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▶ To cite this version:

Betty Chaumet, Nicolas Mazzella, Julie Neury-Ormanni, Soizic Morin. Light and temperature influence on diuron bioaccumulation and toxicity in biofilms. Ecotoxicology, 2020, 29, pp.185-195. 10.1007/s10646-020-02166-8. hal-02610164

HAL Id: hal-02610164 https://hal.inrae.fr/hal-02610164

Submitted on 11 Apr 2021

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- 1 This is a post-peer-review, pre-copyedit version of an article published in <u>Ecotoxicology</u>. The final
- 2 authenticated version is available online at: <u>https://doi.org/10.1007/s10646-020-02166-8</u>.
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4 Light and temperature influence on diuron bioaccumulation and toxicity in biofilms

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8 Abstract

9 Variations of temperature and photoperiod throughout different seasons can affect aquatic 10 communities such as biofilms. Biofilms, generally present at the base of trophic chains in 11 freshwaters, are also subject to organic contamination, and are especially affected by herbicides. 12 Many studies have investigated the effect and interactions of herbicides and environmental factors 13 on biofilms, but never with a toxicokinetic point of view.

The objective of this study was to assess structural and functional changes in biofilms exposed to diuron, and to link them with contaminant accumulation, under the influence of temperature and light variations. To this aim, biofilms were exposed to all possible combinations of three concentrations (0, 5 and 50 µg.L⁻¹) of diuron, two temperatures (10 and 26°C), and two light/dark photoperiods (16/8, 10/14), for durations of 0, 1 and 3 days. Diuron accumulation in biofilms was quantified and structural descriptors (protein and polysaccharide contents, dry weight) and functional endpoints (photosynthetic and enzymatic activities) were analysed.

The results obtained mainly highlighted the influence of temperature on diuron bioaccumulation and the associated toxic impact on biofilms. Bioaccumulation in biofilms exposed during three days at 10°C, at the highest diuron concentration, was in average 1.4 times higher than bioaccumulation on biofilms exposed to 26°C. Accordingly, the photosynthetic yield was more inhibited at lower than at
higher temperatures. Temperature was also the highest impacting factor for metabolism regulation;
for example, at 26°C after three days of exposure, polysaccharide production was boosted under
both photoperiods tested.

28 Keywords

29 Freshwater periphyton, herbicide, microbial ecotoxicology, seasons, toxicokinetics-toxicodynamics

30

31 1. Introduction

32 It is well known that temperature and incident light are two of the main environmental factors able to modulate primary production (Dodds et al., 1996; Gomes & Juneau, 2017; Staehr & Sand-Jensen, 33 2006). In temperate regions, both factors change seasonally, subjecting primary production to 34 35 seasonal variations. Temperature and photoperiod influence organisms at the base of trophic chains 36 in aquatic environments such as biofilms (Flemming et al., 2007) which are composed of microalgae, 37 fungi and bacteria embedded in an EPS matrix (Aguilera et al., 2008; Flemming & Wingender, 2010). 38 The EPS matrix contains polysaccharides, which make-up around 97 % of its total constituents 39 (Aguilera et al., 2008), proteins, humic acids and DNA. Additionally, biofilm integrates and reacts 40 quickly to contamination, making it a good bioindicator (Edwards & Kjellerup, 2013).

Biofilms are increasingly constrained by environmental stresses, coupled with anthropogenic pressures such as organic pollution (DeLorenzo et al., 2001; Guasch et al., 2016; Kim Tiam et al., 2016). In fact, watercourses are among the final receptacles of pollution, including pesticide contamination brought about by runoff or leaching processes (Kohusova et al., 2011). Due to their massive use, pesticides are widely monitored in aquatic environments. This is especially true for diuron, a photosynthesis inhibitor of photosystem II herbicide (Yebra et al., 2004), considered as a priority substance in the European Water Framework Directive (Directive 2000/06/EC). Herbicides, and diuron in particular, have been shown to drastically reduce photosynthetic activity (Kim Tiam et
al., 2015; Pesce et al., 2011; Tlili et al., 2008).

50 Many studies have already tackled the biological impacts of temperature, light and herbicide 51 contamination on biofilm (e.g: Bérard et al., 1999; Larras et al., 2013; Villeneuve et al., 2010). The 52 effects of these parameters on the growth and photosynthetic efficiency of autotrophic organisms 53 are well documented. In fact, it has already been shown that an increase in temperature, or in the 54 length of the photoperiod can increase biofilm resistance to contaminants (Staehr & Sand-Jensen, 55 2006; Herzig & Dubinsky, 1992). Moreover, Duong et al. (2010) and Villeneuve et al. (2011) showed 56 that biofilm community composition could be modified according to season. Also, Wolfstein & Stal 57 (2002) highlighted a variation in EPS content associated with different temperatures and degrees of 58 irradiance exposure. Also, Chalifour et al. (2014) showed that temperature can affect the sensitivity 59 of biofilms to herbicides.

According to the literature, a biofilm exposed to high temperatures, greater irradiance (such as during summer) and herbicides would be less sensitive to toxic effects than a winter biofilm (Chalifour et al., 2014). Variations of temperature and light intensity target processes involved in photosynthesis and metabolism (Guasch et al. 2016). As these environmental factors, pesticides such as herbicides can interact on photosynthesis and metabolism process due to their mode of action (Draber et al., 1991). These combined effects may be synergistic, additional or antagonistic, but remain little known.

As herbicides, light and temperature co-occur in natural environments, we can hypothesise the interaction between all these factors may disturb the normal functioning of biofilm, and potentially more during the winter period. Consequently, it is crucial to study the fate and effect of herbicides in biofilms, and their impact, under environmental factors variations.

To answer this question, the aim of this study was to use a toxicokinetic-toxicodynamic approach to
gain a better understanding of the different steps related to the effect of diuron under contrasting

r3 seasonal conditions. Thanks to this approach, more details would be provided about the time needed
r4 for metabolism processes and variation among herbicide contamination.

The objectives of the study were to investigate i) bioaccumulation by microorganisms in the biofilm, ii) subsequent modification of photosynthetic yield, and finally, iii) overall impact on metabolism via the determination of biochemical contents, and the measurement of complementary heterotrophic enzymatic activities.

79

80 2. Materials and methods

To reach the objectives arising from the problematic of this study, an experimental design was set up
in which a mature biofilm was exposed to a combination of two different temperatures (10 and 26°C)
and photoperiods (light/dark: 16/8 and 10/14), and to three diuron concentrations (0, 5 and 50 µg.L⁻
¹).

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86 2.1. Experimental design

During January-February 2017, bare glass slides (L: 26 cm, l: 6 cm) were placed in Gazinet-Cestas pond (South-West France, geographical coordinates: $44^{\circ}46'30.1''N$, $0^{\circ}41'44.3''W$). They were collected after four weeks of immersion, the time needed for biofilm to colonize the slides. During the colonization period, water temperature was 8.6 ± 0.5 °C (n= 9), and daily hours of sunlight numbered 10h09 ± 6min (n= 50).

The collected biofilm was immediately re-immersed in artificial channels (L: 50 cm, l: 9.2 cm , H: 5 cm) containing recirculating dauta medium (Dauta, 1982) at a pH of 7.5-8, with a flow velocity of 2 cm.s⁻¹, in a thermoregulated room. Two temperatures were tested, with room temperature set at 26°C and 10°C, and for each temperature, two light/dark photoperiods were applied: 16/8 and

96 10/14, to mimic four contrasting "seasonal conditions" (named "Seasons" 1 to 4) as presented in Figure 1. The four experiments were performed independently, starting from Season 1 (S1) on 97 February 13th, to Season 4 (S4), ending on March 9th, with one experiment per week during this 98 99 period. A diuron (CAS: 330-54-1, purity: 99 %, Dr Ehrenstorfer GmbH) stock solution was prepared in acetonitrile (CAS: 75-05-8, purity: 99.97 %, Biosolve) to reach a final concentration of 200 mg.L⁻¹. The 100 101 toxicity of acetonitrile was tested on biofilms prior to the experiment. The NOEC was established at 102 1%, based on the measurement of photosynthesis inhibition (Neury-Ormanni, unpublished data). All 103 exposure conditions were prepared to ensure that the final concentration of acetonitrile reached 104 0.25%, which is below the established NOEC. For each condition, three nominal concentrations of diuron were selected: 0, 5 and 50 µg.L⁻¹ (respectively named Ctrl, 5D and 50D, prepared from stock 105 106 solution in ultrapure water), and the biofilms were exposed to these conditions during one and three 107 days, to ensure that the equilibrium between diuron concentrations in water and in biofilms was 108 reached (Chaumet et al., 2019). Moreover, the 3-day exposure was expected to be enough to 109 highlight likely effects of photoperiod (16/8 photoperiod condition has 37 % more hours of 110 illumination than 10/14 photoperiod condition).



Figure 1: Graphical representation of experimental design and abbreviations associated with eachcondition

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116 **2.2. Biofilm preparation**

117 At each collection time (T₀, one day and three days) for each condition (S1, S2, S3 and S4 for all the 118 diuron exposure: Ctrl, 5D and 50D), four replicates from the same channel were sampled and 119 prepared as described below. Biofilms were scrapped with a razor blade and introduced into a 120 beaker previously filled with 40 mL of dauta medium. Biofilm directly collected after colonisation in 121 the pond (i.e. without exposure in the channels) was also analysed in the same way as those after one and three days of exposure and was named T₀. All samples were homogenized by agitation at 122 room temperature for 30 minutes at 150 rpm (Variomag multi-station agitator, Thermo-Scientific). 123 Several analyses were then performed on biofilms in suspension: determination of dry weight (2.3.2), 124

polysaccharide (2.4.1) and protein content (2.4.2), enzymatic (2.4.3) and photosynthetic activities
(2.4.4), and pesticide quantification (2.3.4).

127

128 **2.3.** Sample processing and physicochemical analyses

129 **2.3.1.** Experimental conditions

Three mL of water from each channel were collected at each sampling time (T_0 , one day and three days) for all conditions (S1, S2, S3 and S4) and exposure conditions (the control and the two contaminated); and filtered on syringe-filters (pore: 0.45 µm, hydrophilic regenerated cellulose membrane, Whatman), in order to quantify pesticide content in the medium. A volume of 10 µL of internal standards was added to 990 µL of the filtrate prior to analysis (see 2.3.4). The solution of internal standards contained Diuron D6 (CAS: 1007536-67-5 – purity: 98 %, provided by Dr. Ehrenstorfer GmbH) prepared at 10 mg.L⁻¹.

137 The temperature in the channels and in the thermoregulated room was measured by data loggers 138 (Tinytag, Radiospare) every ten minutes for each condition. A light meter (LI-COR Li-250, USA) was 139 used to control incident light intensity reaching the slides. The data are listed in Table 1.

140 Table 1: Mean (± standard error) temperatures (n= 475) and light intensity (n=8) during the 141 experiments

Conditions	Water temperature (°C)	Room temperature (°C)	Light intensity (µmol.s ⁻¹ .m ⁻²)
Season 1	na*	na*	
Season 2	22.70 ± 0.59	25.16 ± 0.01	
			7.47 ± 0.42
Season 3	11.60 ± 0.18	11.23 ± 0.00	
Season 4	11.65 ± 0.15	11.17 ± 0.00	

na*: data not available. A technical problem was encountered for the recording of Season 1
temperature data, but the temperature settings of the thermoregulated room were identical
between Season 1 and Season 2.

145

146 **2.3.2.** Dry weight

For dry weight measurement, 8.8 mL of the 40 mL of suspended biofilm were filtered on a Buchner with previously-weighed ashed GF/F filters (Ø 90 mm, pore size: 0.7 μ m, Whatman). Then, filters were frozen for 12 hours, lyophilised, and re-weighed. Dry weight data were used to standardize bioaccumulation data, as described in 2.3.4.

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2.3.3. Biofilm sample preparation for diuron analysis

153 Diuron was extracted from biofilm matrices using Accelerated Solvent Extraction (ASE 2000, Dionex, France). A solution of surrogates was prepared with Monuron d6 (CAS: 217488-65-8) at a final 154 concentration of 10 mg.L⁻¹, Prometryn d6 (CAS: 1705649-52-0) at 1 mg.L⁻¹ and Simazine d5 (CAS: 155 220621-41-0) at 20 mg.L⁻¹, all provided by Dr. Ehrenstorfer GmbH. The ASE cells were filled as 156 follows: the GF/F filter containing the lyophilized biofilm, Fontainebleau sand, and 10 µL of 157 158 surrogates (to control the percentage of recovery) were introduced into the ASE device. The extraction was performed at 70 bars and 100°C, with acetone (CAS: 67-64-1, purity: 99.5 %, Chem-159 160 Lab) as the solvent. Once the extraction complete, acetone samples were evaporated under nitrogen 161 flux with a Visiprep[™] (Supelco) and the extracted pesticides were dissolved in 10 mL of ultrapure 162 water (Synergy UV, Millipore).

163 Samples were purified by Solid Phase Extraction (SPE) on HR-X Chromabond cartridges (Macherey-164 Nagel, Germany) previously conditioned with 5 mL of methanol (CAS: 67-56-1, purity: 99.95 %, Biosolve) and 5 mL of ultrapure water. Samples were percolated through the cartridge, then the cartridge was rinsed with 20 % - 80 % methanol (CAS: 67-56-1, purity: 99.95 %) -ultrapure water buffer, and dried in a vacuum with a VisiprepTM for 30 minutes. The cartridges were eluted with 5 mL of acetonitrile (CAS: 75-05-8, purity: 99.97 %), and 10 μ L of internal standard were added to the eluate collected. The mixture was evaporated until dry in a SpeedVac (Thermo Fisher Scientific, France), and the extracted diuron was dissolved into 1 mL of ultrapure water before analysis (2.3.4).

171

172 **2.3.4.** Diuron quantification in water and biofilm

173 Diuron and its main metabolites (DCPMU and DCPU) in biofilms and water samples were quantified 174 by UPLC-ToF (Ultra-Performance Liquid Chromatography – Time of Flight Mass Spectrometry, Xevo 175 G2-S ToF, Waters) as proceeded in a previous study (see Chaumet et al., 2019). An ACQUITY BEH C18 176 column 50 \times 2.1 mm, 1.7 μ m (Waters) was used for chromatographic separation at 45°C with a binary gradient of ultrapure water (at pH= 5) and methanol (both solutions with ammonium acetate to a 177 178 concentration of 10 mM, CAS: 631-61-8, ULC quality Biosolve), and 20 µL of samples were injected. 179 The gradient was performed with the following range: 98 % of ultra-pure water solution (0–3.6 min), 2 % of methanol solution (3.6–6 min) at a flow rate of 0.45 mL.min⁻¹. 180

Data were processed with MassLynx V4.1 software, and diuron concentrations were inferred via a calibration curve from 0 to 100 μ g.L⁻¹, comprising 10 points. Every 10 samples, 3 quality controls were carried out at 0, 1 and 10 μ g.L⁻¹ on the calibration curve. For biofilm samples, data were normalized by dry weight and then expressed in μ g.g⁻¹. Limits of quantification were established at 5 μ g.L⁻¹ for water samples and at 0.05 μ g.mg⁻¹ for biofilm samples.

186

187 **2.4. Biofilm characterization**

188 **2.4.1.** Polysaccharide analysis

Polysaccharide content was analysed according to the method outlined by Dubois et al. 189 (1956). Aliquots of 500 µL of samples were prepared by adding 12.5 µL of phenol (CAS: 108-95-2, 190 purity: 99 %, Scharlab) and 2.5 mL of sulfuric acid (CAS: 7664-93-9, purity: 95 %, VWR). Samples were 191 192 incubated at room temperature for 10 minutes, and then 20 minutes in a water bath at 30°C. Absorbance was read with a microplate reader (Synergy [™] HT, Biotek, USA) at 485 nm. Absorbance 193 data were converted into μg glucose-equivalent per cm² using a standard curve of D(+) – glucose 194 anhydrous solution (CAS: 50-99-7, ACS Reagent Grade, Scharlab) from 0 to 750 µg.L⁻¹. Concentrations 195 196 were expressed in percentage of T_0 values for each condition, to account for variability of the 197 inoculums used.

198

199 2.4.2. Protein dosage

200 A protocol adapted to low concentrations of proteins was developed based on the methodology 201 presented by Bradford (1976). An aliquot of 500 µL of the suspended biofilm was collected and 4.5 202 mL of methanol were added, then the mixture was sonicated for 20 minutes. Samples were then 203 evaporated close to dryness with a SpeedVac (Thermo Fisher Scientific, France) and filled again with 204 500 µL of ultrapure water and 2.5 mL of Bradford reagent, prepared using 3.3 mg of Brilliant blue G 205 (CAS: 6104-58-1, Sigma), 33.3 mL of methanol, 66.67 mL of phosphoric acid (CAS: 7664-38-2, purity: 206 85 %, Chem-Lab) and 900 mL of ultra-pure water. Samples were gently agitated to homogenize the color thus formed. Absorbance was measured using a microplate reader (Synergy[™] HT, Biotek, USA) 207 at 595 nm. Protein content was determined using a standard curve from 0 to 250 µg.L⁻¹ performed 208 209 with Bovine Serum Albumine (BSA, CAS: 9048-46-8, Sigma). Data obtained in µg of BSA-equivalent 210 per cm^2 were then expressed in percentage of T_0 values for each condition.

211

212 **2.4.3.** Enzymatic activity measurement

213 Two extracellular enzymatic activities related to the heterotrophic component of the biofilm were measured: the β-glucosaminidase (EC 3.2.1.30) and the β-glucosidase (EC 3.2.1.21) activities, carried-214 215 out according to the methodology outlined in Romaní et al. (2008). The two substrates, 4-216 methylumbelliferyl N-acetyl-β-D-glucosaminide (CAS: 37067-30-04, Sigma) and 4-methylumbelliferyl-217 β -D-glucopyranoside (CAS: 18997-57-4, Sigma), respectively associated to β -glucosaminidase and β glucosidase, were prepared at a final concentration of 0.3 mmol.L⁻¹ (corresponding to the saturation 218 219 concentration of the substrate (Romanı et al., 2004). A 150 µL volume of substrate was added to 150 220 µL of sample aliquot, away from light. Samples were incubated under agitation (80 rpm, Orbitron, 221 Infors HT) during one hour in the dark. The reaction was stopped with 5 mL of glycine and NaOH buffer, made with 803.5 mL of NaOH (CAS: 1310-73-2, purity: 98 %, Sigma-Aldrich) solution at 8 g.L⁻¹ 222 223 and 196.5 mL of a glycine (CAS: 17-1323-01, purity: 99.7 %, GE-healthcare - Life sciences) solution at 75.04 g.L⁻¹. Fluorescence was measured with a microplate reader (Synergy[™] HT, Biotek, USA) at 224 excitation-emission wavelengths of 365-455 nm. A standard curve was performed with 7-Hydroxy-4-225 226 methylcoumarin (MUF, CAS: 90-33-5, purity: 97 %, Acros Organics) from 0 to 50000 µg MUF.L⁻¹.h⁻¹ 227 and was used to calculate the percentage of enzymatic activities in comparison with T₀ values for 228 each condition.

229

230

2.4.4. Photosynthetic activity measurement

231 Photosynthetic activity (effective photosystem II quantum yield, Φ_{PSII}) of all samples was assessed by 232 chlorophyll *a* fluorescence measurement with a Pulse Amplitude Modulated fluorimeter (PhytoPAM, 233 Heinz Walz GmbH, Germany) in quartz cuvettes (Emitter-Detector Unit PHYTO-ED). Five replicate 234 measurements per sample were performed on biofilm suspensions after 15 minutes of light 235 adaptation. Every sample was measured with a Photosynthetic Active Radiation of 164 µmol.m⁻².s⁻¹ in 236 Actinic Light mode.

238 2.5. Data analysis

Data obtained were not normal and different conditions tested were independent, consequently, 239 240 non-parametric tests were applied. The aim of the statistical analysis was to highlight if they were 241 significant differences between conditions tested like between the two temperatures, or the two 242 photoperiods, or the three exposure concentrations, or time exposure. A Kruskal-Wallis test followed by a posthoc kruskalmc test [Package pgirmess version 3.6.1] were performed on data from all the 243 244 analyses (in percentage of T_0), and then on each biological descriptor individually. A p-value of 0.05 245 was considered significant. A linear discriminant analysis was applied on the same dataset (in percentage of T_0) with R software [Package *ade4* version 1.6-2], with the aim of individualising data 246 247 into similarly-composed groups.

248

249 3. Results

3.1. Seasonal condition effects on control samples

251 The dry weight of the biofilm samples measured immediately after collection from the pond (T_0) for 252 each season showed high variations. Indeed, biomass regularly increased between S1 and S4, with values 6-fold higher at S4 than S1 (DW_{S1}= 33 \pm 5 μ g.cm⁻² and DW_{S4}= 203 \pm 27 μ g.cm⁻², Table 2) 253 254 highlighting that biofilm colonisation depends on the period of the year (meteorological parameters) 255 in temperate settings. It was observed that the biomass in the inoculum can vary greatly from one 256 sample to another. Therefore, the results obtained for the structural and functional descriptors were 257 standardized by the associated T₀, thus avoiding biomass bias. The samples could then be compared 258 with each other at the level of absolute variations observed after exposure. Variations between dates 259 and diuron exposure treatments were expressed as percentages of T₀, to highlight only the effect of 260 temperature, photoperiod and diuron. Biofilm dry weight generally increased over the three days of 261 channel exposure, and the 3-day increase in biomass was little more pronounced at 26°C. In fact, for season 1 and 2, the growth rate was of 0.68 and 0.69 μ g.cm⁻².h⁻¹ respectively (2.5 times increase in 3 262

days for S1 and 1.9 times greater for S2, Table 2) and of 0.4 and -0.4 for season 3 and 4 respectively
(1.4 times increase in 3 days for S3, Table 2). Even, a slight decrease in dry weight was visible after
three days of exposure for S4. On the other hand, in terms of photosynthetic yield of the control
samples, there were no marked effects of temperature nor of photoperiod, whatever the exposure
time.

268 Protein dosage and β -glucosidase activity measurement showed an increase in percentage at three 269 days compared to T₀ (Table SI 1 and Figure 2). The same trend was visible for polysaccharide results 270 and β -glucosaminidase activity measurements, but only for biofilms exposed to 26°C. For these 271 descriptors, samples exposed to 10°C did not highlight any particular variation (around 100 % T₀).



Figure 2: Polysaccharide and protein contents, and β-Glucosaminidase and β-Glucosidase activities (mean±sd, n=4) in percentage of T_0 (grey line: 100% of T_0 value for each condition). The conditions

are differentiated as follows: Season 1 in red, Season 2 in yellow, Season 3 in green, and Season 4 in
blue.

Generally, for all descriptors, results highlighted an evolution between T₀, one day and three days of 277 278 exposure, with results slightly more marked after three days. For data collected after three days of 279 exposure, the temperature effect was noticeable for all descriptors (Table SI 1 and Figure 2). 280 Nevertheless, data were not statistically different but they allow to highlight a trend because all 281 showed an increase of percentage in comparison to T₀ for samples exposed to 26°C. There were 282 almost no differences between conditions for β-glucosaminidase activity among the different 283 photoperiods. However, β -glucosidase activity was slightly affected by the most extended 284 photoperiod independently of the temperature after three days of exposure. Indeed, we observed an 285 increase of this enzymatic activity with the longer photoperiod (e.g. S1 was 3.8 times higher than S2 286 and S3 1.6 greater than S4, Figure 2, Table SI1). The same trend was visible for polysaccharide 287 percentage, but more moderately, also for protein percentage but only between S3 and S4.

Table 2: Mean (±sd) dry weight in μ g.cm⁻² and photosynthetic yield in Ctrl biofilms. * indicates significant differences within the conditions (Kruscalmc test)

Conditions	Exposure time	-2	
(temperature, photoperiod)	(day)	Mean dry weight (µg.cm ⁻)	Photosynthetic yield
Concer 1	0	33±5	0.41±0.02
Season 1	1	25+6	0 48+0 02
(26°C, 16/8)	Ţ	55±0	0.4810.02
	3	83±10	0.49±0.02
Concer 2	0	57±10	0.44±0.02
Season 2	1	65+11	0 51+0 02
(26°C, 10/14)	T	0511	0.51±0.05
	3	106±18	0.51±0.01

	0	79±10	0.50±0.01
Season 3			
	1	87±11	0.55±0.01*
(10°C, 16/8)			
	3	107±13	0.54±0.01*
	0	203±27	0.53±0.01
Season 4			
	1	139±27	0.51±0.01
(10°C, 10/14)			
	3	175±34	0.51±0.01

291

About biofilm characterization, temperature and photoperiod had an influence on structural biofilm parameters. For biofilms exposed to the highest temperature (26°C), polysaccharide content increased between day 1 and day 3 (Figure 2). This effect was considerably less marked in biofilms exposed at a temperature of 10°C than at 26°C. In general, the polysaccharide percentage from control treatments increased in the following order: S4 < S3 < S2 < S1. This result resembles that observed for other descriptors, like protein content and enzymatic activities. At equal temperature, polysaccharide concentrations were higher with a longer photoperiod (S2 < S1, S4 < S3).

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300 3.2. From bioaccumulation to toxic impact

Diuron concentration in the water channels was quantified at 5.1 ± 0.2 (n= 4) and $43.9 \pm 0.4 \mu g.L^{-1}$ (n= 4) of diuron, respectively, for 5D and 50D treatments which is extremely close to nominal concentrations. The results from control water channels and metabolite concentrations in exposed channels (for water and biofilms samples) were below the limit of quantification. No statistical difference in diuron exposure for each treatment was found between conditions or dates of sampling. The concentrations of the main metabolites of diuron were below the limits of quantification A small amount of diuron was quantified in biofilm samples from control conditions (mean= $88.4 \pm$ 19.9 µg.g⁻¹ of diuron) which could originate from the environment during colonisation. The total amount of diuron measured within biofilms exposed to 10°C was globally higher (but not significantly) than within those exposed to 26°C (Figure 3). This observation was more noticeable for 50D treatments. For 50D, at a given temperature of exposure, bioaccumulation tended to be higher when light exposure time increased.

In general, the results presented in Figure 2 did not show very marked trends in terms of the effect of diuron on functional and structural descriptors. However, although there was no significant difference to confirm these observations, it would appear that for all descriptors, after 3 days of exposure, there was an increase with exposure concentration for biofilms exposed to 10°C, and a slight decrease (proteins, polysaccharides, β -glucosidase activity), or a stability (β -glucosaminidase activity) for biofilms exposed to 26°C.







323 the biofilm in $\mu g.g^{-1}$ (mean±sd), for biofilms exposed in channels during one and three days. The

324 conditions are differentiated as follows: Season 1 in red, Season 2 in yellow, Season 3 in green, and
325 Season 4 in blue. The black line represents the linear regression performed on the whole dataset.
326 The size of the points is proportional to the dose of exposure (small = Ctrl, middle = 5D, large = 50D).

327 The effects of temperature on toxicity were not as clear as on bioaccumulation. Indeed, the 328 photosynthetic yield was lower at 10°C for the 50D treatment and at 26°C for the 5D treatment 329 (Figure 3, also see Table SI2 in the Supplementary Material). However, at 10°C the photosynthetic 330 yield tended to decrease with the luminosity amplitude (S4 < S3), and at 26°C, the contrary was 331 observable, but the photosynthetic yields were quite close between S1 and S2. Photosynthetic 332 activity was negatively correlated with diuron bioaccumulation in the biofilm (R^2 = 0.684, p-value < 333 0.001; Figure 3), correlation coefficients calculated for each condition separately increased as follow: 334 S1 (R²= 0.48, p-value< 0.001) <S2 (R²= 0.52, p-value< 0.001) <S3 (R²= 0.87, p-value< 0.001) <S4 (R²= 0.94, p-value< 0.001) (see Figure SI 1). 335

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3.3. Influence of environmental variation on metabolism

The Kruskalmc test performed on data from all analyses did not show significant differences according to temperature, photoperiod or diuron effect. It was subsequently performed with data from each descriptor, and there were no significant trends with regard to the different environmental exposure conditions and contaminations. The linear discriminant analysis carried out with structural descriptors (proteins, polysaccharides) and enzymatic activities, after three days of exposure, all treatments combined (as diuron had no effect on these descriptors), discriminated the different conditions.

Seasons 1 and 2 were separated along axis 1 (Figure 4), which was mostly explained by an increasing
relative activity of β-glucosidase ("BetaGlu"; mean of three days for all treatments S1= 706 % and S2=
215 %). In fact, conditions were discriminated along this axis in relation to the β-glucosidase activity

as follows: S2 < S4 < S3 < S1. Thus, axis 1 highlighted photoperiod influence on biological responses, mainly related to the amplitude of variation in β -glucosidase activity over three days. Season 2 was also characterized by a slightly higher relative protein content and β -glucosaminidase activity, and Season 1 by slightly higher polysaccharide content and β -glucosidase activity. In contrast to the conditions presenting higher temperatures, Seasons 3 and 4 were anticorrelated with Seasons 1 and 2 along axis 2, due to their slightly lower polysaccharide content. Axis 2 discriminated seasons according to temperature as follows: S2 < S1 < S3 < S4.

Additionally, the fact that conditions sharing a seasonal characteristic (equal temperature or photoperiod) were not fully identical suggests an interaction between the influence of temperature and photoperiod. These results highlight the influence of photoperiod and temperature on biofilm metabolism and illustrate the previous conclusions that these factors influenced the variation over three days in polysaccharide and protein content, as well as in β -glucosaminidase activity over three days (section 3.1).



Figure 4: Linear discriminant analysis of the different conditions after three days of exposure (left panel) based on polysaccharide and protein content, and extracellular enzymatic activities (right panel).

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369 4. Discussion

370 4.1. Diuron impact on PSII

371 The results show that diuron bioaccumulation in biofilm was higher at 10°C than at 26°C (e.g. 27 % 372 more at 10°C) except for S1 with 5D treatment, and consequently, the photosynthetic activity was 373 more inhibited at 10°C for associated samples, at equal photoperiods (Figure 3). Toxic effects 374 observed in this study are in accordance with many others carried out on similar microorganisms 375 (green algae, diatoms) with pesticides. For example, Tasmin et al. (2014) found a 12-17 % increase of 376 photosynthesis inhibition on microalgae with a decrease in temperature from 25 to 10°C for a contamination at 32 µg.L⁻¹ of diuron during three days. These observations are in accordance with 377 our results: an increase of 16 % of photosynthesis inhibition with a decrease in temperature. 378 379 Moreover, Chalifour et al. (2014) measured a photosynthetic yield decrease from 57 to 98 % 380 between 25 and 15°C in green algae exposed to 2.5 μM of norflurazon (a herbicide). Although the 381 literature dealing with combined herbicide and temperature impacts on photosynthetic yield is 382 abundant, there is very little information on bioaccumulation aspects. This work complements the 383 existing knowledge by showing that higher PSII inhibition at lower temperatures is likely explained by 384 higher amounts of accumulated diuron.

385

386

4.2. Metabolism variations with temperature and light

387 Environmental modulation effect

388 Heterotrophic organisms were not accurately identified, however, the choice of the dosage of the 389 two enzymatic activities allowed to provide information on the activity of the heterotrophic 390 component of the biofilm. Indeed, β -glucosaminidase plays a role in the degradation of peptidoglycans (a component of the bacterial wall) and chitin (a polysaccharide derived from fungal 391 392 walls), and β -glucosidase is involved in carbohydrate degradation. In our study, the linear 393 discriminant analysis highlighted that β -glucosidase activity was boosted by longer periods of light, 394 and the contrary for β -glucosaminidase activity, both at higher temperatures. This means that the 395 metabolism of organisms such as bacteria and fungi were intensively activated at 26°C, indicating a 396 favourable environment for them (Lurling et al., 2012).

For autotrophic organisms, photosynthetic activity is directly related to metabolism, itself dependent on temperature (Brown et al., 2004). In this study, polysaccharide quantities produced after three days of exposure were greater at 26°C than at 10°C, suggesting that the biofilm was less productive at colder temperatures. These results are in accordance with those of Wolfstein and Stal (2002) who found a slight increase (around 1.25 - 1.3 times higher) in EPS content (composed of polysaccharides and proteins) after three and nine days of exposure between biofilm exposed from 10°C to 25°C, with a photoperiod of 12/12.

404

405 Interaction of environmental factors effects with the impact of diuron

The effects of environmental factors interacting with diuron were not clearly visible and not significant on heterotrophic organisms. In fact, as diuron is a photosynthetic inhibitor herbicide, it should have a greater influence on autotrophic compartment. Results for β -glucosaminidase activity showed a slight decrease at 10°C and a stability at 26°C after three days of exposure between controls and the most contaminated condition. In contrary β -glucosidase activity was marked by a small increase. Indeed, diuron is a photosynthetic inhibitor herbicide, so it does not directly target bacteria and fungi over such short periods of exposure as strongly as microalgae and their 413 photosynthetic capacity. Because of the very short duration of biofilm exposure (three days), the 414 toxic effect of these factors may not have reached the whole community through cascading effects 415 (Proia et al., 2012). The same phenomenon was found by Proia et al. (2011), who observed that after 416 two days of exposure to diuron at 15 μ g.L⁻¹, the bacteria showed no structural nor functional 417 changes. However, following this exposure and during one week of recovery, they observed indirect 418 effects such as a transient increase in bacterial mortality.

However, this study, combining toxicokinetic and toxicodynamic approaches, showed the evolution of the effects of environmental factors and diuron on biofilm metabolism over time. Although the data showed little difference over a three-day period exposure, they still highlight an evolution and a dynamic system, suggesting a greater impact on longer-term exposure in the natural environment.

Although our results showed no significant differences between conditions, the effect of diuron on algae seemed to be modulated by temperature and light to variable extents, depending on the concentration of exposure. Nevertheless, the results showed that among all the descriptors analysed, the only part of the metabolism significantly impacted by diuron was photosynthetic activity. Contrastingly, polysaccharide and protein production and enzymatic activities were mainly affected by the interaction between temperature and photoperiod (Figure 2).

429 Overall, biofilm appeared to be less impacted and more productive at 26°C than at 10°C. This can be 430 explained by the fact that the biofilm originated from conditions close to Season 4 (10°C, light/dark 431 cycle of 10/14). A priori, the biofilms having experienced the highest degree of disturbance should be 432 the ones from S1, with a drastic change in environmental conditions (+6h of light/day and +16°C). As 433 conditions in S1 seem to be favourable to the activation of metabolism, this could explain the high 434 polysaccharide and protein production under the conditions of this treatment. That means that even 435 if the change of environmental conditions can affect the biofilms (e.g. from around 10°C in the pond 436 to 26°C in the channels), it appears that this case the increase of temperature and photoperiod 437 duration is not the most impacting external stress like cold temperature and dark/light cycle438 reduced.

439 Indeed, in the case of stressful conditions, organisms can slow their metabolism in order to reduce 440 their energy needs (Brown et al., 2004). The results of Chalifour et al. (2014) support this fact by 441 showing that algae use their energy reserves to maintain a growth rate similar to controls in the presence of herbicide (2.5 µM norflurazon) and over 4 days, they observed a strong decrease in 442 443 photosynthetic efficiency. Our results present the same kind of phenomenon for contaminated 444 conditions: at 26°C polysaccharide production was boosted, while at 10 °C, production remained 445 stable. Also, photosynthetic efficiency was greater under 26°C exposure. All this shows that biofilm 446 exposed to low temperatures and a shorter photoperiod is forced to restrict the energy allocated for 447 its growth, hence the lower metabolic activity of the biofilm during Season 4 and its greater 448 sensitivity to diuron.

All the variations observed in this study on biofilm show only trends and not significant differences, which begs the question of what the major changes would be that would take place over longer exposure times, and at the level of community composition.

452

453 **5.** Conclusion

This study focused on the influence of interactions between diuron exposure, temperature and photoperiod on pesticide bioaccumulation and toxic impact on biofilm. Our results suggest some influence of temperature on bioaccumulation, and consequently on photosynthetic activity. They imply that Winter biofilms in temperate regions are more sensitive to diuron exposure than Summer ones.

459 Our results agree with the existing literature dealing with the joint influence of temperature and 460 herbicides on biofilm vulnerability. However, this study also demonstrates the influence of

461	photoperiod duration on several biofilm endpoints, not only related to the autotrophic component of
462	the community. Moreover, this work goes further by coupling toxicokinetic and toxicodynamic
463	aspects, allowing to link the bioaccumulation of pesticides in biofilms with their metabolism.

464 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 Programme, within the Cluster of Excellence COTE (ANR-10-LABX-45).

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469 Conflict of interest
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The authors declare that the research was conducted in the absence of any commercial or financialrelationships that could be construed as a potential conflict of interest.

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