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1 **Monoculture and co-culture tests of the toxicity of four typical**
2 **herbicides on growth, photosynthesis**
3 **and oxidative stress responses of the marine diatoms**
4 ***Pseudo-nitzschia mannii* and *Chaetoceros decipiens***

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41 Sahraoui).

30 Abstract

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2
3 31 The toxicity of four herbicides in mixture (alachlor, diuron, des-isopropyl-atrazine and
4
5 32 simazine) on the growth and the photosynthesis parameters of two marine diatoms *Pseudo-*
6
7 33 *nitzschia mannii* and *Chaetoceros decipiens* have been investigated for 9 days in monoculture
8
9
10 34 and co-culture tests. The catalase (CAT) and guaiacol peroxidase (GPX) were also monitored
11
12 35 to assess the oxidative stress response. In single-species assays, while both species displayed
13
14
15 36 no affected instantaneous growth rate by herbicides, their physiological responses were
16
17 37 different. Chl *a* content of *P. mannii* significantly decreased upon herbicide exposure, due
18
19
20 38 probably to pigment destruction or inhibition of their synthesis. This decrease was associated
21
22 39 with a reduction in the chlorophyll fluorescence parameters (ABS_0/RC , TR_0/RC , ET_0/RC and
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24 40 DI_0/RC). In contrast, *C. decipiens* maintained an effective photosynthetic performance under
25
26
27 41 herbicide exposure, as Chl *a* per cell content and the specific energy fluxes per reaction center
28
29 42 remained unchanged relative to control values. GPX activity was significantly higher in
30
31
32 43 contaminated *P. mannii* and *C. decipiens* monocultures than in controls at early herbicide
33
34 44 exposure (1 day), whereas a significant induction of CAT activity occurred later (from day 3
35
36 45 for *C. decipiens* and at day 9 for *P. mannii*) in response to herbicides. In control co-culture, *P.*
37
38
39 46 *mannii* was eliminated by *C. decipiens*. As observed in the monoculture, the herbicides did not
40
41 47 affect the photosynthetic performance of *C. decipiens* in co-culture, but significantly reduced
42
43
44 48 its instantaneous growth rate. The oxidative stress response in co-culture has similar trends to
45
46 49 that of *C. decipiens* in monoculture, but the interspecies competition likely resulted in higher
47
48
49 50 CAT activity under herbicide exposure. Results of this study suggest that herbicide toxicity for
50
51 51 marine diatoms might be amplified by interspecies interactions in natural communities, which
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53
54 52 might lead to different physiological and growth responses.
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55 *Key words:* diatom, herbicide, growth, photosynthesis, antioxidant enzyme activity.

56 **Introduction**

57 The expansion and intensification of agricultural activities over the last few decades has
58 led to a substantial increase in herbicide use (Mhadhbi et al. 2019; de Suza et al. 2020). These
59 toxicants are not readily adsorbed by soil (Moncada, 2005) and therefore they can reach rivers,
60 lakes and then marine waters, through spraying, leaching and runoff (Carter, 2000; Ramezani,
61 2008). Some herbicides, belonging to triazines and phenylureas are forbidden or restricted in
62 several Mediterranean countries (e.g. atrazine, 2004/141/CE ; simazine 2004/247/CE and
63 diuron, 2007/417/CE) but they are still found in aquatic ecosystems (Nödler et al., 2014;
64 Bancon-Montigny et al. 2019). Herbicides are persistent pollutants in seawater, since they are
65 weakly adsorbed by marine sediment (Thomas et al. 2002; Tissier et al. 2005). Moreover, their
66 degradation is limited and can potentially produce more toxic and/or persistent secondary
67 metabolites (Ortiz-Hernández et al. 2013). Therefore, herbicides constitute a potential threat for
68 marine ecosystems and their phytotoxicity has already been demonstrated on several
69 phototrophic organisms, including phytoplankton (e.g. glyphosate, Smedbol et al. 2017; Lam
70 et al. 2020; triazine, Yang et al. 2019), microphytobenthic biofilms (e.g. diuron and atrazine,
71 Legrand et al. 2006; glyphosate, Lima et al. 2014) and macrophytes (e.g. norflurazon and
72 fluridone, Chalifour, 2014; paraquat, Moustakas et al. 2016; glyphosate, Mudge and Houlahan,
73 2019). Most herbicides, including triazines and phenylureas, are photosynthetic inhibitors.
74 Their active molecules bind to the D1 protein that blocks electron transport from photosystem
75 II (Schuler and Rand, 2008), thus inhibiting Hill's reaction (Leboulanger *et al.* 2001; Dorigo *et*
76 *al.* 2004; Macedo et al. 2008). This further provokes oxidative stress with reactive oxidative
77 species (ROS) formation and accumulation leading to the damage of pigments, proteins, and
78 lipids of the photosynthetic apparatus (Gomes and Juneau, 2017). Other herbicides belonging

79 to chloroacetamide family, such as alachlor and metalachlor, inhibit the biosynthesis of amino-
80 acids and long chain fatty acids (Boger et al. 2003; Yang et al. 2010; Busi, 2014).

81 Several studies have assessed the impact of herbicides on benthic and planktonic
82 microalgae, particularly on diatoms. These silicified microalgae are ubiquitous photosynthetic
83 organisms in marine and fresh waters that account for 20% of global primary production and
84 they are the major contributors to sedimentary organic matter (Nelson et al. 1995; Thornton,
85 2014). Diatoms are at the base of marine food webs, particularly the herbivorous pathway
86 (Sakka Halili et al. 2014; Meddeb et al. 2019) and thus the effects of toxicants on these primary
87 producers can be cascaded to higher trophic levels consequently impacting the function of the
88 whole ecosystem (Relyea, 2009; Pesce et al. 2011; Hashimoto et al. 2019). Diatoms quickly
89 respond to environmental pollutants and hence they are often used as efficient bioindicators to
90 assess the ecological status of aquatic ecosystem (Stevenson and Pan, 2010; Berthon et al. 2011;
91 Pandey, 2017; Pandey et al. 2020). Exposure to herbicides at concentrations ranging from μg
92 L^{-1} to mg L^{-1} , was often followed by either significant decrease in Chl *a*, growth rate,
93 photosynthetic efficiency or lipid content for several marine diatoms (Schmitt-Jansen and
94 Altenburger, 2005; Gatidou and Thomaidis, 2007; Bao et al. 2011; Ebenezer and Ki, 2013;
95 Coquillé et al. 2018). Deformation of diatom frustules and DNA alteration can be also often
96 observed (Schmitt-Jansen and Altenburger, 2005; Debenest et al. 2008; Roubex et al. 2011).
97 However, it is possible to observe no adverse effects on diatoms by herbicides. For example,
98 Debenest et al. (2010) have not detected any impact of atrazine ($25 \mu\text{g l}^{-1}$) on a freshwater
99 diatom community biomass and Hourmant et al. (2009) even found increase in pigment content,
100 photosynthetic rate, and ATP synthesis for *Chaetoceros gracilis* exposed to bentazon with
101 concentration of 0 to $100 \mu\text{g L}^{-1}$. In fact, there is a considerable variability in diatom species
102 sensitivity to herbicides (Larras et al. 2014), even towards the same herbicide (Larras et al.
103 2012).

104 Most of the data on the sensitivity of diatoms to herbicides are provided from single-
1 species bioassays. These allow a wide assessment of herbicide impacts at the molecular, cellular
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5 106 and population levels (Wood et al. 2016; 2019; Chamsi et al. 2019; Demailly et al. 2019).
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7 107 However, there are insufficient conclusive data in view of the effects of herbicides at the
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10 108 community level. In natural communities, interactions between coexisting species may alter
11
12 109 their ability to respond to perturbations. Indeed, it has been shown that diatoms respond
13
14 110 differently to environmental stress depending on whether they are tested in laboratory
15
16 111 monocultures, mixtures or in natural communities (Bérard et al. 1999; Schabhüttl et al. 2012;
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18
19 112 Koedooder et al. 2019).

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21
22 113 The present study aimed to assess the toxicity of four herbicides (diuron, des-isopropyl-
23
24 114 atrazine (DIA), alachlor and simazine) in mixture on two ecologically important marine
25
26 115 diatoms: the pennate species *Pseudo-nitzschia manni* and the centric species *Chaetoceros*
27
28 116 *decepiens*. The first species, known as a neurotoxic producer, was recently reported in
29
30 117 Mediterranean waters (Sakka Hlaili et al. 2016) and the second is a potentially noxious diatom
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33 118 that can form intense blooms (Smayda, 2006; López-Cortés et al. 2015). These diatoms were
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36 119 isolated from the Bizerte Lagoon (SW Mediterranean) in which the four herbicides are
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38
39 120 predominant ($\sum_{\text{diuron+DIA+alachlor+simazine}}: 0.035 \mu\text{g L}^{-1}$, Bancon-Montigny et al. 2019).
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41
42 121 Effects were evaluated with different physiological parameters Chl *a*, growth rate,
43
44 122 photosynthetic efficiency, and antioxidant enzyme activities, to better assess the herbicide
45
46 123 toxicity. Experiments were carried out either as single-species (monoculture) or two-species
47
48
49 124 (co-culture) bioassays.

50 51 52 125 **Material and Methods**

53 54 126 **Microalgal isolation and culture conditions**

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57 127 Cells of *Chaetoceros* (diameter: 24-27 μm , length: 33-36 μm) and *Pseudo-nitzschia*
58
59 128 (width: 1.80-1.90 μm , length: 32-37 μm) were collected in spring 2015 with a Hydrobios 23
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129 μm mesh plankton net by vertical hauls from 3 to 4 m depth to establish laboratory cultures of
130 diatom species. Monoclonal cultures were obtained by single-cell micropipette isolation.
131 Nonaxenic cultures were grown in f/2 medium (Guillard and Ryther, 1962) in 125 mL flasks
132 incubated at 20°C and with a photon flux density of 100 $\mu\text{E m}^{-2} \text{ s}^{-1}$, provided by cool-white
133 fluorescent tubes, with a 12:12 h light:dark cycle in temperature-controlled chamber
134 (Aqualytic, Dortmund, Germany). Diatom cultures were acclimated to these experimental
135 conditions for a minimum of 4-5 generations before dilution with fresh media and the start of
136 the experiments. For this, early exponentially growing cells were transferred on new f/2 media,
137 every 8 days of incubation. Inoculations were carried out in a laminar flow hood and using
138 sterile laboratory material to avoid contamination. All Flasks were swirled by hand at least
139 twice daily, and their positions on the incubator shelves were changed arbitrarily at least once
140 each day to moderate any discontinuities in irradiance or temperature within the incubator.

141 **Species identification**

142 Identification of the two diatom isolates up to the genus level was made by light
143 microscopy under a Zeiss AxioCam HRc light microscope (Zeiss, Oberkochen, Germany).
144 Then, in a second step, transmission electron microscopy was used to identify the two diatoms
145 at the species level. For this, 20 mL subsamples of cultures were filtered then washed with
146 250 mL of distilled water through a 3 μm pore size polytetrafluoroethylene (PTFE) membrane
147 using a Millipore vacuum filtration apparatus. The diatom frustules were then re-suspended in
148 5 mL distilled water and cleaned by adding 10 mL each of concentrated sulfuric and nitric acid
149 in a boiling water bath for 60 min. Frustules were then washed once more with 250 mL of
150 distilled water in the filtration apparatus and re-suspended in a final volume of 5 mL. For
151 electron microscopy examination, one drop of cleaned sample was diluted 20:1 (for cultures)
152 and single drops were dried onto 400-mesh copper grids bearing pure carbon support films, and
153 examined using a Tecnai G2 TEM operating at 60 kV, at the Electron Microscopy Unit (Faculty

154 of Sciences of Bizerte, Tunisia). The species *Pseudo-nitzschia mannii* and *Chaetoceros*
155 *decipiens* Cleve were identified following identification keys of Amato and Montresor (2008)
156 and of Lee et al. (2014), respectively.

157 **Chemicals preparation**

158 The herbicides used in this study are representative of two different modes of action and
159 have different solubility K_{ow} values (Table 1). Stock solutions of herbicides (all dissolved in
160 sterilized Milli-Q water) were used to prepare an herbicide mixture (diuron, DIA, alachlor and
161 simazine) containing $\sim 1 \text{ mg l}^{-1}$ of each herbicide.

162 **Experimental design**

163 Herbicide contamination experiments were carried out on single-species bioassays
164 (monoculture of *Pseudo-nitzschia mannii* or *Chaetoceros decipiens*), as well as on a co-culture
165 of both species. Bioassays were conducted in triplicate in 2 L glass Erlenmeyers flasks,
166 containing 1000 mL of F/2 medium and inoculated with diatom strain cultures in the
167 exponential growth phase. Cultures were set at the initial cell densities of $5 \times 10^3 \text{ mL}^{-1}$ for *P.*
168 *mannii* and $5 \times 10^2 \text{ cells mL}^{-1}$ for *C. decipiens*, respecting the *in situ* concentrations during
169 bloom periods (Sahraoui et al., 2009; 2012). For each species in monoculture or co-culture, two
170 treatments were carried out: control without any herbicide (treatment C) and treatment
171 contaminated with the herbicide blend solution to have the final concentration of $\sim 1 \mu\text{g L}^{-1}$ of
172 each herbicide (treatment Herb). The concentration of total herbicides in contaminated flasks
173 was $4.64 \mu\text{g L}^{-1}$. The chosen herbicide concentration represents ~ 5 times the maximum level
174 observed in Bizerte Lagoon watershed environment ($1.246 \mu\text{g L}^{-1}$, Mhadhbi et al. 2019).

175 All treatments (in mono- and co-cultures) were incubated for 9 days in the temperature-
176 controlled chamber under the same light and temperature conditions described above.
177 Subsamples were taken from all treatments at six time points (t= 0, 1, 2, 3, 6 and 9 days) for
178 growth, physiological, photochemical and biochemical analyses.

179 **Laboratory analyses**

180 **Chlorophyll *a***

181 For chlorophyll *a* (Chl *a*), 10 ml samples were filtered through Whatman GF/F filters.
182 Pigment concentrations were determined using the standard spectrophotometric method
183 (Parsons et al. 1984), following extraction with 10 ml 90% acetone overnight at 4°C in the dark.

184 **Cell density and growth rate calculation**

185 Samples (1.5 ml) used to determine cell density were immediately fixed with 3% acid
186 Lugol's solution. Counts were done in triplicate on 30 µl aliquots under an inverted microscope
187 at magnification of x 40. The instantaneous specific growth rate (μ_{inst} , d⁻¹) was determined by
188 linear regression of the natural logarithm (ln) of cell count vs time over the sampling days.

189 **Measurements of photosynthetic parameters**

190 Samples (10 ml) were dark-adapted for 15 min prior to fluorescence measurements. The
191 AquaPen AP-P100 (Photon Systems Instruments, Czech Republic) was used to measure the
192 rapid polyphasic Chl *a* fluorescence rise during 3 sec by using a LED blue emitter light intensity
193 of 3000 µE m⁻²s⁻¹.

194 Fluorescence yield (F) at investigated fluorescence transients was determined according
195 to Strasser and Strasser (1995). The fluorescence yield at 50 µs was considered as O transient
196 (F_{50µs}) when all reaction centers of PSII are open. The maximal fluorescence yield (F_M) was
197 determined at P transient and the variable fluorescence yields related to J and I transients were
198 determined at 2 ms and 30 ms, respectively. Photosynthetic parameters indicating PSII activity
199 (ABS₀/RC: absorption of photons per active reaction center (RC); TR₀/RC: trapped energy flux
200 per RC; ET₀/RC: electron transport flux per RC and DI₀/RC: non-photochemical energy
201 dissipation per active reaction centre) were evaluated following to Strasser et al. (1999).

202 **Detection of antioxidant enzyme activities**

203 Samples (100 ml) were collected by centrifugation at 6000 g for 30 min at 4°C and then
204 broken down in the phosphate buffer (pH = 7.0) by sonication. Samples were observed under a
205 microscope to make sure the breaking ratio was more than 95%. Finally, the sonicated solution
206 was centrifuged again at 6000 g for 30 min at 4°C and the supernatants were collected to
207 measure the antioxidant enzyme activities.

208 Catalase activity was determined according to the method of Beers and Sizer (1952) by
209 following the H₂O₂ disappearance kinetic for 5 minutes at 240 nm. The reaction medium
210 contained 50 mM phosphate buffer at pH 7.5 and 10 mM H₂O₂. The extinction coefficient was
211 0.036 mM⁻¹ cm⁻¹ and the reaction was initiated by the addition of the protein extract.

212 The guaiacol peroxidase (GPX) activity was determined according to the method of
213 Jiang and Penner (2015) based on the increase in the absorbance at 470 nm due to the
214 polymerization of guaiacol to tetraguaiacol in the presence of hydrogen peroxide for 5 minutes.
215 The reaction medium contained 0.1 M sodium phosphate at pH 6.5, 0.135 mM H₂O₂ and 3.6
216 mM guaiacol. The molar extinction coefficient of tetraguaiacol was 26.6 mM⁻¹ cm⁻¹. The
217 reaction was initiated by the addition of the protein extract.

218 **Statistical analyses**

219 Mean and standard deviation values were calculated for each treatment from three
220 independent replicate cultures and graphed. A two-way ANOVA was used to test the effect of
221 herbicide mixture and culture mode on the instantaneous growth rate of *P. mannii* and *C.*
222 *decipiens* maintained in monoculture and co-culture. The normality of data distribution
223 (Kolmogorov–Smirnov test) and homogeneity of variance (Bartlett–Box test) were respected
224 and differences were accepted as significant when $p < 0.05$.

225 For each experiment (*P. mannii* monoculture, *C. decipiens* monoculture and *P. mannii* +
226 *C. decipiens* co-culture), Student's *t*-tests were performed to detect significant differences in
227 Chl *a* cell content, photosynthetic parameters and antioxidant enzyme activities between control

228 and herbicide treatments and for comparisons between the different cultures (with a p value
1
2 229 <0.05 considered as statistically significant) after having checked the homoscedasticity with
3
4
5 230 Fisher's F-test).

6
7 231 The statistical analyses were performed with the software XLSTAT 18.

9 232 RESULTS

11 233 Response of diatom growth

13 234 Instantaneous growth rate

15 235 At day 1, the instantaneous growth rate of *P. mannii* monoculture was about $\sim 1.10 \text{ d}^{-1}$ for
16
17 236 the control and the herbicide treatments (Fig. 1a). Values then increased to about 1.25 d^{-1} at day
18
19 237 2 and then decreased to $\sim 0.39 \text{ d}^{-1}$ at day 9 for both treatments. The herbicide mixture had no
20
21 238 significant effect on the growth of *P. mannii* maintained in monoculture (two-way ANOVA, P
22
23
24 239 > 0.05 , Fig. 1a). However, the co-culture had a negative effect on the growth of *P. mannii*,
25
26 240 which displayed constantly negative values over the experiment for the control treatment (-0.35
27
28 241 to -0.07 d^{-1}) (two-way ANOVA, $P < 0.05$, Fig. 1a). Negative growth rates were also registered
29
30 242 for *P. mannii* contaminated co-culture, with similar values to those of the control co-culture.
31
32 243 The herbicide mixture did not affect the growth of *C. decipiens* in monoculture (two-way
33
34 244 ANOVA, $P > 0.05$, Fig. 1b) since comparable instantaneous growth rate values were registered
35
36 245 between the control and the herbicide treatment (from ~ 0.5 at day 1 to ~ 0.2 at day 9). In control
37
38 246 co-culture, *C. decipiens* maintained similar growth rates to those of the control monoculture
39
40 247 (two-way ANOVA, $P > 0.05$, Fig. 1b). Conversely, the herbicide mixture has significantly
41
42 248 impacted *C. decipiens* when the species was growing in co-culture (two-way ANOVA, $P <$
43
44 249 0.05 , Fig. 1b) and its instantaneous growth rate values were reduced by 35.41 %, 41.39 % and
45
46 250 61.11 % compared to the control co-cultures, at days 3, 6 and 9, respectively.

51 251 Chlorophyll a content

252 Under monoculture conditions, the Chl *a* per cell content of *P. mannii* (initially of
1
2 253 9.34 ± 0.4 $\mu\text{g cell}^{-1}$) increased significantly in the control to reach a maximum on day 3
3
4
5 254 (18.77 ± 5.75 $\mu\text{g cell}^{-1}$) and then decreased sharply to attain 2.09 ± 0.01 $\mu\text{g cell}^{-1}$ at the end of
6
7 255 the experiment (Fig. 2a). The herbicide mixture induced a significant reduction in Chl *a* cell
8
9 256 content for *P. mannii* monoculture (Student's *t*-test, $P < 0.05$) and values decreased by 55-89%
10
11 257 compared to the control after 2-3 days. Conversely, the addition of herbicides did not provoke
12
13 258 any significant effect on the specific biomass of *C. decipiens* in monoculture (Student's *t*-test,
14
15 259 $P > 0.05$, Fig. 2b) whose mean values varied in a range from 31.76 ± 7.77 to 53.61 ± 7.90 μg
16
17
18
19 260 cell^{-1} .

261 For the diatom co-culture under control conditions, the initial Chl *a* per cell content (9.86
22
23
24 262 ± 0.8 $\mu\text{g cell}^{-1}$) slightly diminished at the beginning (until day 3) to increase thereafter
25
26 263 significantly by 2 to 3-fold compared to the initial level (Fig. 2c). The herbicide mixture induced
27
28 264 a significant increase in Chl *a* cell content compared to the control (Student's *t*-test, $P < 0.05$)
29
30 265 with the most important rise at day 3 (increase by 8 folds compared to the control) (Fig. 2c).

266 **Physiological response of diatoms**

267 **Photosynthetic parameters**

268
37
38
39 269 Whether for the diatom monocultures or co-culture, the maximum quantum yield of PSII
40
41 270 (F_v/F_m) did not show any significant difference between the control and herbicide treatment
42
43 271 (Student's *t*-test, $P > 0.05$, Fig. 3a). This parameter declined from the beginning ($\sim 0.7 - 0.8$
44
45 272 a.u.) to the end of the experiment, reaching values of -20 to -30 % relative to the initial level
46
47 273 (Fig. 3a).

50
51 274 For *P. mannii* in monoculture, the herbicide mixture induced a significant sharp decrease
52
53 275 of ABS_0/RC (Student's *t*-test, $P < 0.05$, Fig. 3b). This reduction was noticeable from day 1,
54
55 276 such that values of this parameter (in the range of 1.38-1.98 a.u. for control) approached the
56
57 277 zero at day 2 (-98.01 % relative to control) Values recovered to control levels at day 3 to
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278 decrease again to approximately -50 % relative to the control at day 9. This ratio was unchanged
279 by the herbicide mixture in the monoculture of *C. decipiens* (Student's *t*-test, $P > 0.05$, Fig. 3b)
280 with values ranging in general between 2 and 3 a.u. For the diatom co-culture, there was also
281 no significant effect of the herbicide mixture on ABS_0/RC (Student's *t*-test, $P > 0.05$, Fig 3b)
282 and values were often in the range of 2 to 3 a.u.

283 Likewise, the ratios of trapping of excitation energy per reaction centre (TR_0/RC) and
284 electron transport per active reaction centre (ET_0/RC) were significantly reduced by herbicide
285 for *P. mannii* monoculture (Student's *t*-test, $P < 0.05$, Fig. 3c, d) as both parameters decreased
286 at day 2 by 80% and 89 % relative to control, respectively. Conversely, TR_0/RC and ET_0/RC
287 were unchanged by the herbicide mixture in *C. decipiens* monoculture (Student's *t*-test, $P >$
288 0.05) with values mostly of about 1.5-1.8 a.u. and 1.12-2.1 a.u., respectively (Fig. 3c, 3d). For
289 the diatom co-culture, there was no significant effect of the herbicide mixture on both ratios
290 (Student's *t*-test, $p > 0.05$, Fig. 3c, d).

291 Herbicide treatment significantly impacted the dissipated energy flux per active reaction
292 center (DI_0/RC) for *P. mannii* monoculture (Student's *t*-test, $P < 0.05$, Fig. 3e), causing 33-
293 98% reduction relative to control. Conversely, no significant effect was observed for this
294 physiological parameter for the *C. decipiens* monoculture (range of values: 0.78-1.31 a.u.) nor
295 for the diatom co-culture (range of values: 0.74-1.18 a.u.) (Student's *t*-test, $P > 0.05$, Fig. 3e).

296 Antioxidant enzyme activity

297 For the *P. mannii* monoculture, the catalase activity was relatively low in the control
298 (range of values: 0.42 – 1.05 U mg^{-1} Protein, Fig 4a). The herbicide mixture did not affect the
299 enzyme activity (Student's *t*-test, $P > 0.05$) except at the end of the experiment where a great
300 increase was observed (roughly 20 times the control value, Fig. 4a). In the *C. decipiens* control
301 monoculture the catalase activity was significantly greater than that of *P. mannii* control
302 monoculture (range of values: 7.14-10.52 U mg^{-1} Protein) (Student's *t*-test, $P < 0.05$). Exposure

303 to herbicide resulted in a significant increase in catalase activity (Student's *t*-test, $P < 0.05$,
1 Fig.4b) and values increased by 3-5 folds relative to control after 3-days treatment. The same
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3 response to herbicides was observed for the diatom co-culture (Student's *t*-test, $P < 0.05$,
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5 Fig. 4c) as catalase activity increased by 4-7 folds in comparison to control after 3-days
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7 treatment.
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11 Overall, the GPX activity, measured in monocultures and co-culture, was not affected by
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13 the herbicide mixture addition (Student's *t*-test, $P > 0.05$, Fig. 4d, e, f) except on day 1, when
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15 the enzyme showed 2-3 folds higher activity in herbicide treatment. The GPX activity values
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17 generally varied within the range of 1.83-22.41 U mg⁻¹ Protein, for all type of cultures (Fig. 4d,
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19 e, f).
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24 313 DISCUSSION

26 314 Herbicide Toxicity towards diatoms in monoculture

29 315 Growth and Chl *a* cell content

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31 Our findings showed that the herbicide mixture did not significantly affect the
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33 instantaneous growth rate of *P. mannii* and *C. decipiens* grown as monocultures ($P > 0.05$, Fig.
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35 1a, b). Literature data on the effect of herbicides on diatom growth are highly contrasted.
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37 Indeed, it seems difficult to distinguish a general response of diatoms to herbicide exposure.
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39 Previously, authors have reported a decrease in growth for freshwater diatoms in response to
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41 herbicides, such as atrazine (1-1000 µg L⁻¹: Larras et al. 2013; 500 µg L⁻¹: Wood et al. 2014),
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43 metolachlor (5-30 µg L⁻¹: Roubex et al. 2011), isoproturon (5-20 µg L⁻¹: Pérès et al. 1996) and
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45 diuron (10 µg L⁻¹: Demailly et al. 2019). All these authors have used herbicides at higher
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47 concentrations than used in this study (Σ herbicides ~ 5 µg L⁻¹), which could explain the lack
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49 of herbicide impact on the growth of *P. mannii* and *C. decipiens* monocultures. However, other
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51 studies have reported positive impact of herbicides on diatom growth (50 µg L⁻¹ atrazine,
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53 Kalopesa et al., 2008; 30 µg L⁻¹ isoproturon, Debenest et al. 2009; and 7 µg L⁻¹ diuron, Ricart
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328 et al. 2009). In our experiment, *P. mannii* and *C. decipiens*, growing as monocultures, seemed
329 to tolerate the herbicide mixture at the tested concentration. Both species originated from
330 Bizerte Lagoon, where noticeable contamination by herbicides has been reported for waters and
331 sediment (Pringault et al. 2016; Bancon-Montigny et al. 2019; Mhadhbi et al. 2019). The
332 ecosystem has long been under the influence of water runoff from adjacent land areas used for
333 agricultural crops cultivation with intensive use of herbicides (Ben Salem et al. 2017).
334 Consequently, it is likely that *P. mannii* and *C. decipiens* isolated from this contaminated
335 ecosystem might have developed tolerance to herbicides through adaptation mechanisms that
336 make them physiologically able to resist to these pollutants.

337 In monoculture, the Chl *a* per cell content of *P. mannii* was significantly affected by Herb
338 treatment ($P < 0.05$, Fig. 2a), although growth was not affected (Fig. 1a). This suggests that the
339 per-cell Chl *a* content would decrease under herbicide contamination probably due to inhibition
340 of pigment synthesis or pigment destruction. In fact, herbicides, particularly the PSII inhibitors
341 (e.g. DIA, diuron and simazine), are known to act by binding to the exchangeable quinone site
342 in the PSII reaction center, thus blocking electron transfer (Gomes and Juneau, 2017; Thomas
343 et al. 2020). The excitation of the PSII complex is therefore directly transferred to O₂, resulting
344 in the formation of singlet oxygen causing uncontrolled oxidation of membranes components
345 in particular chlorophyll pigments, including Chl *a* (Thomas et al. 2020). Furthermore, PSII
346 inhibiting herbicides would be responsible for the accumulation of compounds such as
347 polyenes, phytols and phytophloenes that inhibit chlorophyll synthesis in microalgae (Thomas
348 et al. 2020). Conversely to *P. mannii*, herbicides did not cause any effect on Chl *a* cell content
349 of *C. decipiens* monoculture ($P > 0.05$, Fig. 2b), suggesting that the two species have different
350 levels of tolerance to herbicides. In fact, studies carried out on *Chaetoceros* species reported
351 different results. An increase in Chl *a* content was observed for *C. gracilis* exposed to 50 to
352 60 µg l⁻¹ of Bentazon, while a drastic decrease was found for *Chaetoceros* sp. as a short-term

353 response to 2,4-D dimethylamine (50 to 300 mg l⁻¹) and endosulfan (20 to 100 mg l⁻¹) exposure
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2 354 (Mine and Matsunaka 1975; Hii et al. 2009).
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355 **Photosynthetic response and antioxidant enzymes activity**

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356 Whether for *P. mannii* or for *C. decipiens* grown in monoculture our results showed that
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11 357 the Fv/Fm ratio has been unaltered by the tested herbicide mixture ($P < 0.05$, Fig 3.a),
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13 358 suggesting a relative integrity of photosystem II under the experimental conditions. However,
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15 359 herbicide-induced changes were clearly visible in all the other OJIP parameters (ABS₀/RC,
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17 360 TR₀/RC, ET₀/RC and DI₀/RC) for *P. mannii* monoculture (Fig. 3). Several authors have already
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19 361 pointed out that Fv/Fm is probably not the best parameter to evaluate toxic effects of herbicides
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21 362 due to its relatively low sensitivity (e.g. Juneau et al. 2007; Kargar et al. 2019). Fv/Fm measures
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23 363 the optimal photosynthetic efficiency and does not reflect the true nature of PSII-activity under
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25 364 normal light conditions (Juneau et al. 2007). The decrease in the photosynthetic parameters in
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27 365 *P. mannii* monoculture may indicate an increase in activated fraction of RCs or in antenna size
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29 366 by *P. mannii* to counterbalance the chlorophyll pigment deterioration by herbicides (cf. Fig. 2a)
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31 367 to maintain an efficient light energy transfer. In fact, these ratios are influenced by the ratio of
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33 368 active/inactive RCs of light harvesting complex (LHC) and as the number of active centers
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35 369 increased, they decreased (Mathur et al. 2011; Kumar et al. 2019). None of the specific energy
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37 370 fluxes per RC were affected by herbicides for *C. decipiens* monoculture ($P > 0.05$, Fig 3),
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39 371 suggesting that the species could maintain an effective photosynthetic performance under
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41 372 herbicide exposure, which could explain the lack of effect on its Chl *a* content. Therefore, *C.*
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43 373 *decipiens* seemed to be physiologically more tolerant to herbicides than *P. mannii*. This could
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45 374 be due to the antenna organization, which differs significantly between pennate and centric
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47 375 diatoms (Gundermann et al. 2019).
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376 The enzymes involved in oxidative damage repair have significantly increased under
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59 377 herbicide contamination. However, this stimulation was different between the diatom species.
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378 Indeed, the CAT activity has increased for *P. mannii* only by the end of the experience, whereas
379 *C. decipiens* showed a considerably higher CAT response to the herbicide mixture ($P < 0.05$,
380 Fig. 4). CAT enzyme has been possibly activated to regulate toxic levels of reactive oxygen
381 species (ROS) which production was probably induced by herbicides (Bai et al. 2015; Thomas
382 et al. 2020). For *C. decipiens*, the eventual oxidative damage could be likely induced by the
383 Very-Long-Chain Fatty Acid (VLCFA) inhibitor (alachlor) as the PSII inhibiting herbicides has
384 not impacted its photosynthetic performance ($P > 0.05$, Fig 3). It is widely known that the
385 toxicity of chloroacetamides like alachlor is linked to the oxidation of cell components with the
386 increase of ROS and lipid peroxidation (Maronić et al. 2018). At short term (day 1), the
387 herbicide mixture promptly stimulated the GPX activity for *P. mannii* and *C. decipiens* (Fig.
388 4), probably indicating an oxidative short-term stress in both species under herbicide exposure,
389 as it has been observed with other chemical toxic compounds (Manimaran et al. 2012, Nguyen
390 Deroche et al. 2012). In summary, it seems that the antioxidant enzymatic response to
391 herbicides could provide an efficient protection to *C. decipiens*, probably resulting in the
392 maintenance of its photosynthetic apparatus integrity and Chl *a* per-cell content. On the other
393 hand, the antioxidant defense system of *P. mannii* was only triggered at the short time scale
394 which might result in significant effects on the photosynthetic apparatus integrity.

395 **Herbicide toxicity towards diatoms in co-culture**

396 **Growth and Chl *a* cell content**

397 Under control conditions, diatom species in co-culture have evolved in a different way
398 than when maintained in monoculture, with a slower growth for *C. decipiens* and a decline for
399 *P. mannii*. (two-way ANOVA, $P < 0.05$, Fig. 1). This suggests that *C. decipiens* achieved
400 growth advantage by dominating *P. mannii* due to either a better competition for resources (e.g.,
401 nutrients, light) and/or a chemically mediated interference (i.e. allelopathy). In our study, the
402 mono- and co-cultures bioassays were performed under similar physico-chemical conditions,

403 using the same non-limiting nutrient concentrations, that the exclusion of *P. mannii* in co-
404 culture might probably be explained by allelopathy. Allelopathy was found to play a significant
405 role in dominance, succession, and formation of natural diatom communities (Leflaive and Ten
406 Hage, 2009; Zhang et al. 2014). In addition, the small size of *P. mannii* may increase its
407 sensitivity to allelopathic interactions due to a high surface/volume ratio (Lyzkowski and
408 Karp-Boss, 2014).

409 The single-species exposure to the herbicide mixture, at the tested concentration, showed
410 that *P. mannii* and *C. decipiens* were tolerant, with no significant impact on their instantaneous
411 growth rates (two-way ANOVA, $P > 0.05$, Table 2). However, growth of *C. decipiens* in co-
412 culture was significantly impacted by herbicides (two-way ANOVA, $P < 0.05$, Table 2),
413 suggesting that its resistance to herbicide exposure could be modulated when the species
414 competes in co-culture. This trend may be explained by the classical trade-off theory between
415 tolerance ability and competitive-response ability in phytoplankton (Mohr, 2019; Lüring,
416 2021).

417 The differences in sensitivity to herbicides among *P. mannii* and *C. decipiens* under co-
418 culture conditions might be also explained by differences at the genetic level or in the trophic
419 mode. In fact, some authors have observed that herbicides may cause a decrease in growth of
420 some diatoms, specifically from the centric genera, whereas it has the opposite effect on pennate
421 diatoms (Tang et al., 1997; Bérard et al., 2004). In this sense, Larras et al. (2014) have found a
422 strong phylogenetic signal for diatom sensitivity to herbicides with a major separation within
423 the phylogenetic tree. These authors observed that the most sensitive species were mainly
424 centrics and araphid diatoms whereas the most resistant species were mainly pennates. Other
425 authors asserted that sensitivity differences among diatom species to herbicides is more likely
426 related to their trophic mode (Larras et al. 2012). Indeed it was argued that heterotrophic
427 diatoms were more tolerant than autotrophic diatoms species to PSII inhibitors (Pérès et al.

1996; Bérard et al. 1998; Debenest et al. 2009). For this assumption, authors explained that conversely to autotrophic species, facultative or obligatory heterotrophic species (eventually the case of *P. mannii* in this study) could metabolize other substrata to decrease their dependence on photosynthesis (Larras et al. 2012) and thus to prevent effects of PSII inhibitor herbicides. Heterotrophy has been clearly evidenced for several species of the genus *Pseudo-nitzschia* (Mengelt and Prezelin, 2005, Loureiro et al. 2009, Melliti Ben Garali et al. 2016).

In our experiment, the growth of *P. mannii* was inhibited by *C. decipiens* which dominated the co-culture, substantially on days 6 and 9. The Herbicide induced increase of Chl *a* cell content seemed then to be likely related to *C. decipiens* (Fig. 2). This suggest that the competitive advantage of *C. decipiens* might not affect its Chl *a* response to herbicides. Increased Chl *a* content in response to herbicides has been noted for several microalgae, including *C. gracilis*, contaminated by PSII inhibitors such as diuron (Magnusson et al. 2008; Stachowski-Haberkorn et al. 2013), atrazine (Adler et al. 2007), bentazon (Mine and Matsunaka, 1975), isoproturon and terbutryn (Rioboo et al. 2002). This reaction, also known as “greening”, is widely described as an increase in pigment in antennae in order to maximize light harvesting as a strategy to counterbalance the herbicide action (Magnusson et al. 2008; Ricart et al. 2009; Mansano et al. 2017) and hence maintain an efficient conversion of light energy into chemical energy (Coquillé et al. 2014). This would allow an adaptation of photosynthetic metabolism to herbicide effects.

Photosynthetic response and antioxidant enzymes activity

Maximum quantum yield of PSII (F_v/F_m) as well as all the energy fluxes per RC (ABS_0/RC , TR_0/RC , ET_0/RC and DI_0/RC) were unchanged by the herbicide mixture for the diatom co-culture ($P > 0.05$, Fig. 3), closely retracing evolution of these parameters for *C. decipiens* (Fig. 3), which has by far dominated the diatom co-culture. So, as for Chl *a*, the competitive advantage of *C. decipiens* on co-culture did not also influence its photosynthesis

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453 response to the herbicide mixture. As observed for *P. mannii* or *C. decipiens* monocultures, the
454 GPX activity was stimulated only after one day of contamination in co-culture (Fig. 4),
455 suggesting that this enzyme could be considered as the antioxidant defense at early exposure to
456 herbicides. There was a significant increase of CAT activity by herbicides in co-culture ($P <$
457 0.05 , Fig. 4), especially at late herbicide exposure, similarly to that observed for *C. decipiens*
458 monoculture, providing additional support that the physiological response of the co-culture to
459 herbicides was likely attributed to *C. decipiens*. However, CAT activity levels were
460 significantly higher than those measured for *C. decipiens* monoculture (Student's t -test, $P <$
461 0.05 , Fig. 4) and values at days 3 and 6 were 1.2 to 2 folds higher than in *C. decipiens*
462 monoculture, probably suggesting a higher CAT response due to the competitive stress. These
463 results provided further evidence that GPX and CAT enzymes were triggered by the herbicide-
464 induced oxidative stress, at early and tardy exposure, in order to repair damage and keep the
465 diatom in good physiological state during most period of herbicide contamination.

466 CONCLUSION

467 The study found that response to herbicides differ between the two marine diatoms and
468 between the culture conditions. The diatom *C. decipiens* was more sensitive to herbicides in co-
469 culture than in the single species-pollutant experiment, which suggest that herbicide pollution
470 in natural environments might considerably alter the diatom populations and phytoplankton
471 communities. We link altered growth of *C. decipiens* to a modified physiological profile under
472 herbicide conditions, thereby suggesting that chemical pollutants effects are not only reflected
473 by changes in cell densities but also by changes in the physiology of the cells. In order to attain
474 higher comparability with natural conditions, further studies should focus on the effects of
475 herbicide pollution and other abiotic fluctuations and their interactions with competition
476 between diatoms. More complex diatom combinations could be also tested in order to identify

477 composite ecosystem responses and acquire approaches to apprehend changes that might take
478 place under future environmental scenarios.

479 **DECLARATIONS**

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483 **COMPLIANCE WITH ETHICAL STANDARDS**

484 Conflict of interest: the authors have no competing interests to declare that are relevant to the
485 content of this article.

486 **AVAILABILITY OF DATA AND MATERIAL**

487 All data and materials as well as software application or custom code comply with field
488 standards.

489 **ETHICS APPROVAL**

490 This article does not contain any studies with animals performed by any of the authors

491 **AUTHOR CONTRIBUTIONS**

492 IS, SMB, ZC and ASH contributed to the study conception and design. Laboratory analyses
493 were performed by IS and ZC and CG. The first draft of the paper was written by IS. ASH
494 and OP commented on previous versions of the paper. All authors read and approved the final
495 paper.

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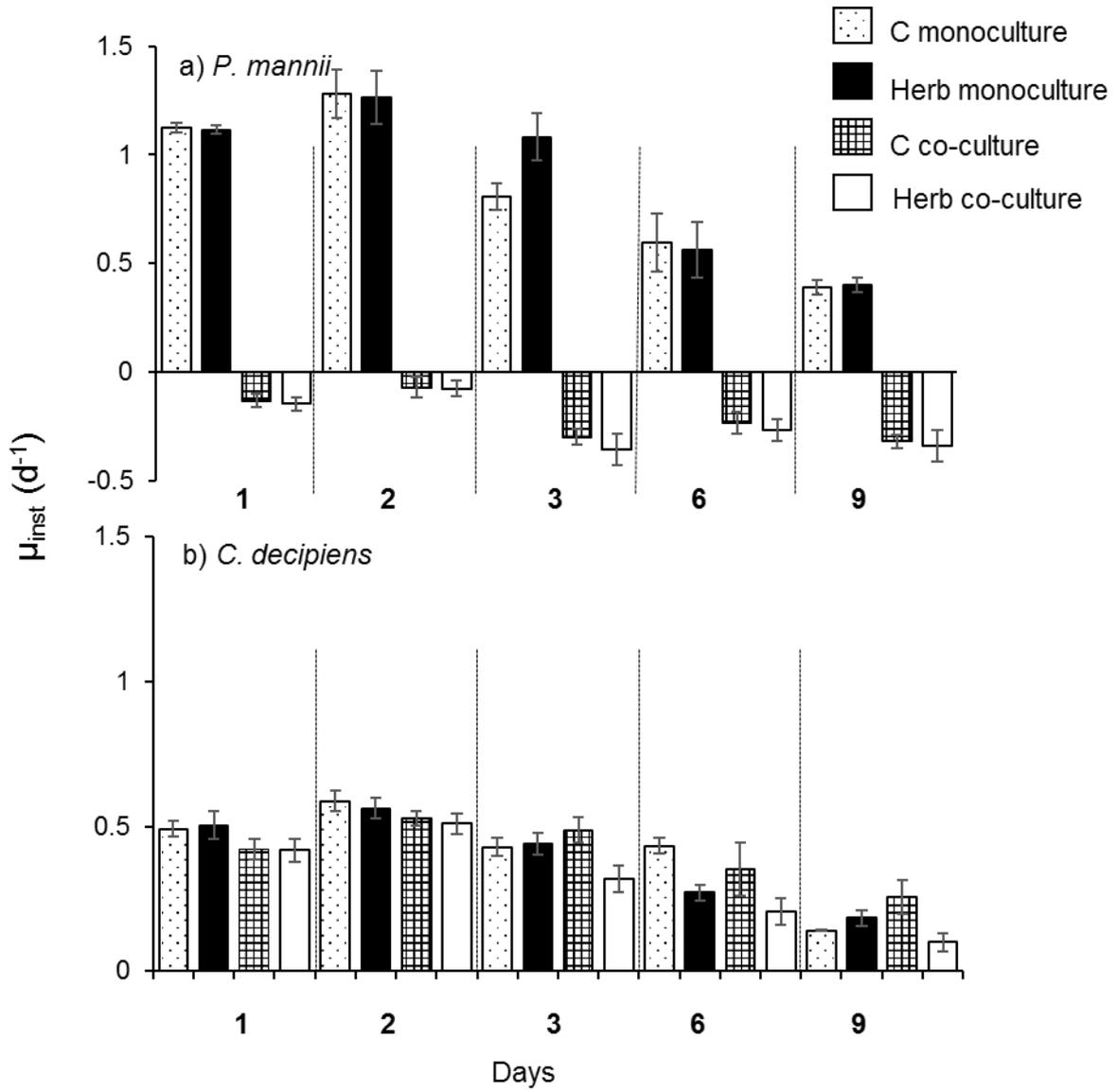
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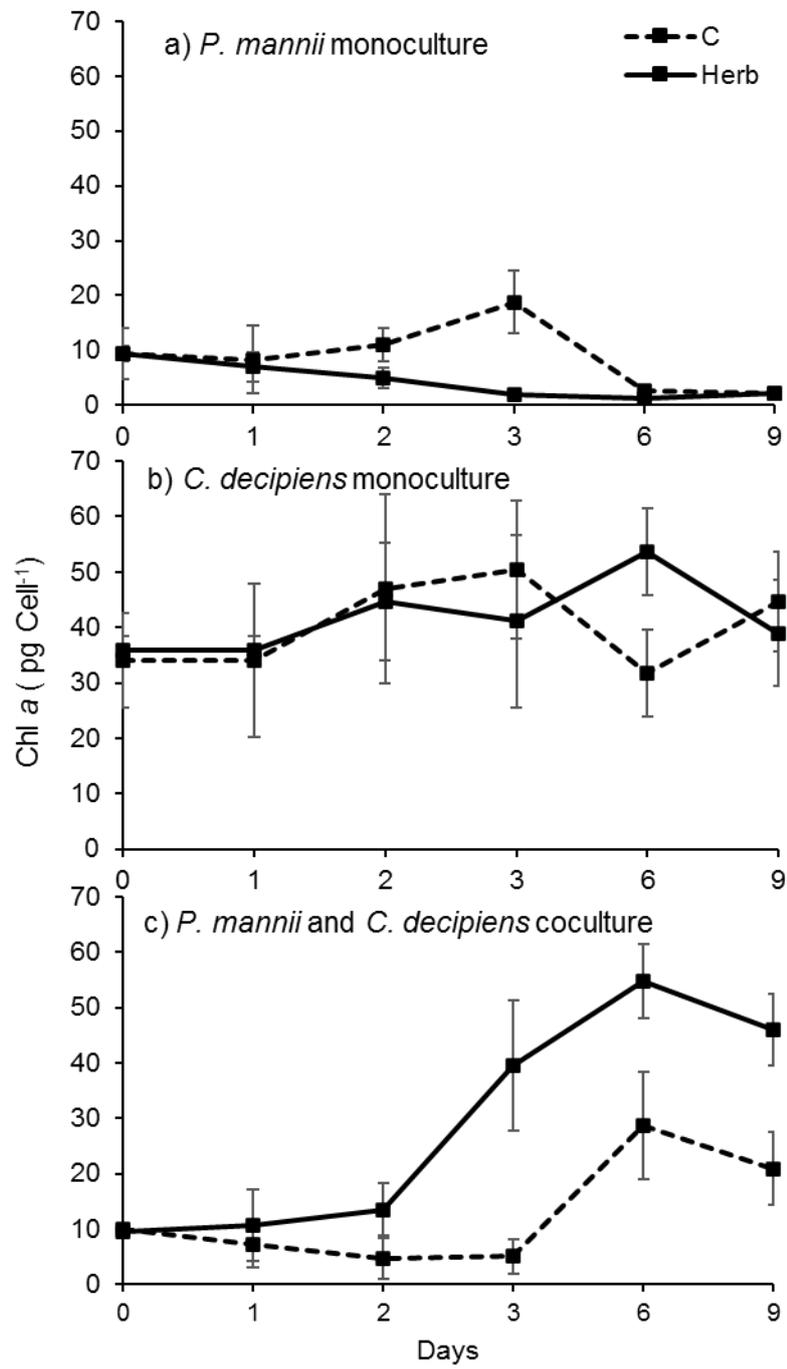
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21 **Figure 2**



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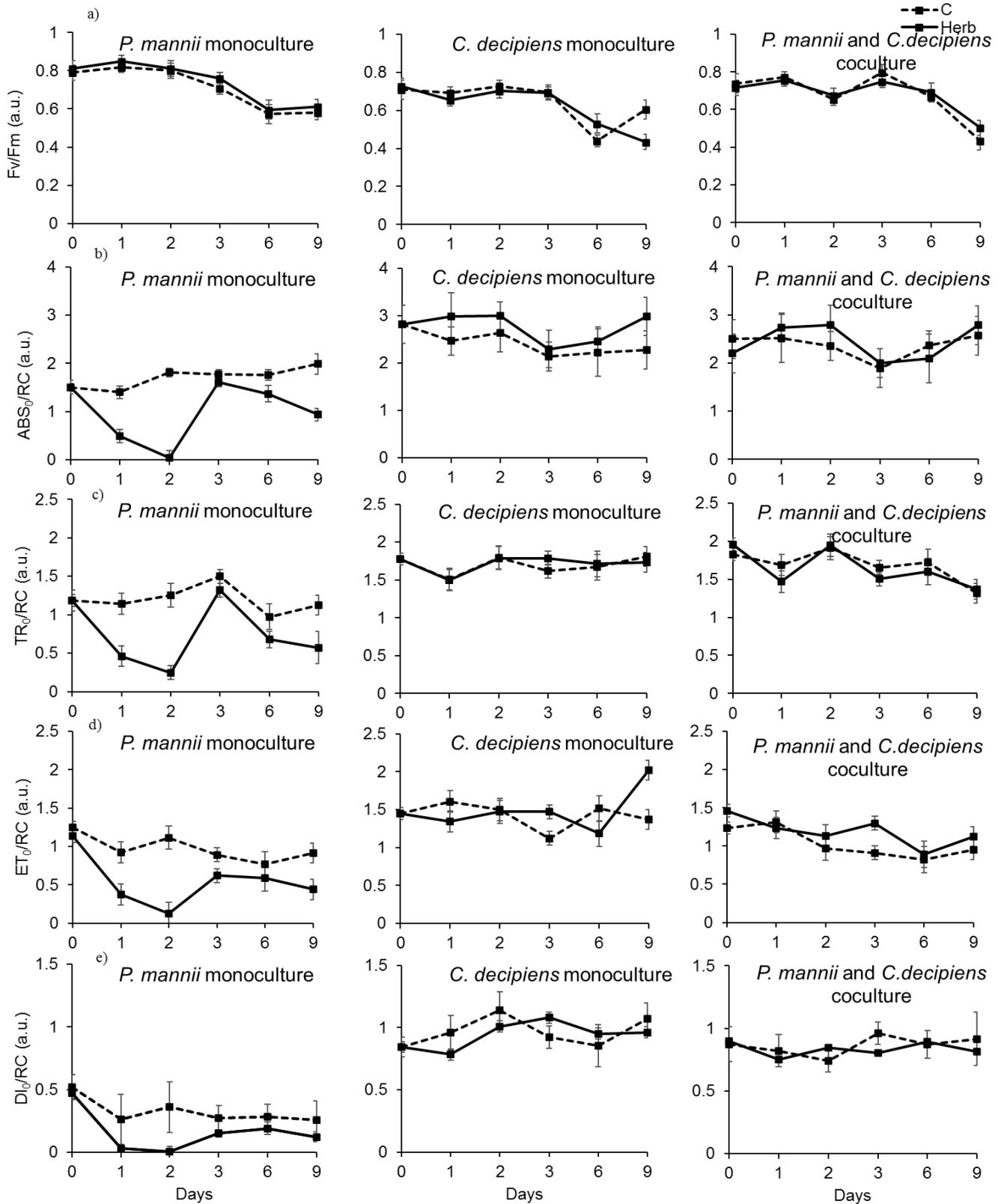
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37 **Figure 4**

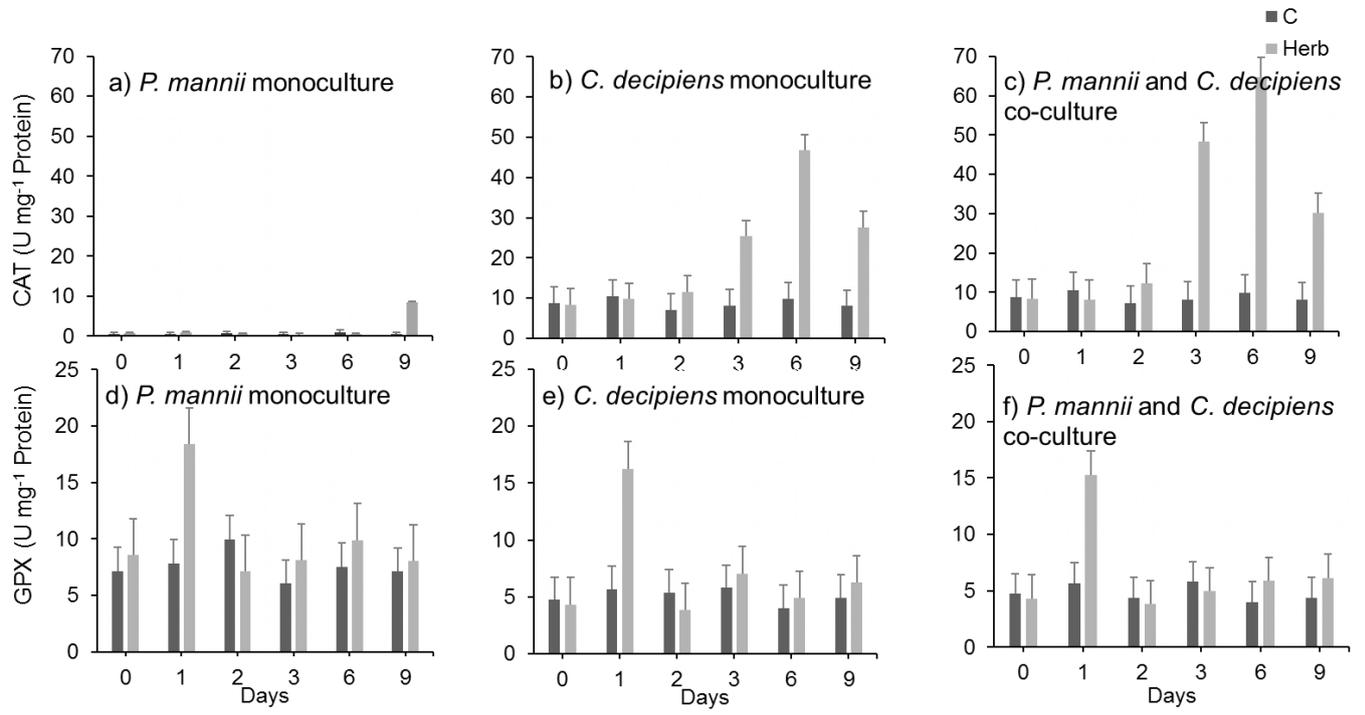


Table 1 Chemical family, physical proprieties and action modes of the different tested herbicides.

Herbicides	Chemical family ^a	Log K _{ow} ^b	Solubility (mg L ⁻¹) ^{b, c}	Mode of action/Site of action ^a
Alachlor	Chloroacetamide	3.52	210 ± 38.62	Growth inhibitor/ Very-Long-Chain Fatty Acid (VLCFA) Inhibitors
DIA	Triazine	1.15	3200	Photosynthesis inhibitor/ PSII inhibitor
Diuron	Phenylurea	2.68	47.30 ± 30	Photosynthesis inhibitor/ PSII inhibitor
Simazine	Triazine	2.18	6.43 ± 4.29	Photosynthesis inhibitor/ PSII inhibitor

^a Sherwani *et al.*, 2015, ^b Finizio et al., 1997, ^c Thurman and Meyer, 1996

Table 2 F ratios of two-way ANOVA analysis testing for effects of treatment (control/herbicide) and culture mode (monoculture/co-culture) on instantaneous growth rates (IGR) of *P. mannii* and *C. decipiens*

	Treatment		Culture mode		Treatment x Culture mode	
	F	<i>Prob</i>	F	<i>Prob</i>	F	<i>Prob</i>
IGR <i>P. mannii</i>	1.943	0.170	152.611	0.000	1.769	0.152
IGR <i>C. decipiens</i>	1.196	0.281	11.687	0.001	19.078	0.000



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**Suppl Mtrls (not cover letter - place cover letter in
"comments")**

Figure captions (Sahraoui et al.).docx