



Freshwater Testate Amoebae (Arcellinida) Response to Eutrophication as Revealed by Test Size and Shape Indices

Andrew L. Macumber^{1*}, Helen M. Roe^{1*}, Stephen V. Prentice¹, Carl D. Sayer², Helen Bennion³ and Jorge Salgado^{4,5}

¹ School of Natural and Built Environment, Queen's University Belfast, Belfast, United Kingdom, ² Pond Restoration Research Group, Environmental Change Research Centre, Department of Geography, University College London, London, United Kingdom, ³ Environmental Change Research Centre, Department of Geography, University College London, London, United Kingdom, ⁴ Facultad de Ingeniería, Universidad Católica de Colombia, Bogotá, Colombia, ⁵ School of Geography, University of Nottingham, Nottingham, United Kingdom

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*Correspondence:

Andrew L. Macumber
andrew.l.macumber@gmail.com
Helen M. Roe
h.roe@qub.ac.uk

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We review the potential for applying traits-based approaches to freshwater testate amoeba, a diverse protist group that are abundant in lakes and are valuable ecological indicators. We investigated the efficacy of geometric morphometric analysis to define Arcellinida test size and shape indices that could summarize freshwater testate amoeba community dynamics along a temporal gradient of eutrophication in Loch Leven, Scotland (United Kingdom). A cluster analysis of test size and shape indices yielded three clusters, each dominated by a single shape: elongate, spherical and ovoid. When plotted stratigraphically, we observed increases in spherical tests, decreases in elongate tests and shrinking of test size coeval with eutrophication in Loch Leven. Decreases in the elongate cluster may reflect benthic conditions with reduced oxygen levels, while increases in the spherical cluster are likely associated with an expanding macrophyte community that promoted pelagic and epibiotic life habits. Shrinking of test size may be a stress response to eutrophication and/or warming temperatures. Tracking community dynamics using test size and shape indices was found to be as effective as using species-based approaches to summarize key palaeolimnological changes, with the added benefits of being free from taxonomic bias and error. The approach thus shows significant potential for future studies of aquatic community change in nutrient impacted lakes.

Keywords: Arcellinida, ecology, lake, morphometrics, paleolimnology, Loch Leven, land-use change

INTRODUCTION

The ever-increasing impact of humans on their environment via urbanization, industrialization and climate change has led to an 80% decline in freshwater biodiversity in the last 50 years (Grooten and Almond, 2018). This emphasizes the need to model the trajectory of impacted natural freshwater systems. Lakes being complex natural systems are best understood by characterizing multiple proxies representing various levels of biological organization and assessing how their functional interactions vary through time and space (Blois et al., 2013).

Testate amoeba are a diverse protist group that enclose their cell body within a test (i.e., a hard shell) that can be used to identify species and preserves well in sediments. They are valuable ecological indicators due to their (1) abundance in freshwater, moist soils and wetlands; (2) sensitivity to environmental conditions; (3) high preservation potential; and (4) rapid generation times (Patterson and Kumar, 2002; Mitchell et al., 2008). In lakes, lobose testate amoeba dominate (i.e., Arcellinida) and have been used as indicators for: lake acidity (Kumar and Patterson, 2000; Patterson et al., 2013); land-use change (Patterson et al., 2002); industrial impacts (Nasser et al., 2016); water quality (Roe et al., 2010); ecosystem health and seasonal environmental change (Neville et al., 2011); nutrient loading (Patterson et al., 2012; Prentice et al., 2018); and climate change (McCarthy et al., 1995; Boudreau et al., 2005), amongst other variables.

Arcellinida studies typically present community dynamics by tracking the abundances of multiple species. The expertise required to identify Arcellinida species and associated taxonomic error and bias limits the comparability of the results (Lamentowicz et al., 2015). Summaries of the ecological function of the group (e.g., life habits, metabolism), as tracked by functional traits, has the potential to offer a complementary approach and potentially provide more robust predictions that could translate across time and space (Blois et al., 2013; Lamentowicz et al., 2015; Marcisz et al., 2020). The success of using functional traits, rather than species abundances, to track community dynamics is demonstrated by studies of peatland testate amoeba where researchers have modeled the response to drier climates (Fournier et al., 2015), autogenous plant succession from lake to bog (Lamentowicz et al., 2015) and peatland disturbance (Jassey et al., 2016; Marcisz et al., 2016). van Bellen et al. (2017) also demonstrated that water table depth reconstructions (i.e., transfer functions) based on functional traits were comparable to species-based reconstructions. Unlike their peatland counterparts, the functional ecology of lake Arcellinida is poorly understood.

Based on four morphological traits (pseudopod characteristics, presence of gas vacuoles, test compression and test composition) Arrieira et al. (2015) observed that functional diversity of Arcellinida communities from the upper Paraná River floodplain, Brazil were controlled by nutrient dynamics. Lansac-Tóha et al. (2014) characterized habitats of Guaraná Lake, Brazil (i.e., epipelagic, epibiotic, and planktic) based on the relative abundance of three Arcellinida test morphologies: elongate, spherical and hemispherical. These modern ecological studies highlight the promise of using Arcellinida functional traits to summarize their ecological function in lakes. One property that both studies share with species-based approaches is they all transform a continuous variable (i.e., test morphology) in to a discrete one (i.e., groupings and labels). Morphological variability was shown to be effectively continuous, with many transitional forms blurring the boundaries between morphotypes (Bobrov and Mazei, 2004). It is still uncertain whether this inherent morphological plasticity (i.e., phenotypic plasticity) is completely random (Wanner, 1999), an adaptive response

to variable environmental conditions (Bobrov and Mazei, 2004) or perhaps species specific. Thus, there is value in capturing morphological variation as a continuous variable to preserve potential environmental signals present in the many transitional forms.

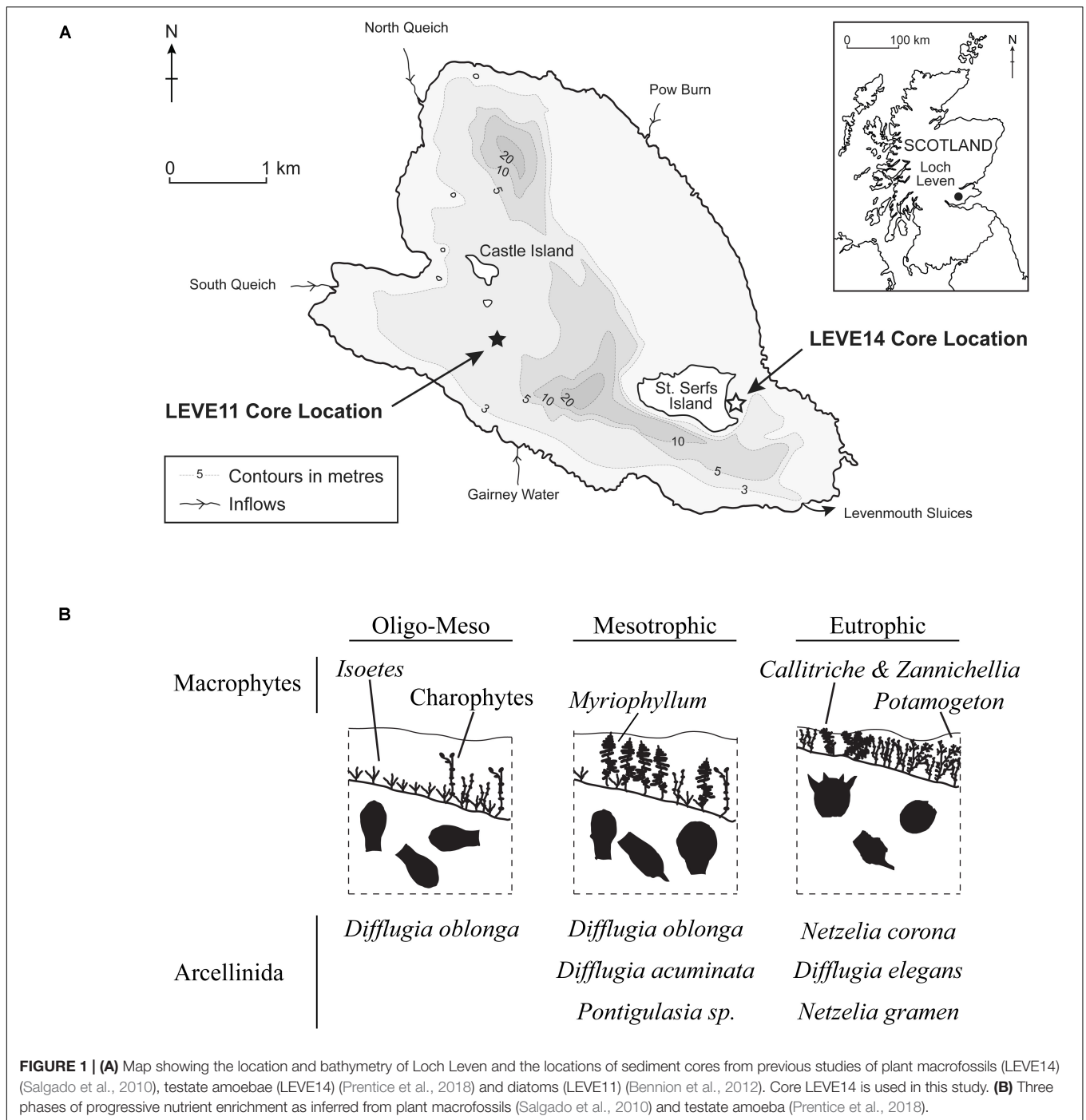
Geometric morphometric analysis has been applied successfully to characterize continuous shape variation in a number of biological and paleontological studies (Zelditch et al., 2012; Adams et al., 2013), including for other shelled microfossil groups (e.g., diatoms; Beszteri, 2005). Macumber et al. (2020) used geometric morphometric analysis to demonstrate that two novel clades of Arcellinida could be differentiated based on test size. Shapes are defined by the geometric configuration of landmarks (fixed anatomically definable locations found on all specimens) and by boundary curves drawn between the landmarks (Zelditch et al., 2012). Geometric morphometric analysis of similarly shaped specimens (i.e., Arcellinida tests) models a morphological space where each test (i.e., shape) occupies a unique place, providing continuous measures of morphological variability (Zelditch et al., 2012). A generalized Procrustes analysis removes variation due to differences in size and orientation, capturing size variation as an independent variable. Multivariate statistical methods can test for shape differences among groups, covariation between shape and other continuous variables and visualize patterns of shape variation (Adams et al., 2013).

As the functional ecology of lake Arcellinida is poorly understood there is value in using continuous measures of morphological variation (i.e., geometric morphometric analysis) to highlight important Arcellinida test traits that are associated with environmental change. Highlighted traits could form the basis of future functional trait studies of lake Arcellinida. We investigated a previously described Arcellinida assemblage from a lake sediment core collected from Loch Leven, Scotland, a large shallow lake which has a well documented history of nutrient enrichment and associated biological change (Salgado et al., 2010; Prentice et al., 2018). Our aims were to (1) characterize test morphological variability in response to environmental change, specifically lake eutrophication induced shifts in the macrophyte community; (2) test the efficacy of geometric morphometric analysis in summarizing community dynamics as compared to conventional paleoecological (i.e., species- and assemblage based) approaches; (3) identify inter- and intraspecific variability in relation to the observed changes in test size and morphology; and (4) make basic inferences regarding the role of shape and size in Arcellinida ecology.

MATERIALS AND METHODS

Study Site

Loch Leven situated in central Scotland (56°11'55" N, 3°22'46" W), is a large (13.3 km²), shallow (mean = 3.9 m, max = 25.4 m), lowland (107 m.a.s.l) freshwater lake (**Figure 1**). It is moderately eutrophic, with mean annual average total phosphorus and chlorophyll *a* concentrations (2008–2010) of 33 and 21 μg L⁻¹, respectively (Dudley et al., 2012). There are four inflows sourcing



water from farms, villages and the nearby town of Kinross (population ~5000).

Core Collection

The sediment core LEVE14, previously studied by Salgado et al. (2010) and Prentice et al. (2018), was collected in shallow-water (depth = 2.2 m) using an adapted Livingstone (7.4 cm diameter) piston corer in May 2006 near the eastern shore of St. Serf's Island in the southeast corner of Loch Leven (Figure 1). The

141-cm core was extruded (i.e., subsampled) in the field at 1-cm intervals.

Chronology

The core was dated radiometrically (^{210}Pb , ^{226}Ra , and ^{137}Cs isotope analysis) using thirteen sediment samples from the top 60 cm (Salgado et al., 2010) using standard methods (Appleby and Oldfield, 1978). The ^{210}Pb chronology suggests a constant sedimentation rate from the late 1920s through the late 1990s

(0.29 cm year⁻¹), although this increased to 1.1 cm year⁻¹ from the early 2000s (Salgado et al., 2010). Extrapolation of this sedimentation rate would give a basal date of AD 1492 (Salgado et al., 2010).

To extend the age depth model, Prentice et al. (2018) obtained three samples for AMS ¹⁴C radiocarbon dating: bulk sediment from 68 to 69 cm (1882 ± 37 year BP), a charcoal sample from 96 to 97 cm (827 ± 34 year BP) and bulk sediment from 134 to 135 cm (2420 ± 34 year BP). The sedimentation rate based on the charcoal date (0.11 cm year⁻¹) estimates a basal date of AD 828. Unfortunately, the AMS ¹⁴C dates do not correspond well with the extrapolated ²¹⁰Pb chronology (see Prentice et al., 2018 for further discussion). Interpretation of the ages is further compounded by a lithostratigraphic change between 120 and 90 cm. Thus, estimating ages for depths greater than 90 cm in the core is contentious, but the basal part of the record is likely older than inferred by the ²¹⁰Pb age-depth model.

Lithology

Fifty one samples were analyzed for loss-on-ignition (LOI) by Salgado et al. (2010) and an additional eighteen samples by Prentice et al. (2018), providing the relative abundance of organic carbon, inorganic carbon and unignitable minerogenic matter (Dean, 1974). Prentice et al. (2018) completed particle size analysis (% sand, silt, clay) of forty-two samples determined by laser diffraction using a Malvern Mastersizer-2000.

Study Design

Geometric morphometric analysis of Arcellinida tests requires more analytical time than conventional Arcellinida enumeration, thus our study was limited to a subset of the 42 Arcellinida samples counted from the LEVE14 core by Prentice et al. (2018). In order to examine morphological variability at key points of change in the sediment history of Loch Leven we targeted core depths immediately above and below CONISS zone boundaries (Grimm, 1987) defined by Prentice et al. (2018). We iteratively compared the CONISS zones based on sample subsets to the zones identified by Prentice et al. (2018) and continued to increase the number of samples in our subset until both agreed. This occurred with a subset of 14 samples (see **Supplementary Table S1** and **Supplementary Figure S1**).

Arcellinida Identification

We enumerated Arcellinida tests from uncounted subsamples from core LEVE14 interval splits [see Prentice et al. (2018) for details of subsample preparation]. Arcellinida tests were quantified in aqueous solution using a Nikon light dissection binocular microscope at x50-93 magnification with reference to various identification keys and published images (**Supplementary Table S2**). Our taxonomic approach differs from Prentice et al. (2018) who employed a strain-based taxonomic approach (Patterson and Kumar, 2002). All specimens in our study were matched with a previously described species; this resulted in some inconsistencies and highlights some of the difficulties with species-based approaches (**Supplementary Table S2**). We identified individual specimens

until our total counts were enough to pass the inflection points of the accumulation curves (see **Supplementary Figure S1**).

Geometric Morphometric Analysis

We used a modified geometric morphometric protocol based on MacLeod (2008) and Zelditch et al. (2012) to capture shape and size indices for the Arcellinida tests. Geometric morphometric analysis creates a shape space in which the morphology of each specimen in a dataset is represented as a unique point and every other point in the shape space represents a hypothetical transitional morphology. Trends in shape variation are reduced to principal components [via principal component analysis (PCA)], the scores from which can be illustrated as hypothetical shape models. It should be noted that because landmarks need to be consistently placed on each specimen (see **Figure 2**) only similarly shaped specimens could be included in the dataset. We therefore focused on Arcellinida species whose test have terminal apertures, and tests that could not be oriented in a consistent manner (e.g., Centropyxidae taxa which are characterized by angled apertures and a random distributions of spines) were excluded from the study.

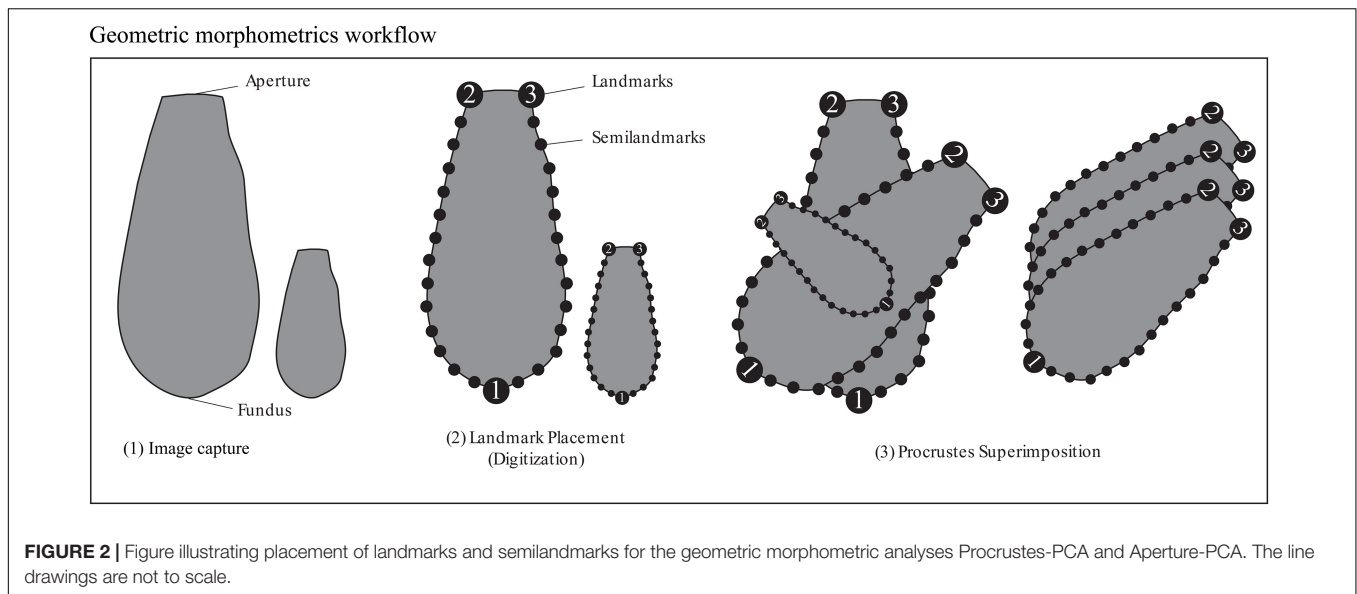
Arcellinida tests ($n = 1505$) were photographed in aqueous solution using a digital camera mounted to a Nikon binocular dissecting light microscope at x93 magnification. Images were digitized using the tps series of software (Rohlf, 2015). We chose three landmark locations on the outer wall of each specimen that could be consistently identified: (1) at the furthest point of the fundus away from the aperture, (2) the left edge of the aperture and (3) the right edge of the aperture (**Figure 2**). Semilandmarks can be anchored and equally spaced between landmarks and are ideal for tracking curves. Two sets of thirteen equally spaced semilandmarks were anchored between landmarks 1 and 2 and landmarks 1 and 3 (**Figure 2**). The combination of landmarks and semilandmarks were used to quantify variability related to test vertical elongation, horizontal widening and tapering.

Statistical Analysis

To capture size indices for the Arcellinida tests we carried out a Procrustes superimposition on the landmark and semilandmark coordinates (**Figure 2**) using sliders to align all specimens and isolate size variation as an independent variable (Zelditch et al., 2012; Rohlf, 2015). Size variation was quantified as the square root of the non-weighted sum of squared distances from the joint centroid (MacLeod, 2008).

To capture shape indices for the Arcellinida tests we modeled the Arcellinida test shape space through a PCA of the Procrustes superimposed landmarks using the “geomorph” R-package (Adams and Otárola-Castillo, 2013). We excluded all principal components that described less than 5% of the variance as this was less than the error of geometric morphometric analysis (see below).

We used a Procrustes ANOVA to characterize error and assess the repeatability of our imaging and landmark placement (Fruciano, 2016). We used Arcellinida tests isolated from a subsample at 80 cm core depth as our test group following the protocol illustrated in **Supplementary Figure S2**. To quantify



the repeatability of our imaging method we compared the mean square error between image replicates (**Supplementary Figure S2** and **Supplementary Table S3**). To quantify the repeatability of our landmark placement we compared the mean square error between landmark placement replicates (**Supplementary Figure S2** and **Supplementary Table S3**). Both the imaging and landmark placement had repeatability measures greater than 95%.

To summarize the principal components of the Procrustes PCA into groupings based on shape indices, we visualized significant breaks in Procrustes principal component 1 and 2 scores using a Wards cluster analysis implemented with the “ward.D” method in the `hclust` function in the `stats` package (R Core Team, 2016). Before cluster analysis we created an Euclidean distance matrix using the `vegdist` function in the `vegan` package in R (Oksanen et al., 2017). Following Borcard et al. (2011), we constrained the number of clusters using the Rousseeuw quality index and the Mantel statistic. To compare within and between species trait variation (i.e., centroid size, PC1 and PC2 scores) we grouped specimens by species and displayed variance in their trait values as boxplots.

We defined temporal communities represented by the CONISS zones as defined by Prentice et al. (2018): Zone 1 (141–94 cm), Zone 2 (94–41 cm), Zone 3 (41–3 cm), and Zone 4 (3–0 cm). Using these temporal communities, we can investigate how shape (PC1 and PC2 scores) and size (centroid size) indices vary in response to eutrophication in a freshwater lake (Salgado et al., 2010; Prentice et al., 2018). To understand the contribution of intraspecific trait variance to the total within-community trait variance (sum of interspecific and intraspecific trait variance), we calculated the relative contribution of intraspecific trait variance to within-community trait variance as described by Siefert et al. (2015). To compare Arcellinida test shape (i.e., median PC1 scores) and size (i.e., median centroid size) to Arcellinida species dynamics (i.e., over time) we used a series of biplots, each representing

specimen values from a temporal community representing different environmental conditions.

RESULTS

Temporal Communities and Environmental Conditions

The palaeolimnological studies of Salgado et al. (2010) and Prentice et al. (2018) which utilized plant macrofossils and Arcellinida, respectively, identified three main long-term phases of ecological change in the Loch Leven (LEVE14) core in response to gradual eutrophication: (1) an early phase of oligo-mesotrophic conditions, (2) an intermediate phase of mesotrophic conditions which began after circa 1200 AD, and (3) an upper eutrophic phase. Prentice et al. (2018) observed a fourth phase in the testate amoeba assemblage captured in the uppermost two samples (0–3 cm). They observed a significant drop in species diversity with a coeval increase in test concentrations (tests/cc). We adopt the four phases (or zones) defined by Prentice et al. (2018) through CONISS analysis of the plant macrofossils and Arcellinida results. The four phases demarcate temporal communities each associated with different environmental conditions and hereafter referred to as Zones 1 to 4.

The sediments of Zone 1 (141–94 cm) are characterized by increasing sand content and decreasing organic matter (LOI = 1–11%) toward Zone 2. The oligo-mesotrophic conditions are characterized by tests of *Diffflugia oblonga* Ehrenberg 1838 (Prentice et al., 2018) and isoetid (e.g., *Isoetes lacustris*) macrophytes (Salgado et al., 2010). Zone 2 (94–41 cm) is characterized by an increase in organic matter and decreased sand content in comparison to Zone 1. The mesotrophic conditions are characterized by a greater diversity in the Arcellinida assemblage with a decreasing trend in *D. oblonga* (Prentice et al., 2018) and a reduction in isoetids

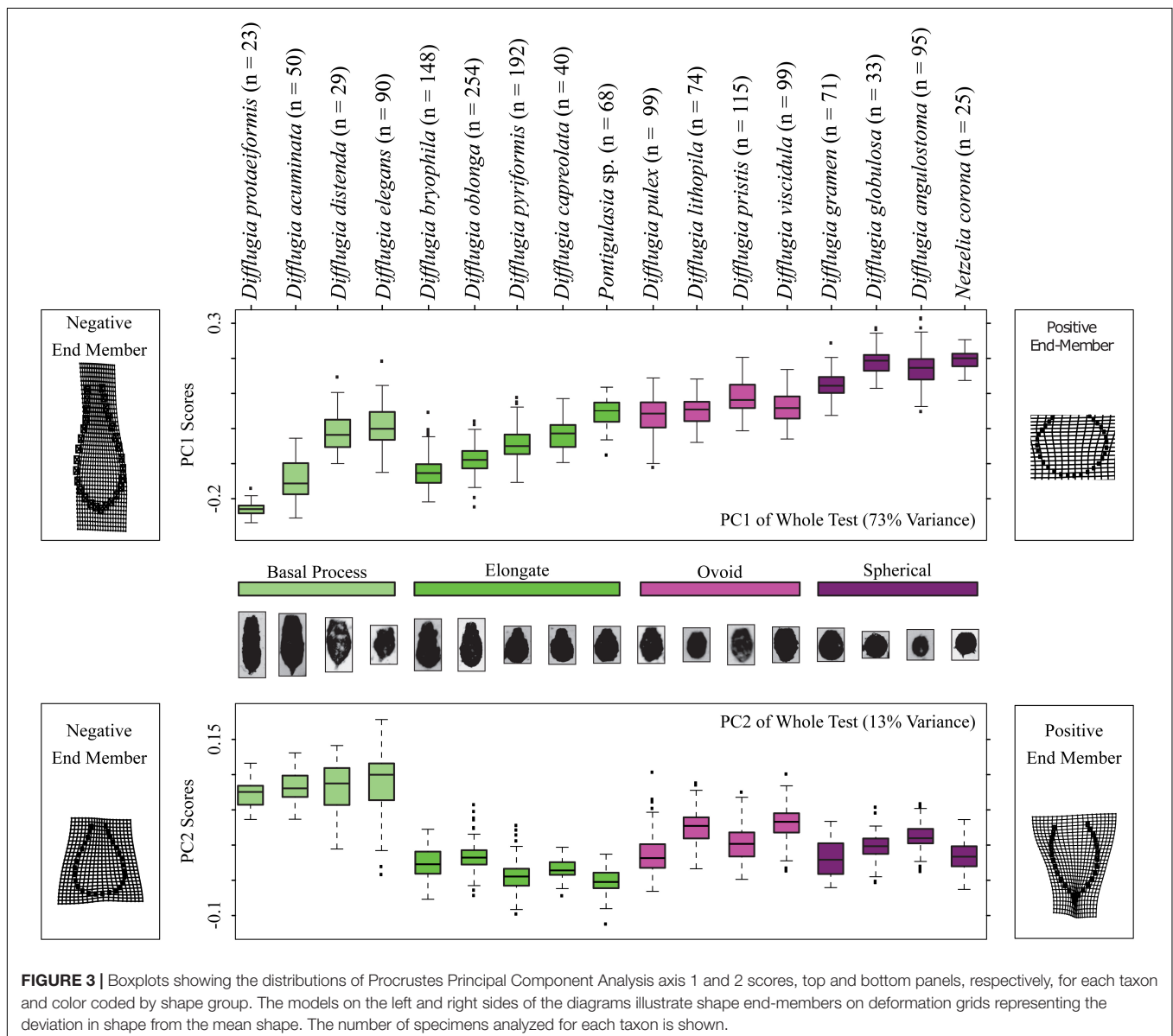
(Salgado et al., 2010). The sediments of Zone 3 (41–3 cm) become less sandy and continue to increase in organics toward Zone 4. The eutrophic conditions are characterized by tests of *Cucurbitella tricuspis* (Carter 1856) and *Mediolus corona* (Wallich 1864) Patterson et al., 2014 (i.e., *Netzelia* sp. see **Supplementary Table S2**), the disappearance of isoetid plants, and the proliferation of elodeid (e.g., *Zannichellia palustris*, *Potamogeton pusillus*) macrophytes. There is no change in the lithology for Zone 4 (3–0 cm) but there is a 15-fold increase in Arcellinida test concentrations compared to Zone 3 and Arcellinida diversity decreased substantially.

Geometric Morphometric Analysis

The Procrustes PCA yielded two axes of shape variation (**Supplementary Table S4**): principal component one (PC1) explained 72.3% and PC2 explained 13.5% of the test shape

variation in the Loch Leven Arcellinida microfossil assemblages. We kept the first two principal components (PC1 and PC2) as all other principal components explained less than 5% of the shape variation, less than methodological error (**Supplementary Table S3, S4**).

Shape change in the Arcellinida tests along PC1 (**Figure 3**) represents the gradual transition from elongated specimens (negative values) to spherical specimens (positive values) as illustrated by the end-members found to the left (negative end member) and right (positive end member) of the panels in **Figure 3**. Important modes of change include compression of the vertical axis, widening of the horizontal axis, and the loss of a distinctive neck (**Figure 3**). Based on PC1 scores, the basal process-type taxa could be divided into an elongate-type (i.e., *Diffflugia protaeiformis* Lamarck 1816 and *Diffflugia acuminata* Ehrenberg, 1838) and the ovoid-type group (i.e., *Diffflugia*



distenda Ogden 1983 and *Diffflugia elegans* Penard 1890). Shape change along PC2 varies between tests with wide bases and narrow apertures to tests with pointed bases and wide apertures, and effectively separates the basal process-type taxa from all other types.

Not all taxa can be distinguished based on shape alone. For example, *D. distenda* and *D. elegans* or *Diffflugia lithophila* Penard 1902 and *Diffflugia viscidula* Penard 1902 can only be differentiated based on centroid size (Figure 4). Several taxa, especially the elongate type but also *D. acuminata* and *Diffflugia globulosa* Dujardin 1837, have wide test centroid size ranges. The elongate-type taxa [except *Diffflugia bryophila* (Penard 1902) Jung 1942] also have the largest test centroid sizes, as do *D. protaeiformis*, *D. viscidula*, and *Netzelia corona* (Wallich 1864) Gomaa et al., 2017. Ovoid-type taxa have narrow ranges and apart from *D. viscidula* have low mean test centroid sizes (Figure 4).

Intraspecific Trait Variation (ITV)

For each Arcellinida test trait (centroid size, PC1, PC2 scores), we calculated the contribution of intraspecific trait variation (ITV)

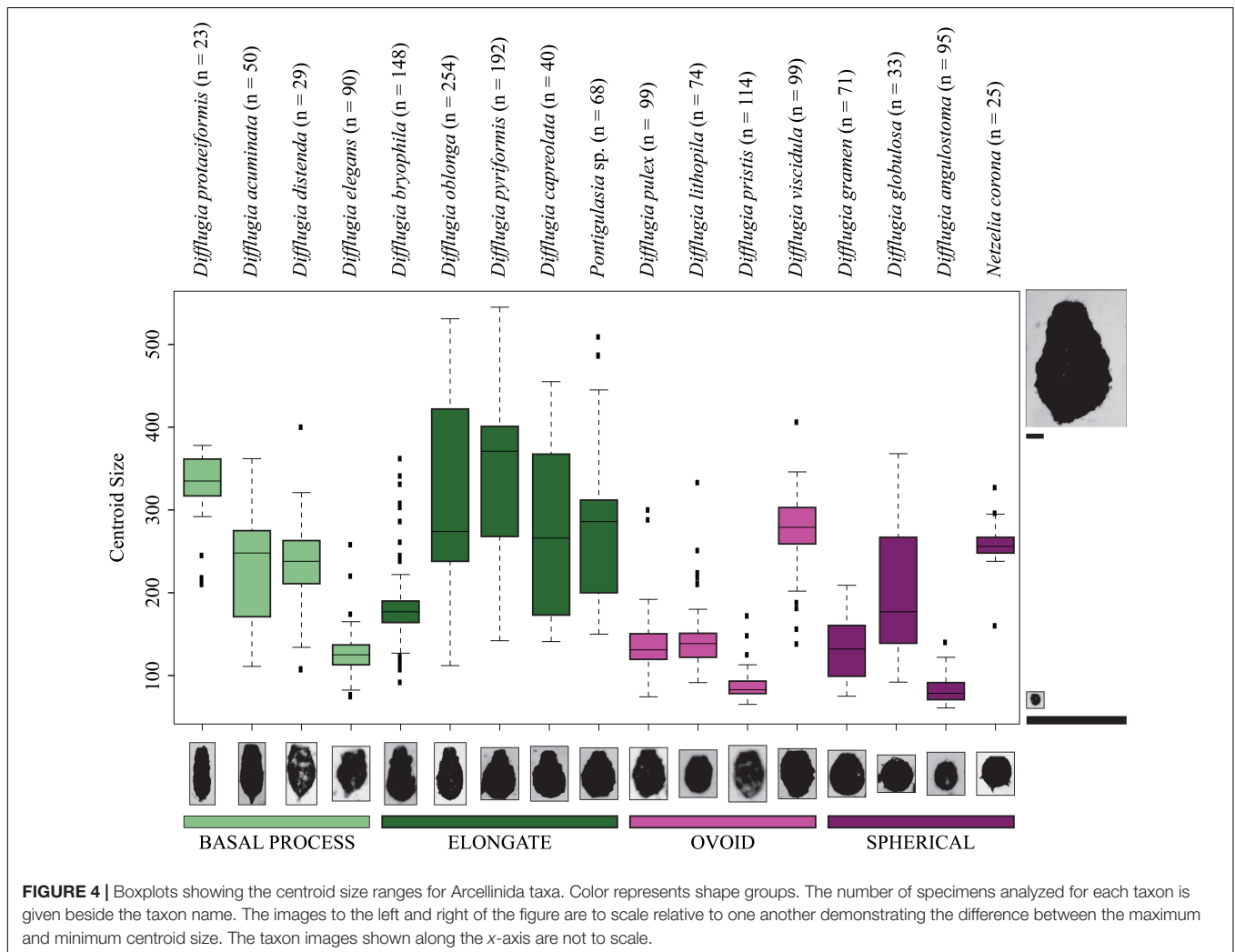
to the total within community trait variation (i.e., the sum of intraspecific and interspecific trait variation; Figure 5).

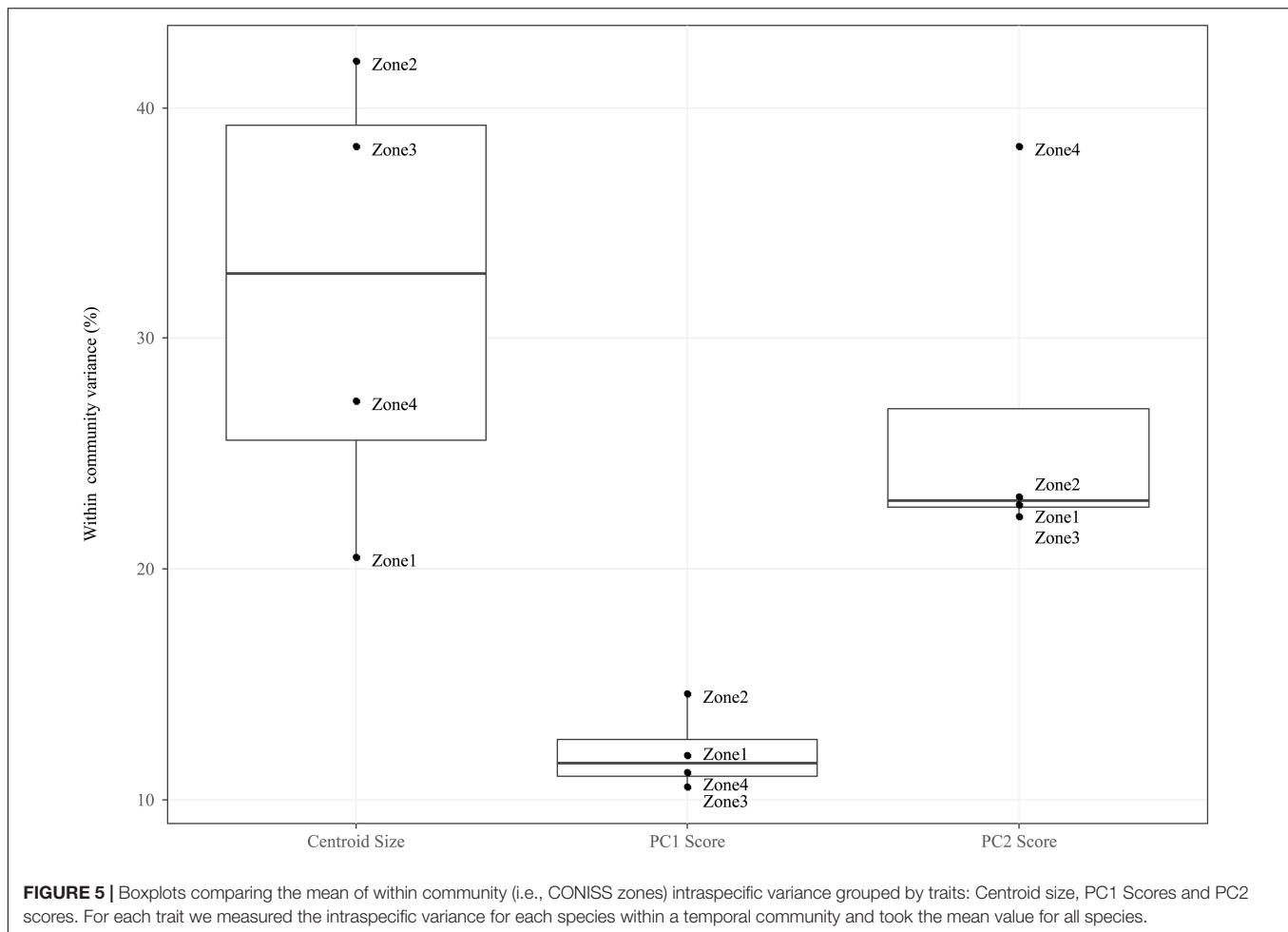
For the PC1 scores, ITV contributes relatively little variation to the total within community trait variation (median = 11.54%, mad = 0.01%), and this is true across all temporal communities. For the PC2 scores, ITV contribution is twice as large (median = 22.67%, mad = 0.01%) as compared to the PC1 scores, with the ITV in Zone 4 being significantly higher (mean = 38.36%) than all other communities that are clustered around the median.

When compared to the shape indices (i.e., PC1 and PC2), ITV was found to explain a greater amount of variation (median = 32.80%, mad = 10.92%) to total within community centroid size variation. In addition, the communities (i.e., Zone 1–4) form two groupings on either size of the median: Zones 1 and 4 (range = 20.52–27.29%) and Zones 2 and 3 (range = 38.32–42.01%).

Traits and Species Dynamics

Figure 6 displays the temporal communities (CONISS zones) as a series of biplots of Arcellinida species with median PC1





score along the x-axis and median centroid size along the y-axis. Interestingly, between Zone 1 to Zone 4 the average community test shape changes from a dominance of elongate-ovoid to ovoid-spherical shape types, while the average test size decreases. The greatest range in both centroid size and test shape is found in Zone 3, followed by Zone 2. Zone 1 is restricted, as compared to Zone 2 and 3, in its shape range, while Zone 4, as compared to all other zones, is restricted in both shape and size.

There are several taxa that display relatively large changes in median centroid size (e.g., *Diffugia capreolata* Penard 1902, *D. distenda*, *D. globulosa* and *D. oblonga*) as compared to the other taxa. The communities differ in their size structure, with Zone 1 comprising two groupings, large (>300) and small tests (<200); in Zone 2 there is the development of a medium test size (200–300), increased representation of the small test size and decrease in the large test size; Zone 3 continues the trend seen in Zone 2, with a further decrease in large tests and an increase in the number of medium sized taxa; finally in Zone 4 both large and medium tests are not found and only small tests remain.

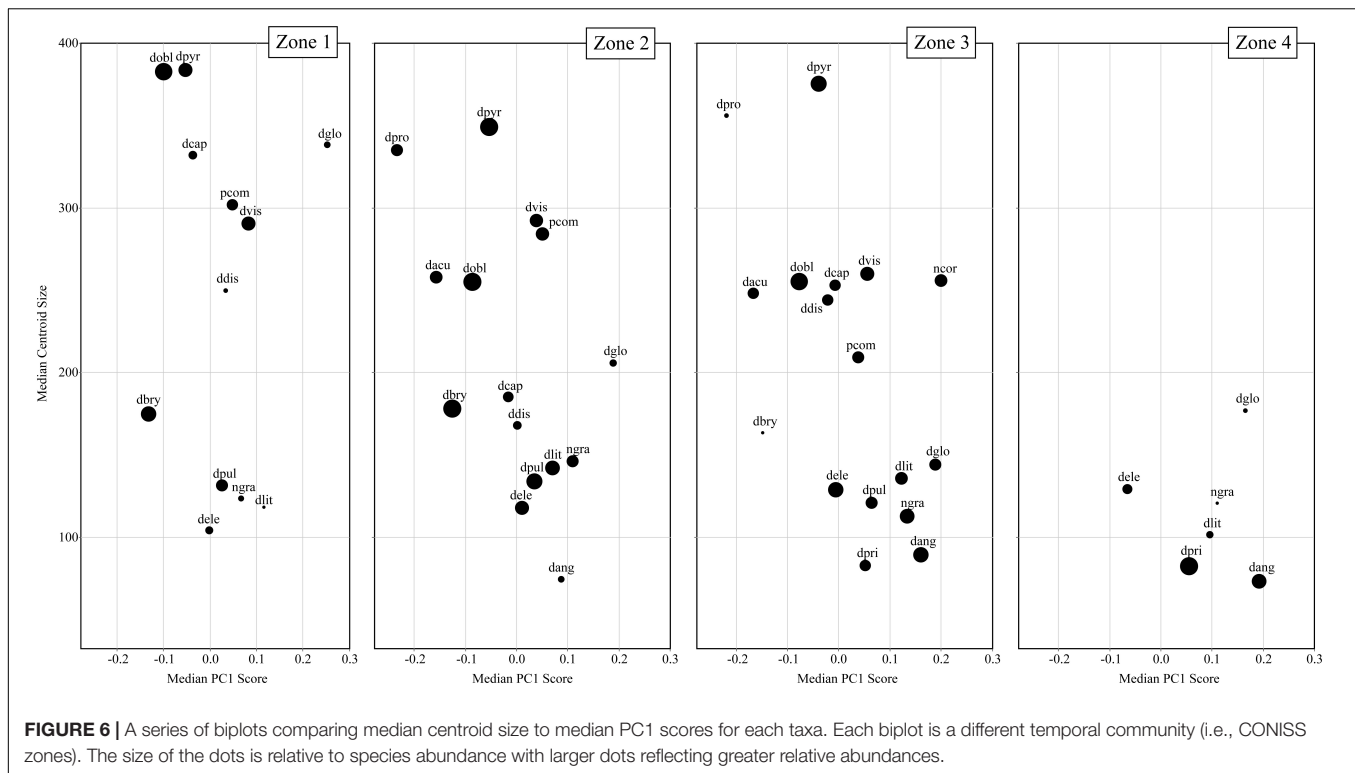
Cluster Analysis

Cluster analysis on the Procrustes PC1 and PC2 scores divided the dataset into three test shape clusters (Figure 7).

Each cluster is dominated (and so named) by a shape-group (i.e., Elongate, Ovoid and Spherical). The cluster analysis further divides the basal process shape group between the Elongate (30%) and Ovoid (69%) clusters. This distinction of taxa based on a simplified morphological descriptor reliably represented the cluster assignment (e.g., elongate-type taxa were assigned to the Elongate cluster); however, several exceptions exist: *D. acuminata*, *D. capreolata*, *Netzelia gramen* (Penard 1902) Gomaa et al. (2017), *D. globulosa* and *Diffugia angulostoma* Gauthier-Lièvre and Thomas (1958) (Figure 7). Cluster analysis divides these taxa into several clusters.

Stratigraphic Variability

In Figure 8 we display the stratigraphic variation of our test size and shape data alongside the species-based results of the Salgado et al. (2010) plant macrofossil and the Prentice et al. (2018) Arcellinida studies. Significantly, our size and shape indices display stratigraphic trends that correspond with the CONISS zone boundaries defined by the testate amoeba and plant macrofossil datasets, especially the initiations of Zones 3 and 4 (Figure 8). We observed four phases of test shape cluster change: (1) a stable phase comprising only the elongate and ovoid tests



through Zones 1 and 2; (2) the appearance of spherical tests at 40 cm (Zone 3); (3) the initiation of a decline in elongate tests at 36 cm; and (4) the disappearance of elongate tests above 6 cm (Zone 4). The median centroid size follows a similar stratigraphic pattern to the elongate tests, with a noticeable decrease in median centroid size above 6 cm.

DISCUSSION

The four phases of ecological change recorded in Loch Leven core LEVE14 in response to gradual eutrophication, as identified by the palaeolimnological studies of Salgado et al. (2010) and Prentice et al. (2018), provide an excellent framework to see whether geometric morphometric analysis applied to freshwater Arcellinida tests can identify functional traits which provide complementary summaries of ecological function without taxonomic expertise. In addition, assess the impact of intraspecific trait variability.

Pre-enrichment Phase (pre-1830)

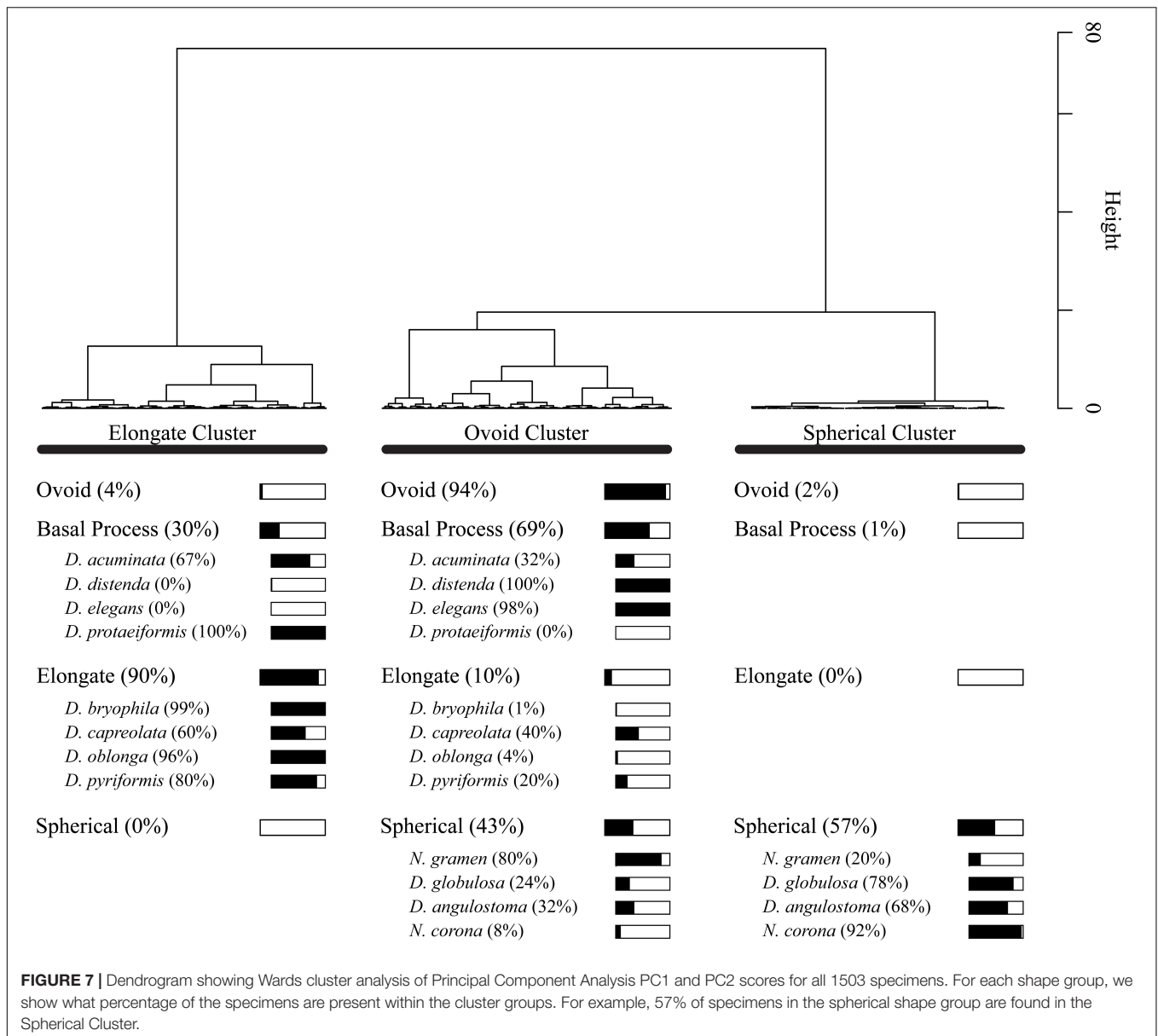
Zone 1 (141–90 cm)

Salgado et al. (2010) inferred that the Zone 1 plant macrofossil assemblage of the LEVE14 sediment core was typical of a nutrient poor oligotrophic-mesotrophic lake, with very few dense stands of macrophytes and relatively deep and open water conditions (Figure 1B). The Arcellinida assemblage is comprised of mainly of elongate tests (60%; Figures 7, 8). Elongate difflugids, are inferred to reflect a benthic life habit (Patterson and Kumar, 2002; Lansac-Tôha et al., 2014; Prentice et al., 2018) and

in fact, their abundances have been observed to decrease as benthic conditions become stressed (e.g., in reduced oxygen environments, Roe et al., 2010).

Taxa in the ovoid cluster make up 30% of the Zone 1 community (Figure 6). Lansac-Tôha et al. (2014), in a study of living testate amoeba from different habitats of a shallow lake in Brazil, observed assemblages composed of “elongate” tests in benthic habitats, also in association with macrophyte habitats (i.e., epibiotic). Several of the taxa classified as “elongate” by Lansac-Tôha et al. (2014) are classified by our geometric morphometric analysis as ovoid (e.g., *D. elegans*; Figure 7). Thus, we infer that taxa of the ovoid cluster might represent the success of an epibiotic life habit (i.e., stands of macrophytes) in addition to an oxygenated benthic environment.

Zone 1 has the greatest median centroid size (Figure 8), with more species characterized by large tests (centroid size >200) than any other temporal community (Figure 6). This is typified by *Difflugia pyriformis* Perty 1849 a large elongate difflugid known to be mixotrophic (Leidy, 1879). Macumber et al. (2020) using high-resolution phylogenetics (NAD9-NAD7 genes) and geometric morphometrics demonstrated two novel clades of elongate type *Difflugia* species that could be distinguished based on test centroid size: a pyriform clade and a lanceolate clade. The “pyriform” clade was characterized by larger centroid sizes and members known to be mixotrophic, while the “lanceolate” clade had smaller test centroid sizes and only heterotrophic taxa. The larger average centroid size and the higher abundance of elongate type taxa recorded in Zone 1 could therefore reflect relatively higher abundances of mixotrophic as compared to heterotrophic taxa. The oligotrophic-mesotrophic conditions inferred for the



loch at this time would have promoted a benthic mixotrophic life habit and thus may well explain the presence of large elongate difflugids.

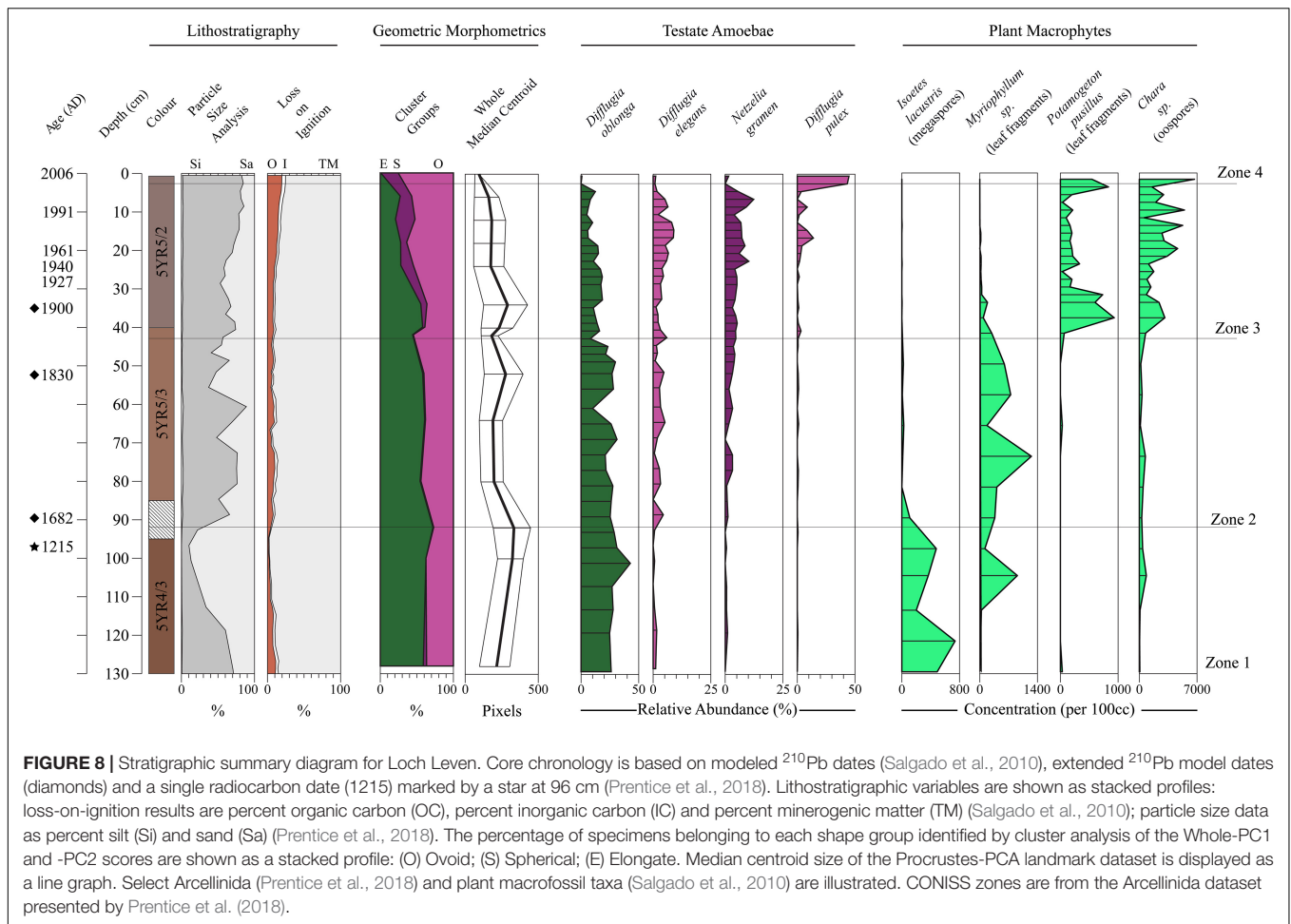
Zone 2 (90–41 cm)

From Zone 1 to Zone 2, macrofossils of the submerged taller growing macrophytes *Myriophyllum* and *Chara* sp., expanded coinciding with a reduction in isoetid plants. These changes were associated with increasing eutrophication of the lake, which was driven by expanding agricultural production in the catchment (Salgado et al., 2010). Denser stands of macrophytes would have provided increased habitat availability for epibiotic taxa such as members of the ovoid cluster. Accordingly, we observe an increase in the ovoid cluster (33 to 44%) as represented by increases in *D. elegans*, *Diffugia pulex* Penard 1902, and

D. lithophila, with the appearance of *D. distenda* (Figures 6, 8). This increase in ovoid taxa could also explain the coeval decrease in median centroid size (Figure 8), which is driven by a decrease in median size of several species, notably *D. oblonga*, *D. protaeiformis*, *D. pyriformis*, and *D. globulosa* (Figure 6). Such shifts in median centroid size for several species would also explain why Zone 2 has the greatest amount of intraspecific centroid size variation (Figure 5).

Water Level Lowering and Nutrient Enrichment Phase (1830–1985)

The 1830s lowering of Loch Leven by 1.5 m was coincident with increased catchment agriculture and was inferred by a sedimentological color change at 41 cm in the LEVE14 core (Salgado et al., 2010; Figure 8). Nutrient enrichment led



to frequent phytoplankton blooms, reducing light penetration and favoring macrophyte canopy forming species that grew close to the water surface (e.g., *Potamogeton* and *Chara* sp.; **Figure 1B**; Salgado et al., 2010). The expansion of dense elodeid stands (e.g., *Myriophyllum* and *Chara* sp.) could have resulted in benthic conditions low in oxygen (Lindholm et al., 2008; Tarkowska-Kukuryk and Kornijów, 2008) as supported by decreases in benthic diatoms (Bennion et al., 2012). Oxygen levels could have been further impacted by stands of *Chara* sp. reducing water flow (Vermaat et al., 2000). Reduced bottom water oxygen levels would have stressed benthic taxa. Indeed, we observe coeval decreases in the relative abundance of the elongate cluster, inferred to reflect benthic life habits (**Figure 8**).

At 40 cm there is also an increase in taxa from the spherical cluster: *N. corona* and *D. globulosa* and other taxa transitioning to more spherical shapes: *D. angulostoma*, *D. lithophila*, and *N. gramen* (**Figures 6, 8**). These shifts in test shape are characterized by a relative low amount of intraspecific variability as compared to centroid size (**Figure 5**). The taxon *N. gramen* (i.e., *C. tricuspis*; **Supplementary Table S2**), assigned to the ovoid and spherical clusters, can switch from benthic to planktic life habits during the summer through the addition of fat droplets

and gas bubbles into the test to aid buoyancy (Schönborn, 1962; Arndt, 1993). In their study of Guaraná Lake in Brazil, Lansac-Tôha et al. (2014) found that planktic testate amoeba assemblages were composed of hemispherical and spherical tests. Wiik et al. (2015) also observed coincident increases in *C. tricuspis* (i.e., *N. gramen*), planktic diatoms and *Daphnia* sp., in response to increasing eutrophication of a small marl lake, Cunswick Tarn (United Kingdom). Interestingly, Han et al. (2008, 2011) described the feeding agility and wide prey range of several planktic Arcellinida taxa including *N. tuberspinifera*, which is morphologically and phylogenetically related to *N. corona* (Gomaa et al., 2017) which is assigned to the spherical cluster (**Figure 7**). Taking these points together, it is likely that the observed increase in the spherical cluster in our study reflects the expansion of planktic and epibiotic life strategies, both for Arcellinida and their prey, driven by the expansion of canopy forming macrophyte forming denser stands in the shallower waters.

The existence of an ecological separation between taxa in the spherical (i.e., planktic or epibiotic) and elongate cluster (i.e., benthic) is mirrored by differences in their phylogenetic placement. Molecular barcoding results demonstrate that spherical cluster taxa (e.g., *Netzelia* sp.) previously classified as

part of the genus *Diffugia* in fact belong to the family Netzellidae and were assigned to the genus *Netzelia* (Kosakyan et al., 2016; Blandenier et al., 2017; Gomaa et al., 2017). The genetic divergence between these two groups could reflect a speciation event related to divergent life habits (i.e., planktic/epibiotic vs. benthic; Weisse, 2008).

The period from 1969 to 1987 represents one of the most productive periods in the history of Loch Leven with cyanobacteria blooms regularly occurring and planktic diatoms dominating the diatom assemblages (Bennion et al., 2012). This period coincided with a peak in tests from the spherical cluster at 12 cm (c. 1986 AD). This was broadly coeval with a reported *Daphnia* sp. bloom in the 1970s (May et al., 2012) suggesting that a zooplankton life strategy was very successful at this time and perhaps why there is an increase in small spherical tests. An increase in the availability of zooplanktonic prey would support planktic carnivory, potentially explaining the increase in *N. corona* (Han et al., 2008, 2011).

Remediation Phase (1985 to Present)

Remediation measures to reduce nutrient loading of Loch Leven have been in place since the mid-1980s (Salgado et al., 2010). Bennion et al. (2012) observed little evidence in the Loch Leven diatom assemblage for remediation success though and hypothesized that it was masked by the increasing influence of climate change. The Arcellinida data support this observation as test morphological trends continue along trajectories established before remediation efforts (Figure 8). The disappearance of the elongate cluster from 2000 AD is inferred to represent the development of an inhospitable benthic zone, while planktic and epibiotic life habits continued to be successful (Figures 6, 8). Prentice et al. (2018) hypothesized that continued *Chara* sp. expansion from 2000 to 2006 AD (Salgado et al., 2010) could have further reduced bottom water oxygen levels.

At c. 2006 AD, very small tests, having a median centroid size 58% less than in Zone 3, dominate the sample at unprecedented concentrations (Figures 6, 8). Prentice et al. (2018) inferred stressed environmental conditions prevailed in the loch at this time based on a decrease in Arcellinida species diversity. Assemblage dwarfing was observed in response to a stressed environment (e.g., fire and peat farming) by Marcisz et al. (2016) in their study of *Sphagnum* peatlands in Poland, as well as in other protist groups after abrupt perturbations (e.g., foraminifera; Hsiang et al., 2016).

Leading up to the interval representing c. 2006 AD centroid size is relatively stable, suggesting that an additional stressor in addition to nutrient enrichment may have impacted the Arcellinida of this uppermost part of the record, for example, warming water temperatures. Spring temperatures are certainly known to have risen in the loch between 1968 and 2007 (Carvalho et al., 2012). A relationship between Arcellinida test size and temperature has been observed experimentally (Wanner, 1999) and in field studies (Collins et al., 1990; Dallimore et al., 2000). A temperature increase in 10°C doubles cellular respiration while oxygen diffusion increases proportionally (Wanner, 1999). Thus, testate lobose amoeba may respond to temperature rises by increasing their surface area

to volume (e.g., smaller cell sizes) and increasing the size of their apertures.

Efficacy of Geometric Morphometrics to Summarize Arcellinida Community Dynamics as Compared to Species-Based Approaches

This study has shown that geometric morphometric analysis of Arcellinida tests was able to capture the key phases of environmental variability recorded in the Loch Leven LEVE14 sediment core using only three variables (i.e., PC1, PC2, and centroid size). This is far fewer variables than a conventional species-based approach and requires less taxonomic expertise, making it more accessible to non-specialists. Our identification of three shape types (ovoid, elongate, and spherical) in freshwater Arcellinida tests adds support to ecological (Lansac-Tôha et al., 2014) and molecular (Gomaa et al., 2017) studies which have shown that there is a link between test morphology and life habit. In the current study, spherical tests have been shown to be associated with the expansion of both planktic and macrophytic habitats, while elongate tests are related to benthic habitat conditions. We recommend future experimental studies to test the relationship between Arcellinida and dissolved oxygen levels, to further observe macrophyte and Arcellinida associations and to undertake lake surveys along nutrient gradients to characterize the test morphological response to nutrient-enrichment.

Size variability, as an independent measure, was found to be valuable for inferring a response to eutrophication and/or warming temperatures and illustrating that shape groups (e.g., the elongate cluster) and species vary greatly in their range of sizes (Figures 4–6). This high degree of infraspecific variation could reflect an environmental response or could be related to the lumping of distinct taxa during the enumeration process. More research is needed to understand whether size is a specific trait and/or an intraspecific response to environmental variability. Other potential evidence of intraspecific variation was observed by certain taxa belonging to more than one shape cluster. The ability to capture this variation is an added benefit of geometric morphometrics, and future studies could assess the importance of this variation as a response to environmental variability.

Future Development

Geometric morphometric analysis requires Arcellinida tests to be photographed and digitized (i.e., landmark placement) in a consistent manner. This increases analytical time, excludes those taxa that cannot be oriented in a consistent manner (e.g., centropxydids) and, depending on the camera resolution, means that fine features may not be captured. Photography and digitization could potentially be automated reducing analytical time (Hsiang et al., 2016; Steele et al., 2020). Using 3D imaging techniques (e.g., nano-ct) could facilitate the inclusion of all test shapes. The sediment record of LEVE14 core, illustrates an environmental history of plant macrofossil community turnover associated with enrichment, resulting in significant habitat changes. Whether morphological changes in freshwater

Arcellinida tests can track other types of environmental changes should be the focus of future research.

CONCLUSION

This study is the first paleoecological study to characterize Arcellinida test morphological variability using geometric morphometric analysis, providing continuous and independent measures of shape and size variation. Four morphological parameters (centroid size, spherical, ovoid and elongate clusters) accorded well with species-based reconstructions of limnological change in Loch Leven in terms of timing and nature. Fewer parameters that do not require taxonomic expertise and associated with ecological functioning provided intuitive results that could be made accessible to non-specialists facilitating their incorporation into larger multi-proxy studies. Our results support and build upon the hypothesis that freshwater Arcellinida test morphology reflects life habit, with spherical tests representing planktic habitat settings, ovoid tests signaling epibiotic living modes and elongate tests associated with benthic habitats. Our research illustrates the promise of freshwater Arcellinida test morphological variability in tracking environmental change.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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AUTHOR CONTRIBUTIONS

AM and HR conceived and designed the analyses. SP and CS provided valuable insight about the previous Loch Leven study and access to the subsamples. HR provided research space and resources. AM performed data collection, analyses, and wrote the manuscript. All authors revised and edited the manuscript, read, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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