to the pellet. The fatty acid methyl esters were obtained
248 according to the protocol described by Le Guédard et al. (2008).

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

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

257

258 **2.4. Statistical analyses**

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

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

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

272

273 **3. Results**

274 ***3.1. "Classical descriptors"***

275 ***3.1.1. Cellular concentration, growth rate, photosynthesis and pigment*** 276 ***contents***

277 Control cultures grew during the seven days of the experiment with an average growth rate of
278 $0.11 \text{ div.day}^{-1}$. The cellular concentration increased between t1 and t7 from $373,000 \text{ cells.mL}^{-1}$
279 1 to $576,400 \text{ cells.mL}^{-1}$. However, the chlorophyll a and carotenoid contents were stable over
280 the time. Chlorophyll a content was in average of $0.84 \pm 0.36 \text{ mg.L}^{-1}$ at t0 and 1.49 ± 0.61
281 mg.L^{-1} at t7 and carotenoid content was $0.19 \pm 0.05 \text{ mg.L}^{-1}$ at t0 and $0.42 \pm 0.17 \text{ mg.L}^{-1}$ at t7.
282 Moreover, the photosynthetic activity was stable over time, with an average effective
283 quantum yield of 0.54 ± 0.01 .

284 S-metolachlor and diuron significantly decreased the growth of *G. gracile* (pvalue<0.05) with
285 a decrease in growth rate from $0.11 \text{ div.day}^{-1}$ to $0.07 \text{ div.day}^{-1}$ and to $0.02 \text{ div.day}^{-1}$,
286 respectively. The cellular concentration of *G. gracile* was significantly lower in both S-
287 metolachlor and diuron-treated cultures than in the controls (pvalue<0.05), being $427,600 \pm$
288 $26,368 \text{ cells.mL}^{-1}$ and $308,600 \pm 57,189 \text{ cells.mL}^{-1}$, respectively.

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

294 Chlorophyll *a* concentration significantly decreased with the two pesticide treatments from
295 $1.49 \pm 0.61 \text{ mg.L}^{-1}$ to $0.80 \pm 0.17 \text{ mg.L}^{-1}$ for diuron ($p\text{value} < 0.05$) and from $1.49 \pm 0.61 \text{ mg.L}^{-1}$
296 to $0.78 \pm 0.19 \text{ mg.L}^{-1}$ for S-metolachlor ($p\text{value} < 0.05$), while carotenoid contents were not
297 impacted by either herbicide.

298

299 **3.1.2. Genetic analyses**

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

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

308

309 **3.2. Fatty acid profiles**

310 **3.2.1. Variability of fatty acid profiles with time**

311 During the seven days of culture without herbicides, changes in the fatty acid composition of
312 diatoms were observed. At the beginning of the experiment (t_0), *G. gracile* was composed
313 mainly of seven fatty acids: EPA ($26.17 \pm 0.08\%$); 16:1 ($18.63 \pm 0.18\%$); 16:0 ($17.57 \pm$
314 0.22%); 16:3n4 ($9.45 \pm 0.06\%$); 14:0 ($7.14 \pm 0.12\%$); 18:1n9 ($5.71 \pm 0.12\%$) and 20:4n6 (5.63
315 $\pm 0.01\%$). At the end of the experiment (t_7), the six major fatty acids were 20:5n3 ($28.49 \pm$
316 0.53%); 16:1 ($18.21 \pm 1.99\%$); 16:0 ($16.57 \pm 0.50\%$), 16:3n4 ($11.16 \pm 0.52\%$); 14:0 ($7.01 \pm$
317 0.33%) and 20:4n6 ($5.67 \pm 0.27\%$). The rest of fatty acids that were not described previously
318 had content lower than 5%.

319 The percentage in 16:0 and 18:1n9 significantly decreased ($p < 0.05$) respectively from
320 $17.57 \pm 0.22\%$ to $16.57 \pm 0.50\%$ and from $5.71 \pm 0.12\%$ to $3.11 \pm 1.78\%$ between the
321 beginning and the end of the experiment.
322 SFA were affected by the seven days of culture with a significant decrease ($p < 0.05$) in
323 their percentage between t_0 and t_7 from $24.71 \pm 0.32\%$ to $23.58 \pm 0.48\%$. In the same way,
324 the percentage in MUFA significantly decreased ($p < 0.05$) between t_0 and t_7 from 24.34
325 $\pm 0.25\%$ to $21.32 \pm 1.21\%$. The percentage in polyunsaturated fatty acids 16:3n4 and 18:4n3
326 significantly increased ($p < 0.05$) respectively from $9.45 \pm 0.06\%$ to $11.16 \pm 0.52\%$ and
327 from $0 \pm 0\%$ to $0.56 \pm 0.02\%$ and significantly decreased ($p < 0.05$) for 18:2n6 from 1.64
328 $\pm 0.02\%$ to $1.44 \pm 0.05\%$ between t_0 and t_7 . Overall, the percentage in PUFA significantly
329 increased ($p < 0.05$) from $16.42 \pm 0.12\%$ to $18.78 \pm 0.94\%$. The percentage in highly
330 unsaturated fatty acid EPA significantly increased ($p < 0.05$) from $26.17 \pm 0.08\%$ to 28.49
331 $\pm 0.53\%$ and the percentage in 22:5n3 significantly decreased ($p < 0.05$) from $2.42 \pm$
332 0.04% to $1.85 \pm 0.20\%$. The overall percentage in HUFA significantly increased
333 ($p < 0.05$) from $34.51 \pm 0.63\%$ to $36.31 \pm 1.13\%$.

334

335 **3.2.2. Variability of fatty acid profiles with herbicide exposure**

336 Under diuron treatment, the major fatty acid classes were HUFA (31.12%); MUFA (28.84%)
337 (Figure 1) and the major fatty acids were EPA (24.88%); 16:1 (20.64%); 16:0 (14.97%);
338 16:3n4 (12.73%); 18:1n9 (8.20%) and 14:0 (6.41%) (Figure 2). Diuron significantly increased
339 the percentage in MUFA from 21.32% to 28.84% and significantly decreased SFA and HUFA
340 percentage from 23.58% to 21.38% and from 36.31% to 31.12%, respectively (Figure 2).
341 Furthermore, a significant increase in the percentage of 18:1n9, 16:2n4 and 16:3n4 was noted
342 between control and diuron conditions from 3.11% to 8.20%, from 3.51% to 3.86% and from
343 11.16% to 12.73%, respectively. A significant decrease in the percentage of 14:0, 16:0,

344 18:2n6, 18:4n3 and EPA was observed from 7.01% to 6.41%, from 16.57% to 14.97%, from
345 1.44% to 0.29%, from 0.56% to 0% and from 28.49% to 24.88%, respectively. Moreover, a
346 non-significant decrease in the percentage of 22:5n3 and 22:6n3 was noted between control
347 and diuron treatment from 1.85% to 0.50% and from 0.30% to 0%, respectively. Also, the
348 percentage of 16:1 increased in a non-significant way from 18.21% to 20.64%.

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

353

354 ***3.3. Correlations between « classical descriptors » and fatty acid profiles***

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

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

366

367

368

369 **4. Discussion**

370 **4.1. “Classical descriptors”**

371 With chemical contamination such as pesticides, reactive centers of diatom photosystems are
372 saturated faster, and light is used less efficiently by diatoms. Therefore, potential damages and
373 the physiological state of the organism can be reflected by the effective quantum yield
374 (Laviale 2008, Bonnineau et al. 2012, Coquillé et al. 2015, Moisset et al. 2015). *G. gracile*
375 cultures exposed to environmental concentration of diuron showed that this herbicide targets
376 photosynthesis and thus cell development. Larras et al. (2013) observed the sensitivity of
377 benthic diatoms to diuron with an establishment of an EC50 value of 9.50 $\mu\text{g.L}^{-1}$ based on the
378 96h growth rate of the population. Diuron acts by blocking electron transport between PSII
379 and PSI and as a consequence, it is considered to be one of the most potent photosynthetic
380 inhibitors (Hall Jr et al. 1999, Thomas et al. 2003).

381 With S-metolachlor contamination, no impact was observed on photosynthetic activity of *G.*
382 *gracile* culture exposed to environmental concentrations. Coquillé et al. (2015) demonstrated
383 the same result on photosynthetic activity of *G. gracile* with this pesticide at 1, 10 and 100
384 $\mu\text{g.L}^{-1}$.

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

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

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

399 As found here, Coquillé et al. (2015) demonstrated a decrease in chlorophyll a content under
400 S-metolachlor at $100\mu\text{g.L}^{-1}$.

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

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

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

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

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

429

430 **4.2. Fatty acid profiles**

431 **4.2.1.1. Variability of fatty acid profiles with time**

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

441 Li et al. (2014) noted this variability with the accumulation of neutral lipids rich in SFA (14:0,
442 16:0) and MUFA (16:1) during a stationary growth phase. These types of lipids are used to
443 store excess carbon accumulated during photosynthesis. Since structural components are

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

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

454

455 **4.2.1.2. Variability of fatty acid profiles with herbicide exposure**

456 Numerous studies demonstrated that marine and freshwater microalgae confronted to acute
457 exposition to pesticides (not only herbicides) responded by a modification of their fatty acid
458 profiles with a decrease of PUFA and HUFA content, particularly EPA and PUFA with 18-C,
459 such as linoleic acid (18:2), linolenic acid (18:3); but also, with an increase of MUFA content
460 with palmitoleic acid (16:1) and oleic acid (18:1) (Sicko-Goad et al. 1989, Sicko-Goad et al.
461 1989, Robert et al. 2007).

462

463 **4.2.1.2.1. MUFA**

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

469 nutrients, photoinhibition, or other sorts of damages, the major electron acceptor for
470 photosynthesis can become depleted and NADPH is consumed in the biosynthesis of fatty
471 acids. The cells deposit fatty acids in triacylglycerol (TAG), which are reserve lipids and
472 contained majority of MUFA, because there is no need to produce new membrane compounds
473 (Thompson Jr 1996, Hu et al. 2008, Converti et al. 2009). Finally, higher neutral lipid content
474 in algae can be a biological reaction to physiological stress and results observed in the present
475 study are likely the result of a modification in lipid metabolism from the synthesis of
476 membrane lipids to storage of neutral lipids (Hu et al. 2008). To confirm this explanation, it
477 could be relevant in future studies to carry out lipid measurement in diatoms using TAG
478 dosage (Lupette et al. 2019) or fluorescent Nile red and BODIPY, for example (Rumin et al.
479 2015).

480

481 **4.2.1.2.2. PUFA and HUFA**

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

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

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

498 Surprisingly, no effect was observed on fatty acid classes under S-metolachlor treatment.
499 However, a significant decrease of 18:2n6, 18:4n3 and 20:4n6 was observed (pvalue<0.05). It
500 has been shown that the chloroacetamide herbicide metolachlor modifies the synthesis of very
501 long chain fatty acids by inhibiting the action of the enzyme involved in the condensation of
502 malonyl-CoA and acyl-CoA to produce CO₂ and 3-ketoacyl-CoA and impacts fatty acid
503 elongase (FAE) in *Melosira cf. moniliformis* with the decrease of the fatty acid content of
504 18:0, 18:1n9, 18:4 and 20:5 (Thakkar et al. 2013). The marine diatom *Melosira cf.*
505 *moniliformis* contains a FAE-type that converts 16:0-ACP into 18:0-ACP (ACP: Acyl Carrier
506 Protein is an important compound in the process of fatty acid synthesis (Berg et al. 2008)),
507 and 18:1 acid is then produced by n9-desaturation. Moreover, the inhibition of n3 and n6-
508 desaturation induces a decrease of linoleic and linolenic acids content in diatoms (Filimonova
509 et al. 2016). In order to confirm these hypotheses, it could be relevant to contrast genetic
510 responses of desaturase and elongase genes at the same time.

511

512 **4.3.Fatty acid relevance as biomarker of water contamination**

513 **4.3.1. Complementarity between “classical descriptors” and fatty acid** 514 **profiles**

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

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 *dl* 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 *dl* 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

544 compounds lower in the present case. Filimonova et al. (2016) studied the impact on the
545 marine diatom *Thalassiosira weissflogii* of the commercial compound Primextra® Gold TZ,
546 which is produced by Syngenta AG and is made-up of two main active ingredients: 17.75%
547 (w/w) of terbuthylazine (TBA) and 30.2% (w/w) of S-metolachlor; 2) the applied
548 concentration was 10 µg.L⁻¹, a much lower concentration compared to other studies enabling
549 the defense mechanisms to be efficient. For example, Robert et al. (2007) studied the impact
550 of metolachlor but the concentrations applied were 5.7; 7.09 and 8.5 mg.L⁻¹; 3) the high
551 concentrations of nutrients provided by the Dauta medium may have stimulated diatoms
552 metabolism and masked, at least partially, pesticide toxicity (Guasch et al. 1998, Guasch et al.
553 2004); 4) the species *G. gracile* is less sensitive than those used in the previous studies. Wood
554 et al. (2016) showed that the mode of action of herbicide did not change the most sensitive
555 benthic diatom response used in their study, except for *G. gracile*. In fact, *G. gracile* was the
556 third most sensitive to the PSII inhibitors but was ranked as less sensitive to herbicide with
557 another mode of action. Larras et al. (2012) reported the same results: PSII inhibitors are more
558 phytotoxic to diatoms than herbicides with other modes of action. In the current study, the
559 finding of diuron being more toxic than S-metolachlor could so be an artefact of the test
560 organism *G. gracile*.

561
562 Finally and according to the results of the PCA analysis, it was shown that fatty acid profiles
563 provide complementary information of herbicide pollution compared to pigment content
564 responses where no impacts were found. Conversely, fatty acid profile responses were
565 correlated with photosynthesis and growth rate responses. However, fatty acid profiles give
566 supplementary information of mode of action of herbicide with especially induction of ROS
567 species, conversely to growth rate and photosynthesis descriptors.

568

569 **4.3.2. Ecological relevance of fatty acid profiles**

570 Fatty acid profiles analyses were often used to identify relationships in trophic chain (El-
571 Sabaawi et al. 2009, Van den Meersche et al. 2009, Allan et al. 2010, Kelly and Scheibling
572 2012). HUFA and especially EPA are necessary in the health and function of all animals at all
573 trophic levels, including plankton invertebrates, fish and humans. Animals are able to convert
574 one form of HUFA to another using elongation and desaturation process but very few can
575 synthesize HUFA *de novo* (Brett and Müller-Navarra 1997). Moreover, it was shown in the
576 literature that bacillariophyceae as diatoms had high contents of EPA and thus were
577 excellent quality food resources for zooplankton (Taipale et al. 2013).

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

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

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

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

598

599 **5. Conclusions**

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

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

616

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618

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625

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