# A new preparation method for testate amoebae in minerogenic sediments

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## SUMMARY

Testate amoebae are one of a few moisture-sensitive proxies available to study Holocene palaeohydrology. Although the majority of research has been conducted on ombrotrophic peatlands in the Northern Hemisphere, the application of testate amoebae in minerogenic sediments, such as minerotrophic peatlands and saltmarshes, holds considerable promise but is often impeded by the low concentration of testate amoebae and by time-consuming counting. Here a new preparation protocol to concentrate testate amoebae is proposed; it removes more minerogenic particles and organic matter, but results in negligible damage to testate amoebae. Sodium pyrophosphate ($Na_4P_2O_7$) is introduced to remove fine particles through deflocculation that, in contrast to the commonly used chemical digestion/deprotonation via an alkaline treatment, is more physically benign to testate amoebae. Furthermore, acetone is introduced as an organic co-solvent to increase the solubility of organic matter in the alkaline treatment. We test the new protocol against standard, water-based methods and find that the addition of sodium pyrophosphate yields the highest concentration and increases the total number of testate amoebae recovered. Statistical analyses (multivariate ANOVA and ordination) suggest that the new method retains the assemblage integrity. We conclude by recommending a protocol combining sodium pyrophosphate, acetone and a mild alkaline treatment, as this combination yields the best slide clarity, reduced counting time and results in negligible damage to testate amoebae.

**KEY WORDS:** laboratory preparation, sodium pyrophosphate, acetone

## INTRODUCTION

The unicellular protists known as testate amoebae have been widely used as a bio-indicator to study ecology and palaeoecology in peatlands (Mitchell *et al*. 2008a). They are ubiquitous in moist environment such as soils, lakes and peatlands, and their tests preserve well in sediments, even dating back to the Late Neoproterozoic (Porter & Knoll 2000). With these features, testate amoebae have been widely used to study palaeohydrology in peatlands, especially in the Holocene (Mitchell *et al*. 2008a) and it has been more than two decades since the first quantitative reconstruction of water-table depth (Warner & Charman 1994).

Despite this capacity, the majority of research has been conducted in ombrotrophic peatlands, particularly in the Northern Hemisphere, and more minerogenic sites have been used much less frequently (Payne 2011, Charman *et al*. 2010, Swindles *et al*. 2016). As an example, Charman *et al*. (2010) managed to count 50 tests in samples from saltmarshes, where testate amoebae have applications relevant to sea-level change (Barnett *et al*. 2017). In our experience, and somewhat counter-intuitively, it is often the silt-sized organic matter in the minerogenic samples from peatlands that impedes quantification of testate amoebae, perhaps because the non-organic components can often be removed using sieving (or micro-sieving) (Hendon & Charman 1997) or differential settling techniques and the organic content in these environments is often structurally more robust. The fragility of some testate amoebae means that strongly basic and strongly oxidising conditions are avoided and hence a high organic content in the preparations for testate amoebae is also clearly an issue in other settings: as an example, Swindles *et al*. (2016) described the concentration of testate amoebae in a tropical peatland as very low owing to poor preservation and obscuration by organic matter.

A high concentration of organic matter in a sample prepared for the quantification of testate amoebae leads to two problems: (1) the organic matter can aggregate (through both physical association and chemical crosslinking), which can obscure testate amoebae; and (2) a relatively low concentration often requires the preparation of many microscope slides and extra counting time. These low concentrations, potentially biased results, low counts


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and statistically unsatisfactory totals (Charman *et al*. 2010) have limited the application of testate amoebae as an independent or complementary proxy to a wider geographical area and to other environmental settings.

As noted, the standard methods to concentrate testate amoebae for quantification are designed to minimise damage and are based on sieving and either involve a mild alkaline digestion or are water-based (Hendon & Charman 1997). Although an alkaline digestion is effective at removing unwanted organic matter, thereby increasing the concentration (Hendon & Charman 1997) and reducing the counting time (Barnett *et al*. 2013), the fragility of testate amoebae means that tests may be damaged and some features missing or altered (Hendon & Charman 1997). For this reason water-based methods are often advocated (Booth *et al*. 2010). However, significantly increasing the concentration and reducing the counting time should be evaluated against the 'cost' of acceptable damage. Based on such considerations, Barnett *et al*. (2013) used a mild (less than 1 % KOH) alkaline treatment for sample solutions on a preheated hotplate, compared to 10 % KOH (in Hendon & Charman 1997), and significantly reduced the average counting time from 16 h to 8 h.

Although it has a long history in palynology as a deflocculant to remove fine mineral particles such as clay and silt (Bates *et al*. 1978, Cwynar *et al*. 1979), sodium pyrophosphate is also an effective dispersant for fine particles of organic matter (Bremner & Lees 1949, Kaur & Fanourakis 2016). In contrast to an alkaline treatment, sodium pyrophosphate is a near-neutral agent and so should not damage testate amoebae, but its effectiveness had not been demonstrated.

This research addressed several of these issues with the over-arching aim of improving techniques to concentrate testate amoebae in more minerogenic sediments and thereby reducing counting times. In particular, the introduction of a sodium-based dispersant and the inclusion of an organic co-solvent (acetone, IUPAC name propan-2-one) to increase the effective interaction between the alkaline digestion and organic matter was investigated. These techniques were assessed against simple water-based methods and this included consideration of the damage to tests under the various protocols.

## METHODS

To test the new methods, sediments collected from Snowy Flat (35° 33' 41" S, 148° 47' 5" E), located at 1618 m a.s.l. in the Australian Capital Territory, were used. Snowy Flat is one of the largest *Sphagnum-Richea-Empodisma* high altitude shrub bogs in Australia (Hope *et al*. 2009) and has an organic-rich sediment that includes minerogenic material from slope runoff and aeolian sources. Three cubic centimetres were sampled from a 3 cm wide section of the core centred on 145, 190 and 217 cm deep, and these were soaked in reverse osmosis (RO) water overnight to disaggregate the sediments. A known concentration of Safranin O stained *Lycopodium clavatum* L. spores (Stockmarr 1971) was added as an exotic marker to track the concentration of testate amoebae before the samples were sieved. In this, and all cases mentioned below, three nested sieves (20, 215 and 250 μm) were used, with the top sieve used as a cushion to reduce the strength of the flushing water, and the material for analysis was collected between the 20 and 215 μm sieves (Zheng *et al*. 2019). Each sample was carefully removed from the sieve, homogenised and subdivided into three subsamples. Although the volume of each subsample did not really matter (owing to the presence of the *Lycopodium* spores), the same volume was used. From that point on, the different subsamples were subjected to different procedures, as represented diagrammatically in Figure 1.

Treatment #1 was processed using the common, simple water-based preparation (Hendon & Charman 1997) that includes sieving only. Treatment #2 was processed using two dispersant steps, separated by sieving. In this research, sodium metapyrophosphate ($Na_4P_2O_7.10H_2O$) was used as the dispersant: the subsample was placed into a 250 ml flask with 100 ml of a 5 % aqueous sodium metapyrophosphate solution, and then placed on a shaker table for 4 hours at 110 rpm. Treatment #3 was processed using an alkaline treatment adapted from Barnett *et al*. (2013). In a 250 ml flask the subsamples were mixed with 20 ml RO water, 25 ml acetone and 5 ml of a 10 % aqueous sodium hydroxide solution (in that order) and then placed on a hotplate, which had been preheated to 80 °C, for 5 mins. (It is important that the sodium hydroxide solution is not added to the acetone until immediately before the treatment; acetone polymerises (albeit slowly) through an aldol reaction under basic conditions.) As described above, acetone acts as a co-solvent to solubilise the organic matter, which may potentially have been deprotonated under the basic conditions. Although a 10 % aqueous sodium hydroxide solution was used, it is diluted in the flask and so overall this is a mild alkaline treatment. Treatment #4 was a combination of the other protocols: the residue from Subsample #1 (water-only treatment) was then processed using two dispersant treatments, sieving after each, followed by the revised alkaline treatment (with acetone).


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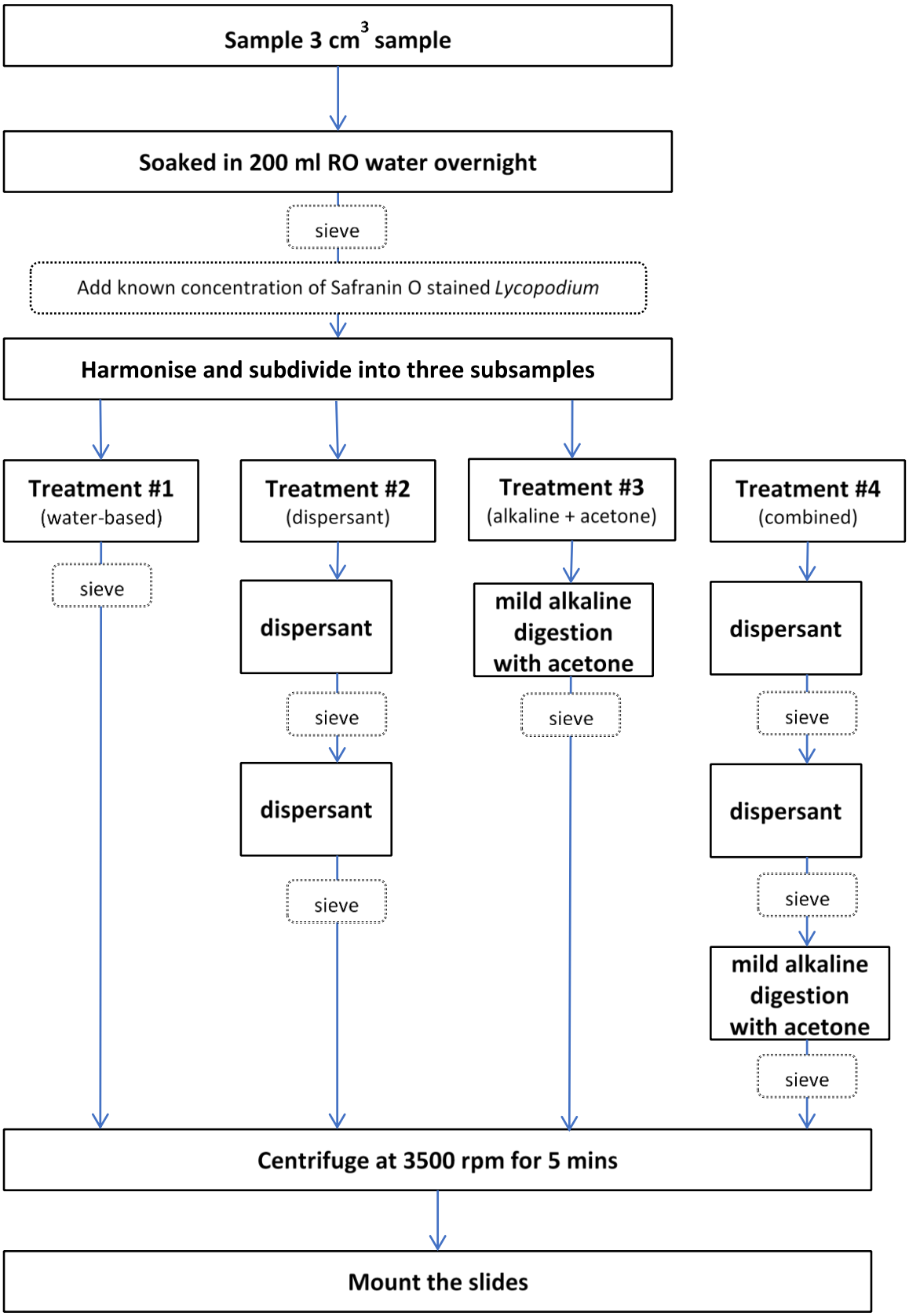


Figure 1. A flowchart summarising the four preparation procedures used in this study. "Sieve" indicates wet sieving using three nested sieves (20, 215 and 250 µm), with the target material collected between the 20 and 215 µm sieves. "Dispersant" indicates adding the subsample to 100 ml of 5 % sodium metapyrophosphate and 4 hours on a shaker table (110 rpm). The "alkaline digestion" was with 20 ml RO water + 25 ml acetone + 5 ml 10 % NaOH + sample, on a 80 °C hotplate for 5 mins.

For better comparison, the samples from the different preparation procedures were mounted using the same protocol (Figure 1). The subsamples were first centrifuged (at 3500 rpm for 5 min), the supernatant poured off, and the solution was then made up to 5 ml with RO water. The same amount of sample was then mounted on a microscope slide (with a 22 × 55 mm coverslip): a very dilute solution was used, in spite of the real concentration of testate amoebae, to avoid potentially overlooking testate amoebae obscured by organic matter. The testate amoebae were counted at 200× or 400× magnification until 200 *Lycopodium* spores were encountered. Damage was also assessed, which was defined as a test with any visible damage, and this was expressed as a percentage of the total count. The number of slides counted for each sample was between 6 and 8. Identification of the testate amoebae followed Sullivan & Booth (2007) and Southern Hemisphere endemic taxa were identified using Patagonian references (van Bellen *et al*. 2014); however, *Certesella martiali* in our research has no ridges around the neck. One un-identified taxon was included in the database as *Nebela* sp. 1 (see Zheng *et al*. 2019 for details).

All the numerical analyses were done in R 3.03 (RCoreTeam 2013) with the package "vegan" (Oksanen *et al*. 2015) or "rioja" (Juggins 2017). Before analyses, the compositional data were transformed into percentages. Chord distance was then used to quantify dissimilarities between the testate amoebae composition in the samples resulting from the different treatments. Chord distance is a well-used method to consider dissimilarity in palaeoecological studies (Overpeck *et al*. 1985, Gavin *et al*. 2003) and is particularly useful for closed compositional data because chord distance downweighs rare taxa, obtains good signal to noise, and emphasises the major patterns in the data (Overpeck *et al*. 1985, Gavin *et al*. 2003). After that, multivariate ANOVA based on dissimilarities (using the adonis function in vegan package) was used to test whether there were statistical differences ($\alpha = 0.05$) between the testate amoebae quantified after the different treatments (Anderson 2001). A principal coordinate analysis (PCoA) based on chord distance was also used to examine any differences among the different treatments and depths (Gower 1966). Finally, any potential effects of the different treatments on the testate amoebae community composition to the reconstruction of water-table depth were also investigated, and for this procedure we used a transfer function recently developed in Australia using the modern analogue technique (MAT) (Zheng *et al*. 2019).

## RESULTS

Table 1 and Figure 2 show raw count data and percentage data for testate amoebae in each sample. The concentration of testate amoebae across all treatments was relatively low compared with the concentrations using the alkaline treatment in Hendon & Charman (1997), ranging from 1692 to 5075 test/cm$^3$ (Figure 3). The water-based treatment (#1) always yielded the lowest concentration of testate amoebae and the dispersant treatment (#2) consistently yielded the highest concentration (Table 1 and Figure 3). Notably, the alkaline treatment with acetone (#3) resulted in only a slightly higher concentration of testate amoebae compared to the water-based treatment.

The sodium metapyrophosphate dispersant acts on the organic matter via deflocculation in a physical way, and no additional damage to the testate amoebae under the dispersant compared with the water-based treatment (Table 1) supports the contention that it does not damage testate amoebae. The total counts for the dispersant treatments always surpassed 50 tests, exceeding the minimum required total for the reconstruction of water-table depth (Payne & Mitchell 2009).

The revised alkaline digestion (with acetone, Treatment #3) generally resulted in a higher percentage of broken testate amoebae compared to the water-based treatment (#1) and the dispersant treatment (#2) (Table 1), reflecting the (harsher) removal of organic matter via chemical digestion. The exception was the sample from 217 cm depth in the Snowy Flat bog, which was the most minerogenic.

The extent of breakage of the testate amoebae was moderate for our combined treatment (#4), which used dispersants and then an alkaline treatment (with acetone). The counts (concentration) for this treatment were about the average, and always exceeded the simple water-based treatment. The real advantage from Treatment #4 was the significant enhancement of slide clarity (e.g. for Subsample 217-4 in Figure 4). In this case, combining the dispersant and revised alkaline treatments reduced organic matter in both size and the amount. This had a large effect as testate amoebae were not obscured by organic matter, and organic matter aggregates were much less apparent. The counting time under this treatment was reduced to 1–2 hours per slide, in contrast to 2–3 hours per slide under other treatments.

The sample from 217 cm depth was purposefully chosen as it came from a clay-rich section of the Snowy Flat bog sediment. This sample had a noticeably lower concentration of testate amoebae (Table 1), but a very clear response to our alternative


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Table 1. Raw counts of testate amoebae in each sample. Samples are grouped according to depth (145, 190 and 217 cm in a core from Snowy Flat bog) and then into the different preparation treatments (1 = water-based preparation; 2 = dispersant; 3 = mild alkaline + acetone; 4 = a combination of dispersant and alkaline + acetone). Damage is the percentage of damaged tests.

| Samples / Taxa | 145 | | | | 190 | | | | 217 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 145-1 | 145-2 | 145-3 | 145-4 | 190-1 | 190-2 | 190-3 | 190-4 | 217-1 | 217-2 | 217-3 | 217-4 |
| *Apodera vas* | | | | | 1 | 2 | | | | | | |
| *Assulina muscorum* | 2 | | | | 7 | 5 | 7 | 4 | 3 | 1 | | 2 |
| *Centropyxis arculeata* type | | | | | | | 1 | | | | | |
| *Centropyxis cassis* type | | 1 | | | | | | | | | | |
| *Centropyxis platystoma* type | 4 | 12 | 7 | 8 | 9 | 4 | 2 | 6 | 3 | 2 | 1 | 2 |
| *Certesella martiali* | 3 | 6 | 5 | 5 | 5 | 3 | 2 | 12 | 4 | 5 | 2 | 3 |
| *Cyclopyxis arcelloides* type | 1 | 6 | 5 | 3 | 9 | 7 | 13 | 14 | 2 | 1 | 3 | 1 |
| *Difflugia pritist* type | 1 | | 2 | 1 | 2 | 1 | 1 | | | 1 | 1 | |
| *Difflugia pulex* | | 4 | 2 | 2 | 4 | 1 | 3 | 3 | | | | |
| *Helelpera sphagni* | | 2 | | | | 1 | | | | 1 | | |
| *Heleopera sylvatica* | | 2 | 1 | 5 | | 8 | 4 | 2 | 3 | 6 | 6 | 8 |
| *Hyalosphenia subflava* | 28 | 60 | 33 | 48 | 29 | 37 | 34 | 35 | 15 | 34 | 24 | 21 |
| *Nebela barbata* | 1 | 1 | | | | | | | | | | |
| *Nebela bohemica* type | | | | | | | 1 | | | | | |
| *Nebela militaris* type | 1 | | | | 6 | | 1 | | 2 | | | |
| *Nebela sp1* | 3 | 2 | | | | | | | 1 | | | |
| *Nebela wailesi* type | 1 | | | | | 1 | | | | | | |
| *Physochila griseola* type | | 3 | 1 | | | | | | | | | |
| *Psedodifflugia fulva* type | 6 | 3 | 8 | 1 | 1 | 6 | 5 | 8 | 1 | 1 | | |
| *Quadrulella symmetrica* | 1 | | | | 1 | | | | | | | |
| *Trigonopyxis arcula* type | | | 2 | | | 2 | | 2 | 1 | 2 | | |
| *Trigonopyxis minuta* type | | 3 | | | | | | | | | 2 | |
| *Trinema/Corythion* type | | | | | | 2 | | | | | | |
| Total count | 52 | 105 | 66 | 73 | 74 | 80 | 74 | 86 | 35 | 54 | 39 | 37 |
| Damage | 8% | 13% | 23% | 16% | 11% | 10% | 18% | 19% | 14% | 7% | 10% | 16% |
| Concentration (test/cm$^3$) | 2513 | 5075 | 3190 | 3528 | 3576 | 3866 | 3576 | 4156 | 1692 | 2610 | 1885 | 1788 |

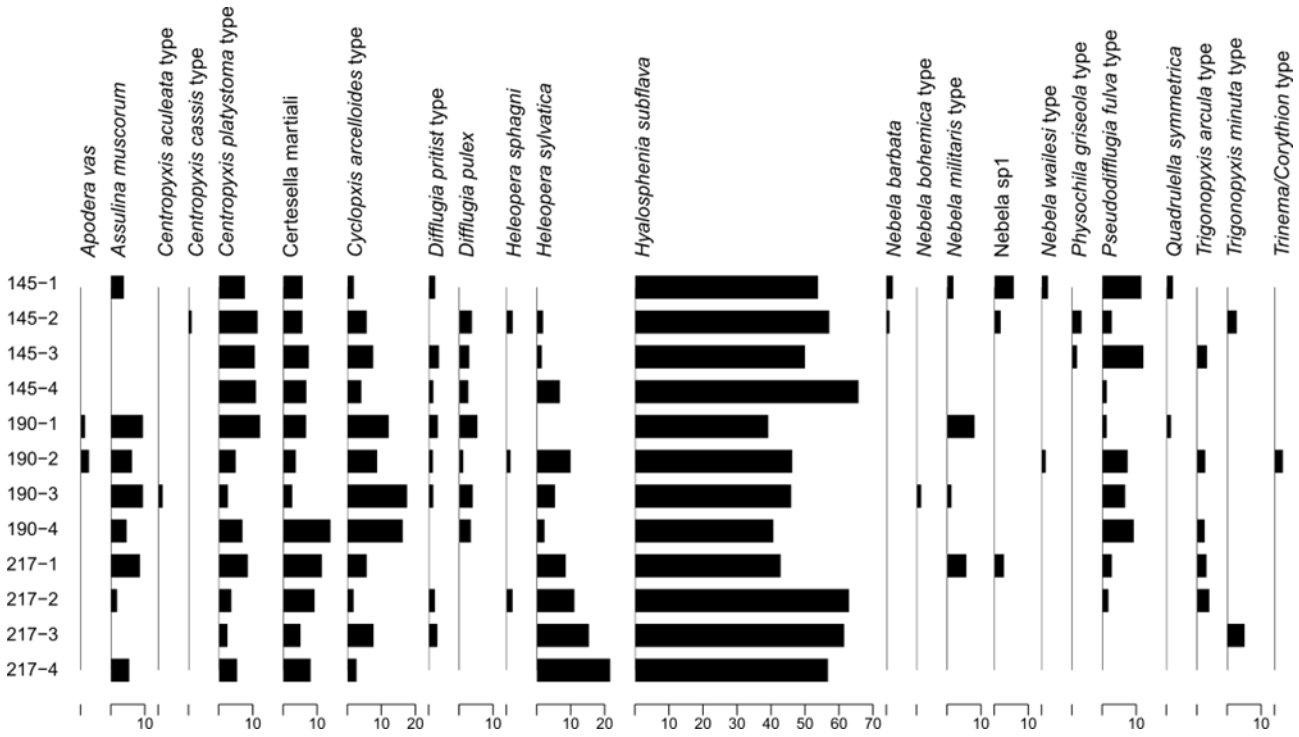


Figure 2. The relative proportion (percentage data) of taxa in each sample. The full names of the taxa are in Table 1. The samples are identified on the basis of their depth (145, 190 and 217 cm in the Snowy Flat bog core) and their treatment (1 = water-based preparation; 2 = dispersant; 3 = mild alkaline + acetone; 4 = a combination of dispersant and alkaline + acetone).

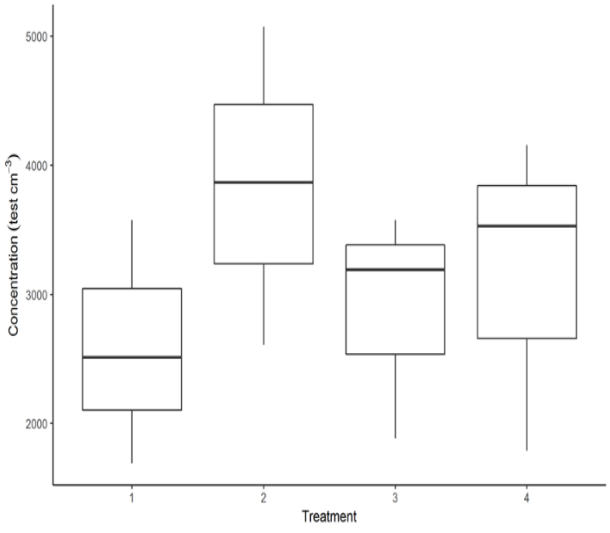


Figure 3. Boxplots showing the concentration of testate amoebae after the different treatments. (1 = water-based preparation; 2 = dispersant; 3 = mild alkaline + acetone; 4 = a combination of dispersant and alkaline + acetone.)

treatments was found, and perhaps not surprisingly, the dispersant treatment (#2) performed exceptionally well. The revised alkaline (+ acetone) treatment (#3) also performed well, and for this treatment (and our combination treatment #4) there was a significant reduction of fine organic particles (Figure 4) and increased clarity of the slides. Despite this enhanced clarity, some fine particles survived both treatments, and so very minerogenic samples are still problematic.

The recovered assemblages under the dispersant (#2) and alkaline plus acetone (#3) treatments were identical to the water-based treatment (Table 2 and Figure 2). *Hyaloshenia subflava* was the dominant taxon in each sample, accounting for about 50 % (Figure 2) of the total community assemblage. The missing or single occurrence of certain taxa in the water-based (#1) treatment, such as *Difflugia pulex* that was missing from 145-1 (in the water-based treatment of the sample from 145 cm depth), *Heleopera sylvatica* missing in 145-1 and 190-1, and one occurrence of *Pesudodifflugia fulva* in 190-1,


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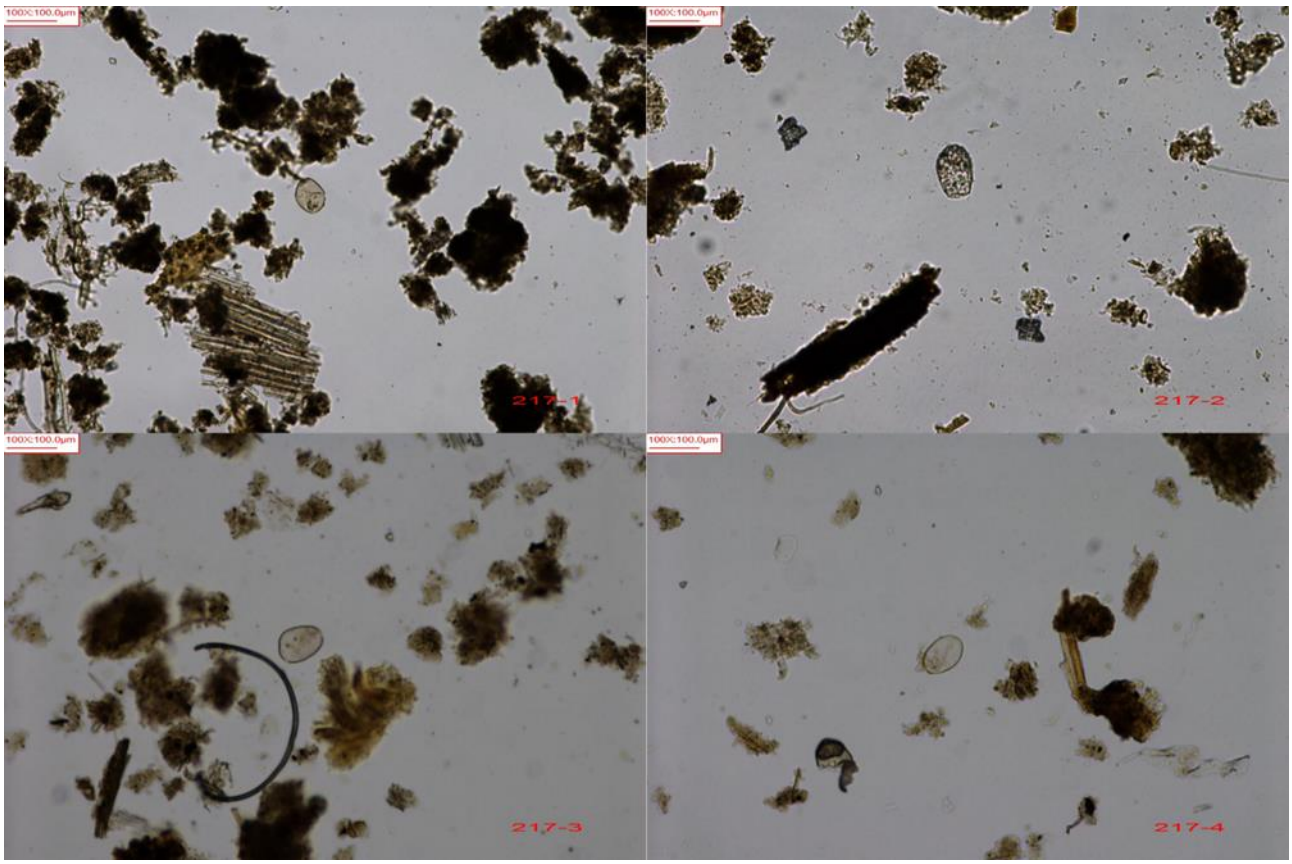


Figure 4. Microscope slide clarity of samples from depth 270 cm with the four different treatments under 100× magnification.

Table 2. Pairwise, multivariate ANOVA on dissimilarities (based on percentages) between the different treatments. (Treatment 1 = water-based preparation; 2 = dispersant; 3 = mild alkaline + acetone; 4 = a combination of dispersant and alkaline + acetone.) P is the calculated significance of the comparison: the results indicate no significant difference ($\alpha = 0.05$) in the community composition between all of the treatments.

| Treatments | $R^2$ | P |
|---|---|---|
| 1–2 | 0.26 | 0.40 |
| 1–3 | 0.21 | 0.60 |
| 1–4 | 0.17 | 0.60 |
| 2–3 | 0.11 | 0.80 |
| 2–4 | 0.07 | 1.00 |
| 3–4 | 0.08 | 0.90 |

implies that small taxa may be easily overlooked under the water-based treatment. Conversely, *Nebela militaris* most commonly only appeared in the water-based (#1) treatment (e.g. in 145-1 while it was not found in 145-2, 145-3 and 145-4; in 217-1 but not in 217-2, 217-3 and 217-4; abundantly in 190-1 but not in the others), which suggests that it was fragile and damaged by the other treatments.

Figure 5 reveals that there was very little overlap among the samples from the three different depths analysed, suggesting different assemblages of testate amoebae at these depths. Figure 5 also depicts considerable overlap between the testate amoebae assemblages after the different treatments tested, although there was no overlap between Treatment #1 and Treatment#2. In contrast, the multivariate ANOVA analysis (Table 2) identified no significant differences between all pairwise treatments. Overall, the multivariate analyses suggest that the integrity of the testate amoebae community composition was maintained across the different treatments. In addition, the reconstructed water-table depths were of a very similar magnitude within each depth sample and shared a consistent (slightly increasing) trend from 145 to 217 cm sample depth (Figure 6).


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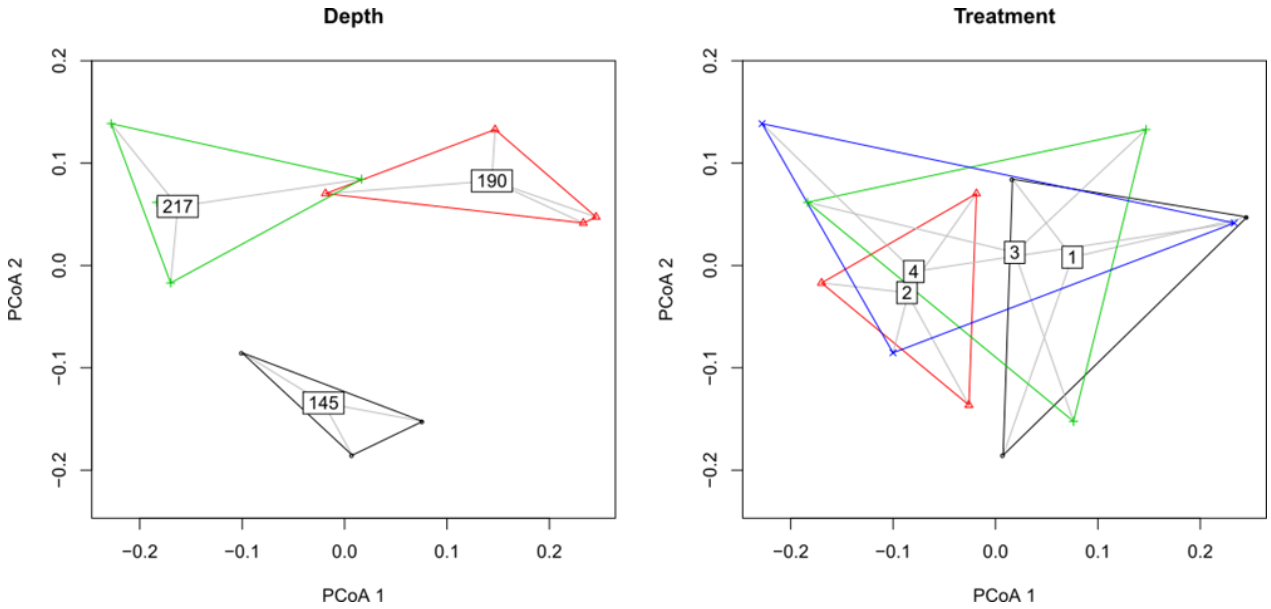


Figure 5. Principal coordinate analysis (PCoA), based on chord distance, among testate amoebae assemblages against depth and treatment.

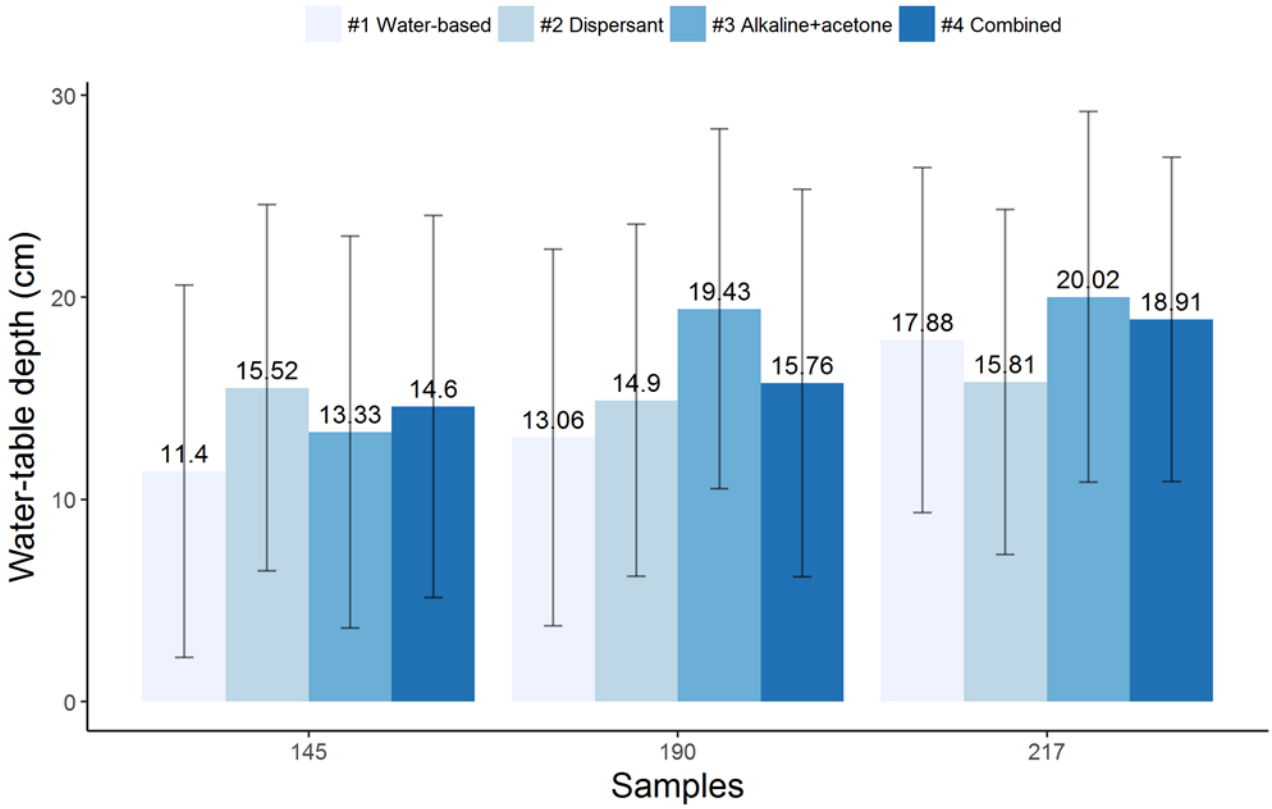


Figure 6. Reconstructed water-table depth using the modern analogue technique method based on a transfer function developed in south-eastern Australia (Zheng *et al*. 2019). Error bars are sample-specific errors (equivalent to 95 % confidence intervals).

## DISCUSSION

It is clear that the revised dispersant (sodium pyrophosphate) treatment worked very well to concentrate testate amoebae, yielding the highest concentration of testate amoebae with the lowest percentage of damage. Although commonly used in cognate disciplines (e.g. palynology, Faegri *et al*. 1989) to remove minerogenic particles, the revised dispersant treatment has been, apparently, previously overlooked in testate amoebae protocols. Sodium pyrophosphate is an effective dispersant for both minerogenic particles and organic matter (Bates *et al*. 1978, Bremner & Lees 1949) as the sodium replaces multivalent cations to stop fine particles from flocculating, while the pyrophosphate anion interacts with the replaced multivalent cations and removes them from suspension. This means that deflocculated, fine particles (smaller than 20 µm in this research) can be removed by wet sieving. This physical process to remove fine organic matter was shown to result in negligible damage to testate amoebae, in contrast to chemical digestion by the mild alkaline treatment.

Notably, the revised dispersant treatment was as effective as, or more effective than, the revised mild-alkaline-digestion in terms of reducing extraneous organic matter. The revised-alkaline-digestion (with acetone as a co-solvent) also increased the concentration of testate amoebae with negligible additional damage. The combination of the dispersant and alkaline treatments yielded the best slide clarity, thereby significantly reducing the counting time, and this was balanced against only a slight increase in damage.

The methods developed here have not yet been tested in other depositional environments and their effect on other testate amoebae is also unknown. The (sub)fossil testate amoebae assemblages in the Snowy Flat bog sediment core are not particularly species rich and were dominated by *H. subflava*. This species belongs to the 'protein + calcium' type, which secretes a proteinaceous test (Mitchell *et al*. 2008b). They have been described as one of the least resistant taxa to decomposition (Lousier & Parkinson 1981). We cannot comment on the effect of the revised dispersant method on taxa of other types (e.g. idiosomes + organic and xenosomes), but they are considered to be more robust as they incorporate organic or mineral particles into their tests (Payne 2007). Although this experiment did not include many taxa from the most vulnerable type 'idiosomes' (Mitchell *et al*. 2008b), such as *Euglypha* or *Trinema*, the physical interaction of the dispersant suggests that there would be less damage compared to an alkaline-based procedure. *Euglypha* are common in modern samples, but their rarity in (sub)fossil samples (Wilmshurst *et al*. 2003, Payne 2007) means they are often not important in any transfer function reconstruction based on the testate amoebae community and have little influence on the reconstruction of interest. Booth & Jackson (2003) excluded taxa absent below the acrotelm from their training set for reconstruction. Nonetheless, more experiments are needed to evaluate comprehensively the effectiveness of this revised dispersant method for concentrating testate amoebae in minerogenic sediments, especially in the Northern Hemisphere where the testate amoebae community differs.

In the protocols tested here, all of the treatments began with overnight soaking in RO water (Figure 1), and this step primarily aimed to disaggregate and release any testate amoebae from the sediment. The findings support the contention that efficiency can be increased by placing the sample in sodium pyrophosphate instead of water (in this initial step). Hence, it is reasonable to use an aqueous solution of dispersant at the first step to aid the disaggregation of the sediment. This small change resulted in our final laboratory method for concentrating testate amoebae, as described in Table 3. It should be emphasised that the dispersant treatment alone may be sufficient to improve slide clarity, and hence experimentation might allow Step 4 in Table 3 to be eliminated. This protocol has been specifically developed for organic sediments that have a more minerogenic nature, but it is also likely to increase slide clarity and reduce counting time for the quantification of testate amoebae in minerogenic-rich sediments such as saltmarshes, without significantly affecting the integrity of the species assemblage/composition.

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Table 3. A recommended laboratory preparation protocol for the concentration of testate amoebae.

| | |
|---|---|
| Step 1 | **Subsampling**<br>Place a known volume (e.g. 1 $cm^3$) of sediment into a 250 ml flask. |
| Step 2 | **1st dispersant treatment**<br>Add 100 ml of a 5 % (by mass) sodium pyrophosphate solution and place on a shaker table overnight at 110 rpm. |
| Step 3 | **2nd dispersant treatment**<br>Wash the sample through three nested sieves (*e.g. 420, 300 and 15 μm), transfer the material between the 300 and 15 μm sieves into 100 ml of a 5 % (by mass) sodium pyrophosphate solution and place on a shaker table for 4 hours at 110 rpm. |
| Step 4** | **Alkaline treatment**<br>Wash the material through the nested sieves and transfer the material isolated between 300 and 15 μm sieves into a beaker and then add 20 ml RO water, 25 ml acetone and 5 ml 10 % NaOH (by mass) in that order. Place this beaker on a preheated (80 ºC) hotplate for 5 mins. |
| Step 5 | **Sieving and centrifuging**<br>Wash the material through the nested sieves. (Add aliquot of stained *Lycopodium* spores at this stage for concentration measure, if desired.) Wash the material isolated between the 300 and 15 μm sieves into centrifuge tubes with RO water and centrifuge at 3000 rpm for 5 mins. |
| Step 6 | **Mounting and storing**<br>Carefully pour off the supernatant, mount the sample onto a microscope slide with a coverslip and seal with nail polish. The remaining sample can be stored in water in a fridge (e.g. 4 ºC). |

* the accepted procedure for the preparation of testate amoeba samples uses 300 and 15 μm mesh sieves. A larger sieve above the 300 μm sieve was used here, to protect the samples during wet-sieving.

** the alkaline treatment can be eliminated if Steps 1–3 are sufficient to provide mounts of acceptable clarity.

## AUTHOR CONTRIBUTIONS

XZ, GH and SDM conceived the study. XZ did the laboratory preparations and identification of testate amoeba. JBH oversaw the chemistry and in particular suggested acetone as a co-solvent. XZ and SDM wrote the first draft of this article, but all authors contributed to later drafts and commented on all versions of the manuscript.

## REFERENCES

Anderson, M.J. (2001) A new method for non-parametric multivariate analysis of variance. *Austral Ecology*, 26, 32–46.

Barnett, R.L., Charman, D.J., Gehrels, W.R., Saher, M.H. & Marshall, W.A. (2013) Testate amoebae as sea-level indicators in northwestern Norway: developments in sample preparation and analysis. *Acta Protozoologica*, 52, 115–128.

Barnett, R.L., Newton, T.L., Charman, D.J. & Gehrels, W.R. (2017) Salt-marsh testate amoebae as precise and widespread indicators of sea-level change. *Earth-Science Reviews*, 164, 193–207.

Bates, C.D., Coxon, P. & Gibbard, P.L. (1978) A new method for the preparation of clay-rich sediment samples for palynological investigation. *New Phytologist*, 81, 459–463.

Booth, R.K. & Jackson, S.T. (2003) A high-resolution record of late Holocene moisture variability from a Michigan raised bog. The Holocene, 13, 863–878.

Booth, R., Lamentowicz, M. & Charman, D. (2010) Preparation and analysis of testate amoebae in peatland paleoenvironmental studies. *Mires and Peat*, 7(02), 1–7.

Bremner, J. & Lees, H. (1949) Studies on soil organic


 DOI: 10.19189/MaP.2018.OMB.380

matter: Part II. The extraction of organic matter from soil by neutral reagents. *The Journal of Agricultural Science*, 39, 274–279.

Charman, D.J., Gehrels, W.R., Manning, C. & Sharma, C. (2010) Reconstruction of recent sea-level change using testate amoebae. *Quaternary Research*, 73, 208–219.

Cwynar, L.C., Burden, E. & McAndrews, J.H. (1979) An inexpensive sieving method for concentrating pollen and spores from fine-grained sediments. *Canadian Journal of Earth Sciences*, 16, 1115–1120.

Faegri, K., Kaland, P.E. & Krzywinski, K. (1989) *Textbook of Pollen Analysis*. Fourth edition, John Wiley & Sons Ltd., Chichester, 328 pp.

Gavin, D.G., Oswald, W.W., Wahl, E.R. & Williams, J.W. (2003) A statistical approach to evaluating distance metrics and analog assignments for pollen records. *Quaternary Research*, 60, 356–367.

Gower, J.C. (1966) Some distance properties of latent root and vector methods used in multivariate analysis. *Biometrika*, 53, 325–338.

Hendon, D. & Charman, D.J. (1997) The preparation of testate amoebae (Protozoa: Rhizopoda) samples from peat. *The Holocene*, 7, 199–205.

Hope, G., Nanson, R. & Flett, I. (2009) *The Peat-Forming Mires of the Australian Capital Territory*. Technical Report 19, The Department of Territory and Municipal Services, ACT Government, Canberra, 57 pp.

Juggins, S. (2017) rioja: Analysis of Quaternary Science Data. R package version (0.9-15). R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

Kaur, A. & Fanourakis, G. (2016) The effect of type, concentration and volume of dispersing agent on the magnitude of the clay content determined by the hydrometer analysis. *Journal of the South African Institution of Civil Engineering*, 58, 48–54.

Lousier, J.D. & Parkinson, D. (1981) The disappearance of the empty tests of litter- and soil-testate amoebae (Testacea, Rhizopoda, Protozoa). *Archiv Fur Protistenkunde*, 124, 312–336.

Mitchell, E.A.D., Charman, D.J. & Warner, B.G. (2008a) Testate amoebae analysis in ecological and paleoecological studies of wetlands: past, present and future. *Biodiversity and Conservation*, 17, 2115–2137.

Mitchell, E.A.D., Payne, R.J. & Lamentowicz, M. (2008b) Potential implications of differential preservation of testate amoeba shells for paleoenvironmental reconstruction in peatlands. *Journal of Paleolimnology*, 40, 603–618.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H. & Wagner, H. (2015) vegan: Community Ecology Package. R package version 2.2-1. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

Overpeck, J.T., Webb, T. & Prentice, I.C. (1985) Quantitative interpretation of fossil pollen spectra: Dissimilarity coefficients and the method of modern analogs. *Quaternary Research*, 23, 87–108.

Payne, R. (2007) Laboratory experiments on testate amoebae preservation in peats: Implications for palaeoecology and future studies. *Acta Protozoologica*, 46, 325–332.

Payne, R.J. (2011) Can testate amoeba-based palaeohydrology be extended to fens? *Journal of Quaternary Science*, 26, 15–27.

Payne, R.J. & Mitchell, E.A.D. (2009) How many is enough? Determining optimal count totals for ecological and palaeoecological studies of testate amoebae. *Journal of Paleolimnology*, 42, 483–495.

Porter, S.M. & Knoll, A.H. (2000) Testate amoebae in the Neoproterozoic Era: evidence from vase-shaped microfossils in the Chuar Group, Grand Canyon. *Paleobiology*, 26, 360–385.

RCoreTeam (2013) R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

Stockmarr, J. (1971) Tablets with spores used in absolute pollen analysis. *Pollen et spores*, 13, 615–621.

Sullivan, M. & Booth, R. (2007) *Key of Testate Amoebae Inhabiting* Sphagnum-*Dominated Peatlands with an Emphasis on Taxa Preserved in Holocene Sediments*. Lehigh University, Bethlehem, Pennsylvania, USA, 46 pp.

Swindles, G.T, Lamentowicz, M., Reczuga, M. & Galloway, J.M. (2016) Palaeoecology of testate amoebae in a tropical peatland. *European Journal of Protistology*, 55 Part B, 181–189.

Van Bellen, S., Mauquoy, D., Payne, R.J., Roland, T.P., Daley, T.J., Hughes, P.D.M., Loader, N.J., Street-Perrott, F.A., Rice, E.M. & Pancotto, V.A. (2014) Testate amoebae as a proxy for reconstructing Holocene water table dynamics in southern Patagonian peat bogs. *Journal of Quaternary Science*, 29, 463–474.

Warner, B.G. & Charman, D.J. (1994) Holocene changes on a peatland in northwestern Ontario interpreted from testate amebas (Protozoa)


 DOI: 10.19189/MaP.2018.OMB.380

analysis. *Boreas*, 23, 270–279.
Wilmshurst, J.M., Wiser, S.K. & Charman, D.J. (2003) Reconstructing Holocene water tables in New Zealand using testate amoebae: differential preservation of tests and implications for the use of transfer functions. *The Holocene*, 13, 61–72.
Zheng, X., Amesbury, M.J., Hope, G., Martin, L.F. & Mooney, S.D. (2019) Testate amoebae as a hydrological proxy for reconstructing water-table depth in the mires of south-eastern Australia. *Ecological Indicators*, 96, 701–710.

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