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The effects of sample storage duration on tardigrade density and community composition in moss samples

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ABSTRACT

In studies on micrometazoans, sample storage and processing methods are mostly decided based on sample quality (e.g., substrate type and moisture level), and the choice of methods may affect the reliability of the data. However, these methods are poorly studied and rarely reported in detail. Our aim was to determine the methodological compromise between efficiency and reliability required for large-scale quantitative meiofaunal ecological studies. Specifically, we tested whether storage duration (necessary for large number of samples) affects the density or community composition of tardigrades in moss samples. We focus on a largely unexplored limnoterrestrial ecosystem – boreal peatlands, where moss moisture levels are naturally variable across different microhabitats and moss species. We collected seven moss samples from a peatland in Central Finland, kept them in a refrigerator and extracted tardigrades using the Baermann wet funnel at 1, 24, 48 and 96 h post sampling. We found a significant decrease in tardigrade density (32 % on average), but no changes in community composition, after the first 24 h of storage. Based on these results, we recommend that samples collected from wet limnoterrestrial habitats should be processed within 24 h to ensure accuracy and comparability of large-scale quantitative data on tardigrade ecology.

1. Introduction

Quantitative ecological research is the main tool in estimating global biodiversity and species distributions. However, the accuracy of quantitative ecological research is highly dependent on the methods (Aceves-Bueno et al., 2017; Cesarz et al., 2019; Czerneková et al., 2018). In addition to sampling design itself, secondary measures such as handling, storing, and transporting samples may affect the reliability of the data. Studying micrometazoans requires extraction of the study organisms from their substrate, for instance, soil or leaf litter (Cesarz et al., 2019; Czerneková et al., 2018). The extraction is never conducted in the field and, therefore, samples are transported and stored for some time before the extraction. The storage time often depends on the length of the transportation (see e.g., Sohlenius et al., 2004) and is rarely reported in papers, although long storage duration could potentially affect the quantity and community composition of micrometazoans.

Tardigrades are micrometazoans that are most famous for their ability to survive extreme conditions by undergoing reversible dormant state; yet they need a layer of water to stay active (Guidetti et al., 2011; Møbjerg et al., 2018, 2011). From the c. 1400 tardigrade species so far described (Degma and Guidetti, 2023) most are found from limnoterrestrial habitats in substrates such as soil, mosses, and lichens (Bartels et al., 2016; Nelson et al., 2018). In ecological studies on tardigrades, animals have to be first extracted from the substrate. Because of their small size (100–1200 μm) and slow movement, tardigrades are commonly extracted by using mechanical methods that rely on passive movement. These methods include, for instance, sieving through a mesh (Guidetti et al., 1999; Guil et al., 2009), Ludox centrifugation (Romano III et al., 2011), and soaking and shaking moss samples in water (Stec et al., 2015). One of the most commonly used methods that rely on active movement of tardigrades is the Baermann wet funnel method with some variations (Baermann, 1917; Czerneková et al., 2018; Jönsson, 2003). The substance specific accuracy and effectiveness of different extraction methods of tardigrades is discussed in some papers (Degma, 2018; Hallas and Yeates, 1972; Nelson et al., 2015), however, systematic studies comparing different methods are rare (but see, e.g., Czerneková et al., 2018). All extraction methods seem to have some disadvantages. The above-mentioned mechanical methods tend to be laborious and time consuming, whereas methods relying on active movement of tardigrades do not extract non-motile animals or eggs

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making tardigrade identification to species level difficult. However, since the Baermann wet funnel method requires the least time and the least (yet consistent) effort per sample, it is therefore better suited for processing the greater number of samples (i.e., hundreds) needed for large-scale quantitative analyses. Therefore, we are looking into this particular method to understand how to achieve the best possible accuracy and effectiveness to extract tardigrades from a large number of moss samples.

The design of the Baermann wet funnel method includes a plastic funnel with a steel net placed in the main cone and a silicone tube in the narrow end of the funnel leading to a vial. Samples are placed on the steel net and covered entirely with water. While soaked in water, tardigrades loosen their grip from the substrate and move into the vial along with other fauna. The quantity of a sample that can be placed in a funnel at once is restricted by the diameter of the funnel and the steel net, as increasing the height of the substrate cover might reduce the extraction efficiency (Cesarz et al., 2019), and the number of samples that can be extracted simultaneously is restricted by the number of funnels in use. Thus, a large number of replicate samples often requires storage of the samples. Moreover, sample storage time is usually dependent on the length of transportation from the sampling site or time-consuming extraction methods and tends to vary greatly between and even within studies yet is rarely reported. Studies on methodology in tardigrade sampling have covered extraction methods (Czerneková et al., 2018), sampling methods in the field and different storage methods (reviewed in Degma, 2018), but to our knowledge, the effects of duration of storage have never been systematically studied.

Long-term tardigrade studies with quantitative replicate samples are rare (but see, e.g., Bartels and Nelson, 2006; Nelson and Bartels, 2013, 2007), but essential for further understanding of the tardigrade ecology (Nelson et al., 2018). Sample size in studies is principally limited by time-consuming methods and sample processing. Therefore, it is important to develop methods that maximize the efficiency of sampling without causing bias of the data. The purpose of this study is to estimate, based on a relatively small number of samples, the across-sample comparability in methods that are needed for large scale sampling of tardigrades in limnoterrestrial habitats with variable moisture conditions, across microhabitats and substrate species, e.g., peatlands. So far, peatland habitats have been overlooked in ecological tardigrade research. This is probably due to their low pH to which tardigrades are known to be sensitive (Massa et al., 2023). However, pH and moisture levels vary both between and within peatland types (Rydin et al., 2013) and consistent and reliable sampling methods for peatland habitats is nonexistent. For the first time, we investigate whether duration of sample storage affects the observed density and community composition of moss living tardigrades. To test this, we use the Baermann wet funnel method for extracting tardigrades from moss samples. The Baermann wet funnel method is less time consuming compared to alternative methods that require active handling of samples one-by-one (e.g., sieving). In addition, the extraction efficiency in the Baermann wet funnel method is less dependent on the individual effort put in each sample. Hence, funnel extraction is more suitable for processing the large number of samples required for large scale quantitative ecological studies. Our aim is not to compare the overall efficiency of different extraction methods but to improve the accuracy and comparability of the Baermann wet funnel method when applied to stored samples in large-scale quantitative ecological studies on moss-living tardigrades.

2. Methods

We collected seven moss samples in early May 2021 from three sites in Central Finland that represented habitats with different kinds of moisture levels. We collected five samples from a pine mire, one from a small bog and one from a spruce mire. Each sample consisted of a single moss species identified in the field. Four samples represented mosses from the family Sphagnaceae: *Sphagnum angustifolium* ((Russow) C.E.O. Jensen), *Sphagnum divinum* (Flatberg & Hassel), *Sphagnum fallax* (H. Klinggr), *and Sphagnum balticum* (Russow) Two samples were *Pleurozium schreberi* ((Willd. ex Brid.) Mitt.) and one was *Hylocomium splendens* ((Hedw.) Schimp), which are both common feather mosses in forested habitats and wooded peatlands and have relatively high tardigrade densities (Boeckner and Proctor, 2004; Jönsson, 2003) (Table 1). After collection, we stored samples in sealable plastic bags of 1 liter volume that was filled entirely with moss. We used plastic bags because of the high range of water content across the samples. We placed samples into coolers and transported them to the University of Jyväskylä.

We extracted tardigrades from the seven samples by using the Baermann wet funnel method with sieves of 1 mm mesh size. From each sample, we took three subsamples by picking up small pieces of moss evenly throughout the sample and covered the steel nets on top of the funnels with a thin layer of moss. This procedure was repeated four times: first within one hour after collecting the samples and 24, 48 and 96 h after sampling. Usually, the duration of extraction in tardigrade studies varies between 12 and 72 h. Because the most efficient extraction seems to happen within the first 24 h (Czerneková et al., 2018; Hallas and Yeates, 1972), we extracted samples for 24 h. After the extraction, we replaced the water in the vials with 70 % ethanol, and the moss material was dried at 60 °C for 48 h and weighed (1 milligram precision; Mettler Toledo XS204 DeltaRange). The dry weight of moss varied from 9.12 g to 14.97 g between samples and from 0.31 g to 1.47 g between subsamples. The latter was taken into account in the statistical analyses (explained in more detail below). Also, the original water content level varied between the moss samples: it tended to be higher in *Sphagnum* samples (Samples 1, 5, 6 and 7) than in feather mosses (Samples 2, 3 and 4). We counted the total number of animals extracted under a dissecting microscope (Olympus SZX9). If present, we randomly chose a maximum of 50 specimens from each sample and mounted them on slides in Hoyer's medium for identification. We did not find any tardigrade eggs. For taxonomic analysis, we identified animals under a phase contrast microscope (Zeiss AXIO, 100x magnification) by using updated taxonomic accounts and descriptions. Since our aim was not to conduct a detailed taxonomic report but to estimate the effects of sample storage duration on community composition, we identified tardigrades to genus level.

3. Statistical analysis

To answer whether sample storage time affects tardigrade abundance, we analyzed the density of tardigrades with Generalized Linear Mixed Model (GLMM: function glmer in R-package lme4 (Bates et al., 2015). We repeated the GLMM with poisson and binomial distribution (with log link) and chose the latter as it had a lower AIC (Akaike Information Criteria). Three samples (Samples 5, 6, and 7) were not included in the analysis because all subsamples had extremely low number of tardigrades compared to the other four samples (< 18 T/g vs. >79 T/g; see results, Fig. 1). The response variable in the model was the number of tardigrades in each subsample of the four considered samples (i.e., $n = 48$ datapoints: 4 samples * 4 time points * 3 subsamples for each time point), with moss dry weight (gram) set as an offset to estimate the density of tardigrades in a standardized amount of substrate (i.e., 1 g) across all subsamples and samples. As the predictor in the model, we used the storage duration time points as a four-level factor variable (1, 24, 48 and 96 h). Our aim was to estimate the change in the density of tardigrades between adjacent extraction time points. Therefore, we used backwards adjacent contrasts in the predictor variable to compare the density of tardigrades between time steps: 24 h vs 1 h, 48 h vs 24 h, and 96 h vs 48 h. We included the sample ID as a random effect to test for the fixed effects using a within-sample design. The proportion of variance explained by the random effect was calculated using R package Performance and the function R2_nakagawa (Nakagawa et al., 2017).

We used non-metric multidimensional scaling (NMDS: function metaMDS in R-package vegan (Oksanen et al., 2022) to visualize the

Table 1
A list of the collected moss samples including moss species, moss dry weight, and the number of specimens found for each tardigrade genus identified. Samples 5, 6, and 7 were not included in the statistical analysis and tardigrades were not identified because of the low number of tardigrades found in the samples.

Site	Coordinates (WGS84)	Sample ID	Moss species	Moss dry weight	Tardigrades N	Tardigrade genus	Specimen N
Pine mire	62° 20' 35,914"/ 25° 20' 48,252"	Sample 1	*S. angustifolium*	9.77 g	1754	*Adropion*	32
						Crenubiotus	141
						Guidettion	1
						Hypsibius	72
						Macrobiotus	111
						Mesobiotus	4
						Milnesium	1
						Murrayon	6
						Pilatobius	4
Pine mire	62° 20' 35,914"/ 25° 20' 48,252"	Sample 2	*P. schreberi*	7.90 g	1813	*Adropion*	9
						Diphascon	18
						Guidettion	3
						Hypsibius	22
						Macrobiotus	416
						Mesobiotus	11
						Milnesium	38
						Minibiotus	3
						Murrayon	5
						Pilatobius	8
						Ramazottius	7
						Ursulinius	2
Pine mire	62° 20' 35,914"/ 25° 20' 48,252"	Sample 3	*H. splendens*	8.30 g	4923	*Adropion*	7
						Diphascon	53
						Guidettion	2
						Hypsibius	12
						Macrobiotus	267
						Mesobiotus	40
						Mesocrista	4
						Milnesium	69
						Minibiotus	14
						Murrayon	26
						Pilatobius	13
						Platicrista	1
						Ursulinius	1
Spruce mire	62° 20' 43,185"/ 25° 21' 7221"	Sample 4	*P. schreberi*	12.86 g	4632	*Adropion*	26
						Crenubiotus	1
						Diphascon	82
						Hypsibius	15
						Macrobiotus	222
						Mesobiotus	3
						Mesocrista	4
						Milnesium	68
						Pilatobius	1
						Platicrista	3
						Ursulinius	1
Pine mire	62° 20' 35,914"/ 25° 20' 48,252"	Sample 5	*S. divinum*	7.60 g	16	*Not identified*	
Pine mire	62° 20' 35,914"/ 25° 20' 48,252"	Sample 6	*S. fallax*	8.95 g	75	*Not identified*	
Bog	62° 20' 42,087"/ 25° 21' 3720"	Sample 7	*S. balticum*	8.98 g	56	*Not identified*	

variation in tardigrade community composition in relation to storage duration time points and across each moss sample with two indices: an incidence-based Jaccard and an abundance-based (i.e., taking the number of individuals into account) Bray-Curtis. For Bray-Curtis index, we used the relative abundance of each genus in relation to the total number of tardigrades found in the samples. All statistical tests and illustrations were done using R software version 4.2.1.

4. Results

We found a total of 13,269 tardigrades, varying between 16 and 4923 (20–6922 per g of moss) per sample and 0–855 (0–969 per g of moss) per subsample. The samples are listed in Table 1 including site information, the total number of extracted tardigrades, moss dry weight, tardigrade genera found, and the number of specimens in each genus.

Across the four samples included in the GLMM, the density of tardigrades extracted 24 h post sampling was significantly lower (32 %) than the 1 h time point (Table 2; Fig. 1). The decrease in tardigrade density was detected especially in three of the four samples (Fig. 1). The 14 % density increase between 24 h and 48 h was not statistically significant. The increase in tardigrade density seemed to happen mostly in Samples 1 and 4 (Fig. 1). However, between 48 h and 96 h post sampling, there was a further and significant decrease of 27 % in density (Table 2, Fig. 1). The Sample ID that was included as a random effect in the model explained 62 % of the variance in tardigrade density.

We found a total of 15 tardigrade genera of which nine occurred in Sample 1, 12 in Sample 2, 13 in Sample 3, and 11 in Sample 4. The most common genus across all samples was *Macrobiotus*, except for Sample 1 where the genus *Crenubiotus* was the most abundant. Neither the abundance-based Bray-Curtis index nor the presence-absence based Jaccard index showed any clear patterns in tardigrade community composition in relation to different storage duration time points (Fig. 2). However, based on the Bray-Curtis index the community composition in Sample 1 was distinct from the other three samples (Fig. 2d).

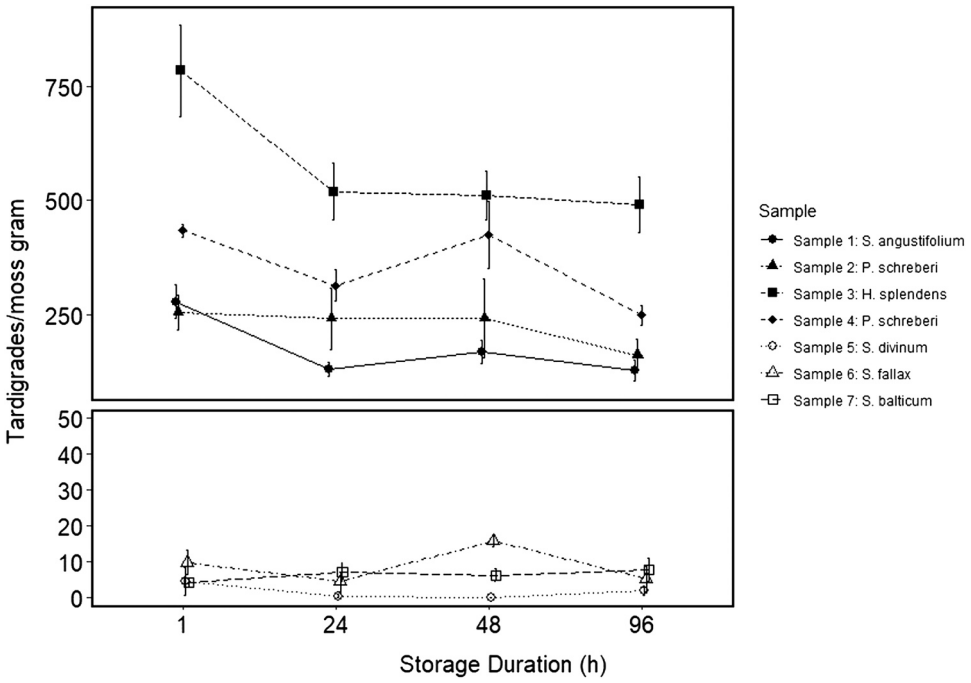

Fig. 1. The effects of the duration of sample storage on tardigrade density (tardigrade number per gram of dry moss processed). The error bars represent the standard error of the mean across three subsamples at each storage duration time point for each moss sample. The moss species are listed in the symbol guide next to corresponding Sample ID.

Table 2
The generalized linear mixed model output comparing the density of tardigrades within samples between adjacent time steps.

	Estimate	SE	Z	P
1 h (intercept)	5.68	0.23	24.66	< 0.001
1–24 h	-0.39	0.12	-3.31	< 0.001
24–48 h	0.14	0.12	1.15	0.251
48–96 h	-0.32	0.12	-2.65	0.008

5. Discussion

In this study, we tested whether the duration of sample storage affects the observed density and community composition of moss living tardigrades when using the Baermann wet funnel method for extracting them from the substrate. In general, we found that the longer the moss samples were stored, the fewer tardigrades were found. The most notable decrease in the density of tardigrades was found in the samples extracted 24 h post sampling; approximately 32% less than what was found in the samples extracted 1 h post sampling. A further drop in the density of approximately 27 % was found between 48 h and 96 h storage duration, where the time interval between measures was doubled compared to others. The community composition did not show any patterns across time intervals. According to the abundance-based Bray-Curtis index (Fig. 2d) the community composition in Sample 1 differed from the other three samples, which is likely due to the higher density of tardigrades belonging to the genus *Crenubiotus* cf. *Macrobiotus*. However, the observed decrease in the density of tardigrades across longer storage duration appears to be random with respect to tardigrade genera.

Limnoterrestrial ecosystems, such as peatlands, with high variation in moisture levels across microhabitats and substrate species have been ignored in tardigrade studies. Therefore, it is yet unknown whether the sample extraction and storage methods that have been used in other ecosystems are suitable for quantitative large-scale studies in such environments. Several methods are used for sample storage in tardigrade studies and the choice of methods depends on the duration of storage, type of the sample and type of the substrate (reviewed in Degma, 2018). For example, relatively dry terrestrial moss samples may be put into paper bags and allowed to dry naturally (Czerneková et al., 2018; Nelson et al., 2020) or samples are kept frozen, especially when collected from low temperature (Sohlenius et al., 2004; Zawierucha et al., 2019). Drying may cause tardigrades to undergo anhydrobiosis (Schill and Hengherr, 2018) whereas freezing induces cryobiosis (Hengherr and Schill, 2018) which are both environmental adaptations that may increase the survival rate of tardigrades in samples. However, the ability to undergo these dormant stages and to withstand environmental extremes varies between tardigrade species (Guidetti et al., 2011; Møbjerg et al., 2011; Roszkowska et al., 2021). Highly moist or aquatic samples are often stored in ethanol or in formaldehyde solution in plastic containers (Bartels and Nelson, 2006; Romano III et al., 2011; Zawierucha et al., 2019, 2015). The choice of storing method also depends on the extraction method to follow, and samples cannot be soaked in ethanol prior to Baermann wet funnel method, since the animals would be washed from the substrate before the extraction.

The specific way the samples were stored could explain the pattern we found. We collected moss samples in sealed plastic bags and stored them in closed bags in refrigerator, without freezing, drying, or adding ethanol. We chose this storage method for two main reasons. (1) This storage method is often used for samples obtained from freshwater habitats or highly moist terrestrial habitats, when samples cannot be dried (Degma, 2018). Therefore, it is the widely applicable for sampling peatland habitats, which naturally have a wide range of moisture conditions (Rydin et al., 2013). (2) This storage method allows the use of the meiofauna extraction method most appropriate for large scale quantitative analyses of tardigrades in wet limnoterrestrial ecosystems: the Baermann funnel (less time yet consistent effort cf. mechanical methods such as sieving samples one-by-one). However, our study found that this protocol's efficiency and comparability has the downside of considerable sensitivity to storage duration, since it precludes storing the moss samples in ethanol. In addition, we cannot know the cause for the observed "loss" of tardigrades from the stored moss: perhaps some specimens are washed out from the substrate if samples were really wet, and/or some could become anoxic or die (i.e., not extractable in the

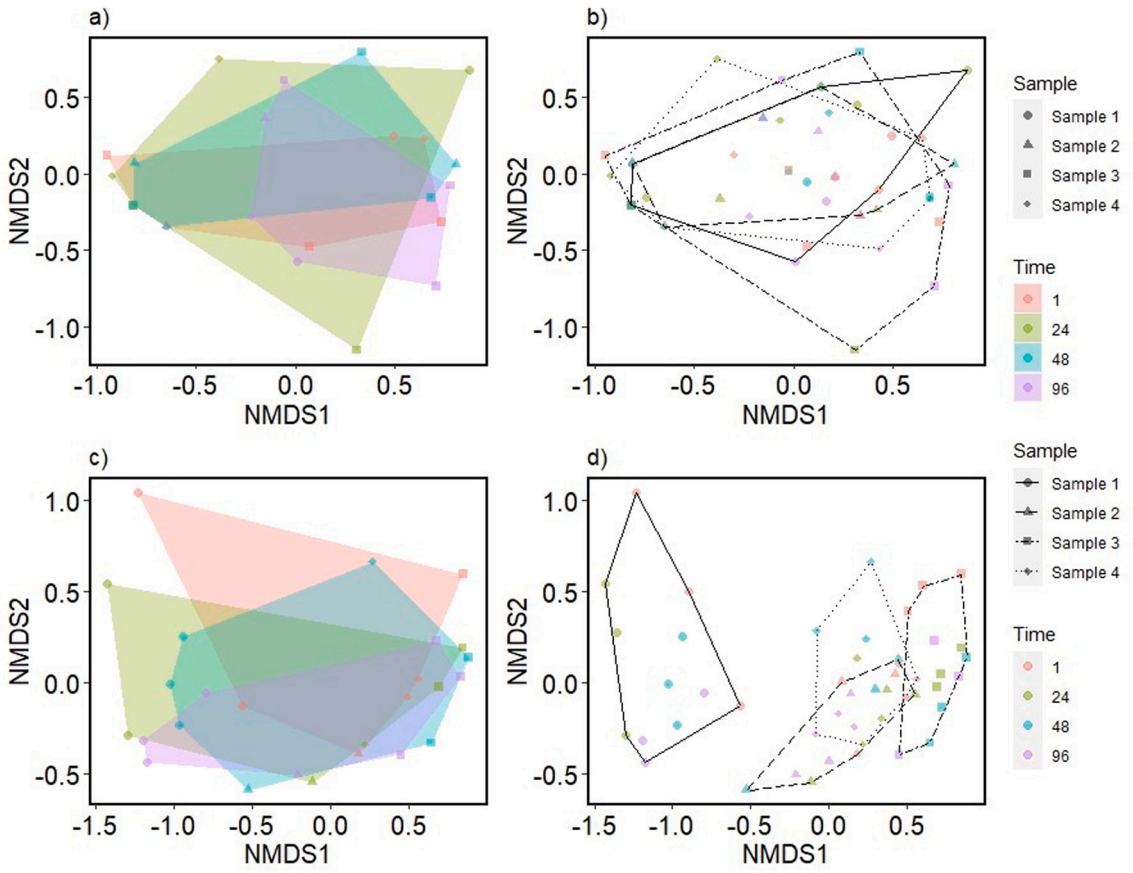

Fig. 2. Non-metric multidimensional scaling of community composition across samples and storage duration time points. Colored hulls in a) and c) reflect the comparison across the four times points (1, 24, 48 and 96 h post-sampling). The different line types in b) and d) represent different samples and variation across them. Figures a) and b) were created using the incidence-based Jaccard index, whereas Figures c) and d) use the abundance-based Bray-Curtis index.

funnels) due to their sensitivity to a potential oxygen deficiency (Møbjerg and Neves, 2021) inside the closed bags.

Sample 1 was moss species *S. angustifolium*: it had higher original water content level than the other samples included in our analysis and showed a notable drop in tardigrade density within the first 24 h. Yet, a similar decrease in density was seen also in drier samples, especially in Sample 3 that was *H. splendens*. Therefore, the original water content level might not be the main cause of the observed drop. All moss species in our study are also known to host variety of fungal species (Kauserud et al., 2008; Kostka et al., 2016) and some fungi are known to infect tardigrades (Loeffelholz et al., 2021). The storage duration in this study was relatively short (max. 96 h) and the most notable drop in tardigrade densities was detected already within the first 24 h of storage. Thus, it is unlikely that the drop in tardigrade density would be explained by fungal infection and no evidence of contamination was detected microscopically. Altogether, different water contents between samples may cause bias in detected tardigrade densities after long storage duration but cannot be avoided when sampling ecosystems with heterogenous moisture levels across microhabitats.

The choice of a single and consistent pre-storage sample processing method is important when conducting standardized large-scale quantitative studies in ecosystems with heterogenous moisture levels across microhabitats and samples (such as the peatlands used in this study), and especially when using the Baermann wet funnel method in extracting tardigrades. Since we found a 1/3 drop in numbers just after 24 h in cold storage, we recommend, when possible, to use appropriate pre-storage processing of the samples (e.g., drying of forest mosses). However, highly moist moss samples cannot be put in paper bags directly in the field or dried prior to transportation or extraction (especially with the Baermann wet funnel method) as can be done with drier moss samples. Therefore, when sampling habitats with heterogenous moisture levels researchers need to make careful logistic decisions to ensure across-sample reliability and comparability. Our results consider specifically tardigrade studies where the Baermann wet funnel method is used to extract tardigrades from moss samples. The Baermann method is less time-consuming and requires less effort per sample than the mechanical methods and is therefore useful for large scale quantitative ecological studies. Since the Baermann method poses limitations on, e.g., extracting tardigrade eggs that are important for identifying tardigrades to species level, mechanical extraction methods are likely to be more suitable for specific taxonomic studies. However, systematic studies comparing accuracy and efficiency between different extraction methods are rare (but see, e.g., Cesarz et al., 2019; Czerneková et al., 2018) and the overall accuracy of the Baermann method compared to other methods remains relatively unknown. Our results help to clarify the best possible protocol when using the Baermann method.

Patchiness in tardigrade density and community composition is a common and well documented phenomenon (Degma et al., 2011, 2005; Miller et al., 1994; Nelson et al., 2018). Concordantly, we found variation in tardigrade density between replicates within time points (Fig. 1) and community composition across moss species and between subsamples within time points (see Figs. 1 and 2). As previously found (Jönsson, 2003), feathermosses such as *P. schreberi* and *H. splendens* (Samples 2, 3 and 4) are particularly rich in tardigrades. Moreover, the variation between subsamples supports the previously documented very small-scale (within moss cushion) patchiness in abundance and community composition (Degma et al., 2011). Note however, that despite the considerable differences in tardigrade densities across moss species, the drop after 24 h was clear in three out of four of the samples (Fig. 1).

Conclusions

Our study highlights the importance of the neglected aspects of sample handling and storing protocols for large scale quantitative ecological studies on tardigrades. According to our results, pre-extraction sample storage time affects tardigrade densities, at least when samples cannot be processed pre-storage (e.g., drying, freezing, or using ethanol) and when labor-intensive mechanical extraction (e.g., sieving) is not possible, i.e., when Baermann wet funnel is the only viable option. This should be considered while making logistic and sample storage decisions in quantitative and ecological tardigrade studies. The methods needed for large-scale ecological studies on tardigrades are time-consuming, which often limits the sampling effort. Therefore, it is important to develop more efficient and accurate protocols. There is, however, considerable variation within and across studies in how samples are collected, stored, processed, and how long they are stored before processing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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