Characterizing the Feeding Habits of the Testate Amoebae Hyalosphenia papilio and Nebela tincta along a Narrow "Fen-Bog" Gradient Using Digestive Vacuole Content and (13)C and (15)N Isotopic Analyses.

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ORIGINAL PAPER

Characterizing the Feeding Habits of the Testate Amoebae *Hyalosphenia papilio* and *Nebela tincta* along a Narrow "Fen-Bog" Gradient Using Digestive Vacuole Content and ^{13}C and ^{15}N Isotopic Analyses

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Population dynamics and feeding habits of the testate amoebae *Nebela tincta* and *Hyalosphenia papilio* were studied along a short "fen" to "bog" gradient in a *Sphagnum*-dominated mire (Jura, France). Samples were collected in living "top segments" (0-3 cm) and early declining "bottom segments" (3-6 cm) of *Sphagnum fallax* peat. Observations of digestive vacuole content and stable isotope analyses (^{13}C and ^{15}N) were used to establish the feeding behavior of both testate amoeba species. Owing to their vertical distribution, the feeding habit of *H. papilio* was described from top segments, and that of *N. tincta* from bottom segments. Among identified food sources, those most frequently ingested by *N. tincta* were spores and mycelia of fungi (55%), microalgae (25%) and cyanobacteria (8.5%). For *H. papilio*, the most frequently ingested prey were ciliates (55%) and microalgae (35%). Nonmetric Multidimensional Scaling analysis clearly demonstrated that the two species did not have the same feeding habit along the "fen-bog" gradient, and furthermore that a significant spatial split exists in the feeding behavior of *H. papilio*. Additionally, isotope analyses suggested that *H. papilio* and *N. tincta* did not have the same trophic position in the microbial food web, probably resulting from their different feeding strategies.

Key words: Ecological gradient; food preference; ^{13}C and ^{15}N isotopes; peatland; *Sphagnum*; testate amoebae.

Introduction

Testate amoebae are abundant and diverse unicellular microorganisms (Protozoa) that are especially common in organic-rich soils, lakes, wetlands, and moss habitats (Booth 2001; Charman and Warner 1992; Mitchell et al. 2008; Ogden and Hedley 1980). Because they represent a common and abundant group of free-living terrestrial protists and a major group of predators in the microbial food web (Gilbert et al. 1998, 2003; Ogden and Hedley 1980), these microorganisms are increasingly recognized as an important component of many ecosystems, especially in peatlands (Mitchell et al. 2008).

Testate amoebae are sensitive to a variety of environmental variables along ecological gradients, including hydrology, pH, or nutrient status (Booth 2008; Heal 1961, 1962, 1964; Mitchell et al. 2000; Mitchell and Gilbert 2004; Opravilova and Hajek 2006). Owing to their decay-resistant shells, testate amoebae are of considerable interest for the study of past and present environmental dynamics in peatlands (Andersson and Schoning 2010; Charman 2001; Markel et al. 2010; Tsyganov et al. 2011). The considerable sensitivity of testate amoeba communities to defined ecological features makes them a useful tool in ecological and paleoecological studies (Charman 2001; Mitchell et al. 2008). However, how different local ecological settings influence their distribution remains unclear. Some data show that testate amoebae may be directly affected by environmental gradients, such as physicochemical factors and/or vegetation composition, which strongly influence their community composition (Booth, 2008; Jassey et al. 2011a; Lamentowicz et al. 2010; Mitchell et al. 2000; Tsyganov et al. 2011). In parallel, other studies also suggest that indirect effects on their community composition may be modulated by microbial food webs (e.g. trophic effect) (Beyens et al. 2009; Jassey et al. 2011b; Mitchell et al. 2003). Indeed, as intermediaries between bacterial and invertebrate soil communities (Gilbert et al. 1998), testate amoebae occupy top positions in the microbial food web. Usually considered as having a wide range of feeding preferences, including small organisms (e.g. bacteria, fungi, algae and other protozoa) (Coûteaux and

Ogden 1988; Coûteaux and Pussard 1983; Gilbert et al. 2000, 2003; Ogden and Coûteaux 1987; Ogden and Hedley 1980; Schönborn, 1965, 1982; Schroeter 2001), and larger organisms (i.e. rotifers and nematodes) (Han et al. 2008; Yeates and Foissner 1995; Wilkinson and Mitchell 2010), testate amoebae are potentially sensitive to changing abundance and community structure in lower trophic levels (Gilbert et al. 2000). However, the understanding of the sensitivity of feeding habit of testate amoebae still suffers from the scarcity of available data concerning the range of foods preferentially ingested by testate amoebae, as well as about their feeding behavior in different ecological settings.

Furthermore, little is known concerning the feeding structure of testate amoeba communities, even for dominant species. Although the general nature of ingested foods have been highlighted in peatlands (Gilbert et al. 2000, 2003), it remains unknown whether species commonly described as omnivores (e.g. *Nebela tincta* or *Hyalosphenia papilio*; Gilbert et al. 2000) share the same trophic position in the microbial food web. In this context, stable carbon and nitrogen isotope signatures (δ^{13}C and δ^{15}N) of testate amoebae have the potential to provide useful and complementary information concerning trophic relationships in soil ecosystems (Hyodo et al. 2010; Post 2002; Vander Zanden and Rasmussen 1999). Both δ^{13}C and δ^{15}N signatures are effective bulk integrators of long-term diet and complex interactions such as omnivory (Anderson and Cabana 2009; Cabana and Rasmussen 1994; Post 2002). Nitrogen isotope ratios are especially useful estimators of the trophic position among predators and omnivores (Anderson and Cabana 2005, 2009; Kohzu et al. 2009a; Post 2002).

The present paper introduces a detailed record of testate amoeba feeding habits spanning different ecological settings. Multi-proxy analyses, i.e. digestive vacuole content, abundance of their prey, predation preference estimations, and stable isotope analyses are exploited to establish the feeding behavior of two commonly dominant testate amoebae in *Sphagnum* peatlands: *Nebela tincta/N. tincta major* complex (for simplicity hereafter referred to here as *N. tincta*) and *Hylosphenia papilio* (Heal 1964; Warner 1987). Their feeding habits

were studied in living *Sphagnum* shoots (living "top segments" and early declining "bottom segments") along a short ecological gradient from a transitional *Sphagnum*-dominated poor fen (hereafter referred to here as "fen") to a *Sphagnum* bog with more pronounced micro-topography (hereafter referred to here as "bog"). We hypothesized that the feeding habit of these species would differ and would vary along this ecological gradient, despite their general characterization as omnivores. Considering the different functional traits of *N. tincta* (heterotrophy) and *H. papilio* (mixotrophy) (Mitchell et al. 2004), we also hypothesized that these two species have a different feeding behavior and occupy different trophic position within their community.

Results

Microbial Structure

Testate amoeba specimens were differentially distributed along *Sphagnum* segments. Active forms of *H. papilio* were significantly more abundant in top segments than in bottom segments along the gradient ("fen" area: an average of 18 000 ± 5400 and 2200 ± 850 ind.g^{-1} DM respectively; "bog" area: an average of 4100 ± 1400 and 680 ± 480 ind.g^{-1} DM respectively). Conversely, active forms of *N. tincta* were significantly less abundant in top segments than in bottom segments ("fen" area: an average of 160 ± 50 and 1500 ± 710 ind.g^{-1} DM respectively; "bog" area: an average of 3300 ± 1500 and 8900 ± 2600 ind.g^{-1} DM respectively) (Table 1; $P < 0.05$, ANOVA tests). Differences among sampling areas were also recorded, since *H. papilio* was more abundant in the "fen" area than in the "bog" area, while *N. tincta* had higher density in the "bog" area than in the "fen" area (Table 1; $P < 0.05$, ANOVA tests). All of these observations were also recorded for the biomass of *H. papilio* and *N. tincta* (Table 1).

The structure of microbial communities along the "fen-bog" gradient differed significantly. The NMDS leading-axis biplot showed that samples of top and bottom segments were clearly separated in the ordination space between the "fen" and the "bog" area (Fig. 1; P = 0.04, ANOSIM). The densities and the biomasses of the different microbial groups were similarly distributed along the "fen-bog" gradient, with the exception of microalgae and fungi which were more abundant in the "bog", and ciliates which were more common in the "fen" area (Fig. 1; Table 1; $P < 0.05$, ANOVA tests). Microalgae were dominated by Chlorophyceae in both areas (e.g. *Eudorina* sp. and *Cylindrocystis brebissonii*). The community of ciliates was dominated by three species in the two sampling area: *Uronema* sp. ("fen": 41.7% of the total density; "bog": 82.8%), *Playtorya sphagni* ("fen": 24.8%; "bog": 7.9%) and *Paramecium bursaria* ("fen": 32.6%; "bog": 6.4%). An increase of the density and the biomass of fungi was also observed between top and bottom segments in the "fen" area, and for testate amoebae and nematodes in the "bog" area ($P < 0.05$; ANOVA tests).

General Feeding Habit of Testate Amoeba Specimens

The frequencies of *H. papilio* specimens associated with a prey was the same in the two sampling areas ("fen": 57.9 ± 10%; "bog": 53.7 ± 4%), while frequencies of *N. tincta* specimens associated with a prey were higher in the "fen" area (89.1 ± 5%) than in the "bog" area (55.8 ± 4%) (Appendix A). The frequency of unidentified prey was < 5% in both sampling areas for both testate amoeba species. The number of specimens observed in association with a prey was positively correlated with the number of active individuals (n = 12, r = 0.78, $P < 0.01$). Because of the vertical microdistribution of the two species in the top and bottom segments (Table 1), the feeding habit of *H. papilio* was investigated in detail from top segments only, and similarly that of *N. tincta* from bottom segments.

Among the identified food sources, those most frequently ingested by *N. tincta* along the "fen-bog" gradient were spores and mycelia of fungi ("fen": 55.6% of the total identified predator-prey associations; "bog": 59.3%; including hyphae of ascomycetes and spores of *Helicoon pluriseptatum*), microalgae ("fen": 27.3%; "bog": 23.9%; primarily *Eudorina* sp. and *Cylindrocystis brebissonii*) and cyanobacteria ("fen": 8.6%; "bog": 9.1%, notably *Anabaena* spp.) (Fig. 2G-J, 3A). Predation of protozoa and micrometazoa such as rotifers and testate amoebae (e.g. *Archerella flavum*) was low along the "fen-bog" gradient. Conversion of these data to total biovolumes ingested modified these proportions considerably: the proportion of fungi decreased ("fen": 25.5%; "bog": 31.8%), and that of ciliates ("fen": 19.6%; "bog": 11.9%) and rotifers ("fen": 21.8%; "bog": 25.1%) increased (Fig. 3B). Preferential predation indices highlighted that *N. tincta* fed evenly on rotifers, microalgae, ciliates and fungi in the "fen" area ($\alpha = 0.2$), while in the "bog" area they fed preferentially on ciliates ($\alpha = 0.6$) and rotifers ($\alpha = 0.2$) (Table 2).

For *H. papilio*, the most frequently identified food sources in the "fen" area were ciliates (58.1% of the total identified predator-prey associations, including *Paramecium bursaria* and *Playtorya sphagni*) and microalgae (34.1%, predominantly *Eudorina* sp. and *Cylindrocystis brebissonii*). In the "bog" area, the most frequently ingested prey were ciliates (46.3%), microalgae (43.4%), spores and mycelia of fungi (7.1%) (Figs 2A-F, 3A). Predation of rotifers (e.g. *Habrotrocha* sp.) and testate amoebae (e.g. *Archerella flavum*) appeared to be low along the "fen-bog" gradient. With consideration to the biovolume ingested by *H. papilio*, ciliates represent an average of 75% of the total identified predator-prey associations in the two sampling areas, microalgae only 15%, and rotifers increased up to 5.4% (Fig. 3B). The preferential predation ratio revealed that ciliates ($\alpha \geq 0.8$) were preferentially ingested by *H. papilio* in the two sampling areas, while the index of preference for microalgae was very low ($\alpha < 0.05$) (Table 2).

168 Spatial Feeding Activity of Testate Amoeba Specimens

169 NMDS ordination of the feeding habit of the two testate amoeba species from the two
170 sampling areas showed that *H. papilio* and *N. tincta* differed markedly between the two
171 sampled areas (P = 0.001, ANOSIM; Fig. 4). This ordination showed that ciliates were
172 essentially associated to *H. papilio* and fungi to *N. tincta*. NMDS also highlighted that feeding
173 habits of *H. papilio* differed only slightly along the "fen-bog" gradient, while no spatial
174 differences of feeding activity at all were detected in *N. tincta*.

175 Figure 5 illustrates variations among the dominant ingested food types along the "fen-
176 bog" gradient: microalgae, fungi (mycelia and spores), ciliates, and other protozoa and
177 micrometazoa (flagellates, testate amoebae, rotifers, nematodes). A significant relationship
178 was identified between the density of fungi and the frequency of their ingestion by *H. papilio*
179 between the "fen" and "bog" areas (r = 0.83, P = 0.01) (Fig. 5A, B). Another significant
180 relationship was found between the densities of ciliates along the gradient and the frequency
181 of their ingestion by *H. papilio* (r = 0.91, P = 0.001) (Figs 5A, B, 6A), and more specifically
182 with the mixotrophic species *Playtorya sphagni* (r = 0.80, P = 0.051) and *Paramecium*
183 *bursaria* (r = 0.94, P = 0.004) (Fig. 6B, C). No correlation was found between the dominant
184 group of ciliate (*Uronema* sp.) and the frequency of ciliate ingestion by *H. papilio* (r = 0.44, P
185 = 0.38) along the ecological gradient (Fig. 6D). On the other hand, a positive correlation was
186 found between the densities of ciliates and *H. papilio* along the ecological gradient (r = 0.84,
187 P = 0.03). Such relationships were not identified between *N. tincta* predation and the ambient
188 densities of various food sources. However, a significant linear correlation exists between the
189 density of fungi and the density of *N. tincta* in both *Sphagnum* ecotypes ("fen": r = 0.71, P =
190 0.03; "bog": r = 0.67, P = 0.04).

191

192

193 Isotopic Composition of Testate Amoeba Specimens

194 Composite testate amoeba samples produced enriched $\delta^{15}N$ and $\delta^{13}C$ values relative to
195 baseline values determined from *S. fallax* foliage (Table 3). The average enrichment is in the
196 order of 9‰ for $\delta^{15}N$ and 3‰ for $\delta^{13}C$. Among the two species considered, *N. tincta* isotopic
197 values are consistently enriched relative to those obtained from *H. papilio*, by 0.9 ‰ for $\delta^{13}C$
198 and 2.4 ‰ for $\delta^{15}N$. This level of consistent inter-specific isotopic variability implies that *N.*
199 *tincta* occupies a slightly higher trophic position than *H. papilio* in the peat-soil microfauna.

200

201 **Discussion**

202 Feeding Behavior of Testate Amoeba Specimens along the Ecological Gradient

203 The feeding behavior of *N. tincta* and *H. papilio* differs markedly between them, irrespective
204 to ecological settings. The variability of their functional traits (heterotrophy vs. mixotrophy,
205 respectively) could explain such variability. Indeed mixotrophic specimens – *H. papilio* –
206 preferentially live in top *Sphagnum* segments, while heterotrophic specimens – *N. tincta* –
207 live in deeper *Sphagnum* segments (Booth 2002; Jassey et al. 2011a; Mitchell and Gilbert
208 2004). In addition to environmental parameters influencing their vertical microdistribution
209 (Jassey et al. 2011a), we showed a certain variability of the biomass and the abundance of
210 their identified prey along *Sphagnum* shoots, which may also influence their distribution and
211 their feeding behavior.

212 Among identified foods, those most frequently ingested by *N. tincta* were fungi (55%)
213 and microalgae (25%). Gilbert et al. (2003) previously identified fungi and microalgae as the
214 primary food source for *N. tincta,* accounting for 36% and 45% of total annual diet,
215 respectively. Our feeding observations now further demonstrate that feeding activity of *N.*
216 *tincta* is essentially unchanged along the ecological gradient despite differences within the

ambient microbial community abundance and structure. The lack of spatial variation is perhaps not surprising given the high density of fungi and microalgae in both sampling areas. We predict that seasonal dynamics of food sources remain a key factor regulating feeding behavior of *N. tincta*, as described by Gilbert et al. (2003).

The positive correlation between the densities of fungi and *N. tincta* within *Sphagnum* shoots, and the high frequency of fungal associations suggested that fungal standing crop was a primary determinant of the ecology of *N. tincta*, as supposed for *Phryganella acropodia* in soils (Coûteaux 1985; Ogden and Pitta 1990; Schröter 2001; Vohnik et al. 2009, 2011). Although these results seem to indicate that *N. tincta* is rather a fungal specialist (Coûteaux, 1985; Coûteaux and Dévaux 1983; Ogden and Pitta 1990), two lines of evidence contrast with such a conclusion. First, grazing by *N. tincta* on the most common co-occurring microorganisms was frequently observed, as well as an opportunistic feeding behavior on protozoa and micrometazoa. Additionally, it remains unclear if mycophagous species preferentially consume hyphae, feed on exudates from hyphae, or ingest bacteria feeding on fungal exudates (Coûteaux 1985; Wilkinson and Mitchell 2010).

Second, it is important to recognize the inherent limitations of studying feeding behavior using light microscopy alone, despite the value of these data with respect to gaining a better understanding of testate amoebae autecology. With consideration to the biovolumes ingested by *N. tincta*, the data highlight the potential role of ciliates or rotifers in its feeding habit. Indeed, an ingested ciliate or rotifer is 10 up to 20 times larger than the pieces of fungal mycelium frequently ingested by testate amoebae. The preferential predation ratios likewise suggested that *N. tincta* preferred to select protozoa and micrometazoa when they were easily available. In addition, fungal mycelia or spores were easily identifiable (even dead) among digestive vacuole content of *N. tincta* because of their rigid cell walls (Ogden and Pitta 1990). Ultimately, their ingestion frequencies were probably quite accurate, whereas those recorded for ciliates or rotifers were most likely underestimated (Gilbert et al. 2003). Unicellular

protozoa may disappear faster from the digestive vacuole of testate amoebae (Gilbert et al. 2000), while fungal mycelia or spores recorded in shells are not always assimilated by testate amoebae and simply ended in the shells by chance (Coûteaux and Déveaux, 1983; Ogden and Pitta 1990). Therefore, the feeding habit of *N. tincta* seems to be rather generalist than fungal specialist and focused on the major sources of carbon and nitrogen.

Information on general feeding behavior in *H. papilio* showed that this species essentially fed on ciliates (52%) and microalgae (38%). Generally, this species is described as having a wide variety of food sources including fungi, cyanobacteria, microalgae, ciliates and metazoans (Gilbert et al. 2000). However, few data are available concerning the frequency of prey ingestion in *Sphagnum* habitats. Together, our results indicate that this species preferentially selected ciliates in the environment. In particular, *H. papilio* associated with ciliates were more closely correlated with the larger mixotrophic species *Playtorya sphagni* and *Paramecium bursaria* than with the smaller yet dominant ciliate *Uronema* sp. Although our results showed that *H. papilio* fed on resources within a wide range of body size, the results on ciliate assimilation seem support the optimal foraging theory which states that organisms forage in such a way as to maximize their net energy intake (Petchey et al. 2008; Stephen and Krebs 1986). The frequent and rapid shifts of *Uronema* in the environment may also explain such results. Because the size of our data set is limited, and ciliates associated with *H. papilio* were not always recognizable, it is difficult to draw strong conclusions regarding the ability of *H. papilio* to select among mixotrophic ciliates. Finally, the significant correlation between the densities of *H. papilio* and ciliates imply that predation was density-dependant along the investigated environmental gradients.

The slight spatial variability of feeding behavior of *H. papilio* means that this species has different ecological niches along the ecological gradient relative to *N. tincta*. Jassey et al. (2011a) showed that the specific environmental features described in the “fen” and the “bog” areas (i.e. a suite of distinct microhabitats with respect to water chemistry, microtopography,

and vegetation cover) clearly affected the distribution of testate amoebae, especially *H. papilio*. In the same way, ciliates were also influenced by these distinct microhabitats. For instance, strong variations in the density of *Paramecium bursaria* and *Playtorya sphagni* were recorded between the two areas. The structure of the *Sphagnum* carpet (i.e. patchiness of vegetation) has been recognized as influencing ciliate community structure and abundance at fine ecological scales (Mieczan 2010). Therefore, vegetation patchiness along the "fen-bog" gradient may directly influence the occurrence of ciliates and *H. papilio*, and indirectly that of *H. papilio* through its feeding behavior.

δ^{13}C and δ^{15}N Trophic Enrichment in Peatland Microbial Food Web

Isotopic signatures of peatland trophic interactions are relatively scarce. A few studies have used δ^{13}C or δ^{15}N in plant tissues to infer past moisture variations, although such relationships remain complex (Andersson and Schoning 2010; Loader et al. 2007; Loisel et al. 2008; Markel et al. 2010). The δ^{13}C and δ^{15}N signatures of *S. fallax* (-27‰ and -6‰, respectively) reported here correspond well to those previously found in peatlands (Andersson and Schoning 2010; Asada et al. 2005; Bragazza et al. 2005, 2010; Loader et al. 2007; Markel et al. 2010; Price et al. 1997).

The δ^{13}C and δ^{15}N signals recorded for testate amoeba specimens were enriched relative to those of *S. fallax* (an average increase of 3‰ and 9‰ respectively, occurred) suggesting trophic enrichment between *Sphagnum* mosses and testate amoebae. As the major source of organic matter in peatlands (Francez and Loiseau 1999; Gilbert and Mitchell 2006), *Sphagnum* represents a potential isotopic baseline of the microbial food web. However, the consumer δ^{13}C values are generally similar (< 1‰ difference) to those of their diet, while consumer δ^{15}N values are about 3‰ higher than those of their diet (Post 2002; Hyodo et al. 2010). These findings indicate that preferential incorporation and accumulation of ^{13}C and ^{15}N from *Sphagnum* to testate amoebae occurs, probably mediated by the microbial food web.

Even though microorganisms are consistently enriched in ^{13}C relative to adjacent plant substrates (Dijkstra et al. 2006; Hyodo et al. 2010), testate amoebae cannot directly feed on *Sphagnum*. *Sphagnum* cells are difficult to assimilate by protozoa and require prior decomposition by fungi or bacteria (Gilbert 1998; Gilbert et al. 2000, 2003; Gilbert and Mitchell 2006). Conversely, and as described in this paper, microalgae and fungi are directly assimilated by both testate amoeba species. In this context, the characterization of $\delta^{13}C$ and $\delta^{15}N$ enrichment at each trophic level of the microbial food chain should be investigated, following the lead taken in other aquatic or terrestrial ecosystems (Hyodo et al. 2010; Kohzu et al. 2009b; Vander Zanden and Rasmussen 1999).

The differential $\delta^{15}N$ enrichment of 2.4‰ between *H. papilio* and *N. tincta* suggests that these two species do not occupy identical trophic positions in the microbial food web, corroborating our previous observations on their feeding habits. Although, there is a still lack of isotopic data addressing the various food sources commonly ingested by these species, one hypothesis is that differences in the $\delta^{15}N$ signature of *N. tincta* and *H. papilio* emerge from their different feeding strategies. Indeed, fungal mycelia are typically enriched in ^{15}N (Bragazza et al. 2010; Hobbie and Colpaert 2004; Lindahl et al. 2007). For example, the $\delta^{15}N$ of fungi in tundra varies between 1.5 and 3‰ (Mayor et al. 2009). Although our results suggest some importance of ciliates and rotifers in the diet of *N. tincta*, more than 50% of predator-prey associations were with fungi. Thus, the ^{15}N enrichment of *N. tincta* may result from this mycophagous behavior. At the same time, peatland ciliates are recognized as bacterivores and algivores that typically retain depleted ^{15}N signatures (Mieczan 2007, 2009). Bacteria have a greater potential for immobilizing nitrate depleted in ^{15}N in bog litter (Bragazza et al. 2010). An alternative hypothesis is that mixotrophy alters the $\delta^{15}N$ signature of *H. papilio*. In addition to ingested food particles, *H. papilio* also contains endosymbiotic algae, which represent a potential alternate source of energy (Wilkinson and Mitchell 2010). Few studies have attempted to quantify the energetic benefits of endosymbiotic algae,

320 although a strong case has been made about the importance of this energy source in *H. papilio*
321 (Schönborn 1965).

322 Because $\delta^{13}C$ and $\delta^{15}N$ data pertaining to peatland microbial food webs are presently
323 limited, more research in this area is warranted. Our data set is also limited, rendering it to
324 draw difficult strong conclusions about the trophic positions of *N. tincta* and *H. papilio*.
325 However, the pooled species-specific isotopic values verify that both *N. tincta* and *H. papilio*
326 in *Sphagnum* peatlands target specific foods types, and these species probably do not occupy
327 the same trophic position in the microbial food web of peatlands. Further measurements are
328 required to assess seasonal variations of feeding behavior among these microbial
329 communities.

330

331 **Methods**

332 **Field sampling and laboratory analyses:** Experiments were conducted on Le Forbonnet
333 peatland, an undisturbed *Sphagnum*-dominated mire situated in the Jura Mountains (Doubs,
334 France, 46°49'35''N, 6°10'20''E) at an altitude of 840 m above sea level (Fig. 7). Cold
335 winters (on average -1.4 °C) and mild summers (on average 14.6 °C) characterized the site.
336 The annual mean temperature measured at the site over a one-year period from 5th November
337 2008 to 30th November 2009 was 6.5 °C and the annual precipitations 1200 mm.

338 Samples of *Sphagnum fallax* were collected on June 26th 2008 within homogeneous
339 and similar plots of *S. fallax* carpet across two adjacent areas selected in relation to their
340 wetness, soil micro-topography, vegetation, and degree of humification (Delarue et al. 2011).
341 The first sampling area (called "fen") was a transitional *Sphagnum*-dominated poor fen,
342 relatively flat and homogeneous, characterized by a moss cover dominated by *S. fallax* and by
343 the lack of *S. magellanicum*. Vascular plants such as *Eriophorum vaginatum, Vaccinum*

oxycoccus and *Andromeda polifolia* were recorded in very low abundance. *Scheuchzeria palustris* and *Carex limosa* occurred outside of the studied plots. The second sampling area (called "bog") was a *Sphagnum* bog directly adjacent to the fen area. Patterns of hummocks with *S. magellanicum, V. oxycoccos, E. vaginatum* and *Calluna vulgaris,* and hollows with lawns of *S. fallax, Carex rostrata* and *A. polifolia* characterized the sampling area. The terms "fen" and "bog" are used for simplicity and to denote the existence of a trophic and wetness gradient inferred from the vegetation. In each of the two sampling areas, three plots were selected in representative surfaces. The maximal distance between the two most distant plots was ca. 30 m. *S. fallax* mosses were collected in each plot around 10 permanent markers. The goal of this sampling design was to obtain a composite sample from each plot and avoid any bias due to spatial heterogeneity (Mitchell et al. 2000).

For microbial preparations, *S. fallax* samples were cut into two levels (sampling depth): 0-3 cm (living "top segments") and 3-6 cm (early declining "bottom segments") of the capitulum. Then, the samples were fixed with glutaraldehyde (2% final concentration) and stored at 4 °C in the dark. Microorganisms were extracted from *Sphagnum* mosses using the method describe in Jassey et al. (2011b). The remaining fraction of *Sphagnum* was dried at 80 °C for 48h and weighted to express microbial density in grams of dry mass (DM) of *Sphagnum*. Microalgae, cyanobacteria, protozoa, rotifers, nematodes and fungi were identified and counted at x200 and x400 magnification using an inverted microscope (OLYMPUS IX71) following Uthermöhl's method (Ütermöhl 1958). For each community, the average biovolume (μm^3) was estimated by assuming geometrical shapes and the biomass of each microbial group was calculated (Gilbert et al. 1998). In parallel, a minimum a 20 specimens of *Hyalosphenia papilio* and *Nebela tincta* (total for this study: 1240 specimens) was observed for each sample. Among active specimens, we distinguished those either with a prey within the tests or those which are feeding on a prey, i.e. any organic matter particle, to determine the feeding habit of these two species, as described in Gilbert et al. (2003).

Subsequently, ingested organisms were expressed as identified prey abundance per gram DM of *Sphagnum* and as total biovolume of ingested prey per grams DM of *Sphagnum*.

For isotope analyses, *Nebela tincta* and *Hyalosphenia papilio* were extracted from fresh mosses by six successive rinsing of *S. fallax* using distilled water and successively filtrated at 100 and 40 μm (Millipore, Nylon net filters). Testate amoebae were picked up randomly and individually using micropipette. In order to obtain reliable measurements for isotope analyses, all samples have been pooled to acquire a final sample of 600 living specimens for each testate amoeba species. Consequently, we were unable to obtain repeatedly isotopic measurements for testate amoeba specimens. To discern trophic position of testate amoebae using ^{13}C and ^{15}N signatures, it is essential to estimate the ^{13}C and ^{15}N baseline values of food web by directly measuring primary producers (Post 2002). Thus, samples of *S. fallax* were also analyzed to obtain the baseline of the ecosystem.

Samples were precisely weighed (0.001 mg) in a tin capsule for stable isotope analysis and were analyzed using an isotope ratio mass spectrometer (Isoprime Micromass, UK) coupled to an elemental analyzer (EuroVector EA 3024, Italy). Stable isotope ratios are expressed in delta (δ) notation, defined as parts per thousand (‰) deviation from a standard material; $\delta^{13}C$ or $\delta^{15}N = ([R_{sample}/R_{standard}] - 1) \times 1000$, where $R = {}^{13}C/{}^{12}C$ or $^{15}N/^{14}N$. A more positive (less negative for carbon) isotopic signature is defined as isotopically enriched, meaning that the sample contains proportionally more of the heavy stable isotope (^{13}C or ^{15}N).

Preferential predation index: An index of prey preferentially selected by testate amoeba specimens was achieved using a preferential predation ratio (α) adapted from Gaucel (2005):

$$\alpha i = (Ci/Ni) / ((Ci/Ni) + (Cj/Nj) + (Ck/Nk) + \ldots + (Cz/Nz))$$

where, αi represent the preferential predation ratio of the ingested microbial group *i; i, j, k, z* the different microbial groups ingested by testate amoebae; *Ci* the total abundance of the microbial group *i* ingested by testate amoebae; *Ni* the total abundance of the microbial group *i*

in the environment. The ratio varies between 0 and 1. A value αi near 1 means that the group *i* is preferentially ingested by testate amoebae. No corrections for biomass were added in this index because we were not always able to estimate the biomass of ingested prey. Thus this index may misrepresent the major source of C and N in testate amoeba feeding habits.

Numerical analyses: Correlations between the density of ciliates and *H. papilio* associated with ciliates in top segments, as well as between the density of fungi and *N. tincta* in two segments along the "fen-bog" gradient were determined using one-way analysis of variance (ANOVA). The normality of the data distribution was examined by plotting residuals of the model, and the homogeneity of variance was examined with a test of variance. The variability among sampling areas and *Sphagnum* segments of microbial communities assemblages was tested using linear mixed-effect model included three factors: (1) blocks (three levels, random), (2) sampling area (two levels, fixed), and (3) sampling depth (two levels, fixed), with n = 3 observations per combination of factor levels. Thereafter, ANOVA was performed for testing the model and interaction among factors. The assumptions of parametric tests were also visualized and tested. Differences among preferential prey ingested by testate amoeba specimens were achieved using Student's *t* tests.

Non-metric multidimensional scaling (NMDS) and analysis of similarities (ANOSIM) using the Bray-Curtis dissimilarity metric were computed to highlight patterns of variations of the microbial communities, and feeding habit of the two testate amoeba specimens along the "fen-bog" gradient. Since rare ingested groups could have a large influence on ordination, microbial groups in less than 1% of the total abundance were excluded from the data set prior to analyses (Lavoie et al. 2009). Homogeneous clusters of habitat groups and feeding behavior using pairwise comparisons in ANOSIM were added on NMDS plots. The output statistic, R, takes a value of 0 if there is no separation of community structure attributable to a

419 factor, and 1 if perfect separation occurs. All statistical analyses were performed using R (R
420 Development Core Team, 2010).

421

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597

598 **Tables**

599 **Table 1:** Densities (per g^{-1} DM x 10^3; mean ± standard error) and biomass (µgC g^{-1} DM;
600 mean ± standard error) of *N. tincta*, *H. papilio* and microbial groups in top and bottom
601 segments along the "fen-bog" gradient of the Forbonnet mire (French Jura; n = 3). For fungi,
602 figures represent the number of fungal hyphae pieces and spores counted in each sampling
603 area.

604

605 **Table 2:** Preferential predation ratios (α) of the different prey ingested by *H. papilio* (HP) and
606 *N. tincta* (NT) specimens along the "fen-bog" gradient.

607

608 **Table 3:** Isotopic ratios (‰) of carbon and nitrogen in *Sphagnum fallax* leaf tissues ($\delta^{13}C$ or
609 $\delta^{15}N$, mean ± standard error, n = 6) and of pooled specimens of *N. tincta* and *H. papilio* ($\delta^{13}C$
610 or $\delta^{15}N$, n = 1) from the Forbonnet mire (French Jura).

611

Figures

Figure 1. Biplot of the two primary axes of the three-dimensional NMDS ordination of microbial community data (final stress = 5.1). Samples are coded by sampling area and by sampling depth, with open symbols represent the "fen" area and filled symbols the "bog" area. Circles represent *Sphagnum*'s top segments and squares *Sphagnum*'s bottom segments. Broken lines indicate homogeneous clusters determined by ANOSIM pairwise comparisons (R = 0.41, *P* = 0.006).

Figure 2. *H. papilio* associated with fungal hyphae **(A)**, testate amoebae (*Archerella flavum*) **(B)**, ciliate **(C, E, F)**, and rotifer **(D)**. *N. tincta* associated with plant cell and ciliate **(G)**, fungal hyphae and (or) pieces of fungal spores **(H, I, J)** and cyanobacteria **(J)**. Scale bars indicate approximately 50 μm.

Figure 3. (A) Relative proportions (%) of the different identified prey categories abundance ingested by *H. papilio* and *N. tincta* specimens along the "fen-bog" gradient. **(B)** Relative proportions (%) of the different identified prey categories ingested by *H. papilio* and *N. tincta* specimens along the "fen-bog" gradient converted into biovolumes.

Figure 4. The first two primary axes of the three-dimensional NMDS ordination of testate amoebae feeding habit along the "fen-bog" gradient (*H. papilio* = HP; *N. tincta* = NT) (final stress = 9.4). Samples are coded by sampling area and by species, with open symbols represent the "fen" area and filled symbols the "bog" area. Circles represent *H. papilio* (HP) and squares *N. tincta* (NT). Broken lines indicate homogeneous clusters determined by ANOSIM pairwise comparisons (R = 0.81, *P* = 0.001).

Figure 5. Spatial relative proportion of variations of the identified prey ingested **(A)** by *H. papilio* and **(C)** by *N. tincta*. Relative proportion of the abundance of the same categories in **(B)** top and **(D)** bottom segments of *Sphagnum fallax*.

Figure 6. Ciliates ingested by *H. papilio* (ind.g^{-1} DM) plotted against **(A)** the density of ciliates (ind.g^{-1} DM), **(B)** the density of *Playtorya sphagni* (ind.g^{-1} DM), **(C)** the density of *Paramecium bursaria* and **(D)** the density of *Uronema* sp. Open symbols represents the "bog" area and filled symbols the "fen" area. Lines are regression line, significant at $P = 0.05$ level (ANOVA tests).

Figure 7. Location of the Forbonnet Peatland with inset showing the location of the two sampling areas ("fen" and "bog").

Supplementary information:

Appendix A: Spatial variations of the relative proportion (%) of *H. papilio* (HP) and *N. tincta* (NT) specimens associated with a prey along the "fen-bog" gradient.