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1 **Demonstrating the need for chemical exposure** 2 **characterisation in a microplate test system: toxicity** 3 **screening of sixteen pesticides on two marine** 4 **microalgae**

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19

20 **Abstract**

21 Pesticides used in viticulture create a potential risk for the aquatic environment due to drift during
22 application, runoff and soil leaching. The toxicity of sixteen pesticides and one metabolite were
23 evaluated on the growth of two marine microalgae, *Tisochrysis lutea* and *Skeletonema marinoi*, in 96-h
24 exposure assays conducted in microplates. For each substance, concentrations of stock solutions
25 were analytically measured and abiotic assays were performed to evaluate the chemical stability of
26 pesticides in microplates. For two chemicals, microalgae exposures were run simultaneously in
27 microplates and culture flasks to compare EC₅₀ calculated from the two exposure systems.

28 Results from chemical analyses demonstrated the low stability of hydrophobic pesticides ($\log K_{ow} > 3$).

29 For such chemicals, EC₅₀ values calculated using measured pesticide concentrations were two-fold
30 lower than those first estimated using nominal concentrations. Photosystem II inhibitors were the most
31 toxic herbicides, with EC₅₀ values below 10 $\mu\text{g L}^{-1}$ for diuron and around double this for isoproturon.

32 Chlorpyrifos-methyl was the only insecticide to significantly affect the growth of *T. lutea*, with an EC₅₀
33 around 400 $\mu\text{g L}^{-1}$. All fungicides tested were significantly toxic to both species: strobilurins showed

34 low overall toxicity, with EC₅₀ values around 400 $\mu\text{g L}^{-1}$, whereas quinoxifen, and spiroxamine,

35 showed high toxicity to both species, especially to *T. lutea*, with an EC₅₀ below 1 µg L⁻¹ measured for
36 spiroxamine in culture flasks. This study highlights the need to perform chemical analyses for reliable
37 toxicity assessment and discusses the advantages and disadvantages of using microplates as a
38 toxicity screening tool.

39

40 **Keywords**

41 Microalgae; microplates; screening; pesticides; toxicity; exposure characterisation

42

43 **1 Introduction**

44 In France, viticulture accounts for about 15% of total pesticide usage, although it covers only 3% of the
45 utilised agricultural area (UAA), thus implying a very high phytosanitary pressure (Butault et al., 2011).
46 While ensuring good harvest quality and food protection, the use of pesticides also poses a risk for the
47 surrounding environment, due to drift during application (Gil and Sinfort, 2005) and runoff (Leu et al.,
48 2004; Brady et al., 2006).

49 Many studies have reported the contamination of watercourses through viticultural activity in European
50 countries (Hildebrandt et al., 2008; Herrero-Hernández et al., 2013; Fernández et al., 2015), including
51 France (Louchart et al., 2001; Rabiet et al., 2010). In the Spanish wine region La Rioja, 62% of
52 surface waters were found to have a cumulative pesticide concentration above 0.50 µg L⁻¹ (Herrero-
53 Hernández et al., 2013). In France, Rabiet et al. (2010) monitored the fate of six pesticides for one
54 year in the Morcille river (Beaujolais) and found concentrations of diuron ranging between 2.00 and
55 12.0 µg L⁻¹ from April to July. In comparison with continental waters, a cumulative annual average
56 concentration of 10.0 µg L⁻¹ (for 56 pesticides monitored) was found in the coastal waters of the Ria
57 Formosa Lagoon in Portugal (Cruzeiro et al., 2015).

58 The term 'pesticides' includes all phytosanitary products (PPs) and biocides. PPs cover a wide variety
59 of chemicals and can be grouped into several classes (such as rodenticides, molluscicides, acaricides,
60 algaecides...etc.), depending on their usage and modes of action (MoA). Among these groups, the
61 three main classes are herbicides, insecticides and fungicides. Pesticides can also be classed
62 according to their chemical families (i.e., phenylureas, organophosphates, strobilurins). Due to the
63 great diversity of MoA, pesticides can affect many non-target species due to physiological similarities
64 with the latter. Herbicides can thus be responsible for harmful effects on aquatic organisms such as

65 phytoplankton (Pérès et al., 1996; Roubéix et al., 2012; Kim Tiam et al., 2014a, 2014b; Moisset et al.,
66 2015). Insecticides can show high toxicity towards zooplankton (Zalizniak and Nugegoda, 2006;
67 Overmyer et al., 2007; López-Mancisidor et al., 2008). Fungicides can be toxic to a wider range of
68 organisms (Ochoa-Acuña et al., 2009) because their MoA are often not specific to fungi (Maltby et al.,
69 2009), but involve enzymes that are highly conserved across fungi, plants and animals (Stenersen,
70 2004).

71 In order to assess the toxicity of such chemicals on microalgae, the present standard procedures
72 (OECD 201, 2011; ISO 10253:2016, 2016) recommend the use of glass testing recipients. Indeed,
73 standard procedures using glass culture flasks are the result of many experiments and years of work,
74 which is why these vessels are still considered one of the most reliable tools for toxicity testing on
75 microalgae. Culture flasks offer numerous advantages, such as a larger volume of culture that makes
76 it possible to perform multiple analyses on a single culture, which is not possible with microplates due
77 to their very small well volumes. However, when the main goal of an experiment is to determine
78 standard toxicity values such as EC₅₀ (which requires at least five concentrations to be tested in
79 triplicate) for numerous chemicals, the use of culture flasks can be time- and space-consuming. In
80 comparison, microplate-based phytotoxicity assays have rapidly become common since their first
81 appearance in the early 80s, as several authors pointed out their reliability for toxicity testing by
82 demonstrating a good agreement of toxicity levels between microplate and culture flask test systems
83 (Blaise et al., 1986; St-Laurent et al., 1992; Rojíčková et al., 1998; Geis et al., 2000; Eisentraeger et
84 al., 2003; Kim Tiam et al., 2014a).

85 Most microplate-based assays using microalgae rely on chlorophyll fluorescence measurement as a
86 proxy for algal growth. However, in some cases, it has been demonstrated that certain contaminants,
87 like glyphosate, paraquat or diuron significantly affect chlorophyll content and/or fluorescence (Prado
88 et al., 2011; Stachowski-Haberkorn et al., 2013; Smedbol et al., 2018). Consequently, the use of
89 chlorophyll fluorescence for algal growth measurement in a microplate test system can be biased in
90 potentially different ways depending on the contaminants tested.

91 Another much discussed issue concerning microplate test systems is the bioavailability of the tested
92 chemicals in the microplate wells. Indeed, several studies have demonstrated that one should be very
93 careful when testing chemicals of high volatility and/or hydrophobicity because effective
94 concentrations might be lower than nominal concentrations (Simpson et al., 2003; Riedl and

95 Altenburger, 2007). In addition to a higher potential sorption of chemicals to plastic (Stadnicka-
96 Michalak et al., 2014) than glass, the high surface/volume ratio in microplates can also induce
97 excessive evaporation and/or sorption (Hörnström, 1990). As a consequence, exposure
98 concentrations can be lower than those targeted, thus leading to an underestimation of the measured
99 toxicity for the tested chemicals. It is therefore recommended to perform chemical analyses when
100 testing chemicals with high volatility and/or hydrophobicity (Riedl and Altenburger, 2007; Tanneberger
101 et al., 2013), although this is still rarely done in ecotoxicological studies nowadays.

102 Phytoplankton account for roughly half of all primary production on Earth (Beardall and Raven, 2016)
103 and form the foundation of oceanic food webs. They are considered as environmental quality
104 indicators because of their worldwide distribution and are widely used as model organisms in standard
105 toxicity assays because of their ease of culture in laboratory and short generation time.

106 In this study, 17 chemicals, including thirteen PPs used in viticulture, three biocides and one PP
107 metabolite, were tested for their toxicity on two marine microalgal species: the haptophyte *Tisochrysis*
108 *lutea* and the diatom *Skeletonema marinoi*.

109 To assess the reliability of a microplate-based toxicity assay for screening pesticides with different
110 physicochemical properties, this study aimed:

- 111 1. To evaluate the stability of the tested chemicals in the culture medium and in the microplate
112 wells over a 96-h experiment period.
- 113 2. To perform the toxicity screening of sixteen pesticides and one metabolite on the two species
114 of microalgae using the microplate-based growth inhibition assay.
- 115 3. To compare EC₅₀ values obtained using: i) nominal vs measured concentrations; ii) microplate
116 and culture flask test systems.

117 To answer these questions, we adapted a 96-h growth inhibition assay (Blaise et al., 1997;
118 Eisentraeger et al., 2003; Riedl and Altenburger, 2007) and conducted this in 48-well microplates.
119 Abiotic assays were carried out to evaluate the stability of the tested chemicals in the microplates by
120 measuring effective exposure concentrations in the culture medium by liquid or gas chromatography
121 tandem mass spectrometry analysis (LC/MS/MS or GC/MS/MS). Microalgal exposure experiments
122 were performed to compare toxicity values obtained in microplates with those obtained in glass flasks.
123 Concentration-response experiments were then done in microplates to determine EC₅₀ values for
124 sixteen selected pesticides and one metabolite on the two microalgal species.

125 The main interest of this study is the combination of the analytical determination of exposure
126 concentrations with the comparison of the toxicity response obtained in both microplates and glass
127 flasks, which allowed us to produce a reliable unbiased toxicity dataset for the tested substances. The
128 application of the proposed microplate toxicity assay to the toxicity screening of pesticides exhibiting
129 various physicochemical properties is discussed, and recommendations are given regarding its use.
130

131 **2 Material and methods**

132 **2.1 Chemical preparation**

133 All chemicals used in this study (Table 1) were purchased from Sigma-Aldrich. Internal standards,
134 diuron-d6, isoproturon-d6, carbofuran-d3, terbutryn-d5, metolachlor-d6, cis-permethrin-13C,
135 chlorpyrifos-d10, chlorpyrifos-methyl-d6, fipronil-13C-15N, imidacloprid-d4, atrazine-d5 and quizalofop-
136 ethyl-d3 were purchased from Cluzeau Info Labo (Sainte Foy la Grande, France).

137 Stock solutions were prepared in pure methanol ($\geq 99\%$) except for glyphosate and AMPA, which were
138 prepared in sterile ultra-pure water, and for flazasulfuron, acrinathrin and spiroxamine, which were
139 prepared in pure acetone ($\geq 99\%$). These stock solutions were diluted in their solvents to make
140 working solutions. The nominal concentrations of the stock and working solutions were calculated from
141 weighed masses of the active substances and solvents. These solutions were then analysed by liquid
142 or gas chromatography tandem mass spectrometry (LC-MS/MS or GC-MS/MS, 2.4) and the measured
143 concentrations made available as supplementary material (Table S1). The measured concentrations of
144 the stock solutions were then used *a posteriori* to correct the nominal exposure concentrations (then
145 called 'corrected nominal concentrations') in the toxicity assays (see section 2.6.1 for further details).

146

147 **2.2 Microalgal cultures**

148 The marine microalga *Tisochrysis lutea* (Haptophyta) CCAP 927/14 was purchased from the Culture
149 Center of Algae and Protozoa (CCAP, Oban, Scotland). The marine diatom *Skeletonema marinoi*
150 (Bacillariophyta) AC174 was purchased from the University of Caen Algalbank (Caen, France).
151 Microalgal cultures were maintained in sterile f/2 (for *T. lutea*) and f/2-silica (f/2-Si; for *S. marinoi*)
152 media (Guillard and Ryther, 1962; Guillard, 1975) at $20 \pm 1^\circ\text{C}$, in a thermostatic chamber at $130 \mu\text{mol}$
153 $\text{m}^{-2} \text{s}^{-1}$ (Quantometer Li-Cor Li-250 equipped with a spherical sensor), with a dark:light cycle of 8:16 h.

154 Cultures were grown in 100 mL round borosilicate sterile glass flasks previously heated to 450°C for
155 6 h, autoclaved for 20 min at 121°C, and then filled with 50.0 mL of culture medium. Cultures were
156 diluted weekly in order to maintain an exponential growth phase.

157

158 **2.3 Exposure experiments**

159 **2.3.1 Microplate toxicity assay**

160 The microplate toxicity assay consisted in concentration-response experiments conducted with each
161 microalga and chemical to be tested. The aim was to calculate the EC₅₀ – the Effective Concentration
162 inducing a 50% inhibition on growth rate of the microalga after a 96-h exposure – for each chemical.
163 For these experiments, transparent polystyrene 48-well microplates (Greiner Bio-One GmbH, cat.
164 677102, untreated) were used, each well being filled with 0.90 mL of sterile f/2 (for *T. lutea*) or f/2-Si
165 (for *S. marinoi*) culture medium. Microplates were covered with their own lid, allowing gas exchanges.
166 Peripheral wells were not used in order to avoid edge effects (Caux et al., 1992; St-Laurent et al.,
167 1992); instead, these were filled with 1.00 mL of sterile 0.20-µm filtered Milli-Q water to prevent
168 evaporation and maintain high humidity. For the toxicity screening experiments, one test was carried
169 out for each substance to be tested. The test consisted in exposure to five nominal concentrations,
170 1.00, 10.0, 100, 500 and 1000 µg L⁻¹ of the pesticide in triplicate (three wells), six solvent controls (SC)
171 containing the highest solvent percentage used in the microplate (max 0.1%) and three controls (C)
172 without solvent (supplementary material: Figure S1). For diuron, because this chemical had already
173 been tested in our preliminary experiments, a refined range was used. When needed, some other
174 chemicals were tested a second time using a refined range of six concentrations in order to improve
175 the determination of their EC₅₀. Prior to their introduction into the triplicate wells of the microplate, both
176 chemical and solvent solutions were prepared at targeted concentrations in sterile glass flasks (one
177 glass flask per tested chemical and concentration) containing 25.0 mL of sterile culture medium.
178 After measurement of cell density by flow cytometry, 0.10 mL of the diluted microalgal culture was
179 added to each assay well to reach a concentration of 20,000 cell mL⁻¹ at the beginning of exposure.
180 The final volume of each well was 1.00 mL. After 96 h, the whole content of each well was recovered,
181 fixed using glutaraldehyde (final concentration 0.25%) and left in the dark for 10 min before being
182 frozen and stored in liquid nitrogen (-196°C) until cell density analysis by flow cytometry.

183

184 **2.3.2 Culture flask toxicity assay**

185 For two selected chemicals (isoproturon and spiroxamine), toxicity assays were also performed in
186 glass culture flasks, using the same culture conditions as the microplate assays. This enabled us to
187 compare the toxicity between the two test systems: glass culture flasks vs microplates. Isoproturon
188 and spiroxamine were selected because of their high toxicity to both algal species, as well as their
189 differing chemical families, carrier solvents (methanol vs acetone, §2.1), and usages. For these
190 experiments, microalgal samples were exposed in triplicate to a range of six chemical concentrations,
191 chosen based on EC₅₀ values obtained during the previous microplate toxicity assay. Three solvent
192 controls were set up containing the highest solvent percentage. It should be noted that six wells were
193 used for the solvent controls in the microplate toxicity assay. Chemical and solvent solutions were
194 prepared at targeted concentrations in 50.0 mL sterile f/2 (for *T. lutea*) or f/2-Si (for *S. marinoi*) culture
195 medium. For each species, a stock culture was analysed by flow cytometry for the measurement of
196 microalgal cell density: then, depending on the species and cell density, between 0.10 and 0.50 mL of
197 stock culture were added to each flask to reach an initial concentration of 20,000 cell mL⁻¹ at the
198 beginning of experiment. During the experiment, each culture was sampled daily (0.50 mL) for cell
199 density analysis by flow cytometry.

200

201 **2.3.3 Chemical stability of tested substances in the microplates**

202 In order to investigate the chemical stability of each substance tested in the microplates for the 96-h
203 duration of the test (supplementary material: Tables S2 and S3), an abiotic assay was run under the
204 same conditions as the microplate toxicity assay (§2.3.1), with strictly the same nominal
205 concentrations, operating mode and experimental design. The only difference was that no microalgae
206 were added to the wells. Triplicate wells were used for solvent control and contaminated wells. For
207 each chemical and concentration, 1.00 mL was pipetted out from the first of the three wells into a
208 clean glass vial at the beginning of the test (0 h), the second well was sampled after 6 h and the third
209 after 96 h. The vials were then stored at -20°C until chemical analysis.

210

211 **2.4 Chemical analysis**

212 For each sample (stock solutions and chemical stability assay), serial dilutions in ultra-pure water were
213 performed to reach a final concentration of 0.20 or 0.10 µg L⁻¹ before analysis.

214 The method used to quantify glyphosate and AMPA was adapted from Fauvelle et al. (2015). Samples
215 containing glyphosate and AMPA were extracted by solid phase extraction (SPE) on Oasis HLB® (60
216 mg, 3.00 mL, Waters®) cartridges after derivatization by FMOC-Cl (9-Fluorenylmethoxycarbonyl
217 chloride). Analyses were then performed by liquid chromatography tandem mass spectrometry (LC-
218 MS/MS; Agilent LC 1290 infinity - 6460 triple quad) with an electrospray ionization (ESI) source used
219 in positive mode.

220 The method used to quantify diuron, isoproturon, flazasulfuron, metazachlor, S-metolachlor,
221 imidacloprid, azoxystrobin, kresoxim-methyl, quinoxyfen and spiroxamine was adapted from Belles
222 et al. (2014). Analyses were performed by direct injection ($V_{\text{injection}} = 40.0 \mu\text{L}$) into a liquid
223 chromatography tandem mass spectrometer (LC-MS/MS; Agilent LC 1290 Infinity - 6460 triple quad)
224 equipped with an ESI source used in positive mode.

225 The method used to quantify α -cypermethrin, acrinathrin, chlorpyrifos, chlorpyrifos-methyl and fipronil
226 was adapted from Belles et al. (2014). Analyses were performed by solid phase microextraction
227 (SPME) coupled with gas chromatography tandem mass spectrometry (GC-MS/MS; Agilent GC 7890 -
228 7000 triple quad).

229 Analytical methods were validated in terms of extraction recoveries (samples of fortified mineral water
230 at $0.20 \mu\text{g L}^{-1}$) and limits of quantification (LoQ: signal to noise ratio ≥ 10). For each analysis series,
231 blank runs (complete procedure but without the matrix) were performed. Control calibrating standards
232 ($0.20 \mu\text{g L}^{-1}$) were also injected every 15 samples and analytical blanks were performed.

233 The detailed analytical method for these analyses is described in the supplementary material (Table
234 S4).

235

236 **2.5 Growth measurement in microplates and culture flasks**

237 As mentioned in the introduction, chlorophyll content per microalgal cell can be modified by exposure
238 to pesticides, as can chlorophyll fluorescence. Absorbance at 750 nm, which could have permitted the
239 avoidance of a potential bias in growth measurement due to chlorophyll alteration, could not be used
240 because the signal was too weak at the beginning of the experiment. Therefore, in order to evaluate to
241 what extent such an effect on chlorophyll fluorescence could skew the growth rate calculation, the
242 microalgal cell growth rate in microplates was determined by both chlorophyll fluorescence (using a

243 microplate reader) and cell density (using a flow cytometer). Due to the volume required to perform
244 flow cytometry analysis (50–100 μL), only the final cell density was measured in this way.

245 Microalgal growth in microplates was measured daily using a SAFIRE microplate reader (TECAN) with
246 the XFluor4beta Excel macro as software. Excitation/emission wavelengths were: 450/684 nm (10 nm
247 bandwidth), nine reads were performed per well from the bottom, with an integration time of 20 μs .
248 Each microplate was shaken for 20 s before making the reading using an Orbis Plus (Mikura Ltd)
249 microplate shaker in orbital mode. For each well, the growth rate was calculated using the following
250 equation: $\mu = \ln (F_t - F_0)/t$, where F_t was the fluorescence (a.u.) of the well at t (hours), μ (hours^{-1}) was
251 the growth rate and F_0 the initial fluorescence intensity at $t = 0$ h.

252 For cell density measurement, samples were run on an Accuri C6 flow cytometer (Becton Dickinson
253 Accuri™) equipped with a blue (488 nm) and a red (640 nm) laser, detectors of forward (FSC) and
254 side (SSC) light scatter, and four fluorescence detectors: 530 ± 15 nm (FL1), 585 ± 20 nm (FL2),
255 > 670 nm (FL3) and 675 ± 12.5 nm (red laser, FL4). FL1 vs FL4 channel density plots were used to
256 analyse both microalgal species and each was gated to avoid counting non-microalgal particles. The
257 growth rate was calculated with the following equation: $\mu = \ln (C_t - C_0)/t$, where C_t is the cell
258 concentration (cell mL^{-1}) at t (hours), μ (hours^{-1}) is the growth rate and C_0 the initial cell concentration
259 at $t = 0$ h.

260

261 **2.6 Statistical analysis**

262 **2.6.1 Concentration-response analysis**

263 Concentration-response analyses were carried out with the *drc* package (Ritz and Streibig, 2005; Ritz
264 et al., 2015) in R software 3.4.3. For each chemical, the single three-parameter log-logistic regression
265 model LL.3 (equation 1) was applied:

$$U = \frac{d}{\left(1 + \left(\frac{x}{\text{EC}_{50}}\right)^b\right)} \quad (1)$$

266 where b is the slope, d the upper-limit determined by the fitted concentration-response model, and U
267 the response, in our case the growth rate (μ), at the concentration x .

268 Based on the chemical analyses performed on the stock solutions (supplementary material: Table S1)
269 and their dilutions in the microplate wells (supplementary material: Table S2), three EC_{50} values were

270 calculated for each chemical (except for diuron and S-metolachlor, as only one concentration of each
271 was measured in the abiotic assays), considering the three possible expressions of the exposure
272 concentrations:

- 273 i. nominal concentrations, calculated theoretically using the nominal stock solution
274 concentrations;
- 275 ii. corrected nominal concentrations, calculated theoretically using the measured stock solution
276 concentrations;
- 277 iii. measured concentrations, the chemical concentrations in the wells reached at the beginning
278 of the exposure as measured by chemical analysis.

279 In addition, a fourth EC_{50} value was calculated using measured concentrations and cell densities
280 (measured at the beginning and end of the experiment; §2.3.1), instead of fluorescence, to assess the
281 potential bias associated with the use of chlorophyll fluorescence to measure growth rate.

282

283 **2.6.2 Principal component analysis**

284 A principal component analysis (PCA) was performed using the R package *FactoMineR* (Lê et al.,
285 2008) to examine the relationships between the concentrations measured in the microplate wells after
286 0, 6 and 96 h of exposure (§2.3.3; supplementary material: Table S3), and the physicochemical
287 properties of the chemicals (Table 1), namely: the octanol-water partition coefficient, $\log K_{ow}$,
288 reflecting hydrophobicity (chemicals with a $\log K_{ow} < 3$ are considered as hydrophilic whereas those
289 with a $\log K_{ow} > 3$ are considered as hydrophobic); Henry's law constant, $\log H$ (in $\text{atm m}^3 \text{mol}^{-1}$),
290 reflecting volatility (chemicals with a $\log H < -5.61$ are considered non-volatile whereas those with a
291 $\log H > -5.61$ are considered volatile) (Tanneberger et al., 2013); the solubility in water at 20°C pH 7
292 (in mg L^{-1}), $\log S$ (higher solubility for higher values). As the physicochemical parameters of the
293 chemicals were not measured in our experiment but taken from a database, these variables were
294 considered as supplementary variables, which means they had no influence on the principal
295 components of the analysis but can help us to interpret the dimensions of variability. Two individuals
296 (out of 65) were considered as outliers and were therefore removed from the dataset before analysis:
297 CYP-3 and KRE-3.

298

299 **3 Results**

300 In the Results and Discussion sections, the substances diuron and isoproturon are considered as
301 belonging to the herbicides group, as their physicochemical properties and MoA are similar to those of
302 herbicides. Similarly, fipronil is considered as belonging to the 'insecticides' group.

303

304 **3.1 Chemical analyses**

305 When comparing the nominal (calculated from weighed mass, §2.1) and measured stock solution
306 concentrations (supplementary material: Table S1), it appears that for almost all herbicides, the
307 percentage difference between the two is commonly below 30%, except for metazachlor solutions S2
308 and S3 and all AMPA solutions. Regarding insecticides, measured concentrations of imidacloprid and
309 fipronil stock solutions, as well as chlorpyrifos solution S1, were quite close to the nominal
310 concentrations. For the other insecticides, the percentage of variation was higher, especially for the
311 two pyrethroids α -cypermethrin and acrinathrin. Finally, differences between nominal and measured
312 stock solutions for fungicides ranged between 30% and 65%, apart from for azoxystrobin and
313 quinoxifen solution S3.

314 Results from the chemical stability study of pesticides tested in microplates showed that the target
315 concentrations (corrected nominal concentrations) at the beginning of the exposure were rarely
316 reached (supplementary material: Tables S2 and S3). The measured concentrations in the microplate
317 wells at 0 h were usually lower than expected. For herbicides, at least 60% of the chemical were
318 recovered in the wells at the beginning of the abiotic experiment. For insecticides, recovery
319 percentages at the beginning of the exposure were lower, especially for α -cypermethrin and
320 acrinathrin (except at concentration C3 where an anomaly was noticed for both of them) and
321 chlorpyrifos (except at concentration C1). For fungicides, the percentages of chemical recovered at the
322 beginning of the exposure were close to those obtained for herbicides (except three lower values for
323 quinoxifen at C2 and C4 and spiroxamine at C3).

324 The percentages of chemical recovered after 6 and 96 h (compared with the corrected nominal
325 concentration introduced at the beginning of the test) also varied among the tested chemicals
326 (supplementary material: Table S3). All herbicides tested showed good overall stability, except
327 flazasulfuron, for which an large fall in concentration was observed after 96 h (5–10% recovered for

328 C1, C2 and C3). In contrast, insecticides had poor stability in the test system, especially pyrethroids α -
329 cypermethrin and acrinathrin for which less than 10% was recovered after 96 h regardless of the
330 concentration added at the beginning of the test. For the organophosphate chlorpyrifos, a large fall in
331 concentration was also observed after 96 h, especially at the highest tested concentrations (C3, C4
332 and C5). Imidacloprid and fipronil seemed to have good stability during the 96-h test period, although
333 only one concentration was tested. For fungicides, the stability was slightly lower than herbicides but
334 higher than insecticides, the percentage recovered after 96 h being generally above 50% for
335 azoxystrobin and kresoxim-methyl (apart from an anomaly at C3), around 40–50% for spiroxamine
336 (apart from a small anomaly at C2), and 20–30% for quinoxifen (apart from an anomaly at C1 after 6
337 h).

338 For all chemicals tested, the percentages recovered at all measurement times (0, 6 and 96 h) were
339 analysed with a PCA, using the physicochemical properties of the chemicals as supplementary
340 variables.

341 On the PCA (Figure 1), the first two axes (Dim1 and Dim2) explain around 90% of the total variance.
342 The first axis (Dim1) represents the overall percentage of recovery. On the left are the least stable
343 chemicals like insecticides α -cypermethrin (CYP), acrinathrin (ACR), with very low percentages of
344 recovery at 0, 6 and 96 h, and some fungicides like quinoxifen (QUI) (Figure 1A). On the right are the
345 most stable chemicals, represented by a majority of herbicides (glyphosate “GLY”, AMPA “AMP”,
346 isoproturon “ISO”, metazachlor “MET”). The vertical axis represents temporal variation: for example, at
347 the bottom-right corner we find the pesticides with a high percentage recovery at 0 and/or 6 h but a
348 very low percentage recovery after 96 h (FLA_1, FLA_2 and ACR_3). Two individuals, CHL_1 and
349 QUI_1 are separated from the rest because of their unexpected behaviour: the percentage recovered
350 after 6 h decreased, then increased again after 96 h (CHL_1) or vice versa (QUI_1). Analysis of the
351 variable factor map (Figure 1B) shows that the percentage of chemical recovered after 96 h (and to a
352 lesser extent after 0 and 6 h) is correlated with the hydrophobicity ($\log K_{ow}$) and solubility ($\log S$) of the
353 chemical: the more hydrophobic (and thus, the less soluble) the chemical is, the lower the percentage
354 recovered. The volatility of the chemicals ($\log H$) had almost no influence on the percentage of
355 chemical recovered.

356 In general, herbicides, which are not highly hydrophobic ($\log K_{ow} < 3$) and thus easily soluble in water
357 were highly stable in the microplates. Four insecticides (α -cypermethrin, acrinathrin, chlorpyrifos and

358 chlorpyrifos-methyl) and one fungicide (quinoxifen) that are highly hydrophobic ($\log K_{ow} > 4$), and thus
359 poorly soluble in water, were recovered at very low concentrations at all measurement times and/or
360 showed a large fall in concentration over time.

361

362 **3.2 Toxicity screening of 17 chemicals**

363 Among the 17 chemicals tested, nine were sufficiently toxic to allow the calculation of an EC_{50}
364 (Table 2): the herbicides diuron, isoproturon, metazachlor and S-metolachlor; the insecticide
365 chlorpyrifos-methyl and all the fungicides. As shown by the different shapes of the concentration-
366 response curves (Figures 2 and 3), the tested chemicals showed various different toxicity patterns.
367 Some, like diuron, azoxystrobin and kresoxim-methyl, showed a progressively descending slope, while
368 others like quinoxifen and chlorpyrifos-methyl showed steep slopes. Comparing the sensitivity of the
369 two species tested, *T. lutea* was not sensitive to the two chloroacetanilides metazachlor and S-
370 metolachlor, but was significantly sensitive to the insecticide chlorpyrifos-methyl, while *S. marinoi* was
371 not sensitive to this chemical, but was significantly sensitive to the two chloroacetanilides. Only diuron,
372 isoproturon and spiroxamine were capable of a 50% inhibition of *T. lutea* growth rate at a
373 concentration below $10 \mu\text{g L}^{-1}$.

374 As stated in the Material and methods (§2.6.1), three different expressions of the exposure
375 concentrations were used to calculate EC_{50} values for each chemical (except for diuron and S-
376 metolachlor). The three different EC_{50} values appear to be within the same range for herbicides, with
377 differences of less than two-fold, for example: 12.1 ± 5.48 , 13.0 ± 6.76 and $8.55 \pm 3.39 \mu\text{g L}^{-1}$ for
378 isoproturon on *T. lutea*; 323 ± 41.4 , 370 ± 43.2 and $245 \pm 24.7 \mu\text{g L}^{-1}$ for metazachlor on *S. marinoi*,
379 using nominal, corrected nominal and measured concentrations, respectively. For insecticides and
380 fungicides, values more than two-fold lower were found using the nominal, corrected or measured
381 concentration, for example: 877 ± 13.7 , 406 ± 6.33 and $449 \pm 6.70 \mu\text{g L}^{-1}$ for chlorpyrifos-methyl on *T.*
382 *lutea*; and 124 ± 82.8 , 68.8 ± 33.0 and $33.0 \pm 8.49 \mu\text{g L}^{-1}$ for quinoxifen on *S. marinoi* using nominal,
383 corrected nominal and measured concentrations, respectively. Concerning the concentration-response
384 curves (Figures 2 and 3), those computed with corrected nominal (dashed line) or measured (dotted
385 line) concentrations are shifted to the right, implying lower EC_{50} values. Looking at the difference
386 between EC_{50} values calculated with corrected nominal and measured concentrations, it appears that,
387 for all the chemicals except chlorpyrifos-methyl, EC_{50} values calculated using the measured

388 concentration were lower. Interestingly, these differences were higher for *S. marinoi* (differences of
389 more than two-fold for quinoxifen and spiroxamine) than for *T. lutea* (differences of less than two-fold).
390 Both fluorescence (measured daily) and cell density (measured at the beginning and end of the
391 exposure) were used to calculate growth rate (Table 2). However, EC₅₀ values using cell density were
392 only calculated based on measured concentrations as these are considered the most accurate. As a
393 consequence, EC₅₀ values based on cell density should only be compared with EC₅₀ values calculated
394 with measured concentrations. The EC₅₀ on *S. marinoi* exposed to metazachlor was two-fold higher
395 when using cell density ($563 \pm 121 \mu\text{g L}^{-1}$) rather than fluorescence ($245 \pm 24.7 \mu\text{g L}^{-1}$) for growth rate
396 determination. On the contrary, EC₅₀ values for azoxystrobin and kresoxim-methyl were much lower
397 for both species when using cell density, with a four-fold difference for azoxystrobin on *T. lutea* ($112 \pm$
398 32.2 and $434 \pm 144 \mu\text{g L}^{-1}$ for cell density and fluorescence, respectively). For isoproturon and
399 quinoxifen, there was almost no difference between the cell density and fluorescence EC₅₀ values
400 with both microalgal species, and similarly for chlorpyrifos-methyl with *T. lutea*. Finally, regarding
401 spiroxamine, an EC₅₀ three-fold lower was found for *T. lutea*, while no significant difference was
402 observed for *S. marinoi*. However, the very poor fit of the log-logistic model in these cases prevented
403 us from making a reliable interpretation.

404

405 **3.3 Comparison between microplate and culture flask exposure systems**

406 For the experiments comparing microplate and culture flask methods, it should be noted that no
407 analytical measurements were made of the concentrations. The EC₅₀ values given below were
408 calculated using corrected nominal concentrations.

409 To evaluate the differences in toxicity that could exist when conducting the assay in microplates rather
410 than in culture flasks, two of the most toxic substances, isoproturon (herbicide) and spiroxamine
411 (fungicide), were tested simultaneously in both systems. Contrasting results were obtained depending
412 on the species and/or chemical, as shown by the different patterns of concentration-response curve
413 (Figure 4). For *T. lutea*, isoproturon showed very similar toxicity between the two test systems, as
414 illustrated when the two concentration-response curves are superimposed, with EC₅₀ values of $9.57 \pm$
415 0.57 and $8.68 \pm 1.40 \mu\text{g L}^{-1}$, for the microplates and culture flasks, respectively. The same experiment
416 conducted with the diatom *S. marinoi* led to a bigger difference in EC₅₀ values, with 10.0 ± 0.50 and
417 $5.90 \pm 0.59 \mu\text{g L}^{-1}$ for the microplates and culture flasks, respectively. The difference was particularly

418 noticeable for the two lowest concentrations tested. Concerning the fungicide spiroxamine, there was
419 a four-fold higher toxicity in culture flasks than in microplates for *T. lutea*, with EC₅₀ values of 2.80 ±
420 0.35 and 0.70 ± 0.10 µg L⁻¹, respectively. In this case, the difference in toxicity is obvious when looking
421 at the concentration curves: the two lowest concentrations induced a two-fold higher effect in culture
422 flasks, and higher concentrations resulted in complete inhibition of microalgal growth, which was not
423 observed in microplates. Finally, spiroxamine induced a higher toxicity to *S. marinoi* in culture flasks,
424 with an EC₅₀ of 19.5 ± 0.68 µg L⁻¹ compared with 31.8 ± 0.92 µg L⁻¹ in microplates, the difference in
425 toxicity being most noticeable at 20 and 30 µg L⁻¹.

426

427 **4 Discussion**

428 Several comparative studies between microplate and flask assay exposure systems in the literature
429 (St-Laurent et al., 1992; Rojíčková et al., 1998; Eisentraeger et al., 2003; Blaise and Vasseur, 2005;
430 Pavlić et al., 2006) show similar results between these two test procedures and highlight the reliability
431 of the microplate test system due to its repeatability and reproducibility. However, chemicals tested in
432 these studies were generally reference toxicants like metals (Cd²⁺, Cr⁶⁺, Cu²⁺, Zn²⁺), phenol, K₂Cr₂O₇,
433 KClO₃ or herbicides, which all are highly soluble in water. In the present study, pesticides with differing
434 volatility (-9.77 < log *H* < -0.32), hydrophobicity (-3.20 < log *K*_{ow} < 5.50) and solubility (from 2.2 µg L⁻¹
435 to 1467 g L⁻¹) were tested, enabling an evaluation of the robustness of the microplate toxicity assay for
436 a wider range of chemicals.

437 Only nine chemicals out of 17 tested here showed significant toxicity within the tested range of
438 concentrations (1 µg L⁻¹ to 1 mg L⁻¹), regardless of the species on which they were tested. We decided
439 not to test concentrations higher than 1 mg L⁻¹ as this level would inevitably exceed the solubility of
440 some of the selected chemicals. For example, acrinathrin, α-cypermethrin and quinoxifen have a very
441 low solubility in water (2.20, 3.97 and 47.0 µg L⁻¹, respectively) while chlorpyrifos (1.05 mg L⁻¹),
442 kresoxim-methyl (2.00 mg L⁻¹) and chlorpyrifos-methyl (2.74 mg L⁻¹) had solubilities close to 1 mg L⁻¹.
443 Moreover, the selected range was sufficient to permit the identification of the most toxic substances.

444 For several chemicals, the concentrations measured in the microplate wells were much lower than
445 those intended and large decreases in concentration were observed after 96 h. This was principally
446 observed for chemicals with log *K*_{ow} > 3, exhibiting low solubility, which is the case of all the
447 insecticides tested except imidacloprid and two fungicides (kresoxim-methyl and quinoxifen). Previous

448 studies (Riedl and Altenburger, 2007; Tanneberger et al., 2013) have already indicated that special
449 attention should be paid to chemicals with a $\log K_{ow} > 3$ and $\log H > -5.61$. This was well illustrated by
450 the PCA, where the percentage of chemical recovered at 96 h (and to lesser extent 0 and 6 h) was
451 correlated with the hydrophobicity and solubility of the chemicals, which are directly related by the
452 general solubility equation (GSE, Ran et al., 2001). Indeed, chemicals with very low water solubility
453 such as α -cypermethrin, acrinathrin and quinoxifen (3.97, 2.20 and 47.0 $\mu\text{g L}^{-1}$, respectively) showed
454 very poor stability. Furthermore, as water solubility decreases with salinity (Voutchkova et al., 2012),
455 using seawater makes it even more difficult to work with such substances. One can however note the
456 absence of correlation with volatility in our study. Other standard values like hydrolysis or photolysis
457 DT_{50} (Dissipation Time, also known as half-life, defined as the time for a chemical to be reduced by
458 half through degradation; Table 1) were not considered in the PCA as, for most chemicals, values
459 were far above the duration of our experiments (4 days). It should, however, be noted that the
460 hydrolysis DT_{50} of flazasulfuron is quite low (11 days) and pH sensitive. As already reported by
461 Couderchet and Vernet (2003), pH variation inducing faster hydrolysis could explain the fall in
462 concentration of this chemical observed after 96 h in our wells. As the pH of the culture medium could
463 increase due to the consumption of dissolved CO_2 by microalgae in this medium, this might explain the
464 degradation of this chemical. However, such a rise cannot be confirmed as the pH of the enriched
465 seawater was not measured during our experiments.

466 In addition to the physicochemical properties of the chemicals themselves, the test system material
467 plays a role in the stability of the tested chemicals. Previous studies had already highlighted stability
468 issues and/or toxicity underestimation due to plastic sorption when testing hydrophobic chemicals in
469 microplates (Brown et al., 2001; Hirmann et al., 2007; Riedl and Altenburger, 2007). Moreover, these
470 issues can be exacerbated due to a higher area to volume ratio, favouring greater substance
471 adsorption to the plastic (Hörnström, 1990). Recently, Stadnicka-Michalak et al. (2014) reported a
472 good correlation between uptake/elimination rate constants of non-volatile chemicals for the plastic
473 compartment and the $\log K_{ow}$, demonstrating that the more hydrophobic the substance is, the more it
474 is subjected to plastic sorption. These findings also corroborate the results obtained between
475 microplate and culture flask test systems. Indeed, EC_{50} values obtained were systematically lower in
476 culture flasks, which could be explained by lower exposure concentrations in microplates due to plastic
477 sorption, although the substances tested (isoproturon and spiroxamine) are not particularly

478 hydrophobic (log K_{ow} of 2.50 and 2.89, respectively). Therefore, it is not possible to generalize
479 whether or not the toxicity obtained in culture flasks is always higher than in microplates without
480 testing a wider range of chemicals in these two test systems.

481 Comparing EC₅₀ values obtained in microplates and culture flasks also implies the use of different
482 methods to estimate growth, which might introduce a bias in toxicity measurement. Indeed, in
483 microplates, growth was estimated using chlorophyll fluorescence, whereas in culture flasks, cell
484 density was measured. Indeed, even though measurement of the cell density is probably more
485 accurate for estimating growth than fluorescence, it is not possible to perform it with microplates:
486 pipetting out at least 0.20 mL from each well every 24 h for a cell density analysis by flow cytometry
487 would excessively reduce the volume of culture in each well throughout the experiment. In addition,
488 measuring the cell density for each well would inevitably be more time-consuming than the
489 measurement of chlorophyll fluorescence. Hence, the time-effective aspect of the microplate-based
490 assay would no longer be an advantage compared with culture flasks. As a linear relationship exists
491 between chlorophyll fluorescence and cell density in steady experimental conditions (Ichimi et al.,
492 2012), using either of these methods should not bias the growth rate calculation. However, this is only
493 true when chlorophyll fluorescence of individual microalgal cells is not modified by the substance they
494 are exposed to, relative to the control culture. In culture flasks, analysis by flow cytometry allows the
495 measurement of the mean individual chlorophyll fluorescence (red fluorescence, FL3 > 670 nm or
496 FL4 675/25 nm) of fresh microalgal cells. Compared with the controls, it allows the measurement of a
497 potential effect of the tested substances on this parameter (supplementary material: Figure S2). For
498 *Tisochrysis lutea*, cells exposed to increasing concentrations of isoproturon (supplementary material:
499 Figure S2A) exhibited a significantly higher and concentration-dependent chlorophyll fluorescence
500 (more than two-fold higher for the three highest concentrations at 96 h). With spiroxamine
501 (supplementary material: Figure S2B), the biggest increase in chlorophyll fluorescence in this species
502 was induced by the three lowest concentrations. When looking at the diatom *Skeletonema marinoi*, a
503 smaller but significant increase in chlorophyll fluorescence was observed following exposure to the
504 three lowest isoproturon concentrations (supplementary material: Figure S2C), while almost no effect
505 was observed upon exposure to spiroxamine (supplementary material: Figure S2D). Consequently,
506 the higher fluorescence measured by the microplate reader for exposed wells could be mistakenly
507 attributed to growth when it is in fact due to an increase in the mean individual fluorescence of the

508 exposed microalgal cells. Higher growth values for exposed cells might therefore give the impression
509 of reduced toxicity in the microplates. One should therefore keep in mind that due to the
510 abovementioned bias, EC₅₀ values determined with two different endpoints, chlorophyll fluorescence
511 or cell density, will probably differ depending on the chemical and species tested. Although this bias
512 cannot explain the lower toxicity obtained in culture flasks on its own, it probably played a non-
513 negligible role in this study, along with the potential lower bioavailability of the chemical and/or higher
514 adsorption on the cells in culture flasks. This could also probably explain the differences observed in
515 EC₅₀ values calculated with fluorescence and cell density (Table 2), although cell density was only
516 measured at the beginning and the end of the experiment, adding another bias. Nevertheless, EC₅₀
517 values calculated with chlorophyll fluorescence and cell density do not differ sufficiently to modify our
518 interpretation of the toxicity; thus, EC₅₀ based on chlorophyll fluorescence can be viewed as reliable.

519 In several recent studies (Ochoa-Acuña et al., 2009; Malev et al., 2012; Nagai et al., 2013, 2016;
520 Nagai and De Schamphelaere, 2016; Nagai, 2017), the toxicity of pesticides towards microalgae was
521 measured using a chlorophyll fluorescence-microplate based assay, similar that performed in this
522 study. However, unlike the present work, none of these studies evaluated the potential bias in growth
523 measurement associated with the alteration of chlorophyll fluorescence and/or content upon exposure
524 of the microalgal cells to the pesticides tested, which is nonetheless thoroughly documented (Prado et
525 al., 2011; Stachowski-Haberkorn et al., 2013; Smedbol et al., 2018).

526 The chemical analyses performed, along with the comparison of the toxicity values between
527 microplates and culture flasks, made it possible to pinpoint different factors that can bias toxicity
528 assessment in the microplate test system. For herbicides, very similar EC₅₀ values were obtained
529 regardless of the exposure concentration set used, which corroborates their good overall stability in
530 microplates, as shown by the PCA (Figure 1A). On the contrary, significant differences in EC₅₀ values
531 were observed for chlorpyrifos-methyl and fungicides, due to a lower stability in microplates. For these
532 classes of pesticides, values two-fold lower were often obtained when using the corrected nominal
533 and/or measured concentration values. Using the corrected nominal concentration instead of the
534 nominal concentration always resulted in EC₅₀ values closer to those obtained using measured
535 concentrations, thus demonstrating that measuring the concentrations of the stock solutions used can,
536 at the very least greatly improve the accuracy of EC₅₀ values. Hence, to obtain the best accuracy of
537 toxicity assessment for a chosen chemical, one should systematically: i) consider the potential bias

538 associated with the hydrophobicity/solubility of the chemical; ii) perform, in similar conditions as the
539 toxicity assay, an analytical determination of the concentrations, at least for the stock solutions and
540 whenever possible for all dilutions made in the test medium. In the case of highly hydrophobic
541 chemicals ($\log K_{ow} > 5$), specific methods like passive dosing should be considered (Brown et al.,
542 2001; Vicquelin et al., 2011; Stibany et al., 2017b, 2017a) to avoid a distorted EC_{50} estimation.

543 Regarding the observed toxicity of the tested pesticides towards the two microalgal species, four
544 herbicides out of six, one insecticide out of six, and all fungicides were sufficiently toxic in the range
545 tested to permit the calculation of an EC_{50} . In the following discussion of these measured toxicities,
546 only EC_{50} values calculated using fluorescence and measured concentrations will be considered, as
547 these are the most reliable.

548 Herbicides were the chemicals most likely to be toxic to microalgae, especially the phenylureas diuron
549 and isoproturon targeting PSII, as this structure is very well-conserved among plants and algae
550 (Readman et al., 1993). These two chemicals were among the three that showed the highest toxicity,
551 with EC_{50} values below $10 \mu\text{g L}^{-1}$, in agreement with values from the literature. For diuron, EC_{50} values
552 of 5.90 and $4.30 \mu\text{g L}^{-1}$ were reported on the growth of *Skeletonema costatum* and *Thalassiosira*
553 *pseudonana*, respectively, (Bao et al., 2011), and Petersen et al. (2014) determined an EC_{50} value of
554 $15.6 \mu\text{g L}^{-1}$ on the growth of *S. pseudocostatum*. For isoproturon, Sjollema et al. (2014), determined
555 EC_{50} values of 8.70, 3.70 and 4.30 on the effective PSII efficiency of *Dunaliella tertiolecta*,
556 *Phaeodactylum tricornutum* and *T. pseudonana*, respectively; the two chloroacetanilides only showed
557 toxicity towards the diatom, with EC_{50} values around 300 and $1000 \mu\text{g L}^{-1}$ for metazachlor and S-
558 metolachlor, respectively, thus indicating different sensitivity in the two microalgal species tested. For
559 S-metolachlor, EC_{50} values on growth for *Tetraselmis suecica* and *Ditylum brightwellii*, were 21.3 mg
560 L^{-1} and $423 \mu\text{g L}^{-1}$, respectively (Ebenezer and Ki, 2013). For metazachlor, data are scarce but FAO
561 (1993) determined EC_{50} values of 1.63 mg L^{-1} and $31 \mu\text{g L}^{-1}$ on the growth of *Chlorella sp.* and
562 *Pseudokirchneriella subcapitata*.

563 Other herbicides like the sulfonyleurea flazasulfuron or the glycine glyphosate (and its main metabolite
564 AMPA), whose MoAs target amino-acid synthesis, did not have any toxic effect in the range tested
565 here. Only one earlier study evaluated the toxicity of flazasulfuron, which was evaluated on the
566 microalga *Chlorella vulgaris* and revealed a significant decrease in chlorophyll content from $100 \mu\text{g L}^{-1}$
567 (Couderechet and Vernet, 2003), although effects on growth were not measured. Regarding

568 glyphosate, the active substance itself was found to exhibit very low toxicity towards microalgae, with
569 EC₅₀ values of several tens of mg L⁻¹ (Ma, 2002; Ma et al., 2006; Vendrell et al., 2009).

570 Insecticides were not likely to induce any toxicity as their MoAs are very specific to structures that are
571 not found in microalgae, nevertheless, the organophosphate chlorpyrifos-methyl induced a 50%
572 inhibition on the growth rate of *T. lutea* at approximately 400 µg L⁻¹. Its mechanism of toxicity in
573 terrestrial plants is thought to be the inhibition of cytochrome P450 monooxygenases (Biediger et al.,
574 1992; Kapusta and Krausz, 1992), although the MoA on microalgae remains unclear (DeLorenzo et
575 al., 2001). Chlorpyrifos-methyl was reported to be mildly toxic to algae, with an EC₅₀ of 570 µg L⁻¹ on
576 the growth of *Raphidocelis subcapitata* (*Pesticide Properties DataBase*, PPDB, Lewis et al., 2016).

577 With fungicides, toxicity was potentially expected due to their MoAs, which are often not specific to
578 fungi, targeting enzymes that are well conserved across animals, fungi and plants (Stenersen, 2004).

579 Data on the toxicity to algae of the fungicides tested in this study are very scarce, or completely absent
580 from databases. The two strobilurines (azoxystrobin and kresoxim-methyl), inhibiting mitochondrial
581 electron transport (Stenersen, 2004), showed low overall toxicity with EC₅₀ values around 400 µg L⁻¹
582 for *T. lutea* and 500–900 µg L⁻¹ for *S. marinoi*, respectively. Ochoa-Acuña et al. (2009) determined an
583 EC₅₀ on growth of 230 µg L⁻¹ for azoxystrobin on the microalga *P. subcapitata*, while Liu et al. (2018)
584 determined an EC₅₀ of 3.06 mg L⁻¹ and 131 µg L⁻¹ for kresoxim-methyl on the growth of the microalgae
585 *C. pyrenoidsa* and *C. vulgaris*, respectively. As for quinoxyfen, the MoA is not common among
586 fungicides and has only been identified recently (Agrow, 2010): it acts by the disruption of signalling
587 processes that are crucial for early stages of powdery mildew development, by targeting numerous
588 pathways that are vital for the pathogen to grow. Though very specific to powdery mildew, this
589 chemical exhibited a higher toxicity to the diatom than the two strobilurines, with an EC₅₀ close to 30 µg
590 L⁻¹; however, its toxicity towards the haptophyte was lower and in a similar range as those of
591 azoxystrobin and kresoxim-methyl. Quinoxyfen was reported to show moderate toxicity to algae, with
592 an EC₅₀ value of 27 µg L⁻¹ on *R. subcapitata* (PPDB, Lewis et al., 2016). Finally, spiroxamine, whose
593 MoA acts by inhibiting the Δ¹⁴-sterol reductase enzyme and consequently sterol biosynthesis including
594 ergosterol, was the most toxic fungicide. It showed very high toxicity towards *T. lutea*, with an EC₅₀
595 around 4 µg L⁻¹ in microplates, and as low as 0.7 µg L⁻¹ in culture flasks (Figure 4), while the diatom
596 was less sensitive, with EC₅₀ values around 20–30 µg L⁻¹ in microplates/culture flasks. Sterols are very
597 important constituents of cellular membranes (Haines, 2001; Ohvo-Rekilä et al., 2002) and ergosterol

598 is one of the major sterols found in microalgae (Martin-Creuzburg and Merkel, 2016; Brumfield et al.,
599 2017). Hence, the high acute toxicity observed with this chemical might be due to cell membrane
600 disruption. Spiroxamine has already been reported as highly toxic to algae, with an EC₅₀ value of 3 µg
601 L⁻¹ on *Scenedemus subspicatus* (PPDB, Lewis et al., 2016).

602 Regarding the potential toxicity of the most toxic pesticides in this study towards the environment, few
603 previous studies have measured any of these substances in estuarine or coastal waters. In The
604 Netherlands, maximum concentrations of diuron and isoproturon of 0.37 and 0.12 µg L⁻¹ were
605 recorded in Western Scheldt (estuarine waters) in 2003, while lower concentrations of 0.030 and 0.088
606 were found more recently, in 2012 (Booij et al., 2015). In the coastal waters of the Ria Formosa lagoon
607 in Portugal, azoxystrobin was found at an average concentration of 2.15 µg L⁻¹ in spring (Cruzeiro et
608 al., 2015). In France, Munaron et al. (2012) followed the concentration of 27 hydrophilic pesticides in
609 Mediterranean coastal waters using POCIS (Polar Organic Chemical Integrative Sampler), and found
610 a maximum time-weighted average concentration of 0.033 µg L⁻¹ for diuron in the Thau lagoon. In
611 Normandy, concentrations of 0.13 and 0.042 µg L⁻¹ of diuron and isoproturon were measured in the
612 Bay of Veys in May (Buisson et al., 2008). Finally, Caquet et al. (2013) measured diuron and
613 isoproturon concentrations of up to 0.075 and 0.094 µg L⁻¹ in the coastal waters of the Bay of Vilaine
614 (France), while higher concentrations of 0.27 and 0.19 µg L⁻¹ were measured upstream in the Arzal
615 river (France). No studies have reported the presence of spiroxamine, one of the most toxic chemicals
616 tested in the present study, in the marine environment. Overall, the environmental concentrations
617 found in these previous studies are about 10-fold or more lower than the calculated EC₅₀ values in the
618 present study. However, the EC₁₀, an analogue of the NOEC (no-effect-concentration) (Beasley et al.,
619 2015), for diuron on *T. lutea* was calculated at around 0.68/0.76 µg L⁻¹ (culture flasks/microplate, data
620 not shown), which is close to the abovementioned environmental concentrations. It is also important to
621 keep in mind that these previous studies only focused on chosen substances and are thus not
622 representative of the 'real' contamination of the area studied. In addition, potential interactive effects
623 due to the mixture of several substances, which might, in some cases, result in increased toxicity
624 (Cedergreen, 2014) were not taken into account in this study.

625 The screening of toxicity on a wide range of pesticides used (for the most part) in viticulture using a
626 microplate assay confirmed the value of microplate-based bioassay as a screening tool while
627 indicating some issues related to chemicals with high hydrophobicity/low solubility. Although

628 herbicides like diuron or isoproturon were expected to be highly toxic, this was not predicted for
629 fungicides. All fungicides tested showed significant toxicity towards both microalgal species tested,
630 particularly spiroxamine, which was highly toxic to *T. lutea* and to a lesser extent to *S. marinoi*.

631

632 **5 Conclusion**

633 A microplate bioassay was combined with chemical analyses to provide a reliable calculation of EC₅₀
634 values through a toxicity screening experiment performed on two marine microalgae. Only nine
635 substances among the 17 tested were sufficiently toxic in the ranges tested to permit the calculation of
636 EC₅₀ values. We therefore make the following recommendations concerning the use of microplate
637 bioassays:

- 638 - At the very least, analytical determination of the stock solution concentrations should be
639 performed to allow correction of nominal exposure concentrations.
- 640 - For hydrophobic chemicals with a log *K*_{ow} > 3, measurement of the effective chemical
641 concentration in the test system is necessary.
- 642 - The microplate bioassay is not suitable for highly hydrophobic chemicals (log *K*_{ow} > 5), which
643 should be tested using specific exposure methods like passive dosing.
- 644 - Whenever possible, cell density measurement should be performed in addition to chlorophyll
645 fluorescence because some chemicals alter chlorophyll fluorescence and consequently bias
646 growth measurement based on this endpoint.

647 Regarding the toxicity induced by the tested chemicals, the herbicides diuron and isoproturon had high
648 acute toxicity (EC₅₀ < 10 µg L⁻¹) for both microalgal species. Insecticides did not generally show any
649 toxicity in the range of concentrations tested, apart from chlorpyrifos-methyl, which caused moderate
650 toxicity to *T. lutea*. Surprisingly, all fungicides tested showed significant toxicity, especially spiroxamine
651 which had very high acute toxicity (EC₅₀ 0.7-4 µg L⁻¹) to *Tisochrysis lutea*. Hence, further studies
652 should be performed to assess spiroxamine contamination of aquatic ecosystems in vineyard areas.

653

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660

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- 917

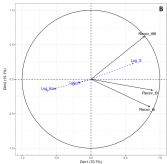
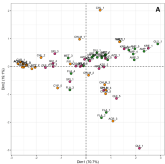
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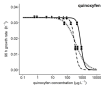
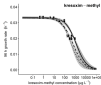
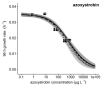
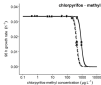
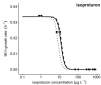
Figure 1 (*to be printed in colour*). Principal component analysis (PCA) illustrating the relationships between concentrations measured in the microplate wells after 0, 6 and 96 h of exposure and physicochemical properties of the chemicals (Log K_{ow} , Log H , Log S) added as supplementary variables. The left-hand graph (A) is the projection of individuals grouped by pesticide class: green for herbicides, orange for insecticides and pink for fungicides. Individuals are named using the first three letters of the chemical and the concentration level (1 for C1 etc.). The right-hand graph (B) is the factor map of variables on the two first axes (supplementary quantitative variables are shown in blue with dashed arrows).

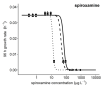
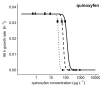
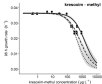
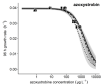
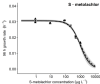
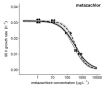
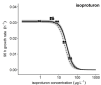
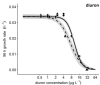
Figure 2: Concentration-response curves for all substances tested on *Tisochrysis lutea*. Points (in triplicate; dots: nominal concentration; squares: corrected nominal concentration; triangles: measured concentration) correspond to the 96-h growth rate. Lines (solid line: nominal concentration; dashed line: corrected nominal concentration; dotted line: measured concentration) correspond to the fitted three-parameter log-logistic model with their 95% confidence intervals shown in grey.

Figure 3: Concentration-response curves for all substances tested on *Skeletonema marinoi*. Points (in triplicate; dots: nominal concentration; squares: corrected nominal concentration; triangles: measured concentration) correspond to the 96-h growth rate. Lines (solid line: nominal concentration; dashed line: corrected nominal concentration; dotted line: measured concentration) correspond to the fitted three-parameter log-logistic model with their 95% confidence intervals shown in grey.

Figure 4: Comparison of concentration-response curves obtained in microplate and culture flasks for *Tisochrysis lutea* (A, B) and *Skeletonema marinoi* (C, D). Points (in triplicates; dots: microplate; triangles: culture flasks) correspond to the 96-h growth rate. Lines (microplate: solid line; culture flasks: dashed line) correspond to the fitted three-parameter log-logistic model with their 95% confidence intervals shown in grey.







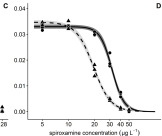
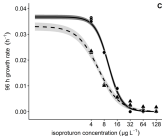
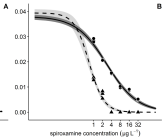
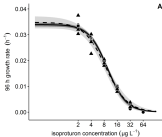


Table 1. Chemicals tested in this study, their physicochemical properties, known modes of action (MoA). Substances displayed as biocides are no longer allowed in any agricultural applications, while all the remaining ones are phytosanitary products (PPs) allowed in viticulture or other agricultural applications. (sources: *Pesticide Properties DataBase*, PPDB, Lewis et al., 2016, E-Phy / ANSES, <https://ephy.anses.fr/> and European chemical agency (ECHA): <https://echa.europa.eu/fr/home>).

Class	Chemical	Family	Log K_{ow}	Log H	Log S	Photolysis DT ₅₀ (d)	Hydrolysis DT ₅₀ (d)	Mode of action
Biocides	diuron	phenylurea	2.87	-7.52	4.54	43.0	stable	inhibition of photosynthesis at photosystem II
	isoproturon	phenylurea	2.50	-4.84	4.85	48.0	stable	inhibition of photosynthesis at photosystem II
	fipronil	phenylpyrazole	3.75	-3.64	3.58	0.33	stable	GABA-gated chloride channel antagonist
Herbicides	flazasulfuron	sulfonylurea	-0.06	-5.59	6.32	stable	11.0	inhibition of acetolactate synthase (ALS)
	metazachlor	chloroacetanilide	2.13	-4.23	5.65	stable	stable	inhibition of elongase and geranylgeranyl pyrophosphate (GGPP)
	S-metolachlor	chloroacetanilide	2.90	-2.66	5.68	stable	stable	inhibition of elongase and geranylgeranyl pyrophosphate (GGPP)
	glyphosate	glycine	-3.20	-6.68	7.02	69.0	stable	inhibition of EPSP synthase
	AMPA	(glyphosate metabolite)	-1.63	-0.80	9.17			
Insecticides	α -cypermethrin	pyrethroid	5.50	-1.16	0.60	6.30	70.00	sodium channel modulator
	acrinathrin	pyrethroid	5.24	-1.97	0.34	2.30	stable	sodium channel modulator
	chlorpyrifos	organophosphate	4.70	-0.32	3.02	29.6	16.0	inhibition of acetylcholine esterase
	chlorpyrifos-methyl	organophosphate	4.24	-0.63	3.44	1.74	21.0	inhibition of acetylcholine esterase
	imidacloprid	neonicotinoid	0.57	-9.77	5.79	0.20	stable	nicotinic acetylcholine receptor agonist
Fungicides	azoxystrobin	strobilurine	2.50	-8.13	3.83	8.70	stable	inhibition of the respiratory chain at the level of Complex III
	kresoxim-methyl	strobilurine	3.40	-3.44	3.30	18.2	35.0	inhibition of the respiratory chain at the level of Complex III
	quinoxifen	quinoline	4.66	-1.50	1.67	0.80	stable	disruption of early cell signalling events
	spiroxamine	morpholine	2.89	-2.42	5.61	50.5	stable	inhibition of sterol biosynthesis

Table 2. EC₅₀ values (\pm 95% confidence interval) computed with the three expressions of exposure concentrations (§2.6.1) (see description below the table) for the two species of microalgae. Note that only chemicals with data permitting the calculation of the EC₅₀ are displayed; data for diuron and S-metolachlor were obtained from preliminary experiments and therefore did not allow the “Measured” and “Cell density” modalities to be computed.

Class	Chemical	<i>Tisochrysis lutea</i>				<i>Skeletonema marinoi</i>			
		EC ₅₀ ($\mu\text{g L}^{-1}$)							
		Nominal ^a	Corrected nominal ^b	Measured ^c	Cell density	Nominal	Corrected nominal	Measured	Cell density
Biocides	diuron	3.79 \pm 0.16	2.20 \pm 0.071			10.3 \pm 0.80	7.26 \pm 1.23		
	isoproturon	12.1 \pm 5.48	13.0 \pm 6.76	8.55 \pm 3.39	8.24 \pm 5.11	26.7 \pm 2.22	26.9 \pm 2.26	21.2 \pm 1.80	18.3 \pm 1.29
Herbicides	metazachlor					323 \pm 41.4	370 \pm 43.2	245 \pm 24.7	563 \pm 121
	S-metolachlor					937 \pm 130	1032 \pm 138		
Insecticides	chlorpyrifos-methyl	877 \pm 13.7	406 \pm 6.33	449 \pm 6.70	390 \pm 19.3				
Fungicides	azoxystrobin	750 \pm 246	624 \pm 207	434 \pm 144	112 \pm 32.2	1799 \pm 462	1504 \pm 386	917 \pm 189	479 \pm 52.5
	kresoxim-methyl	1220 \pm 262	602 \pm 127	414 \pm 65	261 \pm 34.0	1517 \pm 245	753 \pm 119	482 \pm 56.9	386 \pm 44.4
	quinoxifen	820 \pm 19.0	320 \pm 7.1	378 \pm 29.6	295 \pm 39.3	124 \pm 82.8	68.8 \pm 33.0	33.0 \pm 8.49	47.3 \pm 1.20
	spiroxamine	8.28 \pm 2.34	5.04 \pm 1.37	3.37 \pm 0.74	1.10	67.8 \pm 80.4	45.7 \pm 44.6	12.4 \pm 5.63	11.3 \pm 11.7

^a **Nominal**: nominal concentrations calculated using stock solution concentrations calculated by weighing

^b **Corrected nominal**: corrected nominal concentrations calculated using measured stock solution concentrations.

^c **Measured**: measured concentrations in the microplate wells from the chemical stability experiments (§2.3.2)

^d **Cell density**: EC₅₀ was estimated using the cell density measured by flow cytometry at the beginning and at the end of the test (0 and 96 h) instead of fluorescence (used for the 3 modalities above) and measured concentrations. Note that, for spiroxamine with *T. lutea*, no confidence interval could be computed due to a poor fit of the model.