Light and temperature influence on diuron bioaccumulation and toxicity in biofilms

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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.

14   The objective of this study was to assess structural and functional changes in biofilms exposed to

15   diuron, and to link them with contaminant accumulation, under the influence of temperature and

16   light variations. To this aim, biofilms were exposed to all possible combinations of three

17   concentrations (0, 5 and 50 µg.L^{-1}) of diuron, two temperatures (10 and 26°C), and two light/dark

18   photoperiods (16/8, 10/14), for durations of 0, 1 and 3 days. Diuron accumulation in biofilms was

19   quantified and structural descriptors (protein and polysaccharide contents, dry weight) and

20   functional endpoints (photosynthetic and enzymatic activities) were analysed.

21   The results obtained mainly highlighted the influence of temperature on diuron bioaccumulation and

22   the associated toxic impact on biofilms. Bioaccumulation in biofilms exposed during three days at

23   10°C, at the highest diuron concentration, was in average 1.4 times higher than bioaccumulation on

24   biofilms exposed to 26°C. Accordingly, the photosynthetic yield was more inhibited at lower than at

25   higher temperatures. Temperature was also the highest impacting factor for metabolism regulation;

26   for example, at 26°C after three days of exposure, polysaccharide production was boosted under

27   both photoperiods tested.

30

31   **1. Introduction**

32   It is well known that temperature and incident light are two of the main environmental factors able

33   to modulate primary production (Dodds et al., 1996; Gomes & Juneau, 2017; Staehr & Sand-Jensen,

34   2006). In temperate regions, both factors change seasonally, subjecting primary production to

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).

41   Biofilms are increasingly constrained by environmental stresses, coupled with anthropogenic

42   pressures such as organic pollution (DeLorenzo et al., 2001; Guasch et al., 2016; Kim Tiam et al.,

43   2016). In fact, watercourses are among the final receptacles of pollution, including pesticide

44   contamination brought about by runoff or leaching processes (Kohusova et al., 2011). Due to their

45   massive use, pesticides are widely monitored in aquatic environments. This is especially true for

46   diuron, a photosynthesis inhibitor of photosystem II herbicide (Yebra et al., 2004), considered as a

47   priority substance in the European Water Framework Directive (Directive 2000/06/EC). Herbicides,

48 and diuron in particular, have been shown to drastically reduce photosynthetic activity (Kim Tiam et

49 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.

60 According to the literature, a biofilm exposed to high temperatures, greater irradiance (such as

61 during summer) and herbicides would be less sensitive to toxic effects than a winter biofilm

62 (Chalifour et al., 2014). Variations of temperature and light intensity target processes involved in

63 photosynthesis and metabolism (Guasch et al. 2016). As these environmental factors, pesticides such

64 as herbicides can interact on photosynthesis and metabolism process due to their mode of action

65 (Draber et al., 1991). These combined effects may be synergistic, additional or antagonistic, but

66 remain little known.

67 As herbicides, light and temperature co-occur in natural environments, we can hypothesise the

68 interaction between all these factors may disturb the normal functioning of biofilm, and potentially

69 more during the winter period. Consequently, it is crucial to study the fate and effect of herbicides in

70 biofilms, and their impact, under environmental factors variations.

71 To answer this question, the aim of this study was to use a toxicokinetic-toxicodynamic approach to

72 gain a better understanding of the different steps related to the effect of diuron under contrasting

73   seasonal conditions. Thanks to this approach, more details would be provided about the time needed

74   for metabolism processes and variation among herbicide contamination.

75   The objectives of the study were to investigate i) bioaccumulation by microorganisms in the biofilm,

76   ii) subsequent modification of photosynthetic yield, and finally, iii) overall impact on metabolism via

77   the determination of biochemical contents, and the measurement of complementary heterotrophic

78   enzymatic activities.

79

80   **2.   Materials and methods**

81   To reach the objectives arising from the problematic of this study, an experimental design was set up

82   in which a mature biofilm was exposed to a combination of two different temperatures (10 and 26°C)

83   and photoperiods (light/dark: 16/8 and 10/14), and to three diuron concentrations (0, 5 and 50 µg.L^{-}

84   ^{1}).

85

86   **2.1. Experimental design**

87   During January-February 2017, bare glass slides (L: 26 cm, l: 6 cm) were placed in Gazinet-Cestas

88   pond (South-West France, geographical coordinates: 44°46'30.1"N, 0°41'44.3"W). They were

89   collected after four weeks of immersion, the time needed for biofilm to colonize the slides. During

90   the colonization period, water temperature was 8.6 ± 0.5 °C (n= 9), and daily hours of sunlight

91   numbered 10h09 ± 6min (n= 50).

92   The collected biofilm was immediately re-immersed in artificial channels (L: 50 cm, l: 9.2 cm , H:

93   5 cm) containing recirculating dauta medium (Dauta, 1982) at a pH of 7.5-8, with a flow velocity of 2

94   cm.s^{-1}, in a thermoregulated room. Two temperatures were tested, with room temperature set at

95   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

97   Figure 1. The four experiments were performed independently, starting from Season 1 (S1) on

98   February 13th, to Season 4 (S4), ending on March 9th, with one experiment per week during this

99   period. A diuron (CAS: 330-54-1, purity: 99 %, Dr Ehrenstorfer GmbH) stock solution was prepared in

100   acetonitrile (CAS: 75-05-8, purity: 99.97 %, Biosolve) to reach a final concentration of 200 mg.L^{-1}. The

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

105   diuron were selected: 0, 5 and 50 µg.L^{-1} (respectively named Ctrl, 5D and 50D, prepared from stock

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
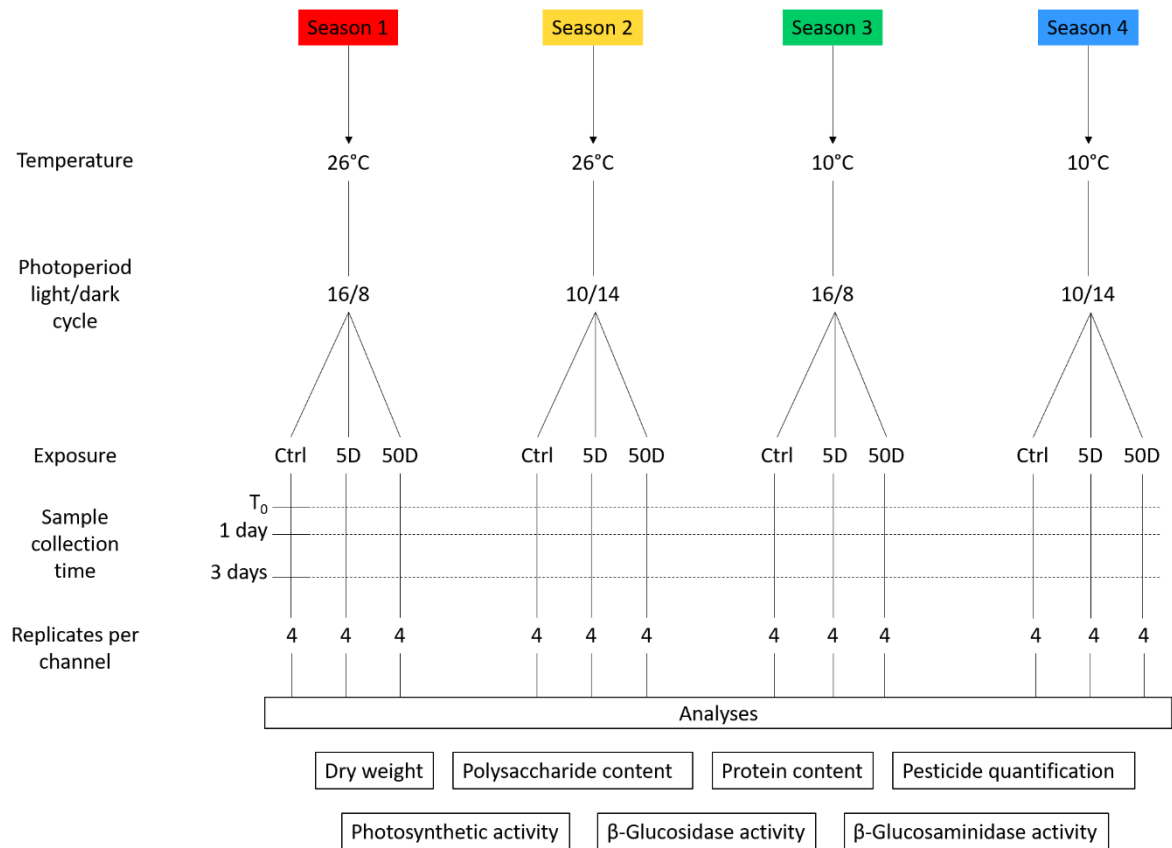
110   illumination than 10/14 photoperiod condition).

111

112

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

114   condition

115

2.2. Biofilm preparation

At each collection time (T_0, one day and three days) for each condition (S1, S2, S3 and S4 for all the

diuron exposure: Ctrl, 5D and 50D), four replicates from the same channel were sampled and

prepared as described below. Biofilms were scrapped with a razor blade and introduced into a

beaker previously filled with 40 mL of dauta medium. Biofilm directly collected after colonisation in

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 T_0. All samples were homogenized by agitation at

room temperature for 30 minutes at 150 rpm (Variomag multi-station agitator, Thermo-Scientific).

Several analyses were then performed on biofilms in suspension: determination of dry weight (2.3.2),

125   polysaccharide (2.4.1) and protein content (2.4.2), enzymatic (2.4.3) and photosynthetic activities

126   (2.4.4), and pesticide quantification (2.3.4).

127

2.3. Sample processing and physicochemical analyses

2.3.1. Experimental conditions

130   Three mL of water from each channel were collected at each sampling time (T_0, one day and three

131   days) for all conditions (S1, S2, S3 and S4) and exposure conditions (the control and the two

132   contaminated); and filtered on syringe-filters (pore: 0.45 µm, hydrophilic regenerated cellulose

133   membrane, Whatman), in order to quantify pesticide content in the medium. A volume of 10 µL of

134   internal standards was added to 990 µL of the filtrate prior to analysis (see 2.3.4). The solution of

135   internal standards contained Diuron D6 (CAS: 1007536-67-5 − purity: 98 %, provided by Dr.

136   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	

142 na*: data not available. A technical problem was encountered for the recording of Season 1

143 temperature data, but the temperature settings of the thermoregulated room were identical

144 between Season 1 and Season 2.

145

146   ### 2.3.2. Dry weight

147 For dry weight measurement, 8.8 mL of the 40 mL of suspended biofilm were filtered on a Buchner

148 with previously-weighed ashed GF/F filters (Ø 90 mm, pore size: 0.7 µm, Whatman). Then, filters

149 were frozen for 12 hours, lyophilised, and re-weighed. Dry weight data were used to standardize

150 bioaccumulation data, as described in 2.3.4.

151

152   ### 2.3.3. Biofilm sample preparation for diuron analysis

153 Diuron was extracted from biofilm matrices using Accelerated Solvent Extraction (ASE 2000, Dionex,

154 France). A solution of surrogates was prepared with Monuron d6 (CAS: 217488-65-8) at a final

155 concentration of 10 mg.L^{-1}, Prometryn d6 (CAS: 1705649-52-0) at 1 mg.L^{-1} and Simazine d5 (CAS:

156 220621-41-0) at 20 mg.L^{-1}, all provided by Dr. Ehrenstorfer GmbH. The ASE cells were filled as

157 follows: the GF/F filter containing the lyophilized biofilm, Fontainebleau sand, and 10 µL of

158 surrogates (to control the percentage of recovery) were introduced into the ASE device. The

159 extraction was performed at 70 bars and 100°C, with acetone (CAS: 67-64-1, purity: 99.5 %, Chem-

160 Lab) as the solvent. Once the extraction complete, acetone samples were evaporated under nitrogen

161 flux with a Visiprep^{TM} (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 %,

165 Biosolve) and 5 mL of ultrapure water. Samples were percolated through the cartridge, then the

166 cartridge was rinsed with 20 % - 80 % methanol (CAS: 67-56-1, purity: 99.95 %) -ultrapure water

167 buffer, and dried in a vacuum with a Visiprep^{TM} for 30 minutes. The cartridges were eluted with 5 mL

168 of acetonitrile (CAS: 75-05-8, purity: 99.97 %), and 10 μL of internal standard were added to the

169 eluate collected. The mixture was evaporated until dry in a SpeedVac (Thermo Fisher Scientific,

170 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 × 2.1 mm, 1.7 μm (Waters) was used for chromatographic separation at 45°C with a binary

177 gradient of ultrapure water (at pH= 5) and methanol (both solutions with ammonium acetate to a

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),

180 2 % of methanol solution (3.6–6 min) at a flow rate of 0.45 mL.min^{-1}.

181 Data were processed with MassLynx V4.1 software, and diuron concentrations were inferred via a

182 calibration curve from 0 to 100 μg.L^{-1}, comprising 10 points. Every 10 samples, 3 quality controls

183 were carried out at 0, 1 and 10 μg.L^{-1} on the calibration curve. For biofilm samples, data were

184 normalized by dry weight and then expressed in μg.g^{-1}. Limits of quantification were established at 5

185 μg.L^{-1} for water samples and at 0.05 μg.mg^{-1} for biofilm samples.

186

187 2.4. Biofilm characterization
188 2.4.1. Polysaccharide analysis

189 Polysaccharide content was analysed according to the method outlined by Dubois et al.

190 (1956).Aliquots of 500 µL of samples were prepared by adding 12.5 µL of phenol (CAS: 108-95-2,

191 purity: 99 %, Scharlab) and 2.5 mL of sulfuric acid (CAS: 7664-93-9, purity: 95 %, VWR). Samples were

192 incubated at room temperature for 10 minutes, and then 20 minutes in a water bath at 30°C.

193 Absorbance was read with a microplate reader (Synergy ^{TM} HT, Biotek, USA) at 485 nm. Absorbance

194 data were converted into µg glucose-equivalent per cm² using a standard curve of D(+) – glucose

195 anhydrous solution (CAS: 50-99-7, ACS Reagent Grade, Scharlab) from 0 to 750 $\mu g.L^{-1}$. Concentrations

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

207 color thus formed. Absorbance was measured using a microplate reader (Synergy^{TM} HT, Biotek, USA)

208 at 595 nm. Protein content was determined using a standard curve from 0 to 250 $\mu g.L^{-1}$ performed

209 with Bovine Serum Albumine (BSA, CAS: 9048-46-8, Sigma). Data obtained in µg of BSA-equivalent

210 per cm² 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

214 measured: the β-glucosaminidase (EC 3.2.1.30) and the β-glucosidase (EC 3.2.1.21) activities, carried-

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-

218 glucosidase, were prepared at a final concentration of 0.3 mmol.L^{-1} (corresponding to the saturation

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

222 buffer, made with 803.5 mL of NaOH (CAS: 1310-73-2, purity: 98 %, Sigma-Aldrich) solution at 8 g.L^{-1}

223 and 196.5 mL of a glycine (CAS: 17-1323-01, purity: 99.7 %, GE-healthcare - Life sciences) solution at

224 75.04 g.L^{-1}. Fluorescence was measured with a microplate reader (Synergy^{TM} HT, Biotek, USA) at

225 excitation-emission wavelengths of 365-455 nm. A standard curve was performed with 7-Hydroxy-4-

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 T_0 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.

237

2.5. Data analysis

Data obtained were not normal and different conditions tested were independent, consequently, non-parametric tests were applied. The aim of the statistical analysis was to highlight if they were significant differences between conditions tested like between the two temperatures, or the two 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 analyses (in percentage of T_0), and then on each biological descriptor individually. A p-value of 0.05 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 into similarly-composed groups.

3. Results

3.1. Seasonal condition effects on control samples

The dry weight of the biofilm samples measured immediately after collection from the pond (T_0) for each season showed high variations. Indeed, biomass regularly increased between S1 and S4, with values 6-fold higher at S4 than S1 ($DW_{S1}= 33 \pm 5$ $\mu g.cm^{-2}$ and $DW_{S4}= 203 \pm 27$ $\mu g.cm^{-2}$, Table 2) highlighting that biofilm colonisation depends on the period of the year (meteorological parameters) in temperate settings. It was observed that the biomass in the inoculum can vary greatly from one sample to another. Therefore, the results obtained for the structural and functional descriptors were standardized by the associated T_0, thus avoiding biomass bias. The samples could then be compared with each other at the level of absolute variations observed after exposure. Variations between dates and diuron exposure treatments were expressed as percentages of T_0, to highlight only the effect of temperature, photoperiod and diuron. Biofilm dry weight generally increased over the three days of 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 $\mu g.cm^{-2}.h^{-1}$ respectively (2.5 times increase in 3

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.

Protein dosage and β-glucosidase activity measurement showed an increase in percentage at three days compared to T_0 (Table SI 1 and Figure 2). The same trend was visible for polysaccharide results and β-glucosaminidase activity measurements, but only for biofilms exposed to 26°C. For these descriptors, samples exposed to 10°C did not highlight any particular variation (around 100 % T_0).
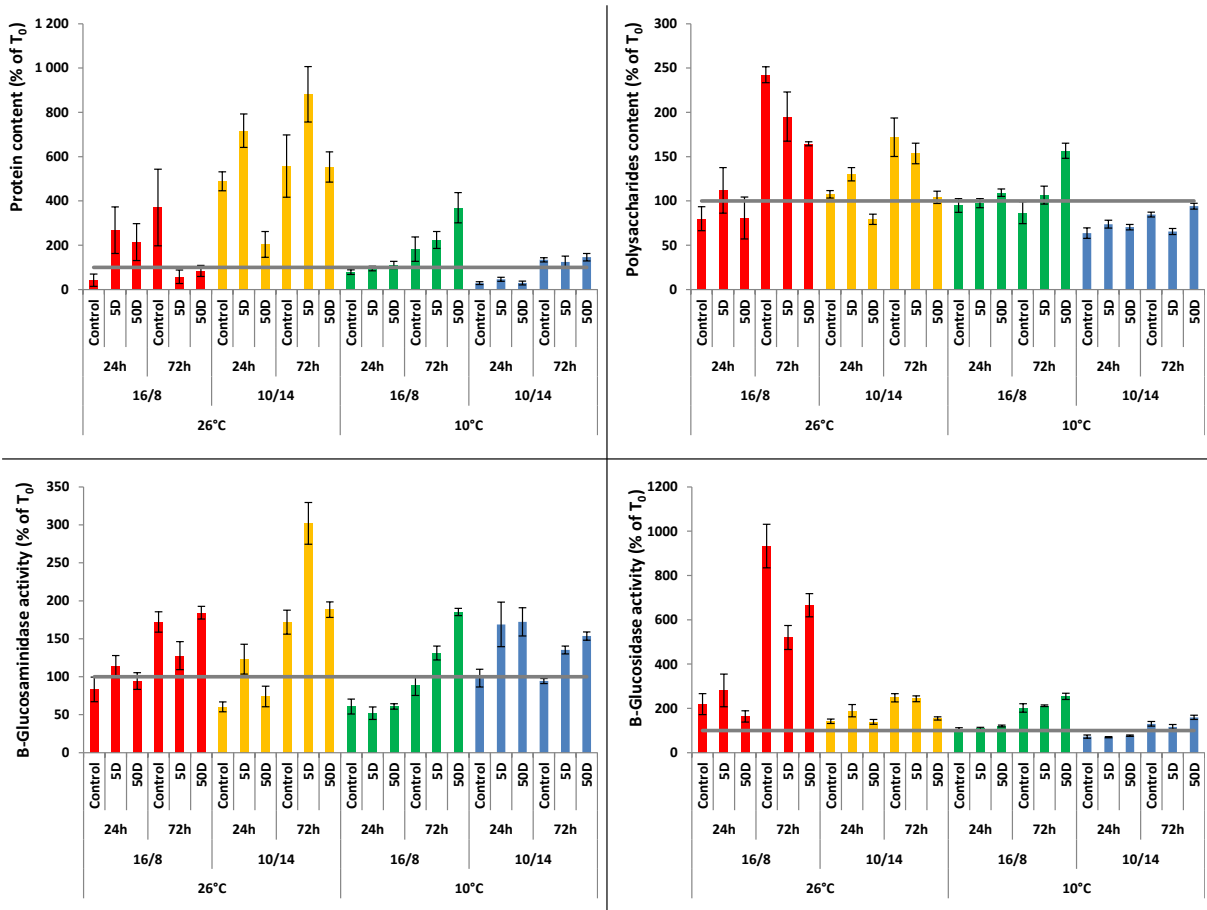

Figure 2: Polysaccharide and protein contents, and β-Glucosaminidase and β-Glucosidase activities (mean±sd, n=4) in percentage of T_0 (grey line: 100% of T_0 value for each condition). The conditions

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

276   blue.

277   Generally, for all descriptors, results highlighted an evolution between T_0, one day and three days of

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 T_0 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.

288

289   Table 2: Mean (±sd) dry weight in $\mu g.cm^{-2}$ and photosynthetic yield in Ctrl biofilms. * indicates

290   significant differences within the conditions (Kruscalmc test)

Conditions (temperature, photoperiod)	Exposure time (day)	Mean dry weight ($\mu g.cm^{-2}$)	Photosynthetic yield
Season 1 (26°C, 16/8)	0	33±5	0.41±0.02
	1	35±6	0.48±0.02
	3	83±10	0.49±0.02
Season 2 (26°C, 10/14)	0	57±10	0.44±0.02
	1	65±11	0.51±0.03
	3	106±18	0.51±0.01

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

291

292 About biofilm characterization, temperature and photoperiod had an influence on structural biofilm

293 parameters. For biofilms exposed to the highest temperature (26°C), polysaccharide content

294 increased between day 1 and day 3 (Figure 2). This effect was considerably less marked in biofilms

295 exposed at a temperature of 10°C than at 26°C. In general, the polysaccharide percentage from

296 control treatments increased in the following order: S4 < S3 < S2 < S1. This result resembles that

297 observed for other descriptors, like protein content and enzymatic activities. At equal temperature,

298 polysaccharide concentrations were higher with a longer photoperiod (S2 < S1, S4 < S3).

299

300 **3.2. From bioaccumulation to toxic impact**

301 Diuron concentration in the water channels was quantified at 5.1 ± 0.2 (n= 4) and 43.9 ± 0.4 $\mu g.L^{-1}$ (n=

302 4) of diuron, respectively, for 5D and 50D treatments which is extremely close to nominal

303 concentrations. The results from control water channels and metabolite concentrations in exposed

304 channels (for water and biofilms samples) were below the limit of quantification. No statistical

305 difference in diuron exposure for each treatment was found between conditions or dates of

306 sampling. The concentrations of the main metabolites of diuron were below the limits of

307 quantification

308   A small amount of diuron was quantified in biofilm samples from control conditions (mean= 88.4 ±

309   19.9 µg.g^{-1} of diuron) which could originate from the environment during colonisation. The total

310   amount of diuron measured within biofilms exposed to 10°C was globally higher (but not

311   significantly) than within those exposed to 26°C (Figure 3). This observation was more noticeable for

312   50D treatments. For 50D, at a given temperature of exposure, bioaccumulation tended to be higher

313   when light exposure time increased.

314   In general, the results presented in Figure 2 did not show very marked trends in terms of the effect of

315   diuron on functional and structural descriptors. However, although there was no significant

316   difference to confirm these observations, it would appear that for all descriptors, after 3 days of

317   exposure, there was an increase with exposure concentration for biofilms exposed to 10°C, and a

318   slight decrease (proteins, polysaccharides, β-glucosidase activity), or a stability (β-glucosaminidase

319   activity) for biofilms exposed to 26°C.

320

321
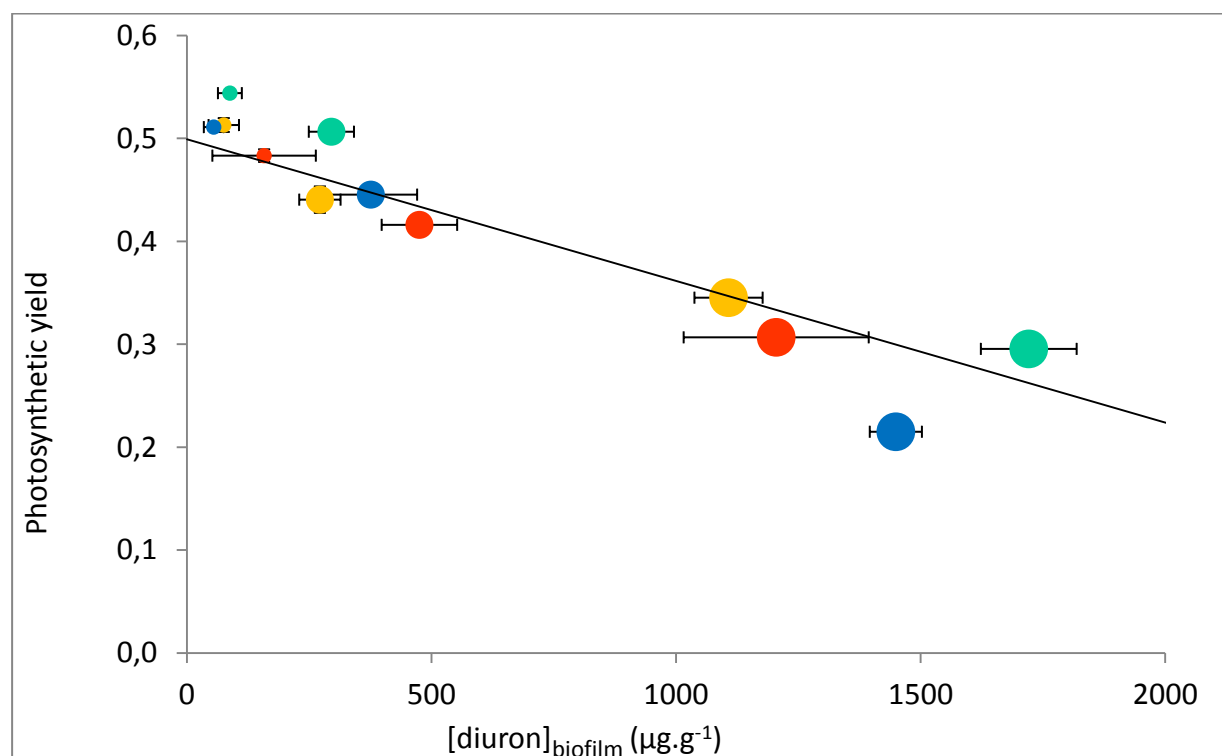322   Figure 3: Mean photosynthetic quantum yield (±sd, n=4) as a function of diuron bioaccumulation in

323   the biofilm in µg.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 (R^2= 0.48, p-value< 0.001) <S2 (R^2= 0.52, p-value< 0.001) <S3 (R^2= 0.87, p-value< 0.001) <S4 (R^2=

335   0.94, p-value< 0.001) (see Figure SI 1).

336

337   **3.3. Influence of environmental variation on metabolism**

338   The Kruskalmc test performed on data from all analyses did not show significant differences

339   according to temperature, photoperiod or diuron effect. It was subsequently performed with data

340   from each descriptor, and there were no significant trends with regard to the different

341   environmental exposure conditions and contaminations. The linear discriminant analysis carried out

342   with structural descriptors (proteins, polysaccharides) and enzymatic activities, after three days of

343   exposure, all treatments combined (as diuron had no effect on these descriptors), discriminated the

344   different conditions.

345   Seasons 1 and 2 were separated along axis 1 (Figure 4), which was mostly explained by an increasing

346   relative activity of β-glucosidase ("BetaGlu"; mean of three days for all treatments S1= 706 % and S2=

347   215 %). In fact, conditions were discriminated along this axis in relation to the β-glucosidase activity

348 as follows: S2 < S4 < S3 < S1. Thus, axis 1 highlighted photoperiod influence on biological responses,

349 mainly related to the amplitude of variation in β-glucosidase activity over three days. Season 2 was

350 also characterized by a slightly higher relative protein content and β-glucosaminidase activity, and

351 Season 1 by slightly higher polysaccharide content and β-glucosidase activity. In contrast to the

352 conditions presenting higher temperatures, Seasons 3 and 4 were anticorrelated with Seasons 1 and

353 2 along axis 2, due to their slightly lower polysaccharide content. Axis 2 discriminated seasons

354 according to temperature as follows: S2 < S1 < S3 < S4.

355 Additionally, the fact that conditions sharing a seasonal characteristic (equal temperature or

356 photoperiod) were not fully identical suggests an interaction between the influence of temperature

357 and photoperiod. These results highlight the influence of photoperiod and temperature on biofilm

358 metabolism and illustrate the previous conclusions that these factors influenced the variation over

359 three days in polysaccharide and protein content, as well as in β-glucosaminidase activity over three
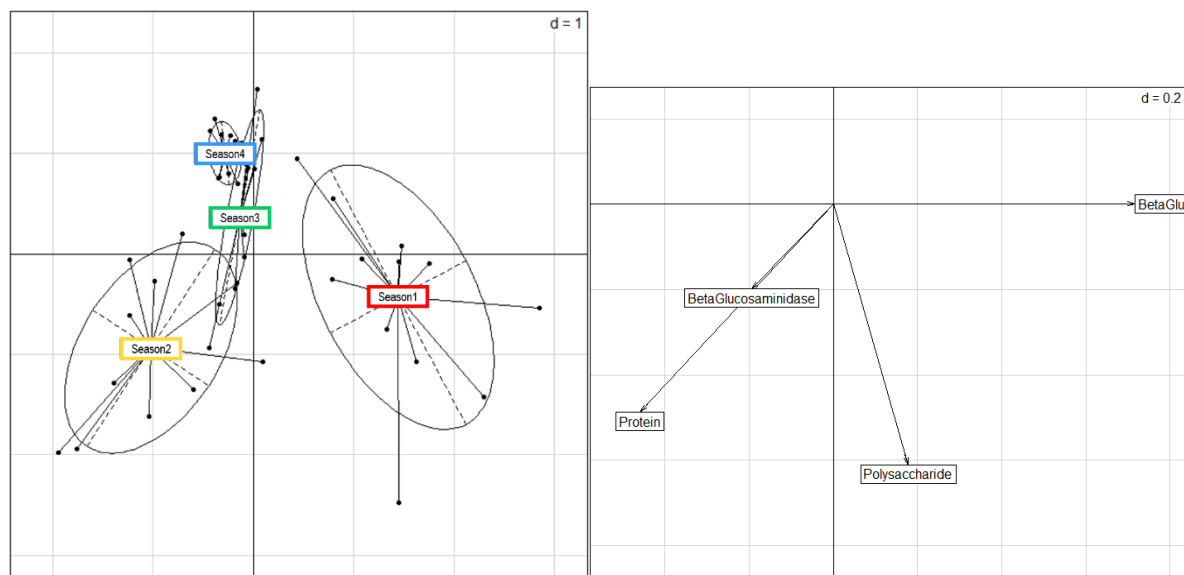
360 days (section 3.1).

361

362 Figure 4: Linear discriminant analysis of the different conditions after three days of exposure (left

363 panel) based on polysaccharide and protein content, and extracellular enzymatic activities (right

364 panel).

365

366

367

368

4. Discussion

4.1. Diuron impact on PSII

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

385

4.2. Metabolism variations with temperature and light

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

391   peptidoglycans (a component of the bacterial wall) and chitin (a polysaccharide derived from fungal

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).

397   For autotrophic organisms, photosynthetic activity is directly related to metabolism, itself dependent

398   on temperature (Brown et al., 2004). In this study, polysaccharide quantities produced after three

399   days of exposure were greater at 26°C than at 10°C, suggesting that the biofilm was less productive

400   at colder temperatures. These results are in accordance with those of Wolfstein and Stal (2002) who

401   found a slight increase (around 1.25 – 1.3 times higher) in EPS content (composed of polysaccharides

402   and proteins) after three and nine days of exposure between biofilm exposed from 10°C to 25°C,

403   with a photoperiod of 12/12.

404

405   ***Interaction of environmental factors effects with the impact of diuron***

406   The effects of environmental factors interacting with diuron were not clearly visible and not

407   significant on heterotrophic organisms. In fact, as diuron is a photosynthetic inhibitor herbicide, it

408   should have a greater influence on autotrophic compartment. Results for β-glucosaminidase activity

409   showed a slight decrease at 10°C and a stability at 26°C after three days of exposure between

410   controls and the most contaminated condition. In contrary β-glucosidase activity was marked by a

411   small increase. Indeed, diuron is a photosynthetic inhibitor herbicide, so it does not directly target

412   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 $\mu g.L^{-1}$, 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.

419 However, this study, combining toxicokinetic and toxicodynamic approaches, showed the evolution

420 of the effects of environmental factors and diuron on biofilm metabolism over time. Although the

421 data showed little difference over a three-day period exposure, they still highlight an evolution and a

422 dynamic system, suggesting a greater impact on longer-term exposure in the natural environment.

423 Although our results showed no significant differences between conditions, the effect of diuron on

424 algae seemed to be modulated by temperature and light to variable extents, depending on the

425 concentration of exposure. Nevertheless, the results showed that among all the descriptors analysed,

426 the only part of the metabolism significantly impacted by diuron was photosynthetic activity.

427 Contrastingly, polysaccharide and protein production and enzymatic activities were mainly affected

428 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 cycle

438   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

442   presence of herbicide (2.5 µM norflurazon) and over 4 days, they observed a strong decrease in

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.

449   All the variations observed in this study on biofilm show only trends and not significant differences,

450   which begs the question of what the major changes would be that would take place over longer

451   exposure times, and at the level of community composition.

452

453   **5. Conclusion**

454   This study focused on the influence of interactions between diuron exposure, temperature and

455   photoperiod on pesticide bioaccumulation and toxic impact on biofilm. Our results suggest some

456   influence of temperature on bioaccumulation, and consequently on photosynthetic activity. They

457   imply that Winter biofilms in temperate regions are more sensitive to diuron exposure than Summer

458   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.

468

469 **Conflict of interest**

470 The authors declare that the research was conducted in the absence of any commercial or financial

471 relationships that could be construed as a potential conflict of interest.

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