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**Loss of testate amoeba functional diversity with increasing frost intensity across a continental gradient reduces microbial activity in peatlands**

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**Abstract**

Soil microbial communities significantly contribute to global fluxes of nutrients and carbon. Their response to climate change, including winter warming, is expected to modify these processes through direct effects on microbial functions through osmotic stress, and changing temperature regimes. Using four European peatlands reflecting different frequencies of frost events, we show that peatland testate amoeba communities diverge among sites with different winter climates, and that this is reflected through contrasting functions. We found that exposure to harder soil frost promoted species  $\beta$ -diversity (species turnover) thus shifting the community composition of testate amoebae. In particular, we found that harder soil frost, and lower water-soluble phenolic compounds, induced functional turnover through the decrease of large species (-68%, > 80  $\mu\text{m}$ ) and the increase of small-bodied mixotrophic species (i.e. *Archerella flavum*; +79%). These results suggest that increased exposure to soil frost could be highly limiting for large species while smaller species are more resistant. Furthermore, we found that  $\beta$ -glucosidase enzymatic activity, in addition to soil temperature, strongly depended ( $R^2 = 0.95$ , ANOVA) of the functional diversity of testate amoebae. Changing winter conditions can therefore strongly impact peatland decomposition process, though it remains unclear if these changes are carried-over to the growing season.

**Keywords:** Beta-diversity; Enzymatic activity; Functional turnover; Mixotrophy; Phenolic compounds; Winter climate change

## Introduction

Global climate is changing, and while substantial research effort seeks to understand the effects of increasing temperatures in summer (Stocker et al., 2014), winter warming, which is comparatively understudied, could be more crucial for key ecosystem processes (Bjerke et al., 2011; 2013; Kreyling, 2010; Kreyling et al., 2011). Winter climatic conditions are indeed changing in many parts of the world, thereby contributing to altering the structure and function of terrestrial ecosystems (Cornelissen and Makoto, 2014; Makoto et al., 2014). Many ecosystems from mid-high latitude are seasonally snow-covered and hence protected against freeze-thaw cycles during the winter. However, as a result of increased winter temperatures, snow cover is significantly declining by c.a.  $31,000 \text{ km}^2 \cdot \text{y}^{-1}$  on average from 1930 to 2005 (Hooper et al., 2013), leaving the vegetation and soil exposed to frost events (Groffman et al., 2001). Although impacts of winter climate change on plants has been confirmed by a number of observations (Bokhorst et al., 2008; 2009; 2015; Hollesen et al., 2015), its effect on soil microbes remains overlooked, despite the potential for drastic modifications of biogeochemical processes (Bokhorst et al., 2010; Durán et al., 2013; 2014; Robroek et al., 2013).

In northern peatlands, testate amoebae (protozoa) play an important functional role both in C mineralization and C assimilation (Lamentowicz et al., 2013; Jassey et al., 2015). As predators of bacteria and fungi (Gilbert et al., 2003; Wilkinson and Mitchell, 2010; Jassey et al., 2012) they indirectly influence decomposition rates and thus represent a key trophic link between organic matter cycling, decomposers, mesofauna and plants (Ledeganck et al., 2003; Krashevskaya et al., 2014; Mieczan et al., 2015b). Furthermore, a recent study highlighted that mixotrophic testate amoebae (i.e. species combining predation and photosynthesis) can significantly contribute to the net C fixation in peatlands (Jassey et al., 2015). Short generation times and large population sizes enable testate amoebae to cope with the harsh winter season (Beyens et al., 1986; Beyens, 1989; Vincke et al., 2004), but decreasing snow cover and repeated exposure to cold temperatures are also costly in terms of development and survival (Bale, 2002). It has been shown that fluctuations in snow depth decreased testate amoeba diversity altering their community composition (Tsyganov et al., 2012). In this context, an important question is whether a decrease in testate amoeba

richness/diversity reduces their functional diversity, i.e. the extent of functional differences among the species in a community (= functional alpha diversity), and whether variation in species composition in response to harder soil frost influences their functional turnover (i.e. functional beta diversity). If species share the same functional traits, redundancy in the community dampens the potential negative consequences of species loss for functional diversity, and hence for ecosystem processes (Fonseca and Ganade, 2001). However, if some species with specific traits are lost or gained from the community, functional diversity may change (i.e. the community host different functional strategies), therefore leading to cascading effects on ecosystem processes (Villéger et al., 2013). Indeed, beyond the changes in species richness and/or composition, modifications of functional diversity may have far-reaching consequences (Milcu et al., 2014; Perkins et al., 2015). Forecasting the changes in species (functional) diversity under different climatic conditions in winter is thus an urgent topic which will help our understanding of climate change effects on peatland functioning, including microbial interactions (e.g. predation) and related biogeochemical processes.

Here we test whether testate amoeba functional diversity (functional alpha- and beta-diversity) differs among four European *Sphagnum*-dominated peatlands experiencing different amount of frost events due to prevailing winter conditions. We characterized testate amoeba functional diversity using a set of six morphological traits associated with their trophic role and feeding habit (see methods). As warmer winter temperatures have been shown to shift microfauna toward larger and heavier organisms (Bokhorst et al., 2012), we hypothesized (1) that snow insulation effect will favour survival of testate amoeba with a large body size. Given that reduced snow-cover will expose testate amoebae to an increasing of frost events, we further hypothesised that this (2) will stress the community (e.g. more encysted species, lower species richness, and/or smaller species). These changes will ultimately impact on testate amoeba community composition and functional diversity, resulting in (3) changes in biogeochemical processes such as microbial hydrolytic enzyme activity.

## Materials and Methods

### *Study sites and sampling*

We selected four sites along an oceanic-continental gradient: Clara, Forbonnet, Kusowo and Store Mosse (Fig. 1). Clara (53°19'N, 07°37'W, 58 m. a.s.l) is an open bog situated in the Irish midlands. *Sphagnum magellanicum* was the most abundant peat moss species, whilst *S. cuspidatum*, *S. rubellum* and *S. fuscum* co-occurred. *Rhynchospora alba*, *Vaccinium oxycoccus*, *Andromeda polifolia* and *Menyanthes trifoliata* were the most common vascular plant species at the sampling location in Clara. Forbonnet is an ombrotrophic peatland situated in the Jura Mountains of France (46°49'35"N, 6°10'20"E, 840 m a.s.l). The moss carpet was co-dominated by *Sphagnum fallax* and *S. magellanicum* while the field layer was dominated by *Eriophorum vaginatum*, *A. polifolia*, *V. oxycoccus* and *Carex rostrata*. Store Mosse is an ombrotrophic peatland in the Store Mosse National Park, Southern Sweden (57°17'N, 14°00'E, 166 m a.s.l). *S. magellanicum* dominated the moss layer whereas. *E. vaginatum*, *Calluna vulgaris*, *A. polifolia*, *Empetrum nigrum*, *V. oxycoccus* and *Erica tetralix* dominated the vascular plant layer. Kusowkie Bagno (Kusowo) is a bog located in northern Poland (53°48'28" N, 16°35'14" E, 150 m a.s.l; Fig. 1) in a nature reserve and is part of the Special Area of Conservation Natura 2000 site "Lake Szczecineckie" (PLH 320009). The moss layer was dominated by *S. magellanicum* while *Drosera rotundifolia*, *V. oxycoccus*, *E. vaginatum* and *Carex limosa* characterized vascular plant vegetation.

Samples were collected within the same week in early-February 2014 as representative of mid-winter conditions (Fig. 1). In each site, we chose five homogeneous *Sphagnum magellanicum* patches (20 x 20 cm, separated at least 5 m from each other to take into account heterogeneity in the microbial communities (Mitchell et al., 2000)). *Sphagnum magellanicum* was chosen because it was the most common *Sphagnum* species in all the sites. Only *Sphagnum* patches with a vascular plant cover lower than 10% were chosen to avoid as much as possible any effect of vascular plants on microbial assemblages and processes (Robroek et al., 2015). We collected samples for testate amoebae, enzymatic activities and water-soluble phenols within the living portion of *Sphagnum magellanicum* (0-5 cm, including the capitula). Climatic variables (air and soil temperature, precipitation) were continuously monitored (every 30 minutes) on site at Forbonnet

(Forbonnet Scientific Research station) and Store Mosse (Store Mosse Research station). For Kusowo and Clara, we used climatic data from the closest meteorological stations (Szczecinek and Mullingard meteorological stations, respectively; data on a daily basis). In all the study sites, snow height was measured during sampling.

#### *Testate amoeba analyses and functional space calculations*

We collected 10 g of fresh *Sphagnum magellanicum* shoots (10 g f. wt, 0-5 cm from the capitulum) in each plot to analyse testate amoeba diversity and community composition. Samples were fixed in 20 mL glutaraldehyde (2% final concentration) immediately after sampling and stored at 4°C in dark conditions. Testate amoebae were extracted from fixed samples following the method described in Jassey et al. (2011b). Testate amoebae were identified at species level by inverted microscopy (x200 and x400; LEICA DMIL) following Utermöhl's method (Utermöhl, 1958). A minimum of 150 individuals was reached to achieve relevant species diversity (Payne and Mitchell, 2009). Living and encysted individuals were taken into account. Next, we compiled six functional traits (Table 1) to characterize the functional space (i.e. a multidimensional space where axes are functional traits along which species are placed according to their functional trait values; (Villéger et al., 2011) of each testate amoeba species: mixotrophy/heterotrophy, biovolume, biomass, body length, diameter of the pseudostome (shell aperture) and body range. Each trait was measured under the microscope for each sample to take into account species-trait plasticity, especially for body size measurements. The biomass was estimated by converting the total biovolume of each species (biovolume x density) into C using the standard conversion factor:  $1 \mu\text{m}^3 = 1.1 \times 10^{-7} \mu\text{gC}$  (Gilbert et al., 1998). Community Weighted Means (CWM) for each trait were calculated as abundance weighted means of species trait values (Garnier et al., 2007).

Per species, we constructed a functional space. We first created a functional distance matrix by applying Gower's distance on each pair of species described by their traits, and then computed a Principal Coordinate Analysis (PCoA) on it. Gower's distance allows mixing of different types of traits (i.e. qualitative and quantitative traits) while giving them equal weights. Then, the first four axes of the PCoA were selected as synthetic functional traits summarizing testate amoeba functional space, and species coordinates in the four-dimensional space defined by the

PCoA were used to calculate functional metrics as synthetic functional trait values (Villéger et al., 2008).

#### *Measuring taxonomic and functional alpha diversity of testate amoebae*

Testate amoeba taxonomic alpha diversity was calculated using Simpson's diversity index. Functional alpha diversity (FD) of communities was calculated using the Rao quadratic entropy (Q) index (Ricotta, 2005):

$$FD_Q = \sum_{i,j}^S d_{ij} p_i p_j$$

where  $d_{ij}$  is the functional difference between the  $i$ -th and  $j$ -th species ( $d_{ij} = d_{ji}$  and  $d_{ii} = 0$ ), and  $p_i$  and  $p_j$  the relative contribution of species  $i$  and  $j$  to the abundance of the whole testate amoebae community.  $d_{ij}$  was calculated as the Gower's distance on each pair of species described by their traits.  $FD_Q$  permits the measurement of the diversity of a given community by combining the species relative abundances described by their functional traits.  $FD_Q$  is unitless, and shows how communities are functionally different, i.e. increasing  $FD_Q$  values indicate functionally increasingly different communities.  $FD_Q$  was calculated in the R statistical environment (R Core Team, 2013) using the *FD* package (Villéger et al., 2008).

#### *Taxonomic and functional beta diversity in testate amoeba assemblages*

For each of the 190 pairs of testate amoeba communities, taxonomic and functional  $\beta$ -diversity and their turnover and nestedness-resultant components were computed following Villéger et al. (2013). Taxonomic  $\beta$ -diversity is defined as the percentage of dissimilarity in species composition between two communities; it equals 0 when two communities are identical in species composition and 1 when the two communities share no species (dissimilar communities). Taxonomic  $\beta$ -diversity can be influenced by species richness; if a certain community has a much larger number of species than another community, taxonomic  $\beta$ -diversity (dissimilarity) between these two communities is close to its maximum value (Baselga, 2010). Therefore, taxonomic  $\beta$ -diversity is defined as the sum of a turnover component (i.e. species replacement in the two communities) and a nestedness-resultant component (i.e. difference in number of species between the two communities compared). Taxonomic turnover and nestedness-resultant components also

vary between 0 and 1. Taxonomic turnover equals 0 when community A hosts the same subset of species than community B, while taxonomic nestedness equals zero when the two communities have the same number of species (more details can be found in Baselga (2010)).

As for taxonomic  $\beta$ -diversity, functional  $\beta$ -diversity equals 0 when two communities are functionally similar (same functional traits can be found; functionally similar communities) and it equals 1 when the two communities share different functional traits (functionally dissimilar communities). A high level of functional  $\beta$ -diversity can result from a high value of functional turnover (i.e. the communities host species with different functional strategies) or a low value of functional turnover (community A hosts a small subset of the functional strategies in community B), in this case the functional nestedness-resultant component is high (i.e. a community fills only a small portion of the functional space filled by the other community; for further details see Villéger et al. (2013)). Taxonomic and functional  $\beta$ -diversity and their respective turnover and nestedness-resultant components were performed in *R* with codes adapted from the *betapart R* package (Baselga and Orme, 2012).

#### *Enzymatic activities, water-soluble polyphenols and pore water analyses*

We used substrates labelled with the fluorophores 7-amino-4-methylcoumarin (MUC) or methylumbelliferone (MUB) to quantify the relative activity (i.e. enzyme activity under saturating substrate conditions) of enzymes responsible for the hydrolysis of one peptide (L-alanine-7-amido-MUC, Alanine), one carbohydrate (4-MUB- $\beta$ -glucopyranoside,  $\beta$ -glucosidase) and one phosphate ester (4-MUB-phosphate, phosphatase; all substrates supplied Sigma-Aldrich Switzerland) (Weedon et al., 2014). Enzymes were extracted following Jassey et al. (2011a) and analysed in microplates. For each sample, 4 pseudo-replicate wells were included in a 96 well microtiter plate. Assay wells received 38  $\mu$ L of extract and 250  $\mu$ L of enzyme substrate. For each sample, 4 pseudo-replicate wells containing 38  $\mu$ L of boiled extract (3h at 90°C) were performed as a control. Then microplates were incubated at 25°C for 3h. Fluorescence was monitored spectrophotometrically with an excitation wavelength of 365 nm and emission detection at 450 nm (Biotek, SynergyMX). All measurements were converted to nanomoles per gram dry weight per min ( $\text{nmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1} \text{ DM}$ ).

We quantified *Sphagnum* water-soluble phenolics as a potential environmental driver of testate amoeba communities. Indeed bryophytes, and especially their phenolic compounds, can be a major driver of testate amoeba community composition (Jassey et al., 2011b; 2014). For moss water-soluble phenolics, 0.05 g dry weight (DW) of *S. magellanicum* was grinded in a mortar, mixed with 10 mL distilled water, bubbled with nitrogen and agitated on a reciprocal shaker (150 rpm) for 3 hours and filtered (Jassey et al., 2011b). Water-soluble phenolics were quantified with the Folin-Ciocalteu reagent and were expressed in mg equivalent gallic acid ( $A_{760}$ ).

### *Statistical analyses*

We performed a redundancy analysis (RDA) to relate climatic variables (air and soil temperatures, precipitation, snow height) to testate amoeba species composition and CWM traits composition. The testate amoeba data set was preliminary Hellinger-transformed (Legendre and Gallagher, 2001) while CWM traits were standardized. Stepwise selection among the climate variables was computed using the 'ordistep' function available in the vegan R package (Oksanen, 2011). This stepwise selection was achieved using AIC (Akaike Information Criterion) and permutation tests. We assessed and tested the spatial dependence of testate amoeba community structure by means of direct multiscale ordination (MSO) applied on the results of the RDA. MSO is a geostatistical tool allowing for spatial partitioning of community variation among distance classes and testing of the spatial community structure (Wagner, 2004). Spatial independences were assessed by means of Mantel test (999 permutations). The 'grain' (i.e. interval size for distance classes) of the variogram was chosen as the truncation threshold (i.e. the longest distance in the minimum spanning tree of the spatial matrix) from a Principal Coordinates of Neighbourhood Matrix (PCNM) analysis applied on the site coordinates (Borcard et al., 2011).

Linear mixed effect models were used to determine whether testate amoeba species richness, diversity, biomass and  $FD_Q$  varied among sites (fixed effect) while accounting for repeated measurements within each site. All models included site as a random effect on the intercept, as to correct for the inflation of the residual degrees of freedom that would occur if we were using repeated measurements within sites as true replicates (Pineiro and Bates, 2000). Similar tests were used for enzymes and phenols. The correlation between taxonomic  $\beta$ -diversity

components and winter climatic dissimilarity between peatlands were tested using Mantel permutational tests. Similar tests were used with functional  $\beta$ -diversity components. Winter climatic dissimilarity was defined as the pairwise Euclidean distance between two peatlands in terms of minimum air temperature, mean winter air and soil temperatures, snow height and precipitation. All computations were performed in R 3.0.1 (R Core Team 2013).

## Results

### *Climatic conditions in the four peatlands*

Winter climatic conditions at Clara bog between first of January and early-February 2014 were very wet (301 mm) and relatively warm with mean air and soil temperature of 4.3°C (minimum air temperature = -4.3°C) and 4.7°C, respectively (Fig. 1). At Forbonnet, they were wet (184 mm) and relatively cold with mean air and soil temperatures of 1.7°C (minimum air temperature = -11.9°C) and 1.9°C, respectively. The moss layer was covered by 6 cm of snow, which had been present for several weeks (Fig. 1). In Store Mosse, the conditions were relatively wet (127 mm) and cold with mean air and soil temperatures of -0.5°C (minimum air temperature = -10.7°C) and -0.4°C, respectively. In Kusowo, winter climatic conditions were very dry (21 mm) and cold with mean air and soil temperatures of -1.3°C (minimum air temperature = -16.9°C) and -0.5°C, respectively. At sampling, *S. magellanicum* was covered by less than 1 cm of snow. Winter climatic dissimilarity analysis, using Kusowo as a reference for pairwise comparisons (highest longitude), showed that a gradient of winter climatic conditions paralleled the longitudinal gradient. Kusowo was closer to Store Mosse ( $d = 2.01$ ) than to Forbonnet ( $d = 3.79$ ) and Clara ( $d = 4.78$ ; Fig. 1). Differences in temperatures and amount of precipitation mainly explained such (dis)similarities (Fig. 1).

To test whether our climatic data recorded in each site from beginning of January to mid-February were representative of global winter climatic conditions, we compared these data to global climatic data available in the WorldClim database (Hijmans et al., 2005), i.e. minimum temperatures, mean winter temperature and precipitations. We found strong correlations between our data and those from WorldClim (minimum temperature:  $r = 0.91$ ,  $P < 0.001$ ; mean winter air

temperature:  $r = 0.94$ ,  $P < 0.001$ ; precipitation  $r = 0.80$ ,  $P < 0.001$ ), which validated our meteorological data as representative of winter climatic conditions for each site.

#### *Variations of water-soluble polyphenols in the different European peatlands*

Water-soluble phenolic compounds significantly differed along the gradient ( $F_{1,3} = 8.4$ ,  $P = 0.003$ ). Water-soluble phenolics showed lower values in Kusowo and Store Mosse and higher values in Forbonnet (Table 2). We further found that water-soluble phenolic compounds increased with rising temperatures, precipitation and snow cover depth (Supplementary Table 1).

#### *Relationships between testate amoeba communities and winter conditions*

Redundancy analysis (RDA) showed clear patterns of testate amoeba species composition along the gradient (Fig. 2A). The best RDA model selected four explanatory variables, which together explained 67.1% of the variance (adjusted  $R^2$ ). Analysis of the ordination space (first two axes were significant at  $P < 0.001$ ) showed that testate amoeba communities aggregated into three groups: Forbonnet, Kusowo and Store Mosse/Clara bogs. Forbonnet group was characterized by a domination of *Nebela* species (*N. tincta*, *N. collaris*, and *N. militaris*) while Kusowo group was dominated by *Heleopera sphagni*, *Hyalosphenia papilio* and *Phryganella* species (Fig. 2A). High abundance of *Archerella flavum* and *Assulina muscorum* characterized the Store Mosse/Clara group. These three groups were related to snow depth ( $F_{1,15} = 18.6$ ,  $P = 0.001$ ), precipitations (Wprec;  $F_{1,15} = 6.2$ ,  $P = 0.002$ ), minimum temperature (TMIN;  $F_{1,15} = 5.5$ ,  $P = 0.002$ ) and *Sphagnum* water-soluble phenolics (phenols;  $F_{1,15} = 12.5$ ,  $P = 0.001$ ). On the first RDA axis, the thicker snow cover and higher amount of phenolics in Forbonnet, as compared to the other bogs, explained the differences in testate amoeba species composition at Forbonnet. On the second axis, peatlands are spread along the temperature gradient, and to a lesser extend along the precipitation gradient (Fig. 2A). The variogram from the MSO (Fig. S1) showed no clear pattern of testate amoeba community structure with distance and no significant autocorrelation was found in any distance class. Species composition from a site was thus spatially independent from a neighbouring site (e.g. Sweden vs. Poland). The MSO also showed that species-environment

relationships were scale invariant and that gradients of temperature, precipitation and snow height were the main drivers of testate amoeba species composition.

In parallel to shifts in species composition, we also found clear patterns of CWM traits of testate amoebae using a redundancy analysis (Fig. 2B). The best RDA model selected two explanatory variables, which together explained 67.2% of the variance (adjusted  $R^2$ ). Along the first axis ( $F_{1,17} = 37.9$ ,  $P = 0.001$ ), CWM traits of testate amoeba communities aggregated into two groups: Forbonnet and Clara/Store Mosse/Kusowo. Snow cover in Forbonnet mainly explained this grouping ( $F_{1,17} = 33.9$ ,  $P = 0.001$ ). Large species (high body length, biovolume, biomass and pseudostome size) characterized the testate amoeba community at Forbonnet while smaller species were in the other bogs (Fig. 2B). In particular, we found that large species decreased by 68% with increasing exposure to frost (no snow and cold soil temperatures), while smaller species increased by 79%. The second axis ( $F_{1,17} = 3.1$ ,  $P = 0.02$ ) is characterized by the gradient of freezing temperature (TMIN;  $F_{1,17} = 7.1$ ,  $P = 0.004$ ), with in order Clara, Store Mosse/Forbonnet, and Kusowo. The gradient of temperature is strongly related to the body range and mixotrophy traits of testate amoebae (Fig. 2B). Furthermore, the proportion of active individuals (non-encysted individuals) strongly decreased with warmer air and soil temperatures or higher snow depth (Supplementary Table 2). The higher proportion of encysted specimens was found in Kusowo (84%) and Store Mosse (51%), and the lower in Forbonnet (22%) and Clara (31%).

#### *Species richness, species diversity, and functional diversity aspects of testate amoeba communities*

We identified 36 testate amoeba species in the four study sites. Testate amoeba species richness (N0) and Simpson's diversity (N1) were significantly higher in Forbonnet bog (N0 = 18 and N1 = 7.6, respectively) compared to the other bogs (on average N0 = 11, N1 = 3.7). Thicker snow cover in Clara, Kusowo and Store Mosse mostly explained such patterns (N0:  $r = 0.82$ ,  $P < 0.01$ ; N1:  $r = 0.64$ ,  $P < 0.01$ ). Taxonomic turnover contributed to 82% ( $\pm 14\%$ ) of taxonomic  $\beta$ -diversity (Fig. 3A x-axis), while nestedness-resultant component represented only 18% ( $\pm 16\%$ ; Fig. 3B x-axis). Furthermore, taxonomic  $\beta$ -diversity significantly increased with winter climatic dissimilarity (Mantel test,  $r = 0.64$ ,  $P < 0.01$ , Fig. 3C) due to taxonomic turnover rather than

taxonomic nestedness-resultant component. More specifically, we found that taxonomic turnover increased with winter climatic dissimilarity (Mantel test,  $r = 0.57$ ,  $P < 0.01$ , Fig. 3E) while taxonomic nestedness-resultant component not (Mantel test,  $r = -0.017$ ,  $P = 0.48$ ).

Testate amoeba functional alpha diversity was the highest in Clara bog ( $FD_Q = 5.2$ ), the lowest in Forbonnet ( $FD_Q = 4.0$ ) and intermediate in Kusowo ( $FD_Q = 4.5$ ) and Store Mosse ( $FD_Q = 4.3$ ;  $F_{1,3} = 20.1$ ,  $P < 0.001$ ). Pearson's correlation tests showed that changes in  $FD_Q$  were positively related to air and soil temperatures and negatively to snow depth (Supplementary Table 2). Functional turnover contributed on average to 62% ( $\pm 30\%$ ) of functional  $\beta$ -diversity (Fig. 3A y-axis), while functional nestedness-resultant component to 40% ( $\pm 35\%$ ; Fig. 3B y-axis). Functional  $\beta$ -diversity was significantly correlated to winter climatic dissimilarity (Mantel test,  $r = 0.57$ ,  $P < 0.01$ , Fig. 3D) and on the whole, was very high ( $0.74 \pm 0.02$ ). Increasing functional  $\beta$ -diversity was related to the response of its functional turnover component ( $0.46 \pm 0.024$ ), which increased winter climatic dissimilarity (Mantel test,  $r = 0.39$ ,  $P < 0.01$ , Fig. 3F). The response of its functional nestedness-resultant component ( $0.28 \pm 0.013$ ) was not related to winter dissimilarity (Mantel test,  $r = -0.04$ ,  $P = 0.58$ ).

#### *Relationships between testate amoeba functional diversity and microbial activity*

Only  $\beta$ -glucosidase activity responded to changing winter climatic conditions along the gradient ( $F_{1,3} = 4.1$ ,  $P = 0.03$ ).  $\beta$ -glucosidase activity was higher in Clara bog (mean  $1001 \pm 98$  nmol min<sup>-1</sup> g<sup>-1</sup> DM), lower in Kusowo (mean  $485 \pm 88$  nmol min<sup>-1</sup> g<sup>-1</sup> DM;  $F = 4.06$ ,  $P = 0.03$ ) and intermediate in Forbonnet ( $866 \pm 153$  nmol min<sup>-1</sup> g<sup>-1</sup> DM) and Store Mosse ( $811 \pm 80$  nmol min<sup>-1</sup> g<sup>-1</sup> DM). Here again, patterns of temperature, precipitation and snow cover depth mainly explained such changes in  $\beta$ -glucosidase activity (Supplementary Table 3). Alanine peptidase ( $F_{1,3} = 0.62$ ,  $P = 0.61$ ) and phosphatase ( $F_{1,3} = 1.83$ ,  $P = 0.19$ ) activities did not change among sites (alanine peptidase: mean =  $694 \pm 50$  nmol min<sup>-1</sup> g<sup>-1</sup> DM, phosphatase: mean =  $4092 \pm 377$  nmol min<sup>-1</sup> g<sup>-1</sup> DM; Table 2).

We further found a strong and significant positive relationship between functional  $\beta$ -diversity of testate amoeba communities and  $\beta$ -glucosidase activity ( $R^2 = 0.95$ ,  $P < 0.01$ ), while no relationship was found between testate amoeba functional alpha diversity and  $\beta$ -glucosidase activity ( $R^2 = 0.10$ ,  $P = 0.68$ ; Fig. 4). Further analyses on traits variation revealed that shifts in the

abundance of mixotrophs mainly explained the link between functional  $\beta$ -diversity of testate amoebae and  $\beta$ -glucosidase activity ( $R^2 = 0.79$ ,  $F_{1,2} = 12.7$ ,  $P = 0.07$ ; Fig. 4B), while traits related to body characteristics had no significant influence ( $R^2 < 0.1$  and  $P > 0.7$  for all). No relationships were found between taxonomic alpha and  $\beta$ -diversity, neither with the activities of the other enzymes nor with functional alpha and  $\beta$ -diversity.

## Discussion

### *Winter climate strongly influences testate amoeba communities*

Deep soil freezing, fluctuations in soil moisture and exposure to strong frost events have been shown to significantly, but inconsistently, influence soil biological communities. Microfauna is usually highly sensitive to freezing soil temperatures because many taxa have no tolerance to freezing (Sømme, 1982; Cannon and Block, 1988; Konestabo et al., 2007). However, some studies also found no significant change in biomass, abundance and/or diversity of microfauna (Bokhorst et al., 2012). Furthermore, and despite these inconsistencies, shifts in soil microfauna communities in response to winter climate change have been poorly quantified at a functional level (but see Bokhorst et al., 2012). Here we show that freezing of the soil surface, exposure to frost (no snow cover) and lower precipitation led to high taxonomic and functional turnover in testate amoeba communities. This result indicates that species replacement in testate amoeba communities along the climatic gradient is mostly between species that are not functionally redundant (Villéger et al., 2013). We found that the presence of snow and/or mean soil temperatures higher than zero is crucial to maintain a high biomass and high functional diversity in testate amoeba communities. This corroborates previous findings of a maintenance or even an increase of testate amoeba density during long period of snow cover (Lousier and Parkinson, 1984). Interestingly, our results are opposite to recent observations in arctic tundra, where low density of active testate amoebae were found under a snow cover (Tsyganov et al., 2012). Snow in arctic tundra often establishes upon an already frozen soil, so considerably limiting the activity of soil protozoa during winter (Laminger, 1978), which probably explains such inconsistency. In accordance with our hypothesis, testate amoebae showed clear signs of stress with freezing conditions. Decreasing snow depth, low precipitations, and frozen soil strongly reduced testate

amoeba activity by favouring cyst formation (+50%, mostly large species). Freezing conditions also reduced the mean body size of the community (e.g. lower body length, biovolume and body range), as previously showed for Collembola (Bokhorst et al., 2012). Although testate amoebae are known to maintain metabolic activity with soil temperatures close to zero (Lousier and Parkinson, 1984), our results showed that freezing temperatures are highly limiting for large species activity and/or survival (e.g. *Nebela* sp., *Heleopera* sp. and *Hyalosphenia* sp.), while smaller species (e.g. *Archerella flavum* and *Assulina muscorum*) remain active and even replace the larger species. These results would indicate that the efficiency of energy transfer from lower to higher trophic levels required to maintain large individuals declined with frost and the decrease of large testate amoebae could be indirectly attributed to a decrease of their preys. However, we did not observe any change in the biomass of bacteria and fungi (Fig. S2), suggesting that changes in winter conditions had a direct effect on large testate amoebae.

In parallel to changes in winter climatic variables, our results revealed that moss chemistry (phenolics) also played a significant role in driving testate amoeba assemblages. While the production of phenolics is considered as an advantageous adaptive mechanism for plant protection against low temperatures (Dixon and Paiva, 1995; Martz et al., 2009), concentration of water-soluble phenolics decreased with low snow depth and low temperatures. Such contrasting results could either come from the phenolic compounds we targeted (i.e. water-soluble compounds instead of cell-bounded compounds) or from a negative effect of extracellular formation of ice on the phenolic metabolism of *Sphagnum* (Solecka and Kacperska, 2003). Reduced water-soluble phenolics were related to lower testate amoeba biomass but also to the lower mean size of the community (e.g. lower body length, pseudostome size and biovolume). This suggests that decreasing phenolic concentrations with decreasing temperatures can alter microbial assemblages in peatlands, as showed with summer warming (Jassey et al., 2013a), and even select for specific microbial functional groups (Hättenschwiler and Vitousek, 2000). These results are consistent with previous findings from tundra soils reporting shifts in phenolic-microbial interactions in freezing soils (Schmidt and Lipson, 2004). Overall, our findings showed that exposure to harder frost events significantly influence the forces structuring microbial communities in peatlands. Such community response is expected to have significant consequences on ecosystem functioning as suggested by

the shifts in functional diversity.

### *Ecosystem consequences*

Our enzymatic assays partly confirmed previous observations that freezing reduces microbial activity in carbon-rich soils (Hartley et al., 2008). Temperature and precipitation are among the key factors controlling microbial activity involved in C cycling in peatlands (Bragazza et al., 2013), and our results support these findings. However, our results also support the hypothesis that such decreases in microbial activity are the result of functional shifts within higher trophic level, i.e. the testate amoebae. As showed for plant functional diversity (Robroek et al., 2015; Steinauer et al., 2015), our findings revealed that  $\beta$ -glucosidase enzymatic activity strongly depended of functional composition and turnover of testate amoebae. This indicates that species replacement within testate amoeba community along the climatic gradient was accompanied by a functional replacement, which strongly determines microbial activity involved in C cycle. This result is particularly significant given the shifts in CWM traits of testate amoebae. Indeed, we found that exposure to harder frost events and reduced precipitation was accompanied by a replacement of large testate amoeba species by the small-body mixotrophic species *Archerella flavum*.

Increasing abundance of mixotrophic species can potentially affect C dynamics of ecosystems (Mitra et al., 2013). As phototrophs, mixotrophic testate amoebae can contribute to photosynthetic C fixation (Jassey et al., 2015), whilst as bacterial/fungal grazers (Jassey et al., 2013b) they can influence organic matter decomposition by controlling bacterial and fungal populations. Hence, higher mixotroph densities can either increase C uptake or C release in peatlands, depending on the relative contribution of phototrophy and heterotrophy in their feeding behaviour. As supported by stable isotope analyses (Jassey et al., 2013b; Mieczan et al., 2015a), mixotrophic testate amoebae mainly acquire their total C through photosynthesis. Consequently, a decrease of the dominance of mixotrophic testate amoebae means that less C is stored by the system and more space is available for strict bacterivores and/or fungivores in the community, as observed in Clara bog with the high abundances of *Phryganella* sps. and *Hyalosphenia elegans* species. Intensified bacterial grazing by protists is known to enhance microbial turnover (Bonkowski, 2004), leading to an overall increased microbial activity (Jílková et al., 2015), which can then cascade to

heterotrophic respiration and nutrient cycling (Petersen and Luxton, 1982). The link we found between the functional diversity of testate amoebae and  $\beta$ -glucosidase activity tends to support such hypothesis and suggest that the shift in the functional structure of testate amoebae promotes microbial activity involved in C cycle. Further experimental work is however needed to fully distinguish whether changes in climatic conditions directly impact microbial activity or indirectly affect activity by shifting species composition.

With current winter climate warming, snow cover will continue to become thinner, and of shorter duration, increasing the exposure of soils to harder frost events. Our findings indicate that exposure to hard soil frost negatively impacts on testate amoeba function and, consequently, on soil microbial activity. Considering that freeze/thaw cycles are expected to increase in the coming decades (Groffman et al., 2001), the associated effects on microbial activity could be rather important for the overall C balance in peatlands (Robroek et al., 2013). Understanding the mechanistic links that shape thermal tolerance of large individuals clearly needs further experimentation and would greatly improve our ability to predict species response to climate changes, and ecosystem processes.

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## Figure captions

**Figure 1:** Map of Europe showing the location of the four peatlands and the magnitude of main climatic variables. Forb: Forbonnet; Kus: Kusowo; SM: Store Mosse. Dot size represents the winter dissimilarity among sites with Kusowo as a reference (highest longitude). Minimum air temperature, mean winter air and soil temperatures, precipitations and snow height plotted against longitude (mean  $\pm$  SD; data from beginning of January to mid-February).

**Figure 2:** (A) Redundancy analysis biplot of Hellinger-transformed testate amoeba communities constrained by snow cover depth, minimum winter air temperature (TMIN), precipitations during winter (Wprec) and water-soluble polyphenols (phenols). Only species with abundance  $> 3\%$  are shown. (B) Redundancy analysis biplot of standardized CWM traits of testate amoebae constrained by snow cover depth and minimum winter air temperature (TMIN). All axes are significant at  $** P < 0.01$  and all explanatory variables were significant at  $P < 0.05$  (permutation tests). Species abbreviations: aflu: *Archerella flavum*; amusc: *Assulina muscorum*; asem: *Assulina seminulum*; cenlae: *Centropyx laevigata*; cordub: *Corythion dubium*; cryovi: *Cryptodifflugia oviformis* type; eugcomp: *Euglypha compressa*; eugstri: *Euglypha strigosa*; helpet: *Heleopera petricola*; helros: *Heleopera rosea*; helsph: *Heleopera sphagni*; helsyl: *Heleopera sylvatica*; hyael: *Hyalosphenia elegans*; hyapap: *Hyalosphenia papilio*; nebcoll: *Nebela collaris*; nebflab: *Nebela flabellulum*; nebmil: *Nebela militaris*; nebtin: *Nebela tinctoria*; phryacro: *Phryganella acropodia*; phrydiss: *Phryganella dissimulatrix* type.

**Figure 3:** Relative contribution of turnover (A) and nestedness-resultant (B) components to taxonomic and functional  $\beta$ -diversity.  $r$  is the correlation coefficient from linear model. Response of  $\beta$ -diversity (community dissimilarity) (C) and functional  $\beta$ -diversity (functional dissimilarity) (D), and their related turnover components (E, F), to changing winter conditions (winter conditions dissimilarity).  $r$  is the Pearson's coefficient from Mantel tests.

**Figure 4:** Relationship between functional alpha (A) and beta (B) diversity of testate amoebae and  $\beta$ -glucosidase enzymatic activity (mean  $\pm$  SEM) in the four European peat bogs. Dot size on lower plot show the average abundance of mixotrophic testate amoebae.  $R^2$  is adjusted R-squared from linear models.

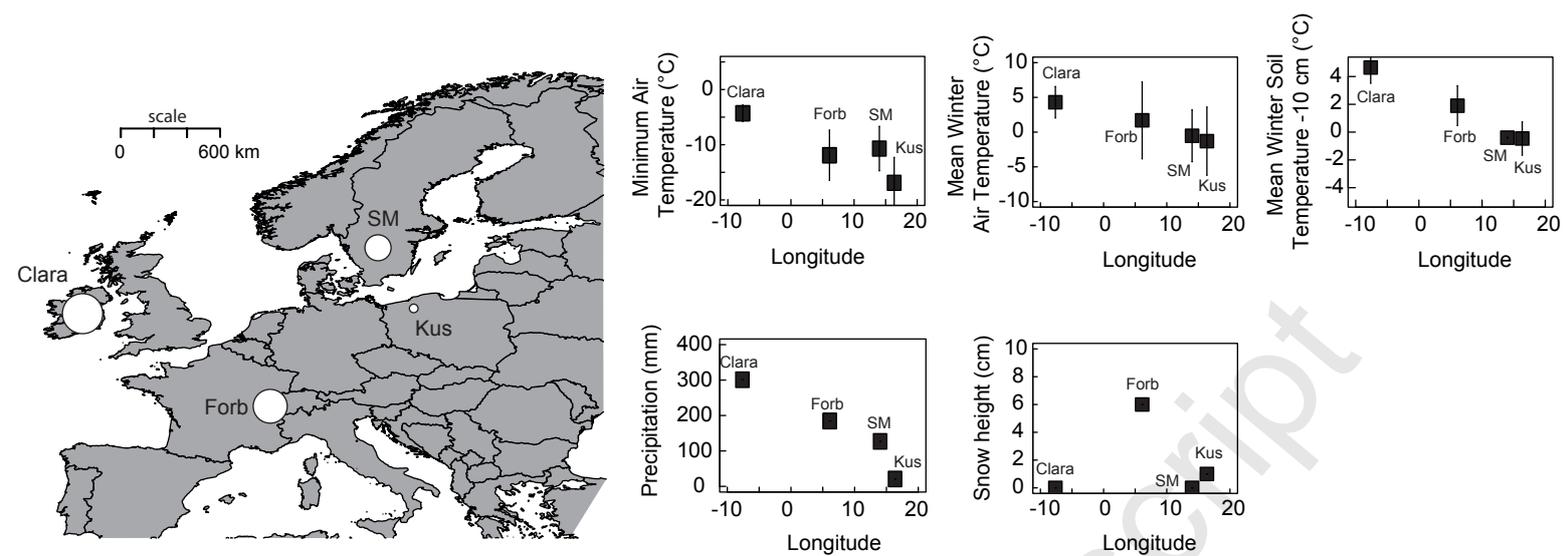
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**Table 1:** Key ecological traits of testate amoebae and related hypotheses on how they might respond to winter climate

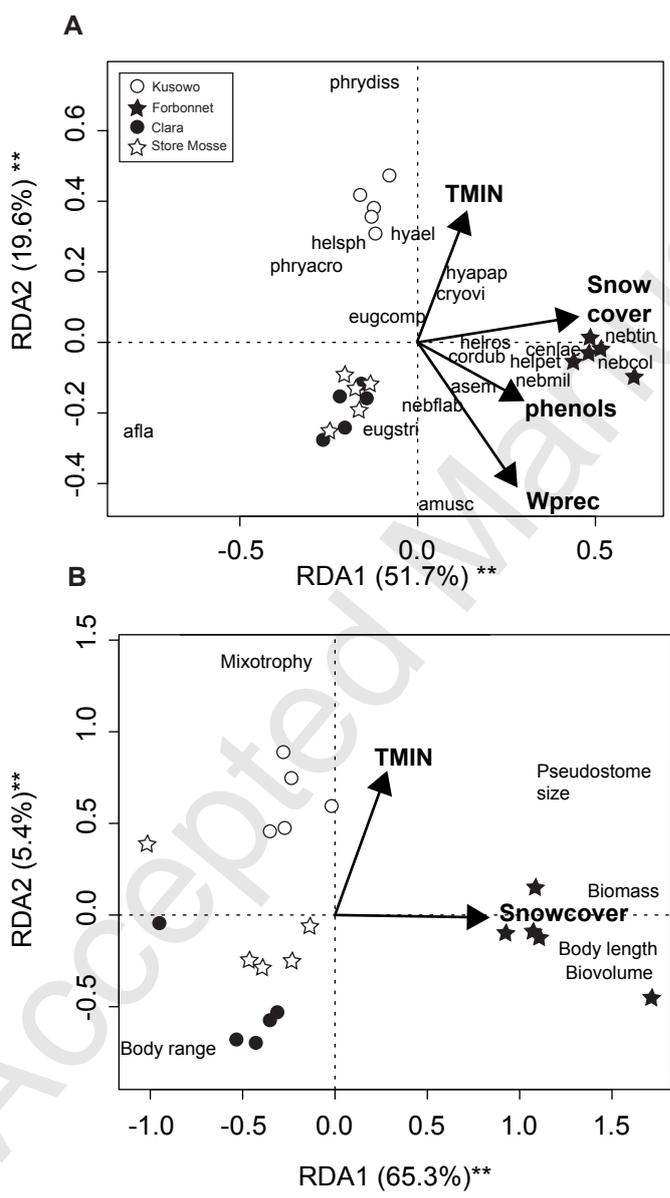
Trait	Description	Potential processes	Function	References
Mixotrophy	Whether a species is heterotroph or mixotroph (combination of phototrophy and heterotrophy)	Phototrophic metabolism is damaged by low temperatures, decreasing the abundance of mixotrophs	Contribution to photosynthetic C fixation of <i>Sphagnum</i> mosses and bacterial grazing	(Jassey et al., 2015)
Biovolume	Volume of a species in $\mu\text{m}^3$ (continuous trait)	Large species might be more represented in colder area because snow cover is likely to protect them from frost	Size and mass of species affects the manner in which they interact with their resources. Large-bodied specimens usually have higher grazing rates; they graze on wider size range of food items and therefore have greater top-down effects on resources than smaller species.	(Carpenter and Kitchell, 1988; Jassey et al., 2013b; 2012)
Biomass	Mass of species in $\mu\text{g C.g}^{-1}\text{ DM}$ (continuous trait)			
Body length	Length of species in $\mu\text{m}$ (continuous trait)			
Body range	(body length species x – maximum body length within the community) / minimum body length within the community			
Pseudostome size (shell aperture size)	Size of the pseudostome in $\mu\text{m}$ (continuous trait)	Two species with equal body length but different pseudostome size may differently respond to frost	Species with large pseudostome are likely to feed on a wider size range of species and therefore have greater top-down effects on resources than smaller species.	(Jassey et al., 2013b)

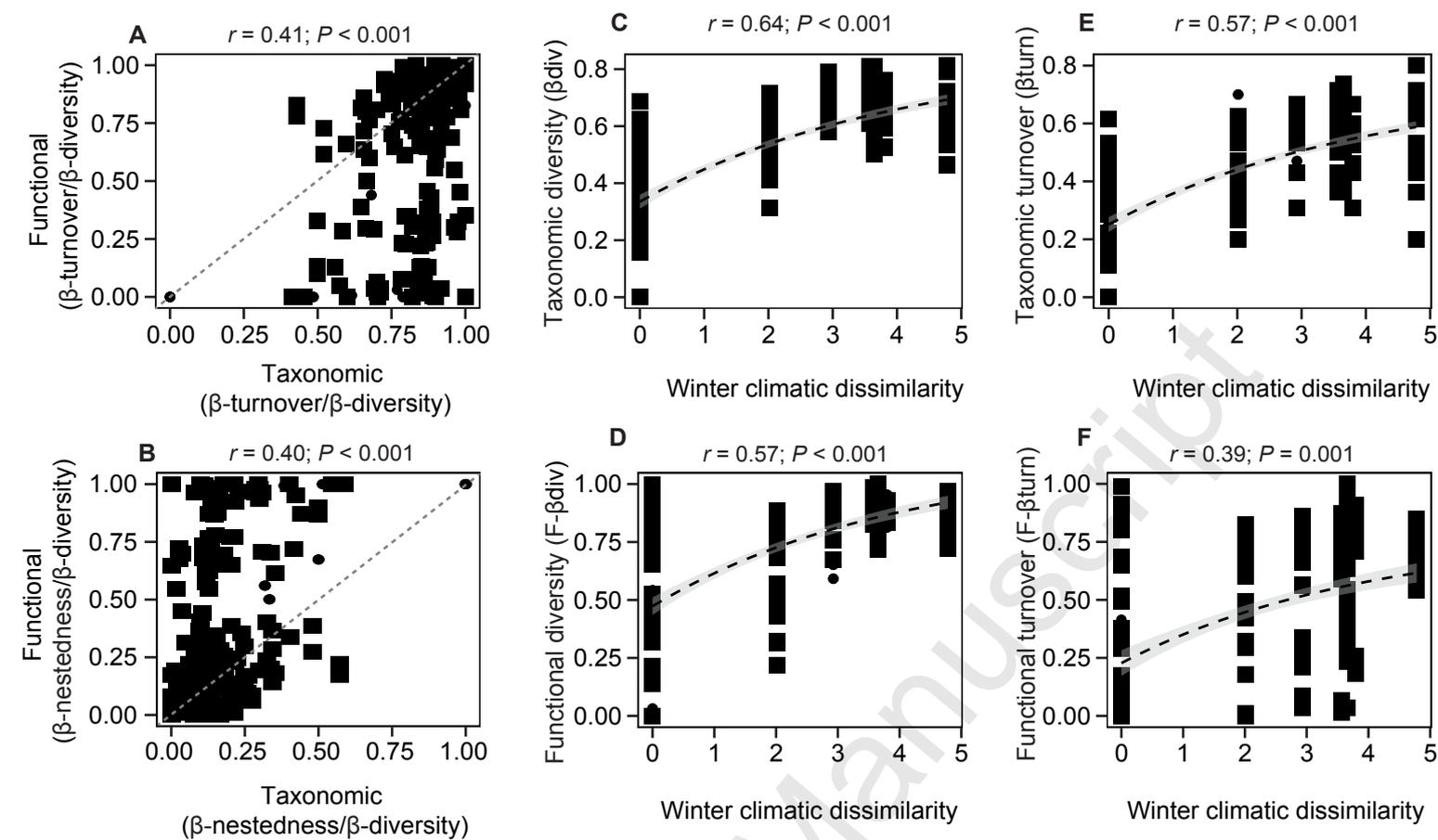
**Table 2:** Mean ( $\pm$  SE) of water-soluble phenolic compounds of *Sphagnum magellanicum* at the four peatlands. Mean ( $\pm$  SE) of potential activity of  $\beta$ -glucosidase, alanine peptidase and phosphatase in the different peatlands. Letters indicate significant differences among sites ( $P < 0.05$ , ANOVA); N = 5 per site.

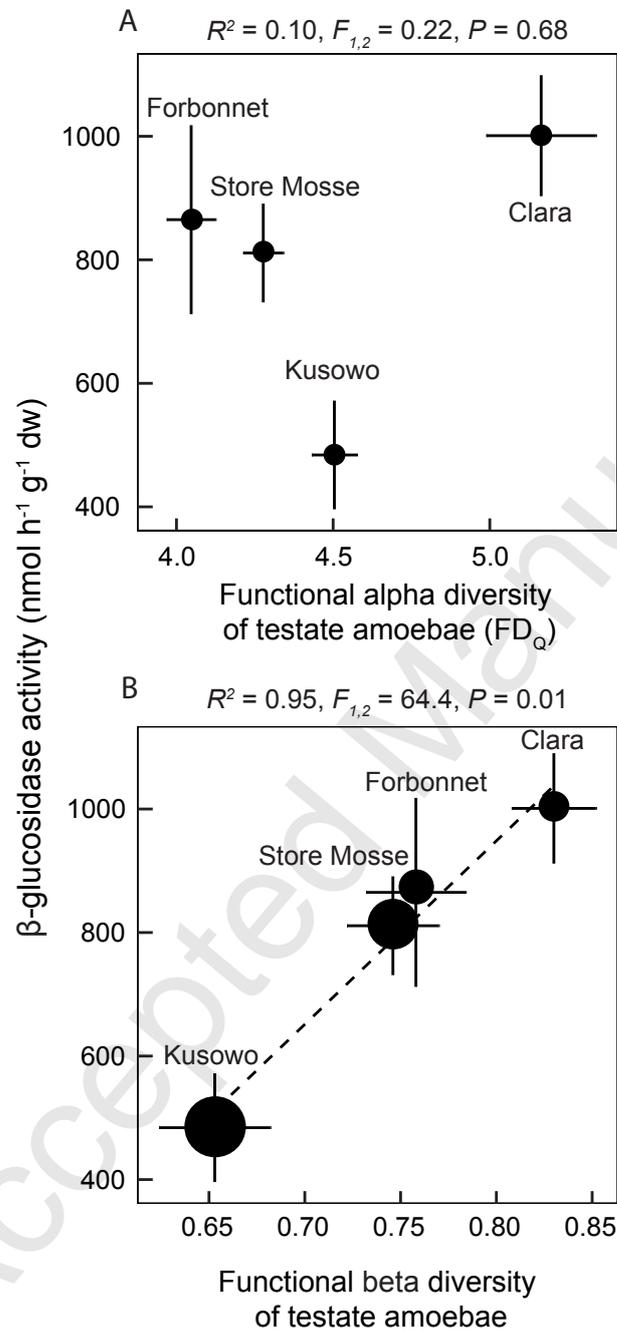
	Clara	Forbonnet	Kusowo	Store Mosse
<b><i>Sphagnum</i> chemistry</b>				
Phenols (mg.g <sup>-1</sup> dw)	1.08 (0.03) ab	1.24 (0.09) b	0.90 (0.05) a	0.90 (0.05) a
<b>Enzymatic activities</b>				
$\beta$ -glucosidase (nmol.h <sup>-1</sup> .g <sup>-1</sup> dw)	1001 (98) c	865 (153) b	484 (88) a	811 (80) b
Alanine peptidase (nmol.h <sup>-1</sup> .g <sup>-1</sup> dw)	716 (104) a	793 (151) a	666 (81) a	600 (51) a
Phosphatase (nmol.h <sup>-1</sup> .g <sup>-1</sup> dw)	4642 (677) a	3586 (924) a	3214 (770) a	4926 (538) a



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Trait	Description	Potential processes	Function	References
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Biovolume	Volume of a species in $\mu\text{m}^3$ (continuous trait)	Large species might be more represented in colder area because snow cover is likely to protect them from frost	Size and mass of species affects the manner in which they interact with their resources. Large-bodied specimens usually have higher grazing rates; they graze on wider size range of food items and therefore have greater top-down effects on resources than smaller species.	(Carpenter and Kitchell, 1988; Jassey et al., 2013b; 2012)
Biomass	Mass of species in $\mu\text{g C.g}^{-1}\text{DM}$ (continuous trait)			
Body length	Length of species in $\mu\text{m}$ (continuous trait)			
Body range	(body length species $\times$ – maximum body length within the community) / minimum body length within the community	Large species might be more represented in coldest testate amoeba communities, thus dominating the community		
Pseudostome size (shell aperture size)	Size of the pseudostome in $\mu\text{m}$ (continuous trait)	Two species with equal body length but different pseudostome size may differently respond to frost	Species with large pseudostome are likely to feed on a wider size range of species and therefore have greater top-down effects on resources than smaller species.	(Jassey et al., 2013b)

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