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Microalgal food sources greatly improve macroinvertebrate growth in detritus-based headwater streams: Evidence from an instream experiment

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1 **Title:** Microalgal food sources greatly improve macroinvertebrate growth in detritus-based
2 headwater streams: Evidence from an instream experiment

3

4 **Running head:** *Microalgae boost invertebrate growth in streams*

5

6 **Authors:** Tiphaine Labeud-Veydert¹, Michael Danger^{2,3}, Vincent Felten², Alexandre Bec¹,
7 Martin Laviale², Maria Cellamare⁴, Christian Desvilettes¹

8

9 ¹ Université Clermont Auvergne, UMR CNRS 6023, Laboratoire Microorganisme, Génome,
10 Environnement, Impasse Amélie Murat, 63170 Aubière

11 ² Université de Lorraine, CNRS, LIEC, F-57000 Metz, France

12 ³ Institut Universitaire de France (IUF), Paris, France

13 ⁴ Phyto-Quality, Paris, France

14

15 **Correspondance to:** C. Desvilettes, christian.desvilettes@uca.fr

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18 Nutritional quality, Compound-Specific Stable Isotopes, epilithic biofilms, *Gammarus pulex*,
19 *Rhithrogena semicolorata*

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35 **Abstract:**

36 1. In forested headwater streams, inconspicuous food resources such as epilithic microalgae can
37 play a major role due to their content of long chain polyunsaturated fatty acids (FAs) that are
38 essential for macroinvertebrate development. Yet, the use of these resources and their
39 consequences for consumers and life history traits remain scarcely studied, especially for non-
40 herbivorous taxa.

41
42 2. Using in instream mesocosms, we aimed to understand how two macroinvertebrate species, a
43 shredder detritivore (*Gammarus pulex*) and a scraper (*Rhithrogena semicolorata*), use available
44 organic resources under light versus shaded conditions (high and low amounts of phototrophic
45 biofilm, respectively). We specifically focused on the origin of carbon and essential lipids
46 (polyunsaturated FAs, sterols) assimilated by the two species and the effects of these
47 compounds on their survival and growth.

48
49 3. When autotrophic biofilms were available (mesocosms exposed to light), both species
50 experienced significantly higher growth than in mesocosms placed in the dark. In *Rhithrogena*
51 nymphs, the survival and imago emergence rates were positively affected by access to
52 autotrophic biofilms.

53
54 4. Using stable isotope analysis ($\delta^{13}\text{C}$ & $\delta^{15}\text{N}$), we demonstrated that under dark conditions both
55 macroinvertebrates assimilated carbon of detrital origin. Under light conditions, most of the
56 carbon assimilated by *R. semicolorata* was derived from autotrophic biofilms and around 11%
57 from fine detrital particles (FPOM). *G. pulex* derived part of its carbon from detrital sources
58 (mainly FPOM) and from autotrophic biofilms.

59
60 5. The results of isotopic analyses ($\delta^{13}\text{C}$) on fatty acids and sterols showed that the scraper
61 *Rhithrogena* was entirely dependent on autotrophic biofilms to meet its dietary FAs and sterol
62 requirements. In contrast, detrital sources were quantitatively important for *G. pulex* both in terms
63 of carbon and sterol supply, irrespective of the conditions tested. For these consumers,
64 microalgae seemed to be a complementary food source, but yet essential to cover a large part of
65 their requirements in long chain polyunsaturated FAs.

66
67 6. This study clearly confirmed the ecological importance of autotrophic biofilms for two
68 representative functional feeding groups of macroinvertebrates in headwater streams providing
69 new insights on the trophic origin of sterols and long chain polyunsaturated FAs in their diet.

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72 **Introduction**

73

74 In temperate regions, headwater stream invertebrates obtain energy from a limited number of
75 organic sources defined either as allochthonous (e.g., leaf litter from riparian vegetation) or
76 autochthonous (epilithic and periphytic biofilms) (Allan & Castillo, 2007). While leaf litter can be
77 quantitatively dominant in the diet, (Collins et al., 2016), it is typically considered to be of poor
78 nutritional quality (Tank et al., 2010). One of the main limitations of the nutritional value of leaf
79 litter is the lack of long-chain polyunsaturated fatty acids (LC-PUFAs) 20:5 ω 3, 20:4 ω 6 (Torres-
80 Ruiz et al., 2007; Brett et al., 2017), regardless of the leaf palatability, which is a function of the
81 tree species considered. Undoubtedly, microorganisms and hyphomycetes colonizing submerged
82 leaves increase nutritional quality by reducing stoichiometric imbalances (Danger et al., 2013),
83 but they do not increase LC-PUFA contents, which remain limited to the presence of 18:2 ω 6 and
84 18:3 ω 3 compounds (Torres-Ruiz et al., 2007). In contrast, the nutritional importance of
85 autotrophic biofilms for stream invertebrates has only recently been highlighted (Guo et al.,
86 2016a) and this is in part due to the higher polyunsaturated fatty acid (PUFA) content of
87 microalgae found in biofilms. LC-PUFAs are particularly abundant in epilithic diatoms in
88 headwater streams (Cashman et al., 2013; Honeyfield & Maloney, 2015; Guo et al., 2016a).
89 Diatoms can also contain sterols in sufficient nutritional quantity for a number of aquatic
90 invertebrates (Gergs et al., 2015), making microalgae a high quality nutritional resource. Over the
91 past decade, studies of numerous aquatic invertebrate species have highlighted the importance
92 of dietary LC-PUFAs (20:5 ω 3, 20:4 ω 6) for their survival, somatic growth and reproduction
93 (Ahlgren et al., 2009; Aguilar et al., 2012; Guo et al., 2016a; Crenier et al., 2017). The LC-PUFAs
94 20:4 ω 6 and 20:5 ω 3 are considered "essential nutrients" in the sense that their bioactive nature
95 has a positive effect on invertebrate growth or other biological responses (Tocher et al., 2019).
96 Macroinvertebrates are therefore expected to recover most of their essential lipid compounds, or
97 even most of their carbon, from microalgae (Brett et al., 2017). Consumption of microalgae rich in
98 20:5 ω 3 and sterols that are precursors of cholesterol synthesis can improve somatic growth in
99 both invertebrate shredders and grazers (Guo et al., 2016a; Martin-Creuzburg & Merkel, 2016;
100 Crenier et al., 2017), provided there is sufficient algal biomass (Kühmayer et al., 2020). This last

101 condition is important because shading in headwater streams can severely limit biofilm growth, in
102 turn limiting invertebrate access to sufficient levels of LC-PUFAs (Hill et al., 2001; Honeyfield &
103 Maloney 2015). It is therefore necessary to determine of resource use when primary production is
104 temporally limited or markedly reduced in forested streams (Alberts et al., 2018; Torrez-Ruiz et
105 al., 2019). Most of the carbon and fatty acids (FA) and even sterols, in invertebrate diets, are
106 derived from microalgae when epilithic biofilms and/or litter biofilms are well developed
107 (Kühmayer et al., 2020). If biofilm development is limited, allochthonous sources will be more
108 important for carbon supply, with microalgae being a secondary food source. Limited access to
109 LC-PUFAs could then become a trophic constraint for macroinvertebrates, the importance of
110 which probably depends on their functional feeding group (FFG) and stage of development.

111 In this context, the aim of our study was to evaluate the use of allochthonous and autochthonous
112 organic sources by two macroinvertebrates species commonly found in temperate headwater
113 streams in Europe, and belonging to contrasting FFGs: the amphipod shredder *Gammarus pulex*
114 and the grazer-scraper *Rhithrogena (semicolorata*-group; Heptageniidae). An experiment was
115 carried-out in early summer under semi-natural conditions, using instream mesocosms placed
116 under either naturally light or dark conditions thereby enabling the development of predominantly
117 autotrophic or heterotrophic biofilms, respectively. Our main objectives were: 1) to understand the
118 origin of carbon and essential lipids (LC-PUFAs, sterols) assimilated by the two species
119 according to their level of access to autotrophic biofilms, and then 2) to assess the impact of
120 essential lipids on their growth and development. We used stable-isotope ratios ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$)
121 to estimate the relative contributions of organic sources to the bulk carbon assimilated by both
122 species. Assuming that $\delta^{13}\text{C}$ values differed sufficiently between organic sources then it should
123 be possible to reveal the relative influence of different sources on the diet assimilated by
124 consumers (Caroll et al., 2016). To trace the origin of PUFAs and sterols, we used stable
125 compound-specific isotope analysis (CSIA).

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131 **Materials and methods**

132 *Experimental devices and procedures*

133 The experiment was performed from 20 June to 11 July 2018 on a controlled-flow reach of the
134 Desges River, a fourth-order stream located in the French Massif Central (Alt: 511m;
135 45°4'43.84"N - 3°31'55.57"E). Six mesocosms were arranged in two blocks of three: one block
136 was exposed to natural light whereas the other block was placed 30 m upstream and kept in the
137 dark using an opaque tarpaulin stretched across the reach. The reach presented an
138 homogeneous morphology (depth: 0.5 m; width: 1.40 m) with sandy substrate and the two blocks
139 of mesocosms were separated by a short distance (30 m) minimising any differences between
140 blocks/treatments associated with stream slope, altitude or similar variables. So, although the
141 light treatment was confounded with the spatial location of blocks in this design, differences in
142 other conditions between the two blocks are likely to be small compared to the treatment effect.

143 Water quality was monitored during the survey using AFNOR guidelines (AFNOR, 1990). Its
144 physico-chemical characteristics were as follows: pH = 7.2, Silicate = $11.4 \pm 0.4 \text{ mg.L}^{-1}$, $P_{\text{total}} = 28$
145 $\pm 1 \mu\text{g.L}^{-1}$ and $N_{\text{total}} = 725 \pm 25 \mu\text{g.L}^{-1}$. Data loggers (HOBO™, PCE Inst., Soultz, France) were
146 used to record the level of light and the temperature inside the flumes and in the reach every hour
147 throughout the experiment. The mesocosms, manufactured by AOT-Plastics® (Thiers, France),
148 comprise a plexiglass flume (L x W x H: 1 x 0.25 x 0.25m) fitted with two movable grids (mesh
149 sizes: 250 μm) for water inlet and outlet and a dorsal hatch that can be fitted with an emergence
150 trap. For our experiment, the bottom of the flume was covered with quartz gravel for invertebrate
151 shelter and ceramic tiles (10 x10 cm) were placed on the gravel to allow autotrophic (light
152 conditions, LC) or heterotrophic (dark conditions, DC) biofilm to develop over a period of three
153 weeks. Four coarse-meshed litter bags (mesh size 10 mm), each containing 6 grams of dry Alder
154 leaves (*Alnus glutinosa*; harvested the previous fall) were also implanted in each flume to be
155 microbially-conditioned over the three-week period.

156 On the day before the start of the experiment, *Gammarus pulex* were collected from a small
157 tributary of the Desges river and *Rhithrogena semicolorata* nymphs were collected from the
158 upstream part of the Desges river. The macroinvertebrates were separated into six groups

159 containing 100 *G. pulex* of homogeneous size (4.94 ± 0.99 mm) and 43 *R. semicolorata* nymphs
160 (6.33 ± 1.41 mm) in similar stages with developing wing pads. At T0, a batch of 100 *G. pulex* and
161 43 *R. semicolorata* was introduced into each mesocosm. Maintenance of the flumes was carried
162 out every two days and *R. semicolorata* emerged imago were counted every three days.

163

164 *Sampling*

165 Sampling of basal sources was carried out at the beginning (T0: June 20) and at the end (T20:
166 July 9) of the experiment. For each replicate, leaf material in the flume was removed from two
167 litter bags and rinsed to remove fine debris and potential micro-invertebrates. Sub-samples of leaf
168 material were used for lipid and stable isotope analysis and stored at -80°C . The fine shredded
169 leaf debris were sieved through a stack of filters with different mesh sizes, yielding the fine
170 particulate organic matter FPOM (fraction 0.10 to 2 mm). FPOM was concentrated on a GF/A
171 filter and frozen at -80°C prior to further analysis. In each replicate flume, ceramic tiles were
172 taken at random and scraped with a toothbrush in order to recover the biofilm microorganisms.
173 During the experiment, a biofilm gradually developed on the plexiglass walls in LC only and was
174 collected from a 169 cm^2 wall surface on each LC flume. The biofilms were divided into sub-
175 samples, half of which was filtered on GF/F filters and frozen at -80°C prior to further analyses,
176 and the other half fixed with a lugol solution for microalgae identification and enumeration.

177 At T0, an initial batch of macroinvertebrates, similar to those introduced into the plexiglass
178 flumes, was kept for analysis. One subsample of 30 *G. pulex* or *R. semicolorata* was fixed in 70%
179 ethanol to determine initial size measurements. Three triplicates of 10 *G. pulex* and 5 *R.*
180 *semicolorata* were frozen at -80°C prior for lipid and stable isotope analysis (SIA). At the end of
181 the experiment (T20), all the invertebrates were collected from each flume, counted and
182 separated into sub-samples. Depending on the number of survivals, *G. pulex* (DC: 3 x 8; LC: 3 x
183 10) and *R. semicolorata* (DC: 3 x 4; LC: 3 x 4) were fixed in 70% ethanol to determine their
184 respective final sizes and gut contents. In both tested conditions the remaining
185 macroinvertebrates were kept for 24 hours in filtered reach water to empty their digestive tracts,
186 divided into triplicates of 3 to 5 individuals each, and frozen at -80°C prior to SIA and lipid
187 analysis.

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Survival and macroinvertebrate growth analysis

Survival was calculated from living invertebrates collected at the end of the experiment minus sampled individuals or emerged subimagos (in the case of *R. semicolorata*). *G. pulex* were photographed under a stereomicroscope (x6 to x40 magnification) in their curved state. Length was recorded from the base of the first antenna to the base of the telson using SigmaScan Image Analysis Version 5.000 (SPSS Inc, Chicago, IL, USA; linear state = 0.88 x curved state [mm]; $r^2 = 0.98$). Total body length of *R. semicolorata* (not including cerci) was measured under a stereomicroscope.

Basal sources

Leaf mass remaining in each bag (n=4) was weighed and compared to the initial mass. Leaf litter decomposition rates (k) in both treatments were estimated using an exponential model ($m_t = m_0 \times e^{-kt}$), where m_t is the leaf mass remaining at t (T20) and m_0 is the initial leaf mass. The counting of biofilm microalgae and taxonomic identification were undertaken using an inverted microscope following the Utermöhl method. Cell measurements were carried out using a digital camera and an image analyser software (Zen 2, C. Zeiss GmbH, Göttingen, Germany) and the results were expressed in terms of biovolume ($\mu\text{m}^3 \cdot \text{cm}^{-2}$).

Macroinvertebrate diet analysis

The diet composition of both species at the end of the experiment was determined by gut content analyses using the method of Felten et al. (2008). Briefly, foregut contents were placed into a drop of water on microscope slides, homogenized, then examined at different magnifications from 100x to 400x to assess the approximate percentage by area of the six items recorded: (i) animal matter, (ii) FPOM (fine amorphous detritus lacking well-defined cellular structure), (iii) diatoms, (iv) filamentous algae, (v) CPOM (coarse particulate organic matter: mainly leaf detritus with brownish palisade cell layers), and (vi) small mineral particles.

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218

219 *Bulk stable isotope analysis*

220 All samples for bulk stable carbon and nitrogen isotope analysis were oven-dried at 60°C for
221 24h-48h, then ground, homogenized, and weighed in a tin capsule. Samples were prepared in
222 triplicate for each condition and injected into a Carlo Erba NC2500 Elemental Analyzer (Milan,
223 Italy) linked to a Thermo Finnigan Delta Plus Mass spectrometer (Les Ullis, France). Isotopic
224 ratios were presented as δ values (‰) expressed relative to the atmospheric N₂ and Vienna Pee-
225 Dee Belemnite for nitrogen and carbon, respectively. Analytical precision (based on the standard
226 deviation of replicates of internal standards) was never > 0.4‰ and 0.23‰ for nitrogen and
227 carbon respectively.

228

229 *Lipid analysis: FA and sterols*

230 All samples were freeze-dried before lipid analysis. The FA composition was determined from
231 total lipids (TL) for basal organic sources and from neutral lipids (NLFA) and polar lipids (PLFA)
232 for *G. pulex* and *R. semicolorata* nymphs. Lipids were extracted and when necessary fractionated
233 using pre-packed silica columns (SPE Strata NH2 Phenomenex®, Le Pecq, France) following the
234 methods described by Koussoroplis et al. (2010). FAs from lipid extracts were converted into FA
235 methyl-esters (FAME) by acid-catalyzed transfersterification (Koussoroplis et al., 2010). FAME
236 were separated and analyzed using an Agilent 6850 gas chromatograph (Les Ullis, France)
237 equipped with a J&W DB-WAX capillary column (Agilent, 30 m × 0.25 mm). Individual FAME
238 were identified by comparing retention times with certified commercial standard mixtures and
239 quantified using 13:0 and 23:0 as internal standards. The identification of all peaks was confirmed
240 or refined using an Agilent 6850 GC coupled to a 5975B Agilent Mass Spectrometer (Les Ullis,
241 France). FAME Spectra separated with a DB wax column were identified using the reference
242 libraries and databases available (NIST/NBS; lipidhome). Analysis of the sterols was undertaken
243 using total lipid extracts obtained from the macroinvertebrate samples and from basal sources.
244 Lipids were saponified with 0.2 mol.L⁻¹ methanolic KOH. Then, the unsaponifiable material was
245 extracted with hexane - diethyl ether, following the detailed procedure of Gergs et al. (2015). After

246 derivatization by silylation, sterol TMS ethers were analyzed by GC-MS using an Agilent 6850 GC
247 coupled to a 5975B Agilent mass spectrometer and equipped with a J&W HP-5MS (Agilent, 30 m
248 × 0.25 mm) capillary column. Each sterol was identified by its mass spectrum and quantitation
249 was carried out using 5 α -cholestane as an internal standard and calibration curves generated
250 with our different laboratory standard sterols.

251

252 *Compound-Specific Isotope Analysis (CSIA)*

253 The stable carbon isotope signatures ($\delta^{13}\text{C}$ values) of FAME and sterol TMS ethers were
254 determined using a Thermo Trace 1310 GC interfaced with a Thermo Finnigan MAT 253 IRMS
255 (Les Ullis, France) via a GC IsoLink II combustion interface. FAME were separated using an
256 Agilent DB-FATWAX capillary column (30m x 0.25mm ID x 0.25 mm film) while sterol TMS ethers
257 were separated in a nonpolar DB-5ms capillary column (60m x 0.25mm ID x 1 mm film). All the
258 isotope values were reported using the δ notation relative to the Vienna Pee-Dee Belemnite
259 reference standard. The measured $\delta^{13}\text{C}$ values were corrected by isotopic mass balance for
260 contributions of carbon incorporated during derivatization of FA and sterols. FAME $\delta^{13}\text{C}$ values
261 were corrected using the formula:

$$\delta^{13}\text{C}_{\text{FA}} = \frac{[(n + 1) \times \delta^{13}\text{C}_{\text{FAME}} - \delta^{13}\text{C}_{\text{MeOH}}] - 1}{n}$$

262 where $\delta^{13}\text{C}_{\text{FAME}}$ and $\delta^{13}\text{C}_{\text{MeOH}}$ are the $\delta^{13}\text{C}$ values of the measured fatty acid methyl ester and
263 methanol used during methylation, respectively. $\delta^{13}\text{C}_{\text{FA}}$ represents the FA $\delta^{13}\text{C}$ before its
264 methylation, and n is the number of carbon atoms in the non-methylated FA.

265 Sterol $\delta^{13}\text{C}$ values were corrected using the formula:

$$\delta^{13}\text{C}_{\text{sterol}} = \frac{[(n\text{CsterolTMS} \times \delta^{13}\text{C}_{\text{sterolTMS}}) - (3 \times \delta^{13}\text{C}_{\text{TMS}})]}{n\text{Csterol}}$$

266 where $\delta^{13}\text{C}_{\text{sterolTMS}}$ and $\delta^{13}\text{C}_{\text{TMS}}$ are the $\delta^{13}\text{C}$ values of the measured sterol TMS ether ester and
267 BSTFA used during silylation, respectively. $\delta^{13}\text{C}_{\text{sterol}}$ represents the sterol $\delta^{13}\text{C}$ before its
268 silylation, and n is the number of carbon atoms in the sterol and its sterol TMS counterpart.

269

270 *Data analysis*

271 All statistical analyses were carried out using Rstudio and open source R packages (Version
272 1.1.456 – © 2009-2018 RStudio, Inc.). The normality and homoscedasticity of the data were
273 tested with the Shapiro-Wilk and Levene tests, respectively. Diets and performance parameters
274 of both macroinvertebrate species were compared between treatments using a proportion test
275 (prop.test Z) while growth parameters were compared with a Mann-Whitney U-Test. Biochemical
276 compound data (FA, sterols, $\delta^{13}\text{C}$ values) were analyzed between and within treatments.
277 Depending on the data distribution, a Mann-Whitney U-Test or a Student t-test was applied to
278 compare paired samples. A one way- ANOVA followed by post hoc multiple comparison
279 (Pairwise) test was used to compare several samples. The significance level was set at $p < 0.05$.
280 For bulk carbon isotopes, we used the mixing model proposed by Rasmussen (2010) to estimate
281 the proportion of terrestrial consumption (leaf litter) versus microalgae consumption by both
282 macroinvertebrates. This author established linear relationships between $\delta^{13}\text{C}$ signatures of river
283 biota, (benthic algae, periphyton, herbivores, gatherers and shredders) along river gradients that
284 allow calculation of the contribution of terrestrial food sources (P_t) to macroinvertebrate functional
285 groups. We used the following equation to estimate the proportion of leaf litter consumed based
286 on the proportion of biofilm microalgae consumed.

$$P_t = (1 - P_a) \text{ and } P_a = \frac{(Y_c - f) - Y_t}{Y_a - Y_t}$$

287 Y_a is the measured $\delta^{13}\text{C}$ signature of the biofilms in each mesocosm; Y_t is the mean measured
288 $\delta^{13}\text{C}$ signatures of the leaf litters; Y_c represents the $\delta^{13}\text{C}$ signature of both macroinvertebrates in
289 each mesocosm; P_t and P_a represent the mixing proportions of allochthonous and
290 autochthonous inputs to macroinvertebrates; f is the trophic signature shift relative to algae
291 estimated for stream invertebrates by Rasmussen (2010) using its gradient model.

292 Concerning total FA, NLFA, and PLFA, the similarities between each basal source and the two
293 macroinvertebrates were analyzed using a Hierarchical Principal Component Classification
294 (Husson et al., 2010). The hierarchical clustering performed on the principal components of the
295 PCA was based on the mean $\delta^{13}\text{C}$ values of all FA and sterols using Euclidean distances.

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300 **Results**

301 *Environmental conditions*

302 Temperatures remained stable during the whole experiment with an amplitude of less
303 than 0.5°C between flumes exposed to light (LC: 13.90 ± 1.87°C) and those kept in the dark (DC:
304 13.46 ± 1.54°C; Supporting information 1). The average amount of light cast on the bottom of the
305 flumes at solar noon (illuminance) were 46 and 2,163 lux in the dark and light conditions,
306 respectively. Thus, darkness was not complete in the dark condition with the conservation of an
307 attenuated Light–Dark cycle.

308

309 *Macroinvertebrate performances: Survival, emergence and growth*

310 After the 20 days of exposure, survival rates of *G. pulex* were close to 50% and were
311 similar in the two tested conditions (Table 1), whereas survival of *R. semicolorata* nymphs was
312 significantly higher in the light (by 53%; prop-test $p < 0.01$). Growth rates of macroinvertebrates
313 were significantly enhanced when reared in the light, by 60% for *G. pulex* (Student t-test $p < 0.001$)
314 and by 400 % for *R. semicolorata* ($p < 0.01$; Table 1). Emergence rates of *R. semicolorata*
315 subimagos were also significantly higher (by 91%; prop-test $p < 0.05$).

316

317 *Food sources: Leaf litter decomposition and biofilm development*

318 Alder decomposition rates were unaffected by light condition and remaining leaf litter after
319 20 days was about 25% of the initial mass (Table 2). Total biofilms biovolumes developed on tiles
320 were stable during the experiment (similar at T0 and T20: see supporting information 2) and
321 systematically higher in the light compared to dark condition ($p < 0.001$ -Student t-test; Table 2). A
322 strong biofilm development was observed at T20 on flume walls only in the light, representing
323 $10^6 \pm 103 \times 10^6 \mu\text{m}^3 \cdot \text{cm}^{-2}$ with diatoms accounting for only 31% and chlorophytes being
324 dominant (62%). On the light-exposed tiles the community was largely dominated by diatom
325 species ($585 \times 10^6 \pm 274 \times 10^6 \mu\text{m}^3 \cdot \text{cm}^{-2}$) accounting for 89% of the total biovolume, with other taxa

326 being chlorophytes and cyanobacteria (Table 2). In the dark, very few diatoms were enumerated
327 in the tile biofilms and their mean biovolumes were only about 2% of those observed in the light
328 ($12 \pm 8 \times 10^6 \mu\text{m}^3 \cdot \text{cm}^{-2}$).

329 *Gut contents of macroinvertebrates*

330 CPOM and FPOM (Figure 1) were the main items found in *G. pulex* and *R. semicolorata*
331 gut contents (representing more than 80%), followed by diatoms. In the gut contents of *G. pulex*
332 and *R. semicolorata* reared in the light, diatoms were significantly more represented to the
333 detriment of CPOM (about - 65% and - 40% for CPOM, 0.5 to 10% and 0.5 to 20% for diatoms,
334 respectively; Figure 1). For the diet of *G. pulex*, significantly higher FPOM and filamentous algae
335 were also observed in the light (33 and 3.60 %, respectively), revealing a change in feeding
336 habits (from shredder to collector – grazer, see Felten et al., 2008). *R. semicolorata* ingested
337 significantly more CPOM in the dark than in the light (prop test $p < 0.01$). Finally, animal preys
338 were found only in *G. pulex*.

339

340 *Bulk stable isotope analysis and basal source assimilation*

341 In both treatments and at each sampling date (T0 and T20), the isotope signatures of leaf
342 litter (Figure 2) were similar (mean $\delta^{13}\text{C}$: $-30 \pm 0.8 \text{‰}$ and $\delta^{15}\text{N}$ values < 0 ; $p = 0.51$ and $p = 0.78$
343 respectively). Compared to leaf litter, the biofilms were enriched in both isotopes and their
344 signatures differed depending on the conditions tested. Temporally stable $\delta^{13}\text{C}$ of -28‰ to -
345 27.9‰ and $\delta^{15}\text{N}$ of $2 \pm 0.6\text{‰}$ were registered in the dark whereas in the light, $\delta^{13}\text{C}$ signatures
346 reached $-20.95 \pm 0.4\text{‰}$ at T20 (Figure 2). $\delta^{13}\text{C}$ values recorded in the wall biofilm (T20) were
347 much higher ($-14.14 \pm 0.6\text{‰}$). At T20, FPOM showed $\delta^{13}\text{C}$ values ($-29.01 \pm 0.1 \text{‰}$) similar to
348 those of leaf litter and biofilm in the dark and intermediate between leaf litter and biofilm in the
349 light ($-25.30 \pm 2.3\text{‰}$). Just before the macroinvertebrates were placed in the flumes (T0), they
350 had similar $\delta^{13}\text{C}$ values ($-25.9 \pm 1.3\text{‰}$) but significant differences in $\delta^{15}\text{N}$ values ($p < 0.001$) (*G.*
351 *pulex*: $6.0 \pm 0.2\text{‰}$; *R. semicolorata*: $3.7 \pm 0.3\text{‰}$; Figure 2). At T20, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures did
352 not differ significantly between the two species in the dark ($\delta^{15}\text{N}$: $4.02 \pm 0.8\text{‰}$ vs $3.84 \pm 0.4 \text{‰}$,
353 $p = 0.8$; $\delta^{13}\text{C}$: $-26.31 \pm 0.3\text{‰}$ vs $26.4 \pm 0.2\text{‰}$, $p = 0.7$). In the light, the $\delta^{13}\text{C}$ values of *R.*
354 *semicolorata* were sharply enriched ($-16.30 \pm 0.6\text{‰}$, close to the wall biofilm $\delta^{13}\text{C}$ signatures,

355 while $\delta^{15}\text{N}$ values remained unchanged throughout the survey (about 4‰). *G. pulex* were
356 depleted in $\delta^{13}\text{C}$ compared to *R. semicolorata* ($-23.2 \pm 0.9\text{‰}$), but enriched compared to *G. pulex*
357 in the dark ($-26.31 \pm 0.3\text{‰}$). Finally, *G. pulex* $\delta^{15}\text{N}$ signature decreased from $6.0 \pm 0.2\text{‰}$ (T0) to
358 $4.3 \pm 0.6\text{‰}$ (T20) (Figure 2).

359 In the light, Pt estimates for *R. semicolorata* indicate a very small contribution of alder
360 leaves to the assimilated carbon (Table 3). The proportion of assimilated litter does not exceed
361 11% ($Pt_2 = 0.11$) and was insignificant ($Pt_1 = 0.00$) when calculated relative to tile biofilm $\delta^{13}\text{C}$
362 signatures. In contrast, *G. pulex* assimilated more carbon from alder leaves with $Pt_1 = 0.22$ and
363 $Pt_2 = 0.56$ depending if the calculation considers tile or wall biofilm signatures (Table 3). In the
364 dark the model cannot discriminate between the two food sources (Table 3), end-member
365 signatures being insufficiently differentiated (less than 3‰, see Rasmussen 2010). In this
366 particular case, most of the carbon can be considered to come directly from the litter or its
367 fragmentation in FPOM.

368

369 *Changes in macroinvertebrate FA amounts and hierarchical clustering of FA mean $\delta^{13}\text{C}$*
370 *values.*

371 Throughout the experiment, no significant changes were observed in the total concentrations
372 of *G. pulex* NLFA and PLFA or between the two treatments (Figure 3). In contrast, *R.*
373 *semicolorata* nymphs showed similar total PLFA concentrations over time and in both treatments
374 but accumulated significantly higher amounts of NLFA (99.75 ± 11.6 vs $24.3 \pm 1.99 \mu\text{g.mg DM}^{-1}$,
375 ANOVA $p < 0.05$) when reared in the light. This higher storage of NLFA was related to a
376 significantly greater accumulation of saturated fatty acids (SFA), Mono-unsaturated fatty acids
377 (MUFA) and PUFA (Figure 3), a feature not observed in nymphs reared in the dark. In *G. pulex*
378 the amounts of SFA, MUFA, and PUFA remained fairly constant between treatments, except for
379 the significantly higher amount of PUFA in *G. pulex* NLFA under light conditions (15.64 ± 2.67
380 $\mu\text{g.mg}^{-1}$ DM) compared to dark conditions ($6.78 \pm 2.70 \mu\text{g.mg}^{-1}$ DM) (ANOVA $p < 0.05$). These
381 higher concentrations of PUFA in both species were related to the significantly higher PUFA
382 concentrations observed in basal food sources sampled in the light (see Table 2), i.e. wall and tile
383 biofilms ($p < 0.001$) and to a lesser extent FPOM ($p < 0.01$). Hierarchical Clustering on Principal

384 Components performed on the average $\delta^{13}\text{C}$ values of all the FAs and sterols detected made it
385 possible to relate the two macroinvertebrate lipid class to the basal sources (Figure 4). For dark
386 conditions, 4 clusters were identified: i) cluster II grouped *G. pulex* isotope signatures (NLFA,
387 PLFA and sterols) with FPOM signatures, ii) cluster III grouped *R. semicolorata* NLFA and PLFA
388 isotope signatures with tile biofilm signatures, while iii) cluster I contained only leaf litter, and iv)
389 cluster IV only contained *R. semicolorata* sterols (Figure 4). In the light, five clusters were
390 identified (Figure 4) : i) cluster IV grouped isotope signatures of *R. semicolorata* NLFA and PLFA
391 with tile biofilm signature, ii) cluster V grouped isotope signatures of *R. semicolorata* sterols with
392 the wall biofilm signature, iii) cluster II grouped *G. pulex* sterol signatures with FPOM signatures,
393 iv) cluster III grouped isotope signatures of *G. pulex* NLFA and PLFA (note that clusters II and III
394 were connected at higher Euclidian distances), while v) cluster I contained only leaf litter (Figure
395 4).

396

397 *Differences in isotopic signatures of FAs*

398 The complete list and details of FAs detected in lipids from basal sources and
399 macroinvertebrates are available as Supporting Information 3 and 4 with the tables S1 (basal
400 sources), S2 (*R. semicolorata*), and S3 (*G. pulex*). In addition, all the available $\delta^{13}\text{C}$ values of FAs
401 are listed as additional information (Tables S4 to S5). To better illustrate the effect of the two
402 conditions tested on the origin and transfer of FAs, we focused on the $\delta^{13}\text{C}$ signatures of several
403 FAs of interest: i) on abundant 16:0 and 18:1 ω 9; ii) on selected diatom markers (exclusive:
404 16:2 ω 4 and 16:4 ω 1; non-exclusive but overabundant: 16:1 ω 7; iii) and on essential PUFAs
405 (18:3 ω 3, 18:2 ω 6) and LC-PUFAs (ARA: 20:4 ω 6, EPA:20:5 ω 3) (see Table 4).

406 In the light, all FA $\delta^{13}\text{C}$ values were depleted in leaf litter (-33.47‰ to -29.19‰), with the
407 exception of ARA (-26.82‰) which was more enriched in ^{13}C (Table 4). In line with bulk $\delta^{13}\text{C}$
408 signatures, FA $\delta^{13}\text{C}$ signatures were highly enriched in tile and wall biofilms (-23.22‰ to -
409 17.46‰) and the ^{13}C enrichment was significant compared to leaf litter FAs (ANOVA $p < 0.05$). In
410 contrast, no statistical difference was found in the FA $\delta^{13}\text{C}$ signatures between the two biofilms
411 (Table 4). FA signatures in FPOM tended to be intermediate between, typically a 18:2 ω 6 $\delta^{13}\text{C}$
412 value identical to the litter's 18:2 ω 6 and an EPA $\delta^{13}\text{C}$ value identical to those in the biofilms. $\delta^{13}\text{C}$

413 values in *Rhithrogena* were markedly enriched in ^{13}C (-23.64 ‰ to -19.55 ‰) and significantly
414 higher than those in gammarids (-30.51‰ to -24.53 ‰) (ANOVA $p < 0.05$), except for 16:4 ω 1 and
415 EPA (Table 4). We found no statistical difference between the ^{13}C values of 18:1 ω 9, 18:2 ω 6,
416 ARA, 18:3 ω 3 and EPA in *Rhithrogena* NLFA and PLFA and both biofilms (ANOVA $p > 0.05$). In *G.*
417 *pulex*, the ^{13}C contents of 18:2 ω 6 and ARA were similar to those in litter and FPOM except for
418 ARA from PLFA which was ^{13}C depleted (-28.62 ± 0.10 ‰). 18:3 ω 3 and EPA $\delta^{13}\text{C}$ values in *G.*
419 *pulex* were similar to those in FPOM (ANOVA $p > 0.05$). Finally, similar trends were observed for
420 16:0, 16:1 ω 7 and 18:1 ω 9, with close values of ^{13}C for *G. pulex* and FPOM, and for *Rhithrogena*
421 and biofilms. Conversely, 16:4 ω 1 was significantly depleted in ^{13}C in both macroinvertebrates (-
422 22.56 ‰ to -22.37 ‰), compared to the two biofilms (-19.43 ‰ to -17.46 ‰). This was also the
423 case for 16:2 ω 4, except for *Rhithrogena* NLFA, where the observed difference with the tile biofilm
424 was not significant.

425 In the dark, there was little contrast in FA $\delta^{13}\text{C}$ values between basal sources (Table 4). Leaf
426 litter tended to have the most depleted values, but only 16:0 and 18:3 ω 3 $\delta^{13}\text{C}$ values were
427 significantly enriched in tile biofilm (-28.64 ‰; -30.27 ‰) compared to litter (-33.58 ‰; -33.80 ‰)
428 (ANOVA $p < 0.05$). FA $\delta^{13}\text{C}$ signatures in FPOM were identical to those in tile biofilm except for
429 EPA. Overall, FA $\delta^{13}\text{C}$ signatures of *G. pulex* and *R. semicolorata* NLFA and PLFA differed little
430 (Table 4). Identical $\delta^{13}\text{C}$ values were found for 16:1 ω 7 18:1 ω 9 and 18:2 ω 6, except in *G. pulex*
431 NLFA where 18:2 ω 6 had a more depleted value (-32.94 ± 0.44 ‰; ANOVA $p < 0.05$). 16:0 exhibited
432 similar $\delta^{13}\text{C}$ values for both macroinvertebrate and FPOM and 18:3 ω 3 $\delta^{13}\text{C}$ values in
433 *Rhithrogena* were not significantly different than those in basal sources (Table 4). More depleted
434 than in *Rhithrogena*, 18:3 ω 3 $\delta^{13}\text{C}$ values in *Gammarus* NLFA (-35.71 ± 0.25 ‰) and PLFA (-
435 33.21 ± 1.07 ‰) were similar to those of litter (ANOVA $p > 0.05$). EPA ^{13}C contents in *G. pulex* (-
436 30.44 ‰ to -29.77 ‰) were depleted compared to *R. semicolorata* (-28.63 ‰ to -27.36 ‰)
437 (ANOVA $p < 0.05$) but similar to the values in tile biofilm. In both species, EPA $\delta^{13}\text{C}$ values in
438 NLFA and PLFA were enriched (from +3.8‰ to +5.2‰) compared to their respective
439 18:3 ω 3 values. No significant difference was found in ARA ^{13}C signatures for the two
440 macroinvertebrates (ANOVA $p > 0.05$).

441

442 *Differences in isotopic signatures of the main sterols*

443 The complete list of sterols detected in the samples and their $\delta^{13}\text{C}$ signatures are available as
444 supporting information 5 with Tables S6 and S7. Due to low amounts of phytosterols detected in
445 macroinvertebrates, reliable isotope fractionation values were only obtained for campesterol, β -
446 sitosterol and cholesterol in both species, values that are reported in table 4.

447 In the dark, isotopic signatures of β -sitosterol were similar in all basal sources (-30.86 ‰ to -
448 30.36 ‰) but significantly depleted in *G. pulex* (-31.58 ± 0.65 ‰; ANOVA $p < 0.05$) and probably in
449 *R. semicolorata* (only one value: -31.39 ‰). The campesterol in *G. pulex* had a $\delta^{13}\text{C}$ value close
450 to that found in biofilm which was more enriched (ANOVA $p < 0.05$) than that in litter and FPOM
451 (Table 4). The $\delta^{13}\text{C}$ value for cholesterol was significantly higher in biofilm (-27.48 ± 0.33 ‰;
452 ANOVA $p < 0.05$) than in litter and FPOM, and the $\delta^{13}\text{C}$ values in *G. pulex* and *R. semicolorata*
453 were intermediate (-29.86 ‰ to -30.38 ‰). In the light, the sterol $\delta^{13}\text{C}$ values were markedly
454 enriched in biofilms (table 4), especially in the wall biofilm (-23.6 ‰ to -19.0 ‰). The ^{13}C content
455 of sterols in FPOM and litter was similar (-33.77 ‰ to -27.29 ‰) and significantly depleted
456 compared to biofilms (ANOVA $p < 0.05$). As observed for FAs, $\delta^{13}\text{C}$ values in *Rhithrogena* sterols
457 were enriched (-23.81 ‰ to -22.86 ‰) and similar to those in both biofilms for cholesterol (-23.43
458 ± 1.4 ‰) and campesterol, but only to tile biofilm for β -sitosterol (table 4). In gammarids, the $\delta^{13}\text{C}$
459 value for cholesterol (-29.31 ± 0.80 ‰) was significantly more depleted than in *Rhithrogena* and
460 intermediate between cholesterol signatures in biofilms and those in litter and FPOM. *G. pulex*
461 $\delta^{13}\text{C}$ values of campesterol and β -sitosterol were more depleted than in *Rhithrogena*.

462

463 **Discussion**

464 Our study highlights the importance that different sources of organic matter may have on
465 the development of macroinvertebrates from different FFG.

466 ***Bulk stable- isotope signatures and origin of the assimilated carbon***

467 In light conditions, *Rhithrogena* nymphs and *Gammarus* had access to an important
468 source of autochthonous organic matter. Similar to results recently observed for another
469 Heptageniidae species, *Ecdyonurus* sp. (Kühmayer et al., 2020), most of *Rhithrogena*'s carbon

470 was derived from autochthonous organic matter (tile and wall biofilm) as estimated by the mixing
471 model and *Rhithrogena*'s ¹³C-enriched signatures. *Gammarus pulex* derived between 22% and
472 56% of its carbon from terrestrial sources, primarily FPOM (based on their gut contents and
473 results from the Hierarchical Clustering model). This percentage of allochthonous food in the
474 gammarid isotope signatures was lower than expected, but must be related to the plasticity of
475 their feeding behavior (Felten et al., 2008). In dark conditions and despite having distant
476 biological traits, *Rhithrogena* nymphs and *Gammarus* had the same trophic positions and similar
477 stable carbon isotope compositions. Assimilated carbon was of detrital origin, likely from fine
478 particles derived from litter fragmentation (FPOM), and from biofilm. This biofilm was
479 predominantly heterotrophic, although a few diatoms may have remained, explaining the slight
480 enrichment in ¹³C compared to FPOM (Risse-Buhl et al., 2012).

481

482 ***Influence of food sources and their PUFA levels on the growth of both species***

483 The absence of well-developed autotrophic biofilms affected the performance of our two
484 macroinvertebrates reared in the dark, in particular the *Rhithrogena* nymphs. In streams, the
485 quality of the food resources can be as limiting as their availability to consumers (Florès et al.,
486 2014) and quality depends on the presence of autochthonous primary production (Torres-Ruiz et
487 al., 2007; Guo et al., 2016b). The content of essential lipid compounds in biofilm microalgae has
488 the greatest influence on nutritional quality (Crenier et al., 2017; Twining et al., 2017; Guo et al.,
489 2018; Torres-Ruiz & Wehr, 2019). In freshwater, essential life history traits such as growth rate,
490 fecundity rate and survival of many invertebrates seem to depend on the nutritional supply of
491 essential PUFAs (Ruess & Müller-Navarra, 2019) and/or phytosterols that can be bioconverted
492 into cholesterol (Martin-Creuzburg & Merkel, 2016). Furthermore, we show that access to these
493 lipids from autotrophic biofilms has impacted some of the life history traits of our two species.
494 When *Rhithrogena* nymphs had access to autotrophic biofilms, all aspects of their performance
495 (survival and emergence rates, growth parameters) were significantly higher than those of their
496 congeners reared in the dark. Although PLFA levels did not differ between nymphs from the two
497 treatments, mayflies reared in the light accumulated huge amounts of NLFA ($99.75 \pm 11.6 \mu\text{g}\cdot\text{mg}^{-1}$
498 ¹ DM). Knowing that in insects most of the NLFA are in the form of triacylglycerols (TAG) (Arrese

499 & Soulages, 2010) this represents approximately $112 \mu\text{mol.g}^{-1}$ DM of TAG. Such concentrations
500 are among the highest observed in late instar of *R. semicolorata* nymphs from mountain streams
501 (mean: $60 \mu\text{mol.g}^{-1}$ DM; max: $118 \mu\text{mol.g}^{-1}$ DM; see in Winkelmann & Koop, 2007). In contrast,
502 dark-reared nymphs contained only $24.30 \pm 2.0 \mu\text{g.mg}^{-1}$ DM of NLFA, representing approximately
503 $27.5 \mu\text{mol.g}^{-1}$ DM of TAG, consistent with the lowest values reported by Winkelmann & Koop
504 (2007). We found that the mean $\delta^{13}\text{C}$ signature of the FA pool in *Rhithrogena* NLFA was indeed
505 derived from biofilms which is in line with Kühmayer et al. (2020), who reported that *Ecdyonurus*
506 sp. acquired its FAs primarily from microalgae. During the last weeks before emergence, energy
507 obtained from food is used to maximize growth with the remainder is stored as TAG and glycogen
508 or used for egg maturation by female *Rhithrogena semicolorata* nymphs (Canavoso et al., 2001;
509 Winkelmann & Koop, 2007). Thus, the amount of lipids stored is intended to cover energy needs
510 during the mating period and depends largely on the quantity and quality of available food
511 sources (Winkelmann & Koop, 2007). While the nymphs certainly fulfilled their FA requirements in
512 the presence of autotrophic biofilms, the lipid and nutritional needs of dark-reared nymphs were
513 certainly not met. In the dark, *Rhithrogena* derived most of their NLFAs from detrital biofilm and it
514 is likely that they ingested large amounts of detrital particles to compensate for the absence of
515 microalgae. Such compensatory feeding behaviour has already been observed in caddisfly larvae
516 facing poor quality food (Florès et al., 2014).

517 Regarding gammarids, access to autotrophic biofilms allowed a significantly higher growth
518 when reared in the light. In crustaceans, growth depends on the nutritional supply of lipid
519 compounds, such as LC-PUFAs (Müller-Navarra et al., 2000; Aguilar et al., 2012) or cholesterol
520 precursor Δ^5 -sterols (Gergs et al., 2015). Results from the hierarchical clustering indicated that
521 NLFA were mainly derived from FPOM in *G. pulex* under both light and dark conditions. The lack
522 of effect on their survival rates and NLFA concentrations is related to their ability to meet their
523 basic metabolic needs by ingesting leaf debris from good quality litter such as alder leaves (Little
524 & Altermatt, 2019). However, we suggest that only the occurrence of microalgae colonizing
525 FPOM can explain the better growth rate observed in light-reared *G. pulex*. In this sense, Crenier
526 et al. (2017) proved that diatoms associated with leaf litter were essential for *Gammarus*
527 *fossarum* growth.

528

529 ***Origin of FAs and essential PUFAs according to the conditions tested***

530 CSIA allowed us to establish the origin of the essential PUFAs as well as the main sterols
531 detected. In *Rhithrogena* reared in the light, the high ^{13}C -enrichments observed in PUFAs, 16:0,
532 18:1 ω 9 and diatom markers (16:1 ω 7, 16:4 ω 1) resembled those observed in the autotrophic
533 biofilms. Undoubtedly, the enriched $\delta^{13}\text{C}$ values of EPA and 18:3 ω 3 in the nymphs NL indicate
534 that such essential FAs originated from biofilms. We suggest that EPA in *Rhithrogena* was
535 directly derived from diatoms in tile biofilms and 18:3 ω 3 came from microalgae in the wall biofilm.
536 The same applies for ω 6 PUFAs and other FAs highly enriched in ^{13}C detected in *R.*
537 *semicolorata*. FAs in *G. pulex* reared in the light may have more diverse origins. As observed in
538 *Rhithrogena*, 16:4 ω 1 was incorporated from diatoms in both types of biofilms with only slight
539 depletion. This confirms that microalgae were indeed assimilated by *G. pulex*. However, with $\delta^{13}\text{C}$
540 signatures between -30.50 ‰ and -29.50 ‰ in *G. pulex*, 18:2 ω 6 could not originate from biofilms
541 so must have come from ingested FPOM. In *G. pulex* ARA and EPA were depleted in ^{13}C relative
542 to FPOM and enriched relative to their respective precursors (18:2 ω 6 and 18:3 ω 3), so these LC-
543 PUFA signatures were likely modified by lipid metabolic activity. Under dark conditions,
544 differences in FA $\delta^{13}\text{C}$ values among basal sources did not exceed 2.7‰, confirming the near-
545 absence of ^{13}C -enriched microalgae. In *Gammarus* and *Rhithrogena*, 18:3 ω 3 and 18:2 ω 6 from
546 both lipid classes were exclusively of detrital origin. The constraint imposed by the lack of
547 microalgae acted on ARA and EPA levels in both species, which were significantly lower in the
548 dark. We think that ARA originated from the bioconversion of 18:2 ω 6 as did EPA from 18:3 ω 3.
549 Both PUFAs were indeed enriched in ^{13}C relative to their precursors, which is consistent with an
550 input of heavy carbon atoms during 18:2 ω 6 and 18:3 ω 3 elongation process (Hayes, 2001;
551 Gladyshev et al., 2012).

552

553 ***Role of microalgae in meeting the LC-PUFAs and sterols requirements of both***
554 ***species***

555 In our opinion, the need to synthesize 20:4 ω 6 and 20:5 ω 3 is likely the main reason for
556 the poorer performance observed in both species under dark conditions. Indeed, experiments

557 examining penaeid shrimp development reported that both dietary PUFAs were important to their
558 survival and development (Merican & Shim, 1996). Growth was generally better when
559 combinations of ARA and EPA were provided in their diet, limiting their energy-intensive
560 synthesis (Glencross et al., 2002). In addition, dietary EPA or ARA are known to improve the
561 reproductive performance of several crustacean species (Schlotz et al., 2013; Ginjupalli, et al.,
562 2015). Sterol deficiency may also have serious consequences for the life cycle of gammarids
563 (Gergs et al., 2015) and most likely also for aquatic insects, based on observations made on
564 terrestrial species (Behmer, 2017). Under day light conditions, the isotopic signatures of dietary
565 campesterol and β -sitosterol detected in both species were unequivocal. Those highly enriched in
566 ^{13}C were derived from biofilm microalgae in *Rhithrogena*, while those depleted in ^{13}C originated
567 from FPOM in *G. pulex*. Low levels of cholesterol were detected in autotrophic biofilms and we do
568 not know whether this was sufficient to cover the needs of *Rhithrogena*. The isotopic signatures
569 being close, the possibility that a substantial part of mayfly cholesterol came from microalgae
570 cannot be ignored, since this sterol can be abundant in certain benthic diatom species (Jaramillo-
571 Madrid et al., 2019). However, the occurrence of its immediate precursor (desmosterol) in
572 *Rhithrogena* may suggest that cholesterol synthesis from dietary sterols also occurred. We also
573 think that *Gammarus* has instead dealkylated dietary β -sitosterol to produce cholesterol. The
574 depleted signature of its residual β -sitosterol and its enriched ^{13}C cholesterol seem to point to this
575 (Hayes, 2001). In the dark, the high abundance of β -sitosterol in detrital resources makes it the
576 probable precursor of cholesterol synthesis in both macroinvertebrates where desmosterol was
577 also detected. However, with access to alder leaf fragments, it is likely that sterol nutritional
578 constraints were limited for both species. Indeed, campesterol and β -sitosterol belong to Δ^5
579 phytosterols that are readily dealkylated by arthropods (Martin-Creuzburg et al., 2014; Behmer,
580 2017). Under light conditions, the ability to produce cholesterol by some benthic diatoms
581 definitely adds to the nutritional value of autotrophic biofilms.

582

583 **Conclusion**

584 Our study clearly indicated that autochthonous food sources were important in meeting
585 the biochemical requirements for LC-PUFAs of both macroinvertebrate species in early summer,

586 leading to higher rates of growth (*G. pulex* and *R. semicolorata*) and emergence (*R.*
587 *semicolorata*). In their last instars, *Rhithrogena* are highly dependent on biofilm microalgae from
588 which they derive most of their carbon, in addition to essential FAs such as ARA and EPA and
589 some dietary cholesterol. Limitation of LC-PUFAs appears to be a major ecological constraint for
590 grazing Heptageniidae in headwater streams when feeding solely on leaf detrital particles
591 because epilithic biofilms are limited by shading. For *G. pulex*, our experiment showed that
592 detrital food sources remain quantitatively important in terms of supply of carbon and sterols. This
593 might be even more important when fine particles come from tree species known for the
594 palatability of their leaf litter (Little & Altermatt, 2019). Microalgae collected in biofilm and/or with
595 FPOM may have usually been considered as a minor complementary food but here they appear
596 to be of paramount nutritional importance to meet a large part of the LC PUFA needs of
597 amphipods. This also emphasizes the importance of fine particles of organic matter, and their
598 exact composition in allochthonous detrital fragments and autochthonous photosynthetic material
599 should be investigated in greater detail in headwater streams as well as their relative contribution
600 to food webs.

601

602

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608

609 **Author contribution statement**

610 Conceptualisation: CD, MD, AB. Developing methods: TLV, CD, MD, VF, AB. Conducting the
611 research: TLV, CD, VF, MD, ML, MC. Data analysis: TLV, CD, MD, VF. Preparation of figures
612 and tables: TLV, CD. Data interpretation and writing: TLV, CD, MD, VF, AB, ML, MC.

613

614 **Data availability statement**

615 Data are available from authors upon reasonable request.

616

617 **Conflict of interest**

618 The authors have no conflict of interest to declare.

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746 Tables

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749 **Table 1** Survival rate, emergence rate, final size and daily growth rate of the two macroinvertebrates
 750 reared under dark conditions (DC) and light conditions (LC). (Prop.test was used for survival and
 751 emergence rates and a Mann-Whitney test for size parameters, *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$;
 ns: not significant).

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752 Analysis	753 Species	T20 - DC mean ± sd	T20 - LC mean ± sd	<i>p</i>	
754 Survival rate (%)	<i>G. pulex</i>	48.3 ± 12.5	55.7 ± 5.7	ns	
	<i>R. semicolorata</i>	36.0 ± 4.9	55.0 ± 1.3	**	
755 Emergence rate (%)	<i>R. semicolorata</i>	9.3 ± 0.0	17.8 ± 5.9	*	
	<i>G. pulex</i>				
756 Sizes (mm) and daily 757 growth rate (mm.d ⁻¹)	Total body length (TBL)	6.118 ± 0.767	6.739 ± 0.867	***	
	Daily growth rate (DGR)	0.010 ± 0.007	0.016 ± 0.008	***	
	<i>R. semicolorata</i>	Total body length (TBL)	7.116 ± 1.068	9.949 ± 1.863	**
		Daily growth rate (DGR)	0.005 ± 0.007	0.025 ± 0.013	**

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Table 2 Main characteristics of basal sources sampled at the end of the experiment in dark (DC) and light conditions (LC) including: 1) remaining litter biomass (ash-free dry mass-AFDM) and litter decomposition rates (*k*) calculated from the start of the survey, 2) the biovolumes of tile biofilm and wall biofilm, 3) the amounts of Saturated Fatty Acid (SFA), Mono-unsaturated Fatty Acids (MUFA) and Polyunsaturated Fatty Acids (PUFA) detected in each basal source. Values are mean ± SD. (Student *t*-test, *: $p < 0.05$; **: $p < 0.001$; ns: not significant).

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Samples	Analysis	T20 - DC	T20 - LC	<i>p</i>
Leaf litter	AFDM (mg)	1.40 ± 0.08	1.52 ± 0.17	ns
	Decomposition rate (<i>k</i>)	0.030 ± 0.001	0.028 ± 0.003	ns
Tile biofilm	Diatoms (x10 ⁶ μm ³ .cm ⁻²)	12 ± 8	449 ± 75	**
	Chlorophyta (x10 ⁶ μm ³ .cm ⁻²)	0	25 ± 28	-
	Cyanobacteria (x10 ⁶ μm ³ .cm ⁻²)	0	24 ± 21	-
Wall biofilm	Diatoms (x10 ⁶ μm ³ .cm ⁻²)		104 ± 75	-
	Chlorophyta (x10 ⁶ μm ³ .cm ⁻²)		206 ± 97	-
	Cyanobacteria (x10 ⁶ μm ³ .cm ⁻²)		19 ± 23	-
Tile biofilm	SFA (μg.mg ⁻¹ DM)	4.35 ± 2.23	7.84 ± 0.99	ns
	MUFA (μg.mg ⁻¹ DM)	1.48 ± 0.78	4.81 ± 0.22	**
	PUFA (μg.mg ⁻¹ DM)	0.25 ± 0.18	2.24 ± 0.22	***

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Table 3 Mixing model estimates (with SE) of allochthonous matter consumption by both macroinvertebrates at T20.

$\delta^{13}\text{C}_{\text{MIV}}$ (i.e. Y_c) is $\delta^{13}\text{C}$ signature in macroinvertebrates ; Y_{a1} and Y_{a2} are the measured $\delta^{13}\text{C}$ signatures of tile and wall biofilms respectively ; Y_t is the mean measured $\delta^{13}\text{C}$ values of the leaf litter in each treatment ; P_{t1} and P_{t2} represent the proportions of allochthonous sources consumed based on the proportion of tile or wall biofilm consumed ; f is the trophic shift relative to periphyton signatures estimated for stream herbivores / grazers (HERB) or shredders (SHR) by Rasmussen (2010) and f is negative ; NA means model not applicable (see text). DC means dark condition and LC means light conditions.

		$\delta^{13}\text{C}_{\text{MIV}}$	P_{t1}	P_{t2}	Y_t	Y_{a1}	Y_{a2}	f values
LC	<i>Rhithrogena semicolorata</i>	-16.3 ‰ ± 0.6	0.00 ± 0.13	0.11 ± 0.03	-29.62 ‰	-20.95 ‰ ± 0.4	-14.14 ‰ ± 0.6	$f_{\text{HER/ALG}}$: - 0.4
LC	<i>Gammarus pulex</i>	-23.2 ‰ ± 0.9	0.22 ± 0.09	0.56 ± 0.06	-29.62 ‰	-20.95 ‰ ± 0.4	-14.14 ‰ ± 0.6	$f_{\text{SHR/ALG}}$: - 0.4
DC	<i>Rhithrogena semicolorata</i>	-26.4 ‰	NA	-	-29.87 ‰	-27.9 ‰ ± 0.1	-	$f_{\text{HER/ALG}}$: - 0.4
DC	<i>Gammarus pulex</i>	-26.03 ‰ ± 0.3	NA	-	-29.87 ‰	-27.9 ‰ ± 0.1	-	$f_{\text{SHR/ALG}}$: - 0.4

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Table 4 $\delta^{13}\text{C}$ values of the selected fatty acids and sterols in basal sources and in *G. pulex* and *R. semicolorata* under Light Conditions or Dark Conditions. Significant differences (one-way ANOVA with $p < 0.05$) are indicated by different letters. NLFA: Neutral lipid fatty acids; PLFA: Polar lipid fatty acids.

Dark conditions	Leaf litter	FPOM	Tile biofilm		NLFA <i>R. semicolorata</i>	PLFA <i>R. semicolorata</i>	NLFA <i>G. pulex</i>	PLFA <i>G. pulex</i>
16:0	-33.58 ± 0.01 ^a	-30.77 ± 2.04 ^{ab}	-28.64 ± 0.85 ^b		-30.64 ± 0.61 ^{ab}	-30.77 ± 2.99 ^{ab}	-30.88 ± 0.26 ^{ab}	-30.91 ± 0.16 ^{ab}
16:1 ω 7	-29.56 ± 0.30 ^a	-28.80 ± 3.95	-30.59 ± 0.49 ^a		-29.79 ± 0.09 ^a	-30.25 ± 3.08 ^a	-30.40 ± 0.25 ^a	-30.30 ± 1.01 ^a
18:1 ω 9	-30.85 ± 1.53 ^{ab}	-29.16 ± 1.00 ^{ab}	-27.70 ± 0.13 ^a		-30.41 ± 0.11 ^b	-30.58 ± 0.77 ^b	-29.83 ± 0.08 ^b	-30.44 ± 0.05 ^a
18:2 ω 6	-30.99 ± 0.35 ^a	-31.14 ± 1.19 ^a	-30.79 ± 0.21 ^a		-31.15 ± 0.90 ^a	-30.75 ± 0.81 ^a	-32.94 ± 0.44 ^b	-30.53 ± 0.41 ^a
18:3 ω 3	-33.80 ± 0.04 ^{ac}	-29.96	-30.27 ± 3.30 ^b		-32.45 ± 0.45 ^{ab}	-32.33 ± 0.54 ^{ab}	-35.71 ± 0.25 ^c	-33.21 ± 1.07 ^{ac}
20:4 ω 6					-29.82 ± 0.67 ^{ab}	-28.89 ± 0.80 ^a	-30.64 ± 0.29 ^b	-29.83 ± 0.15 ^{ab}
20:5 ω 3		-24.42	-30.18 ± 0.89 ^a		-28.63 ± 0.08 ^b	-27.36 ± 0.95 ^c	-30.44 ± 0.30 ^d	-29.77 ± 0.28 ^{ad}

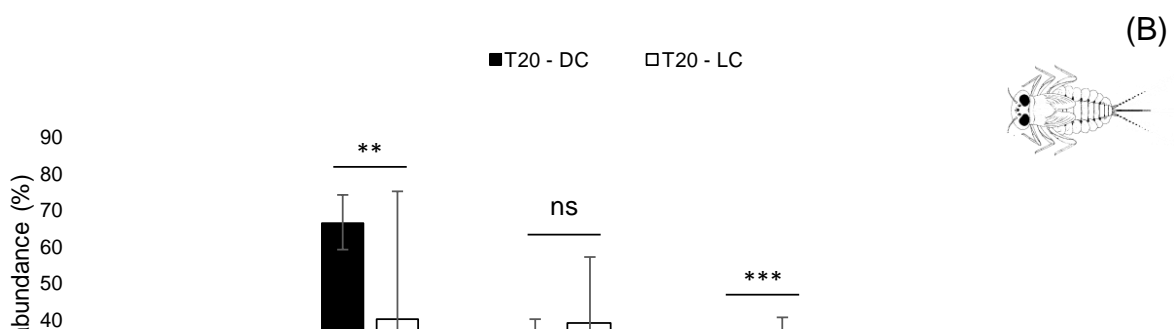
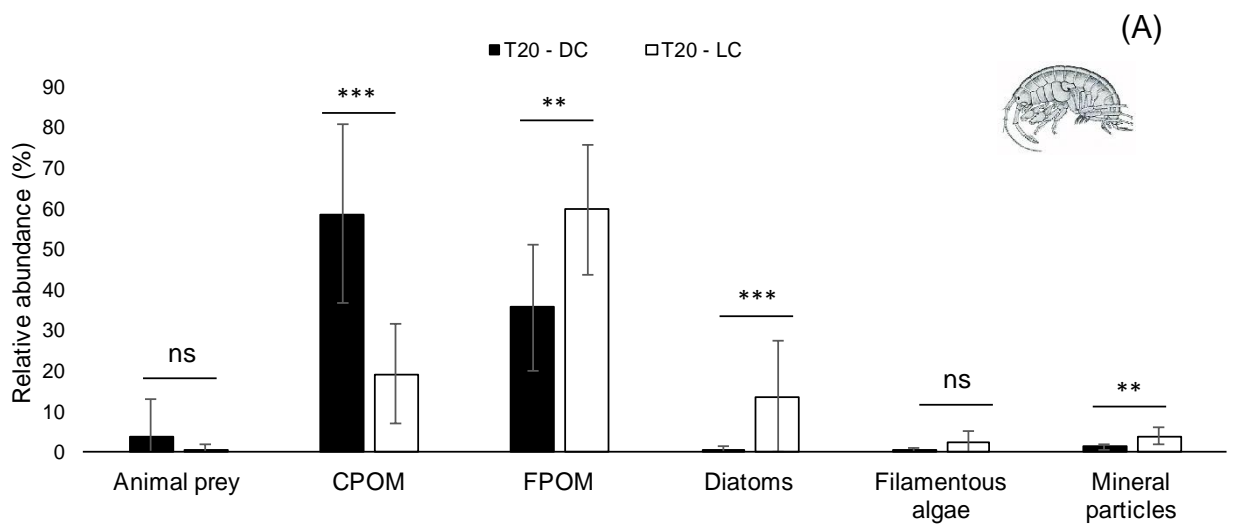
Light conditions	Leaf litter	FPOM	Tile biofilm	Wall biofilm	NLFA <i>R. semicolorata</i>	PLFA <i>R. semicolorata</i>	NLFA <i>G. pulex</i>	PLFA <i>G. pulex</i>
16:0	-33.47 ± 0.31 ^a	-25.71 ± 0.69 ^b	-21.81 ± 0.42 ^b	-22.08 ± 2.05 ^b	-19.76 ± 0.49 ^d	-21.70 ± 0.50 ^c	-27.39 ± 1.33 ^{be}	-27.75 ± 0.72 ^e
16:1 ω 7	-29.19 ± 0.16 ^a	-21.31 ± 1.37 ^b	-18.68 ± 0.98 ^c	-19.12 ± 2.65 ^{bc}	-19.55 ± 0.16 ^{bc}	-19.57 ± 0.53 ^{bc}	-24.59 ± 0.54 ^d	-24.53 ± 0.91 ^d
18:1 ω 9	-30.00 ± 2.06 ^a	-25.89 ± 1.25 ^b	-23.49 ± 0.66 ^{bc}	-22.72 ± 0.82 ^c	-20.96 ± 1.22 ^c	-21.56 ± 0.44 ^c	-27.60 ± 0.15 ^b	-27.75 ± 0.24 ^b
16:2 ω 4	-28.14	-23.25 ± 1.40 ^a	-17.92 ± 2.57 ^a	-17.72	-21.28 ± 0.19 ^a	-23.40 ± 0.71 ^a	-24.14 ± 1.17 ^a	
16:4 ω 1			-19.43 ± 1.83 ^a	-17.46	-22.37 ± 0.79 ^b	-22.47 ± 1.01 ^b	-22.56	
18:2 ω 6	-30.89 ± 0.35 ^a	-29.69 ± 5.00 ^a	-21.57 ± 2.97 ^b	-21.32 ± 3.06 ^b	-22.98 ± 0.29 ^b	-23.84 ± 0.38 ^b	-30.51 ± 1.81 ^a	-29.51 ± a
18:3 ω 3	-33.30 ± 3.96 ^a	-25.12 ± 4.45 ^{bd}	-23.22 ± 0.93 ^{bc}	-19.61 ± 1.86 ^c	-23.19 ± 0.53 ^{bc}	-23.24 ± 0.30 ^{bc}	-27.77 ± 0.12 ^d	-25.99 ± 2.12 ^{bd}
20:4 ω 6	-26.82	-26.42 ± 2.46 ^a	-20.69 ± 0.70 ^b		-21.64 ± 0.36 ^b	-21.94 ± 0.48 ^b	-28.00 ± 0.58 ^{ac}	-28.62 ± 0.10 ^c
20:5 ω 3	-32.70 ± 0.23 ^a	-23.56 ± 2.23 ^{bc}	-19.41 ± 1.71 ^b	-23.18 ± 3.10 ^{bc}	-22.29 ± 0.28 ^{bc}	-21.92 ± 0.10 ^{bc}	-25.33 ± 0.04 ^c	-26.05 ± 0.75 ^c

Dark conditions	Leaf litter	FPOM	Tile biofilm		<i>R. semicolorata</i>	<i>G. pulex</i>
cholesterol	-34.83 ± 0.11 ^a	-34.01 ± 1.24 ^a	-27.48 ± 0.33 ^b		-30.38	-29.86 ± 0.98 ^c
campesterol	-33.03 ± 0.23 ^a	-33.16 ± 0.82 ^a	-30.21 ± 0.93 ^b			-29.76
β -sitosterol	-30.36 ± 0.25 ^a	-30.62 ± 0.41 ^{ab}	-30.86 ± 0.16 ^{ac}		-31.39	-31.58 ± 0.65 ^c

Light conditions	Leaf litter	FPOM	Tile biofilm	Wall biofilm	<i>R. semicolorata</i>	<i>G. pulex</i>
cholesterol	-33.77 ± 0.08 ^a	-32.11 ± 1.56 ^a	-25.12 ± 0.72 ^b	-23.69 ± 0.63 ^b	-23.43 ± 1.40 ^b	-29.31 ± 0.80 ^c

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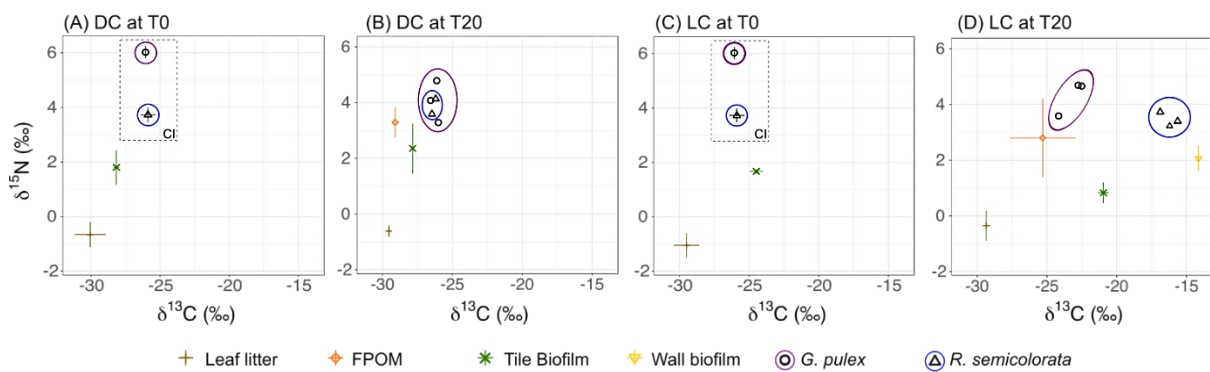
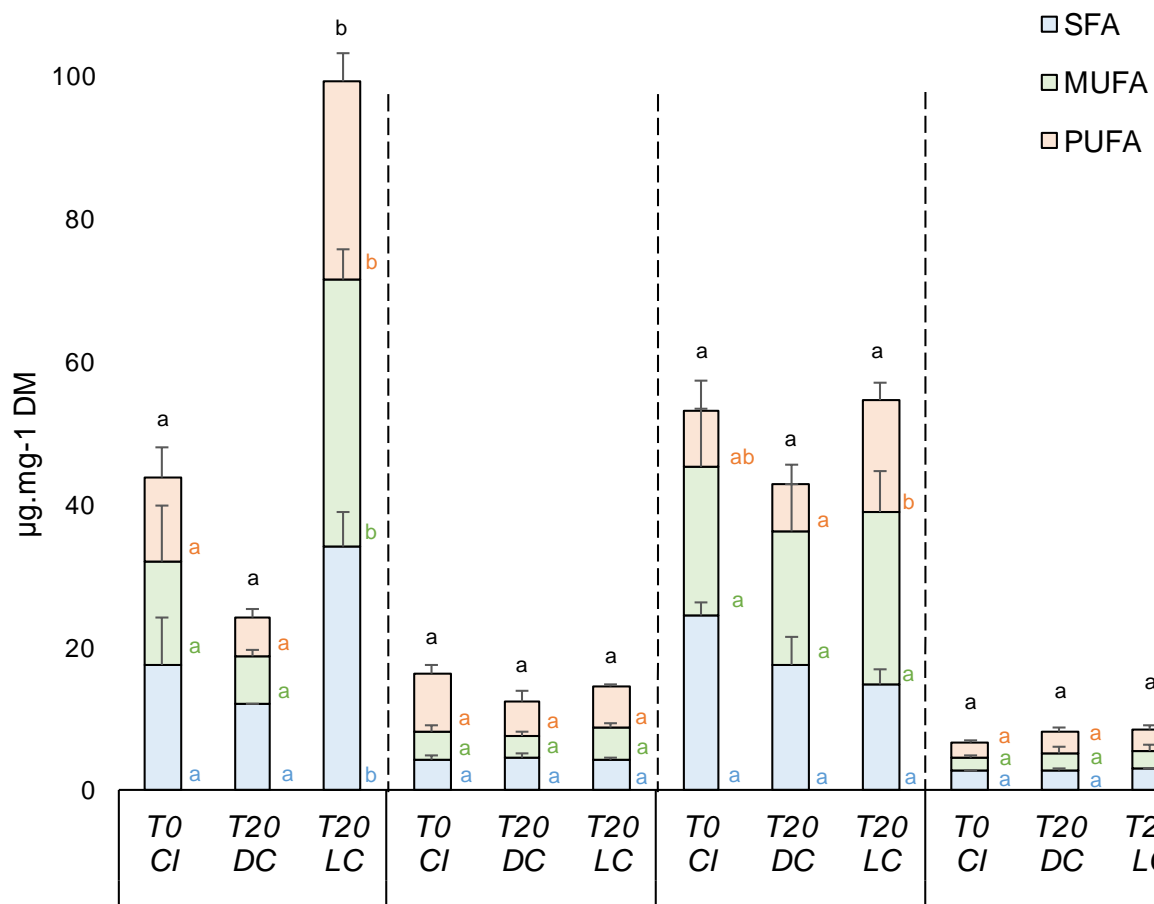


Figure 2 Stable isotope Analysis

$\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ biplot showing the basal sources (mean \pm SD) and individual values of *G. pulex* (orange circles) and *R. semicolorata* (blue circles) in dark conditions at T0 (A) and T20 (B) and in light conditions at T0 (C) and T20 (D). Collected individuals (CI) at T0 are shown for information in the box with a dotted line.

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