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Assessing the ecological value of small testate amoebae (<45 μm) in New Zealand peatlands

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Abstract

Methodological advances are essential for robust ecological research. Quantitative reconstructions of environmental conditions using testate amoebae rely on sound taxonomy. While the taxonomy of large species is relatively well resolved, this is not the case for most small taxa (typically <45 μm long). In New Zealand, peatlands contain a diversity of both cosmopolitan and characteristic large southern endemic taxa, but also have a high abundance of small taxa. The latter are often lumped into morphotypes reducing their value as ecological indicators. In this study, we demonstrate how (a) lumping small taxa versus splitting them into unique types, and (b) including or excluding them from community analysis influenced their ecological inference. We assessed testate amoeba composition in six peat bogs from New Zealand, three that were moderately-to-highly impacted, and three that were non-impacted. Environmental variables were measured at each sampling site and the surface testate amoeba community patterns and community-environment relationships compared. We found a clear division between impacted and non-impacted sites. Several distinct small taxa were more strongly related to water-table depth and conductivity, while the larger taxa were more correlated to pH. These results show that improved taxonomic resolution of small taxa can provide more informed environmental assessment.

Keywords: Bogs; Human impact; New Zealand; Taxonomy; Testate amoebae; Wetlands

Introduction

Testate amoebae are a polyphyletic assemblage of free-living single-celled shelled eukaryotes (Mitchell et al., 2008). Molecular phylogenetic studies based on ribosomal RNA and protein gene sequences show that these organisms belong to the three supergroups Amoebozoa (Nikolaev et al., 2005), Stramenopiles (Gomaa et al., 2014), and Rhizaria (Bhattacharya et al., 1995; Dumack et al., 2016). They are highly diverse, ubiquitous in soil, litter, mosses, lakes, rivers and brackish water environments (Amesbury et al., 2017; Barnett et al., 2017; Charman, 1997; Charman et al., 2007; Fernández et al., 2015; Koenig et al., 2018; Royles et al., 2016; Swindles et al., 2015), and they represent one of the most abundant and diverse groups of terrestrial protists.

Testate amoebae are increasingly being used as models for microbial biogeography (Lara et al., 2016; Mazei et al., 2018; Smith et al., 2007), as indicators of ecological integrity

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in peatlands (Beaulne et al., 2018), as a tool for monitoring restoration success (Swindles et al., 2016; Valentine et al., 2013), and as indicators in ecotoxicology (Amacker et al., 2018; Meyer et al., 2012). They have also been applied as trace evidence in forensic science (Swindles and Ruffell, 2009), and for defining post-mortem intervals (Seppey et al., 2016; Szelecz et al., 2014). As these organisms produce decay-resistant tests, they are commonly applied in palaeoenvironmental research (Amesbury et al., 2016; Lamentowicz et al., 2009; Seddon et al., 2014; Wilmshurst et al., 2003), and have been successfully used to reconstruct past moisture conditions across a broad range of climatic zones (McGlone and Wilmshurst, 1999; Royles et al., 2016; Swindles et al., 2015; Swindles et al., 2016; Wilmshurst et al., 2002). Although the interest in using these organisms in scientific research has notably increased over the last two decades, studies focusing on taxonomy, either morphometrically or phylogenetically, have not increased at the same rate (Roland et al., 2017). This poses a potential problem, as the use of testate amoebae as a bioindicator relies on sound taxonomy.

The necessity of taxonomic improvements for applied uses of testate amoebae analysis has been regularly stressed (Booth and Zygmunt, 2005; Kosakyan et al., 2016a; Lahr et al., 2012; Mitchell et al., 2008). While the taxonomy of large species of testate amoebae is relatively well-resolved based on morphology (Fernández et al., 2016; Fernández et al., 2015; Heger et al., 2013; Lara et al., 2011), and increasingly by using molecular approaches (Gomaa et al., 2012; Kosakyan et al., 2016b; Singer et al., 2015), this is not the case for most "small taxa" (typically less than 45 μm long and hereafter labelled small), which often get lumped into broad morphotypes. Progress is slow due to the limited amount of scientific research on the taxonomy of these morphotypes. This may impede research advances, as significant differences in environmental preferences may exist among different taxa, which could make them even more useful ecological indicators. Such morphological taxonomies are crucial to the field of palaeoecology, as the (sub)-fossil tests are used to reconstruct past communities (Mitchell et al., 2008; Swindles and Roe, 2007; Wilmshurst et al., 2003).

Traditional identification and morphometric analysis of testate amoebae relies on shape and measurements of test dimensions such as size of the shell and apertures, along with variations in the composition of the tests (Ogden and Hedley, 1980). Tests are composed of a proteinaceous matrix (amorpheous, e.g. *Hyalosphenia*, or plate-like, e.g. *Arcella*), which can be reinforced with agglutinated extraneous material (referred to as xenosomes, e.g. *Difflugia*), self-secreted calcareous or siliceous plates (referred to as idiosomes, e.g. *Paraquadrula*, *Euglypha*) or siliceous plates recycled from consumed prey (e.g. *Nebela*). Some taxa can incorporate both xenosomes and idiosomes (e.g. *Netzelia*), or xenosomes and recycled siliceous scales (e.g. *Heleopera*) into their tests.

Monographs widely used by ecologists and palaeoecologists to identify tests tend to describe larger testate amoeba species in greater detail than the smaller taxa, and this is the case for the most frequently cited reference guide by Charman et al. (2000). The existing monographs on individual genera, mostly describe the larger taxa (Chardez, 1969, 1985; Decloître, 1962; Deflandre, 1928; Deflandre, 1929; Deflandre, 1936; Grospietsch, 1958; Thomas and Gauthier-Lièvre, 1959), except for genera *Cryptodifflugia* and *Difflugiella* (Page 1966; Grospietsch 1965). By contrast, knowledge on the diversity of agglutinated xenosomic species is limited (Delaine et al., 2017), making these taxa (such as *Phryganella paradoxa*, *Pseudodifflugia fulva*, *Difflugia pulex* and *Difflugia pristis*) particularly challenging to identify and differentiate. As a result, they are often lumped by community analysts, leaving their value as bioindicators poorly defined.

Most of the work on peatland testate amoebae has been undertaken on Northern Hemisphere *Sphagnum* bogs (Amesbury et al., 2016; Booth and Zygmunt, 2005; Charman et al., 2004; Gałka et al., 2017; Lamentowicz et al., 2008; Mitchell et al., 1999; Swindles et al., 2010). In contrast, testate amoebae remain less studied in the Southern Hemisphere, apart from New Zealand (Charman, 1997; Wilmshurst et al., 2002; Wilmshurst et al., 2003), but recent work is now emerging from Australia (Bamforth, 2015; Meisterfeld et al., 2008; Zheng et al., 2019), South America (Fernández et al., 2016; Fernández et al., 2015; Van Bellen et al., 2014) and Antarctica (Charman et al., 2018; Stelling et al., 2018). The study of testate amoebae from New Zealand started in the early 20th century with the analysis of samples of the 1907–9 British Antarctic Expedition led by Shackelton sent by James Murray to Eugène Penard (Penard, 1911). Later Hoogenraad and De Groot (1948) and Van Oye (1956) provided further taxonomic information on testate amoebae in New Zealand. Van Oye (1956) provided hand drawn illustrations of various species, with basic dimensions, ranging from 20 to 192 μm in length. While these illustrations are extremely valuable, they are less informative, particularly for the smaller tests than more recent high-resolution photomicrographic images. Stout (1958, 1960, 1978, 1984) later explored the micro-fauna of various agricultural soils, grassland and tussocks in New Zealand. Several testate amoebae were identified and linked with soil types; however, no illustrations of the tests were provided. The testate amoebae checklist for New Zealand was further updated with the development of a training set from surface bog samples spanning the North and South Islands by Charman (1997). Sub-fossil tests from peatlands were then successfully used to reconstruct past hydrological conditions from two southern bogs (McGlone and Wilmshurst 1999; Wilmshurst et al. 2003, 2002). The latest work showed that New Zealand peatlands contained a diversity of cosmopolitan as well as characteristic large southern endemic taxa (McGlone and Wilmshurst 1999; Wilmshurst et al. 2003, 2002) and that many sites have a high abundance of small taxa. In the New Zealand testate amoebae training set (Charman, 1997), 20 out of the 62 samples in this study had a community composition where over 75% of the assemblages were composed of small morpho-

types. These small taxa have shown dramatic fluctuations in relative abundance within peat cores suggesting they are sensitive to changing hydrological conditions (Wilmshurst et al. 2003). Small testate amoebae have also been shown to be compositionally dominant in some New Zealand peat cores, for example making up ~67% of the total assemblage in a Holocene peat core taken from Eweburn Bog in the South Island (Wilmshurst et al. 2002).

Small New Zealand testate amoebae are largely composed of agglutinated xenosomes. Charman (1997) noted the high abundance of these taxa in New Zealand peatlands and identified them as: *Difflugia* Type A, *Difflugia* Type B, *Difflugia* Type Wilmshurst et al. (2002, 2003) later identified these agglutinated types as *Pseudodifflugia fulva* (*D*. type A), *Difflugia pulex* (*Difflugia* type B), *Difflugia pristis* (*D*. type C). Our study builds on this work by exploring the potential value of identifying these tests to the highest possible taxonomic level possible (using a light microscope). Such taxonomic improvements may enhance the value and ecological application of testate amoebae by providing more information on species relationships with environmental conditions, and thereby potentially increasing the reliability of predictive models.

The objective of this study is to assess the importance of small taxa (<45 μm long) as environmental indicators in New Zealand peatlands. We test how (a) lumping small taxa into broad morphotypes, and (b) including or excluding them, influence their use as environmental indicators. We hypothesise that greater environmental information could be attained by making the effort to identify the smaller morphotypes to the highest possible taxonomic resolution. Several outcomes are possible from this study: (1) small taxa are too difficult to resolve to species level and too unreliable to provide any meaningful ecological information and would be best left uncounted, (2) small taxa do provide useful information, but this information is also provided more easily by larger taxa with similar environmental relationships; meaning they can be lumped with similar morphotypes (or ignored) to speed up counting time, (3) small taxa provide useful complementary information to the larger taxa (e.g. they are sensitive to other ecological gradients or correspond to other ecological functions).

Our goal is not to conduct a taxonomic revision of the small testate amoebae living in peatlands – this is beyond the scope of this paper – but to assess the bioindication value of these taxa with a taxonomic resolution that can be achieved using light microscopy alone, and hence is applicable to ecological and palaeoecological studies.

Materials and Methods

Study sites

The New Zealand archipelago is in the South Pacific Ocean, spanning latitudes 34°–47° south. The largest island, the South Island, is characterised by the Southern Alps mountain range that runs along the length of the island (maximum altitude 3724 m a.s.l.), creating a steep climatic gradient between the western and eastern sides of the South Island. New Zealand lies within the Southern Hemisphere temperature zone. Prevailing westerlies winds dominate the weather system, which sometimes abate allowing air from either polar or tropical regions to reach the country (Macara, 2018). Temperatures are relatively mild, and rainfall is greatest in the winter months and drier conditions are typically observed in late-summer and early autumn (Macara, 2018). Moisture laden air masses that pass over the Southern Ocean can provide up to >10 m yr^{-1} to the West Coast through orographic rainfall, with lower rainfall in coastal regions (c. 2000–3500 mm $yr^{-1)}$ (Macara, 2016). Temperatures in lowland areas of the West Coast are mild throughout the year (annual range between 0 and 25 °C) and have a median annual temperature of between 11 °C and 13 °C (Macara, 2016). The resulting superhumid and mesothermal climate of the West Coast (Hessel, 1982) provides excellent conditions for wetland development.

It is estimated that 10% of the New Zealand mainland was covered by wetlands before human arrival around 750 cal. yr BP (Ausseil et al., 2011; McGlone, 2009). Although the early indigenous Māori settlers caused extensive deforestation of dryland forests by burning (Argiriadis et al., 2018; McWethy et al., 2010), wetland extent was minimally affected until after European arrival (1800s) when wetland loss accelerated rapidly for development and agriculture (McGlone, 2009). In the last 160 years, logging, fires and drainage have reduced wetland extent to less than 10% of its original cover making wetland ecosystems highly-threatened (Robertson et al., 2015). The most significant loss of wetlands occurred between 1920 and 1980 due to fragmentation and drainage (Taylor and Smith, 1997) to make way for large-scale agricultural activities. The least impacted wetlands in New Zealand are now found in the relatively undeveloped and high-rainfall West Coast region of the South Island (Clarkson et al., 2013).

Field sampling

Six wetlands were selected for our study from the West Coast of the South Island of New Zealand, including three bog systems that were highly to moderately impacted, and three that were relatively non-impacted (Fig. 1; Table 1). Anthropogenic impacts on wetland condition were assessed using the New Zealand Ecological Integrity Index (EII) (Ausseil et al., 2011). This index is a useful tool to place New Zealand wetland conditions in a national perspective. Six human-induced impacts are measured under the EII, including: impervious cover such as urbanisation and roading, nitrate leaching risk, introduced fish, woody weeds, reduced naturalness of the land, and drainage. EEI is ranked 1–0, where 0 indicates complete loss of biodiversity and associated ecological function and 1 indicates pristine (non-impacted)

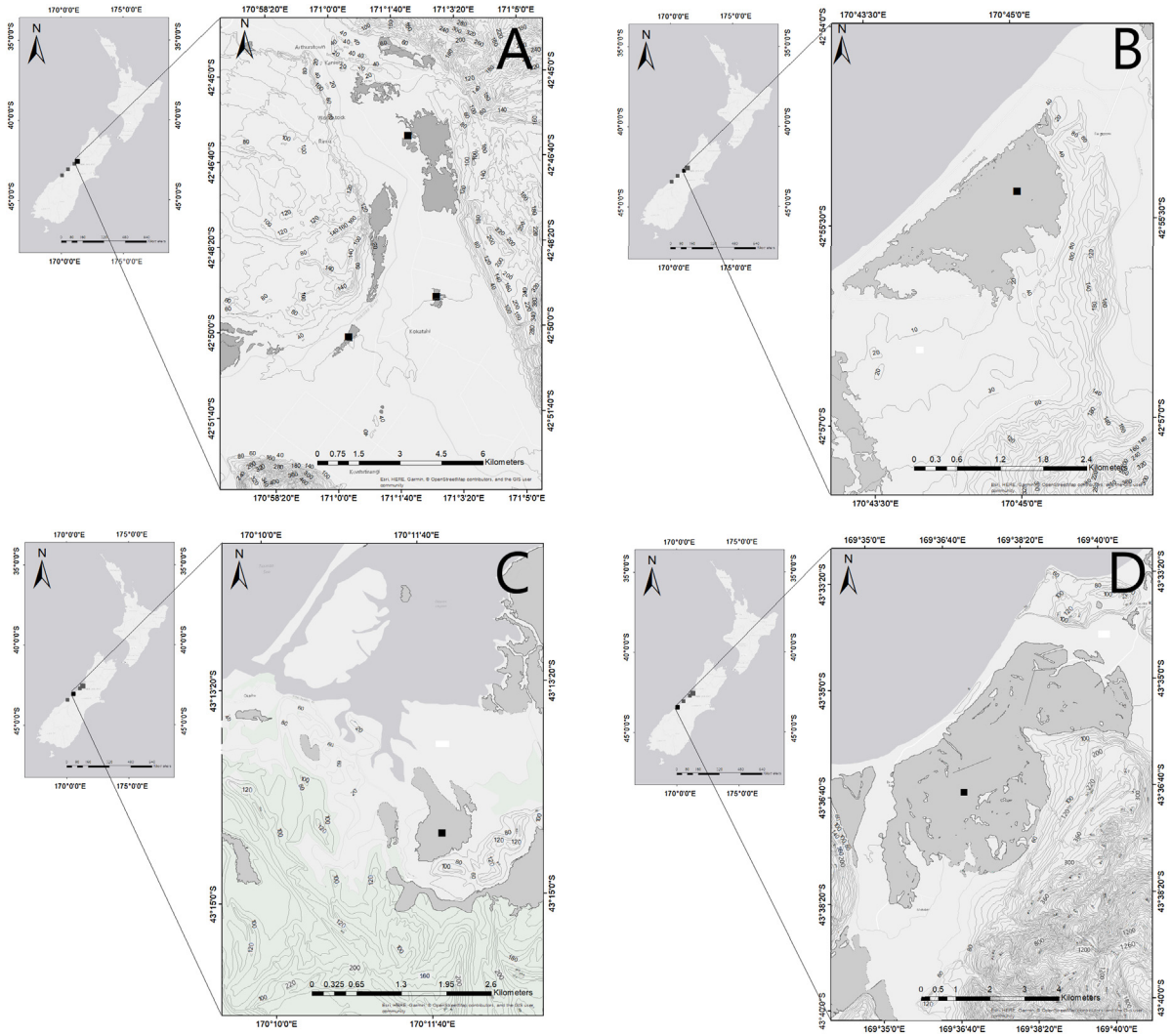

Fig. 1. Maps showing the location of the study sites in New Zealand. A: Hokitika 1–3 (Hokitika 1 is the northernmost site and Hokitika 3 the southernmost), B: Shearer, C: Okarito, D: Bruce Bay. Contour lines: 20 m intervals.

conditions; of the remaining wetlands in New Zealand, 60% have an EII < 0.5 (Ausseil et al., 2011). Our three impacted wetlands had EEI scores ranging from 0.28 to 0.65, compared with the three relatively unimpacted wetlands which ranged from 0.72 to 0.87 (Tables 1 and 2).

Within each bog, three samples were taken to represent a range of micro-topographical conditions including hummocks, hollows and lawns (Table 2). Each sample was arbitrarily chosen from within a randomly selected 5 × 5 m plot. A long knife was used to shorten the living vegetation layer before sampling. The knife was then driven into the surface carpet and a 20-cm deep intact monolith was extracted, inspected and sectioned at the near surface peat to capture the living section of the carpet and the top 0.5 cm of surface peat. This was done to ensure that only modern testate amoeba communities were captured. In all micro-sites, depth to water table (accuracy to 1 mm), pH (accuracy to 0.1), and conductivity (accuracy to 0.01 mS) were measured (Table 2). Depth to water table was from either the top of the moss or the top of the peat, depending on site vegetation and conditions.

Testate amoebae extraction and analysis

To extract testate amoebae, approximately 30 g of the surface vegetation and near surface peat was weighed, placed in a clean sealed container and shaken in tap water for at least 2 min with occasional stirring. The material was then sieved through a 150 μm mesh to separate larger material from the sample and then over a 20 μm mesh to remove fine particles. The 20–150 μm fraction was then recovered in a 50-ml centrifuge tube, centrifuged at 3000 rpm for 3 min to concentrate the tests and the pellet transferred to a 2-ml vial to which 75% ethanol was added to preserve the samples.

Amoeba tests were counted and identified at 200–600× magnification up to a minimum of 120 individuals per sample. As we were interested in comparing the bioindication value of small (<45 μm) vs. large (>45 μm) taxa, we aimed to count at

Table 1. Site and catchment information for bogs sampled for surface testate amoebae in this study.

Site code	Site name	Altitude (m a.s.l.)	Latitude	Longitude	EII[a]	Area (ha)	Dominant vegetation	Catchment information
x18_01	Hokitika 1	13.0	−42.77275	171.03713	0.65	9.6	*Juncus* spp., *Sphagnum cristatum*, *Coprosma areolate*, *Coprosma propinqua*, *Ulex europaeus, Machaerina tenax*	Large area loss, agriculture active in catchment, drainage, feral animal tracks, weed encroachment
x18_02	Hokitika 2	15.8	−42.82347	171.04551	0.30	13.4	*Juncus* spp., *Sphagnum cristatum*, Ulex europaeus, Anthoxanthum odoratum	Large area loss, large scale active agriculture on periphery of wetland, drainage, feral animal tracks, weed encroachment
x18_03	Hokitika 3	19.3	−42.83668	171.00473	0.28	23.9	*Leptospermum scoparium, Coprosma elatirioides, Coprosma dumosa*, *Sphagnum cristatum*, *Carex*, *Blechnum minus*	Large area loss, large scale active agriculture on periphery of wetland, drainage, feral animal tracks, weed encroachment
x18_05	Shearer	0.4	−42.92262	170.75595	0.72	300.2	*Sphagnum cristatum*, *Empodisma minus*, *Gleichenia dicarpa*, *Lepidosperma austral Machaerina rubiginosa*	Area loss, road on periphery of wetland
x18_07	Okarito	76.7	−43.24830	170.21054	0.87	213.8	*Empodisma minus*, *Gleichenia dicarpa*, *Machaerina rubiginosa*	Area loss, feral animal tracks
x18_10	Bruce Bay	7.6	−43.59493	169.61294	0.80	2236.9	*Empodisma minus*, *Gleichenia dicarpa*, *Machaerina teretifolia*	Area loss, agriculture active in catchment, drainage

[a] EII = Ecological Integrity Index. Ranked 1-0, where 0 indicates complete loss of biodiversity and associated ecological function and 1 indicates pristine conditions.

least 50 tests of each of these two categories. We did not reach the 50 count for the small taxa in four samples (Table 2). Light microscopy photographs of all morphotypes were taken in broad view and morphometric features measured. Specimens were identified to the highest possible taxonomic level using several general keys and monographs including (Charman et al., 2000; Corbet, 1973; Deflandre, 1936; Hoogenraad and De Groot, 1948; Leidy, 1879; Mazei and Tsyganov, 2006; Ogden and Hedley, 1980; Penard, 1902; Van Oye, 1956).

Numerical analyses

The community patterns and community-environment relationships were compared among the sites. Statistical analysis was carried out in R version 3.4.4 (R-Core-Team 2018) using the package vegan 2.4.5 (Oksanen et al., 2013). Species richness and Shannon Diversity indices were calculated and plotted for each site. The relationships between the amoeba communities and the three environmental variables that were measured at each sampling site, depth to water table (DWT), pH and conductivity, were explored using principal component analysis (PCA) and Pearson correlations to determine if any significant relationships existed between the site variables. We then investigated the correlation of species richness, Shannon Diversity, and the percentage abundance of <45 μm taxa with these environmental variables using generalised additive models (GAM). Such models allow the discrimination of non-linear relationships (Guisan et al., 2002) between the diversity metrics of small amoebae or the entire community and the environmental variables. The hummock sample from Hokitika 3 was removed from this analysis as it was considered an outlier due to a much higher conductivity reading from this one site compared to the other samples.

Non-metric Multi-Dimensional Scaling (NMDS) was carried out using the Bray–Curtis dissimilarity index to identify the dominant community patterns. Relative abundance data of testate amoebae was subjected to redundancy analysis (RDA) to quantify the correlation between testate amoeba

Table 2. Surface sample information for each micro-topographical location. Negative water-table values indicate the height of surface ponding.

Site code	Site name	Micro-topography	pH	Conductivity (Ms)	Depth to water-table (cm)	Test count (>45 μm)	Test count (<45 μm)
x18_01_SST3	Hokitika 1	Hummock	4.6	78.66	48.0	197	17[a]
x18_01_SST1	Hokitika 1	Lawn	4.8	54.52	0.5	101	73
x18_01_SST2	Hokitika 1	Hollow	5.5	44.26	−13.0	109	57
x18_02_SST3	Hokitika 2	Hummock	5.1	68.11	23.0	167	11[a]
x18_02_SST1	Hokitika 2	Lawn	5.2	35.44	5.0	128	83
x18_02_SST2	Hokitika 2	Hollow	4.9	65.70	0.0	142	53
x18_03_SST2	Hokitika 3	Hummock	3.6	137.30	35.0	179	8[a]
x18_03_SST1	Hokitika 3	Lawn	5.1	60.44	7.0	153	18[a]
x18_03_SST3	Hokitika 3	Hollow	4.3	68.66	2.5	122	63
x18_05_SST2	Shearer	Hummock	4.5	54.40	6.0	119	51
x18_05_SST1	Shearer	Lawn	4.5	54.80	3.0	107	73
x18_05_SST3	Shearer	Hollow	4.7	55.60	0.0	111	61
x18_07_SST2	Okarito	Hummock	4.4	52.12	21.0	165	50
x18_07_SST1	Okarito	Lawn	4.1	40.97	9.0	71	87
x18_07_SST4	Okarito	Hollow	4.0	31.40	1.0	72	50
x18_10_SST2	Bruce Bay	Hummock	4.3	39.60	18.0	54	84
x18_10_SST1	Bruce Bay	Lawn	4.2	39.70	5.0	79	89
x18_10_SST4	Bruce Bay	Hollow	4.1	34.80	0.0	52	107

[a] Samples where we did not enumerate at least 50 tests.

community composition and the three environmental variables, and the significance of these correlations were assessed using Monte Carlo permutation tests (999 unrestricted permutations). The species data were transformed prior to analysis by the Hellinger distance technique (Rao, 1995). Both analyses were carried out on four species matrices:

a) the whole community with high taxonomic resolution of small taxa (<45 μm in length);
b) whole community with low taxonomic resolution of small taxa, i.e., where smaller species were grouped into two morphotypes: (i) agglutinated xenosomes (e.g. small *Difflugia*, *Schoenbornia*, *Pseudodifflugia fulva*, *Phryganella paradoxa* etc.), and (ii) siliceous idiosomes (e.g. *Corythion*, *Euglypha* spp., *Sphenoderia*, etc.). This morphotype grouping was selected as the availability of material and/or the higher cost of self-secretion can constrain species distribution;
c) only the large taxa (>45 μm in length);
d) only the small taxa (<45 μm in length); with high taxonomic resolution.

Results

DWT ranged from -13 (surface sample was 13 cm below the water table) to 48 cm. Conductivity ranged from 31.40 to 137.30 mS, with an average of 56.47. pH ranged from 3.6 to 5.5 (Table 2). The value of the EII index across all sites ranged from 0.28 to 0.80, with the former indicating highly impacted and the latter indicating the least (Table 1). The highly-impacted sites, Hokitika 2 (EII = 0.30) and Hokitika 3 (EII = 0.28), and the moderately impacted site, Hokitika 1 (EII = 0.65), all showed a larger range in DWT across the micro-topographical sites compared to the non-impacted sites (Tables 1 and 2). The impacted sites were also characterised by higher pH and conductivity (Table 2). The non-impacted sites, Shearer (EII = 0.72), Okarito (EII = 0.87), and Bruce Bay (EII = 0.80) all had pH readings less than 4.75 and conductivity was less than 56 mS. Impacted sites also had notable encroachment of woody vegetation on the peatland (Table 1).

The Principal Component Analysis (PCA) applied to the environmental data measured at each site provides a general overview of the pattern of change between sites (Fig. 2a). PCA axis 1 explains 85.5% of the variance in the data; PCA axis 2 explains a further 14.5%. These axes are most closely related with vectors representing conductivity and DWT, respectively. Samples from hummocks are loading negatively on the conductivity and pH vectors in the biplots, and samples from the lawn and hollow tend to load positively (Fig. 2a). The impacted sites (both highly and moderately modified) are more spread in the ordination biplot compared to the sites with a higher ecological integrity index, which appear more clustered. Impacted sites are consistently aligned along the conductivity and pH gradients, having higher conductivity and pH measurements (Fig. 2b). Impacted sites have a larger range in all three variables, with samples from less impacted sites clustered together in all biplots. Conductivity and DWT are the only variables significantly correlated across all sites ($r = 0.48$, $p = 0.05$), where increasing conductivity is positively related to increasing DWT (Fig. 2b). This appears to be driven by the impacted sites ($r = 0.67$, $p = 0.072$). Our data also shows a weak positive relationship between pH and conductivity across all sites ($r = 0.31$, $p = 0.22$). However,

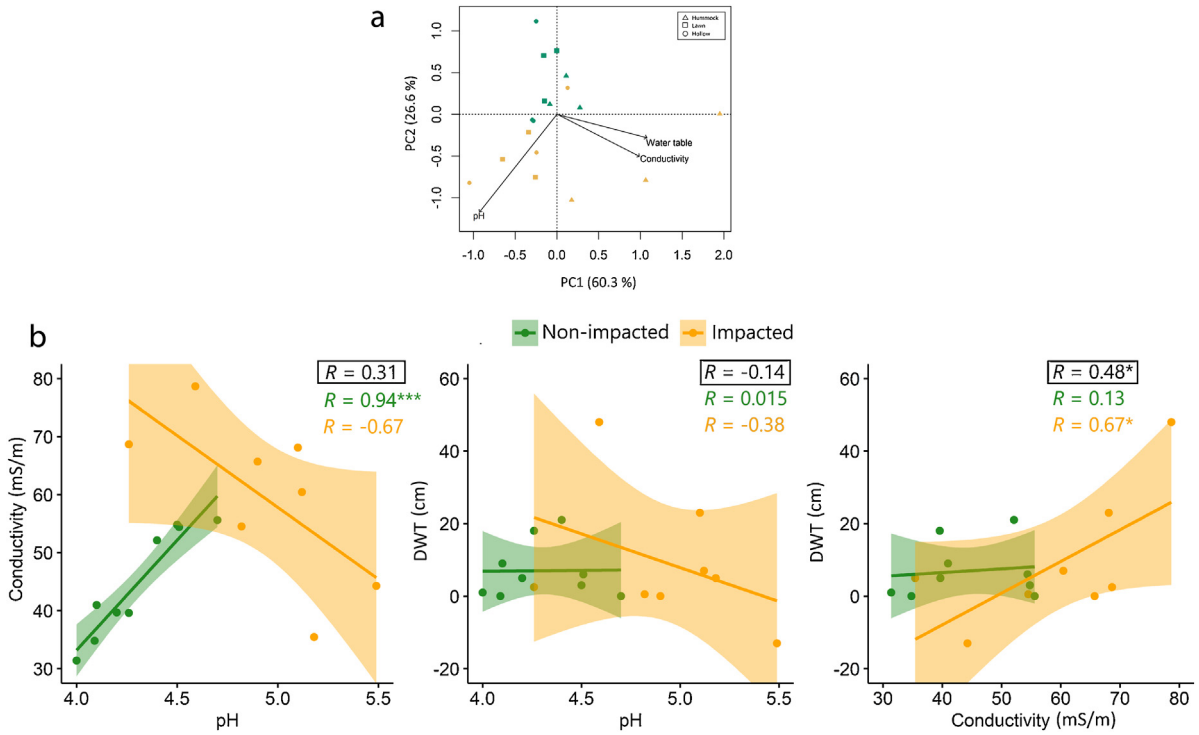

Fig. 2. (a) Bai-plot of principal component analysis (PCA) of samples from impacted (black symbols) and non-impacted (grey symbols) sites classified by microtopography. Environmental variables depth to water table (DWT), pH and conductivity were passively projected in the ordination space. (b) Sbcatter plots of conductivity vs. Ph, DWT vs. Ph, and DWT vs. conductivity. Correlation coefficients presented in black is for all sites, green is for non-impacted sites and orange is for impacted sites. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

non-impacted sites show a strong correlation between the two variables, where decreasing acidity is positively related to increasing conductivity ($r = 0.94$, $p < 0.001$) (Fig. 2b); whereas, the impacted sites show a contradictory relationship ($r = -0.67$, $p < 0.071$). The disassociated here could be related to movement of water in the peatlands facilitated by drainage in the impacted sites.

Detailed observations of small (<45 μm) testate amoebae revealed the existence of multiple taxa within the 18 samples (Fig. 3). The smaller testate amoebae were named only when we were confident that the observed specimens matched the taxonomic descriptions; however, we consider these identifications as tentative given the current state of taxonomic knowledge. We identified *Difflugia pristis* (Penard, 1902), *Difflugia pulex* (Penard, 1902), *Pseudodifflugia fulva* (Archer, 1870), *Phryganella paradoxa* (Penard, 1902), *Sphenoderia fissirostris* (Penard, 1890), *Schoenbornia humicola* (Schönborn et al., 1987), *Corythion dubium* (Taranek, 1871), *Euglypha dolioformis* (Bonnet and Thomas, 1959), *Euglypha rotunda* (Wailes and Penard, 1911), *Euglypha simplex* (Coûteaux et al., 1979). Three taxa were not found to match available descriptions and are referred to as morphotype A, B, and C for this study (Fig. 3).

In the GAM, species richness and diversity were both negatively, although weakly, correlated with DWT ($r^2 = 0.25$, $p = 0.02$, and $r^2 = 0.38$, $p < 0.01$, respectively, Fig. 4); highest values were observed in the 0–10 cm DWT range, while diversity was lower in hummocks. Diversity patterns in relation to pH and conductivity were less clear. The percentage of smaller morphotypes was negatively correlated to DWT ($r^2 = 0.43$, $p = 0.02$) and conductivity ($r^2 = 0.37$, $p < 0.01$). Interestingly, the proportion of small taxa was lower than the overall average in most samples from impacted sites; a high proportion of small taxa thus seems to be indicative of less impacted sites.

All four NMDS ordinations (Fig. 5a–d) show a clear division between impacted and less-impacted sites regardless of the data matrix used; the centroid of the two categories do not overlap except for the ordination scenario of species less than 45 μm in length where they overlap slightly (Fig. 5d). By contrast, the three habitats (hummocks, lawns, hollows/pools) were not clearly separated in any of the four ordinations, except for hummocks in the less-impacted sites for all but the NMDS of small taxa alone.

In the NMDS ordination of the whole community at high taxonomic resolution (Fig. 5a), the samples from impacted and non-impacted sites are clearly spread across Axis 1, and to a lesser extent Axis 2. The only evident pattern among the micro-topographical habitats is observed in the hummock microenvironments in the non-impacted sites; here, the hummock samples are clustered together in the ordination space in all NMDS analyses. While the overall percentage of small

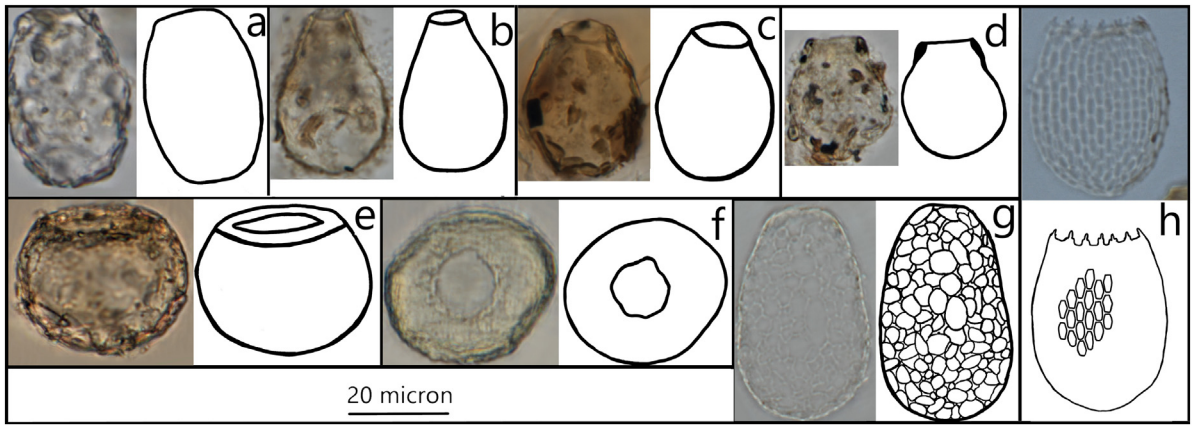

Fig. 3. Light microscope pictures of agglutinated testate amoebae <45 μm in length from peatlands from the west coast of the South Island of New Zealand. Each morphotype image is complemented with an outline of test shape. a = *Difflugia pristis*, b = *Difflugia pulex*, c = *Pseudodifflugia fulva*, d = *Phryganella paradoxa*, e = morphotype A, f = morphotype B, g = *Schoenbornia humicola*, h = *Euglypha dolioformis*. All images of taxa are in lateral view, apart from morphotype B (f), which is flattened and in planar view.

taxa seemed to be indicative of less impacted sites, not all taxa follow this pattern. Indeed, in the NMDS, the small agglutinated taxa, most notably *D. pristis*, *D. pulex*, *Phryganella paradoxa*, and morphotype B, tend to be associated with non-impacted sites while small siliceous species, such as *E. simplex* and *E. rotunda*, are associated with the impacted sites (Fig. 5a,b, and d).

In the RDA ordinations of whole community with high and low taxonomic resolution of small taxa, and of only large taxa (Fig. 6a–c), the impacted and non-impacted sites are well separated across the ordination space. However, in the analysis of small taxa alone, the two groups overlap (Fig. 6d). In all ordinations, the non-impacted sites are more grouped than the impacted sites. The RDA of the whole community at a high taxonomic resolution revealed significant correlation with all three variables (Table 3). Axis 1 and 2 jointly accounted for 50.1% of the variance in the data. The RDA sample biplot shows that conductivity accounted for most of the variance, followed by pH and DWT. The biplot for the whole community shows that impacted sites are more spread across Axis 1 and 2 in comparison to the non-impacted sites (Fig. 6a), and that there is a clear separation of impacted and non-impacted sites. Samples from the impacted sites are loading positively on the pH vector, while almost all samples from the non-impacted sites are loading negatively. This appears to be largely driven by positive relationship of *Centropyxis cassis*-type and *Arcella rotundata* to higher pH values and the preference for *Assulina muscorum* and *Heleopera sylvatica* to more acidic conditions. Samples from the hummock microenvironments in the impacted sites are loading positively on the DWT and conductivity vectors. Here, we observe a negative relationship of small agglutinated morphotypes to DWT and conductivity, most notably *D. pristis* and *P. paradoxa* (Fig. 4). When each variable was independently constrained to Axis 1, all three variables showed a significant relationship with the whole community, with DWT showing the greatest variance (15.3%), followed by conductivity (14.6%) and pH (11.2%) (Table 3).

For the scenario where we examine the whole community where the smaller taxa are lumped into two groups based on their test composition, i.e., agglutinated xenosomes or siliceous idiosomes, the RDA for Axis 1 & 2 showed that all three environmental variables accounted for 50.7% of the variance in the data. When each variable was independently constrained to Axis 1, DWT accounted for 19.7% of the variance, followed by conductivity (19.1%) and pH (12.8%). In the biplot, the samples are spread across both axes; however, samples associated with non-impacted sites are loading negatively along the pH vector, while the samples from the impacted sites are more spread out and loading positively. The lumped small agglutinated morphotypes are negatively related to the DWT and conductivity, while *A. muscorum*, *Nebela tincta*, *Nebela collaris* and *Alocodera cockayni* are positivity related to these three vectors.

For the amoebae data matrix including only large taxa, Axis 1 & 2 accounted for 49.4% of variance in the data (Axis 1 = 12.9%, Axis 2 = 4.6%) (Table 3; Fig. 6c). When each variable was independently constrained to Axis 1, pH was the only variable to show a significant relationship with the variance explaining 16.2%. The impacted and non-impacted samples are spread across Axis 1, with the impacted sites in the general model located to the left of the bi-plot and the non-impacted sites to the right. Samples from impacted sites are more spread across Axis 2, which is largely driven by DWT and conductivity.

Ordination of the data matrix containing only the smaller taxa, showed all three variables accounted for 50.7% of variance in the data (Axis 1 = 13.4%, Axis 2 = 1.0%) (Table 3; Fig. 6d). The biplot for the smaller taxa does not show the clear division between impacted and non-impacted sites observed in the other biplots. It is evident that the agglutinated taxa are negatively related to increasing DWT, most

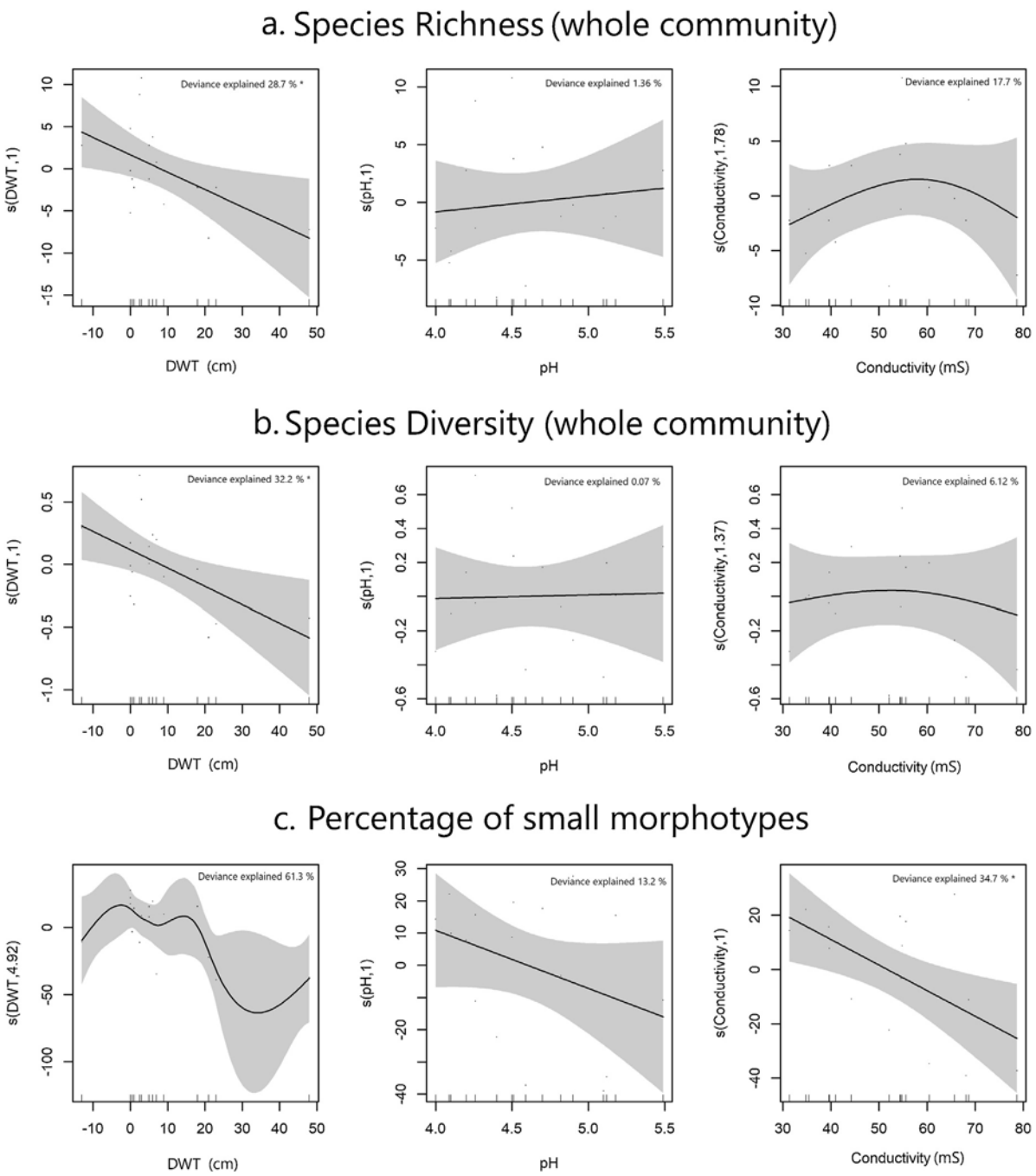

Fig. 4. Smooths of generalised additive models (GAM) terms showing the inferred effect of various variables on (a) testate amoebae species richness of the whole community at a high resolution, (b) testate amoebae species diversity of the whole community at a high resolution, (c) only the percentage of small morphotypes. Locations of observations are shown as vertical lines on the x-axes. Solid lines are estimates of the smooths, shaded areas are standard errors of the estimated smooths, and points of the observation partial residuals. The hummock sample from Hokitika 3 was removed from this analysis as it was considered an outlier due to a much higher conductivity reading.

notably *D. pristis*, *P. paradoxa*, morphotypes A, and B, while *E. simplex* is largely separated from the rest of the taxa and is positively related to the DWT vector. When each variable was solely constrained to Axis 1, the percentage of variance explained increased to 27.0% for DWT, and 23.9% for conductivity.

Discussion

This is the first study, to our knowledge, exploring the value of small testate amoebae as bioindicators in bog envi-

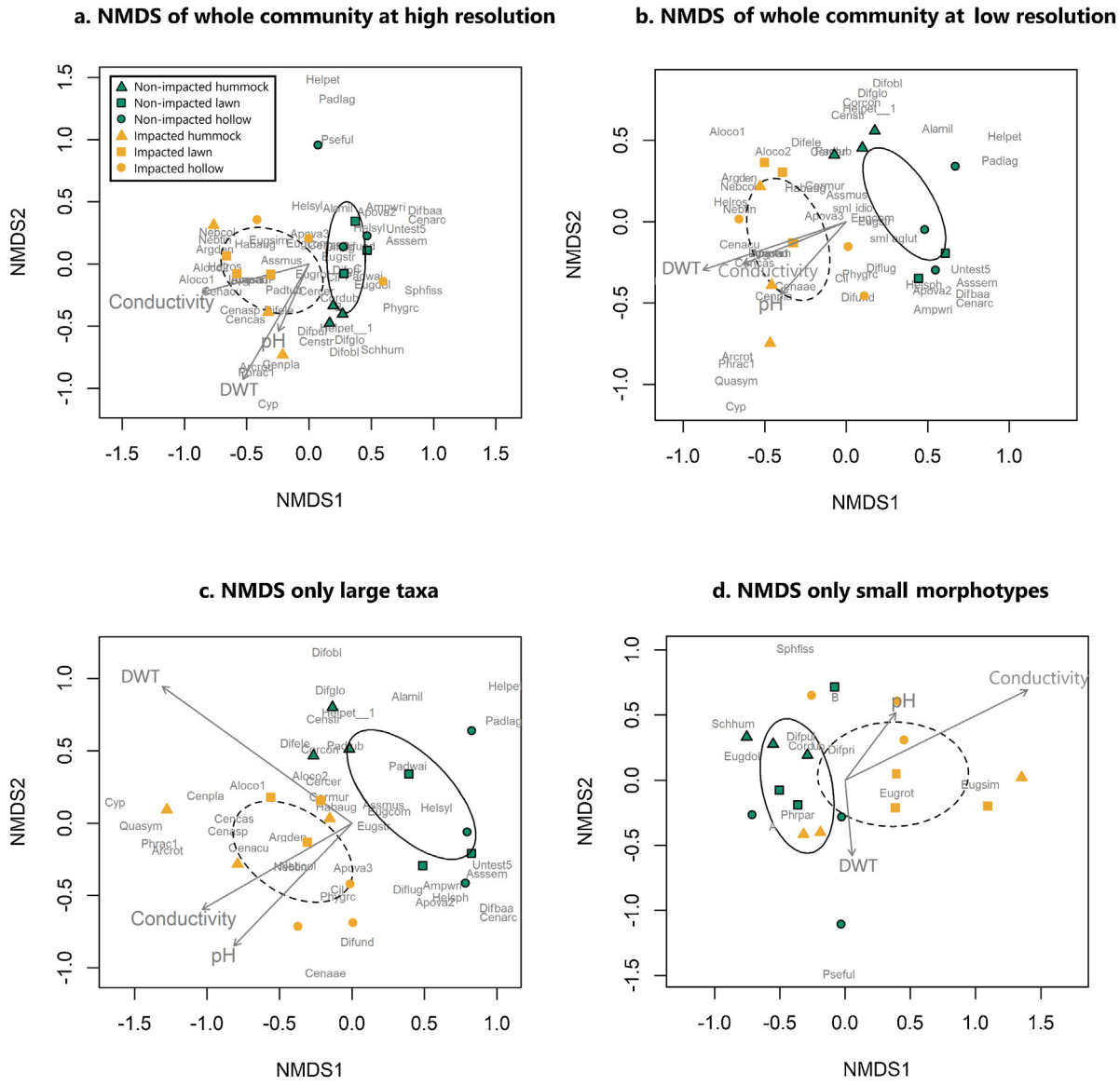

Fig. 5. Bi-plot of the two primary axes of the three-dimensional NMDS ordination of testate amoebae from four different community matrices. Samples are coded by microtopography and impact status. The broken line indicates the centroid of the community from the impacted sites and the solid line represents the centroid of the community from the non-impacted sites. Matrices are as follows: (a) NMDS bi-plot of the whole community where the small (less than 45 μm in length) testate amoebae are identified to a high taxonomic resolution, (b) NMDS bi-plot of the whole community where the small testate amoebae have been lumped into two groups (i) agglutinated (sml aglut), (ii) siliceous (sml idio). (c) NMDS bi-plot of only the large taxa, d. NMDS bi-plot of only the small morphotypes identified to a high taxonomic resolution. Testate amoebae abbreviations are as follows: *Alabasta flabellulum* type = *Alafla*, *Alabasta militaris* = *Alamil, Alocodera cockayni* (wider test) = *Aloco1*, *Alocodera cockayni* = *Aloco2*, *Amphitrema wrightianum* = *Ampwri*, *Apodera vas* (wide) = *Apova1*, *Apodera vas* (keel) = *Apova*2, *Apodera vas* (regular) = *Apova*3, *Arcella rotundata* = *Arcrot*, *Argynnia caudata* = *Argcau*, *Argynnia dentistoma* = *Argden*, *Assulina muscorum* = *Assmus*, *Assulina seminulum* = *Asssem*, *Centropyxis aculeata* = *Cenacu*, *Centropyxis aerophila aerophila* = *Cenaae*, *Centropyxis aerophila sphagnicola* = *Cenasp*, *Centropyxis cassis* type = *Cencas*, *Centropyxis constricta* = *Censtr*, *Centropyxis platystoma* = *Cenpla*, *Certesella* type 1 = *Cercer*, *Certesella* type 2 = *Certy*2, *Certesella* type 3 = *Cermar*, *Corythion constricta* = *Corcon*, *Corythion dubium* = *Cordub*, *Cyclopyxis arcelloides* type = *Cenarc*, *Cyphoderia* = *Cyp*, *Difflugia* undif = *Difund*, *Difflugia bacillariarium* = *Difbaa*, *Difflugia elegans* = *Difele*, *Difflugia globulosa* = *Difglo*, *Difflugia leidyi* type = *Diflei*, *Difflugia lucida* = *Difluc*, *Difflugia oblonga* = *Difobl*, *Euglypha compressa* = *Eugcom*, *Euglypha dolioformis* = *Eugdol*, *Euglypha rotunda* = *Eugrot*, *Euglypha simplex* = *Eugsim*, *Euglypha strigosa* = *Eugstr*, *Habotrocha augusticollis* (a bdelloid rotifer) = *Habaug*, *Heleopera petricola* = *Helpet*, *Heleopera rosea* = *Helros*, *Heleopera sphagni* = *Helsph*, *Heleopera sylvatica* = *Helsyl*, *Heleopora petricola* = *Helpet*, *Heleopora petricola var amethystea* = *Helpet_1*, *Nebela penardiana var minor* = *Nebpem*, *Nebela collaris* complex = *Nebcol*, *Nebela tincta* = *Nebtin*, *Padaungiella lageniformis* = *Padlag*, *Padaungiella tubulata* = *Padtub*, *Padaungiella wailesi* = *Padwai*, *Phryganella acropodia* I = *Phrac*1, *Phryganella paradoxa* = *Phrpar*, *Physochila griseola* = *Phygrc*, *Quadrulella symmetrica* = *Quasym*, *Sphenoderia fissirostris* = *Sphfiss*, Unidentified test 5 = Untest5, Cothurnia (Vaginicolidae) = Cil, *Difflugia pristis* = *Difpri*, *Difflugia pulex* = *Difopul*, *Pseudodifflugia fulva* = *Pseful*, *Schoenbornia humicola* = *Schhum*, morphotype A = A, morphotype B = B.

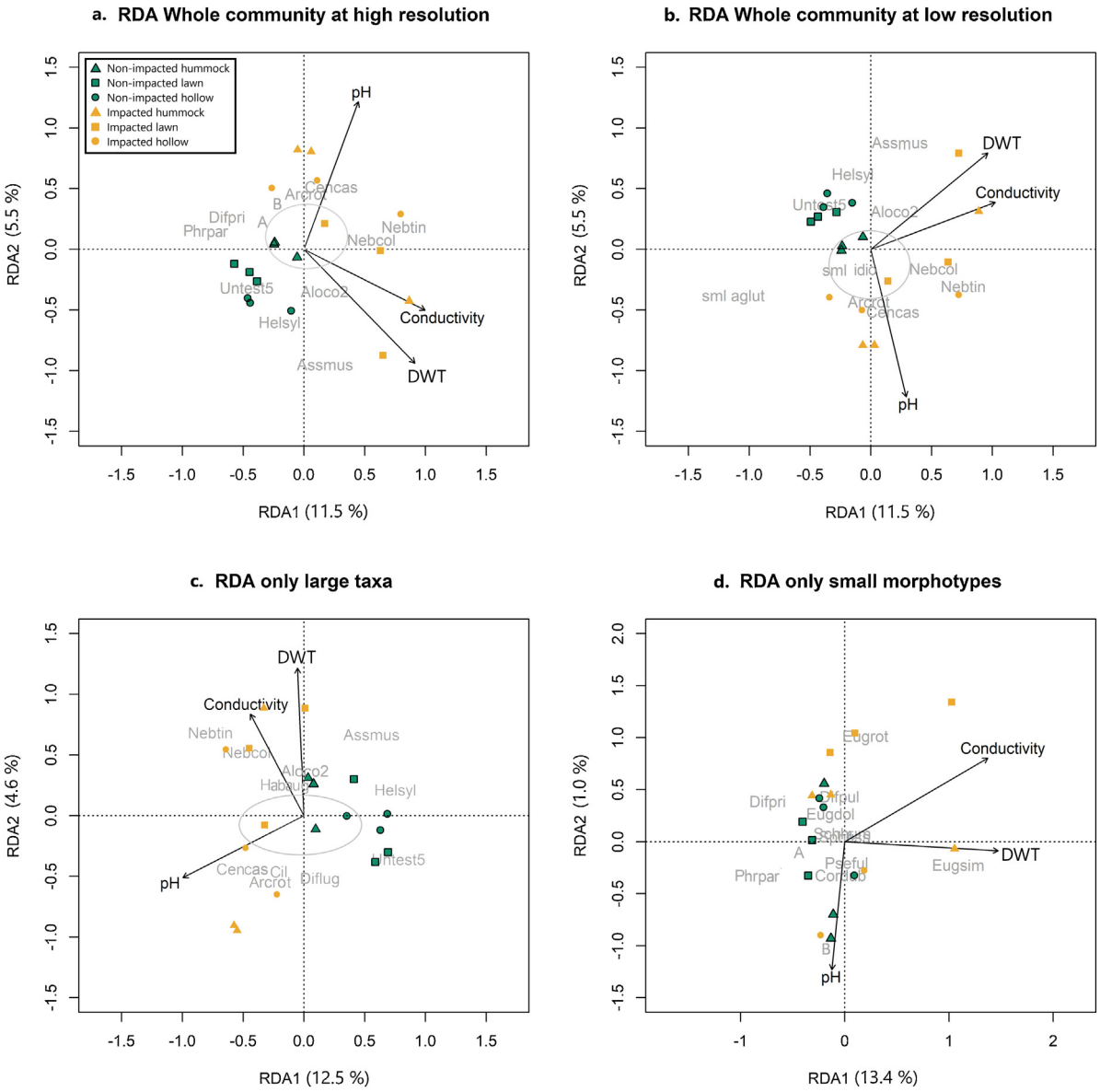

Fig. 6. Bi-plot of the two primary axes of the three-dimensional redundancy analysis (RDA) of testate amoebae from four different community matrices, which are the same as Fig. 5. DWT = Depth to water-table. Samples are coded by microtopography and impact status. The grey circles around the centroid represent taxa that were clustered here and were thus removed for clarity.

ronments. Most of the work on peatland testate amoebae has been undertaken on Northern Hemisphere *Sphagnum* bogs (Amesbury et al., 2016; Booth and Zygmunt, 2005; Charman et al., 2004; Gałka et al., 2017; Lamentowicz et al., 2008; Mitchell et al., 1999; Swindles et al., 2010). In these ecosystems, the smaller taxa such as *Difflugia pulex*, *Cryptodifflugia oviformis*, and small Euglyphida (*Trinema*, *Corythion*, *Euglypha rotunda*-type) are generally associated with dry conditions (Caseldine and Gearey, 2005). In New Zealand, the main peat formers are the restiads *Empodisma minus* and *E. robustum* in the North Island; and graminoids, *E. minus,* and *Sphagnum* spp. in the South Island (McGlone 2009). Here, the smaller *Difflugia* A type and *Difflugia* B type (Charman, 1997), later identified as *Pseudodifflugia fulva* and *D. pulex* species, respectively (Wilmshurst et al., 2002), are associated with wetter conditions in surface peat samples (Charman 1997). Our study further confirms the association of these two small taxa with high water tables. A similar pattern has also been observed in Patagonia (Van Bellen et al., 2014). However, a testate amoeba-based reconstruction spanning the Holocene in the South Island of New Zealand suggests that *D. pulex* can be abundant in communities where taxa typical of drier conditions are dominant, such as *A. muscorum*, *Alocodera* (formerly *Nebela*) *cockayni* (Wilmshurst et al., 2003). This has raised the complexity around the factors at play influencing the ecology of these small taxa and their palaeoecological potential. Charman et al. (2007) discussed this discrepancy and states that *P. fulva* and *D. pulex*

Table 3. Ordination statistics from RDA.

Variable	Whole community small tests high res			Only morphotypes >45 µm			Only morphotypes <45 µm			Whole community small tests low res		
	$\lambda 1$	$\lambda 1/\lambda_2$	% variance	$\lambda 1$	$\lambda 1/\lambda_2$	% variance	$\lambda 1$	$\lambda 1/\lambda_2$	% variance	$\lambda 1$	$\lambda 1/\lambda_2$	% variance
DWT, pH and conductivity	0.12	0.06	50.1^{***}	0.13	0.05	49.4^{***}	0.12	0.01	50.7^{**}	0.12	0.06	55.5^{***}
Variables constrained to Axis 1		$\lambda 1$	% variance		$\lambda 1$	% variance		$\lambda 1$	% variance		$\lambda 1$	% variance
DWT		0.08	15.3^{***}		0.05	7.6		0.11	27.0^{**}		0.09	19.7^{**}
pH		0.06	11.2^{*}		0.1	16.2^{***}		0.01	2.0		0.06	12.8^{*}
Conductivity		0.08	14.6^{**}		0.05	8.2		0.1	23.9^{**}		0.08	19.1^{**}

P significance level for Monte Carlo permutation tests (999 unrestricted permutations).
$^{***}P < 0.001$.
$^{**}P < 0.01$.
$^{*}P < 0.05$.

are associated with shallow water tables in surface samples but can also survive, and dominate in, periods of desiccation. Indeed, Wilmshurst et al. (2003) found that the period of highest *D. pulex* abundance occurred during a period of marked alternations of wet and dry conditions brought on by increasing ENSO variation. Sullivan and Booth (2011) further corroborate these findings and suggest that *D. pulex* and *P. fulva* are more abundant under highly variable moisture conditions and are closely associated with short-term environmental variability. Thus, small taxa can reveal additional environmental information for ecological and palaeoecological research, and that (pseudo)cryptic taxa may be restricted to specific conditions/habitats, which was recently shown for the larger-sized *Nebela tincta* group (Singer et al., 2018).

Our results show that there is greater heterogeneity of testate amoeba communities in micro-topographical gradients from the impacted sites compared to the non-impacted sites. The latter have been drained, fragmented, suffered notable area loss, have large-scale agricultural activities in the surrounding catchment, along with the encroachment of woody-weedy vegetation. Thus, it is not surprising that the environmental measurements and a change in amoebae assemblage is evident between impacted and non-impacted sites. However, what is interesting, is the possible impact this may have on test size.

There is a general correlation between test size and trophic position, and more specifically the size of aperture: testate amoebae with a small aperture size mainly feed on bacteria, fungi, algae and small heterotrophic protists such as flagellates, whereas species with a larger aperture size preferentially feed on larger protists and micro-metazoans such as rotifers and nematodes (Gilbert et al., 2003; Jassey et al., 2012; Mitchell et al., 2000). Wu et al. (2017) state that native wetlands and drained wetlands reclaimed to farmland have different microbial community structure and composition with a higher ratio of fungi to bacteria in the drained wetlands and such a contrast was also observed in comparative study of five European *Sphagnum* peatlands (Mitchell et al., 2003). In our dataset, impacted and non-impacted sites appear to be segregated by pH when samples are constrained to all three environmental variables in the RDA ordinations in the scenarios with (1) the whole community where small taxa are identified to a high taxonomic resolution, (2) the whole community where small taxa are grouped into two categories (low taxonomic resolution), and (3) only large species. The RDA of small taxa only does not show this pattern and the amoeba communities are largely driven by changes in DWT and conductivity, which are positively related in our dataset (Fig. 2b). This relationship is particularly strong in the impacted sites, where deeper water table is significantly related to higher conductivity. The small agglutinated taxa, most notably *D. pristis*, *D. pulex*, *P. paradoxa*, and morphotypes A, and B, tend to be associated with wetter conditions (Fig. 6d), and are more abundant in samples from non-impacted sites (Fig. 5d); although they are present in impacted sites. This is in contrary to the small siliceous taxa, such as *E. simplex* and

E. rotunda, which are more closely associated with deeper water tables in impacted sites (Figs. 5 a & b, and 6 a & b). The lower percentage of smaller amoebae, particularly the agglutinated taxa, in impacted sites (more minerogenic, less acidic bogs, with deeper water tables), may be connected to food-web interactions. This change could be related to a reduction in available bacteria and increase in availability of larger prey (including bacterial grazers keeping bacterial populations in check). This would favour larger testate amoeba species over smaller ones. However, the exact mechanism between the notable change in testate amoeba assemblages across micro-environmental gradients between impacted and non-impacted sites in New Zealand would deserve a more detailed study, and ideally experimental work to fully understand the functional implications of the observed patterns.

Conclusion

Our study builds on and advances previous research and suggests (1) that the general assumption that all small taxa are indicative for dry conditions is not entirely accurate and more complex factors may be at play, and (2) that the true diversity of these small taxa is likely to be much higher than currently believed. Our results demonstrate that identifying small testate amoebae to the highest possible taxonomic resolution can provide more informed environmental assessment of wetlands than by lumping them into groups or ignoring them. This study shows promising results for the use of testate amoebae as indicators of ecological integrity in New Zealand bogs. Testate amoebae community assemblages from wetlands that have been highly impacted by human activities show a clear segregation from less impacted sites. The percentage of testate amoebae less than 45 μm decreases in highly impacted sites and they are found in greater abundance in the wetter hollow and lawn micro-topographical environments, with clear horizontal gradient restrictions.

Despite the relatively low number of sites in this study, we have been able to clearly demonstrate that several distinct small taxa were more strongly related to water table depth and conductivity, while the larger species were more strongly correlated to pH. We suggest the possibility of counting and identifying solely larger taxa, any tests greater than 45 μm, as an indicator of pH; and separately identifying all taxa less than 45 μm to a high taxonomic resolution only, as a tool for reconstructing hydrological conditions. This could be carried out at the preparation stage (sieving at two different sizes) where both larger and smaller sized groups are counted separately.

Author contributions

Michelle M. McKeown is the lead author, she wrote the manuscript, carried out the testate amoebae analysis and statistical modelling. Edward A. D. Mitchell substantially helped with testate amoebae identification and statistical analysis, along with project design. Clément Duckert provided expertise on test identification. Janet M. Wilmshurst and Jamie R. Wood provided information on previous testate amoebae work in New Zealand, along with providing expertise to the introduction and discussion sections. All authors helped edit the manuscript and approve of the manuscripts submission to the Journal of European Protistology.

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