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# **To what extent do food preferences explain the trophic position of heterotrophic and mixotrophic microbial consumers in a *Sphagnum*-peatland?**

Vincent EJ Jassey\* <sup>1,2,3</sup>¶, Caroline Meyer <sup>1</sup>¶, Christine Dupuy <sup>4</sup>, Nadine Bernard <sup>1</sup>, Edward AD Mitchell <sup>5</sup>, Marie-Laure Toussaint <sup>1</sup>, Marc Metian <sup>4</sup>, Auriel P Chatelain <sup>5</sup> and Daniel Gilbert <sup>1</sup>

¶ these authors contributed equally

<sup>1</sup> Université de Franche-Comté, Laboratoire Chrono-Environnement, UMR CNRS/UFC 6249, F-25211 Montbéliard cedex, France

<sup>2</sup> School of Architecture, Civil and Environmental Engineering (ENAC), Ecole Polytechnique Fédérale de Lausanne EPFL, Ecological Systems Laboratory (ECOS), Station 2, 1015 Lausanne, Switzerland

<sup>3</sup> Swiss Federal Institute for Forest, Snow and Landscape Research (WSL), Site Lausanne, Station 2, 1015 Lausanne, Switzerland

<sup>4</sup> Université de La Rochelle, Laboratoire Littoral Environnement et Sociétés, UMR CNRS 7266, F-17000 La Rochelle, France

<sup>5</sup> University of Neuchâtel, Laboratory of Soil Biology, Rue Emile-Argand 11, CH-2000 Neuchâtel, Switzerland

\* Corresponding author, fax +41 (0) 216 935 774; email [vincent.jassey@epfl.ch](mailto:vincent.jassey@epfl.ch) (V.E.J. Jassey).

Running title: Microbial food web structure in peatland

1 **Abstract**

2 Although microorganisms are the primary drivers of biogeochemical cycles, the structure and  
3 functioning of microbial food webs is poorly studied. This is the case in *Sphagnum*-peatlands,  
4 where microbial communities play a key role in the global carbon cycle. Here, we explored  
5 the structure of the microbial food web from a *Sphagnum*-peatland by analysing (1) the  
6 density and biomass of different microbial functional groups, (2) natural stable isotope ( $\delta^{13}\text{C}$   
7 and  $\delta^{15}\text{N}$ ) signatures of key microbial consumers (testate amoebae), and (3) the digestive  
8 vacuole contents of *Hyalosphenia papilio*, the dominant testate amoeba species in our system.  
9 Our results showed that the feeding type of testate amoeba species (bacterivory, algivory or  
10 both) translates into their trophic position as assessed by isotopic signatures. Our study further  
11 demonstrates, for *Hyalosphenia papilio*, the energetic benefits of mixotrophy when the  
12 density of its preferential prey is low. Overall, our results show that testate amoebae occupy  
13 different trophic levels within the microbial food web, depending on their feeding behaviour,  
14 the density of their food resources and their metabolism (i.e. mixotrophy vs. heterotrophy).  
15 Combined analyses of predation, community structure and stable isotopes now allow the  
16 structure of microbial food webs to be more completely described, which should lead to  
17 improved models of microbial community function.

18

19 **Keywords:** 13 Carbon and 15 nitrogen isotopes / Feeding habit / Foraging traits / Natural  
20 stable isotopes / Microbial food web / Testate amoebae

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## 25 **Introduction**

26 The way in which community structure and species attributes determine fluxes of energy in  
27 ecosystems is an important topic in ecology. The study of food webs provides a quantitative  
28 framework in our understanding of links between community structure and ecosystem  
29 functioning [1]. Food webs are networks determining the number and direction of trophic  
30 interactions in ecosystems and provide a natural framework for understanding the ecological  
31 role of species. Identifying how and to what extent these webs are structured is necessary if  
32 we hope to move beyond a “black-box” approach and toward a more mechanistic  
33 understanding of the ecosystem processes and functions that they drive [2, 3, 4, 5, 6].  
34 However, the presence of incompletely described feeding relationships hampers our  
35 understanding of complex and dynamic food webs in nature [7]. A clear example is the  
36 scarcity of existing data on microbial food webs, which, for practical reasons are often limited  
37 to experimental studies using a very limited number of taxa and often in combinations that do  
38 not reflect any natural system [8, 9, 10, 11].

39 A major impediment in determining microbial food web structure probably stems from  
40 the difficulty in identifying interspecific links and collecting the required quantitative dietary  
41 data. Microbial communities are composed of numerous (and often poorly described or  
42 altogether unknown) species that can form complex food webs with numerous feeding links.  
43 Discovering the structure of these food webs is important because they may affect processes  
44 occurring at the population, community, and ecosystem level [7]. The true trophic position  
45 and diversity in trophic roles of different microbial species and/or species group of different  
46 species was until recently all but impossible to determine. The increasing sensitivity of  
47 analytical tools now makes it possible to study ever-smaller organisms using stable isotope  
48 probing [8, 12]. Stable isotope ratios of carbon and nitrogen are increasingly used to provide  
49 time-integrated information about feeding relationships and food web structure, as showed for

50 aquatic food webs [13, 14, 15]. By providing a temporally and spatially averaged estimate of  
51 carbon and nitrogen that is actually assimilated by organisms [16], stable isotopes can  
52 improve our estimates of the major pathways of energy flows through microbial food webs  
53 [17].

54 We focused here on microbial communities living in *Sphagnum*-peatlands. In this  
55 diverse community a group of protozoa, the testate amoebae, are especially abundant. Testate  
56 amoebae represent an ideal model group for microbial food web studies in terrestrial  
57 ecosystems owing to their abundance, diversity, and ubiquity [18]. Testate amoebae are  
58 usually considered as feeding on a wide range of food sources, such as bacteria, fungi,  
59 microalgae, ciliates, rotifers and nematodes [12, 19, 20, 21, 22]. As a result, they are  
60 considered top-predators in microbial food webs [23, 24, 25]. As such, they play an important  
61 role in carbon and nutrient cycling in soils [26, 27, 28, 29]. However, in order to understand  
62 the role of testate amoebae in carbon and nutrient cycling, key aspects of their ecology need  
63 to be understood, including how they obtain energy and nutrients. Precise information on the  
64 feeding habits of testate amoebae is limited to a few species [29]. The broad range of sizes  
65 (ca. 5-500  $\mu\text{m}$  in length, corresponding to volumes spanning six orders of magnitude) and  
66 high diversity of morphologies and functional traits found among testate amoebae [30, 18]  
67 suggest that they play different functional roles in microbial food webs. Species commonly  
68 described as omnivores probably occupy a different trophic level in the microbial network  
69 than specific feeders such as bacterivores or predators. Furthermore, many pigmented  
70 (photosynthetic) microorganisms are known or suspected to be mixotrophic (i.e. organisms  
71 that combine autotrophy and heterotrophy using endosymbiotic zoochlorellae); these include  
72 several testate amoeba species such as *Hyalosphenia papilio* and *Archerella flavum* [31, 32],  
73 species that are commonly found in northern *Sphagnum* peatlands [e.g. 18, 33, 34, 35, 36].  
74 The mixotrophic life trait has significant functional and physiological implications [10, 37,

75 38] and should theoretically translate into distinct isotopic ratios. Hence, the use of isotopic  
76 analyses may provide a mechanistic understanding on how testate amoebae are trophically  
77 structured, highlighting the strategy and functional role of dominant species in food web.

78 Here, we aim to track energy flow through microbial food chains from lower to higher  
79 trophic levels to disentangle the trophic structure of testate amoeba populations in microbial  
80 food web; and to elucidate the role of the mixotrophic strategy in microbial food web  
81 functioning. We hypothesize that the trophic level –based on isotopic enrichment– of testate  
82 amoebae within the microbial network relate to their feeding habit (bacterivory, algivory, and  
83 predation) and metabolism (heterotrophy vs. mixotrophy). Considering the key role of  
84 foraging traits such as density of prey in the feeding habit of testate amoebae [12], we also  
85 hypothesize that prey density affects the trophic position of testate amoebae. For this, we  
86 explored the microbial food web associated with *Sphagnum* moss by combining predation of  
87 testate amoebae (digestive vacuole contents and predatory-prey associations), analyses of  
88 community density and biomass, and isotopic signatures ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) of testate amoebae  
89 and their potential prey, including microalgae, cyanobacteria, rotifers and nematodes.

90

## 91 **Materials and methods**

### 92 Study area and experimental design

93 We sampled one peat monolith (43.5 cm x 33.5 cm, 22 cm in depth) in a macroscopically  
94 homogeneous *Sphagnum fallax* lawn in a peatland in the Jura Mountains (Sur-les-Seignes,  
95 Frambouhans-Les Ecorces, France, 47°18'N, 6°79'E) in late-November 2010. The core was  
96 then placed under controlled conditions to quickly reactivate microbial life, average  
97 temperature of 22°C (day) and 18°C (night), a light cycle of 16h/8h (light/dark) and a light  
98 intensity of 0.029 kW.m<sup>-2</sup>. The depth to water table (DWT) was maintained at constant level  
99 (6.5 cm below the top of the moss carpet) by adding standardized solution (Volvic water = 0.8

100 L every 2 days). We measured the physicochemical conditions of the peat core when the  
101 container was placed under controlled conditions. Temperature of the water table and at the  
102 surface was of 5°C and 12°C, respectively; pH = 4.4; and conductivity = 20.1  $\mu\text{S cm}^{-1}$ . After  
103 15 days these values increased (average temperature of the water table and at the surface of  
104 17°C and 20°C, respectively, pH = 4.9, conductivity = 44.1  $\mu\text{S cm}^{-1}$ ) and stabilised.

105 After 15 days, the *Sphagnum* carpet from the peat monolith was subdivided into three  
106 subsamples. For each subsample, the totality of the living apical green segments of *Sphagnum*  
107 shoots (top 3 cm) was collected. We randomly sampled 5 g of fresh material (ca. fifteen  
108 shoots) in each sub-plot and fixed it in 20 ml of glutaraldehyde (2% final concentration) for  
109 microbial community analysis (density, biomass, and diversity). The remaining material was  
110 used for isotopic analyses. Fully opened healthy capitula of *Sphagnum* (top 3 cm) were also  
111 sampled and lyophilized for isotopic analyses. No moss was taken from the marginal 5 cm  
112 border zone of the peat monolith to avoid possible border effects.

113

#### 114 Microscopic and isotopic analyses

115 All microorganisms were extracted from *S. fallax* following the method used by Jassey et al.  
116 [12]. Microalgae, cyanobacteria, protozoa, rotifers, nematodes and fungi were identified and  
117 quantified at 400x magnification by means of inverted microscopy (Olympus IX71) and  
118 following Uthermöhl's method [39]. Bacterial density was estimated by flow cytometry  
119 (FAC-SCalibur flow cytometer, Becton Dickinson). Fluorescent microbeads (molecular  
120 probes) 1  $\mu\text{m}$  in diameter were added to each sample as an internal standard. Bacteria were  
121 stained with SYBR Green I (1/10,000 final conc.) for 15 min in the dark and samples run at  
122 medium speed (40  $\mu\text{L min}^{-1}$ ). Bacterial size was estimated by epifluorescence microscopy  
123 using image analysis. The feeding habit of the dominant testate amoeba species,  
124 *Hyalosphenia papilio*, was studied in detail. To this aim, a total of 670, 715, and 500

125 specimens of *H. papilio* were observed by inverted microscopy in the three subsamples,  
126 respectively. Among active specimens, we distinguished between individuals with a prey  
127 within the shell and those that were actively feeding (determined as attached to prey or  
128 organic matter particles), to determine the feeding habit of this species, as described in Gilbert  
129 et al. [20]. Such an analysis was not possible for others testate amoeba species due to their  
130 low abundance.

131 The biovolume of each morpho-species or group was estimated using geometrical  
132 shapes and then converted to carbon using the following conversion factors, heterotrophic  
133 bacteria,  $1\mu\text{m}^3 = 5.6 \times 10^{-7} \mu\text{gC}$  [40]; cyanobacteria and microalgae,  $1\mu\text{m}^3 = 1.2 \times 10^{-7} \mu\text{gC}$ ;  
134 ciliates and testate amoebae,  $1\mu\text{m}^3 = 1.1 \times 10^{-7} \mu\text{gC}$  [41]; fungi  $1\mu\text{m}^3 = 2.5 \times 10^{-7} \mu\text{gC}$ ;  
135 nematodes and rotifers,  $1\mu\text{m}^3 = 1.25 \times 10^{-7} \mu\text{gC}$  [42]. These data were expressed as  $\mu\text{gC}$  per  
136 gram of *S. fallax* dry mass ( $\mu\text{gC g}^{-1} \text{DM}$ ).

137 Collection and preparation of microbial samples for isotopic analyses follow Jassey et  
138 al. [12]. We measured the isotopic signatures of cyanobacteria + microalgae (CA), fine  
139 organic matter including bacteria (FOMB), two abundant groups of micro-metazoa, small  
140 nematodes ( $< 200 \mu\text{m}$ ) and rotifers, and the dominant testate amoeba species *Arcella vulgaris*,  
141 *Archerella flavum*, *Bullinularia indica*, *Euglypha compressa*, *Hyalosphenia papilio* and  
142 *Nebela tinctoria*. Microorganisms were extracted from fresh moss by six successive rinsing of *S.*  
143 *fallax* using distilled water and successively filtered at 150, 120, 70, 50, 30, 20 and 11  $\mu\text{m}$   
144 (SEFAR NITEX ®). The fraction under 11 $\mu\text{m}$  was used for FOMB and microalgae. For  
145 FOMB, the fraction was centrifuged at 15,000  $\text{r.min}^{-1}$  for 20 min. Then, the supernatant was  
146 centrifuged again at 10,000  $\text{r.min}^{-1}$  for 10 min and the pellet was used for isotopic  
147 measurements. All other categories were hand picked randomly and individually using  
148 micropipettes. Between 50 and 1,500 living testate amoeba specimens of the selected species  
149 were collected in each sub-sample, depending on their size (Table 1). In order to obtain



150 reliable measurements, 0.1 mg of matter is required for isotopic measurement. To reach this  
151 amount, some samples had to be pooled from the three sub-plots, *Arcella vulgaris*, *Archerella*  
152 *flavum*, *Bullinularia indica*, *Euglypha compressa*, *Nebela tinctoria*, rotifers and nematodes, and  
153 for these no replicated measurements could be obtained. After 3 days, we also collected the  
154 CA biofilm using a micropipette. We estimated trophic positions (TP) following the method  
155 of Post [43]. We used *S. fallax* capitula as the baseline of the network for estimating trophic  
156 positions using  $\delta^{15}\text{N}$ , because we focused on microbial communities living in *Sphagnum*  
157 moss, and *Sphagnum* integrate temporal and spatial variation in isotopic signatures of basal  
158 resources [43].

159

#### 160 Data analysis

161 We used linear mixed effects models to test for relationships between 1)  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$   
162 signatures of microbial species/groups, 2)  $\delta^{15}\text{N}$  and testate amoeba morphological traits (i.e.  
163 body size - length or diameter – shell aperture size and biovolume). These models were built  
164 while accounting for the pseudo-replication measurements in the peat monolith. We fitted all  
165 models including sub-plot as a random effect on the intercept, i.e. we corrected for the  
166 inflation of the residual degrees of freedom that would occur if we were using repeated  
167 measurements as true replicates. Regression diagnostics (leverage and outliers effects) and  
168 global validations of linear model assumptions (normality of the data distribution and  
169 homogeneity of variance) were examined and tested as described in Fox and Weisberg [44].

170 Differences in TP of identified groups or species were assessed using non-parametric  
171 Mann Whitney tests. A hierarchical cluster analysis using the Ward distance method and Bray  
172 Curtis dissimilarity index were computed on  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ , isotopic nitrogen over carbon  
173 ratio, and trophic level values in order to identify feeding types of species and/or microbial

174 groups. The robustness and the significance of the cluster were tested using bootstrap  
175 resampling (i.e. 1000 resampling from the data).

176 All statistical analyses were performed with R 2.15.1 using the *ade4*, *car* and *pvc* packages [44, 45, 46].  
177

178

## 179 **Results**

### 180 Microbial community structure

181 In term of biomass, the microbial community was composed of 53% of decomposers (bacteria  
182 and fungi), 17.3% microbial primary producers (microalgae and cyanobacteria), and 29.8% of  
183 consumers (all other groups; Fig. 1a). Bacteria accounted for almost half of the total biomass  
184 ( $726 \pm 232 \mu\text{gC g}^{-1} \text{DM}$ ), while fungi represented only 6.6% ( $103 \pm 53 \mu\text{gC g}^{-1} \text{DM}$ ; Fig. 1).  
185 Testate amoebae represented about one-quarter of the total biomass ( $413 \pm 176 \mu\text{gC g}^{-1} \text{DM}$ )  
186 and were the dominant group of consumers while rotifers made up only 3.1% of the total  
187 biomass ( $49 \pm 37 \mu\text{gC g}^{-1} \text{DM}$ ; Fig. 1a). Ciliates and nematodes together represented less than  
188 1% of the total biomass ( $3 \pm 1$  and  $2 \pm 0 \mu\text{gC g}^{-1} \text{DM}$ , respectively). Primary producers were  
189 dominated by microalgae (14.7% of the total biomass), while cyanobacteria accounted for a  
190 much lower fraction of the total biomass (ca. 2.6%). The green alga *Cylindrocystis brebissonii*  
191 was the more abundant species of microalgae with more than 90% of the microalgal biomass,  
192 whereas *Aphanothece nidulans* and *Anabaena cylindrica* were the most abundant species of  
193 cyanobacteria (54% and 34% of cyanobacteria biomass, respectively). *Hyalosphenia papilio*  
194 strongly dominated the testate amoeba community and accounted for 89.8% of its biomass  
195 (Fig. 1b).

196

197

198 Feeding habit of *Hyalosphenia papilio*: digestive vacuole contents approach

199 On average 25% of the observed *Hyalosphenia papilio* specimens were associated with prey.  
200 Among the identified food sources, 80% were microbial primary producers (CA = microalgae  
201 and cyanobacteria, e.g. *Aphanothece nidulans*), 12% were fungi (including hyphae of  
202 ascomycetes and spores of *Helicoon pluriseptatum*) and 6% were ciliates (e.g. *Platyophrya*  
203 *sphagni*) (Table 2). Predation on other testate amoebae (e.g. *Archerella flavum*), rotifers (e.g.  
204 *Habrotrochoa* sp. and *Colurella* sp.) and nematodes was observed only rarely (<1% of the  
205 total identified predator-prey associations). These proportions matched very closely the  
206 density of these organisms in the environment (Table 2). These frequencies represented in  
207 term of grazed biomass 9.8% of the biomass of CA, 20.1% of that of fungi and 49.3% of that  
208 of ciliates.

209

210 Isotopic composition of the microbial food web

211 The isotopic values of cyanobacteria and microalgae (CA) ( $\delta^{13}\text{C} = -26.8\text{‰}$ ,  $\delta^{15}\text{N} = -6.7\text{‰}$ )  
212 were almost identical to that of *Sphagnum* ( $\delta^{13}\text{C} = -28.0\text{‰}$ ;  $\delta^{15}\text{N} = -5.4\text{‰}$ ), which is  
213 therefore used as a baseline for further interpretation. By contrast, all other microbial groups  
214 yielded enriched  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values relative to the baseline value determined from *S.*  
215 *fallax* shoots (Fig. 2, Table 1).

216 The  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  signatures of consumers covered a broad range of values, from  
217  $-27.3\text{‰}$  to  $-21.6\text{‰}$  and  $-1.9\text{‰}$  to  $+1\text{‰}$ , respectively. Isotopic values were significantly  
218 higher in consumers (testate amoebae, rotifers and nematodes) as compared to primary  
219 producers (*S. fallax* and CA;  $P = 0.04$ ,  $r = 0.5$ ). For instance, the isotopic diagram showed that  
220 *Archerella flavum* was more enriched in  $\delta^{13}\text{C}$  ( $+1.6\text{‰}$ ) and  $\delta^{15}\text{N}$  ( $+2.9\text{‰}$ ) than FOMB (Fig.  
221 2). Isotopic values of *Arcella vulgaris* ( $\delta^{13}\text{C} = +0.3\text{‰}$ ;  $\delta^{15}\text{N} = +2.1\text{‰}$ ), *B. indica* ( $\delta^{13}\text{C} = +$

222 0.7‰;  $\delta^{15}\text{N} = + 3\text{‰}$ ), and *N. tinctoria* ( $\delta^{13}\text{C} = + 0.3\text{‰}$ ;  $\delta^{15}\text{N} = + 1.7\text{‰}$ ) were also consistently  
223 enriched relative to those of their supposed diet (CA) (Fig. 2, Table 1). Our results also  
224 showed that the mixotrophic species *H. papilio* was highly enriched in  $\delta^{13}\text{C}$  (+ 5.2‰), as  
225 compared to its diet (CA) (Fig. 2, Table 2). This was not the case for the other mixotrophic  
226 species studied (*A. flavum*).

227 Translating these results into trophic position, primary producers (CA) had the same  
228 trophic level as *S. fallax* in the microbial food web (TP = 1), while FOMB (TP = 2) and  
229 consumers (TP ranged from 2.3 to 2.9) had significant higher trophic level than primary  
230 producers ( $P < 0.05$ ) (Table 1, Fig. 3b). In the cluster analysis, microbial groups were split  
231 into primary producers and consumers. The microbial consumers were further split into five  
232 sub-groups corresponding to different feeding strategies (Fig. 3a, b, Table 1). The first sub-  
233 group corresponded to mixotrophic algivores (C1) composed by *H. papilio* (Fig. 3). The  
234 second sub-group included decomposers (FOMB; C2), while the third and fourth sub-groups  
235 corresponded to bacterivores (C3) including *A. flavum* and nematodes, and heterotrophic  
236 algivores (C4) including *A. vulgaris*, *B. indica*, *N. tinctoria* (Fig. 3, Table 1). The fifth sub-group  
237 (C5) included *E. compressa* and rotifers and corresponded to a mix between algivores and  
238 bacterivores (Fig. 3, Table 1). Furthermore, we found that the trophic level of *H. papilio* was  
239 linked to the contribution of ciliates and CA to its total diet (Fig. 4). We found that the trophic  
240 level of *H. papilio* increased with the rise of ciliates and the decrease of CA in its total diet  
241 (Fig. 4a, b).

242 The  $\delta^{15}\text{N}$  values of testate amoebae were negatively correlated to simple  
243 morphological traits such as size of their shell aperture ( $r = -0.86$ ,  $P = 0.02$ ) and their  
244 biovolume ( $r = -0.88$ ,  $P = 0.01$ ), while no significant correlation was found with their body  
245 size (length or diameter) ( $r = -0.72$ ,  $P = 0.07$ , ANOVA).

246

## 247 **Discussion**

248 We investigated the trophic structure of the microbial food web associated to *Sphagnum* moss  
249 by combining analyses of microbial density and biomass, stable isotopes ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) of  
250 microbial groups and digestive vacuole content of key microbial consumers. We found that  
251 the trophic level of microbial organisms inferred from stable isotopes was in line with  
252 expectations; bacteria (FOMB) have a higher trophic level than autotrophs (*S. fallax*, CA),  
253 and microbial consumers a higher trophic level than both decomposers and autotrophs. We  
254 further showed that isotopic signatures of moss and CA did not differ and that both  
255 autotrophic microorganisms and *Sphagnum* mosses have similar directional shifting in food  
256 web space. This agrees with our assumption that autotrophs can be used as the baseline of the  
257 microbial food web associated to *Sphagnum* moss.

258

259 Trophic structure of the microbial food web

260 In most cases, we found isotopic enrichments in line with those of aquatic and soil food web  
261 in which consumer  $\delta^{15}\text{N}$  signatures were higher than 3‰ [43, 47]. Based on these isotopic  
262 enrichments and identified feeding habit, we showed two trophic pathways in our microbial  
263 network: a herbivorous pathway and a detritivorous pathway. Firstly, we found a significant  
264 enrichment in  $\delta^{15}\text{N}$  between microbial autotrophs (CA) and microbial consumers such as  
265 *Nebela tinctoria*, *Euglypha compressa*, *Hyalosphenia papilio*, *Arcella vulgaris* and rotifers,  
266 characterizing the herbivorous pathway. These isotopic enrichments indicated that these  
267 consumers mainly fed on CA in the current study. This agrees with our feeding observations  
268 on *H. papilio*, as well as with previous observations of feeding habits for *Arcella vulgaris*,  
269 *Euglypha compressa* and rotifers [19, 21, 48, 49]. Secondly, the significant  $\delta^{15}\text{N}$  enrichment  
270 between FOMB and consumers such as *Archerella flavum* and nematodes suggests the  
271 existence of a detritivorous pathway. *A. flavum* has been described as exclusively autotrophic

272 [32], but its isotopic signature rather indicated that this species mainly fed on bacteria or fine  
273 organic particles (FOMB). Although food particles are rarely observed in *A. flavum* vacuoles,  
274 this does not exclude the possibility that bacteria occur in their vacuole. Indeed, bacteria are  
275 difficult to identify in testate amoeba vacuoles when using direct observation with  
276 epifluorescence microscopy (pers. observation). Obviously, the recorded isotopic signatures do  
277 not exclude that microbial consumers feed on additional food sources and/or intermediate  
278 trophic level [50, 51], as clearly showed for *H. papilio*.

279 More surprising is the difference between the isotopic signatures of *H. papilio* and *N.*  
280 *tincta* between this study and the one of Jassey et al. [12]. These results reflect inter-site  
281 differences, showing that the isotopic signatures of a given species can be quite variable in  
282 space and/or time depending on environmental conditions [50]. However, the similar  
283 signature of the baseline (i.e. *Sphagnum fallax*) between the two studies also underlines  
284 differences of inter-site food web functioning. The linkages between the trophic position of *H.*  
285 *papilio* and the contribution of main categories of prey to its total diet agree the hypothesis  
286 that differences in prey-availability influence trophic position of testate amoebae. Indeed,  
287 isotopic signatures and trophic levels of top-predators, *H. papilio* and *N. tincta* ( $TP_{H. papilio}$ , 2.3  
288 and  $TP_{N. tincta}$ , 2.4), were significantly lower than those recently obtained by Jassey et al.  
289 (2012;  $TP_{H. papilio}$ , 3.5 and  $TP_{N. tincta}$ , 4.2). Such results are not surprising when we consider the  
290 low density of the potential energetic prey for *H. papilio* and *N. tincta* recorded in this study  
291 (e.g. ciliates, rotifers and nematodes). Because of the very low relative abundances of its  
292 preferential food sources (i.e. ciliates; [12]), our results suggest that *H. papilio* adapts its  
293 feeding behaviour by targeting the most abundant and available food sources (e.g. CA). This  
294 agrees with the predictions of optimal foraging theory, which states that organisms forage in  
295 such a way as to maximize their net energy intake. Optimally-foraging organisms can  
296 minimize the energy cost of food uptake by targeting the most abundant and easily captured

297 prey [52, 53]. We estimated that predation of *H. papilio* on ciliates represented up to 50% of  
298 the total standing biomass of ciliates. This suggests that the density of ciliates was probably  
299 lower than a threshold allowing higher active predation of *H. papilio* on this otherwise  
300 energetically optimal prey.

301 Our results reveal that testate amoeba species occupy different trophic positions in the  
302 microbial food web and that these differences could be related to their feeding type.  
303 Conversely, this study illustrates that it is difficult to reliably infer trophic position of testate  
304 amoebae using relatively simple morphological traits without taking into account prey stocks.  
305 Food webs usually exhibit a surprisingly consistent topological architecture across different  
306 ecosystem types [4, 54] mediated by fundamental constraints where species feed on nearly  
307 contiguous ranges of mostly lower ranked species [55]. In general, species with large body  
308 size occupy high trophic levels and consume many food sources, whereas smaller species  
309 share low trophic levels with more specific food strategies (e.g. bacterivory; [56]). Body size  
310 is thus a fundamental indicator of trophic level in food webs because it places constraints on  
311 many biological variables, including crucial foraging traits (energy content of resources,  
312 handling times, attack rates, and prey density) [57, 58, 59, 60]. However, strong relationship  
313 between body size (here testate amoebae shell length or diameter) and trophic position cannot  
314 be found for consumers that exploit a wide range of prey body sizes [7, 59], as shown here  
315 with *H. papilio* and *N. tincta*. Nevertheless, we found that the size of shell aperture and  
316 biovolume were inversely proportional to the trophic position of testate amoebae in the  
317 microbial network, while usually these morphological traits are considered as indicators of  
318 high trophic level [12, 25, 27]. Again, such results illustrate the importance of foraging traits  
319 such as the density of preferential food sources on trophic position of consumers such as *H.*  
320 *papilio* and *N. tincta*, and therefore on the food web structure. Indeed, if we compare these  
321 results to those of Jassey et al. [12], these data indicate that two morphologically similar

322 populations of the same species (e.g. *H. papilio*) may occupy very different trophic positions,  
323 thus altering any overall relationship between morphology and trophic position in *Sphagnum*-  
324 associated food web. Furthermore, the inverse relationship between morphological trait-sizes  
325 and trophic levels showed that smaller testate amoeba species such as *A. flavum* –found as a  
326 consumer of FOMB– occupied the top of the food web. This suggested that bacterivores  
327 received more translocated nitrogen from the detritivorous pathway than herbivores from the  
328 herbivorous pathway, giving a greater functional role to bacterivores. This isotopic signature  
329 of bacteria –and thus their trophic level– depends of the signature of the detrital source, which  
330 may sometimes be higher than that of autotrophs [50]. Therefore, a switch from an  
331 herbivorous to a detritivorous dominant pathway could break down the morphological  
332 relationships between trait-size and trophic level.

333

334 Algal-endosymbiont contribution to host *Hyalosphenia papilio* diet

335 The highly enriched  $\delta^{13}\text{C}$  content of *H. papilio* (i.e. -22‰ on average) suggested that this  
336 species used another non-identified  $^{13}\text{C}$  baseline than other testate amoeba species (i.e. for  
337 which the average was -26‰), explaining why this species showed such distinct isotopic  
338 patterns. An alternative hypothesis is that *H. papilio* used another source of carbon than CA  
339 and ciliates such as their endosymbiotic algae. Few studies have attempted to quantify the  
340 energetic benefits of endosymbiotic algae of mixotrophic species but Schönborn [32]  
341 suggested that *H. papilio* could probably live without food sources using these endosymbiotic  
342 algae as alternate energy source. The low availability of energetic resources probably led *H.*  
343 *papilio* to use its endosymbionts as complementary energy source, and thus to receive more  
344 translocated carbon from the stimulated activity of its endosymbionts than from CA,  
345 explaining why  $\delta^{13}\text{C}$  signature of *H. papilio* was so high. By analogy to our results,  
346 Bergschneider and Muller-Parker [61] showed that the  $^{13}\text{C}$  signature of zoochlorellae



347 increased from -25‰ to -20‰ when they were used as complementary energy source in  
348 *Anemona elegantissima*. These authors also showed that zoochlorellate anemone exhibited  
349 distinctly lower patterns of <sup>15</sup>N values than non-zoochlorellate anemone indicating that  
350 symbiotic anemones received nutrition from both external (heterotrophic) and internal  
351 (translocated algal products) sources. Although our results do not allow drawing strong  
352 conclusion about the use of endosymbionts as a complementary source of energy in *H.*  
353 *papilio* specimens, they suggest that resource availability may play a fundamental role in the  
354 use of endosymbionts as energy source.

355         The difference between the isotopic signatures of the two-mixotrophic species studied  
356 (*H. papilio* and *A. flavum*) provides evidence in support the hypothesis that resource  
357 availability may play a fundamental role in the use of endosymbionts as energy source.  
358 Although obvious morphological differences in overall size and size of shell aperture occur  
359 between these mixotrophic species, their range of prey available is likely to explain their  
360 different isotopic signatures. These results suggest that mixotrophic species may become  
361 more heterotrophic with abundant and available food sources, which alters their functional  
362 role in food webs and the carbon cycle. Indeed, a shift towards more heterotrophic nutrition  
363 may change the isotopic signature of mixotrophic specimens, and therefore their functional  
364 role from primary production towards consumers [11]. Such results highlight that dynamics in  
365 prey-predator abundance could modify the functional role of mixotrophic species in the  
366 microbial food web. Furthermore, these results also suggest that mixotrophic species can  
367 easily outcompete other competitors when food source are rare in networks by shifting on  
368 their endosymbionts [10]. Hence, our results provide reason to expect that mixotrophic life  
369 history trait play key role for both prey abundance and microbial food web structure  
370 associated to *Sphagnum*.

371 In conclusion, our study demonstrates that nitrogen and carbon isotopic signatures of  
372 microorganisms are useful for estimating food web structure of microbial network associated  
373 to *Sphagnum*. These results show that testate amoeba consumers occupy different trophic  
374 levels within the microbial food web, depending to their feeding behavior, the density of their  
375 food resources and their metabolism (i.e. mixotrophy vs. heterotrophy). Our findings provide  
376 experimental support for the novel hypothesis that mixotrophy has key implications in food  
377 web structure and functioning [62, 63]. Given the key position of mixotrophs in microbial  
378 community in peatlands, changes in their functional role towards primary producers to  
379 consumers (or vice versa) may cascade through food webs, altering species interactions as  
380 well as the magnitude and direction of carbon fluxes.

381

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550 **Table 1:** Number of specimen sampled (n), weight of species sampled (mg, mean  $\pm$  standard error), isotopic ratios (‰, mean  $\pm$  standard error) of  
 551 carbon and nitrogen in *Sphagnum fallax*, microbial groups and testate amoeba species. Trophic level, feeding type and strategy of microbial  
 552 groups and testate amoeba species.

553

Group	Species name	n	Sample weight (mg)	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	Trophic level	Feeding type <sup>a</sup>	Feeding strategy
Bryophyte	<i>Sphagnum fallax</i>		0.93 $\pm$ 0.01	-28.0 $\pm$ 0.2	-5.4 $\pm$ 0.1	1.0		autotroph
Particulate matter + Bacteria			0.83 $\pm$ 0.04	-27.3 $\pm$ 0.2	-1.9 $\pm$ 0.2	2.0	1A	
Microalgae*			0.96 $\pm$ 0.1	-26.8 $\pm$ 0.1	-6.7 $\pm$ 1.4	1.0	0A	autotroph
Testate amoeba	<i>Arcella vulgaris</i> *	250	0.23	-26.8	-0.97	2.3	2C	B, CA, Fu [21]
	<i>Archerella flavum</i> *	4500	0.46	-25.7	1.0	2.9	2A	B Lara, personal communication
	<i>Bullinaria indica</i> *	330	0.19	-26.4	-0.97	2.3	2C	B, CA present paper
	<i>Euglypha compressa</i> *	50	0.07	-25.0	-0.93	2.3	2C	B, CA [19]
	<i>Hyalosphenia papilio</i>	1800	0.51 $\pm$ 0.05	-21.6 $\pm$ 0.5	-1.2 $\pm$ 0.1	2.3	2B	CA, Fu, Fl, Ci, T, M [12]
	<i>Nebela tinctoria</i> *	150	0.07	-25.9	-0.6	2.4	2C	CA, Fu, Fl, Ci, T, M [19, 20, 12]
Rotifers*		300	0.09	-24.3	-0.5	2.4	2C	Filter feeders
Nematodes*		300	0.16	-25.4	0.5	2.7	2A	B [64]

\* pooled species

Food sources: B = bacteria; Fu = Fungi; CA = cyanobacteria and microalgae; Fl = flagellates; Ci = ciliates; T = testate amoebae; M = micrometazoan (rotifers, nematodes)

Feeding type: 0A = photosynthetic organism (protist); 1 = OM feeders; 2A = bacterivores; 2B = primary producer feeders; 2C = bacteria and primary producer feeders

554 **Table 2:** Relative proportion of the identified prey ingested by *Hyalosphenia papilio*.  
 555 Relative proportion of the abundance of microbial groups in *Sphagnum fallax*, excluding  
 556 bacteria. CA = microalgae and cyanobacteria. Relative proportion of the biomass of the  
 557 identified prey ingested by *H. papilio*.  
 558

	Frequency of the preys ingested by <i>H. papilio</i> (%)	Frequency of identified prey (%)	Frequency of ingested organisms in term of biomass (%)
CA	80%	82.4%	9.8%
Fungi	12%	11.8%	20.1%
Ciliates	6%	0.4%	49.3%
Testate amoebae	0%	4.7%	< 0.1%
Rotifers	0.6%	0.5%	0.8%
Nematods	0%	0.2%	< 0.1%

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563 **Figure Legends**

564

565 **Figure 1:** (a) Relative biomass of different microbial groups (% of the total microbial  
566 community) in a *Sphagnum fallax* carpet. (b) Relative abundance (%) of the main testate  
567 amoeba species (% of the total testate amoeba community).

568 **Figure 2:** Stable isotope ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) food web diagram of the major microbial groups and  
569 testate amoeba taxa in *Sphagnum fallax*. Symbols are as follows, Sph\_fal, *Sphagnum fallax*;  
570 CA, cyanobacteria + microalgae; Rot, rotifers; Nem, nematodes; FOMB, fine organic matter  
571 in association with bacteria; Arc\_vulg, *Arcella vulgaris*; Arch fla, *Archerella flavum*;  
572 Bul\_ind, *Bullinularia indica*; Eug\_comp, *Euglypha compressa*; Hya\_pap, *Hyalosphenia*  
573 *papilio*; Neb\_tin, *Nebela tinctoria*. Dark-grey circles represent the herbivory pathway, and light-  
574 grey circles the detritivory pathway.

575 **Figure 3:** (a) Hierarchical clustering (Bray and Curtis index method) of microbial groups and  
576 testate amoeba species from *Sphagnum fallax* based on their  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ , isotopic nitrogen  
577 and carbon ratio, and trophic level (see text for details). (b) Trophic level of the different  
578 testate amoebae species and microbial groups.

579 **Figure 4:** (a) Contribution of food sources to the diet of *Hyalosphenia papilio*. Error bars,  
580 S.E. CA = microalgae + cyanobacteria. (b) Trophic level of *H. papilio* vs. the contribution of  
581 ciliates and CA to total diet of *H. papilio*. This figure includes some results from Jassey et al.  
582 (2012).

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