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Dissolved organic matter modulates the impact of herbicides on a freshwater alga: A laboratory study of a three-way interaction

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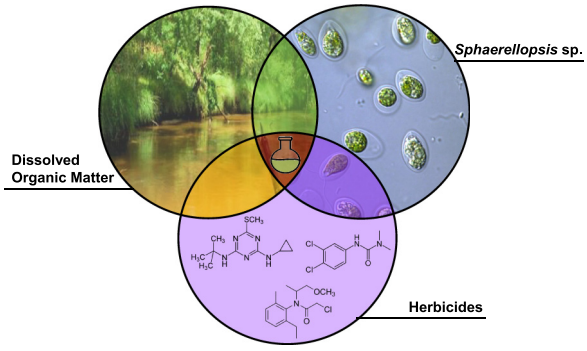
HIGHLIGHTS

- Diuron, irgarol and S-metolachlor were added to *Sphaerellopsis* sp. cultures ± DOM.
- *Sphaerellopsis* sp. growth and physiology were studied for 14 days in the laboratory.
- Herbicide and degradation product concentrations in the culture media were analysed.
- No single or combined herbicide toxicity was seen at peak growth with natural DOM.
- Diuron degradation products were found in cultures with microalgae and natural DOM.
- *Sphaerellopsis* sp. and bacteria may biodegrade herbicides under natural conditions.

GRAPHICAL ABSTRACT



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ABSTRACT

In freshwater environments, microorganisms such as microalgae are influenced by the concentrations of dissolved chemicals but can modify the fate of these substances by biosorption, accumulation and even metabolization. In this laboratory study, we assessed the growth and physiology of non-axenic cultures of the chlorophyte *Sphaerellopsis* sp. exposed to environmental concentrations of diuron, irgarol and S-metolachlor (0.5, 0.5 and 5 $\mu g \cdot L^{-1}$, respectively) singly and in mixture, in the presence or absence of natural dissolved organic matter (DOM). The growth, photosynthetic efficiency and relative intracellular lipid content of *Sphaerellopsis* sp., as were measured after 14 days of exposure, as were the concentrations of bacteria in the cultures. DOM absorbance and fluorescence, and concentrations of the herbicides and their metabolites in the culture medium were also recorded. The growth of *Sphaerellopsis* sp. was very low in the absence of DOM but dramatically enhanced in treatments where DOM was added. As a result, the toxicity of the herbicides observed in treatments without DOM was overcome in those where DOM was added. The chemical characteristics of DOM were modified by the microalgae, and the fate of the herbicides was affected by the interaction between microorganisms

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Metolachlor-ESA
Degradation

(both bacteria and algae) and the DOM. Herbicide concentrations decreased over time, with a simultaneous increase in some of their metabolites, suggesting a biological degradation in the presence of DOM.

1. Introduction

Microalgae form the foundation of aquatic food webs due to their capacity to produce organic carbon from light energy (photosynthesis) (Wetzel, 2001). They interact constantly with their environment and can be influenced by dissolved substances, including those of natural origin (e.g. autochthonous dissolved organic matter, DOM) and those introduced by human activities (e.g. herbicides). In turn, microalgae also play a key role in DOM production, which is itself likely to interact with organic contaminants such as herbicides.

Aquatic DOM is a complex and heterogeneous mixture of autochthonous organic molecules (<0.45 μm) derived from in situ photosynthetic activity and microbial processes, and allochthonous inputs rich in humic substances largely derived from terrestrial environments (Blough and Del Vecchio, 2002; Ogawa and Tanoue, 2003). DOM plays a key role in aquatic biogeochemical and ecological processes (Ahlgren and Merino, 1991; Downing et al., 2009). It has been established that DOM supports aquatic food webs (Taipale et al., 2016; McMeans et al., 2015). Karlsson et al. (2009) showed that, in the natural environment, microalgae can be negatively affected by high turbidity and/or a high concentration of coloured DOM. Conversely, these compounds can protect against UV radiation (for review see Tedetti and Sempéré, 2006). DOM also affects microalgae positively, either directly through its consumption or via benefits from bacterial extracellular enzymatic activities. Indeed, in natural environments as well as in controlled laboratory conditions, bacteria contribute to the bioavailability of various compounds necessary for microalgal development, such as vitamins (Croft et al., 2006). Windler et al. (2014) also showed the crucial importance of the bacteria present in cultures of three freshwater diatoms (*Achnanthidium minutissimum*, *Cymbella affiniformis* and *Nitzschia palea*) for cell size development and morphological integrity. Under controlled conditions, Liu et al. (2009) also showed increased growth in non-axenic cultures of the diatom *Phaeodactylum tricornutum* with the addition of diverse organic compounds such as glucose or acetate, while Campbell et al. (1997) and Millour (2011) demonstrated the ability of the freshwater chlorophyte *Chlorella pyrenoidosa* to adsorb humic substances. Moreover, microalgae themselves are involved in DOM production as both their metabolism and cell death lead to changes in DOM characteristics. In a mesocosm experiment, phytoplankton blooms were shown to enrich natural DOM with autochthonously-produced organic components due to the formation and degradation of dissolved organic carbon by microalgae (Meon and Kirchman, 2001). Other studies performed under controlled conditions (e.g. Henderson et al., 2008; Leloup et al., 2013) have demonstrated that microalgae naturally excrete compounds (such as carbohydrates or lipids) of varying nature and quantity depending on the species and phase of the cellular cycle. These secretions (namely algal organic matter, AOM), and particularly cellular decomposition by bacteria, strongly participate in DOM formation in the aquatic environment (Bertilsson and Jones, 2003).

In the natural environment, microalgae can be impacted by herbicides that enter aquatic systems by runoff, aerial transport, diffusion, drainage, etc., which has led to a generalized contamination of waters in rivers, estuaries, and coastal areas (e.g. Gonzalez-Rey et al., 2015). In southwest France, coastal rivers such as those running into Arcachon Bay are contaminated by a diverse mixture of herbicides used for crop protection, and biocides used in antifouling coatings on boats. Among these, diuron, irgarol and, especially, S-metolachlor are frequently detected in the area (Roubeix et al., 2012). Total herbicide concentrations in the Leyre River, the main river flowing into Arcachon Bay, were generally higher than 500 $ng \cdot L^{-1}$ (and sometimes exceeded 3000 $ng \cdot L^{-1}$) in water samples collected over the 2010–2014 period (REPAR, 2015). Diuron (phenylurea) and irgarol (triazine) are photosynthetic inhibitors, whereas S-metolachlor (chloroacetamide) inhibits fatty acids synthesis. These three herbicides have been shown to exert toxicity on microalgae, but not in the same concentration ranges. For example, Okamura et al. (2003) determined 72 h-EC_{50} values of 1.6 $\mu g \cdot L^{-1}$ irgarol, and 6.6 $\mu g \cdot L^{-1}$ diuron, based on the growth of the freshwater chlorophyte *Selenastrum capricornutum*, and Fairchild et al. (1998) obtained a value of around 84 $\mu g \cdot L^{-1}$ with metolachlor on the same species. Dupraz et al. (2018) determined 96 h-EC_{50} from 0.34 to 0.85 $\mu g \cdot L^{-1}$ and from 3.73 to 10.3 $\mu g \cdot L^{-1}$ for irgarol and diuron, respectively, on three marine microalgae. Synergistic impacts of irgarol and diuron were also recorded on the growth of the freshwater microalga *Selenastrum capricornutum* (Fernandez-Alba et al., 2002). In addition, Dupraz et al. (2018) demonstrated an additive effect of diuron and irgarol, tested in a mixture, on the growth of the marine microalga *Tisochrysis lutea*, and a slightly synergistic effect on *Tetraselmis suecica* and *Skeletonema marinoi*.

Microalgae can thus be indirect targets of herbicides but, in spite of such deleterious impacts on their physiology, some have shown the ability to biodegrade some of these compounds. Indeed, Bi et al. (2012) observed a decrease in growth and photosynthetic effective quantum yield in the chlorophyte *Chlamydomonas reinhardtii* exposed to increasing concentrations of isoproturon, but simultaneously recorded a decrease in isoproturon concentration in the culture medium, an increase of intracellular isoproturon concentration, and an increase in biodegradation as its concentration was increased. Likewise, Kabra et al. (2014) observed a decrease in the growth and chlorophyll *a* content of *Chlamydomonas mexicana* over time with increasing atrazine concentration (0 to 100 $\mu g \cdot L^{-1}$), but also a parallel decrease of bioaccumulation and increase in biodegradation. These capacities of microalgae to biodegrade a number of pesticides involve some well-known enzymatic systems (cytochrome P450, glutathione S-transferase, etc., Torres et al., 2008; Zablotowicz et al., 1998).

Finally, the presence and concentration of DOM may influence the fate, bioavailability, bioaccumulation and toxicity of pesticides (Suzuki and Shoji, 2020). In the natural environment, DOM can increase or decrease the abiotic degradation of pesticides (Lundqvist et al., 2012; McNeill and Canonica, 2016; Pozdnyakov et al., 2018). It can also modify their toxicity. For example, Knauer et al. (2007) observed a decrease of around 10% in photosynthesis with 5 $\mu g \cdot L^{-1}$ diuron and black carbon (a fraction of organic carbon) added to *Pseudokirchneriella subcapitata* cultures. Zhang et al. (2016) demonstrated a modulation of chlorobenzene toxicity with DOM addition (Suwannee River Natural Organic Matter, purchased by the International Humic Substances Society - IHSS) in the freshwater microalga *Chlorella pyrenoidosa*. Likewise, Suzuki and Shoji (2020) showed that the toxicity of chlorophenols to *Chlorella vulgaris* was reduced or enhanced in the presence of humic acid, depending on its concentration. However, studies exploring the influence of natural DOM, which is a major component in aquatic environments, on potential interactions between pesticides and microalgae are scarce (e.g. Nikkilä et al., 2001; Coquillé et al., 2018).

This laboratory study aimed to evaluate i) the effects of natural DOM isolated from the natural environment and three herbicides (irgarol, diuron and S-metolachlor), singly and in mixture, on the growth, photosynthetic efficiency and relative intracellular lipid content of the

freshwater chlorophyte *Sphaerellopsis* sp.; ii) the temporal changes in DOM properties and herbicide concentrations in the presence of this microalga. Based on the available literature, we hypothesized that: i) the addition of natural DOM would enhance microalgal growth and health; ii) herbicides would impact the physiology (particularly photosynthesis for diuron and irgarol, and lipid production for S-metolachlor), and ultimately the growth, of this microalga; iii) DOM addition would mitigate herbicide toxicity; and iv) the presence of microalgae would modify the chemical environment, at least in terms of the DOM properties.

2. Materials and methods

2.1. Biological material and culture conditions

The microalga *Sphaerellopsis* sp. Korshikov, 1925 (Chlorophyta) was isolated from a natural biofilm sample collected in December 2013 in the Rebec stream (southwest France), a small tributary (with limited contaminant inputs, Sandre, 2020) of the Leyre River, the principal river running into Arcachon Bay. The species was isolated by micromanipulation under an inverted microscope and cultured in the laboratory in sterile Dauta medium (Dauta, 1982), in non-axenic conditions at 17 °C in a thermostatic chamber 610 XAP (LMS LTD®, UK), with a dark:light cycle of 8:16 h (average light: 67 ± 0 $\mu mol \cdot m^{-2} \cdot s^{-1}$). The cultures were manually agitated every day to prevent biofilm formation.

Natural DOM was collected in spring 2015 in the Rebec, at the site where the microalga species was originally collected. Stream water (96 L) was pumped, filtered on 0.45-μm Teflon filter cartridges (Whatman, Polycap™ 75TF), and the DOM was concentrated ten times by reverse osmosis (TIA pilot equipped with FILMTEC™ SW30-2540 membrane) as described in Coquillé (2017). Excitation-emission matrix (EEM) fluorescence spectra of the natural DOM collected (see Section 2.4 for detailed protocol), before and after the concentration process, are provided in the Supplementary Information (Fig. SI1).

2.2. Experimental design

The experiment lasted fourteen days and was performed in 100-mL round borosilicate sterile glass flasks previously heated to 450 °C for 6 h and autoclaved for 20 min at 121 °C. These glass flasks were inoculated at 1200 $cell \cdot mL^{-1}$ using stock cultures in exponential growth phase (total volume = 60 mL), under the conditions described in Section 2.1. Ten biotic treatments were made: five without DOM (sterile Dauta medium alone, hereafter referred to as noDOM) and five with DOM added to the culture medium at twice the environmental concentration. For both noDOM and DOM cultures, treatments included a control (no herbicide) plus four herbicide treatments: diuron at 0.5 $\mu g \cdot L^{-1}$, irgarol at 0.5 $\mu g \cdot L^{-1}$, S-metolachlor at 5 $\mu g \cdot L^{-1}$ and a mixture of the three (=I0.5 + D0.5 + S5). Herbicide concentrations were selected to be environmentally realistic (REPAR, 2015).

Stock solutions (500 $mg \cdot L^{-1}$) of diuron (>98%), Irgarol Pestanal® (≥98.4%) and S-metolachlor Pestanal® (≥98.4%), purchased from Sigma-Aldrich, were prepared in pure methanol and diluted in sterile ultrapure water (intermediate solutions) before being used in the treatments. The highest methanol concentration reached in the flasks (0.0012%) was far below the maximum concentration recommended for algal bioassays (Abou-Waly, 2000), so no solvent controls were necessary. NoDOM and DOM control treatments were carried out in quadruplicate and herbicide-exposed cultures in triplicate. All herbicide and DOM combinations were also applied to sterile Dauta medium without microalgae (abiotic treatments): one replicate per treatment without DOM, and duplicates for DOM treatments. During the experiment, samples were taken for analyses of herbicides, DOM and biological parameters, as described in the following sections.

2.3. Herbicide analyses

Herbicides were analysed in all noDOM and DOM treatments (with and without microalgae and with and without herbicide/s) on the first (1–2 h after exposure began) and last days of experiment, based on filtered samples (0.45-μm polyethersulfone filter, VWR, USA). The filtered culture samples (100 μL) were diluted ten-fold using ultrapure water, and 40 μL of these diluted samples were directly analysed by liquid chromatography (1290 Infinity system, Agilent Technologies, USA) coupled to a tandem mass spectrometer (6460 triple quadrupole LC/MS system, Agilent Technologies, USA) after adding internal standards (irgarol-D9, diuron-D6 and metolachlor-D6). The separation was performed using a Kinetex C18 column and using a gradient of 5 mM ammonium acetate with 0.1% acetic acid in ultrapure water and pure methanol as mobile phases, with a flow rate of 0.5 $mL \cdot min^{-1}$. Analyses of pesticides and their metabolites (for diuron and S-metolachlor) were performed in multiple reaction monitoring mode. Quantification limits were 0.24 $ng \cdot L^{-1}$ for irgarol, 1.19 $ng \cdot L^{-1}$ for diuron, 4.43 $ng \cdot L^{-1}$ for DCPMU, 5.29 $ng \cdot L^{-1}$ for 1,2,4-DCPU, 4.26 $ng \cdot L^{-1}$ for 1,3,4-DCPU, 1.11 $ng \cdot L^{-1}$ for S-metolachlor, and 1.23 ng L^{-1}, and 3.13 $ng \cdot L^{-1}$ for metolachlor OA (oxoacetic acid) and ESA (ethanesulfonic acid).

2.4. DOM characterization

After 0.45-μm filtration, all samples were stored at 4 °C in the dark until DOM analyses. Dissolved organic carbon (DOC) concentrations were determined and optical analyses (absorbance and fluorescence) were performed. For DOC concentration measurements, 10-mL samples of filtered cultures were acidified with 2 M hydrochloric acid, then sparged for 6 min with high purity air before injection. DOC concentrations were determined using a Total Organic Carbon Analyzer (Shimadzu TOC-V CSN, Japan), calibrated with solutions of potassium hydrogen phthalate ($C_6H_4(COOK)(COOH)$) and run in non-purgeable organic carbon (NPOC) mode. The DOC concentration result is the mean of the best three to four injections in terms of coefficient of variation (<2%). At least two natural water certified reference materials (CRMs - Environment Canada purchased by ANALAB) were systematically analysed within each sample series in order to validate DOC measurements. The measured values for MISSIPI-03 River Water batch 1010 and CRANBERRY05 Lake Water batch 0411, at 6.42 ± 0.58 $mg \cdot L^{-1}$ and 3.76 ± 0.08 $mg \cdot L^{-1}$, respectively, were within the range expected for these natural water CRMs (6.31 ± 0.71 $mg \cdot L^{-1}$ and 3.60 ± 0.51 $mg \cdot L^{-1}$, respectively).

Three-millilitre samples of filtered cultures were used for absorbance measurements. All absorbance measurements were made using a 1-cm path length fused silica cell (Hellma) and a JASCO V-560 spectrophotometer (JASCO, France) equipped with deuterium and tungsten iodine lamps. The light absorbing properties of DOM were assessed by two optical indices: the specific UV absorbance ($SUVA_{254}$) and the spectral slope ratio (S_R). The $SUVA_{254}$ ($L \cdot mg \cdot C^{-1} \cdot m^{-1}$) index provides information on the aromatic character of DOM (Weishaar et al., 2003); it is calculated as the ratio between UV absorbance at 254 nm and DOC concentration ($mg \cdot L^{-1}$). The S_R parameter, used as a proxy for molecular weight, corresponds to the ratio between the spectral slopes of the 275–295 nm region and the 350–400 nm region of the absorbance spectrum (Helms et al., 2008).

Fluorescence analyses were performed on 3-ml samples of filtered cultures. The spectra were acquired using a 1-cm path length quartz cuvette (Hellma) and a Fluorolog FL3–22 fluorometer (Horiba Jobin Yvon, France) equipped with a xenon lamp (450 W) and a double monochromator at both excitation and emission sides. The EEM spectra were composed of seventeen emission spectra acquired from 260 to 700 nm (with an increment of 1 nm and an integration time of 0.5 s) with excitation wavelengths in the range of 250 to 410 nm (with an increment of 10 nm). Each spectrum obtained was corrected by subtracting an ultrapure water (Milli-Q, Millipore) blank spectrum to

eliminate water Rayleigh and Raman scattering peaks. Spectra were also corrected instrumentally as detailed in Huguet et al. (2009). EEM spectra allowed intensity values to be obtained for the observed fluorescence peaks: α (maximum excitation wavelength, λex, in the 340–370 nm region and maximum emission wavelength, λem, in the 420–480 nm region), β (λex = 310–320 nm and λem = 380–420 nm) and γ (λex = 270–280 nm and λem = 300–350 nm) (Parlanti et al., 2000). The fluorescence intensities were expressed in Raman units (RU). Two additional parameters were calculated from fluorescence EEMs: humification index (HIX) and biological index (BIX). HIX, used to characterize DOM humification in Zsolnay et al. (1999), was calculated as the ratio of the area from emission wavelength 435 to 480 nm divided by the area from 300 to 345 nm for an excitation wavelength of 250 nm. BIX (Huguet et al., 2009) is an indicator of autotrophic productivity (i.e. the recent autochthonous contribution); it was calculated as the ratio of emission intensity at 380 nm divided by emission intensity at 430 nm for an excitation wavelength of 310 nm.

2.5. Biological parameters

Culture samples were analysed on a BD-Accuri C6 flow cytometer (Becton Dickinson Accuri™) equipped with blue (488 nm) and red (640 nm) lasers. Cell density was measured in all cultures, on 300 μL sampled every weekday, fixed using glutaraldehyde (final concentration 0.25%), then frozen (−80 °C) until analysis. Cells were counted on FL1 (green fluorescence, 530/30 nm) versus FL3 (red fluorescence, >670 nm) cytograms. For each culture, the average growth rate (μ, day^{-1}) was calculated from the slope of the regression line of ln ($cell \cdot mL^{-1}$) on time (days).

The relative intracellular lipid content ($FL1_{Lipids}$) was estimated on the last day of the experiment using the green lipophilic fluorochrome $BODIPY^{505/515}$ (Life Technologies®, Carlsbad, CA, USA), following a protocol adapted from Brennan et al. (2012). Briefly, for each culture, 200-μL samples were stained (final $BODIPY^{505/515}$ concentration of 60 $\mu g \cdot L^{-1}$, 1% DMSO) and incubated at room temperature in the dark for 3 min. FL1 values of stained and unstained cells were normalized using FL1 values of 2-μm fluorescent polystyrene microspheres (Flow Check™ High Intensity Alignment Grade Particles 2.00 μm, Polysciences Inc., Warrington, PA, USA), added to all samples, using the formula:

$$FL1_{Lipids} = \frac{FL1_{\text{stained microalgae}}/FL1_{\text{stained microspheres}}}{FL1_{\text{unstained microalgae}}/FL1_{\text{unstained microspheres}}}$$

The bacterial concentrations in the cultures were determined on the last day of the experiment using the fluorochrome SYBR® Green I (Molecular Probes Inc., Eugene, OR, USA), following the protocol of Marie et al. (1997). Briefly, 200-μL samples were stained (final concentration 1×) with a working solution at 100× diluted in sterile Milli-Q water, then incubated for 15 min at room temperature in the dark. Cells were counted from FL1 vs FL4 (red fluorescence, 675/12.5 nm).

The PSII effective quantum yield (Φ'_M) was measured on the last day of the experiment by pulse-amplitude modulated (PAM) fluorescence using a PHYTO-PAM (Heinz Walz, GmbH, Germany) equipped with an emitter-detector unit (PHYTO-EDF). Measurements were performed using a homemade device for reproducible direct measurements on the bottom of the flasks, after homogenization of cultures by agitation and checking that cells were not stuck on the bottom. Final Φ' M values were obtained from the average of 10 measurements per culture.

2.6. Statistical analyses

To compare the changes over time in DOM characteristics and herbicide concentrations between treatments, the results were expressed as the difference (Δ) between the first and last days of the experiment. Type II ANOVAs were carried out to detect significant factors and interactions, with a p-value <0.05 considered as statistically significant. Changes in herbicide concentrations were analysed to determine the contribution and significance of DOM addition and/or biotic vs abiotic conditions. Differences in DOM results were tested by examining the influence of the biotic component (vs abiotic conditions) and/or herbicide exposure. Finally, biological parameters on the last day of the experiment were tested with regard to DOM addition and herbicide exposure. Statistical analyses were performed with R 3.2.2. (Ihaka and Gentleman, 1996), using the *car* package.

3. Results

3.1. Herbicide exposure

Neither diuron nor irgarol were detected in the concentrated natural DOM sample used to enrich the microalgal culture medium for the DOM treatments, but S-metolachlor was quantified, at 9 $ng \cdot L^{-1}$, as were its metabolites ESA and OA, at 417 $ng \cdot L^{-1}$ and 198 $ng \cdot L^{-1}$, respectively (data not shown). Consequently, we assessed the levels of these metabolites in all treatments, including controls, on day 0. Concentrations averaged 74 ± 13 $ng \cdot L^{-1}$ for ESA and 56 ± 3 $ng \cdot L^{-1}$ for OA (Table SI1, Fig. 1C). The dilution factor (of five) and quantification limit of 1.11 $ng \cdot L^{-1}$ explain why S-metolachlor was not detected in any of the treatments where it had not been added as part of a treatment.

On the first day of the experiment, herbicide concentrations measured in the abiotic treatments matched the nominal concentrations: 578 ± 19 $ng \cdot L^{-1}$ for diuron (Fig. 1A), 495 ± 7 $ng \cdot L^{-1}$ for irgarol (Fig. 1B), and 4735 ± 35 $ng \cdot L^{-1}$ for S-metolachlor (Fig. 1C). At the same time, concentrations in the biotic treatments were already significantly lower: −27 ± 4% for diuron, −24 ± 8% for irgarol and −32 ± 2% for S-metolachlor. After 14 days, the concentrations of all herbicides had decreased under abiotic conditions with or without DOM (−17 ± 4% for diuron, −38 ± 4% for irgarol and −9 ± 2% for S-metolachlor, Fig. 1). Temporal changes were also noted in the presence of biotic treatments, with significant time*DOM interactions ($p < 0.05$) for diuron and S-metolachlor and a marginally significant result ($p = 0.078$) for irgarol (Table 1), as DOM and nonDOM treatments differed in their evolution over the experiment. In biotic treatments under noDOM conditions, concentrations on day 14 were higher than they had been on day 0 but were in the same range as the final concentrations of in the corresponding abiotic treatments. Biotic treatments, including those with DOM, were characterized by sharp decreases in diuron and irgarol concentrations over time (−52 ± 5% for diuron, −100 ± 15% for irgarol) and a slight (−11 ± 6%) decline in S-metolachlor. At the same time, no significant rise in the concentration of S-metolachlor or diuron metabolites was found in any of the treatments apart those with DOM, where metolachlor-ESA increased (+45 ± 25%), and where DCPMU (1-(3,4-dichlorophenyl)-3-methylurea) was recorded at an average concentration of 90 ± 21 $ng \cdot L^{-1}$ at the end of the 14-day experimental period (Table 1, Fig. 1).

3.2. DOM characteristics

DOM was characterized on the first and last days of the experiment. Initial DOC concentrations and DOM characteristics under noDOM and DOM conditions are given in Table SI1 and highlight significant quantitative and qualitative differences between the media with and without DOM. Adding DOM increased DOC concentration, DOM aromaticity (shown by higher $SUVA_{254}$ index values and HIX), molecular weight (lower S_R), as well as the intensity of fluorescence peaks characteristic of humic-like substances (α fluorophore), recent production of biological material (β fluorophore and BIX) and protein-like compounds, and bacterial/microbial activity (γ fluorophore).

During the experiment, DOC concentrations increased in the biotic treatments with DOM (Table 2, $p < 0.01$) and $SUVA_{254}$ index decreased over time (Table 2). S_R values remained stable over time in the biotic conditions, except with diuron exposure with noDOM where ΔS_R was

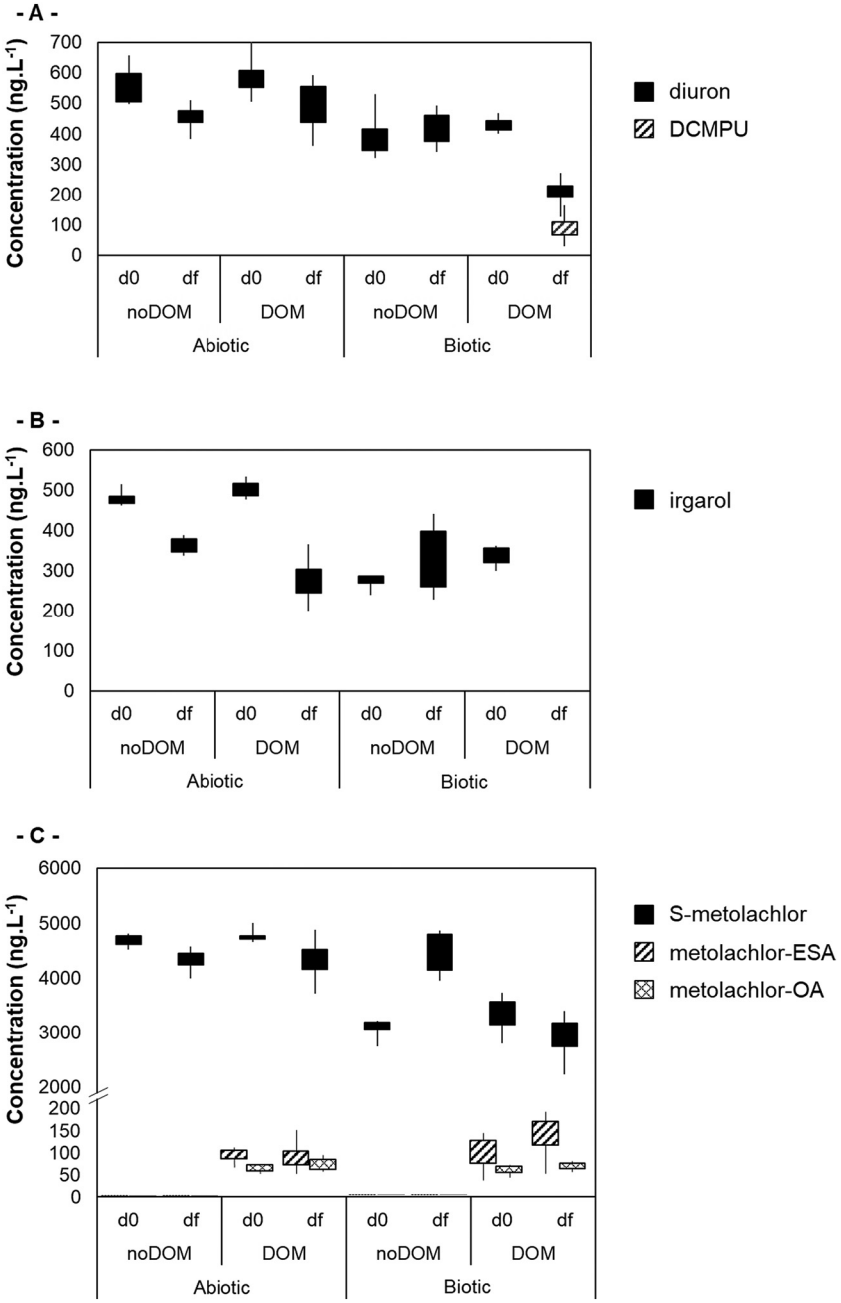

Fig. 1. Herbicide concentrations ($ng \cdot L^{-1}$) measured in the treatments with diuron (A), irgarol (B) and S-metolachlor (C), singly and in mixture, under abiotic or biotic (with microalgae) conditions and without or with DOM addition, on the first (d0) and final (df) days of the experiment.

positive (Table 2). The α fluorophore decreased in most of the DOM treatments (Table 2), while changes in the β fluorophore were impacted by both the presence of microalgae ($p < 0.01$) and that of herbicides ($p < 0.05$) (Table 2). Under herbicide exposure, positive Δβ values were observed only in the biotic treatments. The intensity of the γ fluorophore increased during the experiment in the treatments including microalgae (+924 ± 82% compared with abiotic DOM conditions; Table 2, $p < 0.05$). Neither biotic conditions nor herbicide exposure influenced the temporal changes in humification index (HIX) (Table 2), while S-metolachlor exposure significantly increased ΔBIX (biological index) over the experimental period in noDOM conditions (Table 2).

3.3. Biological parameters

Under noDOM conditions, cell concentration of *Sphaerellopsis* sp. increased from 1200 $cell \cdot mL^{-1}$ to 10,000 ± 1600 $cell \cdot mL^{-1}$ over the 14-day course of the experiment, with a growth rate (μ) of 0.14 ± 0.01 $div \cdot day^{-1}$. The photosynthetic efficiency Φ'_M measured on the last day of the experiment was 0.50 ± 0.01, and the relative intracellular lipid content ($FL1_{Lipids}$ ratio) averaged 6.93 ± 0.38. A concentration of $1.9 \pm 0.2 \times 10^6$ $bacteria \cdot mL^{-1}$ was recorded, corresponding to a bacteria/alga ratio of 165 ± 7. Without DOM addition, herbicide exposure reduced microalgal growth rate and photosynthetic efficiency. Consequently, the bacteria/alga ratio tended to increase, especially with S-metolachlor and the mixture. For most biological parameters, significant DOM and Herbicide effects ($p < 0.001$ and $p < 0.05$, respectively) were observed, except for $FL1_{Lipids}$ ratio, for which only DOM addition led to significant changes ($p < 0.001$, Table 3). Indeed, DOM addition to the culture medium induced a particularly striking increase in cell density, with final values (day 14) of $3.0 \pm 0.2 \times 10^6$ $cell \cdot mL^{-1}$, irrespective of whether the treatment included herbicide exposure. The growth rates averaged 0.55 ± 0.01 $div \cdot day^{-1}$ in the controls, compared

Table 1
Changes (Δ) in herbicide concentrations ($ng \cdot L^{-1}$) between the last and first days of the experiment. All values are mean differences (±standard error) between day 0 and the final sampling date ($n \geq 3$). *P*-values are shown for the significant factors or interactions (DOM addition and/or Bio: biotic component) based on Type II ANOVAs.

			n	ΔDiuron	ΔDCPMU	ΔIrgarol	ΔS-metolachlor	Δmetolachlor-ESA	Δmetolachlor-OA
Control	noDOM	Abiotic	1	0	0	0	0	0	0
		Biotic	4	0	0	0	0	0	0
	DOM	Abiotic	2	0	0	0	0	10	−7
		Biotic	4	0	0	0	0	37 ± 26	5 ± 16
Diuron	noDOM	Abiotic	2	−57	0				
		Biotic	3	−31 ± 45	0				
	DOM	Abiotic	4	−87 ± 42	0				
		Biotic	3	−210 ± 28	100 ± 39				
Irgarol	noDOM	Abiotic	2			−101.5			
		Biotic	3			43 ± 62			
	DOM	Abiotic	4			−221 ± 32			
		Biotic	3			−335 ± 0			
S-metolachlor	noDOM	Abiotic	2				−326		
		Biotic	3				1420 ± 367		
	DOM	Abiotic	4				−482 ± 220	34 ± 29	3 ± 2
		Biotic	3				−135 ± 178	30 ± 12	4 ± 6
Mixture	noDOM	Abiotic	2	−157	0	−132	−381	0	0
		Biotic	3	87 ± 51	0	90 ± 78	1381 ± 277	0	0
	DOM	Abiotic	4	−108 ± 62	0	−232 ± 37	−391 ± 251	−35 ± 13	16 ± 4
		Biotic	3	−235 ± 36	79 ± 31	−335 ± 0	−638 ± 429	50 ± 50	5 ± 6
Type II ANOVAs	Factor(s), p-value			Bio: $p = 0.021$	Bio*DOM: $p = 0.020$	DOM: $p = 0.0001$ Bio*DOM: p = 0.078	Bio*DOM: $p = 0.011$	DOM: $p = 0.026$	/

with the mean value for herbicide-exposed cultures of 0.57 ± 0.01 $div \cdot day^{-1}$. Growth stimulation in the presence of DOM was associated with a lower $FL1_{Lipids}$ ratio than under noDOM conditions (Table 3). The photosynthetic efficiency decreased by 20% when DOM was added to the cultures; however, no significant effect of herbicides was recorded, contrasting with the noDOM conditions where herbicide exposure halved Φ'M values (significant DOM*Herbicide interaction, $p < 0.001$, Table 3). Bacterial concentration was ten times higher in DOM conditions than in noDOM (Table 3). As a consequence of the marked growth of *Sphaerellopsis*, the bacteria/alga ratio dropped to 3 ± 0, with no significant difference between control and herbicide-exposed cultures.

4. Discussion

4.1. DOM addition modulates the toxicity of pesticides towards Sphaerellopsis sp.

Growth results in the noDOM controls suggest that cultures maintained basal metabolism rather than growing efficiently. Microalgae were positively influenced by the presence of DOM in their environment. Indeed, the remarkable stimulation of growth demonstrates that optimal development of this species requires nutrients that are not provided by the Dauta culture medium alone. Nutrient analyses on the first day (Table SI1) showed that DOM effects on growth were not due to a greater quantity of nitrates or phosphates from DOM addition. At the same time, bacteria concentrations in the cultures increased with DOM. However, the bacteria/alga cell ratio dropped, from 165 to 3 $bacteria \cdot cell^{-1}$, confirming that DOM did not introduce high amounts of bacteria or induce a major increase in the bacteria naturally inhabiting the cultures. Bacteria are known to play a role in algal growth and survival and vice versa (Amin et al., 2015; Kim et al., 2014; Windler et al., 2014; Ramanan et al., 2016). Indeed, these heterotrophic organisms degrade and transform organic matter into small molecules necessary for microalgal growth (Croft et al., 2006; Droop, 2007). Additionally, mucus secretion by *Sphaerellopsis* sp. was observed under the microscope; the release of extracellular products by algae has been shown to provide a source of carbon and energy for bacteria (Maurin et al., 1997; Romera-Castillo et al., 2011). Furthermore, several studies have demonstrated the ability of microalgae to use relatively

Table 2
Changes (Δ) in quantitative and qualitative DOM descriptors between the last and first days of experiments. All values are mean differences (±standard error) between final sampling date and day 0 ($n \geq 3$). *P*-values are shown for the significant factors (Herbi: herbicide addition and/or Bio: biotic component) based on Type II ANOVAs.

		n	ΔDOC	Absorbance indices		Fluorescence parameters				
				ΔSUVA254	ΔS_R	ΔHIX	ΔBIX	Δα	Δβ	Δγ
			($mgC^{-1} \cdot L^{-1}$)	($L \cdot mgC^{-1} \cdot m^{-1}$)				(Raman unit)		
Abiotic	noDOM	5	−3.3 ± 3.7	3.30 ± 1.89	0.75 ± 0.47	5.36 ± 3.99	0.01 ± 0.24	0.31 ± 0.21	0.02 ± 0.01	0.09 ± 0.06
	DOM	10	1.4 ± 0.3	−0.43 ± 0.10	0.00 ± 0.01	−6.46 ± 1.00	0.03 ± 0.00	−0.26 ± 0.03	−0.04 ± 0.02	0.13 ± 0.05
Biotic/control	noDOM	4	3.4 ± 2.2	0.43 ± 0.58	−2.11 ± 0.23	0.25 ± 0.10	0.12 ± 0.07	0.00 ± 0.00	0.01 ± 0.00	−0.04 ± 0.03
	DOM	4	12.1 ± 1.2	−1.51 ± 0.11	0.10 ± 0.04	−3.69 ± 0.55	0.03 ± 0.00	−0.25 ± 0.01	−0.03 ± 0.02	1.06 ± 0.23
Biotic/diuron	noDOM	3	−3.5 ± 4.4	0.36 ± 0.37	4.71 ± 0.53	0.27 ± 0.08	0.00 ± 0.03	0.01 ± 0.00	0.00 ± 0.00	0.03 ± 0.00
	DOM	3	12.7 ± 3.6	−1.70 ± 0.25	−0.02 ± 0.01	−2.46 ± 1.31	0.03 ± 0.03	0.06 ± 0.07	0.24 ± 0.02	0.84 ± 0.43
Biotic/irgarol	noDOM	3	0.3 ± 1.4	0.26 ± 0.56	−0.91 ± 0.24	0.19 ± 0.06	0.19 ± 0.05	0.01 ± 0.00	−0.01 ± 0.00	0.03 ± 0.01
	DOM	3	17.1 ± 0.2	−2.17 ± 0.02	0.10 ± 0.01	−8.61 ± 0.16	0.06 ± 0.01	−0.05 ± 0.01	0.15 ± 0.01	1.39 ± 0.10
Biotic/S-metolachlor	noDOM	3	2.0 ± 2.6	−0.43 ± 0.58	−2.61 ± 1.06	0.39 ± 0.02	0.21 ± 0.07	0.01 ± 0.00	0.00 ± 0.00	0.04 ± 0.01
	DOM	3	14.4 ± 2.0	−1.51 ± 0.11	0.10 ± 0.03	−7.27 ± 0.10	0.05 ± 0.00	−0.09 ± 0.02	0.08 ± 0.02	1.26 ± 0.06
Biotic/mixture	noDOM	3	−2.0 ± 0.8	3.11 ± 2.38	−3.60 ± 0.14	0.42 ± 0.02	0.14 ± 0.01	0.01 ± 0.00	0.00 ± 0.00	0.03 ± 0.00
	DOM	3	13.0 ± 3.1	−1.42 ± 0.17	0.08 ± 0.01	−6.15 ± 0.61	0.07 ± 0.00	−0.04 ± 0.01	0.13 ± 0.02	1.55 ± 0.30
Type II ANOVA	Factor(s), p-value		Bio: $p = 0.009$	Bio: $p = 0.049$	/	/	Herbi: $p = 0.039$	/	Bio: p = 0.001 Herbi: $p = 0.029$	Bio: $p = 0.013$

Table 3
Sphaerellopsis sp. growth rate (μ), relative intracellular lipid content ($FL1_{Lipids}$ ratio), operational yield (Φ'_M), bacterial concentration and bacteria-to-alga cell number ratio obtained on day 14 for the five treatments in presence or absence of natural DOM. All values are means (± standard error). *P*-values are shown for the significant factors (DOM addition and/or Biotic component) based on Type II ANOVAs.

		n	μ (day^{-1})	$FL1_{Lipids}$ ratio	Φ'_M	Bacteria concentration (10^6 cells·mL^{-1})	Bacteria/alga ratio
Control	noDOM	4	0.14 ± 0.01	6.93 ± 0.38	0.50 ± 0.01	1.9 ± 0.2	165 ± 7
	DOM	4	0.55 ± 0.01	4.69 ± 0.24	0.40 ± 0.01	9.7 ± 1.5	3 ± 0
Diuron	noDOM	3	0.08 ± 0.01	8.51 ± 1.09	0.24 ± 0.01	1.9 ± 0.1	206 ± 42
	DOM	3	0.54 ± 0.01	4.51 ± 0.42	0.38 ± 0.01	9.7 ± 1.1	3 ± 1
Irgarol	noDOM	3	0.14 ± 0.03	7.11 ± 0.48	0.21 ± 0.01	1.8 ± 0.1	188 ± 15
	DOM	3	0.56 ± 0.01	4.43 ± 0.23	0.37 ± 0.00	11.1 ± 0.3	4 ± 0
S-metolachlor	noDOM	3	0.07 ± 0.03	8.38 ± 0.41	0.15 ± 0.01	2.3 ± 0.3	368 ± 102
	DOM	3	0.56 ± 0.00	5.22 ± 0.48	0.40 ± 0.00	12.4 ± 0.9	3 ± 0
Mixture	noDOM	3	0.11 ± 0.01	8.30 ± 1.22	0.21 ± 0.01	2.7 ± 0.0	310 ± 40
	DOM	3	0.61 ± 0.07	4.28 ± 0.31	0.39 ± 0.00	15.3 ± 0.9	3 ± 1
Type II ANOVA	Factor(s), *p*-value		DOM: $p = 1.5 \times 10^{-9}$ Herbi: $p = 0.048$	DOM: $p = 8.3 \times 10^{-6}$	DOM*Herbi: $p = 1.7 \times 10^{-7}$	*DOM:* $p = 1.8 \times 10^{-10}$ Herbi: $p = 0.008$	*DOM:* $p = 8.3 \times 10^{-9}$ Herbi: $p = 0.0011$

simple molecules such as sugars (glucose: Liu et al., 2009), acetate (Laliberté and de la Noüe, 1993), but also more complex molecules such as humic substances (Campbell et al., 1997). More generally, our results agree with previous observations on several microalgae of different phyla exposed to different concentrations of humic substances (e.g. Pouneva, 2005; Gagnon et al., 2005), where the growth of microalgae (in non-axenic conditions) was stimulated in the presence of DOM components. However, our results do not make it possible to discriminate direct effects of DOM on microalgae via microalgal consumption of such substances from indirect ones mediated through bacterial metabolism because bacteria are able to grow on DOM.

In contrast, the decreases in relative lipid content and photosynthetic efficiency with DOM addition were related to growth results. Due to stimulation of cell division, *Sphaerellopsis* sp. cells were less likely to accumulate energy stock as lipids. Piorreck and Pohl (1984) showed that total lipid content changed over the growth cycle of microalgae, with species-dependent differences. The changes in Φ'_M are in agreement with the results of Liu et al. (2009) and Heifetz et al. (2000). These authors showed a decrease in the photosynthetic efficiency of *Phaeodactylum tricornutum* and *Chlamydomonas reinhardtii* under mixotrophic and non-axenic conditions with glucose, glycerol and acetate, whereas their growth was simultaneously boosted by these molecules. These authors observed a transitory decrease in Φ'_M, which resulted in a stimulation of the respiration caused by organic carbon consumption. Therefore, we hypothesize that the decrease of Φ'_M could also be linked to enhanced respiration, but specific measurements of O_2 consumption would be needed to prove it.

Exposure to herbicides strongly affected the photosynthetic efficiency and increased lipid storage in noDOM cultures, highlighting toxic effects. As stated above, μ values indicated that noDOM cultures grew little under suboptimal conditions, with a likely energetic cost. This may indicate high sensitivity to disturbances such as herbicide exposure. Given the literature available on the toxicity of irgarol and diuron at low concentrations, the observed effects on the growth of *Sphaerellopsis* sp., especially its photosynthesis, were expected. S-metolachlor exposure was expected to have some impact on the lipids. Although this was not observed, the physiology of the culture was affected (Φ'_M), resulting in a sharp decrease in growth rates. Contrastingly, in the presence of DOM, the herbicides did not have any deleterious effects on *Sphaerellopsis* sp. (Table 3). Instead, the growth rate increased under exposure to the mixture (0.61 ± 0.07 day^{-1}) compared with herbicide-free controls (0.55 ± 0.01 day^{-1}). An increase in bacterial concentration was also observed (15.3 ± 0.9 × 10^6 cells·mL^{-1} vs 9.7 ± 1.5 × 10^6 cells·mL^{-1} for DOM controls, Table 3). However, the bacteria per alga ratio was no different. Mean photosynthetic efficiency (all treatments included) was 0.39 ± 0.00 and the $FL1_{Lipids}$ ratio averaged 4.47 ± 0.09. In a previous study, Coquillé et al. (2018) highlighted that the ecotoxicity of these herbicides at the same concentrations increased in the presence of added DOM for the marine microalga *Tetraselmis suecica*, while it decreased for *Chaetoceros calcitrans* with natural DOM. Chlorophytes are among the microalgal taxa most sensitive to herbicides (Mohr et al., 2008; Lewis and Thursby, 2018), and the decrease in the sensitivity of *Sphaerellopsis* sp. exposed to these substances when DOM was added suggests there are interactions between this microalga, the DOM and/or the pesticides. As shown by Zhang et al. (2016), DOM can decrease the bioavailability of herbicides and consequently their toxicity. Using natural DOM from the Suwannee River (IHSS) added to the cultures, these authors found lower toxin-induced growth inhibition of *Chlorella pyrenoidosa* with highly chlorinated chlorobenzenes.

4.2. Changes in DOM in the presence of microorganisms and herbicide contamination

In the noDOM cultures, no significant variation was observed in the intensity of humic-like substances, recent biological material or related protein-like fluorophores (α, β and γ). In the control DOM cultures, γ intensity increased, with a significant effect of the biotic component (Table 2). This increase reveals the activity of microalgae and associated bacteria, i.e. consumption (direct and/or indirect) and/or biotic degradation to different types of DOM compounds. The consumption of various elements can be related to the excretion of enzymes such as proteases, polysaccharidases, and glucosidases (as observed in bacteria by Droop, 2007; De La Rocha and Passow, 2014) or adsorption of molecules such as humic substances (as observed in microalgae by Campbell et al., 1997).

Consumption and/or degradation of DOM substances by bacteria and/or microalgae, as well as the excretion of self-produced molecules, in turn, caused modifications of the chemical environment of the cells. DOC concentration increased during the experiment in noDOM control cultures and in all DOM conditions. This organic carbon production is related to microalgal and bacterial growth and especially to cell excretion. Indeed, several authors (e.g. in non-axenic cultures and mesocosms, Pivokonsky et al., 2006, Schartau et al., 2007) showed that during the exponential growth phase with very low cell mortality, DOC production was mainly due to microalgal metabolism. This organic matter, also known as algal extracellular organic matter, includes exudates and/or extracellular polymeric substances (EPS). The latter are known to be produced by several microalgae and participate in the formation of microbial aggregates (e.g. biofilms, Geesey, 1982). This excretion was also highlighted by the increase of the γ fluorescence intensity over the experiment in the cultures that grew the best, i.e. those with DOM addition. Temporal changes in the aromaticity of DOM were shown by decreasing $SUVA_{254}$ values, corroborating the findings of Henderson

et al. (2008). They observed that DOM was less aromatic and more hydrophilic during exponential growth of *Chlorella vulgaris*, *Microcystis aeruginosa* and *Asterionella formosa* cultivated in non-axenic environment, due to the increased excretion of small simple molecules (generally hydrophilic and of low molecular weight).

Herbicide exposure resulted in the increase of the fluorophore β, corresponding to a labile fraction of DOM produced from recent algal and/or bacteria activity (Parlanti et al., 2000). Positive values of ΔBIX (biological index) under irgarol, S-metolachlor and mixture exposure confirmed recent DOM production from autochthonous origin as shown by Huguet et al. (2009). These results, together with slightly higher values of Δγ, could be a sign of increased excretion by the bacteria and microalgae with herbicide exposure.

4.3. The presence of DOM and microalgae influence the fate of pesticides

The immediately lower herbicide concentrations in the biotic treatments compared with abiotic ones on day 0, with which they showed a difference of a third (Fig. 1), suggest rapid adsorption of the molecules onto cell walls and/or internalization into cells. Based on their log K_{ow} values, these molecules are considered as non-polar, lipophilic and bioaccumulative substances (Tetko et al., 2005), having a high affinity with cell wall phospholipids, lipoproteins and fatty acids (Sandermann Jr, 2003). Nestler et al. (2012) suggested that diuron could be internalized in as little as 1–2 min following its addition to cultures of the freshwater microalga *Chlamydomonas reinhardtii.* No toxicokinetic data are available in the literature to support the hypothesis of rapid biological uptake of the other two molecules. However, the return to initial herbicide concentrations in the noDOM cultures after 14 days while herbicide concentrations continued to decrease in the abiotic treatments suggests ad/absorption by cells and subsequent substance release.

Under abiotic conditions, the decline in pesticide concentrations over time in our experiment (−17 ± 4% for diuron, −38 ± 4% for irgarol and −9 ± 2% for S-metolachlor) could be directly related to abiotic processes such as losses linked to adsorption on walls of flasks and/or evaporation in the headspace of flasks and/or hydrolysis and/or photodegradation. In the case of irgarol (significant effect of DOM, see Table 1), these abiotic phenomena were possibly increased by DOM. Indeed, Sakkas et al. (2002) showed that humic substances promoted the photodegradation of irgarol in controlled conditions. Photolysis is considered to be the main pathway for metolachlor abiotic transformation (Zemolin et al., 2014), and the presence of DOM has been shown to inhibit this photolytic reaction of metolachlor (Dimou et al., 2005). However, our data did not reveal any significant difference in S-metolachlor decrease between noDOM and DOM abiotic treatments. Temporal variations in herbicide concentrations were mostly related to the presence of microorganisms and/or in combination with DOM (except for metolachlor-OA, for which no significant temporal change was found: Fig. 1, Table 1). In the presence of DOM, the concentrations of diuron, irgarol and S-metolachlor decreased markedly, while the metabolites DCPMU and metolachlor-ESA increased in the biotic treatments (Fig. 1).

While diuron concentrations increased in the noDOM cultures (see above), they fell (−52 ± 5%) in the treatments with added DOM, with the simultaneous appearance of DCPMU (not detected in the other treatments). The natural DOM added to the cultures and/or the microbial excretions (highlighted by the recent production of biological material, indicated by positive Δβ values) may have catalysed or promoted diuron photodegradation or hydrolysis (e.g. Luo et al., 2015). Alternatively, biodegradation may have occurred in the DOM cultures in the presence of microalgae and/or bacteria associated with *Sphaerellopsis* sp., as no DCPMU was measured in abiotic or noDOM treatments. This biodegradation phenomenon seems to be related to this particular species and medium (both the alga and the DOM are of freshwater origin), as DCPMU was not quantified in the noDOM conditions where high bacteria/alga ratios were found (and bacterial numbers were more than ten times lower). No DCPMU appeared in a similar experiment with other marine microalgae exposed to the same herbicide treatments in the presence of natural marine DOM (Coquillé et al., 2018). DCPMU results from the loss of a methyl group from diuron (Giacomazzi and Cochet, 2004). Biodegradation is usually performed by Gram positive and negative bacteria (Giacomazzi and Cochet, 2004), both of which cohabit with algae (Ramanan et al., 2016; Romera-Castillo et al., 2011). The photosensitizing role of algal extracellular organic matter in the activation of photolysis of organic chemicals has also been reviewed for antibiotics (Wei et al., 2021). Furthermore, scientific evidence of possible degradation of pesticides by microalgae has been found; for example, Zablotowicz et al. (1998) showed that four microalgae species were able to degrade fluometuron (phenylurea) by *N*-demethylation with cytochrome P450. However, experiments in an axenic environment and/or with other species, coupled with analyses of pesticides (diuron and its metabolites) and enzymatic activities known to participate in detoxification, would be needed to draw firm conclusions (although microalgal growth may be inferior under axenic conditions, Windler et al., 2014). Finally, we cannot exclude co-metabolic degradation as a possible explanation for the diuron degradation. Such microbial degradation mechanisms offer no energy benefits to the organism and occur in situations where another carbon source is available for growth (see review in Tran et al., 2013) through organic substrate consumption by microalgae and/or bacteria naturally inhabiting the cultures (Sutherland and Ralph, 2019). In the case of our study, we cannot draw a conclusion about this hypothesis. Nevertheless, the fact that the remaining orthophosphate concentration in the DOM cultures on the last day of the experiment was 0.15 ± 0.02 $mg \cdot L^{-1}$ (vs 10.77 ± 0.06 $mg \cdot L^{-1}$ in the noDOM cultures, data not shown) could argue for the use of alternative nutrient sources. More insight could be provided by experiments in an axenic environment, including other, 'simple' substrates such as glucose or acetate, and a comparison of their results with those obtained with more complex DOM.

Irgarol had almost disappeared from the DOM cultures by the end of the experiment. Only part of this decrease can be explained by abiotic processes (−38 ± 4%, see above), and it can be hypothesized that the decrease of the remainder (62%) was probably due to biological activity (microalgae and/or bacteria; see the marginally significant interaction between DOM and Biotic conditions in Table 1). Photodegradation could have been catalysed/promoted by the DOM together with the dissolved organic substances excreted by microorganisms, as mentioned for diuron. Indeed, our analyses of dissolved organic carbon showed an increase of DOC concentration over time in the biotic treatments exposed to irgarol (Table 2). As for diuron, another likely hypothesis is the biodegradation by bacteria (INERIS, 2012) and/or microalgae, but irgarol metabolites were not analysed in our study. To be conclusive about the organisms responsible for such degradation, experiments in both axenic and non-axenic conditions would be necessary, as mentioned for diuron. Another option would be to conduct the same experiment detailed in the present paper, but with other algal species, in order to compare pesticide losses over time. In any case, to draw conclusions about biodegradation, measurements of enzymatic activities (cytochrome P450) and concentrations of the parent-molecule and its metabolites in the cells would also be necessary.

The patterns were observed for S-metolachlor were similar to those of the other herbicides: in the presence of DOM and microalgae (significant interaction), concentrations decreased over time. Here, abiotic processes (mainly photolysis, Zemolin et al., 2014) explained a large part of the decrease. However, under these conditions, the concentrations of its ESA metabolite increased. Metolachlor is converted to metolachlor-ESA by microbial degradation mediated by glutathione-S-transferase (GST) (Huang et al., 2017). GST production is, however, a ubiquitous detoxification mechanism found in diverse kingdoms, including algae. Metolachlor biodegradation generally occurs through a co-metabolic process (Zemolin et al., 2014), but Munoz et al. (2011) demonstrated the ability of some microorganisms to use it as a carbon

source. BIX (biological index) values increased significantly over the present experiment in the cultures including DOM exposed to S-metolachlor (Table 2), highlighting recent production of biological material associated with microbial activity. The hypothesis of biodegradation by bacteria (Huang et al., 2017) and/or microalgae is supported by the increase in concentration of metolachlor-ESA, associated with non-significant variations in metolachlor-OA, suggesting specific degradation pathways. Again, complementary experiments are required to improve our understanding of S-metolachlor degradation pathways.

5. Conclusions

The aim of this laboratory study was to assess how natural DOM interacts with microalgae and their associated bacteria in the presence of herbicides. Our results showed:

- the ability of *Sphaerellopsis* sp. to use molecules in their immediate surroundings, issued from natural DOM and/or derived from its degradation by the bacteria associated with cultures, favouring an increase in their growth rate;
- significant changes in DOM characteristics caused by metabolic excretion and direct or indirect consumption by microalgae and their associated bacteria.

This study, therefore, proves that microalgae and their associated bacteria are major players in DOM modification (concentration and composition) through consumption (direct or indirect) and production. It also highlights the influence of DOM on photoautotrophs (not only heterotrophs), confirming the key role of DOM in aquatic ecosystems.

While herbicide toxicity to the freshwater microalga *Sphaerellopsis* sp. was highlighted in the absence of DOM, the present study showed no toxic effect of diuron, irgarol and S-metolachlor, tested at environmental concentrations (Fauvelle, 2012; REPAR, 2015), when DOM was added. This result may reveal a low sensitivity of this alga to the chemicals tested when growth conditions are optimal, but could also be related to changes in herbicide bioavailability in the presence of DOM. It also suggests that *Sphaerellopsis* sp. cultured in non-axenic conditions, may be able, with its associated bacteria and in the presence of natural DOM, to biodegrade herbicides in freshwater environments.

More generally, our study highlights the major role played by natural DOM in chemical and ecotoxicological interactions involving microorganisms. This component should be, therefore, taken into greater consideration in ecotoxicological assessment when integrating environmental conditions.

CRediT authorship contribution statement

Soizic Morin: Conceptualization, Methodology, Sample analysis, Writing – Final manuscript, Supervision, Project administration, Funding acquisition. **Nathalie Coquillé**: Methodology, Investigation, Sample analysis, Writing – Original draft. **Mélissa Eon**: Sample analysis. **Hélène Budzinski**: Conceptualization, Sample analysis. **Édith Parlanti**: Conceptualization, Sample analysis, Methodology, Writing – Review & Editing, Supervision, Funding acquisition. **Sabine Stachowski-Haberkorn**: Conceptualization, Sample analysis, Methodology Writing – Review & Editing, Supervision, Funding acquisition.

Declaration of competing interest

None.

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Appendix A. Supplementary data

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