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1 **Mixed light and biocide pollution affects lipid profiles of**
2 **periphyton communities in freshwater ecosystems**

3
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11
12 **Highlights**

- 13
14 • BAC 12 exposure leads to glycolipid disappearance and a drastic decrease in PUFAs,
15 indicating noticeable impacts on phototrophic organisms within the biofilm.
16 • The information provided by the fatty acids deduced from polar lipids did not allow
17 decoupling the effect of light conditions from BAC 12 exposure.
18 • In contrast, lipid molecular species analysis could show an increase in green algae and
19 cyanobacteria when compared to diatoms under the effect of photoperiod.
20 • It may also be proposed that certain molecular species, particularly those from PCs and
21 DGDGs, might act as more accurate biofilm-scale markers of light duration.
22
23

24 **Abstract**

25

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

42 **Keywords**

43 Artificial light at night, quaternary ammonium compound, biofilm, microalgae, lipidomics,
44 fatty acids.

45

46 1. Introduction

47

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

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

88

89

2. Experimental section

2.1. Chemicals and reagents

The following polar lipid standards were purchased from Avanti Polar Lipids: 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine or PC (16:0/18:1) (850457), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine or PE (16:0/18:1) (850757), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) or PG (16:0/18:1) (840457), 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine or PC (17:0/17:0) (850360), 1,2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine or PE (17:0/17:0) (830756), 1,2-dipentadecanoyl-sn-glycero-3-phosphoethanolamine or PE (15:0/15:0) (850704), and 1,2-diheptadecanoyl-sn-glycero-3-phospho-(1'-rac-glycerol) or PG (17:0/17:0) (830456), L- α -phosphatidylserine (Soy, 99%) (sodium salt) (870336) for the phospholipid standards, and monogalactosyldiacylglycerol (840523), digalactosyldiacylglycerol (840524) and sulfoquinovosyldiacylglycerol (840525) from plant extracts as glycolipid standards. Ammonium acetate (LiChropur) were provided by Sigma-Aldrich. Palmitic acid (76119), oleic acid (O1008), heptadecanoic acid (H3500) and eicosapentanoic acid (44864) analytical standard grades (purity \geq 98 %) were purchased from Sigma-Aldrich. Acetonitrile, methanol (MeOH) tert-Butyl methyl ether (MTBE) and isopropanol HPLC grades were purchased from Biosolve Chimie, France. Ultrapure water (UPW) was obtained from Direct-Q[®] Water Purification System (Merck Millipore). Dodecylbenzyltrimethylammonium chloride (BAC 12, purity \geq 99 %) and Benzyl-2,3,4,5,6-d₅-dimethyl-n-dodecylammonium chloride (purity $>$ 98 %) were obtained from Sigma-Aldrich and Cluzeau (France), respectively.

2.2. Experimental design

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

115 After five months, colonized substrates were removed from the natural environment and were
116 randomly placed in experimental channels (on day 0 = T0) under laboratory conditions as
117 described in Vrba et al. (2023). Briefly, four experimental conditions were set up, each in
118 pseudo-triplicates (i.e., separate 10-L channels fed by a common 10-L tank). The tanks and
119 channels were filled with filtered (20 μm) pond water, at a room temperature of $20.5 \pm 0.1^\circ\text{C}$
120 and water temperature of $18.7 \pm 0.2^\circ\text{C}$. The four conditions were as follow: two treatments
121 under uncontaminated conditions (controls, CTRL) and two treatments exposed to 30 mg L^{-1}
122 of BAC 12 (BAC). All channels were exposed either to normal light (NL) corresponding to a
123 14h day/10h night photoperiod ($20 \mu\text{mol m}^{-2} \text{ s}^{-1}$) or to continuous light (CL). Treatments will
124 be referred as CTRL-NL, CTRL-CL, BAC-NL and BAC-CL. On day 0, biofilms were collected
125 to assess their initial lipid composition, prior to exposure to either BAC or CL. On day 10 (T10),
126 biofilms were collected to analyse qualitative and quantitative changes in lipid profiles.
127 Biofilms were immediately quenched in liquid nitrogen, and then collected by scraping the
128 glass slides with a razor blade. The samples were freeze-dried (Benchtop Ro 8LZL BTF) and
129 kept at -80°C until the extraction step.

130 **2.3. Nutrient and BAC12 dosing in exposure water**

131 Nutrients and mineral salts were monitored and analyzed using a Metrohm 881 Compact
132 Ionic Chromatograph pro (Metrohm). Anion analysis (PO_4^- , NO_3^- , NO_2^- , Cl^- and SO_4^{2-}) was
133 performed using a Supp 4/5 Guard/4.0 precolumn followed by a Metrosep A Supp5 – 250/4.0
134 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
135 of $1 \text{ mmol L}^{-1} \text{ NaHCO}_3$. Cation analysis (Na^+ , K^+ , Ca^{2+} , Mg^{2+} and NH_4^+) was performed using
136 a C4 Guard/4.0 precolumn followed by a Metrosep C6 - 250/4.0 column). The eluent used was
137 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
138 (PCDA). Calibration ranges were from 20 to $1000 \mu\text{g.L}^{-1}$. BAC 12 concentrations in the water
139 were monitored at the beginning (T0) and the end of experiment (T10). Three samples of 20

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

151 **2.4. Lipid extraction**

152 The biofilm samples (10-20 mg of dry mass) were weighed using a Mettler Toledo
153 NS204S precision balance and placed in 2 mL microtubes with 150 mg of microbeads. The
154 biphasic extraction procedure involved addition of 1 mL of a MTBE:MeOH (3:1, v/v) mixture
155 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)
156 solution containing $100\text{ ng }\mu\text{L}^{-1}$ was added as a surrogate. Samples containing microbeads were
157 mechanically homogenized and extracted (3 cycles of 15 s) with the solvent mixtures by using
158 a MP Biomedicals FastPrep-24 5G. The upper lipophilic phase (MTBE) was separated from
159 the lower hydrophilic phase (UPW and MeOH) by centrifugation at 12,000 RPM. $600\text{ }\mu\text{L}$ of
160 the lipophilic phase was collected. A second extraction step extracted (3 cycles of 15 s) was
161 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
162 mixture 3:1 (v/v). The supernatant (MTBE) was collected again and added to the previous one.
163 Only organic lipophilic phases (1.1 mL) were kept for further polar lipid analysis. Extracts were
164 stored at -80°C and solvent underwent extraction as a procedural blank to verify the absence of

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

168 **2.5. HPLC-ESI-MS/MS analysis**

169 Lipid extracts were also analyzed with a Dionex Ultimate 3000 HPLC (Thermo Fisher
170 Scientific, France) coupled with an API 2000 triple quadrupole mass spectrometer (Sciex,
171 France). Chromatographic separation of both glycolipids and phospholipids was performed on
172 a Luna NH₂ HILIC column (3 μm, 100 × 2 mm) with a Security Guard cartridge NH₂ (4 × 2.0
173 mm). The injection volume and temperature were set to 20 μL and 40°C, respectively. The
174 chromatographic separation and mass spectrometry conditions are described in Mazzella et al.
175 (2023a), (2023b). Quantitation of phosphatidylcholine (PC), phosphatidylethanolamine (PE)
176 and phosphatidylglycerol (PG) were respectively carried out with: PC (16:0/18:1), PE
177 (16:0/18:1), PG (16:0/18:1). Quantitation of glycolipids was carried out with MGDG
178 (16:3_18:3) (63 % of the total MGDG standard), DGDG (18:3/18:3) (22 % of the total MGDG
179 standard), and SQDG (34:3) (78 % of the total SQDG standard). The internal standards utilized
180 were PC (17:0/17:0) for PC phospholipids, PE (17:0/17:0) for PE phospholipids and both
181 MGDG and DGDG glycolipids, and PG (17:0/17:0) for PG and SQDG. Concentrations for both
182 phospholipids and glycolipids were reported in nmol mg⁻¹ (dry weight), and the limits of
183 quantification were typically between 0.02-0.05 nmol mg⁻¹, depending on analyte response.

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

189 2.6. Phospholipid and glycolipid nomenclatures

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

198 2.7. Conversion of phospholipids and glycolipids to fatty acid equivalents

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

203 Equation 1 1 mole of MGDG(16:3/18:3) \rightarrow 1 mole of 16:3 + 1 mole of 18:3

204 Equation 2 1 mole of PG(18:1/18:1) \rightarrow 2 moles of 18:1

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

208 2.8. Data analyses

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

212 comparison methods (Conover and Iman) were respectively carried out with a global p-value
213 ≤ 0.05 and Bonferroni corrections. Multivariate analysis of variance (MANOVA) and
214 subsequent analysis of variance (ANOVA) were performed with Welch test corrections.

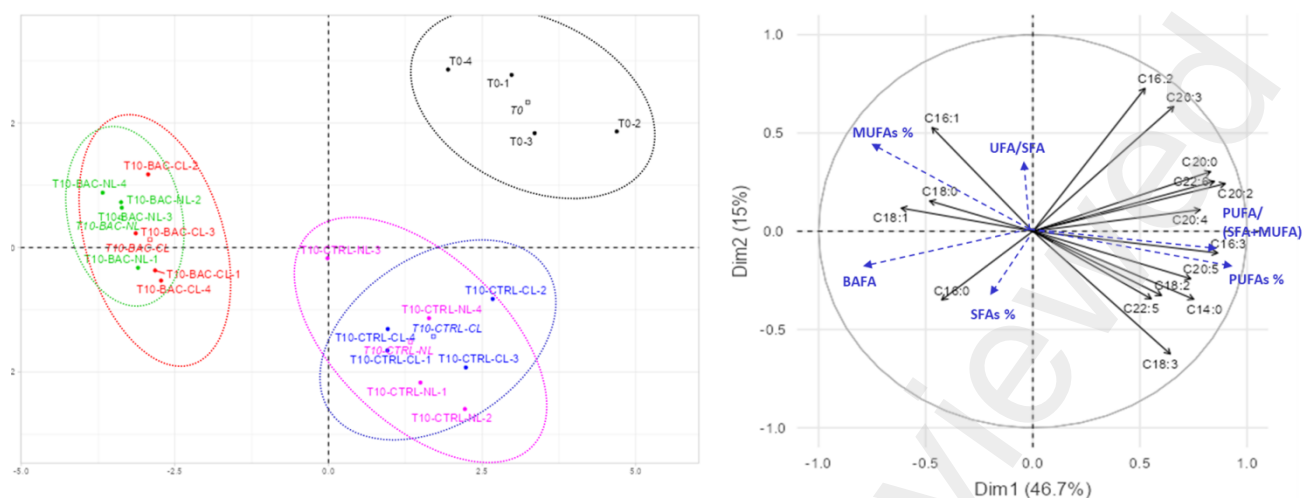
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216 **3. Results**

217

218 **3.1. Fatty acids from polar lipids**

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



233

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

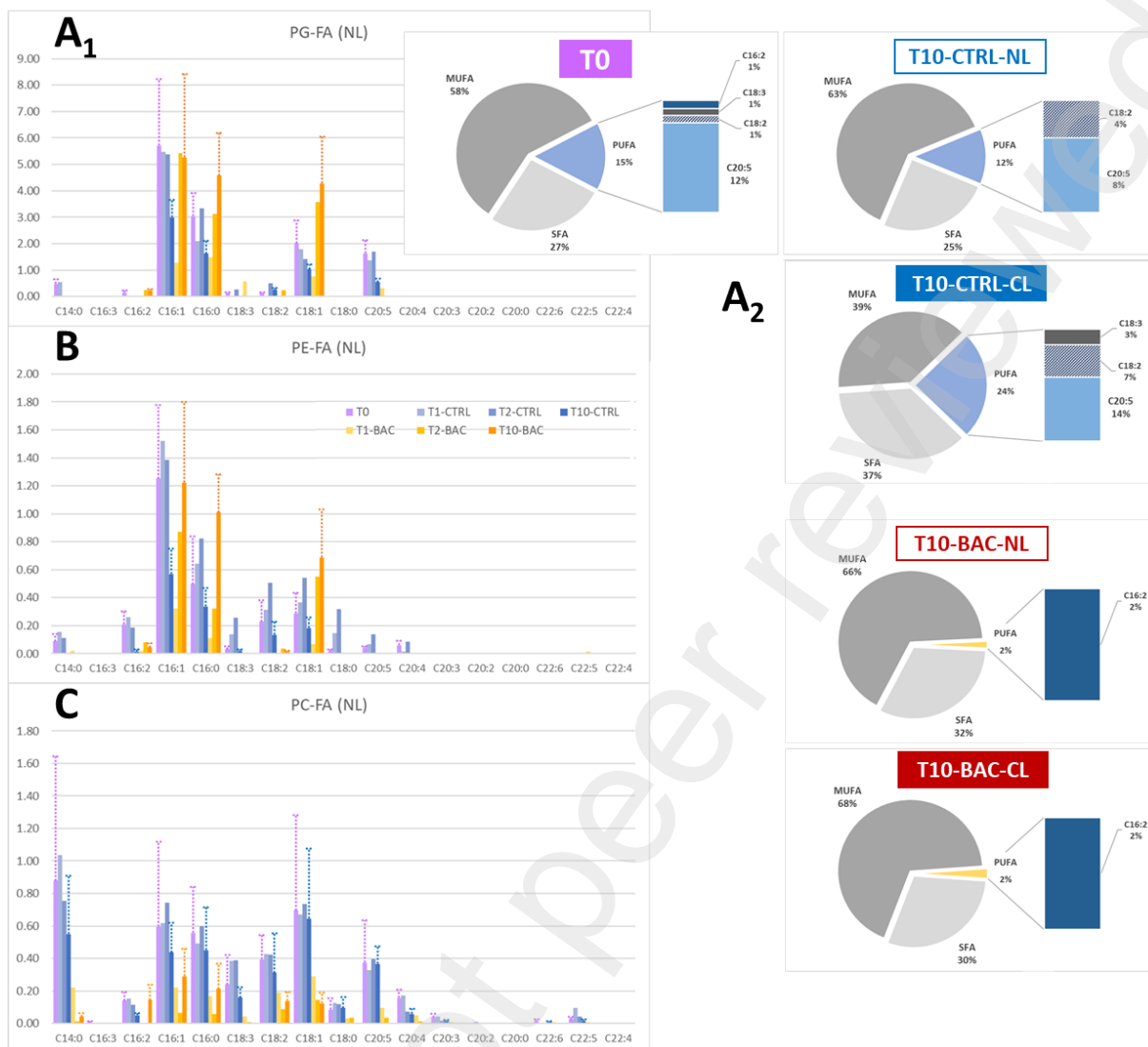
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241 The amounts of fatty acids (in nmol mg⁻¹ of dry biofilm) obtained from the molecular
 242 species of the phospholipids PG, PE and PC are presented in of Figure 2 (A1, B and C). These
 243 barplots are presented only for the alternating photoperiod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

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

256 The initial fatty acid composition (T0) from PE (Figure 2, panel B), as well as the
257 composition after 2 and 10 days of experiment without BAC12 contamination, differed from
258 that of PG where C18:2 and C18:3 were abundant and where C20:5 and C20:4 showed very
259 low concentrations. Within the same phospholipids, and as observed for PG, an increase in
260 MUFAs with 16 or 18 carbon atoms was observed, as well as the SFA C16:0. Finally, PC was
261 characterized by an equivalent distribution of C18:2, C18:3 and C20:5 within the PUFAs from
262 this class of membrane lipids, with contents essentially between 0.2 and 0.4 nmol mg⁻¹ (Figure
263 2, panel C). We did not observe an increase in SFAs or MUFAs as a result of decreasing PUFAs.
264 The results rather suggest that it is the fatty acids from PC that decreased in absolute values.

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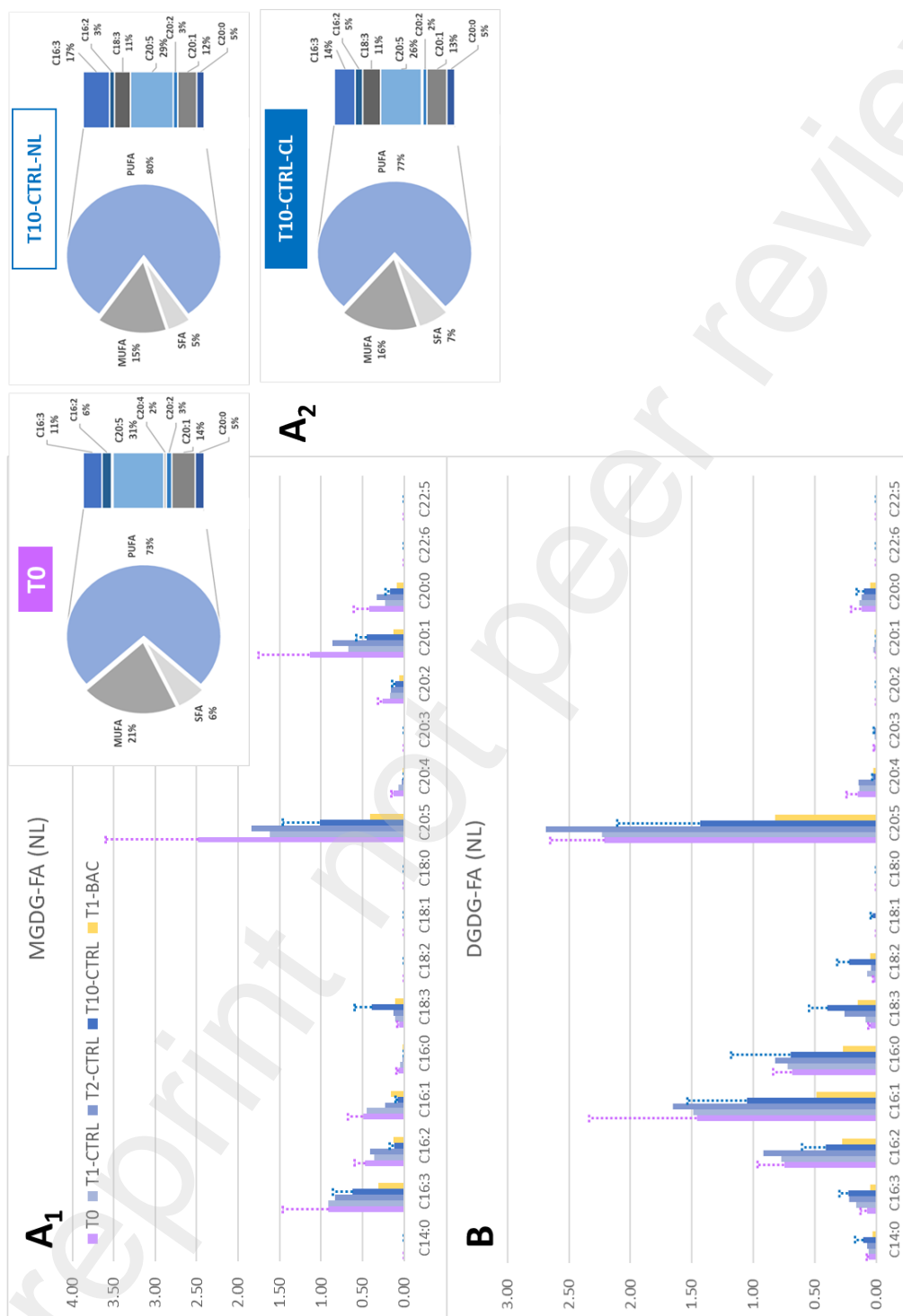
267 Figure 2. Evolution of fatty acids (nmol mg⁻¹) deduced from the molecular species of
 268 phospholipids (A1) PG, (B) PE and (C) PC. Different times are reported here with 1, 2 and 10
 269 days of culture with or without BAC 12 and under normal light conditions. Part A2 on the right
 270 illustrates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

272

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

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

281



282
 283 Figure 3. Evolution of fatty acid concentrations (nmol mg⁻¹) calculated from the molecular
 284 species of glycolipids (A1) MGDG, (B) DGDG. Panel A2 illustrates fatty acids (% mol)
 285 within SFA, MUFA and PUFA for MGDG as well as individual PUFAs.

286

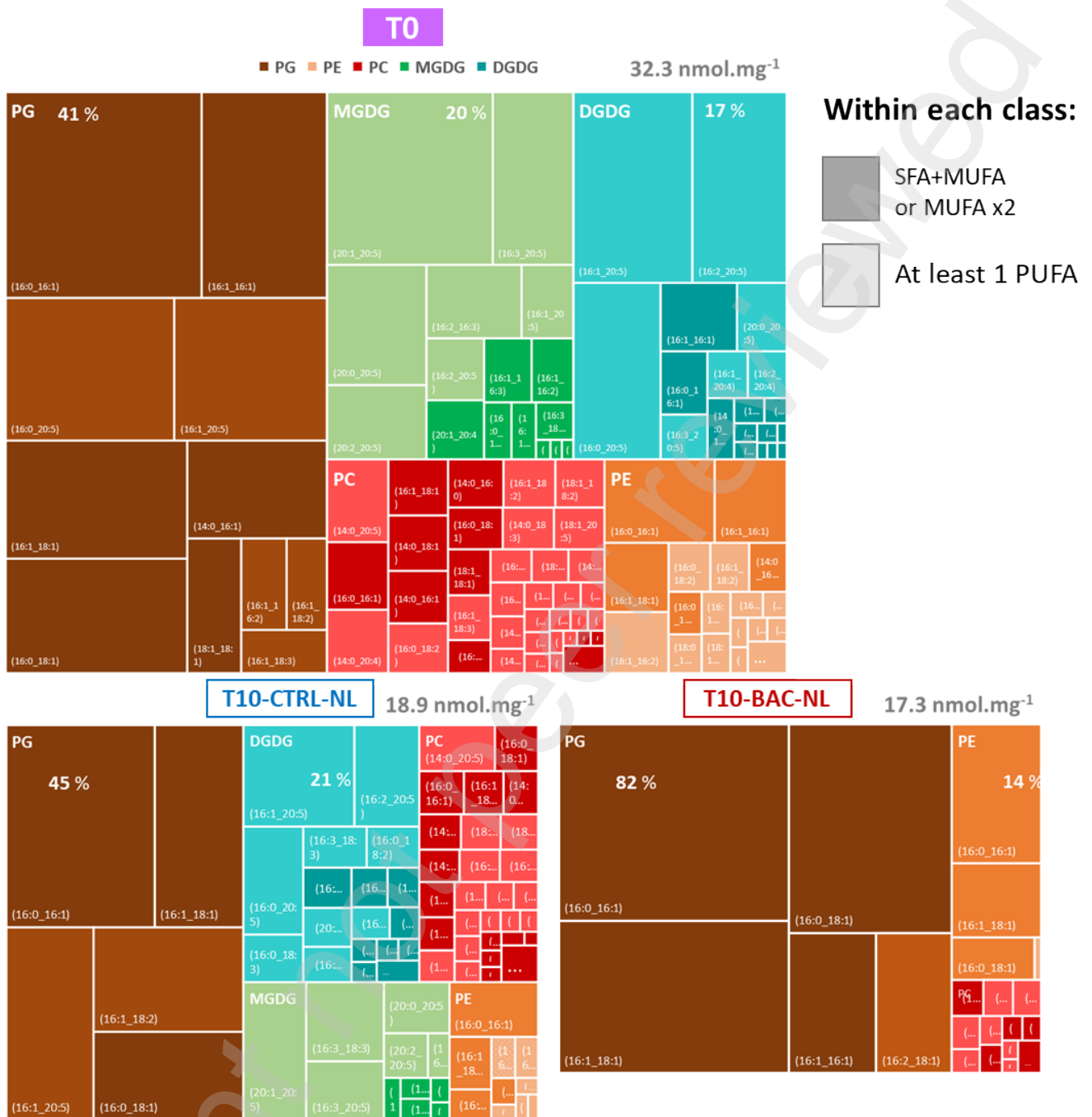
287 In the absence of contamination, C20:5 was the most abundant fatty acid in MGDG with
288 both photoperiods (Figure 3, panel A2), followed by C16:3, C16:2 and C16:1 and long chain
289 fatty acids such as C20:1 and C20:0. All fatty acids decreased over time with the exception of
290 C18:3, which became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

299 **3.2. Molecular species from polar lipids**

300 The molecular species identified within the main classes of PG, PE, PC, MGDG and
301 DGDG are presented in Figure 4. The area of each rectangle is proportional to the relative
302 amount in molar %. Only T0 and T10 under normal light conditions, with or without exposure
303 to BAC 12, are shown. A very similar trend was observed under continuous light conditions
304 and was consequently not illustrated here. At T0, PG clearly dominated with approximately
305 41%, then MGDG and DGDG with 20 and 17%, respectively. These polar lipids are generally
306 associated with thylakoid membranes in plants. Together, PC and PE represented less than a
307 quarter of the polar lipids. These compounds are more representative of cytoplasmic
308 membranes in plants. As with the fatty acids, we observed several molecular species containing
309 at least one PUFA on each of the two acyl chains of each glycerolipid. In the case of PG, and
310 especially MGDG and DGDG, we observed that most fatty acids were a combination between

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

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



323

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

327

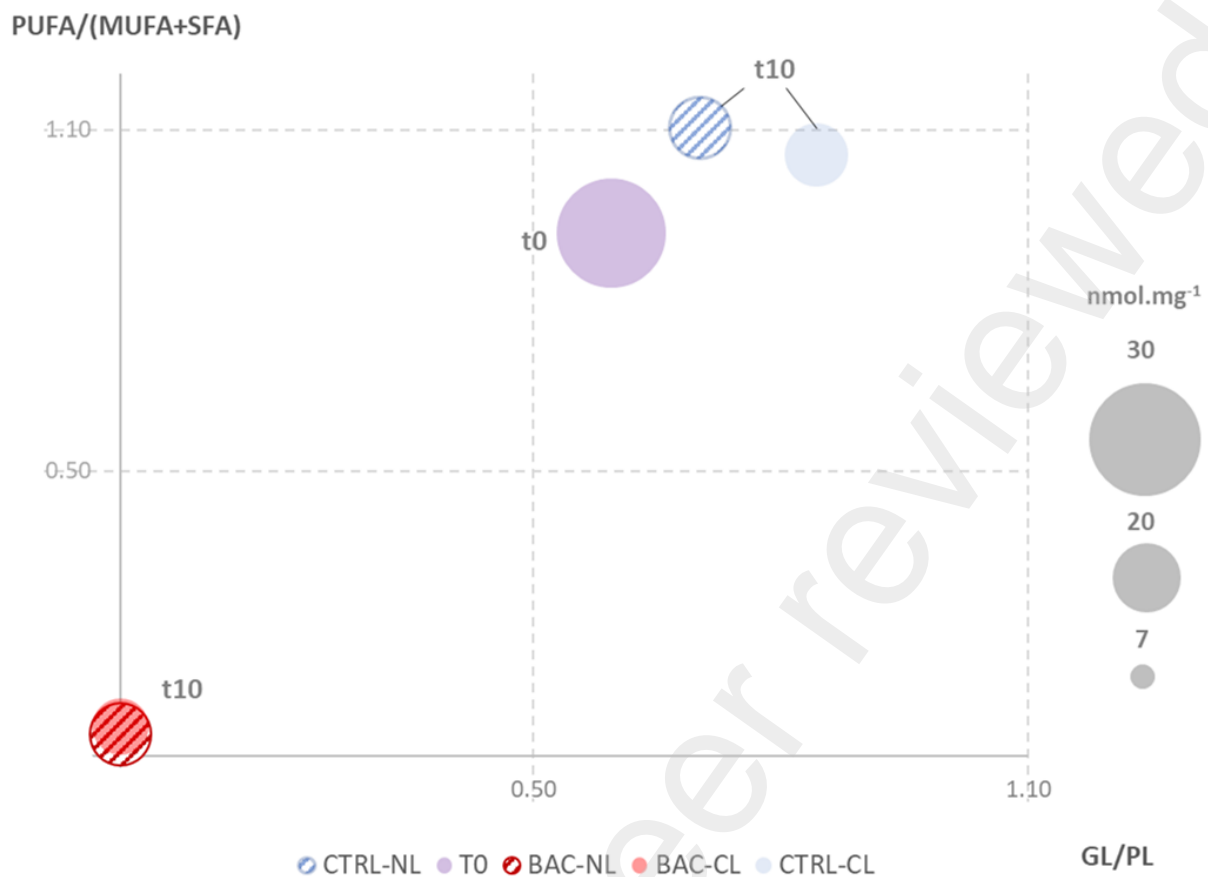
328 3.3. Covariation of polar lipid classes and fatty acid categories

329 A clear effect of BAC 12 on the evolution of both absolute and relative amounts of lipid

330 content in freshwater biofilms has been shown here at the fatty acid level, as previously reported

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

351



352

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

356

357

4. Discussion

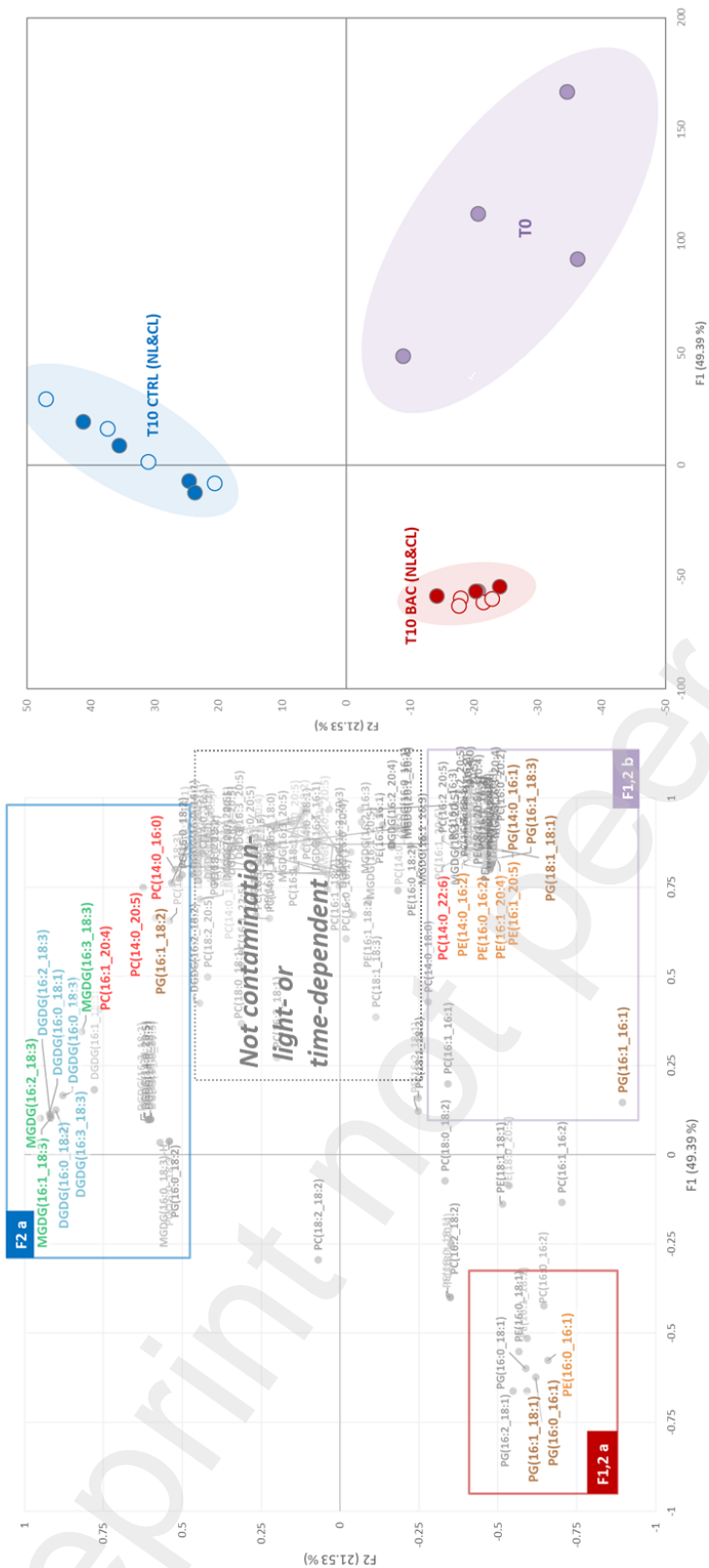
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359

4.1. BAC 12 effects over the time

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

383 to other fatty acid categories (Filimonova et al. 2016, Demailly et al. 2019, Fadhlouli et al.
384 2020).

385 An additional data treatment consisted in a PCA using the molecular species
386 composition of MGDG, DGDG, PG, PE and PC, in order to better distinguish the likely effects
387 of BAC12 and light condition at the algal group level. The variables described in the F1-F2
388 plane of the PCA (Figure 6) were sorted according to their decreasing contribution to these two
389 axes, including only those with $\cos^2 > 0.7$ (Table 1). A clustering of these filtered variables was
390 then performed using a k-means classification (Figure A 4), resulting into three “variable
391 clusters”, namely F2a, F1,2a and F1,2b. A fourth cluster has been identified, but when projected
392 onto the variable plot (Figure 6), it did not appear to provide any sample- or condition-specific
393 information. Additionally, we attempted to reassign molecular species to different autotrophic
394 groups based on information from the literature (Opute 1974, Dunstan et al. 1993, Bergé et al.
395 1995, Lang et al. 2011, Coniglio et al. 2021, Mazzella et al. 2023a). For example, molecular
396 species containing C14:0, C20:5 or C22:6 were preferentially related to diatoms. In contrast,
397 compounds with C18:2 or C18:3 associated with another 16- or 18-C SFA or MUFA were
398 preferentially linked to contributions from green algae or cyanobacteria. Finally, when the
399 molecular species appeared to be non-specific to a particular microbial groups (e.g. associations
400 primarily among C16:0, C16:1 and C18:1), we indicated that it was an undetermined origin.
401 Actually, it is possible to find such molecular species in all eukaryotic algae (Guschina and
402 Harwood 2006), in fungi (Bhatia et al. 1972) as well as in prokaryotic organisms (Zelles 1997,
403 Doumenq et al. 1999, Mazzella et al. 2005, Mazzella et al. 2007, Sohlenkamp and Geiger 2015).



404

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

410 Table 1. Molecular species filtered from the PCA results, considering the 20% of variables
 411 contributing the most to the F1 and F2 axes, as well as with a $\cos^2 > 0.7$ for the sum F1+F2.
 412 Three clusters of variables (F2a, F1,2a and b) were defined, and likely attributions of the
 413 variables was proposed according to the fatty acid composition highlighted within the selected
 414 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.

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

416 Cluster F2a was associated with the T10 CTRL samples, regardless of the photoperiod
 417 condition, while cluster F1,2b was associated with the initial samples at T0. Cluster F1,2b
 418 seemed to be more strongly related to the exposure to BAC 12 during the entire 10 days of
 419 exposure. This result suggests that the presence of such a biocide exerts a selective pressure on
 420 the different microorganisms constituting the biofilm, with a progressive elimination of
 421 microalgae in favor of fungi or prokaryotes (Sakagami et al. 1989). These findings observed at
 422 the molecular species level thus confirm the conclusions drawn from the taxonomic
 423 observations formulated by Vrba et al. (2023). We may thus propose some potential biomarkers
 424 by grouping increasing response of PC molecular species such as (14:0_16:0), (14:0_20:5) and
 425 (16:1_20:4), assumed to be here diatom-originating, in samples not contaminated with BAC 12
 426 (Figure 7). However, it would be interesting to investigate further the degree of specificity of

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

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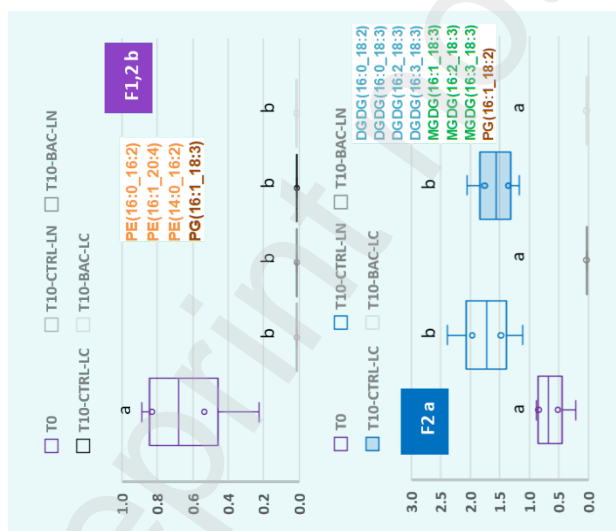
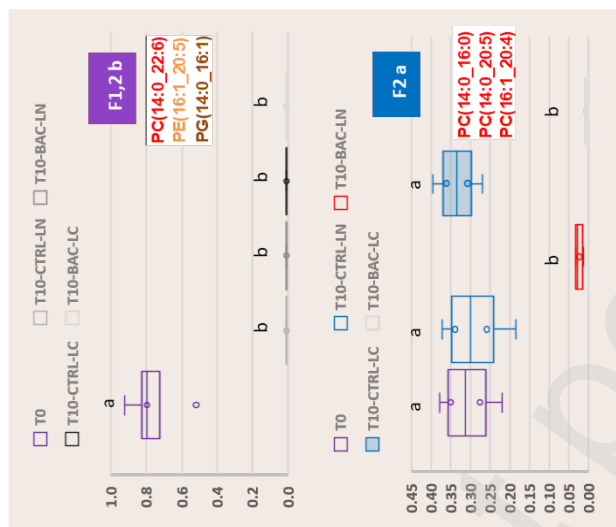
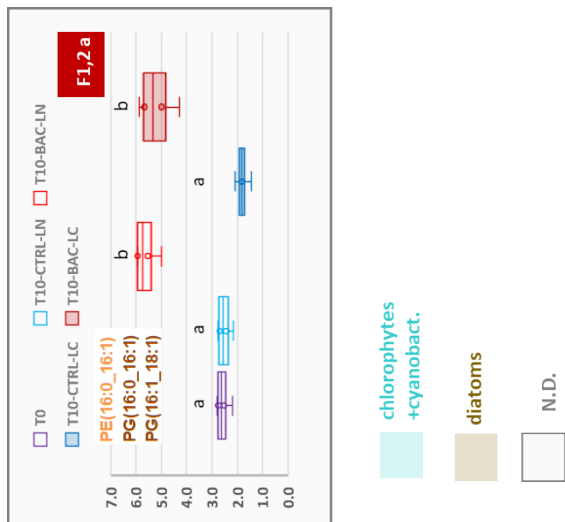
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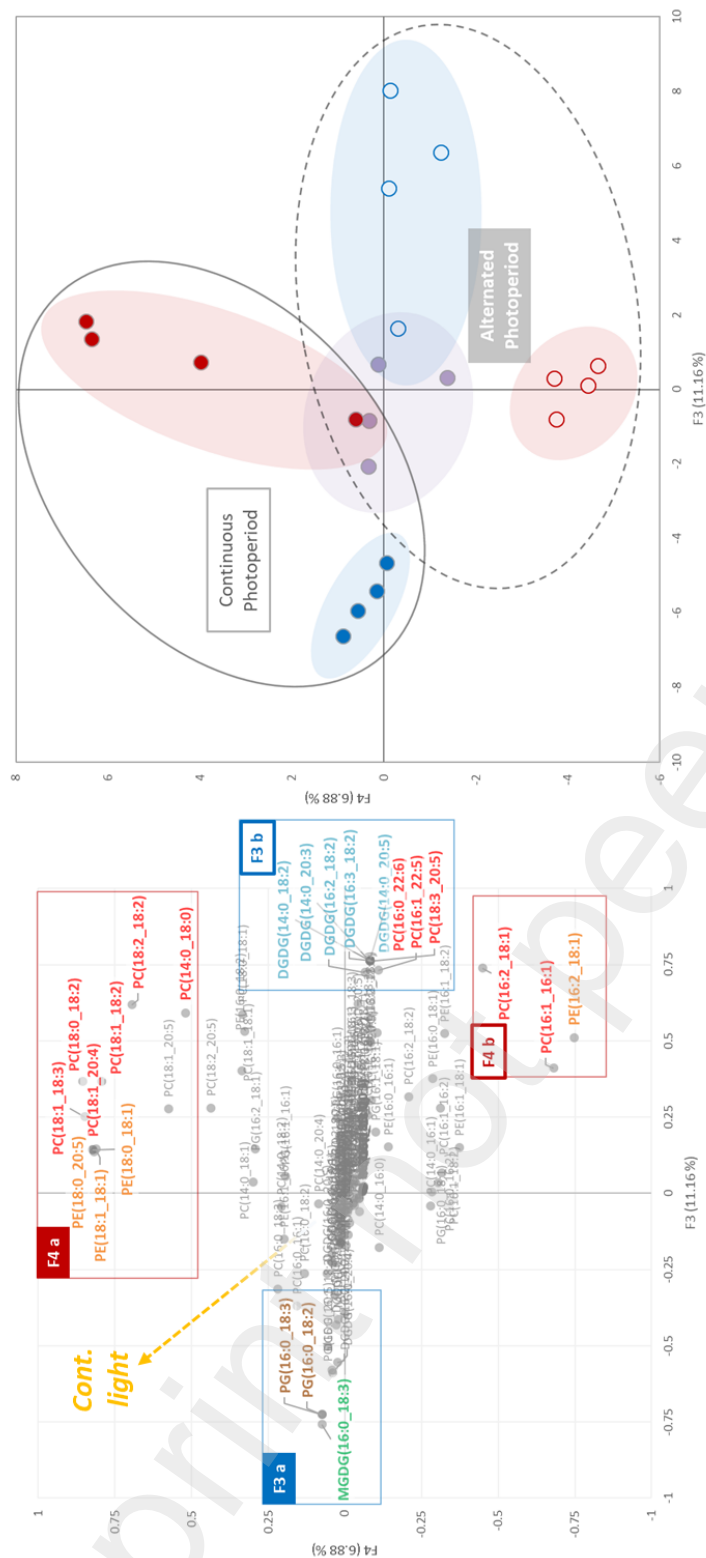


436

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

442 **4.2. Continuous versus alternated photoperiods**

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



460

461 Figure 8 F3- F4 planes of the PCA with the initial samples (T0), the controls after 10 days (T10
 462 CTRL) under continuous (CL) or normal (NL) light, as well as the samples contaminated with
 463 BAC 12 after 10 days (T10 BAC) under the two light conditions (CL and NL). The variables
 464 shown on the left correspond to the set of molecular species associated with polar lipids. The
 465 continuous light vector is a supplementary variable.

466

467 Focusing on F3 and F4 axes of the PCA, a more specific discrimination related to the
468 photoperiod was observed, as shown by the projection of this additional variable in the left part
469 the graph (Figure 8). We then filtered the variables best represented in this F3-F4 plane of the
470 PCA (Table 2) and defined four clusters (Figure A 4), as well as the probable assignment to
471 specific algal groups. It is interesting to note that this analysis allowed the distinction between
472 the samples that have undergone a continuous photoperiod from those that have been treated
473 with an alternating photoperiod, as they appeared to be separated according to the first bisector
474 associated with axes 3 and 4. Thus, the presence or absence of BAC 12 under the NL condition
475 (i.e. alternating photoperiod) was distinguished by the two clusters F3b and F4b. On the other
476 hand, the presence or absence of BAC 12 resulted in two other clusters, F3a and F4a, which
477 were more indicative of the CL condition. Moreover, according to this clustering of variables,
478 it seems that the continuous photoperiod may induce a relative increase in molecular species
479 associated with green algae and cyanobacteria to the detriment of those originating from
480 diatoms. In other words, the change in lipid composition revealed by molecular species analysis
481 indicated that the CL condition would promote the growth of certain photoautotroph groups
482 over time, regardless of the contamination pressure. Wang and Jia (2020) studied the
483 photoprotective mechanisms of *Nannochloropsis oceanica* in response to light, mainly from
484 the point of view of lipid and fatty acid classes, in parallel with the study of pigment
485 composition. These authors showed that at higher intensities, there was a fairly marked decrease
486 in MGDG and DGDG, but also in phospholipids. They also observed a decrease in most of the
487 fatty acids associated with polar lipids, but this did not appear to be specific to certain categories
488 such as PUFAs. The notable difference with our study is that the authors conducted their
489 experiment on an algae culture while we studied the response of biofilms. In addition, they
490 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
491 continuous photoperiod only.

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

497

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

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

499 The lipid content of algae is also significantly affected by light cycles. For example,
 500 Brown et al. (1996) studied the effects of different light regimes on the lipids of the diatom
 501 *Thalassiosira pseudonana* where 100, 50 and 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ under respective 12:12, 24:0 and
 502 24:0 h light/dark cycles were used. Cells grown at the high light intensity and 12:12 photoperiod
 503 exhibited higher concentrations of PUFAs and lower concentrations of both SFAs and MUFAs.
 504 Although it is very likely that the duration of the photoperiod also affected the autotrophs found
 505 in our biofilms, attributing changes to primary physiological effects at the level of each
 506 individual organism in terms of fatty acid (or molecular species) content alone seems rather
 507 uncertain. The change in lipid composition would appear here to be more closely tied to overall
 508 changes in community structure because we investigated a complex biofilm. Fatty acids alone
 509 may not provide sufficient information as they could, masking weaker effects like photoperiod

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

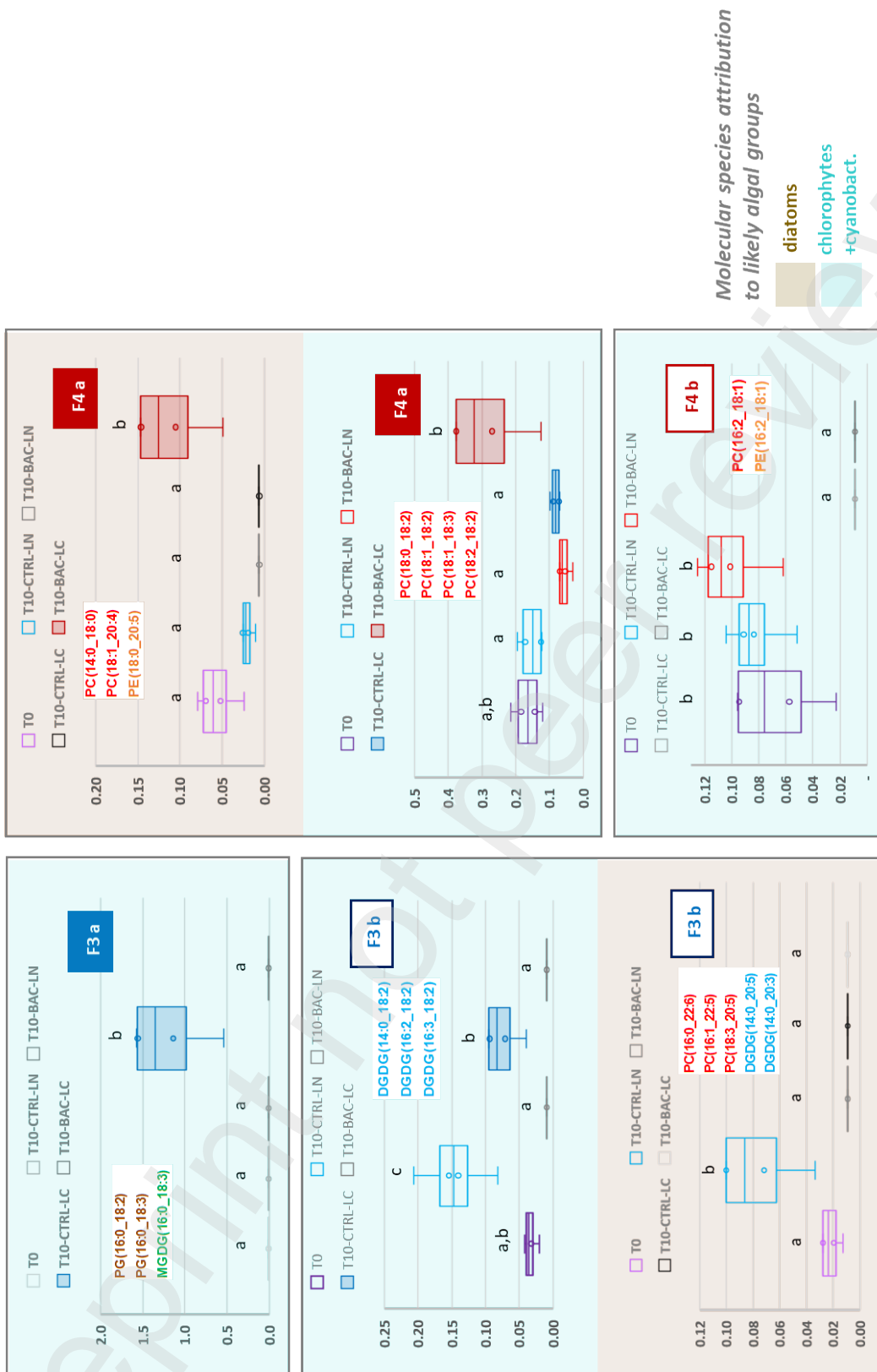
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521 Figure 9. Variation of molecular species (nmol mg^{-1}) clusters selected for each group of
 522 variables (F3 a and b, F4 a and b) as a function of time (T0 or T10), BAC 12contamination
 523 (CTRL or BAC) and light condition (CL or NL). The distinction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

526 **Conclusions**

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

539

540

541 **Author contributions**

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

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

544 Aurélie MOREIRA: Lipid and micropollutant analyses

545 Nicolas CREUSOT: Writing - review & editing.

546 Mélissa EON: Sample preparation, Physico-chemical analysis

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

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

549 Soizic MORIN: Funding acquisition, Supervision, Conceptualization, Writing - review &
550 editing.

551

552 **Conflicts of interest**

553 None

554

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663 **Appendices**

664

665 Table A 1. BAC 12 concentrations (mg L⁻¹) in the four experimental conditions at T10. BAC
 666 12 = contaminated biofilm; CTRL = non-exposed biofilm; NL = alternated photoperiod; CL =
 667 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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670 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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674 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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678 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

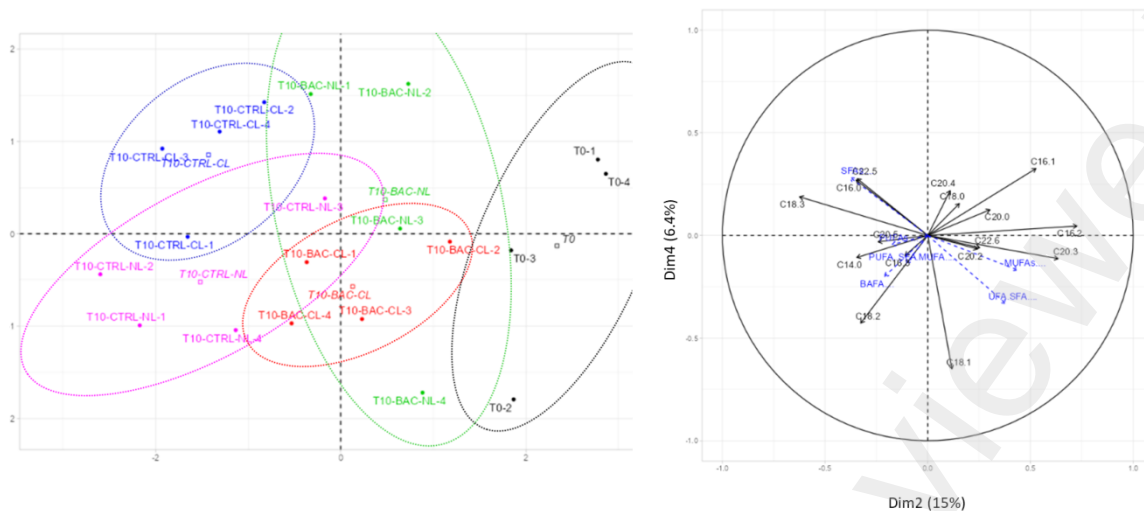
SFAs (%)	20%	18%	42%	15%	44%	44%	26%	13%	56%	44%	32%	11%
MUFAs (%)	55%	25%	29%	54%	31%	21%	51%	46%	42%	51%	65%	86%
PUFAs (%)	26%	56%	28%	30%	26%	35%	23%	41%	2%	5%	4%	2%
PUFA/(SFA+MUFA) (%)	0.34	1.29	0.40	0.43	0.34	0.54	0.30	0.70	0.02	0.05	0.04	0.02
UFA/SFA (%)	4.04	4.44	1.36	5.51	1.29	1.25	2.91	6.62	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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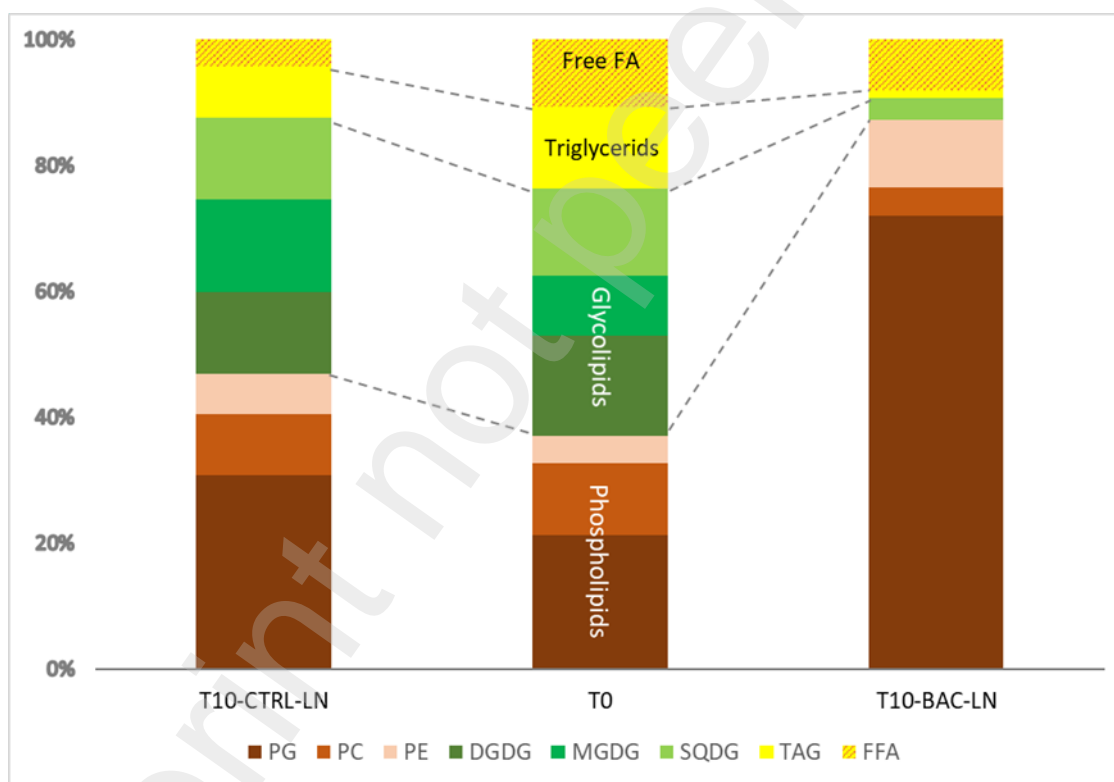
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682 Figure A 1. Dimensions 2 and 4 of the PCA for fatty acids.

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685 Figure A 2. Average lipid classes and FFA relative proportions.

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689 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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695 Table A 6. Multivariate analysis of variance for both PUFA/(MUFA+SFA) and GL/PL ratios.

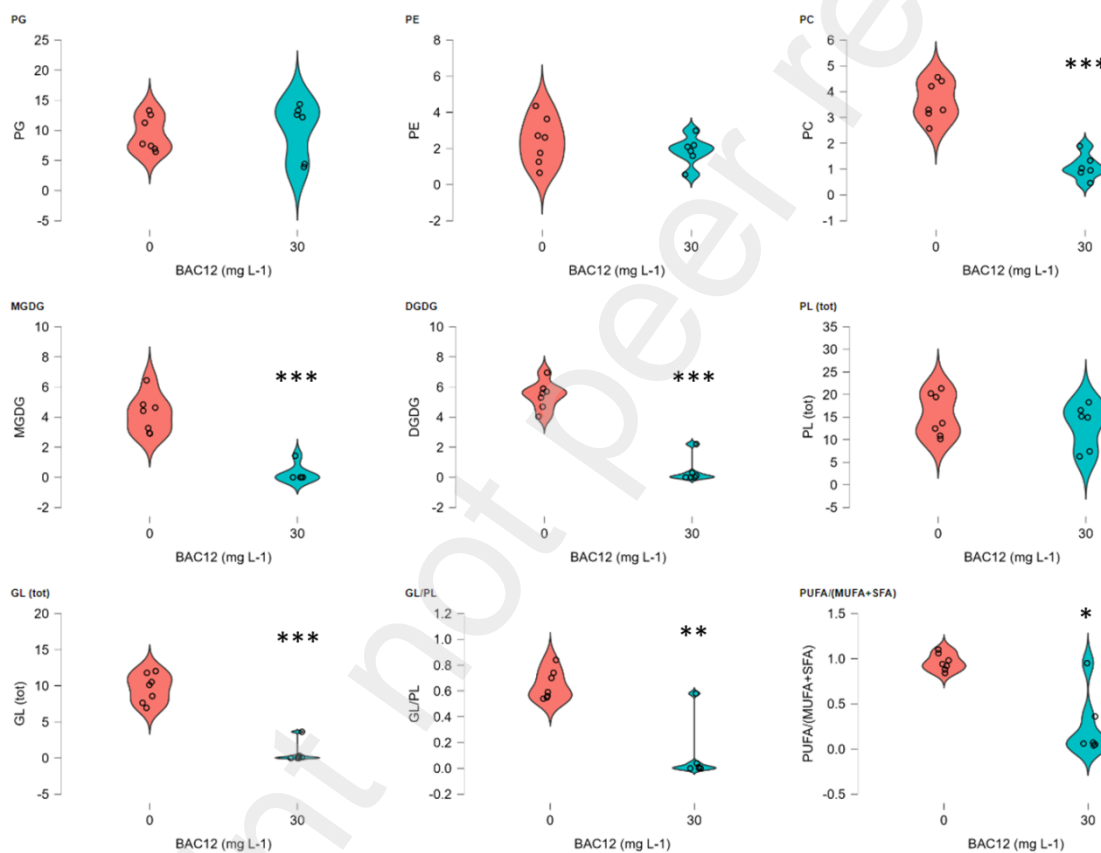
MANOVA

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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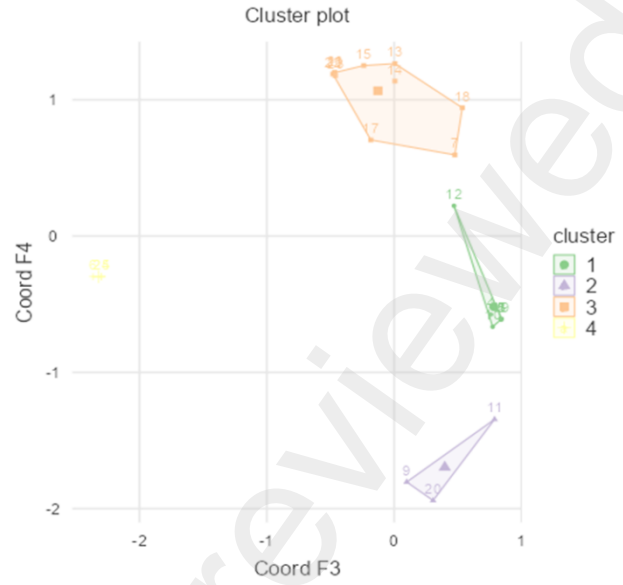
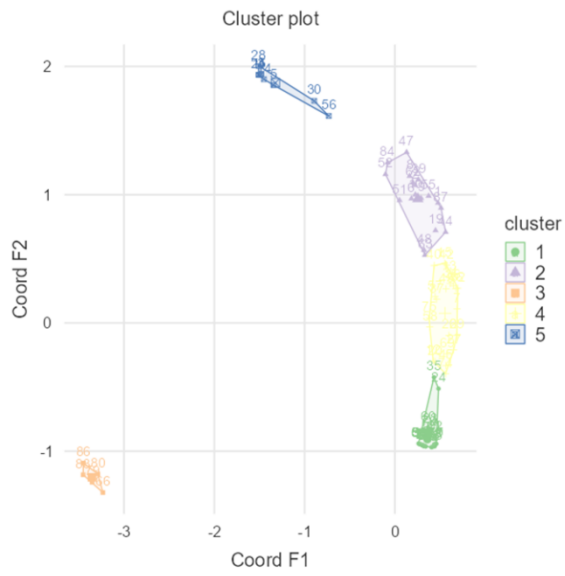


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699 Figure A 3. Univariate (Welch's ANOVA) representation for each polar lipid class as well as
 700 PUFA/(MUFA+SFA) and GL/PL ratios according to BAC 12 exposure. * for p<0.05, ** for p<0.01,
 701 *** for p<0.001.

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

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