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ORIGINAL PAPER

# Refining the palaeoecology of lacustrine testate amoebae: insights from a plant macrofossil record from a eutrophic Scottish lake

Stephen V. Prentice · Helen M. Roe · Helen Bennion · Carl D. Sayer · Jorge Salgado

**Abstract** Arcellinida (lobose testate amoebae) preserve well in lake sediments and have shown great potential as palaeolimnological indicators, but a limited understanding of their ecology prevents more in-depth interpretations of both contemporary and fossil assemblages. Loch Leven, Scotland, has a well-documented history of nutrient-enrichment and associated lake biological change, creating a temporal gradient along which testate amoeba ecology can be investigated. Plant macrofossil data from a dated sediment core (LEVE14) indicate that a transition from oligo-mesotrophic to eutrophic conditions occurred over at least the last 200 years, enabling direct comparisons to be drawn with fossil testate amoeba assemblages from the same core. Cluster analysis and principal components analysis show that testate amoebae and macrophytes responded synchronously to an early phase of nutrient-enrichment after ca. AD 1200, and to anthropogenic water-level lowering and further enrichment after ca. AD 1830. The results of indicator species analysis (IndVal) support the suggestion inferred previously that *Cucurbitella tricuspis* is an indicator of nutrient-enrichment, while the association of some less abundant taxa, specifically *Difflugia amphora* and *Mediolus corona*, with eutrophic environments is also corroborated. Overall, the testate amoeba assemblage response appears to reflect a complex interaction of factors which were ultimately driven by eutrophication. These include variations in microhabitat associated with changing plant structure and sedimentary conditions influenced by macrophyte community succession. In particular, a shift from an oligo-mesotrophic macrophyte assemblage composed of isoetid plants to one dominated by *Potamogeton* spp. and dense stands of Characeae is suggested to influence the relative proportions of sediment dwelling and plant-associated testate amoebae. The results highlight the utility of testate amoebae as benthic indicators of lake nutrient-enrichment and demonstrate the value of using multi-proxy palaeolimnological data to refine the ecology of lesser-studied palaeoecological groups.

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## Introduction

The fossil remains of many biological groups preserve well in lake sediments and have been used successfully to reconstruct long-term ecological changes in shallow lakes, especially associated with eutrophication. These include plant macrofossils (Davidson et al. 2005; Sayer et al. 2010b), diatoms (Anderson et al. 1993; Bennion et al. 2011), cladocerans (Brodersen et al. 1998; Davidson et al. 2011), chironomids (Brooks et al. 2001; Zhang et al. 2012) and non-pollen palynomorphs (NPP) (Drljepan et al. 2014; Volik et al. 2016). Due to a lack of instrumental monitoring of lake conditions, palaeolimnological approaches are essential for assessing long-term trends, determining pre-disturbance conditions (Bennion et al. 2011) and for informing conservation strategies (Kowalewski et al. 2013). Multi-proxy approaches are increasingly being used in palaeolimnological studies (Sayer et al. 2010b; Wiik et al. 2015; Sayer et al. 2016) to provide a more comprehensive understanding of community-wide changes resulting from eutrophication and other anthropogenic drivers in lakes, including the sequence and mechanisms of biological change (Sayer et al. 2010a; Bennion et al. 2015). Typical groups used in multi-proxy work include diatoms, plant macrofossils, cladocerans and algal pigments and invariably the addition of each group enhances understanding. To further this research it is important to explore other potential indicators of changing lake biological structure and ecology and one such group are testate amoebae.

Testate amoebae are a group of shelled protozoans common to a range of aquatic and terrestrial environments, from lakes and ponds to saltmarshes and peatlands (Roe et al. 2002; Roe and Patterson 2006; Amesbury et al. 2016). In lake environments lobose forms of the group dominate (Rhizopoda; Arcellinida). Testate amoebae are useful in palaeolimnological studies due to their rapid generation times, enabling high-resolution sampling (Medioli and Scott 1983), and in particular good preservation and high abundances in sediments (McCarthy et al. 1995). In addition, as bottom-dwelling organisms, they occupy a critical habitat for which environmental reconstructions are widely lacking, excepting chironomid-oxygen studies (Quinlan and Smol 2002). Despite an expanding geographical range of lake testate amoeba research that initially centred on Europe (Asioli et al.

1996; Wall et al. 2010b) and North America (Collins et al. 1990; Patterson and Kumar 2002; Reinhardt et al. 2005) and more recently Asia (Ju et al. 2014) and South America (Caffau et al. 2015), UK lake studies are very few (Ellison 1995; Wiik et al. 2015). Within the UK, research has largely been confined to peatlands and saltmarshes, with the focus generally on quantitative palaeohydrological reconstructions (Swindles et al. 2009) and sea-level change (Gehrels et al. 2001; Barnett et al. 2017). In lakes, testate amoebae are likely to be especially sensitive to changes at the sediment–water interface, such as water temperature (Collins et al. 1990), salinity (Roe and Patterson 2006; 2014), metal contamination (Kihlman and Kauppila 2012; Nasser et al. 2016), pH (Patterson and Kumar 2000a; Escobar et al. 2008; Patterson et al. 2013), dissolved oxygen (Drljepan et al. 2014) and/or variations in substrate characteristics (Reinhardt et al. 2005; Kihlman and Kauppila 2012). In summarising early research (Schönborn et al. 1965; Schönborn 1967; Laminger 1973), Tolonen (1986) noted that, amongst other drivers, eutrophication may be an important factor controlling testate amoeba assemblages. Recent studies of the modern distribution of lobose forms of testate amoebae in lakes along urban–rural (Roe et al. 2010) and nutrient (Ju et al. 2014) gradients have extended this early work and confirmed that a strong association exists between testate amoebae and phosphorus and nitrogen. Down-core analyses of testate amoeba assemblages have been used to infer lake trophic status change, both qualitatively (Reinhardt et al. 2005; Qin et al. 2009; Drljepan et al. 2014) and quantitatively (Patterson et al. 2012). However, a limited understanding of testate amoeba ecology, in particular the causal mechanisms for community change associated with eutrophication and other processes, prevents a more in-depth interpretation of both modern and fossil assemblages.

This paper aims to improve the understanding of testate amoeba ecology within the context of a shallow, eutrophic lake with a well-documented history of nutrient-enrichment and associated lake biological change, while expanding the limited body of UK lake testate amoeba research. Our focal site, Loch Leven, has been the subject of several previous palaeolimnological investigations, including analyses of plant macrofossils (Salgado et al. 2010) and diatoms (Bennion et al. 2012) which reveal a history of

enrichment from oligo-mesotrophic to eutrophic conditions. Furthermore, long-term monitoring of water chemistry, macrophytes, algae, zooplankton, macroinvertebrates, fish and wildfowl, spanning more than 40 years (May and Spears 2012), permit a detailed understanding of eutrophication in Loch Leven and subsequent recovery in the late twentieth century. The objectives of this research are: (i) to compare the timings of changes in the testate amoeba record with those of existing plant macrofossil records from the same core and with wider knowledge of lake changes derived from palaeo-diatom studies and long-term monitoring data; and (ii) based on these comparisons, to provide an improved understanding of testate amoeba ecology.

## Study site

Loch Leven ($56°11'55''N$, $3°22'46''W$) is a large (13.3 km$^2$), shallow (mean depth 3.9 m), lowland (107 m.a.s.l.) lake situated near the town of Kinross in central Scotland (Fig. 1). The loch is currently alkaline and moderately eutrophic, with mean annual average total phosphorus (TP) and chlorophyll *a* (chl *a*) concentrations (2008–2010) of 33 and 21 µg L$^{-1}$, respectively (Dudley et al. 2012a). The surrounding catchment comprises 80% agricultural land (Castle et al. 1999), with water entering the loch via four main inflows (Fig. 1) from farms, villages and the adjacent town of Kinross.

Historical (Jupp et al. 1974; Dudley et al. 2012b) and palaeoecological (Haworth 1972; Salgado et al. 2010; Bennion et al. 2012) records reveal progressive nutrient-enrichment in Loch Leven throughout the nineteenth and twentieth centuries from oligo-mesotrophic to eutrophic conditions. In particular, the loss of *Isoetes lacustris* prior to ca. 1850 and a shift to an elodeid dominated macrophyte community post-1910 indicate an early influence of eutrophication (Salgado et al. 2010). A marked shift to a more planktonic diatom community throughout the twentieth century is also evident in several sediment cores from the site (Haworth 1972; Bennion et al. 2012), particularly since around 1940 (Bennion et al. 2012). This suggests enhanced pelagic primary production relative to the benthic zone caused by increased turbidity impeding photosynthesis on the lakebed.

The main sources of nutrients to the loch are agricultural runoff and sewage discharge (May et al. 2012). Consequently, a catchment management plan was implemented in the late 1980s and 1990s aimed at reducing point sources of pollution (D'Arcy et al. 2006), which resulted in a 60% reduction in catchment TP (May et al. 2012). This can be attributed to a number of factors, including the upgrading of sewage treatment works and the cessation of effluent discharge from a woollen mill in 1988 (D'Arcy et al. 2006). In addition to a dynamic trophic history, Loch Leven has undergone significant morphological change as water levels were lowered by 1.5 m in 1830 to permit agricultural expansion along its shores (Morgan 1970). This was achieved by cutting a new channel for the loch's main outflow, the River Leven.

## Methods

### Core collection and chronology

As part of a previous palaeolimnological study (Salgado et al. 2010), a 141-cm shallow-water (2.2 m) sediment core (LEVE14) was collected in May 2006 off the eastern shore of St. Serf's Island in the southeast corner of Loch Leven (Fig. 1), using an adapted Livingstone (7.4-cm diameter) piston corer. A further sediment core (LEVE11) was collected from a more central lake location (Fig. 1) at a depth of 4 m as part of a palaeolimnological study investigating diatom responses to eutrophication and lake recovery (Bennion et al. 2012). Diatom assemblages from core LEVE11 are used in this study to provide further context for understanding lake-wide changes in eutrophication, while core LEVE14 provided material for testate amoeba analysis and a direct comparison with the plant macrofossil record of Salgado et al. (2010).

Core LEVE14 was dated radiometrically using $^{210}$Pb, $^{226}$Ra and $^{137}$Cs analysis, the results of which are fully described in Salgado et al. (2010). In an attempt to further constrain the chronology of the LEVE14 core, we obtained three accelerator mass spectrometry (AMS) $^{14}$C dates from the middle and basal sections of the core. Owing to a lack of terrestrial macrofossils, bulk sediment and charcoal samples were selected for AMS dating (Electronic Supplementary Material ESM1), which was undertaken at the $^{14}$CHRONO Centre for Climate the Environment and Chronology, Queen's University Belfast.
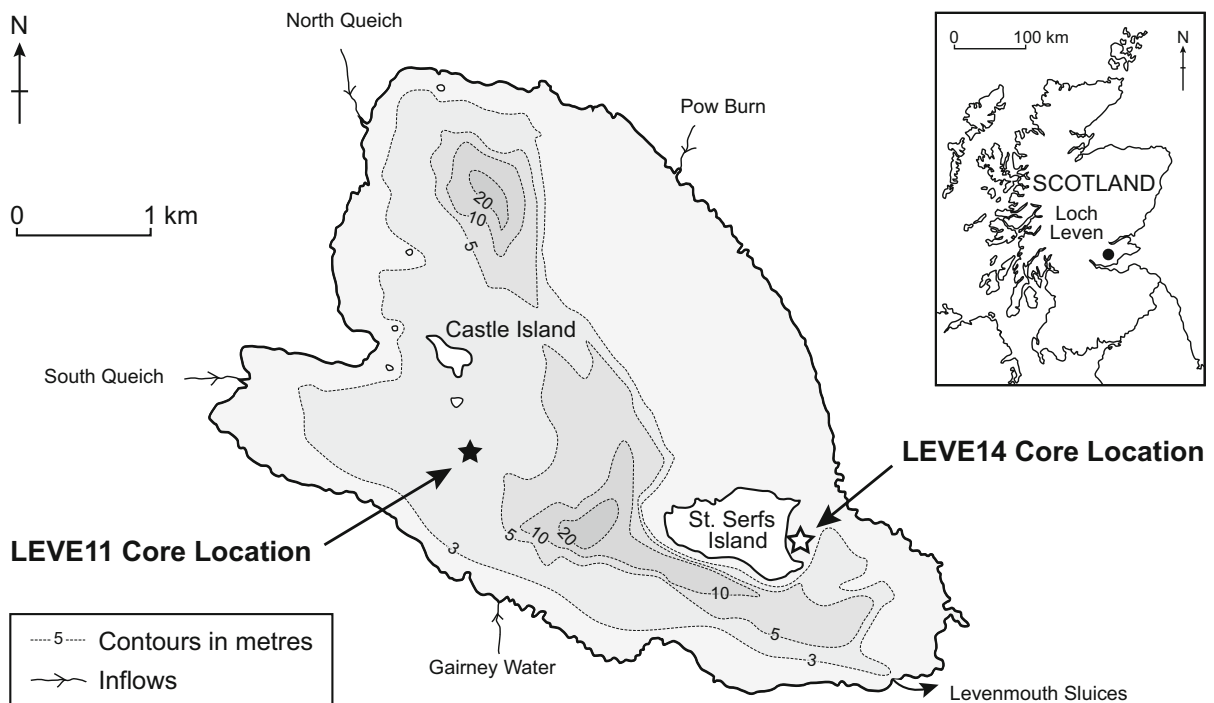
**Fig. 1** Map showing the location and bathymetry of Loch Leven and the locations of sediment cores from previous studies of plant macrofossils (LEVE14) (Salgado et al. 2010) and diatoms (LEVE11) (Bennion et al. 2012). Core LEVE14 is used in this study for testate amoeba analysis

### Sediment and fossil analyses

Thirty-one samples were analysed for plant macrofossils and 51 for loss-on-ignition (LOI) from core LEVE14 by Salgado et al. (2010). Forty-two subsamples from core LEVE14 were selected and prepared for testate amoeba and particle size analyses (2-cm intervals above 50 cm and 4–10-cm intervals below 50 cm). In this study an additional 18 core samples were subjected to LOI analysis (Dean 1974) to match the other analyses undertaken in this study.

Preparation of testate amoebae followed established protocols, previously developed for lake testate amoebae (Patterson and Kumar 2002). Samples of 0.5 cm$^3$ were agitated for one hour using a platform shaker to separate cohesive sediments, and wet-sieved though 300 μm and 37 μm meshes to remove coarse organics and fine silts and clays. Although sieve sizes as small as 15 μm have been used to retain the smallest of specimens in peatland studies (Swindles et al. 2009), 37 μm is generally accepted as an appropriate sieve size to retain the majority of lake taxa (Patterson and Kumar 2000b).

This size fraction was also chosen to ensure comparability with recent lacustrine studies (Qin et al. 2009; Roe et al. 2010; Nasser et al. 2016). The 37–300 μm sieved residues were split into eight aliquots using a wet-splitter (Scott and Hermelin 1993) and fossil testate amoebae were quantified in aqueous solution using a Nikon light dissection binocular microscope at ×50–63 magnification.

Testate amoebae have been shown to display significant amounts of ecophenotypic morphological variability (Medioli et al. 1987). Following many previous lake studies (Asioli et al. 1996; Patterson and Kumar 2000a; Kihlman and Kauppila 2012; Roe and Patterson 2014), a strain-based nomenclature was implemented. Morphotypes of the same species are designated informal 'strain' names to avoid potentially unwarranted descriptions of new species (Asioli et al. 1996; Patterson and Kumar 2002). Although these 'strains' are not formally recognised under the International Code of Zoological Nomenclature, many studies have proved their worth in terms of ecological indication (Boudreau et al. 2005; Escobar et al. 2008; Roe et al. 2010; Nasser et al. 2016). Testate amoebae

were identified with reference to published scanning electron micrographs (Roe et al. 2010; Patterson et al. 2012), illustrations (Medioli and Scott 1983) and lacustrine testate amoebae keys (Kumar and Dalby 1998). Where possible, and to gain statistically significant counts, >150 specimens were quantified per sample (Patterson and Fishbein 1989; Wall et al. 2010a). Selected specimens were mounted for scanning electron micrograph (SEM) imaging, obtained using a JEOL 6500 FEG SEM at Queen's University Belfast Electron Microscopy Unit and a Tescan Vega-II XMU VP SEM at Carleton University, Ottawa Nano Imaging Facility (Fig. 2).

To investigate the influence of changing sediment composition on testate amoeba assemblages, particle size distributions (% sand, silt, clay) were determined by laser diffraction using a Malvern Mastersizer-2000. Sediments were treated with a hydrogen peroxide ($H_2O_2$) digestion to remove organic matter prior to analysis (van Hengstum et al. 2007). Samples were subsequently rinsed in de-ionised water, centrifuged and the supernatant was decanted. Subsamples of the treated sediment were ultrasonically disaggregated and analysed by laser diffraction.

Data analysis

Testate amoeba counts were converted to relative abundance and plant macrofossil data standardised as concentrations (per 100 $cm^3$). The construction and zonation of stratigraphic diagrams was performed using TILIA and TILIAGRAPH (Grimm 1991). Zones were identified for testate amoeba and plant macrofossil assemblages using constrained incremental sum of squares (CONISS) cluster analysis (Grimm 1987). In accordance with Birks (1986), only testate amoeba taxa >5% abundance were included in the analysis.

Indicator taxa were identified for each stratigraphic zone in the testate amoeba assemblage using the IndVal procedure of Dufrêne and Legendre (1997). Those taxa retained had significant ($p < 0.05$) $IndVal_j$ scores based on permutation tests with 1000 random iterations.

To measure changes in testate amoeba taxonomic diversity over time, the Shannon Diversity Index (SDI) was applied (Shannon 1948). Environments are considered stressed where SDI is <1.5, in transition between 1.5 and 2.5 and stable between 2.5 and 3.5 (Magurran 1988; Patterson and Kumar

2000b). The counting of entire aliquots to determine testate amoeba sample concentrations can result in different count sizes above the minimum threshold of 150. To check whether this caused variation in the SDI values, the testate amoeba data were subjected to rarefaction (Birks 2012) and standardised to 150 specimens per sample. The rarefied samples were found to display very similar SDI values to those derived from the original counts (ESM2), suggesting that variations in count sizes above the minimum threshold of 150 do not significantly bias the SDI values. Consequently, the original SDI values were retained.
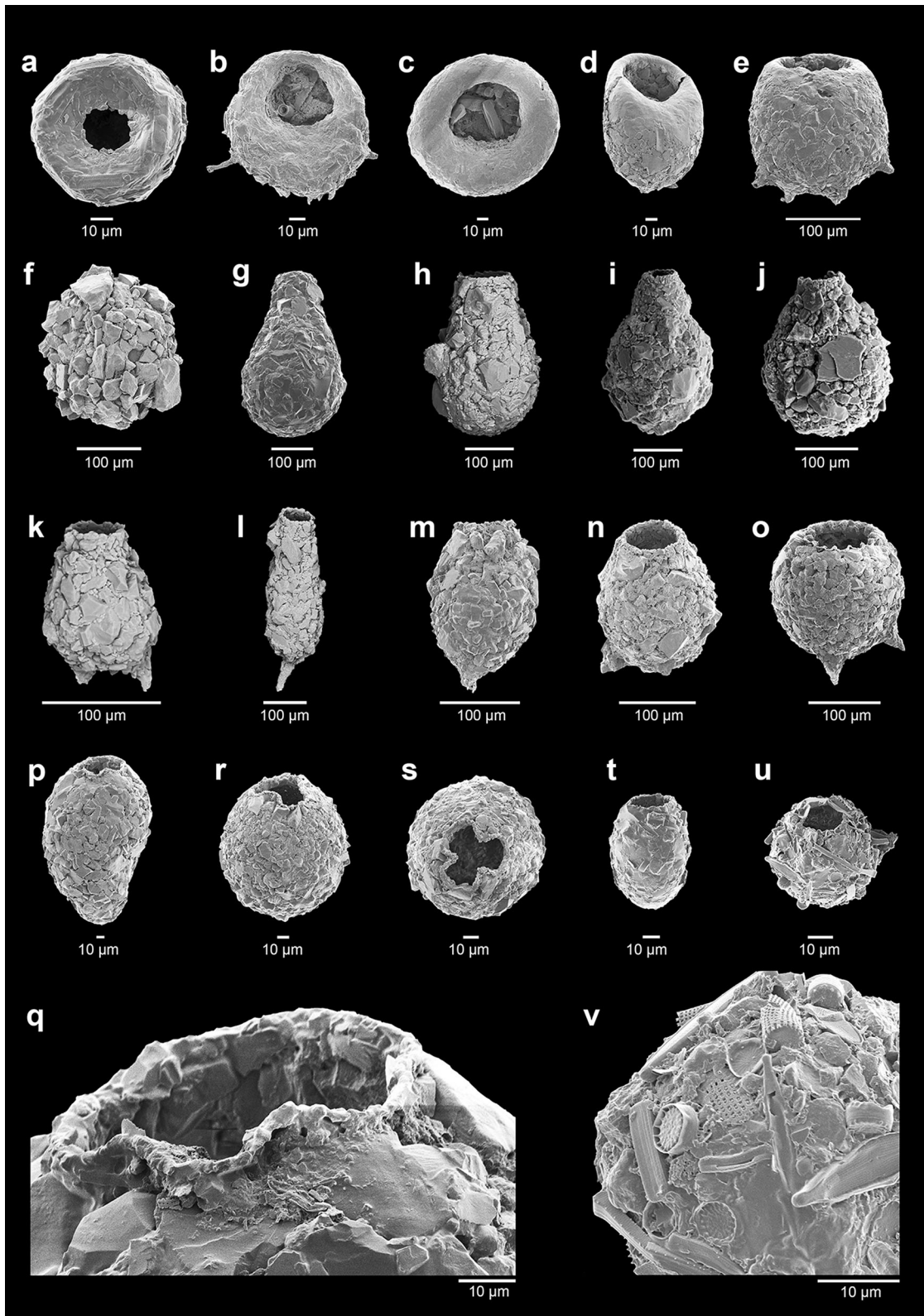
Following an initial detrended correspondence analysis (DCA) of both the plant macrofossil and testate amoeba data, axis 1 gradient lengths for both groups were 2.7 SD and 3.2 SD, respectively. Species' responses were therefore not seen to be unimodal (Lepš and Šmilauer 2003) and principal components analysis (PCA) was chosen as an appropriate method of ordination to determine major gradients of change within the biological assemblages. To make the data suitable for Euclidean-based ordination methods (Legendre and Birks 2012), plant macrofossil concentrations and the testate amoeba relative abundance data were subjected to a Hellinger transformation (Legendre and Gallagher 2001). The differential production of plant macrofossils (for example, leaf remains versus seeds) can lead to an imbalanced representation in palaeoecological records. Therefore, the plant macrofossil data were centred and standardised in order to reduce any potential asymmetry in the data. Significant PCA axes were identified using the broken stick model (Jackson 1993).

All statistical analyses were performed in R version 3.3.2 (R Development Core Team 2016) using the vegan (Oksanen et al. 2017) and labdsv (Roberts 2016) packages.

Results

Core chronology

The results of the AMS $^{14}$C analyses are given in ESM 1. The bulk sediment AMS $^{14}$C date from 68 to 69 cm (1882 ± 37 year BP) predates the charcoal sample from 96 to 97 cm (827 ± 34 year BP), which may

reflect contamination of the bulk sediment sample by older carbon. Similarly, the bulk date from 134 to 135 cm (2420 ± 34 year BP) is much older than expected based on the extrapolation of $^{210}$Pb-inferred sedimentation rates from the late 1920s (0.27 cm year$^{-1}$), which would give a basal date of AD 1492 (Salgado et al. 2010). While the charcoal sample from 96 to 97 cm indicates that the core base may indeed be older than the estimated age, the sedimentation rate for the basal section of the core, indicated by the bulk date from 134 to 135 cm is too low (0.04 cm year$^{-1}$) for the latter date to be correct. As a result, only the date from the charcoal sample at 96–97 cm is included in the stratigraphic diagrams, in addition to the $^{210}$Pb dates from Salgado et al. (2010).

## Testate amoeba assemblages

The preservation of fossil testate amoebae was good in all samples enabling counts of >150 specimens, and in many cases >200 specimens, to be achieved. Interpretation of the CONISS cluster analysis resulted in identification of four testate amoeba assemblage zones (Fig. 3): LLTA-1 (Loch Leven testate amoeba 1), LLTA-2, LLTA-3 and LLTA-4. The assemblage composition of these zones is described below and compared to corresponding changes in lithostratigraphy and the plant macrofossils (Fig. 4). The results of the IndVal analysis (Table 1) reveal a number of significant indicator taxa for each of the four zones identified by CONISS. Those with the highest

indicator values ($IndVal_j$) are highlighted within their respective zones below.

### LLTA-1 (139–94 cm)

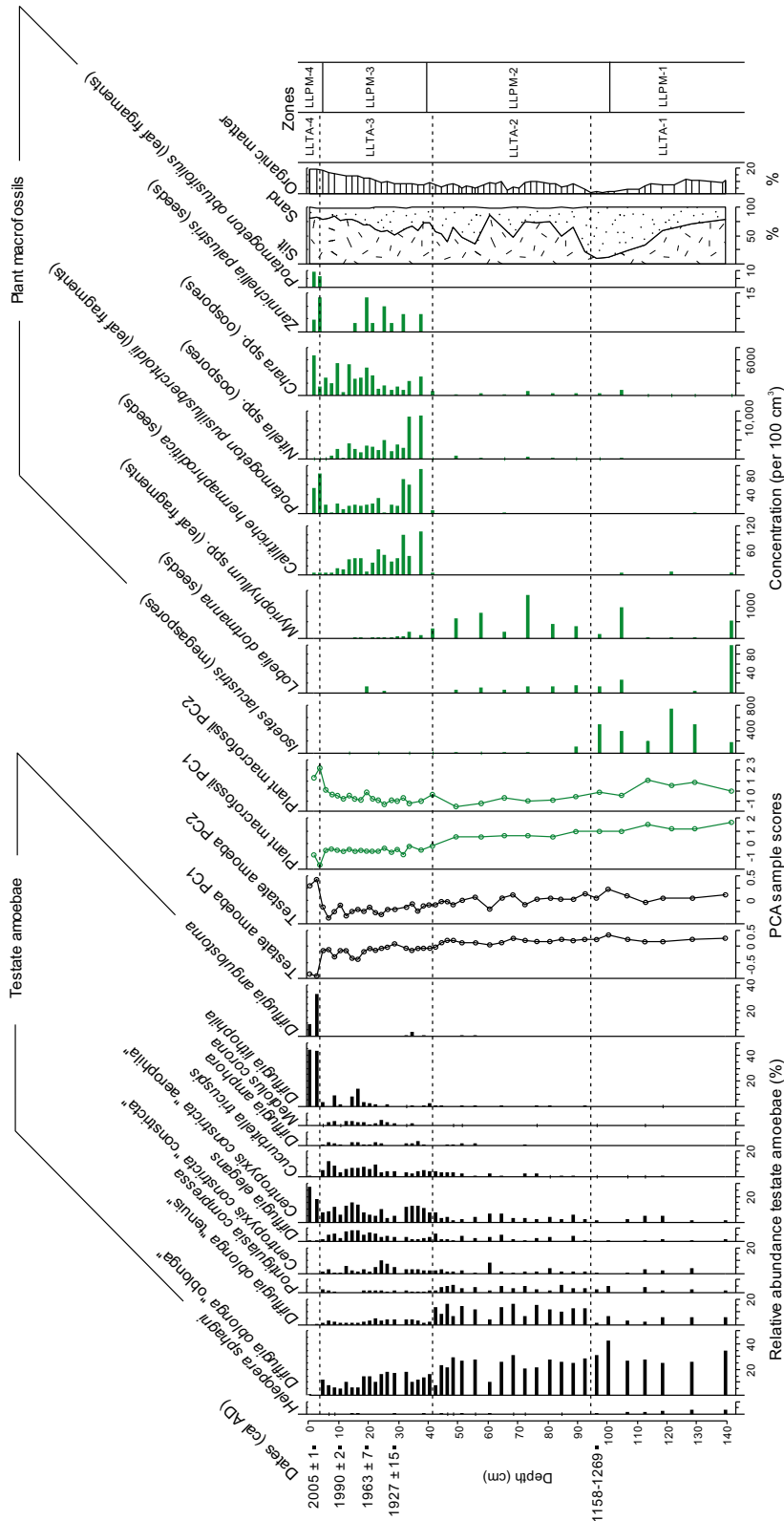The sediments of this zone are characterised by silty gyttja, which becomes less organic (LOI = 1–11%) and sandier towards the boundary with LLTA-2. The preservation of fossil testate amoebae is good (177–1522 specimens cm$^{-3}$) and diversity (SDI = 1.9–2.3) can be classed as intermediate (Patterson and Kumar 2000b). The assemblage is characterised by a dominance of *Difflugia oblonga* Ehrenberg 1832 strain "oblonga" (25–43%) and a mixture of diffugid and centropyxid taxa (Fig. 3). Throughout this zone, proportions of *Centropyxis aculeata* (Ehrenberg 1832) strain "discoides" (9–25%) and *Difflugia glans* Penard 1902 strain "glans" (6–20%) fluctuate, while *Centropyxis aculeata* (Ehrenberg 1832) strain "aculeata" declines from a peak of 22% at 128 cm to 8% at the zone boundary. Other centropyxid and diffugid taxa are present in lesser abundances (typically <5%), as are *Heleopera sphagni* (Leidy 1874), *Cyclopyxis kahli* Deflandre 1912, *Lagenodifflugia vas* Leidy 1874, *Pontigulasia compressa* (Carter 1864) and *Plagiopyxis disclivis* Bonnet 1955. Of those identified as significant ($p < 0.05$) by IndVal, *H. sphagni* ($IndVal_j = 0.67$) and *D. oblonga* "oblonga" ($IndVal_j = 0.46$) have the highest indicator values (Table 1).

### Zone LLTA-2 (94–41 cm)

Sediment organic matter increases compared to LLTA-1 and varies between 1 and 10% throughout LLTA-2, while proportions of silt and sand fluctuate, indicating a less stable benthic environment. *Difflugia oblonga* "oblonga" (8–31%) remains dominant, although proportions decline at 60 cm and 42 cm to 10 and 8%, respectively. Centropyxid taxa, namely *C. aculeata* "aculeata" (6–27%) and *C. aculeata* "discoides" (4–14%), also fluctuate and remain in lesser proportions. However, compared to the previous zone, diversity (SDI = 2.2–2.6) and test concentrations (628–2272 specimens cm$^{-3}$) increase and there are notable expansions in *Difflugia oblonga* Ehrenberg 1832 strain "tenuis" (4–16%) as well as *Difflugia elegans* Penard 1890 (0–6%), *L. vas* (1–10%), *Centropyxis constricta* (Ehrenberg 1843) strain

**Fig. 3** Percentage abundance testate amoeba diagram for core LEVE14 from Loch Leven showing the core lithostratigraphy, dates ascribed by ^{210}Pb and AMS ^{14}C dating, Shannon's Diversity Index (SDI), test concentration, total proportions of Difflugid and Centropyxid taxa and zones identified by CONISS cluster analysis. Taxa are ordered ecologically from left to right to emphasise species turnover throughout the stratigraphic sequence

**Fig. 4** Stratigraphic summary diagram for Loch Leven showing the relative abundance of selected significant indicator testate amoeba taxa and concentrations (per 100 cm³) of selected plant macrofossils in core LEVE14 from Salgado et al. (2010). The principal components analysis (PCA) axis 1 and 2 sample scores for both groups are shown along with the dates ascribed by ²¹⁰Pb and AMS ¹⁴C dating and zones identified by CONISS cluster analysis (LLTA = testate amoebae; LLPM = plant macrofossils)

**Table 1** The indicator value ($Indval_j$) and significance of indicator taxa associated with the four stratigraphic zones in core LEVE14

| Taxa | Group (Zone) | $IndVal_j$ | Significance ($p$ value) |
|------|------|------|------|
| *Heleopera sphagni* | LLTA-1 | 0.6669 | 0.008** |
| *Difflugia oblonga* "oblonga" | LLTA-1 | 0.4590 | 0.001*** |
| *Centropyxis aculeata* "discoides" | LLTA-1 | 0.4289 | 0.002** |
| *Cyclopyxis kahli* | LLTA-1 | 0.4215 | 0.048* |
| *Difflugia glans* "glans" | LLTA-1 | 0.3809 | 0.031* |
| *Difflugia oblonga* "tenuis" | LLTA-2 | 0.6157 | 0.001*** |
| *Difflugia protaeiformis* "claviformis" | LLTA-2 | 0.5464 | 0.04* |
| *Pontigulasia compressa* | LLTA-2 | 0.5104 | 0.001*** |
| *Mediolus corona* | LLTA-3 | 0.7390 | 0.001*** |
| *Difflugia amphora* | LLTA-3 | 0.6459 | 0.004** |
| *Cucurbitella tricuspis* | LLTA-3 | 0.6440 | 0.001*** |
| *Difflugia elegans* | LLTA-3 | 0.5167 | 0.001*** |
| *Difflugia glans* "distenda" | LLTA-3 | 0.4970 | 0.009** |
| *Centropyxis constricta* "constricta" | LLTA-3 | 0.4874 | 0.027* |
| *Lesquereusia spiralis* | LLTA-3 | 0.4665 | 0.044* |
| *Difflugia angulostoma* | LLTA-4 | 0.9823 | 0.001*** |
| *Difflugia lithophila* | LLTA-4 | 0.9280 | 0.001*** |
| *Centropyxis constricta* "aerophila" | LLTA-4 | 0.5883 | 0.001*** |

Significance level:
*** $p \leq 0.001$,
** $p = 0.001$–$0.01$,
* $p = 0.01$–$0.05$

"aerophila" (1–8%), *P. compressa* (2–6%) and *Difflugia protaeiformis* Lamarck 1816 strain "claviformis" (0–7%). *Cucurbitella tricuspis* Carter 1856 (0–5%) is also present and increases in abundance towards the top of the zone. *Difflugia oblonga* "tenuis" ($IndVal_j = 0.62$), *D. protaeiformis* "claviformis" ($IndVal_j = 0.55$) and *P. compressa* ($IndVal_j = 0.51$) are identified as significant indicator taxa according to IndVal.

*Zone LLTA-3 (41–3 cm)*

There is a gradual change in the lithostratigraphy in this zone as the sediments become less sandy and more organic (LOI = 7–19%) towards the top of the core. A concurrent shift in the testate amoeba assemblages is also observed. Compared to previous zones, diversity again increases (SDI = 1.7–2.8) and test concentrations decrease (207–1648 specimens cm$^{-3}$). *Difflugia oblonga* "oblonga" decreases in abundance (5–19%), but co-dominates with *C. aculeata* "aculeata" (3–46%), *C. aculeata* "discoides" (5–17%) and *C. constricta* "aerophila" (3–15%). These centropyxid taxa display broadly synchronous fluctuations throughout LLTA-3. There are also notable expansions compared to LLTA-2 in *Difflugia glans* Penard 1902 strain "distenda" (0–8%) and *Difflugia lithophila*

Penard 1902 (0–14%). *Cucurbitella tricuspis* (3–12%) gradually increases in abundance towards the top of the core, with peaks at 22 and 6 cm. The relative abundance of *Mediolus corona* (Wallich 1864) (0–5%) and *Difflugia amphora* (Leidy 1874) (0–3%) also increase, while *P. compressa* (0–2%) and *D. oblonga* "tenuis" (1–5%) decrease. Of the taxa identified as significant by IndVal, *M. corona* ($IndVal_j = 0.74$), *D. amphora* ($IndVal_j = 0.65$) and *C. tricuspis* ($IndVal_j = 0.64$) have the highest indicator values.

*Zone LLTA-4 (3–0 cm)*

The lithostratigraphy remains similar to the top of the previous zone, comprising organic silty gyttja (LOI = ca. 20%). However, a significant change in the testate amoeba assemblage occurs. There is a 15-fold increase in test concentrations (about 16,500 specimens cm$^{-3}$) compared to LLTA-3 and diversity decreases substantially (SDI = 1.3 and 1.6). In contrast to the previous zones, three smaller taxa dominate the assemblage, *D. lithophilia* (43–45%), *Difflugia angulostoma* Gauthier-Lièvre and Thomas 1958 (9–33%) and *C. constricta* "aerophila" (18–27%). *Difflugia glans* "glans" (1–10%) remains present, along with *C. aculeata* "aculeata" and *C. aculeata* "discoides", but in much smaller proportions (<2%),

and there are substantial decreases in the relative abundances of all other difflugid and centropyxid taxa compared to LLTA-3. *Difflugia lithophila* ($IndVal_j = 0.98$), *D. angulostoma* ($IndVal_j = 0.93$) and *C. constricta* "aerophila" ($IndVal_j = 0.59$) are identified as significant indicator taxa according to IndVal.

## Comparisons with plant macrofossil assemblages

A summary diagram that includes key plant macrofossil and testate amoeba data from core LEVE14 is given in Fig. 4. The diagram is divided into four zones (dashed lines), based on CONISS cluster analysis of the testate amoeba data. Interpretation of an additional CONISS cluster analysis of the plant macrofossil data resulted in the identification of four zones, prefixed with LLPM (Fig. 4). When compared, the zone boundaries of both groups are closely aligned, particularly at around 40 cm and 3 cm. The lower zone boundaries at 94 cm and 101 cm, for testate amoebae and plant macrofossils respectively, are slightly offset. Exact relative timings of community changes based on the zone boundaries are not possible due to different sample positions and resolutions between the groups analysed.

Testate amoeba zone LLTA-1 predates ca. AD 1200 and corresponds with an oligo-mesotrophic macrophyte assemblage (Salgado et al. 2010) dominated by isoetid plants, *I. lacustris* and *Lobelia dortmanna*, with the additional presence of *Myriophyllum* spp. The shift from sandy to siltier gyttja and a more diverse and abundant testate amoeba community in zone LLTA-2 after ca. AD 1200 is accompanied by increased concentrations of elodeid and charophyte taxa, namely *Myriophyllum* spp., *Chara* spp. and *Nitella* spp. A reduced concentration of *I. lacustris* and *L. dortmanna* are indicative of the progression of eutrophication in Loch Leven. Increased organic matter content and testate amoeba diversity in zone LLTA-3 is synchronous with a rapid increase in the concentration of charophyte oospores and leaf fragments of fine-leaved *Potamogeton* spp. The remains of other elodeid taxa, namely *Zannichellia palustris* and *Callitriche hermaphroditica,* also increase, while the concentration of isoetid remains are further reduced compared to the previous zone. These changes are thought to represent increasingly eutrophic and shallower lake conditions (Salgado et al. 2010), following

lowering of the lake water levels ca. 1830. Testate amoeba zone LLTA-4 (ca. 2004–2006) comprises only two samples and corresponds with a macrophyte assemblage dominated by *Chara* spp., *Potamogeton* spp. and *Z. palustris*. In particular, *Potamogeton obtusifolius* is unique to the upper 4 cm.

## Principal components analysis

The total variance in the testate amoeba and plant macrofossil assemblages identified by PCA was 19.7 and 14.0% respectively (Fig. 4). Using the broken stick model (Jackson 1993), four principal component (PC) axes were identified as significant for testate amoebae ($\lambda_1 = 0.343$, $\lambda_2 = 0.153$, $\lambda_3 = 0.095$ and $\lambda_4 = 0.069$) and two for plant macrofossils ($\lambda_1 = 0.356$ and $\lambda_2 = 0.174$). The PC axis 1 and 2 sample scores for the testate amoeba and plant macrofossil assemblages are given in Fig. 4. These indicate synchronous compositional changes between the two groups, particularly around 113 cm and in the uppermost 4 cm.

The plant macrofossil PCA bi-plot (Fig. 5a) emphasises that zone LLPM-1 is associated with isoetid plants, namely, *I. lacustris* and *L. dortmanna,* and varying proportions of organic matter. The transition to LLPM-2 is characterised by a strong association with *Myriophyllum* spp. and *L. dortmanna,* with moderate amounts of organic matter. In addition, zones LLPM-3 and LLPM-4 and their associations with elodeid and charophyte macrofossils and more organic sediments suggest compositionally distinctive eutrophic macrophyte assemblages. The ordering of assemblage zones along PC1 suggests this axis represents a strong eutrophic gradient in the plant macrofossil data.

Interpretation of the testate amoeba PCA bi-plot (Fig. 5b) indicates that PC1 shows a strong positive association with strains of *D. oblonga*, while PC2 shows a strong negative correlation with *C. tricuspis*. Furthermore, there is a distinct grouping of the LLTA-3 samples scores which, based on comparisons with LLPM-3 and greater proportions of organic matter, suggests a distinct eutrophic assemblage. The distribution of samples scores along PC1 and PC2 suggests that either axis could represent a eutrophic gradient in the testate amoeba data. Indeed, the pattern indicates the influence of a strong secondary control that may co-vary with eutrophication. Lastly, the clustering of

**◄ Fig. 5** Principal components analysis (PCA) species-sample bi-plots for (a) plant macrofossils and (b) testate amoebae from core LEVE14. The assemblage zones identified by CONISS cluster analysis are highlighted according to colour (*blue* = LLPM-1 and LLTA-1; *red* = LLPM-2 and LLTA-2; *green* = LLPM-3 and LLTA-3; *black* = LLPM-4 and LLTA-4) and sample plotting symbol is proportional to percentage organic matter. CAA = *Centropyxis aculeata* (Ehrenberg 1832) strain "aculeata"; CAD = *C. aculeata* (Ehrenberg 1832) strain "discoides"; CCA = *C. constricta* (Ehrenberg 1843) strain "aerophila"; CCC = *C. constricta* (Ehrenberg 1843) strain "constricta"; CCS = *C. constricta* (Ehrenberg 1843) strain "spinosa"; CK = *Cyclopyxis kahli* Deflandre 1912; CT = *Cucurbitella tricuspis* Carter 1856; DA = *Difflugia amphora* (Leidy 1874); DAA = *Difflugia angulostoma* Gauthier-Lièvre and Thomas 1958; DB = *Difflugia bidens* Penard 1902; DE = *Difflugia elegans* Penard 1890; DGD = *D. glans* Penard 1902 strain "distenda"; DGG = *D. glans* Penard 1902 strain "glans"; DL = *Difflugia lithophila* Penard 1902; DOLA = *D. oblonga* Ehrenberg 1832 strain "lanceolata"; DOO = *D. oblonga* Ehrenberg 1832 strain "oblonga"; DOTE = *D. oblonga* Ehrenberg 1832 strain "tenuis"; DPA = *D. protaeiformis* Lamark 1816 strain "acuminata"; DPC = *D. protaeiformis* Lamark 1816 strain "claviformis"; DPP = *D. protaeiformis* Lamark 1816 strain "protaeiformis"; DU = *D. urceolata* Carter 1864 strain "urceolata"; HS = *Heleopera sphagni* (Leidy 1874); LS = *Lesquereusia spiralis* (Ehrenberg 1840); LV = *Lagenodifflugia vas* Leidy 1874; MC = *Mediolus corona* (Wallich 1864); PC = *Pontigulasia compressa* Carter 1864; PD = *Plagiopyxis disclivis* Bonnet 1955. (Color figure online)

both LLPM-4 and LLTA-4 confirms that these zones contain markedly different faunal assemblages compared to the other plant macrofossil and testate amoeba zones.

## Discussion

### Palaeo-inferred testate amoeba ecology

The oligo-mesotrophic macrophyte assemblage in zone LLTA-1, containing *I. lacustris* and *L. dortmanna,* is typical of many northern European freshwater lakes prior to nutrient-enrichment (Palmer et al. 1992). The corresponding testate amoeba assemblage is dominated by *D. oblonga* "oblonga", a common taxon which occurs in a range of lacustrine environments, from oligotrophic marl lakes (Wiik et al. 2015) to eutrophic kettle lakes (Roe et al. 2010). Strains of *Difflugia* are known to thrive in sediments rich in organic matter that are thought to sustain high population carrying capacities (Patterson and Kumar 2000a). They are therefore often abundant in lake

gyttja (Collins et al. 1990; McCarthy et al. 1995), likely explaining their abundance in the Loch Leven palaeo-record. However, the organic matter variable on the PCA plot (Fig. 5b) indicates that *D. oblonga* "oblonga" is not associated with the most organic sediments in core LEVE14. Indeed, the peak abundance occurs around 100 cm where sediments are sandy and some of the least organic in the core (Fig. 4). This aligns with the findings of Torigai et al. (2000) who reported an abundance of *D. oblonga* in inorganic lake sediments from Lake Winnipeg, Canada. Overall, this suggests that this taxon can tolerate a wide range of sedimentary conditions and that multiple factors may influence its distribution.

Although *H. sphagni* was found in relatively low abundance (<4%), it has the highest indicator value in the inferred oligo-mesotrophic zone (LLTA-1) and is therefore important to consider. *Heleopera* spp. are often associated with peat mosses like *Sphagnum* spp. (Schönborn 1967; Scott et al. 2001), but are also found in lake sediments (Ellison 1995; McCarthy et al. 1995; Wall et al. 2010b). During the earliest phase of the LEVE14 palaeo-record *H. sphagni* may therefore be associated with sub-littoral *Sphagnum* spp., which often occur in oligotrophic to mesotrophic lake conditions (Raven 1988; Palmer et al. 1992). Similarly, Schönborn (1962) finds *Heleopera* spp. in oligotrophic lake environments and reports them absent in more eutrophic lakes.

*Cyclopyxis kahli* is present throughout zone LLTA-1 and often thought to be indicative of oligotrophic environments (Schönborn 1967; Wall et al. 2010b). However, despite fluctuations, the abundance of *C. kahli* remains relatively consistent throughout most of the palaeo-record despite a macrofossil-inferred transition in Loch Leven to mesotrophic and eutrophic conditions in zones LLTA-2 and LLTA-3 respectively. Others have reported this species in soil environments (Warner 1994) and some have suggested that its presence in lake sediments may indicate secondary derivation from terrestrial in-washing (Roe and Patterson 2006, 2014). Nonetheless, a loyal association of *C. kahli* with oligotrophic lake conditions is not verified by our data. The diversity and abundance of fossil testate amoebae found in zone LLTA-1 confirms that numerous species can withstand and thrive in oligo-mesotrophic conditions. This finding accords with previous work where oligotrophic and mesotrophic lakes dominated by

Difflugid taxa display a similarly high diversity (SDI = approximately 2.1) (Roe and Patterson 2014).

The diatom record from core LEVE11 (Bennion et al. 2012), estimated to extend back about 700 years, includes a mixture of planktonic and periphytic taxa indicative of relatively stable oligotrophic to meso-trophic conditions. This is further substantiated by the mesotrophic plant assemblage identified in LLPM-2 (after ca. 1200 AD). As the depositional environment in zone LLTA-2 became predominantly silt-rich and organic matter increased, there was a corresponding shift to a more diverse and abundant testate amoeba community. *Cucurbitella tricuspis*, often cited as a eutrophic indicator taxon (Torigai et al. 2000; Rein-hardt et al. 2005; Kihlman and Kauppila 2012) due to its association and symbiotic relationship with *Spir-ogyra* algae (Medioli et al. 1987; Patterson and Kumar 2002), increased in this zone. *Difflugia oblonga* "tenuis" and *P. compressa* are highly significant (*p* < 0.001) indicator taxa for this mesotrophic phase. Other palaeolimnological studies have also reported an abundance of *P. compressa* during inferred mesotrophic periods as delineated by pollen and NPP from Lake Simcoe, Canada (Volik et al. 2016) and Sluice Pond, USA (Drljepan et al. 2014). Thus, *P. compressa* may have an affinity for mesotrophic lake conditions. Variations in testate amoeba assemblage composition throughout LLTA-2 are highlighted by fluctuating PCA axis scores (Fig. 4). Given the known sensitivity of testate amoebae to changing substrate characteristics (Kihlman and Kauppila 2012), these changes may be linked to fluctuating proportions of sand and silt (Fig. 4) as well as the influence of eutrophication.

The continued eutrophication of Loch Leven throughout zone LLTA-3 (after ca. 1830) is evidenced by significant expansions of *Potamogeton pusillus/ berchtoldii* and *Z. palustris,* species that often dom-inate in highly eutrophic lakes (Sayer et al. 2010b; Bennion et al. 2015). This accords with the core LEVE11 diatom record (Bennion et al. 2012), which shows a marked shift to a planktonic dominated assemblage throughout the twentieth century, includ-ing *Aulacoseira ambigua* and *Stephanodiscus hantzschii.* Moreover, historical macrophyte surveys from 1905 to 2008 (Dudley et al. 2012b) attest to the continued eutrophication of Loch Leven during much of the twentieth century. Nutrient-enrichment was driven by increased inputs of TP to the loch which rose from an estimated 6 t TP year$^{-1}$ in the early 1900s to around 20 t TP year$^{-1}$ in 1985 (May et al. 2012).

Concomitant changes in the testate amoeba com-munity in zone LLTA-3 are evident as diversity again increases and *D. oblonga* "oblonga" no longer dominates the assemblage. A continued expansion of *C. tricuspis* during the twentieth century and a highly significant indicator value (*p* < 0.001) confirms that this taxon was associated with a more nutrient-enriched lake. Furthermore, the introduction in this zone of two less abundant but statistically significant indicator taxa, *D. amphora* (*p* < 0.01) and *M. corona* (*p* < 0.001), is important to note. Tolonen (1986) provides an overview on the ecology of *M. corona* and *D. amphora*, both of which are linked to eutrophic conditions. These ecological inferences are in accor-dance with other studies from lakes in Ontario, Canada (Volik et al. 2016), Wuhan, China (Qin et al. 2009) and Massachusetts, USA (Drljepan et al. 2014) that report increases in the abundance of *M. corona* associated with nutrient enrichment. Conversely, *D. amphora* is rarely discussed in this regard.

The most significant point of change in the testate amoeba assemblage occurs in zone LLTA-4, which sees a substantial increase in test concentrations and much lowered diversity. Indeed, SDI values in this zone are at their lowest for all the samples in core LEVE14 which may indicate a more unstable and stressed environment (Patterson and Kumar 2000b). The distinct testate amoeba assemblage is dominated by *D. angulostoma* and *D. lithophila*, both of which have very high and significant (*p* < 0.001) indicator values. These taxa have been found in a range of lacustrine environments (Ellison 1995; Yang et al. 2005; Alves et al. 2010) and their presence has not been associated with any specific lake conditions. A number of possible factors may explain this distinctive surficial assemblage, including dissolution of these smaller specimens down-core or a significant recent alteration of the testate amoeba environment. As with most Arcellinida preserved in lakes, the tests of *D. angulostoma* and *D. lithophila* are composed of xenosomes. These types of test are thought to be the most robust (Lousier and Parkinson 1981), so it seems unlikely that differential dissolution might explain the observed pattern. The shift coincides with a significant plant assemblage change in the upper 4 cm, as demonstrated by plant macrofossil PCA axis scores and the CONISS zone boundary. Indeed, Difflugiidae have shown an affinity for a range of

microhabitats in lakes (Hardoim and Heckman 1996) and food preferences are also known to vary among taxa (Han et al. 2011). Therefore, a more suitable explanation may be a substantial change to the habitat in which they are living or to the microbial food-web, which favoured these smaller species, such as changing predation patterns, food availability and/or habitat structure. Additional research on lake testate amoeba taphonomy and habitat preferences is required in order to state more conclusively the cause of the observed changes.

When considered as a whole, the testate amoeba assemblages at Loch Leven showed gradual increases in diversity as the lake transitioned to mesotrophic and eutrophic conditions. This is consistent with other lake testate amoeba studies along eutrophic gradients. For example, Ju et al. (2014) reported a unimodal relationship for species diversity along a nutrient gradient for 51 lakes/reservoirs across China. Similarly, in the Yunnan Plateau, China, Yang et al. (2005) recorded the highest diversity of testate amoebae in mesotrophic lakes and the lowest in hypereutrophic lakes. Furthermore, a palaeolimnological study of Sluice Pond, Massachusetts, USA, (Drljepan et al. 2014) reported increased SDI values associated with eutrophication. However, given the different taxonomic approaches often used among testate amoeba researchers (Kosakyan et al. 2016), caution must be taken in directly comparing diversity between studies. This calls for a standardisation of taxonomic practices among lacustrine testate amoebae researchers in order to develop a coherent taxonomy that would increase the comparability of studies across wide spatial scales.

Mechanisms of change

A close alignment of zone boundaries and PCA axis scores of testate amoebae and plant macrofossils suggest that both groups are responding to nutrient-enrichment, particularly since ca. 1830. However, the plant macrofossils show a greater species turnover compared to the testate amoebae. Indeed, many taxa persist across the eutrophication gradient, such as *D. oblonga* "oblonga", *D. glans* "glans" and several centropyxid taxa. Given this more dampened signal, it is possible that the testate amoeba response to eutrophication could be partially indirect, driven by habitat changes associated with enrichment. This is supported by the distribution of sample scores in the testate amoeba PCA bi-plot (Fig. 5b) which suggests a strong

secondary control on the assemblage that co-varies with eutrophication. In a palaeo-study of chironomid-macrophyte relationships from shallow, eutrophic Lake Søbygaard, Denmark (Brodersen et al. 2001), changing macrophyte assemblage structure was identified as a key likely driver of chironomid community changes. Given the range of lake microhabitats occupied by testate amoebae (Lansac-Tôha et al. 2014) and the synchronous response of both groups demonstrated here, it seems likely that similar relationships may also exist between testate amoebae and aquatic macrophytes.

Flößner et al. (1985) describe the diverse range of habitats occupied by testate amoebae in Lake Stechlin, Germany including not only the sediment but also the periphyton of submerged and floating leaved vegetation. Indeed, macrophytes are thought to provide a significant contribution to testate amoeba microhabitats in lakes (Alves et al. 2010), affording shelter from physical disturbance and increased structural complexity and habitat diversity (Lansac-Tôha et al. 2014). It must be considered, therefore, that any change in plant community structure might also impact the testate amoebae.

In addition to the taxa previously discussed as indicators for zone LLTA-3, *D. elegans* and *C. constricta* "constricta" were also identified as statistically significant in this respect (Table 1). Flößner et al. (1985) identified *Centropyxis gibba* (synonymous here with *C. constricta* "constricta") as inhabiting stands of *Typha* and *Potamogeton*, while *D. elegans* was found amongst beds of Characeae. Lansac-Tôha et al. (2014) also noted that *D. elegans* was a significant indicator of macrophytes, as opposed to sediment or open water habitats. The prevalence of *D. elegans* and *C. constricta* "constricta" in the eutrophic inferred zone (LLTA-3) may therefore be linked with increased macrophyte density associated with elodeid and charophyte vegetation development during the twentieth century.

Many testate amoebae, including *D. oblonga* and *P. compressa* are thought to be strongly associated with sediment microhabitats in lakes (Flößner et al. 1985; Alves et al. 2010; Lansac-Tôha et al. 2014). Occupying the benthos, these taxa will be sensitive to changes in substrate characteristics, including the oxygen status and biochemistry of the sediment water interface (Asioli et al. 1996; Roe et al. 2010; Kihlman and Kauppila 2012). It has been well documented that isoetid plants, such as *L. dortmanna* and *I. lacustris*,

release oxygen via their roots during photosynthesis (Sand-Jensen et al. 1982; Smolders et al. 2002). This process, known as radial oxygen loss (Smolders et al. 2002), can increase sediment oxygen levels depending on the density of isoetid plants. The abundance of *I. lacustris* and, in particular, *L. dortmanna* throughout LLTA-1 and LLTA-2 may therefore facilitate favourable high oxygen conditions for sediment dwelling testate amoebae like *D. oblonga* "oblonga", *D. oblonga* "tenuis" and *P. compressa,* which are significant indicator taxa for these zones. In contrast, a sustained decline in isoetids and the proliferation of charophytes in zone LLTA-3 is coincident with lower abundances of strains of *P. compressa* and strains of *D. oblonga*. A similar trend was noted by Wiik et al. (2015), who reported a decreased abundance of *D. oblonga* "oblonga" in a core from Cunswick Tarn (Cumbria, UK) during charophyte-dominated phases. Similarly, dense beds of *Chara* spp. have been shown to impact benthic chironomids, resulting in the exclusion of many bottom species under lower dissolved oxygen conditions (Tarkowska-Kukuryk and Kornijów 2008). This is due to strong oxygen consumption by microbes during the decomposition of dense beds of vegetation (Lindholm et al. 2008) and the reduced flow of water through charophyte meadows (Vermaat et al. 2000).

To confirm the aforementioned potential structuring mechanisms and determine the directness of the links between eutrophication, macrophytes and testate amoebae modern ecological work is clearly needed.

## Conclusions

This study has employed palaeolimnological techniques to investigate the response of testate amoebae to nutrient enrichment in Loch Leven Scotland, a lake with a well-documented history of eutrophication. Comparisons of testate amoebae with plant macrofossil assemblages from the same core reveal synchronous compositional change across the two groups in response to nutrient enrichment. In addition, the indicator taxa identified for the eutrophic zone (post ca. AD 1830) confirm the previously determined ecological associations of *C. tricuspis, M. corona* and *D. amphora* with eutrophic lake conditions. We hypothesise that sediment dwelling testate amoeba such as *D. oblonga* "oblonga", *D. oblonga* "tenuis" and *P. compressa* are

more abundant in oligo-mesotrophic and mesotrophic zones due to highly oxygenated sediment conditions, maintained by the presence of isoetid plants. We suggest that isoetid decline and a shift to high charophyte biomass during eutrophication (post ca. AD 1830) may have lowered sedimentary oxygen, reducing the abundance of benthic testate amoebae and giving rise to plant associated taxa like *D. elegans* and *C. constricta* "constricta". Our study highlights the key importance of considering macrophyte community succession and plant structure when interpreting fossil testate amoeba assemblages in lakes and demonstrates the value of using palaeoecology to inform ecology. Further research is needed to better understand the influence of aquatic macrophytes on testate amoebae and to examine other potential causal mechanisms of community change.

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