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1 Urban stressors impact lipid profiles in freshwater periphytic communities

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9 10 Abstract

11
12 The composition of lipids in algae are significantly influenced by environmental factors,
13 including light intensity. Exposure to organic and inorganic contaminants can also disrupt the
14 synthesis of fatty acids, changing the lipid composition of microalgae in periphytic
15 communities. In this study, we looked at how a biocide such as
16 dodecylbenzyltrimethylammonium chloride (BAC 12) and two photoperiod durations can affect
17 a biofilm's polar lipidome in a microcosm experiment. The heterotrophic compartment
18 appeared to be raised by exposure to BAC 12 at the expense of phototrophic organisms.
19 Additionally, the overall decline in polyunsaturated fatty acids indicated that the biofilm's
20 phototrophic organisms were all severely impacted. However, it may be difficult to differentiate
21 the effects of contamination from those of light, since there was no observable effect of
22 photoperiods on the conventional fatty acid determination. The molecular species composition
23 of both glycolipids and phospholipids was investigated in additional multivariate analyses. It
24 was suggested that some molecular species may serve as more specific markers of light duration
25 at the biofilm scale. Lastly, we recommend applying a similar lipidomic approach with
26 monospecific cultures of microalgal strains in future research to support these findings, as the
27 methodology used in this study would be applicable to other biofilm-derived microorganisms.

28 Keywords

29 Artificial light at night, dodecylbenzyltrimethylammonium chloride, biofilm, microalgae,
30 lipidomics, fatty acids.

31 1. Introduction

32

33 Freshwater biofilms are composed of a large variety of microorganisms, covering all
34 kingdoms of life (bacteria, archaea, fungi, plantae, protista and animalia). Lipids can be
35 considered as markers of the structure of a community of microorganisms. Fatty acids in
36 autotrophic organisms, such as diatoms, green algae, cyanobacteria, and fungi, have distinct
37 lipid profiles. Diatoms primarily consist of 14-, 16-, and 20-carbon saturated and unsaturated
38 fatty acids (FA), while they produce minor or negligible 18-carbon FAs (Opote 1974).
39 Chlorophyceae and cyanophyceae produce more octadecadienoic and octadecatrienoic fatty
40 acids, while green algae produce hexadecatrienoic and hexadecatetraenoic acids more
41 significantly (Zäuner et al. 2012). Thylakoid membranes in algae mainly consist of glycolipids,
42 while phospholipids like phosphatidylethanolamine (PE), phosphatidylcholine (PC) and
43 phosphatidylglycerol (PG) are less specific due to their presence in both microalgae and
44 prokaryotic cells (Zulu et al. 2018, Li-Beisson et al. 2019). Algal lipid content is also strongly
45 influenced by environmental factors. For instance, at low temperatures, the degree of
46 unsaturation increases to maintain membrane fluidity and integrity, leading to an increase in
47 polyunsaturated fatty-acids (PUFA) compared to saturated fatty-acids (SFA) and monosaturated
48 fatty-acids (MUFA) (Fuschino et al. 2011). Light conditions are also known to affect lipid
49 composition in algae. As lipids are important components of thylakoid membranes, they are
50 involved in the regulation of photosynthetic capacity (Wacker et al. 2015). Lastly, exposure to
51 organic and inorganic contaminants can likewise interfere with fatty acid synthesis, leading to
52 a change in algae and biofilm lipid content (Robert et al. 2007, Filimonova et al. 2016,
53 Fadhlouli et al. 2020). Recent works have focused on fatty acids as algal biomarkers of
54 environmental stress because their composition is very sensitive to stressors and environmental
55 modifications (Arts et al. 2001, Demailly et al. 2019). However, the combined effects of

56 stressors in an urban environment and the subsequent reactions of microorganisms within
57 biofilms remain poorly understood. An earlier research aimed at determining the individual and
58 combined effects of urban stressors, namely dodecylbenzyltrimethylammonium chloride (BAC
59 12) contaminant and Artificial Light at Night (ALAN) on autotrophic organisms in the biofilm
60 (Vrba et al. 2023). This previous work revealed the predominant effects of the biocidal activity
61 on autotrophic organisms in the environment, both at the scale of algal group (i.e., green algae,
62 diatoms and cyanobacteria) and at the level of photosynthetic response. The evolution of major
63 lipid classes, including phospholipids such as PE, PC and PG, glycolipids such as
64 monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and
65 sulfoquinovosyldiacylglycerol (SQDG) was also determined. These previous results are
66 complemented here by the determination of the molecular species composition within PE, PC,
67 PG, MGDG and DGDG classes. Identification and quantification of the molecular species also
68 allowed estimation of the major fatty acid fractions. More precisely, the following study will
69 (i) address the effects of BAC 12 on the fatty acid composition of polar lipids through the study
70 of molecular species, and (ii) discuss the possible effect of light (i.e., alternating or continuous
71 photoperiod) that seemed difficult to uncouple from the strong and overlapping effect of the
72 biocide.

73

74

75 2. Experimental section

76

77 2.1. Chemicals and reagents

78 The following polar lipid standards were purchased from Avanti Polar Lipids: 1-
79 palmitoyl-2-oleoyl-glycero-3-phosphocholine or PC (16:0/18:1) (850457), 1-palmitoyl-2-
80 oleoyl-sn-glycero-3-phosphoethanolamine or PE (16:0/18:1) (850757), 1-palmitoyl-2-oleoyl-
81 sn-glycero-3-phospho-(1'-rac-glycerol) or PG (16:0/18:1) (840457), 1,2-diheptadecanoyl-sn-
82 glycero-3-phosphocholine or PC (17:0/17:0) (850360), 1,2-diheptadecanoyl-sn-glycero-3-
83 phosphoethanolamine or PE (17:0/17:0) (830756), 1,2-dipentadecanoyl-sn-glycero-3-
84 phosphoethanolamine or PE (15:0/15:0) (850704), and 1,2-diheptadecanoyl-sn-glycero-3-
85 phospho-(1'-rac-glycerol) or PG (17:0/17:0) (830456), L- α -phosphatidylserine (Soy, 99%)
86 (sodium salt) (870336) for the phospholipid standards, and monogalactosyldiacylglycerol
87 (840523), digalactosyldiacylglycerol (840524) and sulfoquinovosyldiacylglycerol (840525)
88 from plant extracts as glycolipid standards. Ammonium acetate (LiChropur) were provided by
89 Sigma-Aldrich. Palmitic acid (76119), oleic acid (O1008), heptadecanoic acid (H3500) and
90 eicosapentanoic acid (44864) analytical standard grades (purity \geq 98 %) were purchased from
91 Sigma-Aldrich. Acetonitrile, methanol (MeOH) tert-Butyl methyl ether (MTBE) and
92 isopropanol HPLC grades were purchased from Biosolve Chimie, France. Ultrapure water
93 (UPW) was obtained from Direct-Q® Water Purification System (Merck Millipore).
94 Dodecylbenzyltrimethylammonium chloride (BAC 12, purity \geq 99 %) and Benzyl-2,3,4,5,6-ds-
95 dimethyl-n-dodecylammonium chloride (purity $>$ 98 %) were obtained from Sigma-Aldrich and
96 Cluzeau (France), respectively.

97 2.2. Experimental design

98 Glass slides (individual surface area: 150 cm²) were placed in a small hypereutrophic
99 pond in Cestas (Bordeaux, France)(Chaumet et al. 2019) for colonization by microbial biofilms.

100 After five months, colonized substrates were removed from the natural environment and were
101 randomly placed in experimental channels (on day 0 = T0) under laboratory conditions as
102 described in Vrba et al. (2023). Briefly, four experimental conditions were set up, each in
103 pseudo-triplicates (i.e., separate 10-L channels fed by a common 10-L tank). The tanks and
104 channels were filled with filtered (20 μm) pond water, at a room temperature of $20.5 \pm 0.1^\circ\text{C}$
105 and water temperature of $18.7 \pm 0.2^\circ\text{C}$. The four conditions were as follow: two treatments
106 under uncontaminated conditions (controls, CTRL) and two treatments exposed to 30 mg L^{-1}
107 of BAC 12 (BAC). All channels were exposed either to normal light (NL) corresponding to a
108 14h day/10h night photoperiod ($20 \mu\text{mol m}^{-2} \text{ s}^{-1}$) or to continuous light (CL). Treatments will
109 be referred as CTRL-NL, CTRL-CL, BAC-NL and BAC-CL. On day 0, biofilms were collected
110 to assess their initial lipid composition, prior to exposure to either BAC or CL. On day 10 (T10),
111 biofilms were collected to analyse qualitative and quantitative changes in lipid profiles.
112 Biofilms were immediately quenched in liquid nitrogen, and then collected by scraping the
113 glass slides with a razor blade. The samples were freeze-dried (Benchtop Ro 8LZL BTF) and
114 kept at -80°C until the extraction step.

115 **2.3. Nutrient and BAC12 dosing in exposure water**

116 Nutrients and mineral salts were monitored and analyzed using a Metrohm 881 Compact
117 Ionic Chromatograph pro (Metrohm). Anion analysis (PO_4^- , NO_3^- , NO_2^- , Cl^- and SO_4^{2-}) was
118 performed using a Supp 4/5 Guard/4.0 precolumn followed by a Metrosep A Supp5 – 250/4.0
119 column. The mobile phase was a mixture of a solution of $3.2 \text{ mmol L}^{-1} \text{ Na}_2\text{CO}_3$ and a solution
120 of $1 \text{ mmol L}^{-1} \text{ NaHCO}_3$. Cation analysis (Na^+ , K^+ , Ca^{2+} , Mg^{2+} and NH_4^+) was performed using
121 a C4 Guard/4.0 precolumn followed by a Metrosep C6 - 250/4.0 column). The eluent used was
122 a mixture of $2.5 \text{ mmol L}^{-1} \text{ HNO}_3$ and a solution of 1.7 mmol L^{-1} 10,12-Pentacosadynoic acid
123 (PCDA). Calibration ranges were from 20 to $1000 \mu\text{g.L}^{-1}$. BAC 12 concentrations in the water
124 were monitored at the beginning (T0) and the end of experiment (T10). Three samples of 20

125 mL were collected from each channel and stored at -20°C together with the stock solution until
126 analysis. The samples were analyzed using an Ultimate 3000 HPLC coupled with an API 2000
127 triple quadrupole mass spectrometer. A Gemini® NX-C18 column (Phenomenex) was used as
128 a stationary phase. The mobile phase was 90:10 (5 mM ammonium acetate:acetonitrile, v/v).
129 The chromatographic separation was done in isocratic mode with a flow rate of 0.6 mL min^{-1} .
130 The injection volume was set at $20\text{ }\mu\text{L}$. An internal standard of benzyl-2,3,4,5,6- d_5 -dimethyl-
131 n-dodecylammonium chloride was used, and its concentration in sample vials was typically 100
132 ng mL^{-1} . Samples were diluted and the calibration range was from 1 to $200\text{ }\mu\text{g L}^{-1}$. Quality
133 controls were regularly injected at concentrations of 5 and $25\text{ }\mu\text{g L}^{-1}$, as well as analytical
134 blanks. Concentration values for either BAC 12 (Table A 1) or nutrients are available in Vrba
135 et al. (2023).

136 **2.4. Lipid extraction**

137 The biofilm samples (10-20 mg of dry mass) were weighed using a Mettler Toledo
138 NS204S precision balance and placed in 2 mL microtubes with 150 mg of microbeads. The
139 biphasic extraction procedure involved addition of 1 mL of a MTBE:MeOH (3:1, v/v) mixture
140 and $650\text{ }\mu\text{L}$ of a UPW:MeOH (3:1, v/v) mixture. Prior to extraction, $50\text{ }\mu\text{L}$ of a PE (15:0/15:0)
141 solution containing $100\text{ ng }\mu\text{L}^{-1}$ was added as a surrogate. Samples containing microbeads were
142 mechanically homogenized and extracted (3 cycles of 15 s) with the solvent mixtures by using
143 a MP Biomedicals FastPrep-24 5G. The upper lipophilic phase (MTBE) was separated from
144 the lower hydrophilic phase (UPW and MeOH) by centrifugation at 12,000 RPM. $600\text{ }\mu\text{L}$ of
145 the lipophilic phase was collected. A second extraction step extracted (3 cycles of 15 s) was
146 carried out after adding $700\text{ }\mu\text{L}$ of MTBE:MeOH mixture 3:1 (v/v) and $455\text{ }\mu\text{L}$ UPW:MeOH
147 mixture 3:1 (v/v). The supernatant (MTBE) was collected again and added to the previous one.
148 Only organic lipophilic phases (1.1 mL) were kept for further polar lipid analysis. Extracts were
149 stored at -80°C and solvent underwent extraction as a procedural blank to verify the absence of

150 contamination during extracting procedures. Further details regarding the whole extraction
151 procedure can be found in Mazzella et al. (2023b). The samples were then diluted in appropriate
152 solvent injection (typical volume of 1 mL) and stored at -18°C until analysis within one week.

153 **2.5. HPLC-ESI-MS/MS analysis**

154 Lipid extracts were also analyzed with a Dionex Ultimate 3000 HPLC (Thermo Fisher
155 Scientific, France) coupled with an API 2000 triple quadrupole mass spectrometer (Sciex,
156 France). Chromatographic separation of both glycolipids and phospholipids was performed on
157 a Luna NH2 HILIC column (3 µm, 100 × 2 mm) with a Security Guard cartridge NH2 (4 × 2.0
158 mm). The injection volume and temperature were set to 20 µL and 40°C, respectively. The
159 chromatographic separation and mass spectrometry conditions are described in Mazzella et al.
160 (2023a), (2023b). Quantitation of phosphatidylcholine (PC), phosphatidylethanolamine (PE)
161 and phosphatidylglycerol (PG) were respectively carried out with: PC (16:0/18:1), PE
162 (16:0/18:1), PG (16:0/18:1). Quantitation of glycolipids was carried out with MGDG
163 (16:3_18:3) (63 % of the total MGDG standard), DGDG (18:3/18:3) (22 % of the total MGDG
164 standard), and SQDG (34:3) (78 % of the total SQDG standard). The internal standards utilized
165 were PC (17:0/17:0) for PC phospholipids, PE (17:0/17:0) for PE phospholipids and both
166 MGDG and DGDG glycolipids, and PG (17:0/17:0) for PG and SQDG. Concentrations for both
167 phospholipids and glycolipids were reported in nmol mg⁻¹ (dry weight), and the limits of
168 quantification were typically between 0.02-0.05 nmol mg⁻¹, depending on analyte response.

169 In addition to intact lipids, free fatty acids were analyzed by RPLC-ESI-MS/MS using
170 the same analytical equipment. However, in that case, chromatographic separation was
171 performed on a Kinetex C8 column (2.8 µm, 100 x 2.1 mm). Heptadecanoic acid (C17:0) was
172 used here as an internal standard. Finally, the chromatographic and acquisition parameters are
173 given in the appendices (Table A 2 and Table A 3).

174 **2.6. Phospholipid and glycolipid nomenclatures**

175 Polar glycerolipids are constituted of a glycerol backbone esterified by two fatty acids
176 on the sn-1 and sn-2 positions. The moiety linked to the sn-3 position refers to the polar head
177 group (e.g., sn-phospho-3-glycerol for the PG, a β -D-galactosyl group for MGDG). Each polar
178 head group defines a phospholipid or glycolipid class, and each class can be divided into several
179 molecular species according to the fatty acyl chain composition and distribution. When the fatty
180 acyl chain structures are resolved but the sn-1 and sn-2 positions remain unclear, then the
181 phospholipids or glycolipids are designated PL (C:n_C:n), with C referring to the sum of the
182 number of carbon atoms and n to the number of double bonds for each fatty acyl chain.

183 **2.7. Conversion of phospholipids and glycolipids to fatty acid equivalents**

184 Following the analysis of the different classes of polar lipids, and using the molecular
185 species within each class, the different fatty acids were determined from the acyl chains
186 previously identified. To this purpose, each mole of each molecular species was converted into
187 its fatty acid equivalent.

188 Equation 1 $1 \text{ mole of MGDG}(16:3/18:3) \rightarrow 1 \text{ mole of } 16:3 + 1 \text{ mole of } 18:3$

189 Equation 2 $1 \text{ mole of PG}(18:1/18:1) \rightarrow 2 \text{ moles of } 18:1$

190 Equation 1 illustrates the case where the acyl chains are asymmetric (i.e. the fatty acid at *sn-1*
191 is different from that at *sn-2*), while Equation 2 corresponds to the case where two fatty acids
192 with both the same numbers of carbons and unsaturations are present.

193 **2.8. Data analyses**

194 Data were processed using R 4.2.2 software (R Core Team, 2022) as well as Excel 2016
195 with XLstat 2010 add-on statistical software. Each Principal Components Analysis (PCA) was
196 performed with Pearson correlations. Non-parametric tests (Kruskal-Wallis) and multiple

197 comparison methods (Conover and Iman) were respectively carried out with a global p-value
198 ≤ 0.05 and Bonferroni corrections. Multivariate analysis of variance (MANOVA) and
199 subsequent analysis of variance (ANOVA) were performed with Welch test corrections.

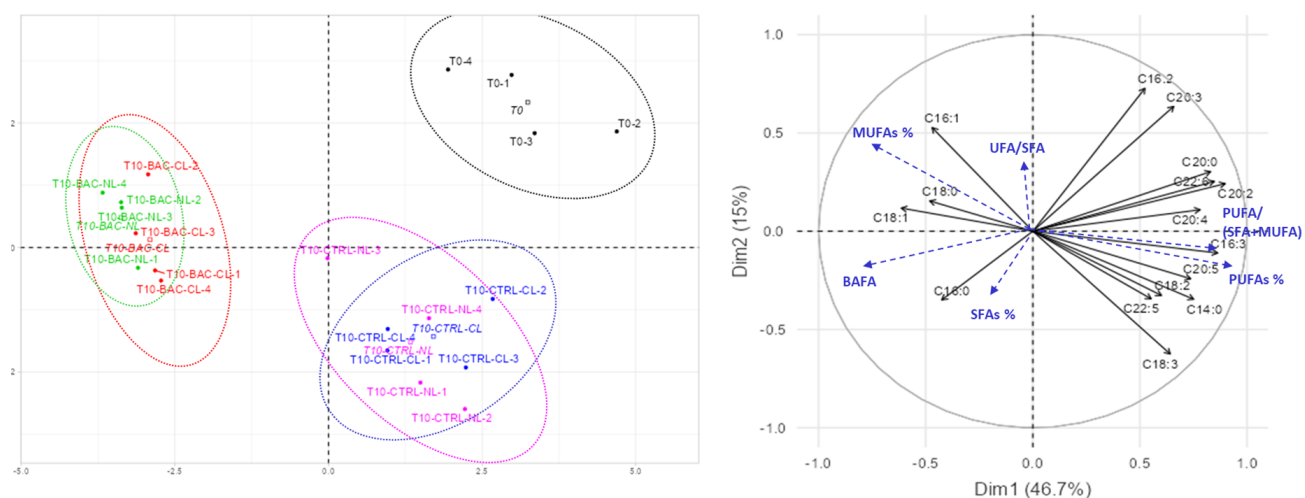
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201 **3. Results**

202

203 **3.1. Fatty acids from polar lipids**

204 Fatty acids obtained from PG, PE, PC, MGDG and DGDG, as well as free fatty acids
205 (FFA) (Table A 4), were plotted on a Principal Component Analysis (PCA) (Figure 1 and Figure
206 A 1). It should be noted that FFA generally represented a small proportion with 3 to 12% of the
207 total fatty acids quantified in the various sample extracts, regardless of the condition applied
208 (Figure A 2). Polyunsaturated fatty acids (C16:3, C18:3, C20:2, C20:3, C20:4, C20:5, C22:5
209 and C22:6) loaded along axis 1. These PUFAs appeared to be more abundant in the samples
210 under CTRL conditions, both at the initial time (T0) and after 10 days of exposure. Conversely,
211 two saturated fatty acids and monounsaturated fatty acids (16:1, 18:0 and 18:1) were most
212 abundant after exposure to BAC12 for 10 days (Vrba et al. 2023). The second axis of the PCA
213 only explained 15.0 % of the total inertia compared to 46.7 % for the first axis. This second
214 axis did not allow for a clear discrimination of other factors, such as a possible effect of normal
215 light (NL) or continuous light (CL) conditions. Another representation can be obtained with a
216 combination of the second and the fourth axes (6.4 %), with however a consequent overlapping
217 of the confidence intervals for T10 CTRL-NL and-CL samples (Figure A 1).



218

219 Figure 1. PCA (dimensions 1 and 2) with initial samples (T0), controls after 10 days (T10
 220 CTRL) according to continuous (CL) or normal (NL) light, as well as samples contaminated
 221 with BAC 12, after 10 days (T10 BAC) and also with the two light conditions (CL and NL).
 222 The variables represented on the right correspond to fatty acids determined from polar lipids,
 223 as well as free fatty acids. % SFAs, MUFAs and PUFAs as well as BAFA, UFA/SFA and
 224 PUFA/(SFA+MUFA) ratios are represented as supplementary variables in blue.

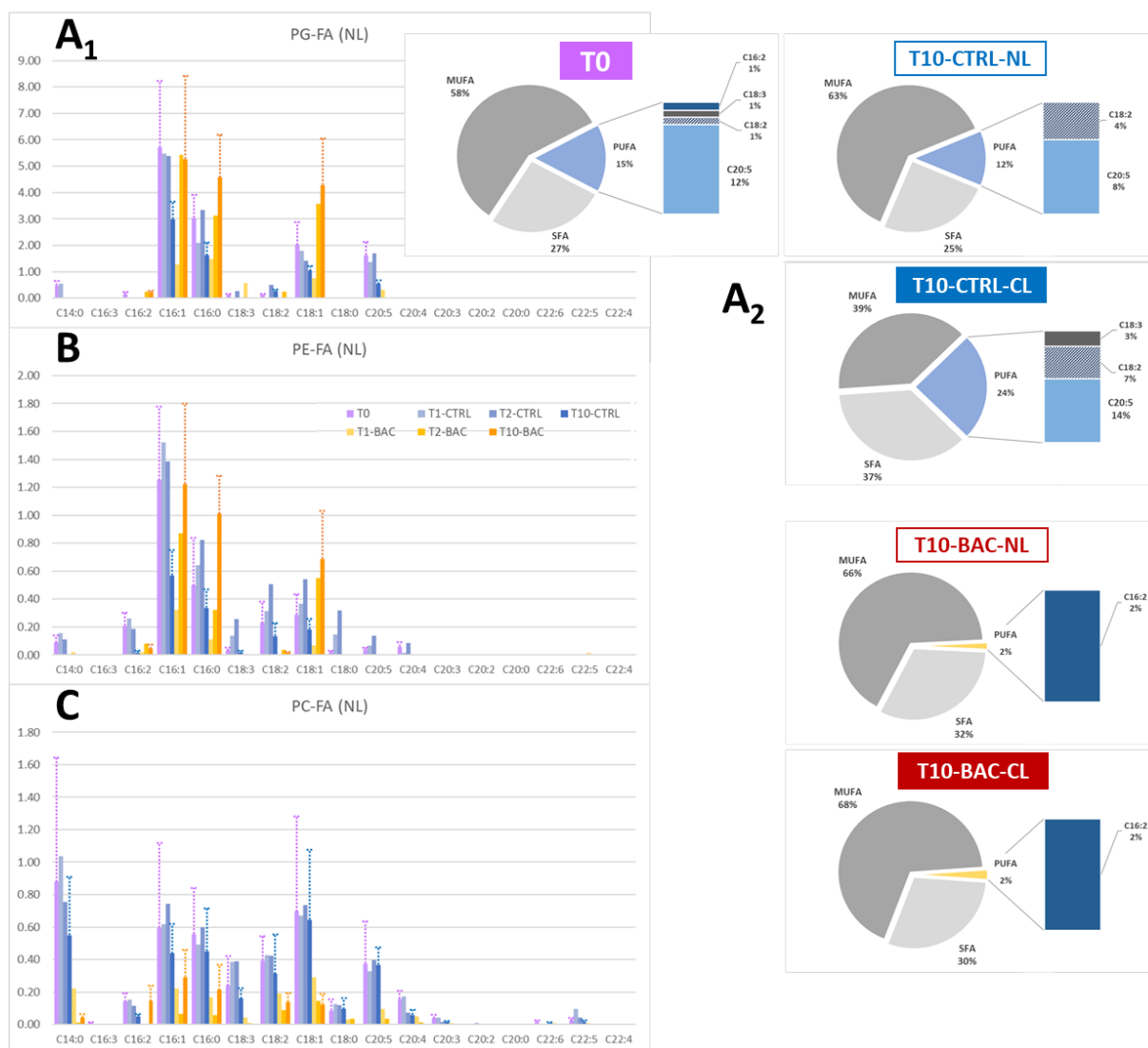
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226 The amounts of fatty acids (in nmol mg⁻¹ of dry biofilm) obtained from the molecular
 227 species of the phospholipids PG, PE and PC are presented in of Figure 2 (A1, B and C). These
 228 barplots are presented only for the alternating photoperiod NL condition because the PCA
 229 previously indicated that photoperiod did not markedly contribute to biofilm fatty acid
 230 composition. The results presented in Figure 2 suggest a near disappearance of C20:5 within
 231 PG, while C16:0, C16:1 and C18:1 highly increased in the presence of BAC 12. Panel A2
 232 presents fatty acids from PG according to the categories SFAs, MUFAs and PUFAs. For the
 233 uncontaminated controls, MUFAs seemed to be in the majority (58-63%) under normal light
 234 condition at both T0 and T10. However, MUFAs appeared to be in equivalent proportion to
 235 SFAs (39 and 37%, respectively) in the continuous light condition. PUFAs also seemed to
 236 increase in the T10-CTRL-CL, however, this remains a trend as no significant differences were
 237 found according to a non-parametric Kruskal-Wallis test. When considering either T10-BAC-
 238 NL or CL samples a drastic decrease in PUFAs, essentially in favor of MUFAs, was observed

239 in the presence of BAC 12. It should be noted that C20:5, which composed the majority of the
240 PUFAs in the CTRL samples, disappeared completely with exposure to the biocide.

241 The initial fatty acid composition (T0) from PE (Figure 2, panel B), as well as the
242 composition after 2 and 10 days of experiment without BAC12 contamination, differed from
243 that of PG where C18:2 and C18:3 were abundant and where C20:5 and C20:4 showed very
244 low concentrations. Within the same phospholipids, and as observed for PG, an increase in
245 MUFAs with 16 or 18 carbon atoms was observed, as well as the SFA C16:0. Finally, PC was
246 characterized by an equivalent distribution of C18:2, C18:3 and C20:5 within the PUFAs from
247 this class of membrane lipids, with contents essentially between 0.2 and 0.4 nmol mg⁻¹ (Figure
248 2, panel C). We did not observe an increase in SFAs or MUFAs as a result of decreasing PUFAs.
249 The results rather suggest that it is the fatty acids from PC that decreased in absolute values.

250



251

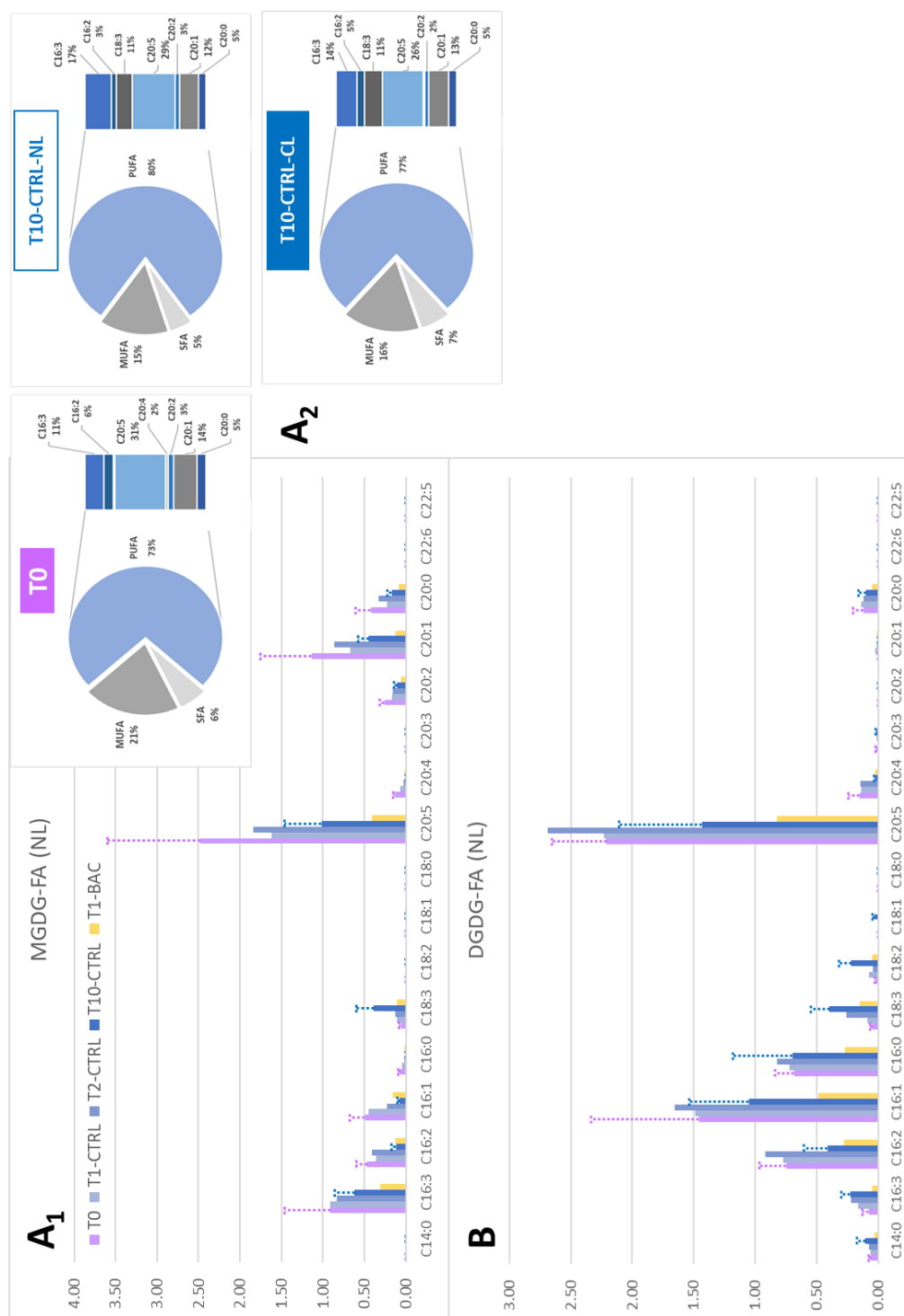
252 Figure 2. Evolution of fatty acids (nmol mg⁻¹) deduced from the molecular species of
 253 phospholipids (A1) PG, (B) PE and (C) PC. Different times are reported here with 1, 2 and 10
 254 days of culture with or without BAC 12 and under normal light conditions. Part A2 on the right
 255 illustrates for PG the grouping of fatty acids (% mol) within SFA, MUFA and PUFA, and then
 256 the details for PUFAs only, for T0 and T10 samples.

257

258 Afterward, we considered the fatty acids associated with MGDG and DGDG. As
 259 explained in Mazzella et al. (2023a), the method we used did not allow for the identification of
 260 the acyl chains of SQDG, and therefore did not allow for an accurate determination of
 261 associated fatty acids. Panels A1 and B of Figure 3 shows the evolution of the content of each
 262 fatty acid within MGDG or DGDG. The most striking response was the near disappearance of
 263 all fatty acids upon exposure to BAC 12, whatever the light conditions, and this was observed

264 from the first day. This may result from the sharp decline in MGDG and DGDG under those
 265 same conditions, as previously observed in Vrba et al. (2023).

266



267

268 Figure 3. Evolution of fatty acid concentrations (nmol mg⁻¹) calculated from the molecular
 269 species of glycolipids (A1) MGDG, (B) DGDG. Panel A2 illustrates fatty acids (% mol)
 270 within SFA, MUFA and PUFA for MGDG as well as individual PUFAs.

271

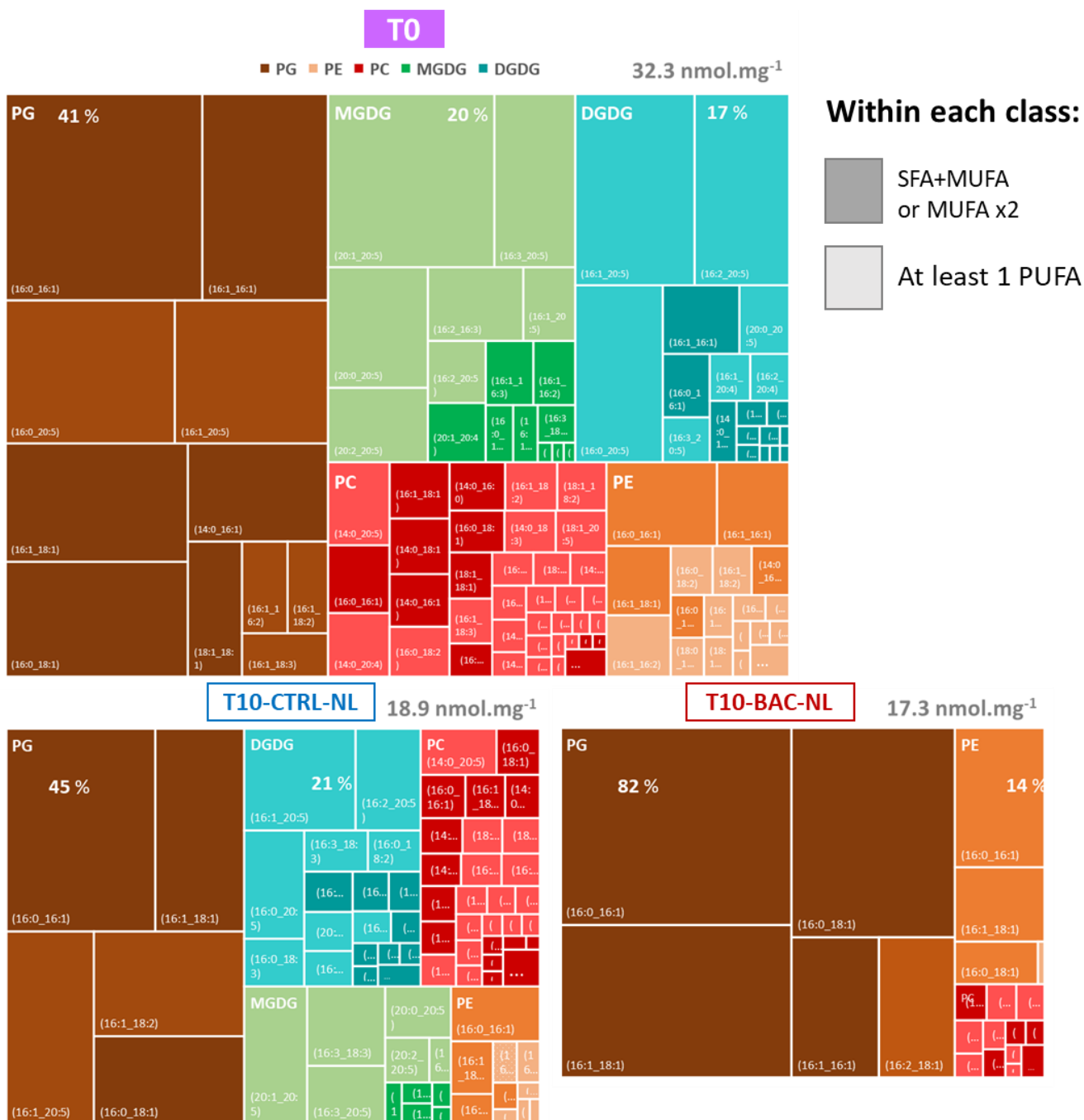
272 In the absence of contamination, C20:5 was the most abundant fatty acid in MGDG with
273 both photoperiods (Figure 3, panel A2), followed by C16:3, C16:2 and C16:1 and long chain
274 fatty acids such as C20:1 and C20:0. All fatty acids decreased over time with the exception of
275 C18:3, which became more abundant at T10. Panel A2 of Figure 3 shows a noticeable stability
276 in SFA, MUFA and PUFA after 10 days of growth in the artificial river channels. Looking at
277 the relative proportions of each PUFAs, we could see a clear increase in C18:3 at the final
278 sampling time with a relative proportion of almost 11 % compared to less than 1 % at the initial
279 time. The fatty acid composition of DGDG was quite distinct from that of MGDG, with a more
280 abundant pool of 16-carbon fatty acids, particularly centered around C16:1. The SFA, MUFA
281 and PUFA categories are not represented here, however they appeared rather similar with a
282 clear majority of PUFAs, as well as an equally stable composition over time in the samples not
283 exposed to BAC 12.

284 **3.2. Molecular species from polar lipids**

285 The molecular species identified within the main classes of PG, PE, PC, MGDG and
286 DGDG are presented in Figure 4. The area of each rectangle is proportional to the relative
287 amount in molar %. Only T0 and T10 under normal light conditions, with or without exposure
288 to BAC 12, are shown. A very similar trend was observed under continuous light conditions
289 and was consequently not illustrated here. At T0, PG clearly dominated with approximately
290 41%, then MGDG and DGDG with 20 and 17%, respectively. These polar lipids are generally
291 associated with thylakoid membranes in plants. Together, PC and PE represented less than a
292 quarter of the polar lipids. These compounds are more representative of cytoplasmic
293 membranes in plants. As with the fatty acids, we observed several molecular species containing
294 at least one PUFA on each of the two acyl chains of each glycerolipid. In the case of PG, and
295 especially MGDG and DGDG, we observed that most fatty acids were a combination between

296 eicosapentaenoic acid (EPA or C20:5) and a SFA or a MUFA such as C16:0, C16:1, C20:0 and
297 C20:1. To a lesser extent, C20:5 appeared to be associated with C16:2 or C16:3.

298 After a 10-day exposure in the channels under normal light (T10-CTRL-NL), MGDG
299 decreased in proportion comparable to DGDG and PC. Overall, we still observed many
300 molecular species containing 1 or 2 PUFAs, but there was a slight increase in the representation
301 of combinations between 16:3, 18:2 and 18:3 compared to the initial condition (T0). With the
302 exposure to the biocide BAC 12 (T10-BAC-NL), we observed a radical change in both classes
303 (i.e. MGDG and DGDG), with the general disappearance of the two glycolipids, as well as an
304 apparent decrease in the number of compounds containing at least one PUFA. Thus, we were
305 able to discern the presence of PG (16:0_16:1), PG (16:0_18:1) or PG (16:1_18:1). The same
306 applies to PE, the second most abundant phospholipid (PG and PE representing almost 96% of
307 the initial polar lipids), with essentially mostly combinations between C16:0, C16:1 and C18:1.



308

309 Figure 4. Tree-map of molecular species, belonging to each of the polar lipid classes, detected
 310 for samples at T0 and T10, contaminated or not with BAC, and for alternating/normal light
 311 (NL) only. The surface of each block is proportional to the amount in nmol mg⁻¹.

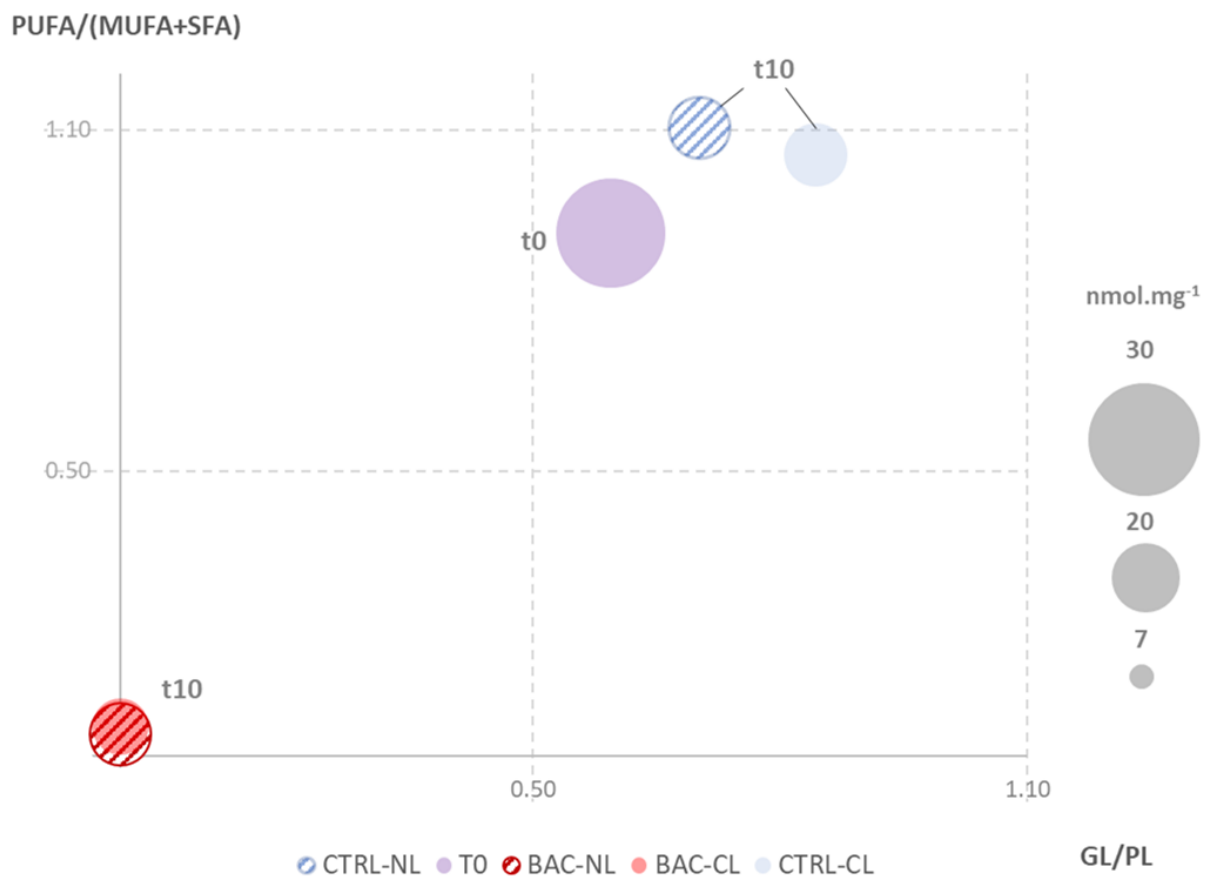
312

313 3.3. Covariation of polar lipid classes and fatty acid categories

314 A clear effect of BAC 12 on the evolution of both absolute and relative amounts of lipid
 315 content in freshwater biofilms has been shown here at the fatty acid level, as previously reported

316 at the lipid class level in Vrba et al. (2023). For each date and condition, we have plotted on
317 abscissa the ratio between the average amount of glycolipids (GL), represented here by all
318 molecular species of MGDG and DGDG, and the average amount of phospholipids (PL) such
319 as PG, PE and PC (i.e. GL/PL ratio). The ordinate is another ratio comprising all PUFAs (from
320 all the classes) over the sum of the MUFAs and SFAs determined simultaneously in the same
321 samples (i.e. (PUFA/(MUFA+SFA) ratio). In the top right-hand quadrant, for GL/PL and
322 PUFA/(MUFA+SFA) ratios between 0.5 and 1.1, we can observe all the samples over the time,
323 and whatever the photoperiod applied, corresponding to non-contaminated conditions. The
324 unique and significant effect of BAC 12 was supported with a MANOVA for the two ratios
325 (Table A 6). These are probably the highest values that the two indices can reach, indicating at
326 the same time a proportion of PUFAs between one third and one half of all fatty acids, and a
327 proportion of glycolipids between 30 and 40% of all polar lipids of both thylakoids and cell
328 membranes. On the other hand, the lower left quadrant, with proportions that are both close to
329 zero, i.e. the near disappearance of glycolipids and PUFAs, consists exclusively of samples
330 contaminated with BAC 12. It should also be noted that the samples did not appear to be
331 differentiated, whatever the light condition applied (NL or CL). These results suggested that
332 PUFAs, even when considered as a whole, are essentially associated with glycolipids in this
333 biofilm. In other words, the joint disappearance of MGDG and DGDG would lead to a
334 consequent and sharp decrease in this category of fatty acids. The results of corresponding
335 Welch's ANOVA was provided as supplementary information (Figure A 3).

336



337

338 Figure 5. Two-dimension scatter plot of samples over time, contaminated or not with BAC 12,
 339 and with two light conditions. The size of the circle corresponds to the mean quantity of polar
 340 lipids per sample and per condition.

341

342

343 4. Discussion

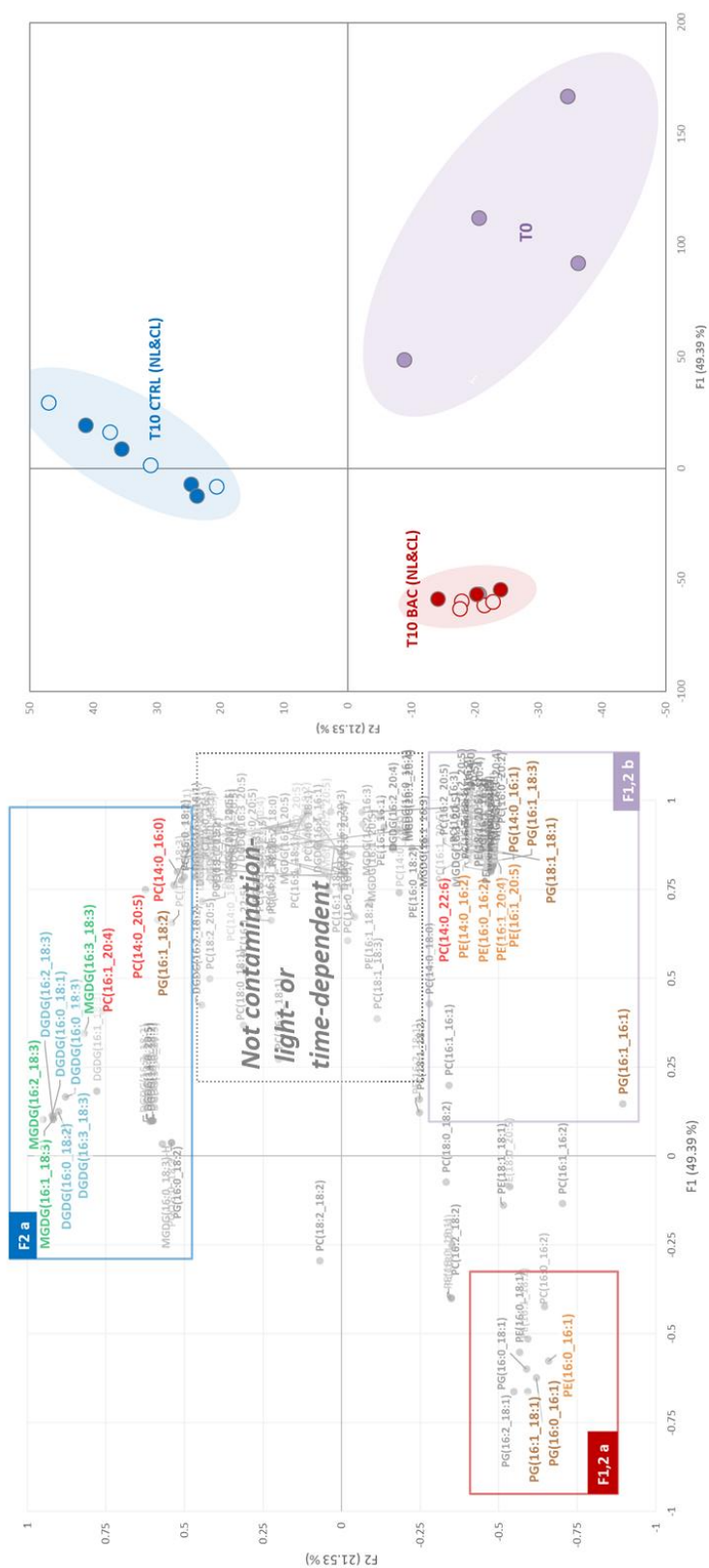
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345 4.1. BAC 12 effects over the time

346 The results presented in Figure 1, Figure 2 and Figure 3 showed that the samples
347 contained polyunsaturated fatty acids in the absence of BAC 12, indicating that the biofilms
348 contained photoautotrophs belonging to the bacillariophyceae, chlorophyceae and
349 cyanophyceae groups (Vestal and White 1989, Guschina and Harwood 2009). This was also
350 confirmed by fluorimetry and microscopy analyses (Vrba et al. 2023). Bacterial FA (BAFA
351 index) with the sum of some SFAs and MUFAs like C15:0, C15:1, C16:0, C17:0, C17:1, C18:0
352 and C18:1n-7 (Napolitano 1999, Dalsgaard et al. 2003) can also be used to estimate the
353 contribution of bacteria to the biofilm community. The projection of the BAFA index among
354 the additional variables in the PCA (Figure 1) could indicate an increase of the heterotrophic
355 compartment over the time, under BAC 12 exposure, at the detriment of phototrophic
356 organisms. In Figure A 1, axis 4 seems to allow for the separation between the continuous and
357 alternating light conditions only in the T10-CTRL samples. This subtle distinction seems to be
358 attributed to C16:0 and C18:3, which contributed more markedly to the CL condition, whereas
359 C18:1 and C18:2 contributed more markedly to the NL condition. However, certain of these
360 fatty acids (i.e., C18:2 and C18:3) were also potentially impacted by the presence or absence of
361 BAC12 and, therefore, it becomes difficult to clearly disentangle the two factors solely based
362 on the fatty acid composition of the polar lipids. The disappearance of certain classes of lipids
363 (e.g., MGDG and DGDG) during exposure to BAC 12 has already been observed in the
364 previous work conducted by Vrba et al. (2023). The overall decrease in PUFAs observed here
365 on the same samples, also suggests a drastic decrease of all phototrophic organisms within the
366 biofilm. It should be noted that other contaminants such as S-metolachlor, diuron, nickel or
367 copper also caused a significant decrease in PUFAs in microalgal cultures or biofilms compared

368 to other fatty acid categories (Filimonova et al. 2016, Demailly et al. 2019, Fadhlouli et al.
369 2020).

370 An additional data treatment consisted in a PCA using the molecular species
371 composition of MGDG, DGDG, PG, PE and PC, in order to better distinguish the likely effects
372 of BAC12 and light condition at the algal group level. The variables described in the F1-F2
373 plane of the PCA (Figure 6) were sorted according to their decreasing contribution to these two
374 axes, including only those with $\cos^2 > 0.7$ (Table 1). A clustering of these filtered variables was
375 then performed using a k-means classification (Figure A 4), resulting into three “variable
376 clusters”, namely F2a, F1,2a and F1,2b. A fourth cluster has been identified, but when projected
377 onto the variable plot (Figure 6), it did not appear to provide any sample- or condition-specific
378 information. Additionally, we attempted to reassign molecular species to different autotrophic
379 groups based on information from the literature (Opute 1974, Dunstan et al. 1993, Bergé et al.
380 1995, Lang et al. 2011, Coniglio et al. 2021, Mazzella et al. 2023a). For example, molecular
381 species containing C14:0, C20:5 or C22:6 were preferentially related to diatoms. In contrast,
382 compounds with C18:2 or C18:3 associated with another 16- or 18-C SFA or MUFA were
383 preferentially linked to contributions from green algae or cyanobacteria. Finally, when the
384 molecular species appeared to be non-specific to a particular microbial groups (e.g. associations
385 primarily among C16:0, C16:1 and C18:1), we indicated that it was an undetermined origin.
386 Actually, it is possible to find such molecular species in all eukaryotic algae (Guschina and
387 Harwood 2006), in fungi (Bhatia et al. 1972) as well as in prokaryotic organisms (Zelles 1997,
388 Doumenq et al. 1999, Mazzella et al. 2005, Mazzella et al. 2007, Sohlenkamp and Geiger 2015).



389

390 Figure 6. F1- F2 plan of the PCA with the initial samples (T0), the controls after 10 days (T10
 391 CTRL) under continuous (CL) or alternating (NL) light, as well as the samples contaminated
 392 with BAC 12 after 10 days (T10 BAC) under the two light conditions (CL and NL). The
 393 variables shown on the left correspond to the set of molecular species associated with polar
 394 lipids.

395 Table 1. Molecular species filtered from the PCA results, considering the 20% of variables
 396 contributing the most to the F1 and F2 axes, as well as with a $\cos^2 > 0.7$ for the sum F1+F2.
 397 Three clusters of variables (F2a, F1,2a and b) were defined, and likely attributions of the
 398 variables was proposed according to the fatty acid composition highlighted within the selected
 399 molecular species.

| Molecular species | Top 20 % contributions axis F1+F2 | Cos ² > 0.7 axis F1+F2 | Correlation significance ¹ | Variable clusters | Likely algal attributions |
|-------------------|-----------------------------------|-----------------------------------|---------------------------------------|-------------------|---------------------------|
| DGDG(16:0_18:1) | 3.010 | 0.863 | *** | F2 a | N.D. |
| DGDG(16:0_18:2) | 2.981 | 0.854 | *** | F2 a | Chloro+Cyano |
| DGDG(16:0_18:3) | 2.766 | 0.802 | *** | F2 a | Chloro+Cyano |
| DGDG(16:2_18:3) | 2.980 | 0.853 | *** | F2 a | Chloro+Cyano |
| DGDG(16:3_18:3) | 2.888 | 0.830 | *** | F2 a | Chloro+Cyano |
| MGDG(16:1_18:3) | 2.980 | 0.853 | *** | F2 a | Chloro+Cyano |
| MGDG(16:2_18:3) | 3.203 | 0.916 | *** | F2 a | Chloro+Cyano |
| MGDG(16:3_18:3) | 2.547 | 0.791 | *** | F2 a | Chloro+Cyano |
| PC(14:0_16:0) | 1.940 | 0.911 | *** | F2 a | Diatoms |
| PC(14:0_20:5) | 2.235 | 0.950 | *** | F2 a | Diatoms |
| PC(16:1_20:4) | 2.300 | 0.747 | * | F2 a | Diatoms |
| PG(16:1_18:2) | 1.888 | 0.785 | ** | F2 a | Chloro+Cyano |
| PE(16:0_16:1) | 2.038 | 0.768 | ** | F1,2 a | N.D. |
| PG(16:0_16:1) | 1.945 | 0.773 | ** | F1,2 a | N.D. |
| PG(16:1_18:1) | 1.902 | 0.789 | *** | F1,2 a | N.D. |
| PC(14:0_22:6) | 1.962 | 0.990 | * | F1,2 b | Diatoms |
| PE(16:0_16:2) | 1.971 | 0.993 | *** | F1,2 b | Chloro+Cyano |
| PE(16:1_20:4) | 1.962 | 0.989 | *** | F1,2 b | Chloro+Cyano |
| PE(16:1_20:5) | 1.953 | 0.967 | *** | F1,2 b | Diatoms |
| PG(14:0_16:1) | 1.949 | 0.977 | *** | F1,2 b | Diatoms |
| PG(16:1_16:1) | 2.846 | 0.821 | *** | F1,2 b | N.D. |
| PE(14:0_16:2) | 1.980 | 0.988 | *** | F1,2 b | Chloro+Cyano |
| PG(16:1_18:3) | 1.882 | 0.924 | * | F1,2 b | Chloro+Cyano |
| PG(18:1_18:1) | 1.963 | 0.974 | *** | F1,2 b | N.D. |

400 ¹p-value <0.05 (*), <0.01 (**), <0.001 (***) for correlations with F1 or F2

401 Cluster F2a was associated with the T10 CTRL samples, regardless of the photoperiod
 402 condition, while cluster F1,2b was associated with the initial samples at T0. Cluster F1,2b
 403 seemed to be more strongly related to the exposure to BAC 12 during the entire 10 days of
 404 exposure. This result suggests that the presence of such a biocide exerts a selective pressure on
 405 the different microorganisms constituting the biofilm, with a progressive elimination of
 406 microalgae in favor of fungi or prokaryotes (Sakagami et al. 1989). These findings observed at
 407 the molecular species level thus confirm the conclusions drawn from the taxonomic
 408 observations formulated by Vrba et al. (2023). We may thus propose some potential biomarkers
 409 by grouping increasing response of PC molecular species such as (14:0_16:0), (14:0_20:5) and
 410 (16:1_20:4), assumed to be here diatom-originating, in samples not contaminated with BAC 12
 411 (Figure 7). However, it would be interesting to investigate further the degree of specificity of

412 this type of response to a specific contaminant in benthic microalgae at the level of lipid
413 molecular species, especially with axenic culture conditions.

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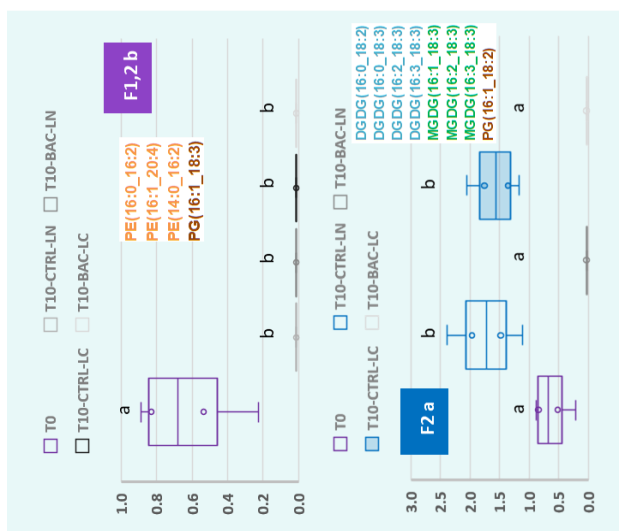
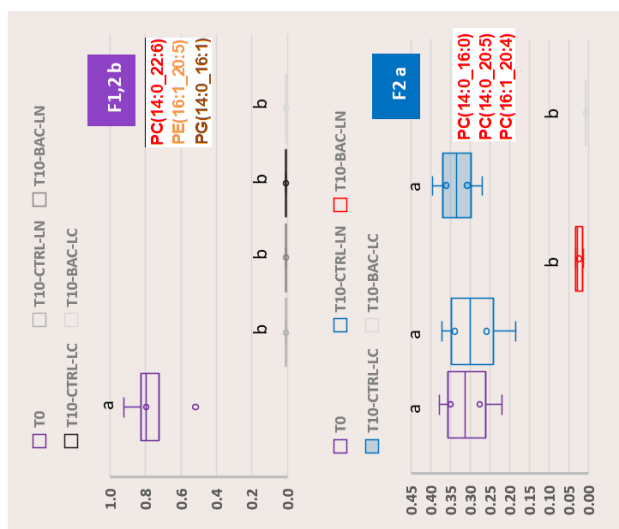
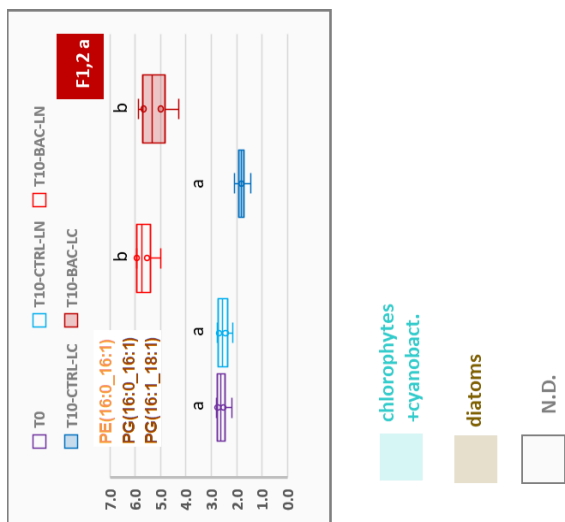
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422 Figure 7. Variation of selected molecular species clusters (nmol mg⁻¹) for each group of
 423 variables (F2 a, F1,2 a and b) as a function of time (T0 or T10), BAC 12 (CTRL or BAC) as
 424 well as the continuous or normal light conditions (CL or NL). The distinction was also made
 425 based on the likely origin of the molecular species, according known and representative fatty
 426 acids of certain autotroph groups.

427 **4.2. Continuous versus alternated photoperiods**

428 No significant effect of the light condition (i.e., NL or CL) was observed on fatty acids
429 derived from polar lipids. Vrba et al., 2023 came to the same conclusion based on analyses of
430 the same samples at the level of lipid classes. This results in a difficulty to dissociate a possible
431 effect of light condition from that of the contaminant. Because this study address also the
432 molecular species, it is possible to keep information associated with the various fatty acids that
433 can be attributed to specific microorganism groups from the biofilm. Actually, it become
434 possible to attribute them either a phototrophic origin (i.e. PUFAs acyl chains of the MGDGs
435 and DGDGs) or a heterotrophic origin (e.g. C16:0, C16:1 or C18:1 acyl chains of the PGs or
436 PEs). Furthermore, in relation to light conditions, MGDG is thought to play an important role
437 in the operation of the xanthophyll cycle in the thylakoid membranes of algae, including
438 diatoms (Goss and Jakob 2010). MGDG is also present in cyanobacteria, the ancestors of
439 chloroplasts in other photosynthetic organisms, even if the prokaryotic thylakoids do not
440 operate a xanthophyll cycle, as found in algae. In addition, the development of thylakoid
441 membrane networks, and therefore an effective photosynthesis, depends on a coordinated
442 biosynthesis of thylakoid lipids with chlorophylls and photosynthetic proteins during
443 chloroplast biogenesis, and both MGDG and DGDG plays a key-role during these processes
444 (Wada and Murata 1998).

452 Focusing on F3 and F4 axes of the PCA, a more specific discrimination related to the
453 photoperiod was observed, as shown by the projection of this additional variable in the left part
454 the graph (Figure 8). We then filtered the variables best represented in this F3-F4 plane of the
455 PCA (Table 2) and defined four clusters (Figure A 4), as well as the probable assignment to
456 specific algal groups. It is interesting to note that this analysis allowed the distinction between
457 the samples that have undergone a continuous photoperiod from those that have been treated
458 with an alternating photoperiod, as they appeared to be separated according to the first bisector
459 associated with axes 3 and 4. Thus, the presence or absence of BAC 12 under the NL condition
460 (i.e. alternating photoperiod) was distinguished by the two clusters F3b and F4b. On the other
461 hand, the presence or absence of BAC 12 resulted in two other clusters, F3a and F4a, which
462 were more indicative of the CL condition. Moreover, according to this clustering of variables,
463 it seems that the continuous photoperiod may induce a relative increase in molecular species
464 associated with green algae and cyanobacteria to the detriment of those originating from
465 diatoms. In other words, the change in lipid composition revealed by molecular species analysis
466 indicated that the CL condition would promote the growth of certain photoautotroph groups
467 over time, regardless of the contamination pressure. Wang and Jia (2020) studied the
468 photoprotective mechanisms of *Nannochloropsis oceanica* in response to light, mainly from
469 the point of view of lipid and fatty acid classes, in parallel with the study of pigment
470 composition. These authors showed that at higher intensities, there was a fairly marked decrease
471 in MGDG and DGDG, but also in phospholipids. They also observed a decrease in most of the
472 fatty acids associated with polar lipids, but this did not appear to be specific to certain categories
473 such as PUFAs. The notable difference with our study is that the authors conducted their
474 experiment on an algae culture while we studied the response of biofilms. In addition, they
475 increased light intensities from 50 to 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (20 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for our study) with a
476 continuous photoperiod only.

477 Table 2. Molecular species filtered from PCA results considering 30% of variables contributing
 478 the most to the plane described by F3 and F4, as well as with a $\cos^2 > 0.5$ for the sum F3+F4.
 479 Four clusters of variables (F3a and b, F4a and b) were defined, and likely attributions of the
 480 variables was proposed according to the fatty acid composition highlighted within the selected
 481 molecular species.

482

| Molecular species | Top 30 % contributions axis F3+F4 | $\cos^2 > 0.5$ axis F3+F4 | Correlation significance ¹ | Variable clusters | Likely algal attributions |
|-------------------|-----------------------------------|---------------------------|---------------------------------------|-------------------|---------------------------|
| PG(16:0_18:2) | 3.648 | 0.534 | *** | F3 a | Chloro+Cyano |
| PG(16:0_18:3) | 3.648 | 0.534 | *** | F3 a | Chloro+Cyano |
| MGDG(16:0_18:3) | 3.979 | 0.582 | *** | F3 a | Chloro+Cyano |
| PC(14:0_18:0) | 5.355 | 0.619 | ** | F4 a | Diatoms |
| PC(18:0_18:2) | 8.991 | 0.868 | *** | F4 a | Chloro+Cyano |
| PC(18:1_18:2) | 7.840 | 0.763 | *** | F4 a | Chloro+Cyano |
| PC(18:1_18:3) | 8.371 | 0.784 | *** | F4 a | Chloro+Cyano |
| PC(18:1_20:4) | 7.614 | 0.699 | *** | F4 a | Diatoms |
| PC(18:2_18:2) | 7.909 | 0.864 | ** | F4 a | Chloro+Cyano |
| PE(18:0_18:1) | 7.606 | 0.698 | ** | F4 a | N.D. |
| PE(18:0_20:5) | 7.505 | 0.689 | *** | F4 a | Diatoms |
| PE(18:1_18:1) | 7.415 | 0.681 | *** | F4 a | N.D. |
| PC(16:0_22:6) | 3.992 | 0.584 | *** | F3 b | Diatoms |
| PC(16:1_22:5) | 3.751 | 0.545 | *** | F3 b | Diatoms |
| PC(18:3_20:5) | 3.991 | 0.584 | ** | F3 b | Diatoms |
| DGDG(14:0_20:5) | 4.017 | 0.587 | *** | F3 b | Diatoms |
| DGDG(14:0_18:2) | 4.018 | 0.588 | *** | F3 b | Chloro+Cyano |
| DGDG(14:0_20:3) | 4.011 | 0.587 | * | F3 b | Diatoms |
| DGDG(16:2_18:2) | 3.596 | 0.527 | *** | F3 b | Chloro+Cyano |
| DGDG(16:3_18:2) | 4.026 | 0.589 | *** | F3 b | Chloro+Cyano |
| PC(16:1_16:1) | 6.217 | 0.629 | ** | F4 b | N.D. |
| PC(16:2_18:1) | 5.906 | 0.745 | * | F4 b | Chloro+Cyano |
| PE(16:2_18:1) | 7.895 | 0.816 | *** | F4 b | Chloro+Cyano |

483 ¹ p-value <0.05 (*), <0.01 (**), <0.001 (***) for correlations with F3 or F4

484 The lipid content of algae is also significantly affected by light cycles. For example,
 485 Brown et al. (1996) studied the effects of different light regimes on the lipids of the diatom
 486 *Thalassiosira pseudonana* where 100, 50 and 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ under respective 12:12, 24:0 and
 487 24:0 h light/dark cycles were used. Cells grown at the high light intensity and 12:12 photoperiod
 488 exhibited higher concentrations of PUFAs and lower concentrations of both SFAs and MUFAs.
 489 Although it is very likely that the duration of the photoperiod also affected the autotrophs found
 490 in our biofilms, attributing changes to primary physiological effects at the level of each
 491 individual organism in terms of fatty acid (or molecular species) content alone seems rather
 492 uncertain. The change in lipid composition would appear here to be more closely tied to overall
 493 changes in community structure because we investigated a complex biofilm. Fatty acids alone
 494 may not provide sufficient information as they could, masking weaker effects like photoperiod

495 duration in favor of other environmental factors (i.e. simultaneous contamination exposure).
496 Therefore, our suggestion is that some molecular species, especially those from PCs and
497 DGDGs here (Table 2), may be more specific markers of light duration at a biofilm scale. In
498 addition, the literature remains sparse at this level of molecular information, and we suggested
499 to use similar lipidomic approach with monospecific cultures of microalgal strains to strengthen
500 our preliminary results.

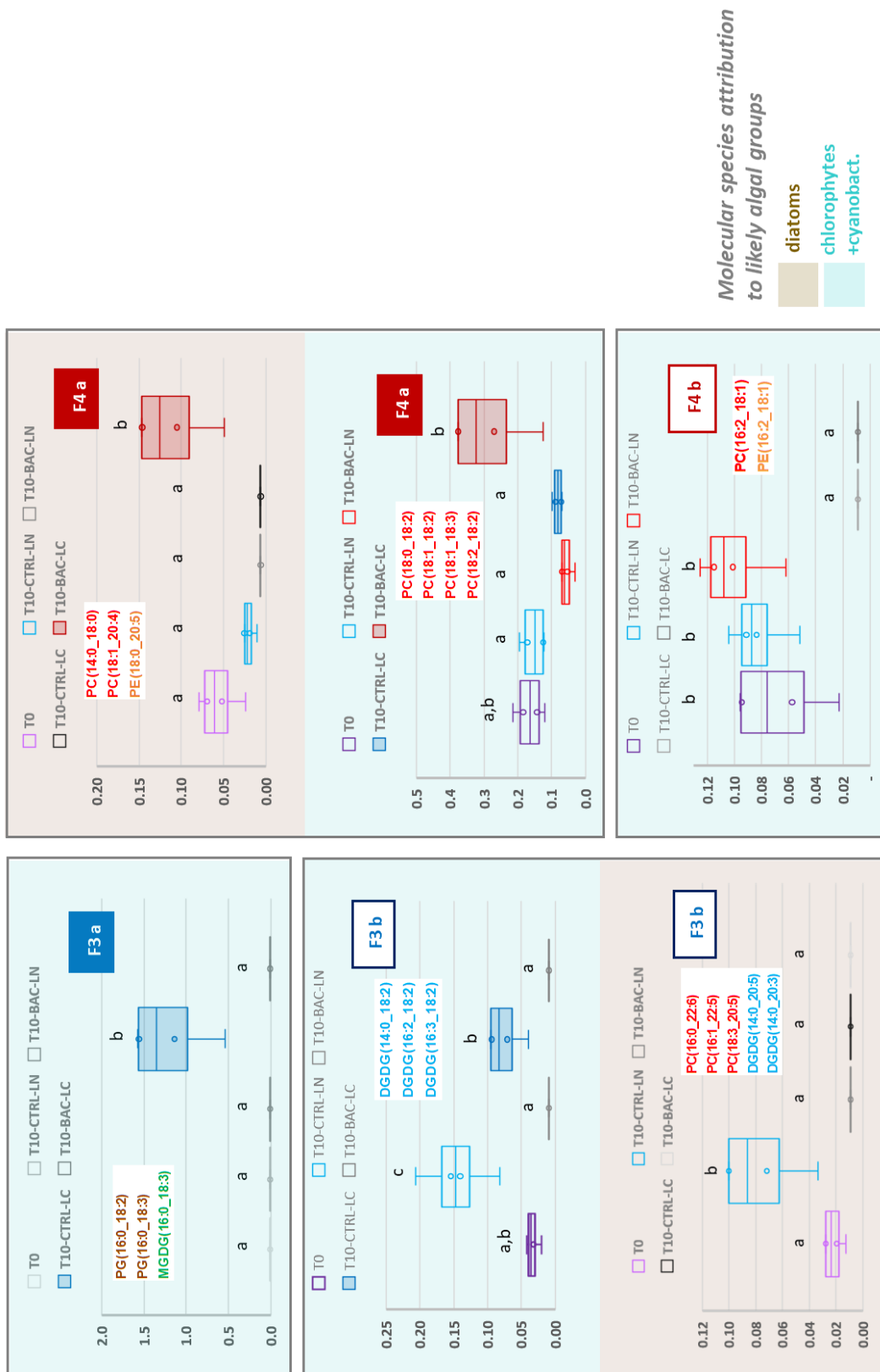
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506 Figure 9. Variation of molecular species (nmol mg^{-1}) clusters selected for each group of
 507 variables (F3 a and b, F4 a and b) as a function of time (T0 or T10), BAC 12contamination
 508 (CTRL or BAC) and light condition (CL or NL). The distinction was also made based on the
 509 probable origin of the molecular species, according to the known and representative fatty acids
 510 of certain photoautotroph groups.

511 **Conclusions**

512 After exposing biofilm to BAC 12, the fatty acid deduced from polar lipid analysis
513 suggested that the heterotrophic compartment would likely increase at the expense of
514 phototrophic organisms. The overall reduction in PUFAs found on the same samples points to
515 a sharp decline in all phototrophic organisms present in the biofilm. To more clearly separate
516 the co-occurring effects of biocide exposure and light condition at the algal group level, the
517 molecular species compositions of MGDG, DGDG, PG, PE, and PC were examined. The most
518 representative molecular species were clustered, and it was proposed that certain molecular
519 species, particularly those from PCs and DGDGs, could potentially act as more accurate
520 markers of light duration at the biofilm scale. To strengthen our early results, it would be
521 beneficial to use a similar lipidomic approach with monospecific cultures of microalgal strains,
522 since the literature is still lacking at this level of both molecular in-depth details and
523 understanding of the physiological mechanisms.

524

525

526 **Author contributions**

527 Nicolas MAZZELLA: Lipid analysis, Data analysis, Writing - original draft, review & editing.

528 Romain VRBA: Conceptualization, Investigation, Methodology, Sample preparation

529 Aurélie MOREIRA: Lipid and micropollutant analyses

530 Nicolas CREUSOT: Writing - review & editing.

531 Mélissa EON: Sample preparation, Physico-chemical analysis

532 Débora MILLAN-NAVARRO: Physico-chemical and micropollutant analyses

533 Isabelle LAVOIE: Funding acquisition, Supervision, Writing - review & editing.

534 Soizic MORIN: Funding acquisition, Supervision, Conceptualization, Writing - review &
535 editing.

536

537 **Conflicts of interest**

538 None

539

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648 **Appendices**

649

650 Table A 1. BAC 12 concentrations (mg L⁻¹) in the four experimental conditions at T10. BAC
 651 12 = contaminated biofilm; CTRL = non-exposed biofilm; NL = alternated photoperiod; CL =
 652 Continuous photoperiod.

| Conditions | BAC 12 concentrations |
|---------------|-----------------------|
| T10-CTRL-NL | < 0.01 |
| T10-BAC-NL | 27.03 ± 12.12 |
| T10-CTRL-CL | < 0.01 |
| T10-BAC 12-CL | 17.20 ± 7.47 |

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655 Table A 2. Mass spectrometry parameters (single ion monitoring) for free fatty acid analysis.

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| Fatty acid | Q1 (m/z) | dwel time (ms) | DP (V) | CE (V) |
|------------|----------|----------------|--------|--------|
| C14:0 | 243 | 30 | 70 | 20 |
| C15:0 | 257 | 30 | 70 | 20 |
| C16:4 | 263 | 30 | 70 | 20 |
| C16:3 | 265 | 30 | 70 | 20 |
| C16:2 | 267 | 30 | 70 | 20 |
| C16:1 | 269 | 30 | 70 | 20 |
| C16:0 | 271 | 30 | 70 | 20 |
| C17:1 | 283 | 30 | 70 | 20 |
| C17:0 | 285 | 30 | 70 | 20 |
| C18:4 | 291 | 30 | 70 | 20 |
| C18:3 | 293 | 30 | 70 | 20 |
| C18:2 | 295 | 30 | 70 | 20 |
| C18:1 | 297 | 30 | 70 | 20 |
| C18:0 | 299 | 30 | 70 | 20 |
| C19:0 | 313 | 30 | 70 | 20 |

| | | | | |
|-------|-----|----|----|----|
| C20:5 | 317 | 30 | 70 | 20 |
| C20:4 | 319 | 30 | 70 | 20 |
| C20:1 | 325 | 30 | 70 | 20 |
| C20:0 | 327 | 30 | 70 | 20 |
| C22:6 | 343 | 30 | 70 | 20 |
| C22:5 | 345 | 30 | 70 | 20 |

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659 Table A 3. Chromatographic analytical gradient for free fatty acids separation.

| Time (min) | % of A (5 mM ammonium acetate) | % of B (acetonitrile:isopropanol, 50:50) | Flow rate ($\mu\text{L min}^{-1}$) |
|-------------------|---------------------------------------|---|--|
| 0 | 50 | 50 | 300 |
| 0.3 | 50 | 50 | 300 |
| 5.8 | 1 | 99 | 300 |
| 8.8 | 1 | 99 | 300 |
| 9.1 | 50 | 50 | 300 |
| 11 | 50 | 50 | 300 |

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663 Table A 4. Polar lipid-derived and free fatty acids data.

| Polar + free FA C (nmol/mg) | T0 | | | | T10-CTRL-NL | | | | T10-BAC-NL | | | |
|--------------------------------|------|------|------|------|-------------|------|------|------|------------|------|------|------|
| | rep1 | rep2 | rep3 | rep4 | rep1 | rep2 | rep3 | rep4 | rep1 | rep2 | rep3 | rep4 |
| C14:0 | 0.5 | 0.9 | 2.9 | 1.1 | 1.7 | 1.2 | 1.3 | 0.9 | 0.4 | 0.1 | 0.1 | 0.1 |
| C16:3 | 1.6 | 0.9 | 1.4 | 1.2 | 0.8 | 1.3 | 0.4 | 1.1 | - | - | - | - |
| C16:2 | 2.3 | 1.6 | 3.1 | 3.1 | 0.2 | 0.6 | 0.6 | 0.2 | 0.4 | 0.4 | 0.6 | 0.4 |
| C16:1 | 15.9 | 3.2 | 9.5 | 15.8 | 4.2 | 1.7 | 11.8 | 6.0 | 9.7 | 4.9 | 8.7 | 8.2 |
| C16:0 | 4.6 | 1.9 | 10.0 | 3.2 | 7.2 | 6.8 | 5.6 | 1.1 | 13.1 | 4.6 | 6.2 | 2.1 |
| C18:3 | 0.6 | 0.3 | 1.0 | 0.9 | 1.7 | 1.4 | 0.8 | 0.5 | - | - | - | - |
| C18:2 | 1.1 | 1.4 | 1.4 | 0.4 | 1.0 | 1.2 | 1.0 | 1.4 | 0.1 | 0.1 | 0.2 | 0.1 |
| C18:1 | 2.7 | 2.1 | 0.9 | 3.5 | 2.3 | 2.2 | 2.6 | 2.5 | 0.7 | 0.6 | 4.6 | 9.2 |
| C18:0 | 0.1 | 0.1 | 0.1 | 0.3 | 0.0 | 0.1 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| C20:5 | 1.2 | 6.2 | 1.9 | 3.8 | 1.1 | 1.8 | 3.3 | 4.0 | - | - | - | - |
| C20:4 | 0.9 | 0.4 | 0.3 | 0.2 | 0.0 | 0.2 | 0.2 | 0.1 | - | - | - | - |
| C20:3 | 0.1 | 0.0 | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | - | - | - | - |
| C20:2 | 0.4 | 0.2 | 0.3 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | - | - | - | - |
| C20:0 | 1.6 | 1.0 | 2.2 | 0.9 | 0.3 | 0.3 | 0.1 | 0.2 | - | - | - | - |
| C22:6 | 0.7 | 0.8 | 0.8 | 0.8 | 0.4 | 0.1 | 0.1 | 0.4 | - | - | - | - |
| C22:5 | 0.0 | - | - | 0.1 | - | 0.0 | 0.0 | 0.0 | - | - | - | - |
| Sum (nmol/mg) | 34.0 | 20.8 | 35.9 | 35.6 | 21.0 | 18.9 | 28.2 | 18.5 | 24.5 | 10.8 | 20.6 | 20.2 |

| | | | | | | | | | | | | |
|---------------------|------|------|------|------|------|------|------|------|------|------|------|------|
| SFAs (%) | 20% | 18% | 42% | 15% | 44% | 44% | 26% | 13% | 56% | 44% | 32% | 11% |
| MUFAs (%) | 55% | 25% | 29% | 54% | 31% | 21% | 51% | 46% | 42% | 51% | 65% | 86% |
| PUFAs (%) | 26% | 56% | 28% | 30% | 26% | 35% | 23% | 41% | 2% | 5% | 4% | 2% |
| PUFA/(SFA+MUFA) (%) | 0.34 | 1.29 | 0.40 | 0.43 | 0.34 | 0.54 | 0.30 | 0.70 | 0.02 | 0.05 | 0.04 | 0.02 |
| UFA/SFA (%) | 4.04 | 4.44 | 1.36 | 5.51 | 1.29 | 1.25 | 2.91 | 6.62 | 0.80 | 1.25 | 2.17 | 7.76 |
| BAFA (%) | 21% | 19% | 31% | 20% | 45% | 48% | 30% | 21% | 57% | 50% | 54% | 57% |

| Polar + free FA C (nmol/mg) | T10-CTRL-CL | | | | T10-BAC-CL | | | |
|--------------------------------|-------------|------|------|------|------------|------|------|------|
| | rep1 | rep2 | rep3 | rep4 | rep1 | rep2 | rep3 | rep4 |
| C14:0 | 1.3 | 0.7 | 0.9 | 0.9 | 0.1 | 0.2 | 0.1 | 0.1 |
| C16:3 | 0.4 | 0.8 | 0.4 | 0.4 | - | - | - | - |
| C16:2 | 0.8 | 0.7 | 0.5 | 0.3 | 0.5 | 0.5 | 0.2 | 0.6 |
| C16:1 | 7.0 | 4.9 | 7.7 | 5.1 | 4.4 | 10.8 | 4.6 | 5.1 |
| C16:0 | 6.1 | 4.8 | 3.2 | 5.6 | 7.6 | 1.4 | 3.0 | 9.8 |
| C18:3 | 1.1 | 1.4 | 1.8 | 1.3 | 0.0 | 0.0 | 0.0 | 0.0 |
| C18:2 | 1.3 | 0.4 | 1.3 | 0.0 | 0.6 | 0.5 | 0.2 | 0.5 |
| C18:1 | 2.3 | 0.2 | 0.5 | 2.0 | 3.0 | 3.4 | 3.9 | 5.8 |
| C18:0 | 0.1 | 0.1 | 0.1 | 0.1 | 0.2 | 0.1 | 0.1 | 0.0 |
| C20:5 | 5.8 | 4.1 | 3.8 | 4.6 | 0.1 | 0.2 | 0.2 | 0.1 |
| C20:4 | 0.3 | 0.3 | 0.3 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 |
| C20:3 | - | - | - | - | - | - | - | - |
| C20:2 | 0.2 | 0.1 | 0.2 | 0.0 | - | - | - | - |
| C20:0 | 0.2 | 0.9 | 0.3 | 0.8 | - | - | - | - |
| C22:6 | - | 0.5 | 0.2 | 0.4 | - | - | - | - |
| C22:5 | 0.0 | 0.0 | 0.0 | 0.0 | - | - | - | - |
| Sum (nmol/mg) | 26.8 | 20.0 | 21.1 | 21.8 | 16.6 | 17.1 | 12.4 | 22.1 |

| | | | | | | | | |
|---------------------|------|------|------|------|------|------|------|------|
| SFAs (%) | 29% | 32% | 21% | 34% | 47% | 10% | 26% | 45% |
| MUFAs (%) | 35% | 25% | 39% | 33% | 45% | 83% | 68% | 49% |
| PUFAs (%) | 37% | 42% | 40% | 34% | 8% | 7% | 6% | 6% |
| PUFA/(SFA+MUFA) (%) | 0.58 | 0.73 | 0.67 | 0.50 | 0.08 | 0.07 | 0.06 | 0.06 |
| UFA/SFA (%) | 2.51 | 2.09 | 3.71 | 1.95 | 1.11 | 8.77 | 2.86 | 1.22 |
| BAFA (%) | 32% | 26% | 18% | 35% | 65% | 29% | 57% | 71% |

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674 Table A 5. Fatty acids as variables filtered by \cos^2 values.

| Polar lipid FA | $\cos^2 > 0.5$ (F1+F2) |
|-----------------------|---|
| C20:0 | 0.881 |
| C16:3 | 0.875 |
| C16:2 | 0.867 |
| C22:6 | 0.807 |
| C20:5 | 0.761 |
| C22:5 | 0.752 |
| C20:4 | 0.728 |
| C20:3 | 0.727 |
| C18:3 | 0.615 |
| C18:1 | 0.552 |
| C20:2 | 0.545 |
| C16:1 | 0.545 |
| C18:0 | 0.522 |

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680 Table A 6. Multivariate analysis of variance for both PUFA/(MUFA+SFA) and GL/PL ratios.

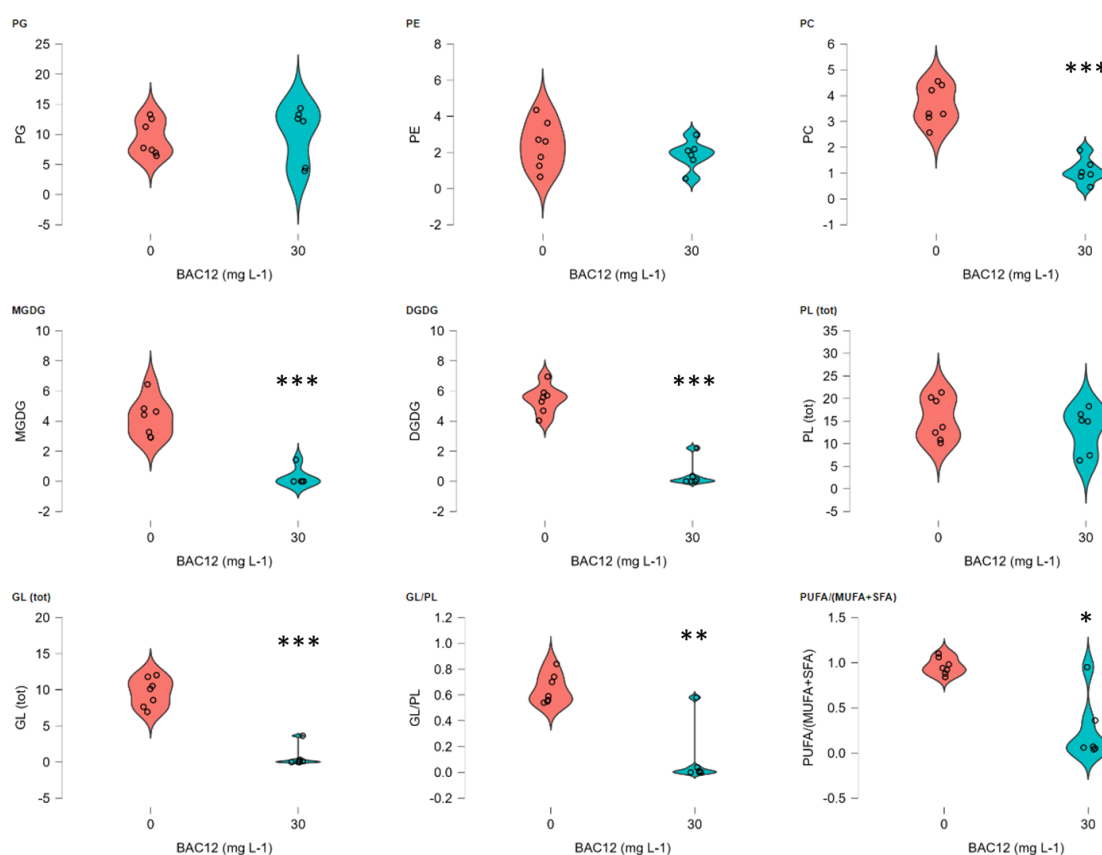
MANOVA

MANOVA: Pillai Test ▼

| Cases | df | Approx. F | Trace _{Pillai} | Num df | Den df | p |
|------------------------|----|-----------|-------------------------|--------|--------|------------------------|
| (Intercept) | 1 | 32.123 | 0.889 | 2 | 8.000 | 1.504×10 ⁻⁴ |
| Light | 1 | 0.977 | 0.196 | 2 | 8.000 | 0.417 |
| BAC12 (mg L-1) | 1 | 14.321 | 0.782 | 2 | 8.000 | 0.002 |
| Light * BAC12 (mg L-1) | 1 | 3.904 | 0.494 | 2 | 8.000 | 0.068 |
| Residuals | 9 | | | | | |

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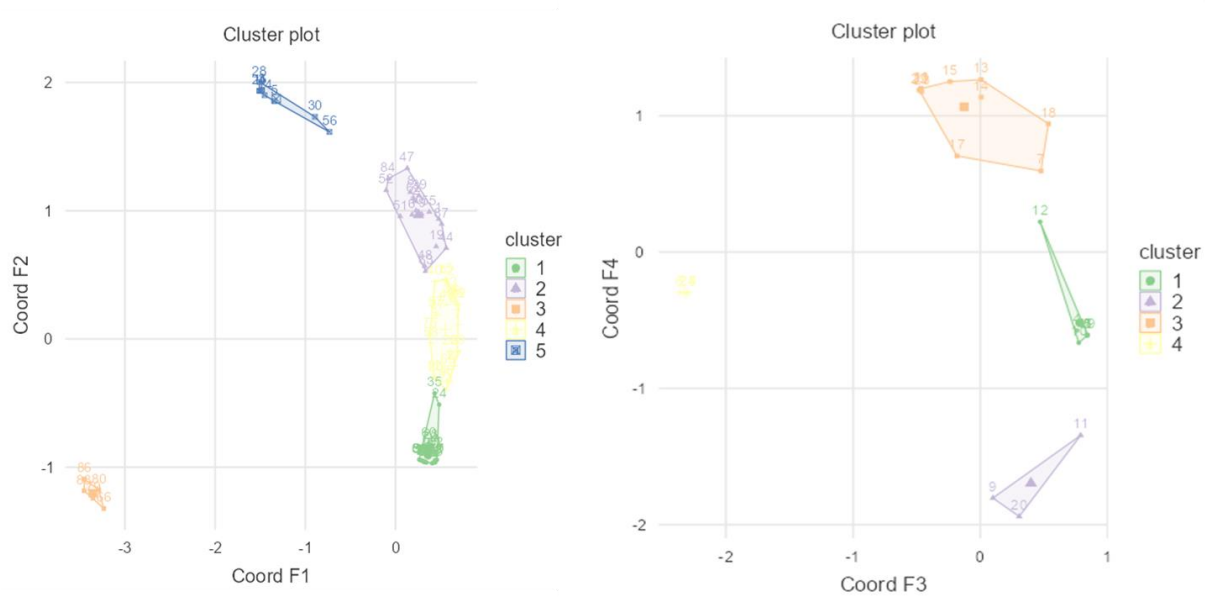


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684 Figure A 3. Univariate (Welch's ANOVA) representation for each polar lipid class as well as
 685 PUFA/(MUFA+SFA) and GL/PL ratios according to BAC 12 exposure. * for p<0.05, ** for p<0.01,
 686 *** for p<0.001.

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690 Figure A 4. k-means clustering of variables best represented in either F1-F2 or F3-F4 plans.

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