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Abstract

Pesticides used in viticulture create a potential risk for the aquatic environment due to drift during application, runoff and soil leaching. The toxicity of sixteen pesticides and one metabolite were evaluated on the growth of two marine microalgae, *Tisochrysis lutea* and *Skeletonema marinoi*, in 96-h exposure assays conducted in microplates. For each substance, concentrations of stock solutions were analytically measured and abiotic assays were performed to evaluate the chemical stability of pesticides in microplates. For two chemicals, microalgae exposures were run simultaneously in microplates and culture flasks to compare EC_{50} calculated from the two exposure systems.

Results from chemical analyses demonstrated the low stability of hydrophobic pesticides (log K_{OW} > 3). For such chemicals, EC_{50} values calculated using measured pesticide concentrations were two-fold lower than those first estimated using nominal concentrations. Photosystem II inhibitors were the most toxic herbicides, with EC_{50} values below 10 µg L^{-1} for diuron and around double this for isoproturon. Chlorpyrifos-methyl was the only insecticide to significantly affect the growth of *T. lutea*, with an EC_{50} around 400 µg L^{-1}. All fungicides tested were significantly toxic to both species: strobilurins showed low overall toxicity, with EC_{50} values around 400 µg L^{-1}, whereas quinoxyfen, and spiroxamine,

showed high toxicity to both species, especially to *T. lutea*, with an EC_{50} below 1 µg L^{-1} measured for spiroxamine in culture flasks. This study highlights the need to perform chemical analyses for reliable toxicity assessment and discusses the advantages and disadvantages of using microplates as a toxicity screening tool.

Keywords

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

1 Introduction

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

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

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

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

In order to assess the toxicity of such chemicals on microalgae, the present standard procedures (OECD 201, 2011; ISO 10253:2016, 2016) recommend the use of glass testing recipients. Indeed, standard procedures using glass culture flasks are the result of many experiments and years of work, which is why these vessels are still considered one of the most reliable tools for toxicity testing on microalgae. Culture flasks offer numerous advantages, such as a larger volume of culture that makes it possible to perform multiple analyses on a single culture, which is not possible with microplates due to their very small well volumes. However, when the main goal of an experiment is to determine standard toxicity values such as EC_{50} (which requires at least five concentrations to be tested in triplicate) for numerous chemicals, the use of culture flasks can be time- and space-consuming. In comparison, microplate-based phytotoxicity assays have rapidly become common since their first appearance in the early 80s, as several authors pointed out their reliability for toxicity testing by demonstrating a good agreement of toxicity levels between microplate and culture flask test systems (Blaise et al., 1986; St-Laurent et al., 1992; Rojîéková et al., 1998; Geis et al., 2000; Eisentraeger et al., 2003; Kim Tiam et al., 2014a).

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

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

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

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

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

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

1. To evaluate the stability of the tested chemicals in the culture medium and in the microplate wells over a 96-h experiment period.
2. To perform the toxicity screening of sixteen pesticides and one metabolite on the two species of microalgae using the microplate-based growth inhibition assay.
3. To compare EC_{50} values obtained using: i) nominal vs measured concentrations; ii) microplate and culture flask test systems.

To answer these questions, we adapted a 96-h growth inhibition assay (Blaise et al., 1997; Eisentraeger et al., 2003; Riedl and Altenburger, 2007) and conducted this in 48-well microplates. Abiotic assays were carried out to evaluate the stability of the tested chemicals in the microplates by measuring effective exposure concentrations in the culture medium by liquid or gas chromatography tandem mass spectrometry analysis (LC/MS/MS or GC/MS/MS). Microalgal exposure experiments were performed to compare toxicity values obtained in microplates with those obtained in glass flasks. Concentration-response experiments were then done in microplates to determine EC_{50} values for sixteen selected pesticides and one metabolite on the two microalgal species.

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

2 Material and methods

2.1 Chemical preparation

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

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

2.2 Microalgal cultures

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

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

2.3 Exposure experiments

2.3.1 Microplate toxicity assay

The microplate toxicity assay consisted in concentration-response experiments conducted with each microalga and chemical to be tested. The aim was to calculate the EC_{50} – the Effective Concentration inducing a 50% inhibition on growth rate of the microalga after a 96-h exposure – for each chemical. For these experiments, transparent polystyrene 48-well microplates (Greiner Bio-One GmbH, cat. 677102, untreated) were used, each well being filled with 0.90 mL of sterile f/2 (for *T. lutea*) or f/2-Si (for *S. marinoi*) culture medium. Microplates were covered with their own lid, allowing gas exchanges. Peripheral wells were not used in order to avoid edge effects (Caux et al., 1992; St-Laurent et al., 1992); instead, these were filled with 1.00 mL of sterile 0.20-µm filtered Milli-Q water to prevent evaporation and maintain high humidity. For the toxicity screening experiments, one test was carried out for each substance to be tested. The test consisted in exposure to five nominal concentrations, 1.00, 10.0, 100, 500 and 1000 µg L^{-1} of the pesticide in triplicate (three wells), six solvent controls (SC) containing the highest solvent percentage used in the microplate (max 0.1%) and three controls (C) without solvent (supplementary material: Figure S1). For diuron, because this chemical had already been tested in our preliminary experiments, a refined range was used. When needed, some other chemicals were tested a second time using a refined range of six concentrations in order to improve the determination of their EC_{50}. Prior to their introduction into the triplicate wells of the microplate, both chemical and solvent solutions were prepared at targeted concentrations in sterile glass flasks (one glass flask per tested chemical and concentration) containing 25.0 mL of sterile culture medium.

After measurement of cell density by flow cytometry, 0.10 mL of the diluted microalgal culture was added to each assay well to reach a concentration of 20,000 cell mL^{-1} at the beginning of exposure. The final volume of each well was 1.00 mL. After 96 h, the whole content of each well was recovered, fixed using glutaraldehyde (final concentration 0.25%) and left in the dark for 10 min before being frozen and stored in liquid nitrogen (-196°C) until cell density analysis by flow cytometry.

2.3.2 Culture flask toxicity assay

For two selected chemicals (isoproturon and spiroxamine), toxicity assays were also performed in glass culture flasks, using the same culture conditions as the microplate assays. This enabled us to compare the toxicity between the two test systems: glass culture flasks vs microplates. Isoproturon and spiroxamine were selected because of their high toxicity to both algal species, as well as their differing chemical families, carrier solvents (methanol vs acetone, §2.1), and usages. For these experiments, microalgal samples were exposed in triplicate to a range of six chemical concentrations, chosen based on EC_{50} values obtained during the previous microplate toxicity assay. Three solvent controls were set up containing the highest solvent percentage. It should be noted that six wells were used for the solvent controls in the microplate toxicity assay. Chemical and solvent solutions were prepared at targeted concentrations in 50.0 mL sterile f/2 (for *T. lutea*) or f/2-Si (for *S. marinoi*) culture medium. For each species, a stock culture was analysed by flow cytometry for the measurement of microalgal cell density: then, depending on the species and cell density, between 0.10 and 0.50 mL of stock culture were added to each flask to reach an initial concentration of 20,000 cell mL^{-1} at the beginning of experiment. During the experiment, each culture was sampled daily (0.50 mL) for cell density analysis by flow cytometry.

2.3.3 Chemical stability of tested substances in the microplates

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

2.4 Chemical analysis

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

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

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

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

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

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

2.5 Growth measurement in microplates and culture flasks

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

$$U = \frac{d}{\left(1 + (\frac{x}{\mathrm{EC50}})^{b}\right)} \quad (1)$$

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

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

i. nominal concentrations, calculated theoretically using the nominal stock solution concentrations;
ii. corrected nominal concentrations, calculated theoretically using the measured stock solution concentrations;
iii. measured concentrations, the chemical concentrations in the wells reached at the beginning of the exposure as measured by chemical analysis.

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

2.6.2 Principal component analysis

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

3 Results

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

3.1 Chemical analyses

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

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

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

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

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

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

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

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

3.2 Toxicity screening of 17 chemicals

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

As stated in the Material and methods (§2.6.1), three different expressions of the exposure concentrations were used to calculate EC_{50} values for each chemical (except for diuron and S-metolachlor). The three different EC_{50} values appear to be within the same range for herbicides, with differences of less than two-fold, for example: 12.1 ± 5.48, 13.0 ± 6.76 and 8.55 ± 3.39 µg L^{-1} for isoproturon on *T. lutea*; 323 ± 41.4, 370 ± 43.2 and 245 ± 24.7 µg L^{-1} for metazachlor on *S. marinoi*, using nominal, corrected nominal and measured concentrations, respectively. For insecticides and fungicides, values more than two-fold lower were found using the nominal, corrected or measured concentration, for example: 877 ± 13.7, 406 ± 6.33 and 449 ± 6.70 µg L^{-1} for chlorpyrifos-methyl on *T. lutea*; and 124 ± 82.8, 68.8 ± 33.0 and 33.0 ± 8.49 µg L^{-1} for quinoxyfen on *S. marinoi* using nominal, corrected nominal and measured concentrations, respectively. Concerning the concentration-response curves (Figures 2 and 3), those computed with corrected nominal (dashed line) or measured (dotted line) concentrations are shifted to the right, implying lower EC_{50} values. Looking at the difference between EC_{50} values calculated with corrected nominal and measured concentrations, it appears that, 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 quinoxyfen 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, EC_{50} values using cell density were
392 only calculated based on measured concentrations as these are considered the most accurate. As a
393 consequence, EC_{50} values based on cell density should only be compared with EC_{50} values calculated
394 with measured concentrations. The EC_{50} on *S. marinoi* exposed to metazachlor was two-fold higher
395 when using cell density (563 ± 121 µg L^{-1}) rather than fluorescence (245 ± 24.7 µg L^{-1}) for growth rate
396 determination. On the contrary, EC_{50} 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 ±
398 32.2 and 434 ± 144 µg L^{-1} for cell density and fluorescence, respectively). For isoproturon and
399 quinoxyfen, there was almost no difference between the cell density and fluorescence EC_{50} values
400 with both microalgal species, and similarly for chlorpyrifos-methyl with *T. lutea*. Finally, regarding
401 spiroxamine, an EC_{50} 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 EC_{50} 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 EC_{50} values of 9.57 ±
415 0.57 and 8.68 ± 1.40 µg 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 EC_{50} values, with 10.0 ± 0.50 and
417 5.90 ± 0.59 µg L^{-1} for the microplates and culture flasks, respectively. The difference was particularly

noticeable for the two lowest concentrations tested. Concerning the fungicide spiroxamine, there was a four-fold higher toxicity in culture flasks than in microplates for *T. lutea*, with EC_{50} values of 2.80 ± 0.35 and 0.70 ± 0.10 µg L^{-1}, respectively. In this case, the difference in toxicity is obvious when looking at the concentration curves: the two lowest concentrations induced a two-fold higher effect in culture flasks, and higher concentrations resulted in complete inhibition of microalgal growth, which was not observed in microplates. Finally, spiroxamine induced a higher toxicity to *S. marinoi* in culture flasks, with an EC_{50} of 19.5 ± 0.68 µg L^{-1} compared with 31.8 ± 0.92 µg L^{-1} in microplates, the difference in toxicity being most noticeable at 20 and 30 µg L^{-1}.

4 Discussion

Several comparative studies between microplate and flask assay exposure systems in the literature (St-Laurent et al., 1992; Rojîéková et al., 1998; Eisentraeger et al., 2003; Blaise and Vasseur, 2005; Pavlić et al., 2006) show similar results between these two test procedures and highlight the reliability of the microplate test system due to its repeatability and reproducibility. However, chemicals tested in these studies were generally reference toxicants like metals (Cd^{2+}, Cr^{6+}, Cu^{2+}, Zn^{2+}), phenol, $K_2Cr_2O_7$, $KClO_3$ or herbicides, which all are highly soluble in water. In the present study, pesticides with differing volatility (-9.77 < log *H* < -0.32), hydrophobicity (-3.20 < log K_{OW} < 5.50) and solubility (from 2.2 µg L^{-1} to 1467 g L^{-1}) were tested, enabling an evaluation of the robustness of the microplate toxicity assay for a wider range of chemicals.

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

For several chemicals, the concentrations measured in the microplate wells were much lower than those intended and large decreases in concentration were observed after 96 h. This was principally observed for chemicals with log K_{OW} > 3, exhibiting low solubility, which is the case of all the insecticides tested except imidacloprid and two fungicides (kresoxim-methyl and quinoxyfen). 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 quinoxyfen (3.97, 2.20 and 47.0 µg 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

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

Comparing EC_{50} values obtained in microplates and culture flasks also implies the use of different methods to estimate growth, which might introduce a bias in toxicity measurement. Indeed, in microplates, growth was estimated using chlorophyll fluorescence, whereas in culture flasks, cell density was measured. Indeed, even though measurement of the cell density is probably more accurate for estimating growth than fluorescence, it is not possible to perform it with microplates: pipetting out at least 0.20 mL from each well every 24 h for a cell density analysis by flow cytometry would excessively reduce the volume of culture in each well throughout the experiment. In addition, measuring the cell density for each well would inevitably be more time-consuming than the measurement of chlorophyll fluorescence. Hence, the time-effective aspect of the microplate-based assay would no longer be an advantage compared with culture flasks. As a linear relationship exists between chlorophyll fluorescence and cell density in steady experimental conditions (Ichimi et al., 2012), using either of these methods should not bias the growth rate calculation. However, this is only true when chlorophyll fluorescence of individual microalgal cells is not modified by the substance they are exposed to, relative to the control culture. In culture flasks, analysis by flow cytometry allows the measurement of the mean individual chlorophyll fluorescence (red fluorescence, FL3 > 670 nm or FL4 675/25 nm) of fresh microalgal cells. Compared with the controls, it allows the measurement of a potential effect of the tested substances on this parameter (supplementary material: Figure S2). For *Tisochrysis lutea*, cells exposed to increasing concentrations of isoproturon (supplementary material: Figure S2A) exhibited a significantly higher and concentration-dependent chlorophyll fluorescence (more than two-fold higher for the three highest concentrations at 96 h). With spiroxamine (supplementary material: Figure S2B), the biggest increase in chlorophyll fluorescence in this species was induced by the three lowest concentrations. When looking at the diatom *Skeletonema marinoi*, a smaller but significant increase in chlorophyll fluorescence was observed following exposure to the three lowest isoproturon concentrations (supplementary material: Figure S2C), while almost no effect was observed upon exposure to spiroxamine (supplementary material: Figure S2D). Consequently, the higher fluorescence measured by the microplate reader for exposed wells could be mistakenly attributed to growth when it is in fact due to an increase in the mean individual fluorescence of the

exposed microalgal cells. Higher growth values for exposed cells might therefore give the impression of reduced toxicity in the microplates. One should therefore keep in mind that due to the abovementioned bias, EC_{50} values determined with two different endpoints, chlorophyll fluorescence or cell density, will probably differ depending on the chemical and species tested. Although this bias cannot explain the lower toxicity obtained in culture flasks on its own, it probably played a non-negligible role in this study, along with the potential lower bioavailability of the chemical and/or higher adsorption on the cells in culture flasks. This could also probably explain the differences observed in EC_{50} values calculated with fluorescence and cell density (Table 2), although cell density was only measured at the beginning and the end of the experiment, adding another bias. Nevertheless, EC_{50} values calculated with chlorophyll fluorescence and cell density do not differ sufficiently to modify our interpretation of the toxicity; thus, EC_{50} based on chlorophyll fluorescence can be viewed as reliable.

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

The chemical analyses performed, along with the comparison of the toxicity values between microplates and culture flasks, made it possible to pinpoint different factors that can bias toxicity assessment in the microplate test system. For herbicides, very similar EC_{50} values were obtained regardless of the exposure concentration set used, which corroborates their good overall stability in microplates, as shown by the PCA (Figure 1A). On the contrary, significant differences in EC_{50} values were observed for chlorpyrifos-methyl and fungicides, due to a lower stability in microplates. For these classes of pesticides, values two-fold lower were often obtained when using the corrected nominal and/or measured concentration values. Using the corrected nominal concentration instead of the nominal concentration always resulted in EC_{50} values closer to those obtained using measured concentrations, thus demonstrating that measuring the concentrations of the stock solutions used can, at the very least greatly improve the accuracy of EC_{50} values. Hence, to obtain the best accuracy of toxicity assessment for a chosen chemical, one should systematically: i) consider the potential bias

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

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

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

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

glyphosate, the active substance itself was found to exhibit very low toxicity towards microalgae, with EC_{50} values of several tens of mg L^{-1} (Ma, 2002; Ma et al., 2006; Vendrell et al., 2009).

Insecticides were not likely to induce any toxicity as their MoAs are very specific to structures that are not found in microalgae, nevertheless, the organophosphate chlorpyrifos-methyl induced a 50% inhibition on the growth rate of *T. lutea* at approximately 400 µg L^{-1}. Its mechanism of toxicity in terrestrial plants is thought to be the inhibition of cytochrome P450 monooxygenases (Biediger et al., 1992; Kapusta and Krausz, 1992), although the MoA on microalgae remains unclear (DeLorenzo et al., 2001). Chlorpyrifos-methyl was reported to be mildly toxic to algae, with an EC_{50} of 570 µg L^{-1} on the growth of *Raphidocelis subcapitata* (*Pesticide Properties DataBase*, PPDB, Lewis et al., 2016). With fungicides, toxicity was potentially expected due to their MoAs, which are often not specific to fungi, targeting enzymes that are well conserved across animals, fungi and plants (Stenersen, 2004). Data on the toxicity to algae of the fungicides tested in this study are very scarce, or completely absent from databases. The two strobilurines (azoxystrobin and kresoxim-methyl), inhibiting mitochondrial electron transport (Stenersen, 2004), showed low overall toxicity with EC_{50} values around 400 µg L^{-1} for *T. lutea* and 500–900 µg L^{-1} for *S. marinoi*, respectively. Ochoa-Acuña et al. (2009) determined an EC_{50} on growth of 230 µg L^{-1} for azoxystrobin on the microalga *P. subcapitata*, while Liu et al. (2018) determined an EC_{50} of 3.06 mg L^{-1} and 131 µg L^{-1} for kresoxim-methyl on the growth of the microalgae *C. pyrenoidsa* and *C. vulgaris*, respectively. As for quinoxyfen, the MoA is not common among fungicides and has only been identified recently (Agrow, 2010): it acts by the disruption of signalling processes that are crucial for early stages of powdery mildew development, by targeting numerous pathways that are vital for the pathogen to grow. Though very specific to powdery mildew, this chemical exhibited a higher toxicity to the diatom than the two strobilurines, with an EC_{50} close to 30 µg L^{-1}; however, its toxicity towards the haptophyte was lower and in a similar range as those of azoxystrobin and kresoxim-methyl. Quinoxyfen was reported to show moderate toxicity to algae, with an EC_{50} value of 27 µg L^{-1} on *R. subcapitata* (PPDB, Lewis et al., 2016). Finally, spiroxamine, whose MoA acts by inhibiting the Δ^{14}-sterol reductase enzyme and consequently sterol biosynthesis including ergosterol, was the most toxic fungicide. It showed very high toxicity towards *T. lutea*, with an EC_{50} around 4 µg L^{-1} in microplates, and as low as 0.7 µg L^{-1} in culture flasks (Figure 4), while the diatom was less sensitive, with EC_{50} values around 20–30 µg L^{-1} in microplates/culture flasks. Sterols are very 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 EC_{50} 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 EC_{50} values in the
618 present study. However, the EC_{10}, 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

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

5 Conclusion

A microplate bioassay was combined with chemical analyses to provide a reliable calculation of EC_{50} values through a toxicity screening experiment performed on two marine microalgae. Only nine substances among the 17 tested were sufficiently toxic in the ranges tested to permit the calculation of EC_{50} values. We therefore make the following recommendations concerning the use of microplate bioassays:

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

Regarding the toxicity induced by the tested chemicals, the herbicides diuron and isoproturon had high acute toxicity (EC_{50} < 10 µg L^{-1}) for both microalgal species. Insecticides did not generally show any toxicity in the range of concentrations tested, apart from chlorpyrifos-methyl, which caused moderate toxicity to *T. lutea*. Surprisingly, all fungicides tested showed significant toxicity, especially spiroxamine which had very high acute toxicity (EC_{50} 0.7-4 µg L^{-1}) to *Tisochrysis lutea*. Hence, further studies should be performed to assess spiroxamine contamination of aquatic ecosystems in vineyard areas.

6 Acknowledgments

This study was carried out with financial support from the French National Research Agency (ANR) in the framework of the Investments for the Future program, within the Cluster of Excellence COTE (ANR-10-LABX-45). We would also like to thank Dominique Ménard and Julien Rouxel for their

658 technical assistance. We thank Helen McCombie for the English correction. We also thank the
659 anonymous reviewer for his comments, which helped us to improve the quality of this manuscript.

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661 7 References

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917

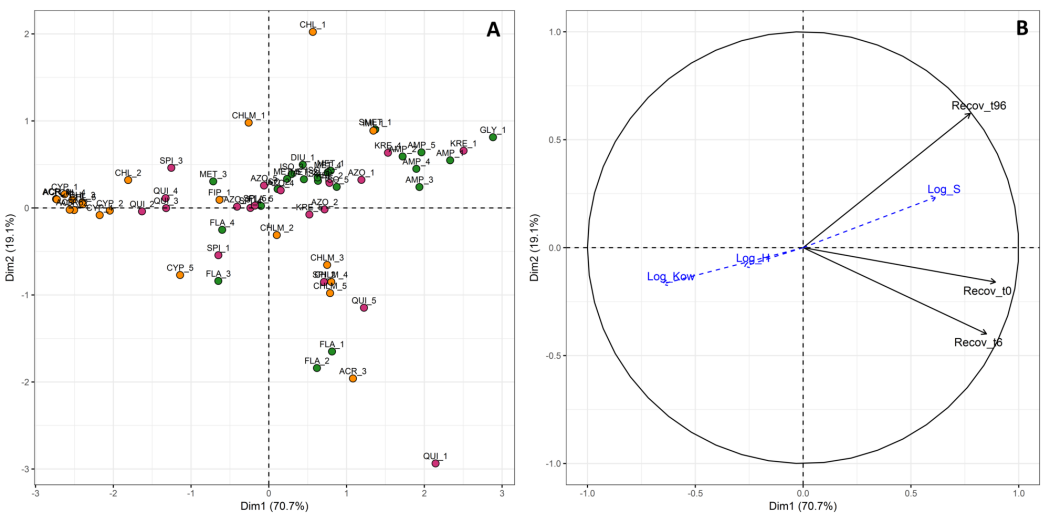
Figure captions

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.


A
B
Dim1 (70.7%)
Dim2 (19.1%)
CHL_1
CHLM_1
SMET_1
GLY_1
KRE_1
AMP
AMP_4
AMP_3
SPI_3
CHL_2
MET_3
QUI_4
FLA_4
CHLM_2
CHLM_3
SPI_1
CYP_5
FLA_3
QUI_5
FLA_1
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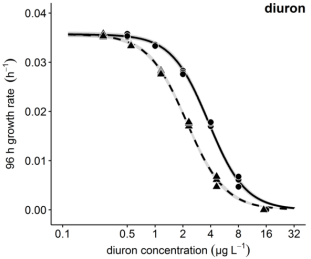
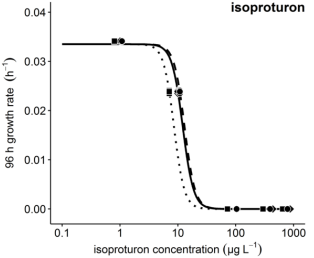
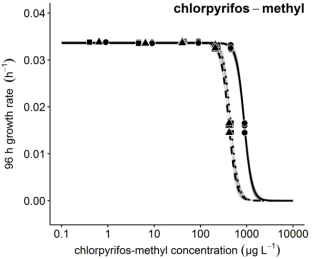
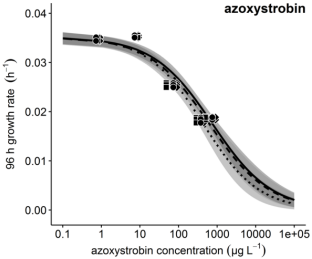
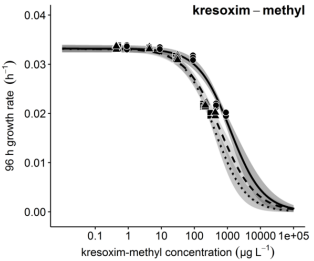
ACR_3
QUI_1
Recov_t96
Log_S
Log_H
Log_Kow
Recov_t0
Recov_t6

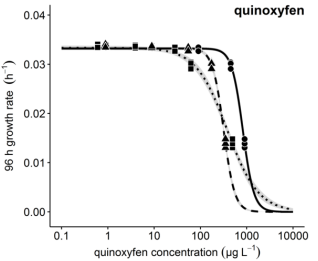

diuron
96 h growth rate (h⁻¹)
0.04
0.03
0.02
0.01
0.00
0.1
0.5
1
2
4
8
16
32
diuron concentration (µg L⁻¹)

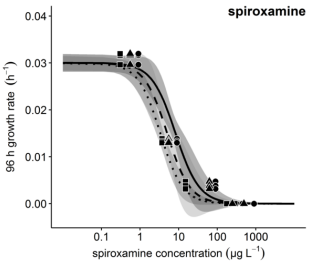

isoproturon
96 h growth rate (h⁻¹)
0.04
0.03
0.02
0.01
0.00
0.1
1
10
100
1000
isoproturon concentration (µg L⁻¹)

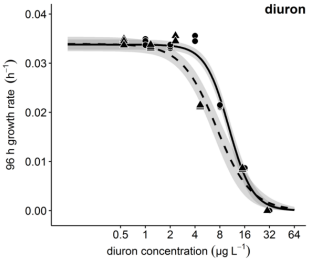

chlorpyrifos – methyl
96 h growth rate (h⁻¹)
0.04
0.03
0.02
0.01
0.00
0.1
1
10
100
1000
10000
chlorpyrifos-methyl concentration (µg L⁻¹)

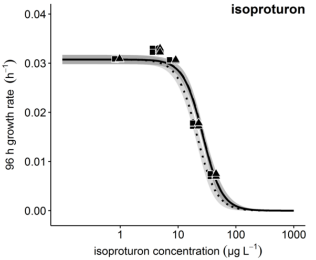

azoxystrobin
96 h growth rate (h⁻¹)
0.04
0.03
0.02
0.01
0.00
0.1
1
10
100
1000
10000
1e+05
azoxystrobin concentration (µg L⁻¹)

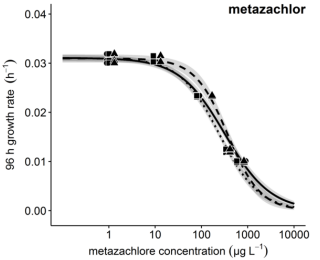

kresoxim – methyl
96 h growth rate (h⁻¹)
0.04
0.03
0.02
0.01
0.00
0.1
1
10
100
1000
10000
1e+05
kresoxim-methyl concentration (µg L⁻¹)

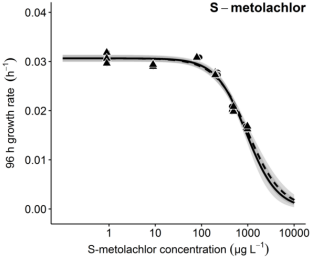

quinoxyfen
96 h growth rate (h⁻¹)
0.04
0.03
0.02
0.01
0.00
0.1
1
10
100
1000
10000
quinoxyfen concentration (µg L⁻¹)

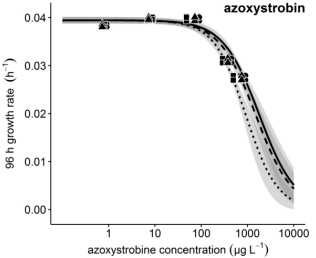

spiroxamine
96 h growth rate (h⁻¹)
0.04
0.03
0.02
0.01
0.00
0.1
1
10
100
1000
spiroxamine concentration (µg L⁻¹)

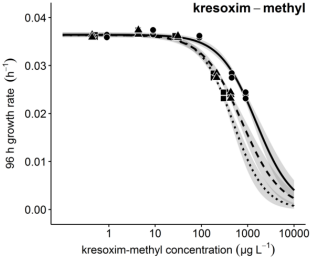

diuron
96 h growth rate (h⁻¹)
0.04
0.03
0.02
0.01
0.00
0.5 1 2 4 8 16 32 64
diuron concentration (µg L⁻¹)

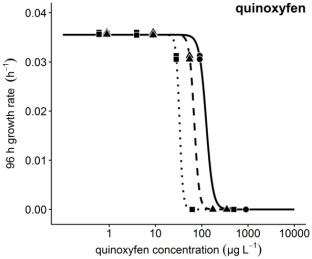

isoproturon
96 h growth rate (h⁻¹)
0.04
0.03
0.02
0.01
0.00
1 10 100 1000
isoproturon concentration (µg L⁻¹)

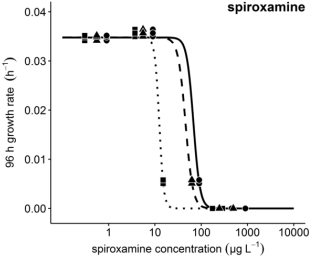

metazachlor
96 h growth rate (h⁻¹)
0.04
0.03
0.02
0.01
0.00
1 10 100 1000 10000
metazachlore concentration (µg L⁻¹)


S – metolachlor
96 h growth rate (h⁻¹)
0.04
0.03
0.02
0.01
0.00
1 10 100 1000 10000
S-metolachlor concentration (µg L⁻¹)


azoxystrobin
96 h growth rate (h⁻¹)
0.04
0.03
0.02
0.01
0.00
1 10 100 1000 10000
azoxystrobine concentration (µg L⁻¹)


kresoxim – methyl
96 h growth rate (h⁻¹)
0.04
0.03
0.02
0.01
0.00
1 10 100 1000 10000
kresoxim-methyl concentration (µg L⁻¹)


quinoxyfen
96 h growth rate (h⁻¹)
0.04
0.03
0.02
0.01
0.00
1 10 100 1000 10000
quinoxyfen concentration (µg L⁻¹)


spiroxamine
96 h growth rate (h⁻¹)
0.04
0.03
0.02
0.01
0.00
1 10 100 1000 10000
spiroxamine concentration (µg L⁻¹)

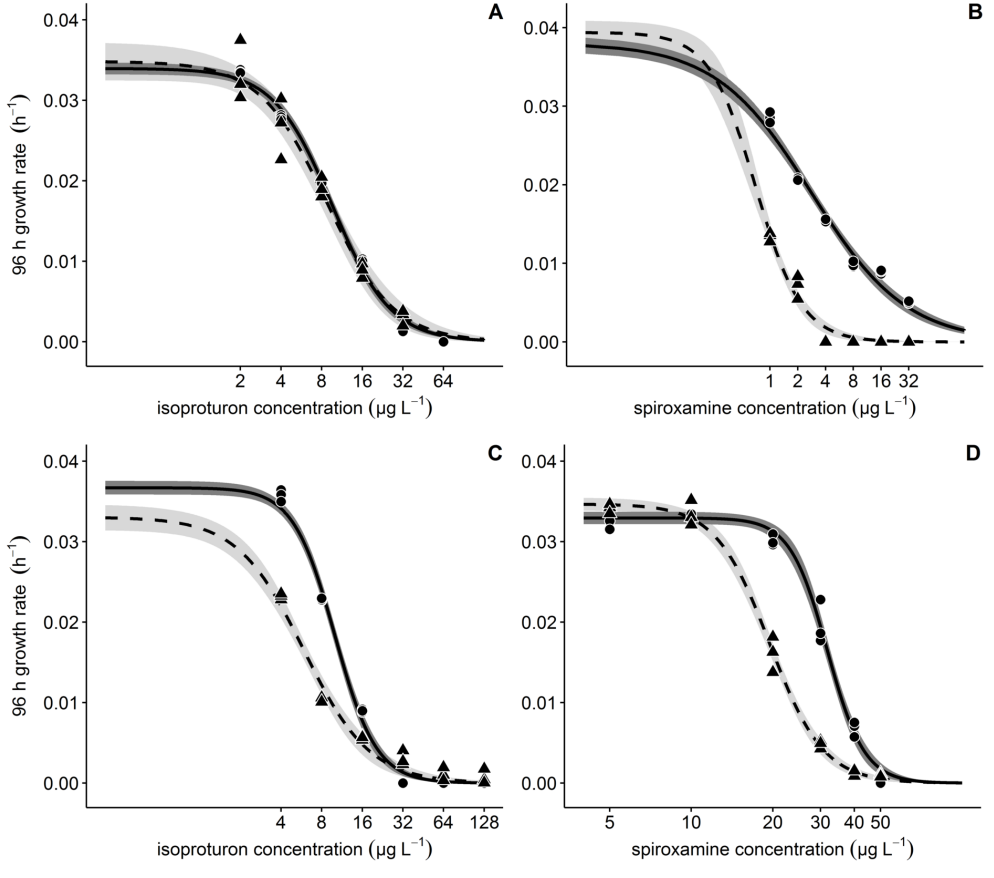

A
B
C
D
96 h growth rate (h^{-1})
0.04
0.03
0.02
0.01
0.00
2 4 8 16 32 64
isoproturon concentration (µg L^{-1})
1 2 4 8 16 32
spiroxamine concentration (µg L^{-1})
4 8 16 32 64 128
isoproturon concentration (µg L^{-1})
5 10 20 30 40 50
spiroxamine concentration (µg L^{-1})

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	sulfonlyurea	-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
	quinoxyfen	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. EC_{50} values (± 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 EC_{50} 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.

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