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1 Experimental testing of two urban stressors on freshwater biofilms

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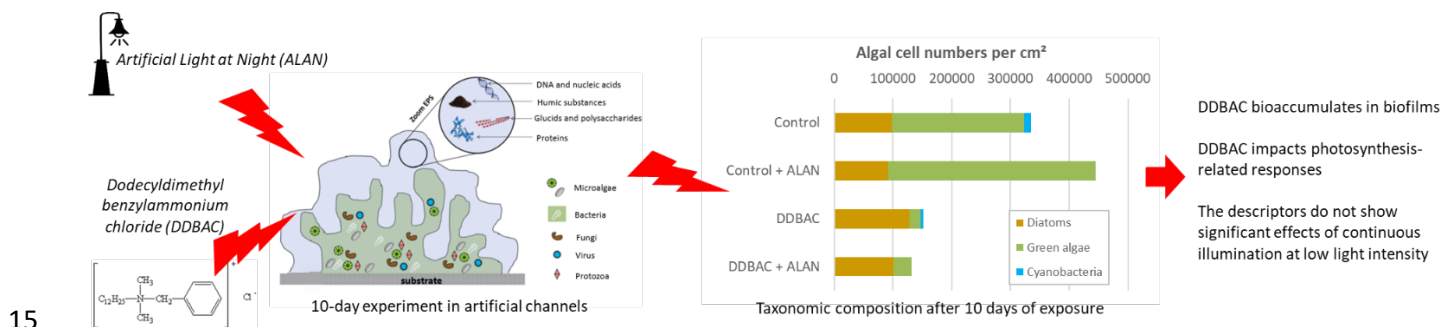
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13

14 Graphical abstract



17 Abstract

18 Aquatic ecosystems and their communities are exposed to numerous stressors of various
19 natures (chemical and physical), whose impacts are often poorly documented. In urban areas,
20 the use of biocides such as dodecyltrimethylbenzylammonium chloride (DDBAC) and their
21 subsequent release in wastewater result in their transfer to urban aquatic ecosystems. DDBAC
22 is known to be toxic to most aquatic organisms. Artificial light at night (ALAN) is another
23 stressor that is increasing globally, especially in urban areas. ALAN may have a negative impact
24 on photosynthetic cycles of periphytic biofilms, which in turn may result in changes in their
25 metabolic functioning. Moreover, studies suggest that exposure to artificial light could
26 increase the biocidal effect of DDBAC on biofilms. The present study investigates the
27 individual and combined effects of DDBAC and/or ALAN on the functioning and structure of
28 photosynthetic biofilms. We exposed biofilms in artificial channels to a nominal concentration
29 of 30 mg.L⁻¹ of DDBAC and/or ALAN for 10 days. ALAN modified DDBAC exposure, decreasing
30 concentrations in the water but not accumulation in biofilms. DDBAC had negative impacts on
31 biofilm functioning and structure. Photosynthetic activity was inhibited by > 90% after 2 days
32 of exposure, compared to the controls, and did not recover over the duration of the
33 experiment. Biofilm composition was also impacted, with a marked decrease in green algae
34 and the disappearance of microfauna under DDBAC exposure. The integrity of algal cells was
35 compromised where DDBAC exposure altered the chloroplasts and chlorophyll content.

36 Impacts on autotrophs were also observed through a shift in lipid profiles, in particular a
37 strong decrease in glycolipid content was noted. We found no significant interactive effect of
38 ALAN and DDBAC on the studied endpoints.

39 **Keywords:** Biocide, ALAN, biofilm, photosynthetic efficiency, bioaccumulation, lipids.

40 1- Introduction

41 Biocides are widely used in different areas of activity including agriculture and industry, as
42 well as in a multitude of domestic products (e.g. pharmaceutical, personal care and household
43 products) (Abbott et al., 2020). Among these biocides, dodecyldimethylbenzylammonium
44 chloride (DDBAC) is commonly used as an active substance in medical disinfection products,
45 as well as in household products such as detergents. Recently, following the regulation of
46 some broadly used disinfectant agents such as triclosan, quaternary ammonium compounds
47 like DDBAC have become popular substitutes (Sreevidya et al., 2018; Arnold et al., 2023).
48 Moreover, DDBAC and all other benzalkonium chloride derivatives have been shown to be
49 very effective against the SARS-CoV virus (Rabenau et al., 2005), resulting in their widespread
50 use as disinfectants during the Covid 19 pandemic (US EPA, 2020). This multi-purpose product
51 eventually reaches wastewaters, where its concentration varies greatly depending on the land
52 use, population and wastewater treatment facilities, among other factors. For example, a
53 concentration of 6 mg.L⁻¹ was recorded in hospital effluents (Kümmerer et al., 1997) due to
54 the intensive use of DDBAC as a disinfectant. In municipal wastewater effluents, DDBAC
55 concentrations were observed to range from just a few ng per liter up to 170 µg.L⁻¹ (Clara et
56 al., 2007; Martínez-Carballo et al., 2007; Zhang et al., 2015).

57

58 Once in the aquatic environment, DDBAC is relatively stable to photodegradation and its half-
59 life in surface waters can reach 180 days, although the presence of photosensitizers (e.g.
60 natural organic matter, Hora & Arnold 2020, Mohapatra et al., 2024) can reduce this half-life
61 to a week (US EPA, 2006). The organic carbon-water partition coefficient (K_{oc}) of DDBAC is 5.43
62 and, therefore, it has a high adsorption ratio on sewage sludge, sediments or humic
63 substances (van Wijk et al., 2009). Although the transfer of DDBAC to urban aquatic
64 ecosystems has been recognized, the ecotoxicity of DDBAC is poorly documented. The toxicity
65 of DDBAC to cells is due to its quaternary ammonium polar head, which has the capacity to
66 bind to the surface membrane, while the alkyl lipophilic chain alters the phospholipid bilayer.
67 This alteration can rapidly lead to membrane disruption and progressive lysis of the cell (Eich
68 et al., 2000). Regarding aquatic organisms, there is redundancy in the biological models used
69 for toxicity assessment where most studies have focused on *Daphnia magna* to determine the
70 acute toxicity of DDBAC (Kreuzinger et al., 2007; Leal et al., 1994; Chen et al., 2014; Lavorgna
71 et al., 2016). To date, *D. magna* is the most sensitive organism to DDBAC, where the
72 concentration needed to immobilize 50% of organisms (EC₅₀) was of 5.9 µg.L⁻¹ (US EPA, 2006).
73 As a result, the US EPA established a non-observed adverse effect concentration (NOAEC) of
74 4.15 µg.L⁻¹ for aquatic invertebrates. Phytoplanktonic organisms seem to be less sensitive to
75 DDBAC than aquatic invertebrates. Indeed, other studies investigated the effects of DDBAC on
76 various microalgae species and the range of EC₅₀ based on growth was found to vary between

77 58 $\mu\text{g}\cdot\text{L}^{-1}$ for *Skeletonema costatum* (Kreuzinger et al., 2007) to 203 $\mu\text{g}\cdot\text{L}^{-1}$ for *Chlorella vulgaris*
78 (Sütterlin et al., 2008). However, to our knowledge, the chronic effects of DDBAC exposure on
79 natural aquatic communities has not been considered.

80

81 In this study, we focused on stream biofilms (periphyton), which are complex structures
82 housing autotrophic microorganisms (e.g. cyanobacteria, green algae, diatoms), bacteria,
83 fungi, and other heterotrophic organisms such as microfauna (Battin et al., 2016). Biofilms are
84 ubiquitous in aquatic environments, including urban streams and ponds. They form the basis
85 of aquatic food webs, providing a source of energy for primary producers, including essential
86 fatty acids (Brett and Müller-Navarra, 1997). Biofilms can also be found downstream of
87 wastewater treatment plants (WWTPs) and have been used as ecological indicators of stress
88 (Tamminen et al., 2022; Tlili et al., 2020). The early detection of ecosystem impairment using
89 autotrophic biofilms can be based on functional (e.g., photosynthesis) and structural
90 endpoints describing biodiversity based on taxonomy as well as pigments or lipid profiles
91 (Sabater et al., 2007; Morin & Artigas, in press). Biofilms are also efficient bioaccumulators of
92 organic substances carried by waters under dissolved or particulate forms (Bonnineau et al.,
93 2021). Given the toxic mode of action of DDBAC, impacts on biofilm fatty acids, particularly on
94 membrane phospholipids, are likely to occur. The attack on phospholipid membrane may also
95 result in the disruption of key functions of biofilm such as photosynthesis. For example, Pozo-
96 Antonio and Sanmartin (2018) showed a significant decrease in chlorophyll *a* and
97 photosynthetic efficiency of phototrophic biofilms from church walls after a DDBAC
98 treatment. The effect of DDBAC as a biofouling removal agent was significantly enhanced
99 when combined with artificial light or UV irradiation.

100

101 Artificial light at night (ALAN) has become a global pollution concern and, as of 2014, more
102 than 20% of the world's land surfaces between 75°N and 60°S were exposed to a light-polluted
103 sky (Falchi et al., 2016). Most urban areas have developed along rivers and coastlines,
104 increasing the exposure of urban aquatic environments to ALAN. Concerns about the impact
105 of ALAN on aquatic ecosystems and research on this topic are quite recent (Perkin et al., 2011).
106 Indeed, ALAN was only recognized as harmful for freshwater ecosystems in the early 2000s
107 (Longcore and Rich, 2004). ALAN could lead to disruptions in nycthemeral cycles of
108 autotrophic organisms in biofilms by inducing variability in the maximum efficiency of
109 photosynthesis (Maggi and Serôdio, 2020). ALAN can also alter taxonomic composition by
110 favouring certain autotrophic groups over others, thereby exerting differential selection, as
111 shown in a study where cyanobacteria proportions in biofilm decreased under ALAN (Grubisic
112 et al., 2017).

113

114 The objective of this study was to determine the individual and combined effects of two
115 anthropogenic stressors typical from urban aquatic ecosystems (DDBAC and ALAN) on
116 autotrophic organisms within biofilms, using structural and functional descriptors of impact.
117 To this aim, we performed a 10-day experiment in which natural autotrophic biofilms were
118 exposed in laboratory channels to DDBAC and/or ALAN. We hypothesized that, given the Koc

119 of the substance, DDBAC would bioaccumulate in the biofilms, generating toxicity to
120 microorganisms. Functional (photosynthetic efficiency) and structural (taxonomic
121 composition, pigment and lipid profiles) descriptors were predicted to be impacted by DDBAC
122 exposure. ALAN was expected to modify the development of microalgae, thus to impact the
123 proportions of algal groups, to alter photosynthetic efficiency to a certain extent, and to
124 increase the sensitivity of microalgae to additional stress. As a consequence, we hypothesized
125 that co-exposure to ALAN and DDBAC would enhance the toxic effects of the biocide.

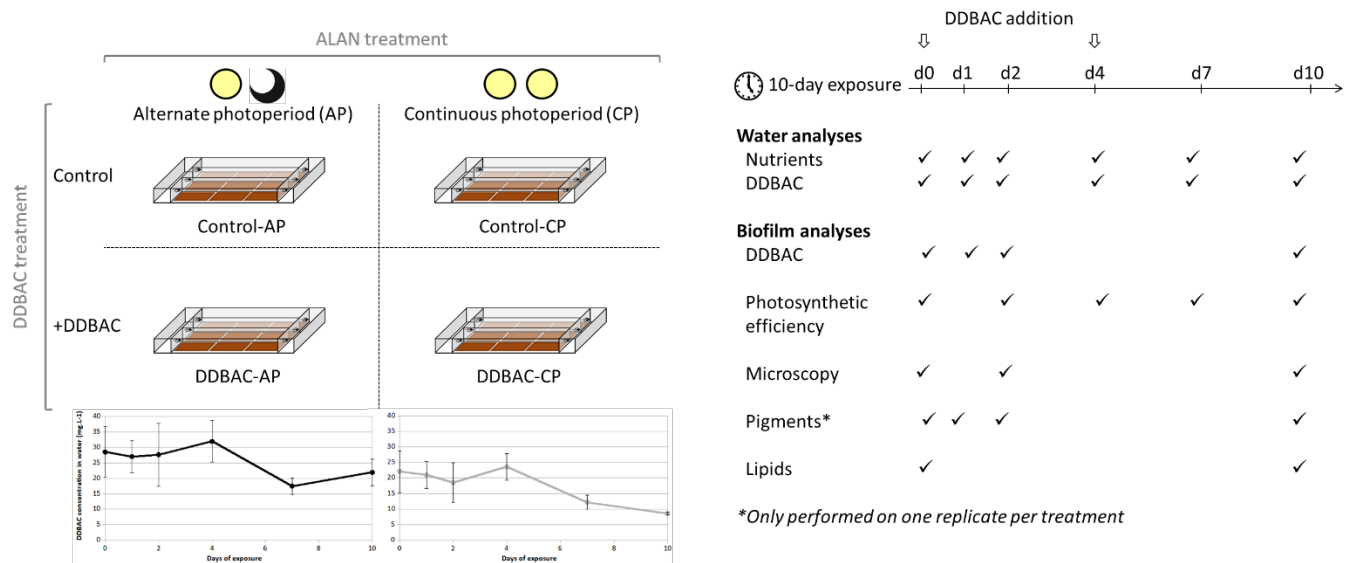
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127 **2- Material and methods**

128 **2.1- Experimental design**

129 Biofilms were grown in a DDBAC-free pond in Cestas, near Bordeaux, France. A previous study
130 classified this small water body as a hypereutrophic pond (Chaumet et al., 2019; Neury-
131 Ormanni et al., 2020). Glass slides (~200 cm²) were immersed in the photic zone, at a depth
132 of 30-50 cm. Colonization took place in winter (December 2020 to April 2021), during five
133 months to collect sufficient amounts of biomass. At this period, daylight irradiance on the top
134 surface on the water (5 cm deep) was below 100 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$. At the end of the colonization
135 period, 36 glass slides covered by mature biofilms were randomly distributed among four
136 series of experimental units (EU) for the experiment. The EUs consisted of experimental
137 channels of about 30 L filled with pond water that had been previously filtered (20 μm) to
138 remove suspended material and most planktonic organisms. To specifically assess the impacts
139 of urban factors (DDBAC and ALAN), while avoiding possible inter-channel variability likely to
140 influence biofilm responses, three parallel channels were set up and connected to individual
141 10-L tanks (one per EU, making the slides pseudoreplicates for each condition). Biofilms
142 corresponding to day 0 (d0, 5 slides) were collected immediately after their recovery from the
143 pond.

144 On d0, two EUs were contaminated with a solution of dodecyldimethylbenzylammonium
145 chloride (DDBAC; Sigma Aldrich, France; CAS: 139-07-1, purity: >99%) to reach a concentration
146 of 30 $\text{mg}\cdot\text{L}^{-1}$. This concentration was selected to elicit an effect on photochemical efficiency
147 while using as low a concentration as possible, based on the observed EC₅ of preliminary dose-
148 response experiments (Appendix A). In order to assess only the effect of altered photoperiod,
149 not light level, a non-stressful incident light intensity of 20 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ was selected. Light was
150 kept on overnight and then from day 1 (d1), one control series and one DDBAC series were
151 exposed to an alternating 14 h day / 10 h night photoperiod (alternated photoperiod, AP) and
152 the other two series (one control, one DDBAC) were exposed to a 24 h day photoperiod
153 (continuous photoperiod, CP) throughout the course of the experiment (Figure 1). Room
154 temperature was maintained constant at 20.5 \pm 0.1°C, while water temperature was kept at
155 18.7 \pm 0.2°C. The tanks were refilled with 3 L of filtered pond water contaminated at 30 $\text{mg}\cdot\text{L}^{-1}$
156 DDBAC on day 4 (d4) to compensate for water evaporation.



157
158 Figure 1: Experimental design and sampling strategy.

159

160 2.2- Water chemistry measurements

161 On d0, d1, d2, d4, d7 and d10, 20 mL water samples were taken from all channels, filtered on
162 1-µm PTFE filters and stored at 4°C in the dark until analysis (performed within 48 h of
163 collection). Nutrients and mineral salts were analysed as described in Chaumet et al. (2019),
164 using a Metrohm 881 Compact Ionic Chromatograph pro (Metrohm). Anion analysis (PO₄⁻,
165 NO₃⁻, NO₂⁻, Cl⁻ and SO₄²⁻) was performed using a Supp 4/5 Guard/4.0 precolumn followed by a
166 Metrosep A Supp5 – 250/4.0 column. The mobile phase was a mixture of a solution of 3.2
167 mmol.L⁻¹ Na₂CO₃ and a solution of 1 mmol.L⁻¹ NaHCO₃. Cation analysis (Na⁺, K⁺, Ca²⁺, Mg²⁺ and
168 NH₄⁺) was performed using a C4 Guard/4.0 precolumn followed by a Metrosep C6 - 250/4.0
169 column). All precolumns and columns were provided by Metrohm. The eluent used was a
170 mixture of 2.5 mmol.L⁻¹ HNO₃ and a solution of 1.7 mmol.L⁻¹ 10,12-Pentacosadynoic acid
171 (PCDA). The limits of quantification (LOQ) of the different ions analysed are shown in Table 1.

172 DDBAC concentrations in the water were monitored frequently over the experiment, at d0,
173 d1, d2, d4, d7 and d10. Three samples of 20 mL were collected from each channel and stored
174 at -20°C together with the stock solution until analysis. The samples were analysed using an
175 Ultimate 3000 HPLC coupled with an API 2000 triple quadrupole mass spectrometer. We used
176 a Gemini® NX-C18 column from Phenomenex as a stationary phase. The mobile phase was
177 90:10 5 mM ammonium acetate/acetonitrile. We worked in isocratic mode, so the
178 composition of the mobile phase was constant during the analysis. The flow rate was set at
179 0.6 mL.min⁻¹ and the injection volume was set at 20 µL. An internal standard of benzyl-
180 2,3,4,5,6-d5-dimethyl-n-dodecylammonium chloride (DDBAC-d5) was used (Cluzeau, France;
181 CAS: 139-07-1, purity: >98%) at an equivalent of 100 µg.L⁻¹ in both samples and standard
182 solutions for calibration. The mass spectrometer was operated in multiple reaction monitoring
183 (MRM) mode with a total cycle time of 0.8 seconds. The MRM transitions were 304>91
184 (quantitation) and 304>212 (confirmation) for DDBAC. For DDBAC-d5, the MRM transitions
185 were 309>96 and 309>212. The declustering potential was set to 65 V and the collision

186 energies were set either to 45 or 30 V for the quantitation or confirmation transition,
187 respectively. Samples were diluted and the calibration range was from 1 to 200 $\mu\text{g}\cdot\text{L}^{-1}$. Quality
188 controls were regularly injected at concentrations of 5 and 25 $\mu\text{g}\cdot\text{L}^{-1}$, as well as analytical
189 blanks.

190

191 **2.3- Biofilm biological endpoints**

192 ***2.3.1- Photosynthetic efficiency***

193 We sampled 2 cm^2 of biofilm from three different glass slides for each treatment on d0, d2,
194 d4, d7, and d10, corresponding to three pseudoreplicates per treatment per sampling. Each
195 biofilm sample was then suspended in 3 mL of channel water and gently shaken for
196 homogenization. Photosynthetic activity (effective photosystem II quantum yield) was
197 assessed on suspensions of biofilm (Genty et al. 1989) within one hour after collection using
198 a Pulse Amplitude Modulation fluorimeter (Phyto-PAM, Heinz Walz GmbH, Germany) in
199 quartz cuvettes under continuous agitation (Emitter–Detector Unit PHYTO-ED). Biofilm
200 suspensions were adapted for 15 min to light ($164 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) before fluorescence
201 measurements at the same active radiation, in Actinic Light mode. The quantum yield values
202 reported are mean values of five measurements per replicate, averaged for the three pigment
203 groups using the device's reference spectra. After photochemical efficiency measurements,
204 samples were preserved for further microscopic analyses by adding a few drops of Lugol
205 solution then stored in the dark at 4°C.

206 ***2.3.2- Microscopic analyses***

207 For microscopic observations, a Nageotte counting slide (Marienfeld, Germany) was used with
208 125 μL biofilm suspension samples collected on d0, d2 and d10 to determine initial taxonomic
209 composition as well as to assess early structural changes and chronic effects of ALAN and
210 DDBAC. Observations were made at x400 magnification under an optical microscope
211 (Olympus BX51) equipped with a digital camera as described in Morin et al. (2010). Ten fields
212 of view were scanned for enumeration of the main taxonomic groups observable on preserved
213 material: diatoms, green algae, cyanobacteria and microfauna. Solitary and colonial algae and
214 cyanobacteria were recorded and counted as cell numbers to allow for density comparisons.
215 Microfauna organisms were recorded as individuals. All densities were expressed as a function
216 of colonized biofilm surface (i.e., per cm^2). Live (intact cell content) and dead (empty frustules)
217 diatoms were counted separately to estimate mortality (Morin et al. 2010).

218 ***2.3.3- Analyses from freeze-dried biofilms***

219 Biofilms were scraped from glass slides on d0, d1, d2 and d10 (4 slides per treatment) and
220 were frozen in liquid nitrogen to prevent lipid degradation. The samples were then lyophilized
221 for 24 hours, and used to determine lipid content, DDBAC bioaccumulation, and
222 concentrations of photosynthetic pigments. Analyses focused on neutral lipids (storage lipids:
223 triacylglycerides) and on polar lipids. Polar lipids correspond to phospholipids (lipids typical
224 from cytoplasmic membranes: phosphatidylcholine-PC, phosphatidylethanolamine-PE, and
225 phosphatidylglycerol-PG) and to glycolipids (characteristic from chloroplastic membranes:

226 digalactosyldiacylglycerol-DGDG, monogalactosyldiacylglycerol-MGDG,
227 sulfoquinovosyldiacylglycerol-SQDG). Lipids were extracted from the biofilms (20 mg dry
228 weight) using a mixture of MTBE-methanol (3:1 %v/v) and UPW-methanol (3:1 %v/v) with 150
229 mg of microbeads. Samples were then homogenized using a FastPrep (MP Biomedicals). The
230 upper organic fraction of the samples (i.e. MTBE) was recovered by centrifugation for lipid
231 analysis, while the lower hydrophilic fraction (mixture of UPW and methanol) was used to
232 determine DDBAC bioaccumulation.

233 **2.3.3.1- Lipid quantification**

234 For lipid analysis, samples from d0 and d10 were evaporated and injection solvent was added
235 before analysis by HPLC-MS/MS following the protocol detailed in Mazzella et al. (2023a).
236 Briefly, different stationary and mobile phases were used for the analysis of
237 phospholipids/glycolipids and triglycerides. For the phospholipid and glycolipid analyses, a
238 LUNA® NH2 HILIC column (100 x 2 mm, 3 µm) from Phenomenex was used as the stationary
239 phase, and a mixture of acetonitrile and 40 mM ammonium acetate buffer as the mobile
240 phase. The flow rate was set at 400 µL.min⁻¹. The proportions of these two solutions are given
241 in Appendix C. For the analysis of triglycerides and betaine lipids (i.e.
242 diacylglyceryltrimethylhomo-Ser, DGTS), a KINETEX® C8 column (100 x 2.1 mm, 2.6 µm) from
243 Phenomenex was used as the stationary phase, and the mobile phase was a mix of a solution
244 of acetonitrile/water/40 mM ammonium acetate buffer (600/390/10, v/v/v) and a solution of
245 isopropanol/acetonitrile/1 M ammonium acetate buffer (900/90/10, v/v/v). The flow rate was
246 set at 300 µL.min⁻¹. The proportions of these two solutions are given in Appendix C. Results
247 were then pre-treated with ANALYST® 1.6.2 software from Sciex. For polar lipids (i.e.
248 glycolipids and phospholipids), the limits of quantification ranged from 0.1 to 0.5 nmol.mg⁻¹,
249 depending on the lipid classes. For triglycerides and DGTS, the limits of quantification reached
250 were 0.01 and 0.1 nmol.mg⁻¹, respectively. Results were expressed as nmol.g⁻¹ freeze-dried
251 biofilm.

252 **2.3.3.2- DDBAC accumulation in biofilms**

253 The same analytical method used for water samples was used to determine bioaccumulated
254 DDBAC in the previously extracted hydrophilic fraction. It was generally necessary to perform
255 significant dilutions (i.e. 10,000-fold) in order to stay within the calibration range. Results were
256 then expressed in the log10 value of the bioconcentration factor (i.e. log(BCF)). The BCF was
257 calculated according to the following formula:

$$258 \quad BCF = \frac{\text{Concentration of DDBAC in biofilm (mg.kg}^{-1}\text{)}}{\text{Concentration of DDBAC in water (mg.L}^{-1}\text{)}} \text{ (Eq.1)}$$

259 **2.3.3.3- Photosynthetic pigments**

260 On selected freeze-dried samples (one replicate per treatment), pigment analyses were also
261 performed following the French standard NF T90-117 (1999). Ten mg of dry biofilm were put
262 in solution using 10 mL of acetone. After 20 minutes of ultrasonication, the mix was filtered
263 on a Büchner filter to remove the solid phase. Absorption was measured at the wavelengths
264 630 nm, 647 nm, 664 nm, 665 nm and 750 nm with a UV-1800 (Shimadzu) spectrophotometer,
265 and was then remeasured at the same wavelengths after acidification of the samples. The

266 concentrations of chlorophyll pigments and phaeopigments were determined following the
267 equations of Lorenzen (1967) and expressed as $\mu\text{g pigments.mg}^{-1}$ freeze-dried biofilm.

268

269 **2.4- Data analyses**

270 ANOVAs were performed after verification that homogeneity of variances and normality were
271 met. A one-way ANOVA was used to test the effect of ALAN on DDBAC degradation, and two-
272 way ANOVAs were used to assess the individual effect of ALAN and DDBAC, as well as their
273 interaction, on nutrient concentrations. Two-way ANOVAs with sampling dates as repeated
274 measures were conducted to assess the individual and interactive effects of ALAN and DDBAC
275 (categorical variables with 2 levels: yes/no) on biofilm endpoints. . Data were processed using
276 R software (R Core Team, 2022).

277

3- Results**278 3.1- Water chemistry**

279 **Table 1. Principal ions and DDBAC concentrations (mg.L⁻¹) in the four experimental treatments along the ten days of exposure. Results are expressed as the mean and**
 280 **standard error for the whole experiment. Two-way ANOVAs were conducted, with ALAN and DDBAC as the two factors, except for DDBAC concentrations (One-way**
 281 **ANOVA assessing the effect of ALAN). DDBAC = contaminated biofilm; Control = non-exposed biofilm; AP = alternated photoperiod; CP = continuous photoperiod; LOQ =**
 282 **Limit of quantification.**

Treatment	DDBAC	NO ₂ ⁻	NO ₃ ⁻	PO ₄ ³⁻	SO ₄ ²⁻	Cl ⁻	NH ₄ ⁺	Na ⁺	K ⁺	Ca ²⁺	Mg ²⁺
Control-AP	<LOQ	0.04 ± 0.03	1.69 ± 0.27	0.03 ± 0.02	21.0 ± 1.8	36.4 ± 2.7	0.09 ± 0.05	22.0 ± 1.9	4.80 ± 0.43	57.8 ± 5.1	4.90 ± 0.42
DDBAC-AP	26.6 ± 2.5	0.02 ± 0.00	0.66 ± 0.17	0.03 ± 0.02	18.9 ± 0.8	35.5 ± 1.4	0.11 ± 0.05	19.7 ± 0.9	4.34 ± 0.20	52.2 ± 2.5	4.41 ± 0.20
Control-CP	<LOQ	0.02 ± 0.01	1.35 ± 0.23	0.01 ± 0.00	17.5 ± 0.3	30.3 ± 0.4	0.09 ± 0.06	18.5 ± 0.3	4.12 ± 0.06	48.4 ± 0.6	4.09 ± 0.06
DDBAC-CP	18.5 ± 2.0	0.02 ± 0.00	0.75 ± 0.10	0.03 ± 0.02	17.8 ± 0.3	33.0 ± 0.6	0.10 ± 0.06	18.8 ± 0.9	4.21 ± 0.11	49.5 ± 1.2	4.18 ± 0.09
LOQ	0.001	0.005	0.01	0.01	0.005	0.01	0.005	0.01	0.0025	0.15	0.15
Significant factor(s) and interaction(s)	ALAN	None	DDBAC	NA	DDBAC	DDBAC	NA	DDBAC	NA	DDBAC	DDBAC
p	<0.001	>0.05	<0.001	NA	<0.05	<0.01	NA	<0.05	NA	<0.05	<0.05

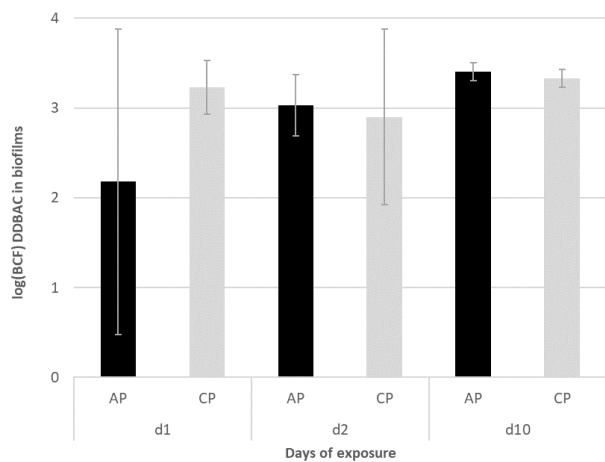
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284 In the control channels, DDBAC concentrations were always below quantification limit (Table
 285 1), confirming that no cross-contamination occurred. Contaminant concentrations averaged
 286 $26.6 \pm 2.5 \text{ mg.L}^{-1}$ (AP) and $18.5 \pm 2.0 \text{ mg.L}^{-1}$ (CP) in the DDBAC treatments. Differences in
 287 DDBAC concentrations were observed between the DDBAC treatment channels exposed to an
 288 alternated photoperiod and those exposed to a continuous photoperiod ($F_{[1,10]} = 232.72$; $p <$
 289 0.001 , also see Figure 1), suggesting that degradation occurred under continuous
 290 photoperiod. Nutrient concentrations were also modified by DDBAC exposure (Table 1).
 291 Indeed, DDBAC addition resulted in lower concentrations of nitrate, sulfate, Cl^- , Na^+ , Ca^{2+} and
 292 Mg^+ than in the control (Table 1), while ALAN showed no effect on nutrient concentrations.

293

294 3.2- Bioaccumulation of DDBAC in biofilms

295 **Figure 2. Evolution of DDBAC bioaccumulated in biofilms (expressed in log(BCF)) between the different**
 296 **ALAN treatments (n=3). AP = alternated photoperiod CP = continuous photoperiod**



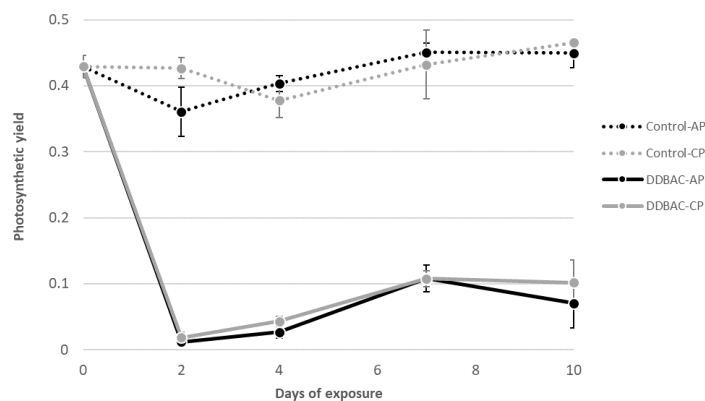
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298 Figure 2 shows the log(BCF) for the hydrophilic fraction of biofilm samples at d1, d2 and d10
 299 for AP and CP treatments. DDBAC was recovered from biofilms for every treatment and every
 300 sampling time, showing that this compound bioaccumulates well in autotrophic biofilms, with
 301 a mean log(BCF) of 3.22 ± 0.25 . The amount of DDBAC bioaccumulated in the biofilms showed
 302 no significant changes between sampling times ($F_{[2,28]} = 2.38$; $p = 0.11$) nor ALAN treatments
 303 ($F_{[1,28]} = 0.28$; $p = 0.60$), and no Time x ALAN interaction ($F_{[2,28]} = 0.025$; $p = 0.98$).

304

305 3.3- Photosynthetic efficiency

306



307

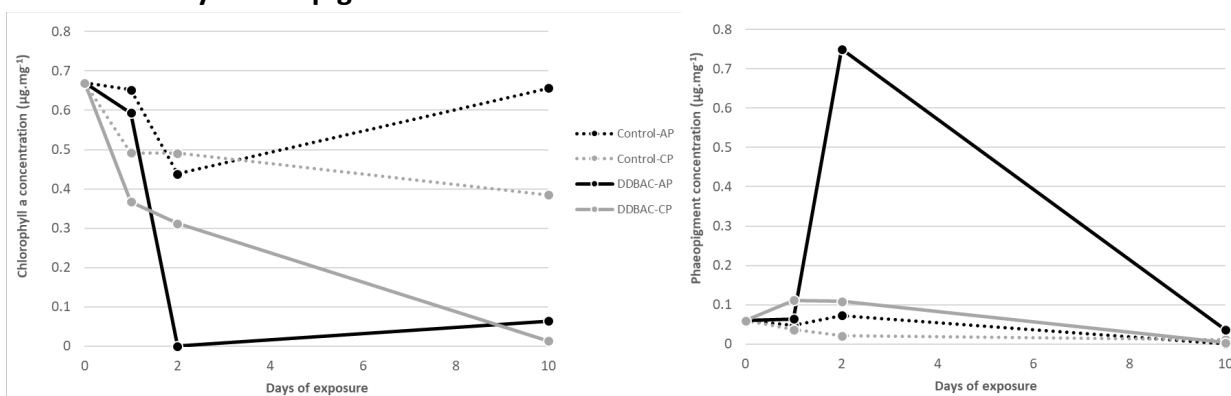
308 **Figure 3. Evolution of photosynthesis yield in biofilms along the ten days of the experiment (d0: n=5, d2 and**
 309 **d10: n=3). Control = non-exposed biofilm; DDBAC = contaminated biofilm; AP = Alternated**
 310 **Photoperiod; CP = Continuous Photoperiod**

311

312 Figure 3 shows that photosynthetic activity was stable over the time of the experiment, with
 313 a mean value of 0.42 ± 0.01 in the control AP and CP channels, regardless of the ALAN
 314 treatment ($F_{[1,42]} = 2.65$; $p > 0.05$). Biofilms exposed to DDBAC showed an almost complete
 315 inhibition of photosynthetic yield starting from d2 and lasting until the end of the experiment
 316 ($F_{[1,42]} = 1279$; $p < 0.001$). In the same way as for control channels, photosynthetic activity
 317 showed no difference between AP and CP for contaminated channels ($F_{[1,42]} = 0.18$; $p = 0.67$).

318

319 **3.4- Photosynthetic pigments**



320

321 **Figure 4. Changes in photosynthetic pigments content in biofilms along the ten days of the experiment (n =**
 322 **1). Control = non-exposed biofilm; DDBAC = contaminated biofilm; AP = alternated photoperiod; CP =**
 323 **continuous photoperiod.**

324

325 Chlorophyll *a* concentrations decreased over time in the DDBAC contaminated samples,
 326 compared to the control samples (Figure 4). At d2, no chlorophyll *a* was detected in the
 327 DDBAC-AP samples, and only phaeopigments were measured. At the end of the experiment,
 328 pigment concentrations in DDBAC samples were very low compared to the controls.
 329 Unfortunately, the lack of replication does not allow for any conclusion on the effect of the
 330 tested treatments on chlorophyll *a* or phaeopigments.

331

332

333 **3.5- Community composition**

334

Table 2. Evolution of the taxonomic composition of biofilms during the experiment. Densities are expressed as individuals $\times 10^3$ / cm², i.e. number of cells for microalgae, and number of organisms for microfauna. Diatom mortality is expressed in %. Results are expressed as mean and standard error for each date and treatment (d0: n=5, d2 and d10: n=3). DDBAC = contaminated biofilm; Control = non-exposed biofilm; AP = Alternated Photoperiod; CP = Continuous Photoperiod

335

Date	Treatment	Diatom density	Diatom mortality	Green algae density	Cyanobacteria density	Microfauna density
d0	Control	81.3 \pm 33.7	7 \pm 1	4.1 \pm 2.3	3.8 \pm 2.0	0.3 \pm 0.0
d2	Control-AP	141.3 \pm 9.1	3 \pm 0	26.7 \pm 4.4	1.6 \pm 2.0	0.6 \pm 0.3
d2	DDBAC-AP	113.2 \pm 11.5	3 \pm 1	11.8 \pm 2.8	0	0.2 \pm 0.2
d2	Control-CP	132.1 \pm 68.8	3 \pm 1	51.7 \pm 46.0	0	0.3 \pm 0.3
d2	DDBAC-CP	145.0 \pm 26.1	2 \pm 1	6.2 \pm 1.7	0	0
d10	Control-AP	98.2 \pm 11.7	5 \pm 2	225.6 \pm 133.5	11.2 \pm 7.8	0.9 \pm 0.5
d10	DDBAC-AP	128.5 \pm 26.0	2 \pm 0	18.9 \pm 15.0	4.8 \pm 3.0	0
d10	Control-CP	91.3 \pm 14.2	6 \pm 1	354.2 \pm 83.1	0	1.0 \pm 0.2
d10	DDBAC-CP	101.0 \pm 27.0	1 \pm 0	30.7 \pm 8.2	0	0
	Significant factor(s) and interaction(s)	None	DDBAC	DDBAC \times Time	None	DDBAC \times Time
	p	>0.05	<0.05	<0.001	>0.05	<0.01

336

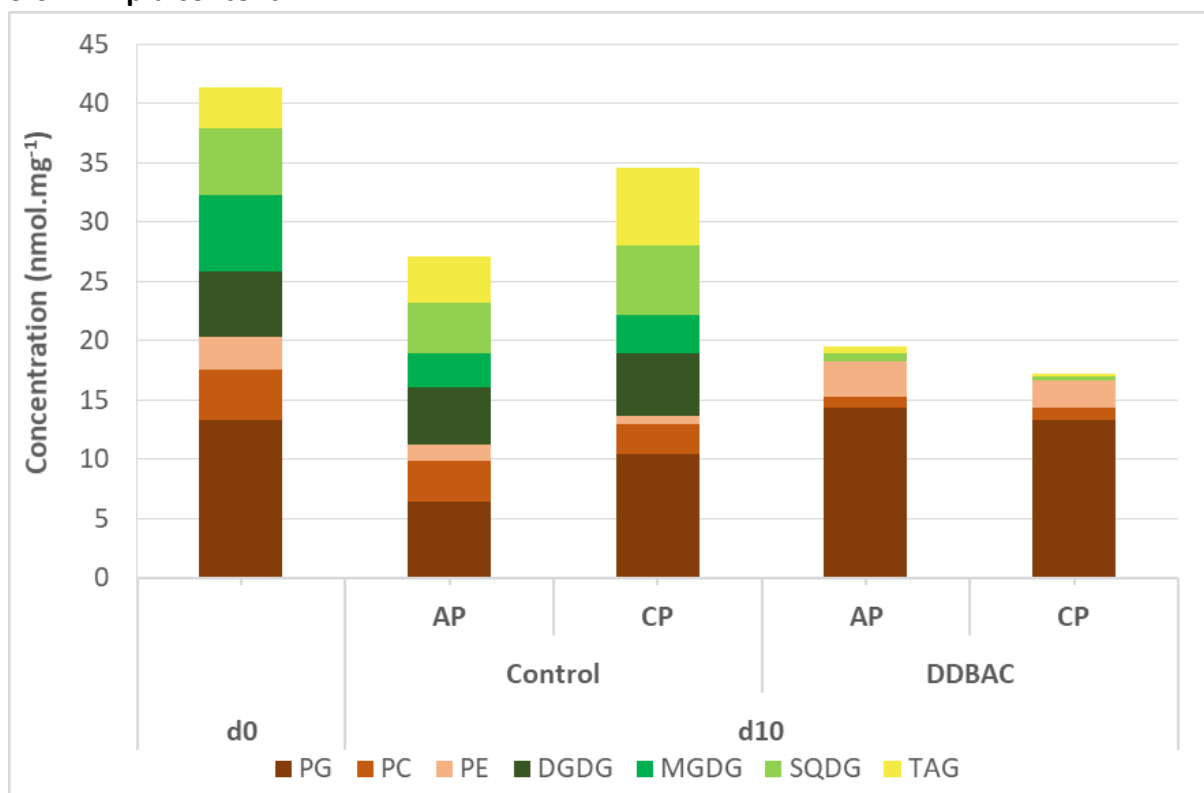
337 Diatoms (91%) dominated the biofilm community at d0, whereas low proportions of green
 338 algae (5%), cyanobacteria (4%) and microfauna (1%) were observed (Table 2). In the control
 339 channels, green algae developed markedly and became the main algal group in terms of
 340 density by d10, with no significant effect of the ALAN treatment (e.g. 67% in AP channels and
 341 79% in CP). The density of green algae slightly increased from d0 to d10 in the DDBAC
 342 contaminated channels, but not to the same extent as in the control channels. Indeed, a
 343 significant Time \times DDBAC interaction was highlighted for green algae densities ($p < 0.001$;

344 Table 2). Microfauna increased in control channels, but had completely disappeared from
 345 biofilms exposed to DDBAC by d10. For this component, a significant Time × DDBAC interaction
 346 was also noted ($p < 0.01$; Table 2). ALAN treatments did not have a significant impact on the
 347 biofilm taxonomic composition.

348 Diatom mortality values (based on the ratio between frustules without cell content and total
 349 diatom frustules) significantly decreased under DDBAC exposure (Table 2). In addition,
 350 microscopic observations highlighted differences in the aspect (shape, colour) of chloroplasts
 351 in diatoms and chlorophytes exposed to DDBAC, becoming highly granular and darker (Figure
 352 B.2). These were, however, not considered as ‘dead’ cells in estimating diatom mortality.
 353

354

355 3.6- Lipid content



356

357 **Figure 5. Effects of DDBAC and continuous photoperiod on the proportion of total lipids and on lipid classes**
 358 **(n=4). DDBAC = contaminated biofilm; Control = non-exposed biofilm; AP = alternated photoperiod; CP =**
 359 **continuous photoperiod; PE = phosphatidylethanolamine; PC = phosphatidylcholine; PG =**
 360 **phosphatidylglycerol; DGDG = digalactosyldiacylglycerol; MGDG = monogalactosyldiacylglycerol; SQDG =**
 361 **sulfoquinovosyldiacylglycerol; TAG = triacylglycerides.**

362

363 On d0, total lipid concentration reached 42.6 nmol.mg⁻¹ of biofilm dry weight. Almost half of
 364 the lipids were phospholipids, and the other half were glycolipids, while neutral lipids (TAG)
 365 represented a minor portion (Table 3). By d10, total lipid concentration had decreased in all
 366 treatments down to an average of 23.6 ± 7.53 nmol.mg⁻¹ of biofilm dry weight (Table 3). No
 367 significant differences were found between AP and CP, while DDBAC significantly affected

368 glycolipids over time (significant Time × DDBAC interaction; $p < 0.01$). Figure 5 shows lipid
369 contents under DDBAC and ALAN exposure on d0 and d10. At the beginning of the experiment,
370 phosphatidylglycerol (PG) was the predominant lipid in the biofilms, while phospholipids and
371 glycolipids in the biofilms were evenly distributed. Total lipid amounts decreased over time in
372 the controls, but the proportions of phospholipids and glycolipids remained similar to those
373 at d0, all the main classes of lipids being present. Total phospholipids remained stable over
374 time, whatever the treatment ($p > 0.05$), but phosphatidylethanolamine (PE) and PG were
375 higher in biofilms exposed to DDBAC compared to controls (Figure 5, Table 3). In the samples
376 exposed to DDBAC, glycolipids such as monogalactosyldiacylglycerol (MGDG) and
377 digalactosyldiacylglycerol (DGDG) decreased significantly, or even became barely detectable,
378 with significant Time × DDBAC interactions (respectively, $p < 0.01$ and 0.001).
379 Diacylglyceryltrimethylhomo-Ser (DGTS) was never detected and, therefore, is not mentioned
380 further.

Table 3: Evolution of lipid content of biofilms (in nmol.g⁻¹ freeze-dried biofilm) between the beginning and the end of the experiment. Results are expressed as mean and standard error for each date and treatment (n=4). DDBAC = contaminated biofilm; Control = non-exposed biofilm; AP = Alternated Photoperiod; CP = Continuous Photoperiod; PE = phosphatidylethanolamine; PC = phosphatidylcholine; PG = phosphatidylglycerol; DGDG = digalactosyldiacylglycerol; MGDG = monogalactosyldiacylglycerol; SQDG = sulfoquinovosyldiacylglycerol; TAG = triacylglycerides. Two-ways ANOVAs on repeated measures were applied on data.

381

Date	Treatment	Neutral lipids (TAG)	PG	PE	PC	Σ Phospholipids	DGDG	MGDG	SQDG	Σ Glycolipids	Total lipids
d0	Control	4.2 ± 2.5	13.7 ± 3.1	2.8 ± 0.5	4.6 ± 0.2	21.0 ± 3.3	5.6 ± 1.2	6.4 ± 1.6	5.3 ± 1.1	17.3 ± 2.4	42.6 ± 3.2
d10	Control-AP	3.9 ± 2.0	6.4 ± 1.0	1.3 ± 0.5	3.5 ± 1.5	11.2 ± 2.4	4.8 ± 0.8	2.9 ± 0.5	4.2 ± 0.7	12.0 ± 0.4	27.1 ± 4.1
d10	DDBAC-AP	0.2 ± 0.3	14.3 ± 2.6	3.0 ± 0.4	0.9 ± 0.4	18.2 ± 2.6	0	0	0.7 ± 0.2	0.7 ± 0.2	19.1 ± 2.6
d10	Control-CP	6.6 ± 2.9	6.9 ± 6.7	0.7 ± 0.1	2.6 ± 1.1	10.1 ± 7.8	5.3 ± 0.2	3.3 ± 1.1	5.8 ± 1.2	14.4 ± 2.2	31.1 ± 8.1
d10	DDBAC-CP	0.1 ± 0.1	13.3 ± 3.5	2.3 ± 1.0	1.0 ± 1.0	16.6 ± 5.2	0	0	0.4 ± 0.2	0.4 ± 0.2	17.1 ± 5.3
	Significant factor(s)	DDBAC × Time	DDBAC × Time	DDBAC × Time	DDBAC × Time	None	DDBAC 12× Time	DDBAC × Time	DDBAC × Time	DDBAC × Time	DDBAC × Time
	p	0.01	<0.05	<0.01	<0.01	>0.05	<0.001	<0.01	<0.001	<0.001	<0.001

382

383 4. Discussion

384 4.1- Experimental conditions and DDBAC exposure

385 In this experiment, natural biofilms were transferred to controlled laboratory conditions
386 under ALAN and DDBAC treatments. Changes in environmental conditions were likely to affect
387 biofilm characteristics; control conditions were however favourable to the development of
388 autotrophic biofilms, as highlighted by continuous growth from d0 to d10 and by stable
389 physiological state (assessed through photosynthetic efficiency). During the 10 days, most
390 physicochemical parameters remained stable, and no nutrient depletion was observed at the
391 end of the experiment in any treatment. However, DDBAC seemed to have an indirect effect
392 on the concentrations of certain nutrients over the 10-day exposure period of our experiment.
393 For example, under DDBAC exposure, concentrations of NO_3^- were lower than in the control
394 (Table 1).

395 Contrary to the hypothesis of Pozo-Antonio and Sanmartin (2018), who suggested that
396 artificial light could lead to an increase in internalization of the contaminant, we found no
397 significant difference in DDBAC concentrations in biofilm between ALAN treatments (Figure
398 2). Thus, our results do not support the hypothesis of a higher uptake of the substance from
399 the water through increased bioaccumulation under ALAN exposure. Lower concentrations of
400 DDBAC under CP compared to AP (Figure 1) might be linked to the differential development
401 of microorganisms exposed to ALAN. Some bacterial strains such as *Aeromonas hydrophila*
402 can biodegrade DDBAC under N-depleted conditions and use the degradation product as a
403 source of nitrogen (Patrauchan and Oriel, 2003). The above-mentioned decrease in certain
404 nutrient concentrations in the water under DDBAC treatment may have provided favourable
405 conditions for such strains able to grow consuming DDBAC as a nutrient source. Moreover,
406 the low light intensity used in the experiment may have enhanced the growth of such
407 heterotrophs in the absence of a strong competition with autotroph organisms from the
408 biofilm..

409 The potential for DDBAC exposure on biofilm consumers (related to bioaccumulation in the
410 biofilm) was similar between ALAN treatments, independently of differences in DDBAC
411 concentrations in the water. DDBAC accumulation rapidly stabilized to average $\log(\text{BCF})$ of
412 3.22 (Figure 2), falling in the range measured for organochlorine substances in biofilms, and
413 greater than previous observations for other pesticides or pharmaceuticals (Bonnineau et al.
414 2021). Our results demonstrate the retention capacity of biofilms for this compound,
415 suggesting a risk of transfer to primary consumers.

416

417 4.2- Effects of DDBAC on the biofilm

418 The first effect of DDBAC observed was the marked and rapid decrease in photosynthetic
419 efficiency (more than 90% compared with the initial yield; Figure 3). This sudden inhibition of
420 photosynthesis was not expected because the exposure concentration was based on the EC_5
421 for photosynthesis inhibition after four hours of exposure determined in a preliminary
422 experiment ($\text{EC}_5 = 30 \text{ mg.L}^{-1}$; Figure A). However, this result is in line with the high ecotoxicity
423 of DDBAC to freshwater and estuarine/marine algae reported by Arnold et al. (2023), with EC_{50}
424 values as low as $14 \mu\text{g.L}^{-1}$. Based on our microscopic observations, diatom mortality was low
425 in all samples (<10%). It should be noted that, following Morin et al. (2010), only diatoms that

426 no longer had chlorophyll in their cell content were considered as dead cells, while cells with
427 chloroplasts were counted as live diatoms. However, many of the cells where chloroplastic
428 content was observed seemed to have suffered critical alteration of their photosynthetic
429 equipment (Figure B.2). Their integrity was potentially severely affected, which could explain
430 the loss of photosynthetic yield in biofilms exposed to DDBAC. This deterioration of cell
431 integrity may be due to the fact that DDBAC is able to quickly penetrate the cell (Severina et
432 al. 2001). Our results highlight, in agreement with Wood et al. (2014) and Pandey et al. (2017),
433 the ecological relevance of microscopic observations of fresh material to better assess the
434 ecotoxicity of biocides.

435 Marked differences in the autotrophic community composition were highlighted by
436 microscopy counts between d2 and d10 under DDBAC exposure. Indeed, densities of green
437 algae were 10 times lower than under control conditions (Table 2). Despite the lack of
438 replication for pigment analyses, the degradation of chlorophyll pigments in DDBAC
439 treatments (Figure 4) is in line with the impairment of the algal component of the biofilms as
440 highlighted through photosynthetic inhibition and chloroplast alteration. The striking
441 decrease in lipid content in biofilms exposed to DDBAC (Table 3), particularly glycolipids found
442 mainly in thylakoid membranes within photosynthetic cells (Zulu et al., 2018), supports the
443 idea that the contaminant is internalized within phototrophic cells, with deleterious impacts
444 on the autotrophic component. Diatom-rich biofilms are generally source of essential
445 polyunsaturated fatty acids such as eicosapentaenoic acid (Zulu et al., 2018), ensuring a high
446 nutritional quality of this food resource for primary consumers (Brett and Müller-Navarra,
447 1997). Demailly et al. (2019) also demonstrated that diatom fatty acid composition may be
448 altered by organic substances such as pesticides. More recently, Mazzella et al. (2023b)
449 highlighted a decrease in eicosapentaenoic acid content, and more generally in
450 polyunsaturated fatty acids, in DDBAC-exposed biofilms collected from the present study.
451 DDBAC-driven changes in biofilm community structure and impact on glycolipids could
452 therefore lead to a decrease in the nutritional quality of biofilms (decrease in essential
453 polyunsaturated fatty acids, such as certain omega-3 and omega-6) and subsequently affect
454 the trophic chain (Brett and Müller-Navarra, 1997), worsened by DDBAC accumulation.

455 In parallel to the decrease in glycolipids noted under DDBAC contamination, we observed an
456 increase in phospholipids. It is difficult to clearly explain this increase because the
457 phospholipid groups analysed (i.e. PE, PC and PG) are not specific to any taxonomic group and
458 can be found in both the plasma membranes of microalgae (Zulu et al., 2018) and in
459 prokaryotic cells (Li-Beisson et al., 2013). One hypothesis to explain this increase could be the
460 development of DDBAC-resistant bacteria, as mentioned above. Indeed, the mechanism of
461 resistance to benzalkonium chloride identified in *Pseudomonas aeruginosa* consists of an
462 increase in the percentage of phospholipids (Sakagami et al. 1989). Considering the impact of
463 DDBAC on biofilm bacteria would be required to determine if the increase in phospholipids
464 observed in our study reflects the development of specific bacteria under DDBAC exposure.

465 Sensitivity to DDBAC differed among biofilm taxa, whose proportions changed over time, and
466 with DDBAC concentration. This result suggests that the biofilm community acclimated to the
467 chemical stress and that only resistant/tolerant species survived DDBAC exposure according

468 to the Pollution-Induced Community Tolerance concept (Blanck et al., 1988). Diatoms
469 appeared to be minimally affected based on growth and mortality, even though the alteration
470 of their photosynthetic structure and cell content suggests a marked impact (Figure B.2).
471 Green algae, which experienced rapid growth in non-contaminated channels, appeared to be
472 negatively affected by DDBAC at d10. DDBAC also had a strong effect on heterotrophic
473 microfauna naturally present in the biofilms (e.g. rotifers), suggesting that they are highly
474 sensitive to this contaminant. Our dataset does not allow to disentangle whether such impacts
475 derive from direct (through water contamination) or indirect (trophic transfer from
476 contaminated biofilms) effects. However, such results confirm the high sensitivity of aquatic
477 microfauna and invertebrates to DDBAC, as already demonstrated for acute exposure with
478 *Daphnia magna* (Kreuzinger et al., 2007; Leal et al., 1994; Chen et al., 2014; Lavgogna et al.,
479 2016).

480

481 **4.3- Effects of ALAN on the biofilm**

482 Our experimental conditions were based on constant light intensities over time, which does
483 not represent realistic conditions. In the field, daylight variations in irradiation are important
484 drivers of biofilm growth and photosynthetic performance (Laviale et al. 2009). Under our
485 exposure conditions, ALAN did not significantly modify the structure or physiology of the
486 autotrophic organisms in the biofilm based on the parameters that we investigated. The low
487 light intensity chosen, and the use of similar light levels during day and night, may have not
488 been enough to generate stress during continuous light exposure, at the timescale of the
489 experiment. Indeed, using biofilms originating from the same pond and exposed to higher
490 irradiance ($\sim 200 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$), Roux et al. (2024) highlighted that ALAN decreased the daily
491 photosynthetic yield after 14 and 30 days of exposure.

492 Concerning the polar lipid classes, an increase in MGDG or DGDG is often observed during low
493 light intensity exposures (Gushina and Harwood, 2009). In the present experiment, while light
494 was low, the duration of exposure differed, which could explain the absence of a significant
495 effect on these two glycolipids over time. Conversely, high light levels can lead to a decrease
496 in polar lipids, especially phospholipids, in favour of TAGs, especially in the case of filamentous
497 green algae such as *Cladophora* spp. (Gushina and Harwood, 2009). Changes in lipid content
498 with ALAN were not clearly observed here, contrarily to what was observed based on a more
499 detailed assessment of fatty acids conducted on the same samples (Mazzella et al. (2023b).
500 Amini Khoeyi et al. (2011) also showed that a prolonged photoperiod can decrease the content
501 of monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids in the microalgae
502 *Chlorella vulgaris*. Considering the constant low light conditions we used, it is likely that the
503 apical endpoints we measured did not allow to observe subtle changes occurring at the
504 individual or subindividual scales. This could be due to compensation phenomena or to the
505 fact that we averaged lipids across different organisms present within a natural biofilm, which
506 differs from the analysis of an isolated chlorophyte strain.

507

508 **4.4- Combined effects of DDBAC and ALAN on the biofilm**

509 Contrary to our hypotheses, DDBAC and ALAN exposure did not show interaction effects on
510 any of the biological endpoints monitored in this experiment. However, ALAN impacted the
511 fate of DDBAC by decreasing exposure concentrations in the medium under our experimental
512 conditions. This result can hardly be extrapolated to natural light regime conditions, calling for
513 further investigations under more realistic light scenarios. DDBAC exposure caused significant
514 impacts on biofilm function and structure, especially impacting autotrophs. Given the wide
515 range of uses of DDBAC, our study highlights that its transfer to the aquatic environment may
516 result in a significant impairment of the functioning of aquatic ecosystems. As the high
517 concentration of DDBAC tested drove most of the changes observed in biofilm composition
518 and physiology, we cannot exclude that DDBAC exposure masked possible interactions with
519 ALAN. Further experiments combining lower concentrations of DDBAC with more realistic light
520 regimes would be required to completely rule out any interaction effects on aquatic biofilms.

521

522

523 **5- Conclusion**

524 In this experiment, we demonstrated that DDBAC exposure strongly impacts river biofilms.
525 DDBAC accumulated quickly in biofilms and damaged the photosynthetic material, in turn
526 altering photosynthesis. There was also evidence for an impact on the structure of the
527 biofilm as changes in its taxonomic composition were observed. Indeed, even though the
528 mortality index did not show greater mortality in the exposed diatoms compared with the
529 controls, microscopic observations suggest that a marked number of individuals were
530 strongly impacted on a physiological level (degraded cell content) and were potentially not
531 viable. This hypothesis is supported by the results obtained during the lipidomic analysis,
532 highlighting a strong decrease in the lipid classes associated with thylakoid membranes
533 specific to microalgae. At elevated concentrations, we highlighted striking effects of DDBAC
534 on the aquatic biota. As DDBAC is a common contaminant with multiple domestic uses,
535 complementary research is needed to characterize its impacts at environmentally relevant
536 concentrations.

537 In contrast, ALAN did not lead to any significant change in biofilms, even though the ALAN
538 treatment modified their exposure to DDBAC. In view of the likely increase in DDBAC
539 concentration in urban waters (downstream of WWTPs) where ALAN is often common, it
540 would also be necessary to investigate whether the effects identified in the present study are
541 manifested at environmental concentrations, under more realistic light regimes, in order to
542 reassess the risk posed by DDBAC to aquatic biodiversity.

543

544

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546

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550

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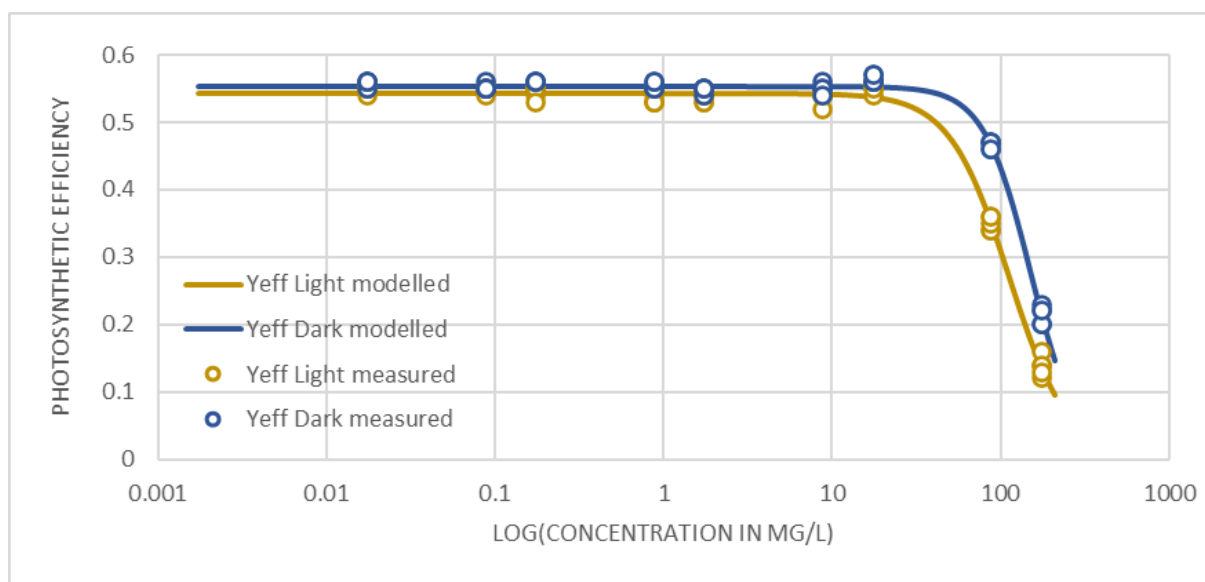
760 **Appendices**

761 **A- Preliminary experiment**

762

763 This preliminary experiment aimed to test the sensitivity of our biofilm to
764 dodecyldimethylbenzylammonium (DDBAC) to select a sub-lethal concentration to be used in
765 the main experiment. We ran a dose-response experiment in which we tested nine
766 concentrations ranging between $17.5 \mu\text{g.L}^{-1}$ and 175mg.L^{-1} (following a logarithmic increase)
767 for 4 hours. Each concentration was tested twice, one test at a mean light level of 16.86
768 $\mu\text{mol.s.m}^{-2}$ and the other kept in the dark.

769 Glass slides ($26.5 \text{ cm} \times 6 \text{ cm}$) colonized by biofilms for three months were scraped and the
770 biofilms put into 500 mL of water. For each concentration, we contaminated 1.5 mL of the
771 biofilm solution. After four hours of contamination, we analysed the photosynthetic efficiency
772 of the samples with a Phyto-PAM (see section 2.3).



773

774 **Figure A. Dose-Response curve for photosynthetic efficiency in biofilms exposed to light or dark and**
775 **increasing DDBAC concentration for 4 hours. Yeff = Photosynthetic efficiency.**

776

777 The dose-response curve suggests an EC_{50} of $112 \pm 3 \text{ mg.L}^{-1}$ and a EC_5 of $34 \pm 3 \text{ mg.L}^{-1}$ for
778 photosynthesis inhibition in the biofilm exposed to light. For the biofilm kept in the dark, we
779 found EC_{50} of $151 \pm 3 \text{ mg.L}^{-1}$ and a EC_5 of $58 \pm 3 \text{ mg.L}^{-1}$. This result allowed us to choose the
780 concentration of 30 mg.L^{-1} for the 10 days biofilm exposure which is the lowest concentration
781 with a minimum effect on photosynthetic efficiency.

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786 **B- Effects of DDBAC on chloroplasts**

787



788

789 **Figure B.1. Chloroplasts of diatoms in non-contaminated biofilm. x400 magnification**

790



791

792 **Figure B.2. Chloroplasts of diatoms in DDBAC contaminated biofilm after 10 days of exposure. x400**

793

794

795 **C- HPLC gradients for the lipidomic analysis**

796

797

Table C.1. HPLC gradients for phospholipid and glycolipid analysis.

798

Time (min)	40 mmol.L ⁻¹ Ammonium acetate buffer (%)	Acetonitrile (%)
0	5	95
2	5	95
7	30	70
10	30	70
11	5	95
13.7	5	95

799

800

Table C.2. HPLC gradients for triglyceride analysis.

801

Time (min)	Solvent A* (%)	Solvent B* (%)
0	50	50
0.3	50	50
5.3	1	99
7.3	1	99
8.3	50	50

9.8	50	50
-----	----	----

802 * Solvent A: solution of acetonitrile/water/40 mmol.L⁻¹ ammonium acetate buffer
803 (600/390/10, v/v/v)

804 Solvent B: solution of isopropanol/acetonitrile/1 mol.L⁻¹ ammonium acetate buffer
805 (900/90/10, v/v/v)

806

807 **D- Lipid standards**

808 Polar lipid standards were purchased from Avanti Polar Lipids. Quantitations of
809 phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) were
810 respectively carried out with 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine or PC (16:0/18:1)
811 (850457), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine or PE (16:0/18:1)
812 (850757), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) or PG (16:0/18:1)
813 (840457).

814 For glycolipids, monogalactosyldiacylglycerol (840523), digalactosyldiacylglycerol (840524)
815 and sulfoquinovosyldiacylglycerol (840525) from plant extracts were used as standards.
816 Quantitation was performed with the following molecular species: MGDG (16:3_18:3) (63% of
817 the total MGDG standard), DGDG (18:3_18:3) (22% of the total MGDG standard), and SQDG
818 (34:3) (78% of the total MGDG standard).

819 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine or PC (2x17:0) (850360) was used as
820 internal standard for PC phospholipids, 1,2-diheptadecanoyl-sn-glycero-3-
821 phosphoethanolamine or PE (2x17:0) (830756) was used as internal standard for PE
822 phospholipids, and 1,2-diheptadecanoyl-sn-glycero-3-phospho-(1'-rac-glycerol) or PG
823 (2x17:0) (830456) was used as internal standard for PG phospholipids, and both MGDG, DGDG
824 and SQDG glycolipids.

825 1,2-dipalmitoyl-sn-glycero-3-O-4'-(N,N,N-trimethyl)-homoserine or DGTS (2x16:0) (857464)
826 was used for the diacylglyceryltrimethylhomo-Ser (DGTS) lipids. 1,2-dipalmitoyl-sn-glycero-3-
827 O-4'-[N,N,N-trimethyl(d9)]-homoserine or DGTS-d9 (2x16:0) (857463) was used as internal
828 standard for DGTS lipids

829 Triglycerides were purchased from Sigma-Aldrich. Tristearin or TAG (3x18:0) (≥99%, T5016)
830 was used as the calibration standard while TAG (3x17:0) (≥99%, T2151) was used as the
831 internal standard.

832

833

834

835 **E- Mass spectrometry parameters for lipid analysis**

836

837 **Table E.1. Mass spectrometry parameters for phospholipid and glycolipid analysis.**

838

	Curtain gas	CAD	IonSpray	Temperature	Ion source gas 1	Ion source gas 2	Declustering potential	Collision energy
MGDG	30 psi	3	-4500 V	450°C	30 psi	60 psi	-61 V	-28 V
DGDG	30 psi	3	-4500 V	450°C	30 psi	60 psi	-61 V	-28 V
SQDG	30 psi	3	-4500 V	450°C	30 psi	60 psi	-126 V	-66 V
PE	30 psi	3	-4500 V	450°C	30 psi	60 psi	-50 V	-50 V
PG	30 psi	3	-4500 V	450°C	30 psi	60 psi	-100 V	-50 V
PC	30 psi	3	-4500 V	450°C	30 psi	60 psi	-100 V	-50 V

839

840

841 **Table E.2. Mass spectrometry parameters for triglyceride analysis.**

	Curtain gas	CAD	IonSpray	Temperature	Ion source gas 1	Ion source gas 2	Declustering potential	Collision energy
Triglycerides	30 psi	3	+5000 V	450°C	30 psi	45 psi	50 V	38 V

842