SOIL MICROBIOLOGY

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Small-scale Variation of Testate Amoeba Assemblages: the Effect of Site Heterogeneity and Empty Shell Inclusion

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Abstract

Studies on testate amoeba species distribution at small scales (i.e., single peatland sites) are rare and mostly focus on bogs or mineral-poor *Sphagnum* fens, leaving spatial patterns within mineral-rich fens completely unexplored. In this study, two mineral-rich fen sites of contrasting groundwater chemistry and moss layer composition were selected for the analysis of testate amoeba compositional variance within a single site. At each study site, samples from 20 randomly chosen moss-dominated plots were collected with several environmental variables being measured at each sampling spot. We also distinguished between empty shells and living individuals to evaluate the effect of empty shell inclusion on recorded species distribution. At the heterogeneous-rich *Sphagnum*-fen, a clear composition turnover in testate amoebae between *Sphagnum*-dominated and brown moss-dominated samples was closely related to water pH, temperature and redox potential. We also found notable species composition variance within the homogeneous calcareous fen, yet it was not as high as for the former site and the likely drivers of community assembly remained unidentified. The exclusion of empty shells provided more accurate data on species distribution as well as their relationship with some environmental variables, particularly moisture. Small-scale variability in species composition of communities seems to be a worthwhile aspect in testate amoeba research and should be considered in future sampling strategies along with a possible empty shell bias for more precise understanding of testate amoeba ecology and paleoecology.

Keywords Testate amoebae · Fens · Peatlands · Empty shells · Small-scale variability

Introduction

There is a growing body of literature on the ecology of testate amoebae in peatlands, covering broad range of habitat types worldwide and exploring diversity patterns at spatial scales from 40×60 cm *Sphagnum* carpet [1] to whole mountain ranges [2]. The majority of studies, however, explored testate amoeba communities on regional scales [3–8]. Conversely, spatial variability of testate amoebae within single sites is poorly known and studies dealing with species distribution and composition variation in this context are focused

primarily on the *Sphagnum* microforms in bogs [9–11]. However, such knowledge might be of consequence for both paleoecological interpretation and overall understanding of the ecology of individual species and communities over larger scales. Also, apart from being scarce, existing studies on small-scale variability in testate amoeba assemblages remain limited to *Sphagnum*-dominated habitats such as bogs and acidic (mineral poor) fens [1, 12], resulting in the complete lack of information on this subject from more mineral-rich fens.

Unlike in bogs with a distinct and stable microtopography, water table in more alkaline fens with tighter relationship to groundwater supply tends to be more dynamic over the season. Combined with local changes in mineral richness and more diversified vegetation [13], these fen types often display remarkable variability of conditions within the surface moss layer. Despite this apparent heterogeneity, studies concerning testate amoebae in alkaline fens were primarily focused on community changes along the poor-rich gradient [5, 6, 8, 14] with little regard for species distribution on finer spatial scales. Nonetheless, more detailed studies on fens might be

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valuable, as they are known to harbor remarkable testate amoeba diversity [5, 14], but also due to the endangered status of these habitats, which became very rare across the globe [15, 16].

In this study, we selected two model fen sites of contrasting small-scale heterogeneity in terms of mineral richness and vegetation composition to explore the compositional variance of testate amoebae across two 20-m transects. We hypothesized that the variance of testate amoeba communities would reflect small scale environmental variation and we expected a higher species turnover among more environmentally distinct plots. As microorganisms have short generation times and potentially a high dispersal ability, we also decided to take into consideration the proportion of empty shells in our samples to obtain more realistic information on community structure and dynamics within our study sites. Shells without protoplasm accumulate in the uppermost parts of moss layers due to mortality caused by unfavorable change of conditions, predation [17], reproduction [18, 19], and passive dispersal caused by wind [20, 21] or animal/human activity [22]. Despite their likely potential to bias species composition and response to environmental factors, their effect has never been properly addressed in ecological studies. Empty shells have been regularly included into community description [9, 23–25] and preferential use of living individuals is applied quite marginally, mostly in experimental studies [26–29] and less in the field studies focused on the community ecology [12, 17, 30, 31]. Here, we specifically aim to evaluate the effect of empty shells on species composition of the surface samples and to find out whether and to which extent their exclusion modifies species correlation to measured environmental variables. This topic seems to be of unexplored nature but with potential implications for future testate amoeba research.

Material and Methods

### Study Site, Sample Collection, and Processing

The sites chosen for sample collection were two minerotrophic mires located in the Western Carpathian flysch zone (Fig. 1). The first site was a small calcareous spring fen near the village of Jasenka in Hostýn-Vsetín Mts., Czech Republic (49° 22′ 4″ N, 18° 01′ 2″ E), characterized by a strong tufa (i.e., calcium carbonate) precipitation caused by extreme mineral-rich groundwater supply. The vegetation composition of the site belongs to the *Caricion davallianae* alliance and the moss layer is formed exclusively by brown mosses with the overall appearance being rather homogeneous. The second sampling site was a mineral-rich *Sphagnum* fen in the Obidová Nature Reserve (Moravskoslezské Beskydy Mts., Czech Republic; 49° 31′

02″ N, 18° 31′ 26″ E). Central European rich (*Sphagnum-*)fens are characterized by vegetation of the *Sphagno warnstorfii-Tomentypnion nitensis* alliance and have been known to host some of the richest plant and moss fen communities [13, 16]. The moss layer is quite heterogeneous, compared to the first site, consisting of a spatial matrix of brown mosses and calcium-tolerant *Sphagnum* species which form a variety of microhabitats with diverse biotic and abiotic conditions. The study sites will be referred to as the "homogeneous fen" and the "heterogeneous fen" throughout the text.

To explore the species distribution of testate amoebae at the studied fens, 20 moss tufts were collected at each site. The area for sample collection was chosen to cover most of the microhabitat diversity of the fens and was delimited by two perpendicular (i.e., crossing in the middle) transects of 20 m each. Random selection of sampling plots with minimal subjective influence of the collectors was achieved by using a wooden frame (1 × 1 m) divided into a 10 × 10 cm grid, which was placed in a zigzag manner along each transect using 2 m spacing. The coordinates of a square section on the grid were then randomly generated and at the respective spot living parts of moss stems were sampled. Prior to sample collection, moisture conditions at each sampling spot were evaluated using a semi-quantitative scale (1, dry; 2, moderately wet; 3, wet; and 4, submerged). After sampling pH, conductivity, redox potential, and water temperature were measured using a portable instrument (HACH HQ40d). For further details on the environmental variables and moss species sampled see Online Resource (Table 3).

In the laboratory, the collected moss stems were washed with distilled water, the suspension was filtered through a 250-μm sieve and fixed with formaldehyde. The moss stems were dried and kept for further identification (for full species list see Online Resource, Table 4). For better distinction between empty shells and living individuals at the time of sampling, 1 ml of 0.2% Rose Bengal stain solution was added into 10 ml of sample suspension. Samples were then left to stain for 2 weeks following the FOBIMO protocol [32]. Testate amoebae were identified under × 200 and × 400 magnification and empty and living shells were tallied separately throughout the scanning. For each sample, two species datasets consisting of 150 individuals were obtained. The first dataset labeled as "ALL" included all shells regardless of being empty or alive. The second dataset labeled as "LIVING" included only shells with protoplasm (i.e., active as well as encysted). No more than 50 shells were examined per slide, so that the community description was always based on at least three replicates. For testate amoeba identification, multiple identification keys were used [19, 33–38] along with the *Microworld* web pages [39]. The species-level identification of individuals was based on shell morphology. The nomenclature follows [19, 40], with some critical taxa being pooled and labeled as "type" (see Online Resource, Tables 1 and 2).

Fig. 1 Location and photographs of the study sites. 1) The homogenous fen: a calcareous spring fen near the village of Jasenka in Hostýn-Vsetín Mts., Czech Republic. 2) The heterogeneous fen: a mineral-rich *Sphagnum* fen in the Obidová Nature Reserve in Moravskoslezské Beskydy Mts., Czech Republic
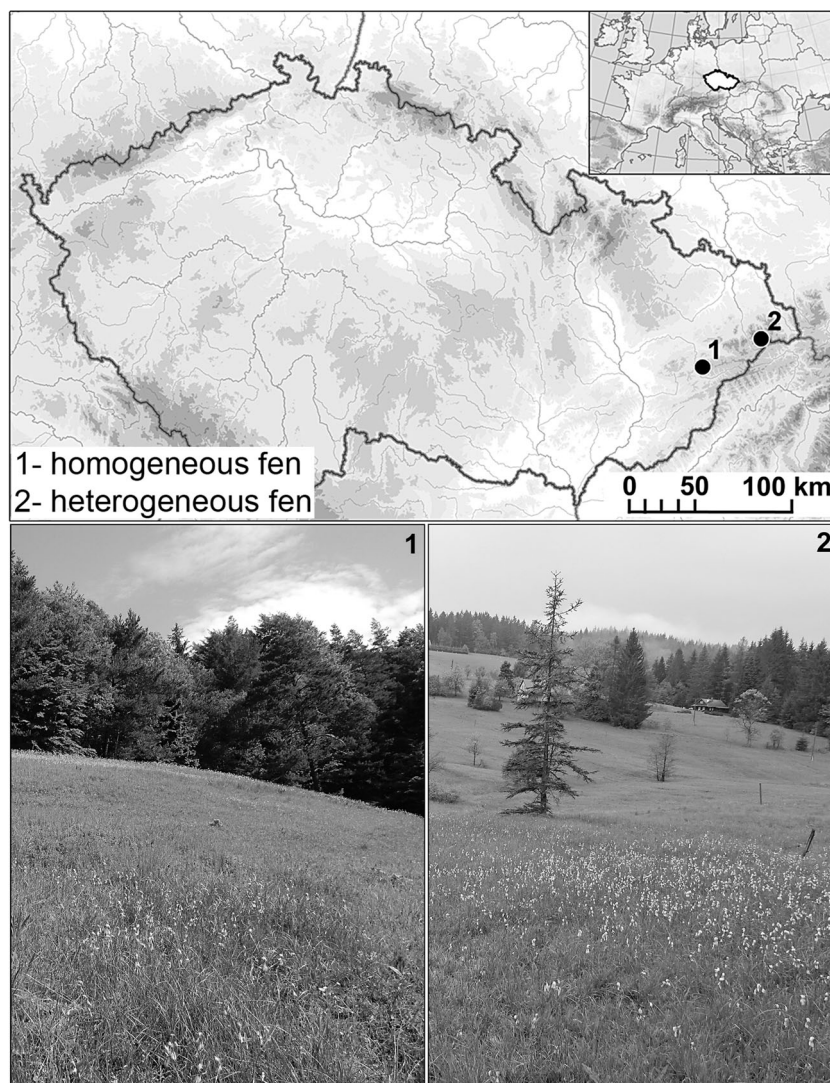
Data Analyses

Although 20 samples were taken at each of the studied fens, in case of the heterogeneous fen, only 19 samples were included in the analyses due to the loss of one sample during processing. All data analyses were performed pairwise for the two fen types as well as the "ALL" and "LIVING" species data sets. Samples from the heterogeneous fen were further sorted based on the two functional groups of mosses present. To test the effect of moss type, the *Sphagnum*/brown moss ratio was assessed from the rinsed moss tufts and scored on the following scale: 0%, $1 \leq 30\%$, $31 \leq 70\%$, and $71 \leq 100\%$ of *Sphagnum* stems. The threshold of 30% *Sphagnum* proportion was further adopted for the distinction between *Sphagnum*- and brown moss-dominated samples. For a comparison between groups of samples, Mann-Whitney U test or Wilcoxon signed-rank test were used. All analyses were performed in R software [41] using the "vegan" [42] and "packfor" [43] packages.

To explore the differences in species composition among samples, we employed distance-based multivariate techniques. In order to reduce the influence of dominant taxa, the species data were log transformed and Bray-Curtis dissimilarity index was applied. For both sampled fens, testate amoeba assemblages were displayed using two-dimensional NMDS ordination analysis and environmental variables measured at each sampling spot were projected onto the resulting ordination diagrams. The variables were fitted using the regression-based "envfit" function and the significance was tested with 4999 permutations. To quantify the changes in community composition after the exclusion of empty shells, we opted for the PCoA based "betadisper" method, so that the distances between samples and their group centroids could be compared between the "ALL" and "LIVING" data sets.

Redundancy analysis (RDA) was used to quantify the correlation between environmental variables and species distribution. Water pH, temperature, conductivity, redox

potential, and moisture at the sampling spot were included in the analyses at both study sites. In case of the heterogeneous fen, Sjörs's correction for conductivity was applied due to low pH values [44], and the proportion of *Sphagnum* stems was added as the sixth variable. All variables were tested together in one model as well as separately. The significance of results was assessed using permutation test with 4999 runs. The explained variability values were always converted into adjusted R^2 and the resulting values were further used as the threshold in the forward selection process.

Results

Species Diversity and Live/Empty Shell Ratio

Overall, 64 testate amoeba taxa were identified, with 40 species found at the homogeneous fen and 55 species at the heterogeneous fen (for complete species lists see Online Resource Tables 1 and 2). About a half of all the species (31) were shared. For both sites, the number of species did not differ between "ALL" and "LIVING" data sets, with 40 and 39 species at the homogeneous fen, and 52 at the heterogeneous fen. The number of species per sample was rather variable, ranging between 16 and 27 species at the homogeneous and 9 and 33 species at the heterogeneous fen. The change in number of species after empty shell exclusion was significant mostly at the homogeneous fen where the number of species decreased by two species per sample on average (Table 1 and Fig. 2a). At the heterogeneous site, the average number of species per sample differed substantially between *Sphagnum*- and brown moss-dominated patches (14 ± 2.8 vs. 25 ± 4.5, see Table 1). In the case of *Sphagnum* samples, the difference between "ALL" and "LIVING" data sets was also significant (Fig. 2b). In general, brown mosses displayed higher species richness, but the proportion of living individuals in these samples was notably lower as compared to *Sphagnum* samples (Fig. 3).

## Community Composition and Environmental Variables

The variability in species composition at both sampling sites was explored by NMDS and PCoA ordination plots (Figs. 4 and 5). Despite the macroscopic homogeneity of the calcareous fen, species distribution at this site was spatially variable and this variance significantly increased after empty shell exclusion (see Figs. 5a, b and 6). However, it was still significantly lower than at the heterogeneous site (MW U test, $p < 0.001$, not shown in the figures). At the heterogeneous fen, the clear distinction between *Sphagnum* and brown moss samples was found, although assemblages also varied notably within each of the two moss groups. Unlike in the homogeneous fen, the shifts in species composition between the "ALL" and "LIVING" data sets did not prove significant for this sampling site (Figs. 5c, d and 6).

Although species composition at the homogeneous fen was clearly not uniform among samples, we failed to detect any likely drivers for these compositional changes. While the projection of explanatory variables in the NMDS diagrams suggested a correlation with conductivity and moisture for the "ALL" and "LIVING" data set respectively (Fig. 4a, b; Table 2), RDA results for this site were not significant (Table 3). In contrast, variables tested in the RDA explained approximately 24% of the variance at the heterogeneous fen (Table 3), and when tested individually four variables were significant in "ALL" data set and five variables in "LIVING" data set (Table 4). In the RDA with forward selection, the proportion of *Sphagnum* in the sampled patches was found to be the principal predictor of testate amoeba community composition (Table 4) and was also strongly correlated with other environmental variables such as pH, redox potential, and temperature (Table 4, Fig. 4c, d). Total variability explained by environmental variables was comparable in "ALL" and "LIVING" data sets (Table 4), however, moisture emerged as an additional significant factor in the "LIVING" data set in both NMDS and RDA analyses (Tables 2 and 4).

Table 1 Number of testate amoeba species recorded in samples based on all shells (i.e., living and empty) and living individuals only. Minimum, median, mean, and maximum values are shown for both studied fens and in case of the heterogeneous fen the values are also shown separately for *Sphagnum*- and brown moss-dominated samples

	ALL				LIVING			
	Min.	Median	Mean	Max.	Min.	Median	Mean	Max.
Homogeneous fen	16	21	21	27	14	19	19	25
Heterogeneous fen	9	18	19	33	10	16	18	34
Sphagnum	9	14	15	19	10	12	14	18
Brown mosses	19	25	25	33	16	24	25	34
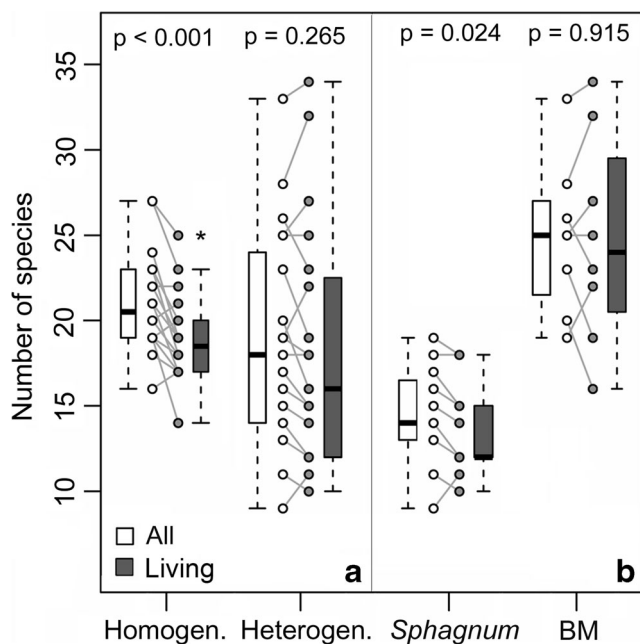
Fig. 2 Comparison of the number of testate amoeba species recorded in samples based on all shells (i.e. living and empty, white) and living individuals only (gray). **a**) Overall situation at both fens. **b**) For the heterogeneous fen, *Sphagnum*- and brown moss-dominated samples are also compared separately. Circles show values for individual samples while lines indicate the shifts in species richness between pairs of samples based on all or living shells only. The significance of differences was tested by Wilcoxon signed-rank test. In the box plots, box edges represent the 1st and 3rd quartiles, middle lines indicate the median and whiskers contain the values found in the interval of 1.5 × interquartile range. If present, outliers are marked with an asterisk
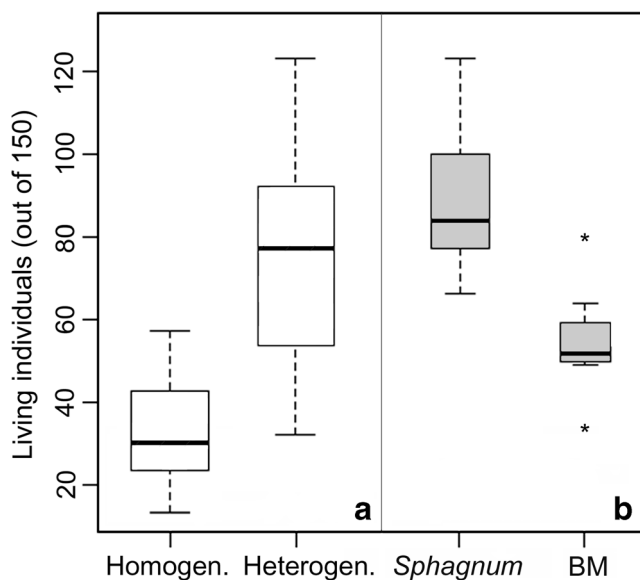
Fig. 3 Number of living individuals recorded among 150 analyzed shells. **a**) Overall situation at both sampled fens. **b**) For the heterogeneous fen, *Sphagnum*- and brown moss (BM)-dominated samples are treated separately. For detailed information on the box plots see the caption for Fig. 2

Discussion

Species Composition Variance on Small Spatial Scales

Studies using a complex sampling with a higher number of repetitions per site are rare in testate amoeba research, even though they often provide important information concerning these organisms [1, 12] and lead to more precise diversity estimates as compared to traditional sampling of representative micro- or mesohabitats [45]. In this study, a repetitive sampling strategy at small spatial scales proved useful in demonstrating that microhabitats which seem macroscopically alike may harbor different assemblages. Similar results were previously reported from acidic *Sphagnum*-dominated peatlands [11, 12], yet have never been described from highly alkaline conditions. Our results provide the first evidence for spatially variable testate amoeba composition at a calcareous fen with a topographically uniform brown moss carpet. Nonetheless, as the measured variables expressed only small portion of variation among samples, we seemingly failed to determine the environmental factors responsible for the compositional variance in the testate amoeba assemblages at this study site. At the environmentally heterogeneous mineral-rich *Sphagnum*-fen, however, the within-site variability in testate amoeba assemblages was notably higher and primarily corresponded with the changes of the moss layer formed by two functional groups of bryophytes, resembling the situation observed along the poor-rich gradient [5, 6, 14]. The dominance of *Sphagnum* or brown mosses was associated with small-scale changes in abiotic conditions (e.g., water pH, redox potential and temperature) and though the major compositional turnover occurred between *Sphagnum* and brown mosses, this factor itself accounted for 21–24% of variability in the species data. Variation within the samples from the two moss types at the heterogeneous fen as well as the brown moss-dominated fen points to additional, not detected factors shaping the species composition of testate amoeba assemblages at the microhabitat scale [4].

Surprisingly, moisture only explained a minor fraction of the variance in our analyses, even though it is often reported as one of the principal variables shaping testate amoeba assemblages in peatlands [2, 3]. This might be caused by a minimal topographical variation at our sites and consequent short moisture gradient due to missing waterlogged microhabitats or pools. Also, grade-scale estimates were used to assess the moisture conditions while depth to water table measurements would be more appropriate here. In addition to moisture, there are other potentially significant factors, especially water chemistry characteristics, which were not explored in this study but were reported as significant by some previous studies focused on community changes along the poor-rich gradient. These include concentrations of silica and phosphorus [46], calcium [5, 8], zinc and iron [47], and sodium and

Fig. 4 Two-dimensional NMDS ordination plots comparing species composition of testate amoeba assemblages collected at the homogeneous fen (**a**, **b**) and the heterogeneous fen (**c**, **d**). The diagrams are given for species data based on all shells (i.e. living and empty; **a**, **c**) or living individuals only (**b**, **d**). For the heterogeneous fen, symbols indicate the type of sampled microhabitats (brown mosses or *Sphagnum*) while the shading of symbols reflects the percentage of *Sphagnum* stems in each moss sample. Vectors represent a passive projection of environmental variables (i.e. water pH, proportion of *Sphagnum* stems, redox potential, temperature, conductivity, and moisture), which were significantly associated with the sample scores on the NMDS ordination axes (4999 permutations, $p < 0.05$). For more detailed information on the environmental variables see Tab. 2
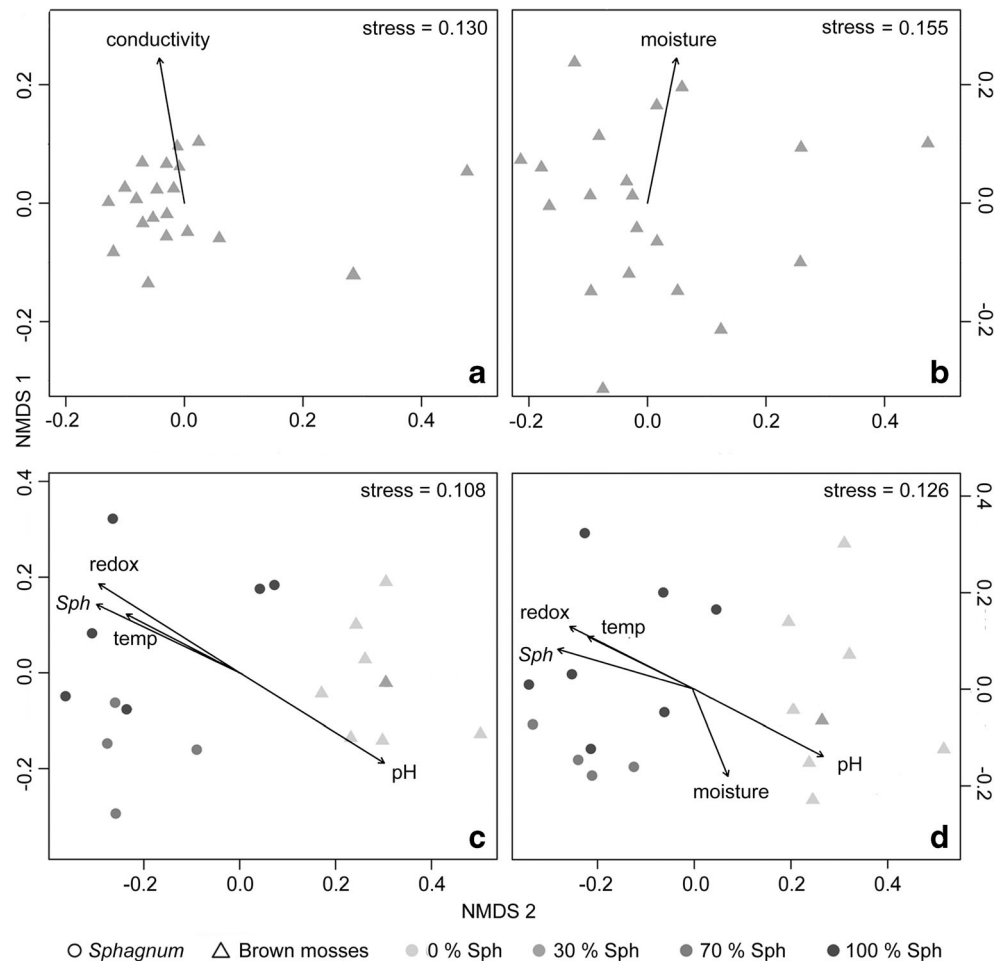

magnesium [5]. Sulfate concentration was also reported as an important factor influencing testate amoebae [29, 48] and an indirect impact via modification of the food web was suggested by Payne et al. [29]. Nonetheless, the influence of these factors is not consistent among studies and there is not enough information on the variability in ground-water chemistry on smaller spatial scales, again completely missing from alkaline fens. But, based on the results of Ulanowski and Branfireun [49], who documented a significant variability in sodium, calcium, magnesium, and sulfate concentrations within the range of a 7×7 m plot over a 2-week period in a fen of neutral reaction, we can assume that the groundwater properties are possibly quite unstable in fens in general.

Besides the chemical properties of the groundwater, discrete difference in testate amoeba assemblages from brown moss- and *Sphagnum*-dominated patches can be also attributed to biotic factors such as phenolic compounds released by *Sphagnum*. Jassey et al. [12] suggested a direct physiological effect of *Sphagnum* mosses on testate amoebae and also a possible indirect influence via their impact on prey organisms. In our samples, we noticed a strong affiliation of *Hyalosphenia papilio* to *Sphagnum*-dominated microsites,
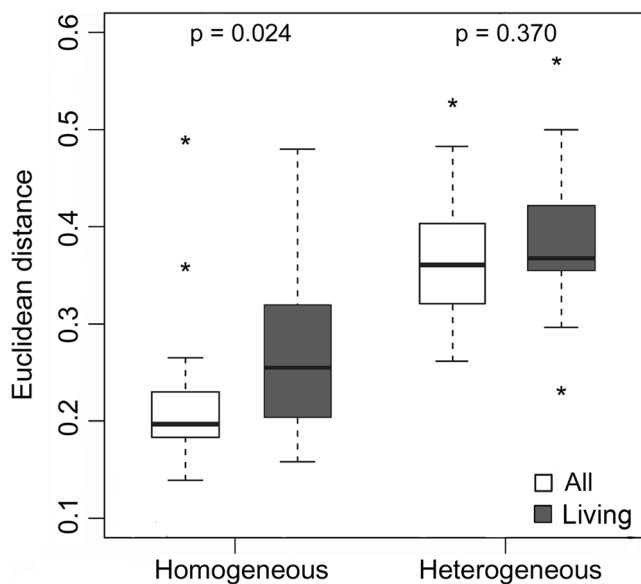
occasionally comprising up to 70% of all identified individuals. Tight relationship between *H. papilio* and other mixotrophic testate amoebae was also confirmed in previous studies, e.g., [11, 50, 51]. However, in two of our *Sphagnum* samples, this species was missing completely with no obvious differences in abiotic conditions being found between these and our other *Sphagnum* microsites. One might thus speculate if the effect of phenolic compounds or other factors alternating the food web were responsible. Our alternative explanation here, however, is linked to possibly differing successional age of the sampled microsites. If some of the *Sphagnum* patches were recently formed, they might not yet had been colonized by all species from the local species pool. This is in line with rather low dispersal potential of *H. papilio* [50]. Of course, this species belongs to the larger testate amoeba taxa and perhaps the compact structure of *Sphagnum* may hamper the dispersal of the shells. Nonetheless, being the most abundant species at our heterogeneous fen (accounting for more than 20% of all identified individuals), some sort of mass effect could be expected here. Still, the mobility appears to be quite limited as *H. papilio* shells were also only present in two of our brown moss samples.

Fig. 5 PCoA ordination diagrams representing testate amoeba assemblages at the homogeneous fen (**a**, **b**) and the heterogeneous fen (**c**, **d**). The diagrams are given based on all shells (i.e., living and empty; **a**, **c**) and living individuals only (**b**, **d**). Empty symbols indicate the type of sampled microhabitat (*Sphagnum* or brown mosses) and full black circles represent the group centroids in multidimensional space. For more information see also Fig. 6
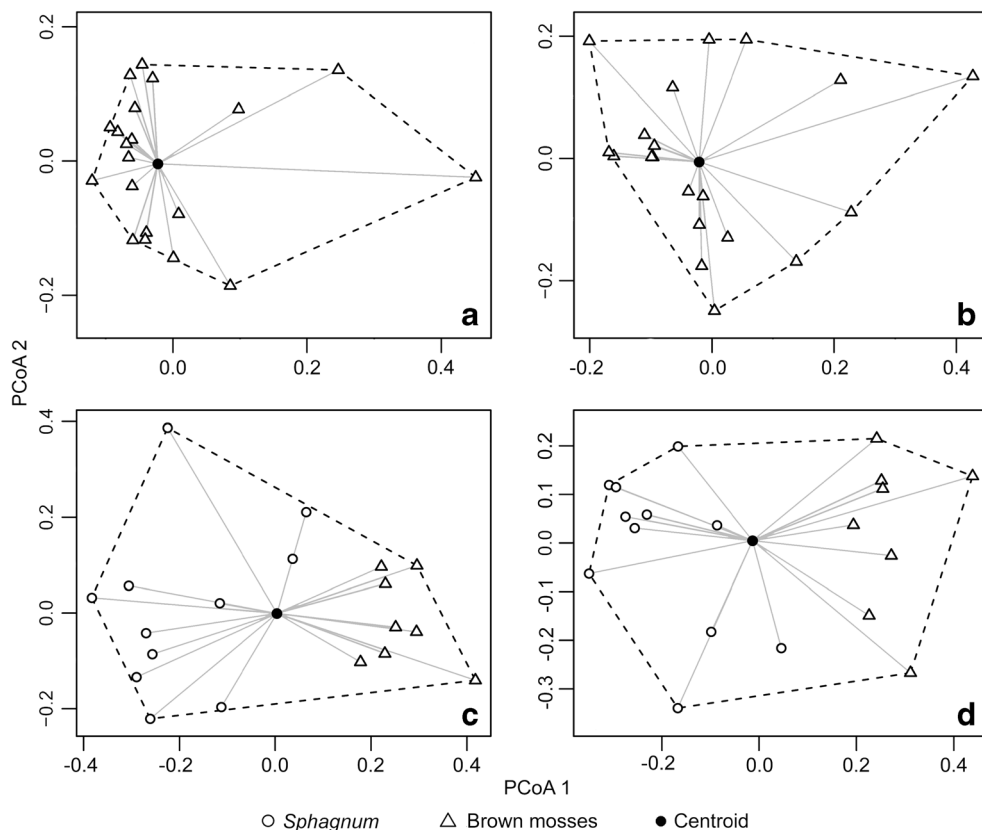
Considering spatial distribution of individual species at the homogeneous fen, the occurrence of *Difflugia geosphaira* was

Fig. 6 Variation in sample distance to the group centroids calculated based on the position of samples in PCoA ordination space. For both studied fens, the results are shown pairwise based on all shells (i.e., living and empty, white) and living individuals only (gray). The significance of the differences between data sets was tested by Mann-Whitney *U* test. For detailed information on the box plots, see the caption for Fig. 2

the most intriguing. This species was found only in five samples located in sequence along one of the transects while forming a dominant component of the community in two of those samples. As this species was not found in any of the remaining 15 samples, not even as empty shells, it seems that it did not recently thrive in other (sampled) parts of the site. However, we were not able to identify possible causes of this limited spatial distribution. Although the reasons behind spatially limited occurrence of species such as *D. geosphaira* and *H. papilio* remain unexplained, we learned that the dispersal of shells across both our study sites seems to be much lower than expected over such short distances.

Effect of Empty Shells

Testate amoebae are often considered as good indicator organisms due to their short generation times and quick response to changing environmental conditions [52]. It was shown that some factors may vary considerably within the time of just a few weeks [49] and seasonal shifts in testate amoeba communities from *Sphagnum*-dominated peatlands were previously reported by several authors [11, 25, 30, 45]. These were often related to changes in hydrology throughout the vegetation season, yet more factors appear to be involved and there is still not enough consistent information available concerning the topic of short-term variability. While changes of

Table 2 Regression coefficients between site scores on the first two NMDS axes of testate amoebae samples and of explanatory variables. The fit of each factor into the ordination space, i.e., percentage variation in the factor explained by site scores on NMDS axes in multiple linear regression (r^2) and significance (p) of the result based on 4999 permutations are shown

	NMDS1	NMDS2	r^2 (%)	p (>r)	NMDS1	NMDS2	r^2 (%)	p (>r)
	Homogeneous fen, ALL				Homogeneous fen, LIVING			
pH	0.160	−0.987	1.14	0.869	−0.369	0.930	0.15	0.990
Conductivity	−0.198	0.980	43.56	0.015	−0.562	0.827	29.04	0.065
Redox	−0.088	0.996	13.40	0.250	−0.278	0.961	02.96	0.766
Temperature	−0.149	−0.989	5.76	0.596	−0.601	−0.799	5.97	0.607
Moisture	0.419	0.908	13.72	0.317	0.232	0.973	37.84	0.021
	Heterogeneous fen, ALL				Heterogeneous fen, LIVING			
pH	0.849	−0.529	73.9	0.001	0.874	−0.486	67.9	0.001
Conductivity	0.953	0.304	7.6	0.543	0.482	0.876	10.3	0.432
Redox	−0.849	0.529	70.4	0.001	−0.885	0.470	62.1	0.001
Temperature	−0.902	0.432	40.2	0.021	−0.887	0.462	45.9	0.006
Moisture	0.481	−0.877	18.3	0.209	0.421	−0.907	40.2	0.019
Sphagnum %	−0.871	0.491	74.0	0.001	−0.937	0.349	74.0	0.001

conditions seem to be the most common source of empty shells, little is also known about the role of predation, reproduction, and dispersal over small and large spatial scales caused by wind [20, 21], wild animals, and even humans [22]. Moreover, better understanding of the vertical distribution of living/dead individuals within the surface peat layers is also needed [53].

Meanwhile, the use of undifferentiated (i.e., living and dead) shells is quite habitual in the testate amoeba research, e.g., [9, 23, 25, 54], according to a common assumption that the potential bias caused by empty shells is negligible. Undifferentiated or even dead assemblages are, for example, often used in transfer function development for paleoecological reconstructions [55–57]. However, the information on species ecology might be skewed when the matter of living and dead individuals is disregarded. Our data showed that the proportion of living and dead individuals can vary substantially among samples, even within a single site. We further documented lower percentages of living individuals in brown mosses (9–53%) as compared to those in *Sphagnum* (44–82%), suggesting that brown mosses might be more prone to variation in conditions, such as DWT and ground-water chemistry, given their loosened spatial structure and lower buffering capacity. Besides, depending on the conditions, higher growth rates can be expected for *Sphagnum*

mosses [58] and surface layers with brown mosses might thus contain shells accumulated over longer periods of time. It is likely that the differentiation between living and empty shells would be more useful in more alkaline conditions than in *Sphagnum*-dominated habitats. However, we are not aware of any study that would focus specifically on the effect of empty shells on distributional pattern assessment in any peatland habitat so far. It is common that some species show different optima among studies which is often explained by taxonomical discrepancies. Perhaps, this could also be due to the inclusion of dead shells of species that did not tolerate the conditions recorded at the time of the sampling.

Here, we tested the effect of empty shells exclusion assuming this will lead to some shifts in community composition between the "ALL" and "LIVING" data sets. We further expected an increase in explained variability, as the "LIVING community should correspond better with our single-shot measurements. At the homogeneous fen, the exclusion of empty shells had indeed a significant impact on the species composition of the assemblages, however, the testing of environmental variables showed non-significant relationships in both cases. At the heterogeneous fen, the main compositional changes in testate amoeba communities occurred between *Sphagnum* and brown moss-dominated patches which formed

Table 3 Redundancy analysis (RDA) summary. Explained variability values are given as adjusted r^2; permutation test with 4999 permutations was used to test the significance of the results. Values are given for all shells (i.e., living and empty) and living individuals only. Results of the model with all variables included

RDA	Total inertia (%)	Explained variability (r^2)	Adjusted r^2	p (>F)
Homogen. fen, ALL	15.59	0.255	−0.012	0.544
Homogen. fen, LIVING	19.43	0.283	0.027	0.315
Heterogen. fen, ALL	30.22	0.490	0.235	0.002
Heterogen. fen, LIVING	30.63	0.496	0.244	0.002

Table 4 Redundancy analysis (RDA) performed individually for each of the variables tested at the heterogenous fen. Values are compared with the forward selection results. Explained variability values are given as adjusted r^2; permutation test with 4999 permutations was used to test the significance of the results. Results are given for all shells (i.e., living and empty) and living individuals only

Heterogeneous	Adj r^2 RDA (p (> F))				Forward selection			
	ALL		LIVING		ALL		LIVING	
Sphagnum %	0.237	(< 0.001)	0.215	(< 0.001)	0.237	(< 0.001)	0.215	(0.002)
pH	0.231	(< 0.001)	0.194	(< 0.001)	n.s.		n.s.	
Redox potential	0.228	(< 0.001)	0.191	(< 0.001)	n.s.		n.s	
Temperature	0.131	(0.002)	0.118	(0.002)	n.s.		n.s.	
Moisture	0.031	(0.131)	0.057	(0.042)	n.s.		0.036	(0.038)
Conductivity	0.019	(0.199)	0.024	(0.167)	n.s.		n.s.	
Total adj r^2	0.235	(0.002)	0.244	(0.002)	0.237		0.251	

the major environmental gradient here. Even though the presence of the two bryophyte groups can be considered stable over the vegetation season, the shift in species composition between the "ALL" and "LIVE" datasets might still be caused by mass effect with species (or their empty shells) spreading from source areas to sites with less favorable conditions. However, based on the species distribution among our samples, we learned that passive dispersal at our study sites was not as frequent as we would expect, and no significant changes in species composition occurred after empty shell exclusion. On the other hand, we did observe a difference in relation to moisture which emerged as significant variable for the "LIVING" data set in both NMDS and RDA. In fact, the moisture content of the moss layer was the only factor with a potential for short-term variability during the season that we included here. Unfortunately, only a coarse grade scale was used to assess the moisture conditions at our sampling spots and more precise description such as DWT measurements would possibly yield more robust results. We thus believe that, despite the low r^2 values and significance levels, especially the result of forward selection supports our hypothesis that the inclusion of empty shells might indeed lead to biased results when it comes environmental variables that are more prone to spatio-temporal fluctuation.

Conclusion

In this study, we aimed to bring new insights into the ecology of testate amoebae inhabiting minerotrophic mires, as well as to draw attention to some methodological aspects that we believe have not yet been adequately addressed. Our results proved that, although marginally studied, minerotrophic mires represent unique environments in terms of testate amoeba assemblages. Using a small-scale approach, we found that macroscopic variability (or homogeneity) of the sampling sites does not correspond to the situation at the microscopic level

and the complexity of testate amoeba spatial patterns within these habitats is greater than assumed. However, our data did not allow us to describe properly the mechanisms and factors shaping the compositional variation of testate amoebae at individual microsites. Still, we can conclude that the heterogeneity of conditions in the moss layer proved to be much higher than recognized by vegetation composition changes observed in the field. We find our results challenging for the traditional sampling methods focused primarily on the most representative micro- or mesohabitats, which might lead to severe loss of information and biased understanding of testate amoebae ecology. On the other hand, the applied approach of randomly selected sampling spots is not thoroughly convenient as some microhabitats might be skipped using this technique. Similar sampling strategies, nonetheless, allow to explore spots that would otherwise be considered unsuitable, redundant or simply unappealing and would probably never get sampled. In this context, Mitchell et al. [59] suggested that sampling at more unconventional spots might perhaps be the key to finding analogous communities for those present in peat cores yet missing in the modern data sets.

Additionally, our results proved that exclusion of empty shells can provide more accurate information on species distribution as well as their relationship with certain environmental variables, especially those with higher spatio-temporal variability (e.g., moisture). Further work elaborating this issue is still needed to fully assess the consequences of different approaches used for community description in ecological studies. Of course, the need for different methods may vary depending on the study objectives and, admittedly, the time cost of sample processing must be considered. In our samples, the proportion of living individuals ranged between 9 and 82%, with lower numbers usually found in brown moss-dominated (micro)habitats. These also tend to yield samples with higher content of inorganic sediments. Thus, aiming for more precise community description based on living individuals can result in a fairly time-consuming process in more alkaline fens. On

the other hand, we believe that these habitats, often recognized for their ecological uniqueness, need more thorough exploration when it comes to testate amoebae.

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