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2 **Testate amoebae response to acid deposition in a Scottish peatland.**

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11

12 **ABSTRACT**

13

14 Peatlands around the world are exposed to anthropogenic or volcanogenic sulphur
15 pollution. Impacts on peatland microbial communities have been inferred from
16 changes in gas flux but have rarely been directly studied. In this study the impacts of
17 sulphuric acid deposition on peatland testate amoebae were investigated by analysis of
18 experimental plots on a Scottish peatland almost seven years after acid treatment.
19 Results showed reduced concentration of live amoebae and changes in community
20 structure which remained significant even when differences in pH were accounted for.
21 Several possible explanations for the impacts can be proposed including taphonomic
22 processes and changes in plant communities. Previous studies have inferred a shift
23 from methanogenic archaea to sulphate reducing bacteria in sulphate-treated peats; it
24 is possible that the impacts detected here might relate to this change, perhaps through
25 testate amoeba predation on methanotrophs.

26

27 **KEYWORDS:** Protists, Mires, Wetlands, Volcanic Impacts, Sulphate deposition,
28 Methanogenesis.

29

30 **INTRODUCTION**

31

32 Many peatlands in, or downwind of, industrialised regions have been exposed
33 to acidic sulphur pollution over recent centuries. Impacts have been suggested in
34 terms of changes to the pH (Proctor and Maltby 1998, Skiba *et al.* 1989) and

1 decomposition rates of peats (Hemond et al. 1980, Sanger et al. 1994), DOC flux
2 (Sanger et al. 1994), methane production (Nedwell and Watson 1995; Watson and
3 Nedwell 1998; Gauci et al. 2002) and the metabolic processes (Ferguson and Lee
4 1979), growth rate (Ferguson and Lee 1980; Rochefort et al. 1990) and community
5 structure (Tallis 1964; Ferguson and Lee 1980) of peatland plants. The potential scale
6 of such impacts is nowhere more apparent than the Pennine blanket mires of northern
7 England where heavy sulphur-loading combined with other pollutants since the
8 beginning of the industrial revolution has led to the near-total elimination of
9 *Sphagnum* and consequent drastic landscape change (Tallis 1964; Ferguson and Lee
10 1983; Lee 1998).

11 Although impacts of sulphur deposition on peatland microbial communities
12 have been inferred from changes in gas flux, few studies have directly investigated
13 microbial community change. This study focuses on testate amoebae, a group of
14 unicellular micro-organisms (protists) which are highly abundant in damp to fully
15 aquatic habitats around the world, and particularly in peatlands. Testate amoebae are
16 increasingly being recognised as an important component of many ecosystems by
17 virtue of their high abundance (up to 30% of microbial biomass in peatlands: Mitchell
18 et al. 2003) and rapid turnover (e.g. Aoki et al. 2007). As testate amoebae lie towards
19 the top of the microbial foodweb and as a group have broad feeding preferences
20 (Gilbert et al. 2000) it is likely that testate amoebae will be sensitive to changed
21 abundance and community structure in many groups at lower trophic levels.

22 Recent studies have highlighted the sensitivity of testate amoebae to pollution,
23 including deposition of heavy metals (Patterson et al. 1996; Reinhardt et al. 1998;
24 Nguyen-Viet et al. 2007; 2008), nutrients (Gilbert et al. 1998a&b, Mitchell 2004), and
25 atmospheric pollutants (Nguyen-Viet et al. 2004, Balik 1991). This work suggests
26 both the potential of testate amoebae for biomonitoring and also that pollution, both
27 anthropogenic and natural, may complicate the use of testate amoebae as indicators of
28 other variables.

29 Given the potential impacts of sulphate deposition on both the abiotic and
30 biotic environment of testate amoebae in peatlands it seems probable that sulphate
31 deposition would lead to changes in abundance and community structure. There is
32 some evidence from field surveys for a relationship between testate amoeba
33 communities and sulphate concentrations. Opravilova and Hajek (2006) and Mitchell
34 et al. (2000b) found sulphate to explain a statistically significant proportion of

1 variance in testate amoeba communities. Swindles et al. (2009) looking at peatlands in
2 northern Ireland and Lamentowicz et al. (2008) looking at a peatland in Poland found
3 sulphate was not a significant environmental variable. In the Polish study this result
4 might be explained by limited variance as only one site was considered, in the
5 northern Irish study the result might be explained by low sulphate concentrations in
6 relatively unpolluted peatlands. Other possible evidence for a relationship between
7 SO₄ and testate amoebae comes from an association with Sr, which is correlated with
8 SO₄ in separate analyses, in an Israeli wetland (Payne et al in press).

9 In an experimental approach Payne et al. (2009) investigated the testate
10 amoeba communities of experimental plots in a Scottish peatland subject to sulphate
11 deposition. Sodium sulphate was applied for a period of 18 months and 25 samples
12 extracted from each of six plots (three treated and three control) after more than ten
13 years. Results showed statistically significant differences between treated and control
14 plots, particularly characterised by reduced abundance of small bacterivorous taxa
15 (*Euglypha rotunda* type, *Corythion dubium*, *Trinema lineare* and *Trinema*
16 *complanatum*). Also apparent was a reduced concentration of live amoebae and
17 proportion of tests occupied by living amoebae.

18 In the pre-industrial era the heaviest sulphate loadings on peatlands would
19 have derived from volcanic eruptions. Such impacts are little-considered but the
20 historical record shows extremely severe impacts of volcanic acid-loading on plant
21 communities even at great distance from volcanic sources (Grattan and Charman
22 1994; Grattan and Gilbertson 1994; Grattan and Pyatt 1999) and the presence of
23 (crypto)tephra deposits preserved in peatlands around the world testifies to the large
24 areas of peatlands which are within reach of volcanic products. Some
25 palaeoecological records have shown testate amoeba community changes coincident
26 with tephra deposition which might represent a response to volcanogenic sulphate
27 deposition (Dwyer and Mitchell 1997; Payne and Blackford 2008). Although
28 contemporary volcanic sulphate emissions contribute a minority of total sulphur
29 emissions these still constitute a major supply of sulphur to peatlands in many regions
30 (Langmann and Graf 2003).

31 The experimental study of Payne et al. (2009) may not be a good analogue for
32 the impacts of volcanic sulphate on peatlands. Sulphate was applied over a period of
33 eighteen months and although it is possible for volcanic eruptions to produce
34 extended periods of sulphate deposition (as for the well-documented 1783-4 eruption

1 of Laki in Iceland), in most distal regions sulphate deposition episodes will be much
2 briefer lasting a matter of hours, days or weeks. Furthermore, the sulphate was applied
3 as sodium sulphate. In real volcanic eruptions much of the sulphur deposited is likely
4 to be as sulphuric acid. By applying only sodium sulphate the previous study did not
5 simulate plant mortality and morbidity, which may well result from volcanic acid
6 deposition and would be likely to affect microbial communities. The use of sodium
7 sulphate also makes it difficult to entirely exclude the possibility that impacts arose
8 from the application of sodium rather than sulphate.

9 This study aims to determine the impact of sulphuric acid deposition on
10 peatland testate amoeba communities with a particular focus on the possible response
11 to volcanogenic pollution events. The study uses previously established experimental
12 plots on a Scottish peatland, comparing the testate amoeba communities of a plot
13 treated with sulphuric acid with control plots.

14 15 SITE and METHODS

16
17 Experiments were conducted on the Moss of Achnacree, a large raised bog in
18 Argyll and Bute, western Scotland (UK Grid Reference NM9134). Peat deposits cover
19 around 7 Km² and average 1.9m depth, the area has a cool temperate climate with an
20 annual rainfall of around 1500mm. Major plant species of the site include *Calluna*
21 *vulgaris*, *Eriophorum vaginatum*, *Cladonia portentosa* and various *Sphagnum* species
22 (including *S. capillifolium*, *S. magellanicum* and *S. papillosum*). The site has been
23 subject to some peripheral peat-cutting and areas of the site have been drained for
24 agriculture. Experiments were conducted in an uncut area towards the west of the
25 main peat area, approximately 100m from South Ledaig Farm (Fig. 1).

26 A sequence of fourteen, 1x1m plots was established on the site and plots
27 subjected to deposition of acids and/or volcanic tephra in May 2002. Experiments
28 were designed to approximate possible acid deposition in Scotland following the 2310
29 ± 20 BCE (Pilcher et al. 1995) eruption of Hekla in Iceland (Hekla-4), which has been
30 implicated in major vegetation change (Blackford et al. 1992). Scenarios were derived
31 by extrapolating the scenario of Grattan and Gilbertson (1994) to the highest levels of
32 tephra deposition noted in northern Scotland (see Payne and Blackford 2005 for
33 details). The plots were re-visited at regular intervals over the subsequent two years
34 and observations of plant communities and measurements of various environmental

1 parameters undertaken (Payne and Blackford 2005, Payne et al. 2005). Drastic
2 impacts on plants were noted with most plants killed in the heaviest treated plots, but
3 the cover-estimates were insufficiently precise to allow small abundance changes to
4 be monitored (Payne and Blackford 2005). For selected plots testate amoeba
5 communities were also analysed through the experimental period but no consistent
6 changes were noted. Reasons for this lack of detectable response probably include an
7 insufficient sampling density to account for the high spatial variability of testate
8 amoeba communities and an inadequate time period given that most of the tests
9 counted were probably accumulated prior to the experimental period (Payne and
10 Blackford 2005). This study attempts to account for these problems by analysing the
11 amoeba communities of experimental plots almost seven years after acid deposition
12 and using a much higher sampling intensity.

13 At the end of the main study period in 2004, the experimental infrastructure
14 was removed in accordance with an agreement with the then landowner. The site was
15 revisited in April 2009, almost seven years after establishment of the experiments. Of
16 the three plots with the heaviest sulphuric acid treatment (0.7 mol m^{-2}) only one could
17 be positioned with sufficient accuracy. This plot (MAC11) was not subject to tephra
18 deposition. The impacts of the experimental treatment on plant communities could
19 still be readily determined with more bare ground than surrounding areas, no
20 *Sphagnum* and only immature *Calluna vulgaris* (see Table 1 for species composition).
21 20 samples of approximately 2x2x5 cm of surface peat were extracted from across the
22 experimental plot and returned to the laboratory. At each sampling spot depth to water
23 table (DWT) was determined by making a small hole and measuring DWT after at
24 least an hour for the water table to equilibrate. Plant species in the immediate vicinity
25 of the sampling location were also recorded. A further 10 samples were extracted
26 from one of the control plots established in the original study (MAC2) and treated
27 with only deionised water. This plot is 8m from MAC11 while testate amoeba
28 communities have been shown to exhibit spatial variability on a very fine scale
29 (Mitchell et al. 2000a). To account for this, 20 further samples were extracted from an
30 additional 1 m^2 area (here termed MAC30) situated 1 m N of MAC11. This area was
31 not a control plot in the previous studies so has not been subject to the disturbance in
32 previous sampling that plots MAC11 and MAC2 have experienced. All plots are
33 situated on hummocks and in most cases surface peat samples consisted of relatively
34 dense, humified peat.

1 In the laboratory approximately 1 cm³ of the upper 1 cm of the samples
2 (regardless of surface vegetation) was removed and volume measured by
3 displacement in deionised water. Samples were made up to 30ml with deionised water
4 and pH measured after approximately two hours. Preparations for testate amoebae
5 followed a method based on that of Hendon and Charman (1997) but without the use
6 of back-sieving as recommended by Payne (2009). Samples were allowed to soak for
7 48 hours before being stirred to disaggregate the peat matrix but were not boiled to
8 avoid killing live amoebae. Samples were subsequently sieved at 300µm and a
9 *Lycopodium* innoculum added (Stockmarr 1971). The <300 µm fraction was retained
10 and stored in water, samples were refrigerated until analysis. Slides were prepared by
11 mixing a drop of the prepared sample with glycerol and examined at 400X
12 magnification. 100 tests per sample were identified and counted (*cf.* Payne and
13 Mitchell 2008) and tests with visible cytoplasm (termed 'live individuals' although
14 truly live individuals could not be distinguished from tests with dead but undecayed
15 cellular contents) differentiated from empty (dead) tests. Taxonomy follows Charman
16 et al. (2000) except where modified by Payne et al. (2009).

17 Differences in amoeba concentration, proportion of occupied tests, species
18 richness, diversity (Shannon's 'H') and environmental variables between the treated
19 and control plots were tested using Mann-Whitney tests in PAST ver. 1.84 (Hammer
20 et al. 2001). An initial test of the difference between the testate amoeba community of
21 the treated and control plots used Analysis of Similarity (ANOSIM: Clarke 1993)
22 with a Bray-Curtis distance measure. Subsequently the multivariate data was
23 investigated using ordination techniques in Canoco vers. 4.53 (Ter Braak and
24 Šmilauer 1997-2004). Species data were Hellinger distance transformed (Rao 1995;
25 Legendre and Gallagher 2001) and taxa present in four or fewer samples were
26 removed from the dataset. Initially the data structure was investigated using Principal
27 Components Analysis (PCA); subsequently Redundancy Analysis (RDA) was used to
28 test the significance of a nominal variable for experimental treatment. pH and DWT
29 were introduced as co-variables to allow their influence to be accounted for.
30 Significance was assessed using Monte Carlo permutation tests (999 permutations).
31 These analyses were each applied to data based on percentages of all tests,
32 percentages of live individuals, concentrations of all tests and concentrations of live
33 individuals. As an additional exploration of the data structure and differences between

1 plots the percentage total tests data was subjected to cluster analysis using the Paired
2 Group Method with a Bray-Curtis similarity matrix in PAST ver. 1.84.

3 As a test of testate amoeba community changes since the end of the previous
4 studies, the amoeba community of plot MAC11 in 2009 was compared to previous
5 analyses from six intervals between 2002 and 2004, beginning one month after
6 treatment and continuing to 24 months after treatment (Payne and Blackford 2005).
7 Due to the probable issues with intra-plot spatial variability in amoeba communities
8 these samples were treated as a single group, ignoring any changes within that period.
9 Only data based on percentage of total tests was used for these analyses. Taxonomic
10 harmonisation was carried out and minor taxa eliminated from the dataset. Difference
11 between the two datasets was tested using RDA, as above, including a nominal
12 variable 'Age' for sampling period.

16 RESULTS

18 Twenty eight testate amoebae taxa (plus the rotifer *Habrotrochoa*
19 *angusticollis*, which was included in calculations) were encountered in the 50
20 samples, of which the most abundant were *Assulina muscorum* (21.9% of total tests),
21 *Nebela tinctoria* type (20.9%), *Corythion dubium* (9.4%) and *Phryganella acropodia*
22 type (9.3%). Some differences in amoeba community between treated and untreated
23 plots are relatively clear even in the overall abundance data (Table 2) including
24 greater abundances of *Diffugia pristis* type, *Hyalosphenia subflava* and *Trigonopyxis*
25 *arcula* in the treated plot and greater abundance of *Corythion dubium* in the control
26 plots. There are also differences in abundance of some taxa between the two control
27 plots (notably *Heleopera rosea*).

28 Mann-Whitney tests showed significant differences between treated and
29 control plots for amoeba concentrations (whether based on total individuals or only on
30 live individuals) and percentage of occupied tests ($P < 0.001$). While the overall test
31 concentration was higher in the treated plot, the concentration of live amoebae and the
32 proportion of occupied tests were greater in the control plots. There was a significant
33 difference in pH between the treated and control plots ($P < 0.001$) with lower values in

1 the treated plot (Fig. 2), there were no significant differences in species richness or
2 diversity between the plots.

3 Principal components analysis shows very clear differences between the
4 treated and control samples. For data based on percentage of all tests axis one
5 effectively divides the samples into two groups with very little overlap (Fig. 3), other
6 datasets give similar results. There is good coincidence between the two sets of
7 control samples (MAC2 and MAC30) with MAC2 samples having a slight tendency
8 to higher scores on axis two. *Trinema lineare*, *Euglypha rotunda* type and *Corythion*
9 *dubium* are positively correlated with pH and negatively correlated with the
10 experimental treatment. *Hyalosphenia subflava*, *Diffflugia pristis* type, *Trigonopyxis*
11 *arcula*, *Heleopera petricola* and *Pseudodiffflugia fulva* type are positively correlated
12 with the experimental treatment and negatively correlated with pH. Post-hoc Mann-
13 Whitney tests showed significant ($P < 0.05$) difference in abundance (% all tests)
14 between treated and control plots for all these taxa except *P.fulva* type.

15 In Redundancy Analyses the treatment nominal variable explained a
16 significant proportion of variance with all datasets (Table 3). With 'treatment' the sole
17 environmental variable in the analysis up to 18.4% of variance was explained
18 ($P = 0.001$). Both pH and DWT were also significant environmental variables but
19 DWT lost significance when pH was partialled out, showing co-variance between the
20 two. Consequently, only pH was used as a co-variable when testing the effect of the
21 experimental treatment. With pH partialled out the treatment nominal variable
22 explained between 4.8 and 6.7% of variance ($P = 0.001$). More variance was explained
23 using concentration data than percentage data, suggesting that there are absolute, not
24 just relative changes in abundance. That strong relationships are apparent when using
25 data based only on living individuals is slightly surprising given that counts were low
26 (mean=10 individuals). ANOSIM shows statistically significant differences between
27 treated and control samples using all data sets ($P < 0.001$), R_{ANOSIM} varies between 0.29
28 and 0.45. Cluster analysis results show a general correspondence of identified
29 groupings to treated and control plots but also quite marked differences among the
30 samples of the treated plot with two samples clearly differentiated from all others
31 (Table 4).

32 There is a significant difference between the 2009 and 2002-2004 testate
33 amoeba community of plot MAC11, a nominal variable explains 24% of variance
34 ($P = 0.001$). Some of the differences between treated and control plots noted in the

1 analysis of 2009 data seem to be matched by changes over the period since previous
2 analysis (Fig. 4). So, *Euglypha rotunda* type and *Corythion dubium* [the *Corythion-*
3 *Trinema* type recorded in 2002-4 probably only represents *C. dubium*] are reduced in
4 abundance both relative to the control plots and to the 2002-4 data. Similarly,
5 *Diffflugia pristis* type is much increased in abundance relative to the control plots and
6 2002-4 data. These changes could be taken as indicating a continuing impact of the
7 experimental treatment in the period 2004-2009. However other changes are in
8 marked contrast to the differences to the control plots, most notably *Hyalosphenia*
9 *subflava* which in 2009 was more abundant in the treated than control plots, but much
10 less abundant than in 2002-4. It is recommended that these results are treated with
11 particular caution due to: 1. The small sample size of the 2002-2004 dataset. 2. The
12 difference in sample preparation methods, with fine-sieving used in 2002-4 and likely
13 to lead to underestimation of the abundance of the smallest taxa (Payne 2009), 3. The
14 lack of data on concentrations and differentiation of live from dead individuals in the
15 2002-4 data, 4. The probability of changes occurring within the 2002-4 period. 5. The
16 impact of non-treatment variables, particularly climatic variability over the
17 intervening period.

18

19 DISCUSSION

20

21 It is important to recognise the limited scale of this experiment. Although the
22 sampling intensity is relatively high there is no replication at plot scale as only one of
23 the treated plots could be accurately located. Complications due to prior differences
24 between plots cannot be ruled out and comparisons between plots may be complicated
25 if accumulation rates differ so the samples represent differing time periods. Results
26 should be treated with caution and further studies will be desirable to replicate the
27 findings presented here. Furthermore, the extent to which the experimental scenario
28 used here is an accurate representation of reality is also open to question (see
29 discussion in Payne and Blackford 2005), these results should probably be viewed as
30 indicating the nature of the testate amoebae response, but not necessarily the scale of
31 the response.

32 However, with caveats stated, this study does provide interesting results. The
33 difference between acid-treated and control plots emerges very strongly in the
34 analyses. The unconstrained ordination plot shows a near-perfect divide between

1 treated and untreated samples along axis one and the constrained ordination shows
2 that a treatment nominal variable explains a significant, and sizeable, proportion of
3 variance with all datasets. Despite the limitations of the experimental design the initial
4 similarity between the treated and control plots, the distinctiveness of the changes and
5 the similarities of the results with the experiment of Payne et al. (2009, discussed
6 below) strongly suggest that the differences between treated and control plots are due
7 to the experimental additions and not to any prior differences.

8 The univariate data analyses show a statistically significant difference in both
9 concentration of tests and proportion of occupied tests. However, while the proportion
10 of occupied tests and concentration of live amoebae are less in the treated than control
11 plots, the overall concentration of tests is greater in the treated than control plots. This
12 presents a curious dichotomy, suggesting a less active amoeba community but higher
13 concentrations of tests. As total test concentrations are dependent on the degree of
14 decomposition of the peat matrix the explanation for this result might be that surface
15 peat in the treated plots has decomposed more than in control plots, increasing
16 apparent test concentration. Although enhanced decomposition is a conceivable
17 impact of sulphuric-acid treatment this was not suggested by alkali-extraction
18 determined humification of near-surface peats in 2002-4 (Payne and Blackford 2005).
19 The reduced abundance of live testate amoebae here parallels response to nutrient and
20 CO₂ enrichment in peatlands (Gilbert et al. 1998a&b, Mitchell et al. 2003, Mitchell
21 2004) and H₂SO₄ treatment in a simulated stream system (Costan and Planas 1986). It
22 appears that a wide variety of environmental perturbations may lead to a reduced
23 abundance of testate amoebae.

24 In both this study and that of Payne et al. (2009) the same three taxa are
25 strongly negatively associated with the treatment: *Corythion dubium*, *Trinema lineare*
26 and *Euglypha rotunda* type. By contrast there is little agreement in the taxa which
27 respond positively. In this study the strongest positive response to sulphuric acid
28 deposition was in *Diffflugia pristis* type, *Hyalosphenia subflava* and *Trigonopyxis*
29 *arcula*. In the experiments of Payne et al. (2009) the taxa showing strongest positive
30 association with sodium sulphate treatment were *Hyalosphenia papilio*, *Arcella*
31 *arenaria* type and *Cryptodiffugia oviformis*. Of these taxa *A. arenaria* type was
32 absent and both *H. papilio* and *C. oviformis* were minor occurrences in this study
33 (0.06% and 0.9% respectively). Of the taxa showing a positive response in this study,
34 two (*D. pristis* type and *H. subflava*) were not found at all by Payne et al. (2009) and

1 the third (*T. arcula*) was a very minor presence, accounting for only 0.04% of total
2 tests. The difference in detected response may therefore relate to initial differences in
3 community composition between the sites.

4 The three testate amoeba taxa which are deleteriously affected by the
5 experimental treatment (*E. rotunda* type, *C. dubium* and *T. lineare*) form a coherent
6 ecological group. All three taxa are small, with idiosome tests and are believed to be
7 largely or exclusively bacterivorous (Gilbert et al. 2000). There is relatively little
8 information on the autecology of *T. arcula*, *H. subflava* and *D. pristis* type. The
9 compilation of Gilbert et al. (2000) suggests *T. arcula* feeds on fungi and organic
10 material. There is no information on the feeding preferences of *D. pristis* type and *H.*
11 *subflava* but other species of *Diffflugia* and *Hyalosphenia* have broad feeding
12 preferences ranging from cyanobacteria to micro-metazoa. All of these three taxa are
13 generally considered typical of dry conditions and are frequently found in hummocks.
14 However, differences in wetness cannot explain the differences in abundance of these
15 taxa observed here, there is no significant difference in DWT between plots MAC11
16 and MAC2 (P=0.8) while there are very significant differences in abundance of all
17 these taxa (P<0.005). Curiously, the increased abundance of *D. pristis* type in these
18 experiments is in marked contrast with the experiment of Costan and Planas (1986)
19 where acidification with H₂SO₄ in a lotic system reduced *D. pristis* concentrations by
20 an order of magnitude. However it should be noted that the difficulties in testate
21 amoeba taxonomy, particularly in the genus *Diffflugia*, are such that it is impossible to
22 be certain that these are the same taxa in both studies, particularly given the difference
23 in environment.

24 Several possible explanations can be proposed for the mode of impact of the
25 experimental treatment on testate amoebae. The simplest possibility for reduced
26 concentration of live amoebae and preferential loss of some taxa would be that they
27 are unable to cope with acid-stress, possibly through H⁺ interference with enzyme or
28 membrane function. Costan and Planas (1986) speculate that acid-shock may perturb
29 the osmotic regulation mechanism of testate amoebae leading to mortality. Over the
30 initial two-years of the experiment there was no overall trend of increased acidity. pH
31 values of samples from the treated plot here are lower than the control plots, but the
32 pH of the treated plot is not highly acidic by the standard of ombrotrophic peatlands
33 (even given the dilute measurement solutions). In the redundancy analysis ‘treatment’

1 explained variance independent of pH differences so acidification alone cannot
2 explain the changes observed.

3 It is notable that the taxa most reduced in abundance have idiosome tests while
4 the taxa most increased in abundance have secretion or xenosome tests. One possible
5 explanation for this result could be decomposition of idiosome tests in a more acidic
6 environment. Swindles and Roe (2007) and Payne (2007) have experimentally
7 demonstrated the dissolution of such tests in strong mineral acids, and these tests are
8 also disproportionately lost from the palaeoecological record (Mitchell et al. 2008).
9 However, in this study reduced abundance of *E. rotunda*, *T. lineare* and *C. dubium*
10 was also apparent when only considering live amoebae. Unless lower pH conditions
11 somehow reduce the bioavailability of Si for test construction this counts against a
12 taphonomic explanation for the changes.

13 Impacts on testate amoeba communities might be related to impacts on plant
14 communities. Over the 2002-4 study period Payne and Blackford (2005) noted near-
15 total plant mortality in plot MAC11 with no new growth noted until a year after acid
16 treatment and differences still apparent when these samples were extracted seven
17 years later. Although the relationships between plant and testate amoeba communities
18 are under-researched the two are likely to be closely linked. In field surveys plant
19 community composition explains variance in testate amoeba communities even when
20 other major controls are accounted for (e.g. Payne and Mitchell 2007). Important
21 mechanisms of plant influence on testate amoeba communities may include litter
22 chemistry (Sutton and Wilkinson 2007); root exudates and the provision of physical
23 niches (for instance the smallest taxa might be able to enter *Sphagnum* hyalocysts).
24 Recent research by Vohník et al. (2009) has even suggested a possible impact of plant
25 communities on testate amoeba taphonomy with mycorrhizal fungi associated with
26 *Rhododendron* spp. using testate amoeba tests (particularly *Centropyxidae* and
27 *Trigonopyxidae*) as a nutrient source. How plant community change would manifest
28 itself on testate amoeba communities is uncertain. A related possibility is that
29 enhanced supply of dead plant material might boost the abundance of testate amoebae
30 which either directly feed on organic matter, or feed on lower micro-organisms that
31 do. That *D. pristis* type, *T. arcula* and *H. subflava* are all associated with drier
32 conditions and peat hummocks might suggest they could be associated with aerobic
33 decomposition. *T. arcula* has been observed to directly feed on organic matter while

1 *Hyalosphenia subflava* is associated with drained peatlands where aerobic
2 decomposition is active, which might support this idea (Tolonen 1986).

3 That the same taxa are deleteriously affected by H₂SO₄ deposition in this study
4 as by Na₂SO₄ deposition in the study of Payne et al. (2009) suggests that the cause of
5 this change is most likely the input of SO₄²⁻ rather than Na⁺ or H⁺ (either directly or
6 indirectly). Recent studies have shown a reduction in methane efflux in sulphate-
7 exposed peatlands (Dise and Verry 2001; Gauci et al. 2002). The mechanism for this
8 change is believed to be sulphate reducing bacteria (SRB) out-competing
9 methanogenic archaea (MA) for electron donors as using sulphate as an electron
10 acceptor is a more energetically favourable pathway. A limited pulse of sulphate may
11 produce a prolonged impact on methane production due to recycling of sulphur in the
12 upper peat (Wieder et al. 1990; Gauci et al. 2005). In sulphate-treated plots on the
13 Moidach More site studied by Payne et al. (2009) methane efflux suppression
14 simultaneous with sulphate reduction has been demonstrated (Gauci et al. 2002; Gauci
15 and Chapman 2006). While these processes were not directly investigated in the
16 previous study of these experimental plots the distinctive odour of H₂S was noted
17 during core extraction from plots subject to the heaviest H₂SO₄-treatment but not in
18 any of the control plots during 2002-4 (Payne and Blackford 2005). It therefore
19 appears that in this site too sulphate reduction has been stimulated. That the testate
20 amoeba taxa most deleteriously affected in both studies are bacterivores indicates that
21 the reduced abundance of these taxa may be due to a change in their food source. The
22 changes in testate amoeba community in both studies may well be linked to the
23 putative MA-SRB shift. The link between these prokaryotes and testate amoebae -if
24 any exists- is unlikely to be direct predation as in theory anaerobic bacteria and
25 archaea should not co-exist with aerobic protists (although the potential influence of
26 hydrological variability and testate amoeba motility are uncertain). One possible
27 mechanism linking the two groups could be testate amoeba predation of
28 methanotrophs, recently demonstrated in naked amoebae and flagellates (Murase and
29 Frenzel 2008).

30 The possible mode of impact of the experimental treatment on testate amoeba
31 communities cannot be conclusively determined on the basis of this evidence alone.
32 Several explanations are possible, however, the similarity in response to the study of
33 Payne et al. (2009) does suggest a common forcing, and this common forcing could
34 well relate to the putative MA-SRB shift.

1 That peatland testate amoebae respond to sulphate deposition appears
2 increasingly clear. This suggests that testate amoebae might have a role as
3 bioindicators, potentially allowing monitoring of both the effects of sulphate pollution
4 on peatland microbial communities and subsequent recovery. The preservation of tests
5 in peats may allow such processes to be studied over longer time-frames. However,
6 this is likely to be complicated by selective test decomposition and the dominant
7 control of hydrology. It would be of particular interest if a known testate amoeba
8 response could be firmly tied to a MA-SRB shift as the preservation of tests in peats
9 might then allow this response to be detected in palaeoecological sequences.
10 Detecting any sulphate-signal in the palaeoecological record is likely to be difficult in
11 practise and may not be possible other than where outside evidence (for instance the
12 presence of a tephra layer or historical information on the occurrence of sulphate
13 pollution) suggest the possibility. The increasing number of environmental variables
14 suggested to be controls on testate amoebae communities urge against a simplistic
15 view of palaeoecological data solely in terms of hydrological change. Non-
16 hydrological controls are likely to be particularly important in peatlands exposed to
17 air pollution over recent centuries.

18
19

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21

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30

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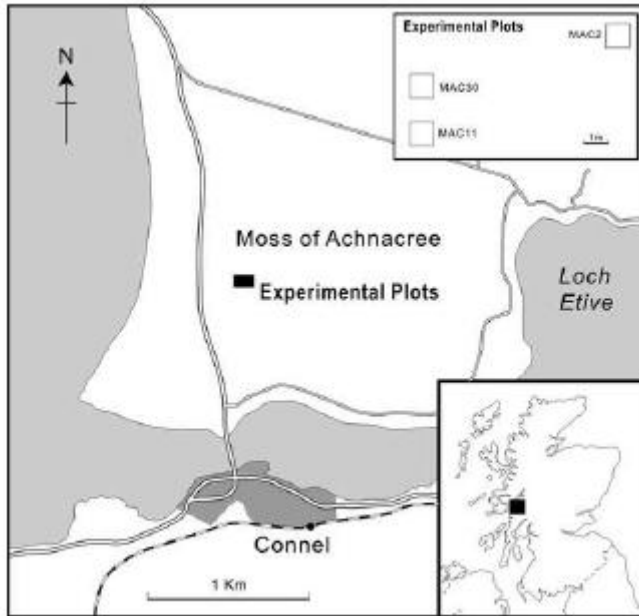
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1 FIGURES

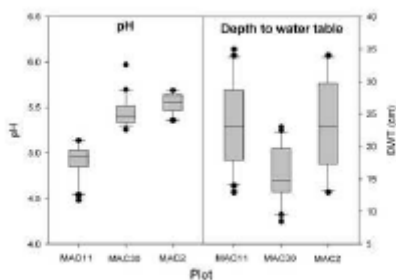
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3 Figure 1. Location map of Moss of Achnacree site and relative position of treated
4 plots within the experimental area.



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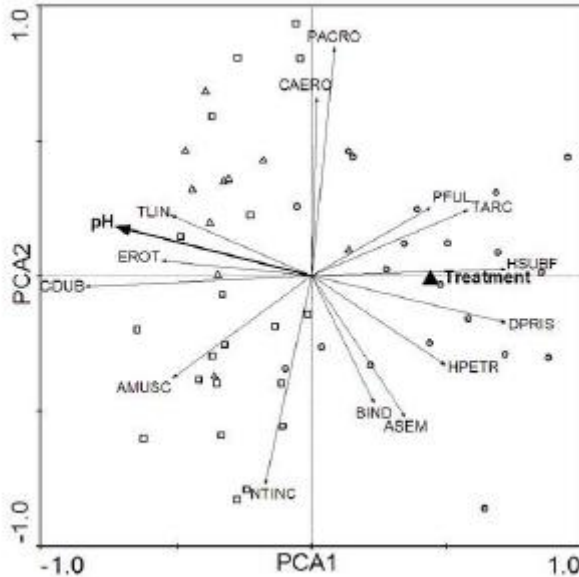
6 Figure 2. Environmental data for the three experimental plots, showing pH of peat
7 suspension in water and depth to water table at time of sampling. Box plots show
8 median (central line), first and third quartiles (grey box), tenth and ninetieth
9 percentiles ('whiskers') and all outliers (dots).



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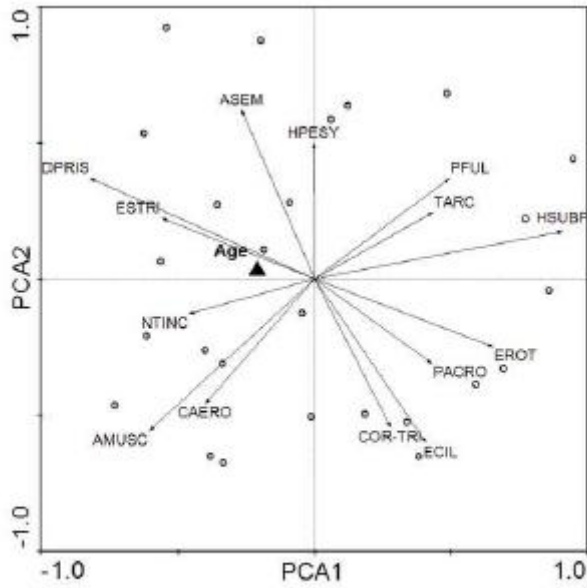
11 Figure 3. Principal components analysis of Hellinger-transformed testate amoebae
12 data (percentages of all tests, excluding taxa $n \leq 4$) for samples from experimental
13 plots. Filled circles show MAC11 samples (acid treated); triangles show MAC2
14 samples (control) and squares show MAC30 samples (additional control). Species
15 codes:- AMUSC: *Assulina muscorum*, ASEM: *Assulina seminulum*, BIND:
16 *Bullinularia indica*, CAERO: *Centropyxis aerophila* type, CDUB: *Corythion dubium*,
17 DPRIS: *Diffflugia pristis* type, EROT: *Euglypha rotunda* type, HPETR: *Heleopera*

- 1 *petricola*, HSUBF: *Hyalosphenia subflava*, NTINC: *Nebela tincta* type, PACRO:
- 2 *Phryganella acropodia* type, PFUL: *Pseudodiffugia* type, TARC: *Trigonopyxis*
- 3 *arcula*, TLIN: *Trinema lineare*.



4
 5 Figure 4. Principal components analysis of Hellinger-transformed testate amoeba data
 6 comparing samples from plot MAC11 in 2009 (filled circles) to samples from the
 7 same plot extracted between 2002 and 2004 (unfilled circles). ‘Age’ is a nominal
 8 variable for the 2009 samples. Species codes as for Fig.3 and Table 2 with the
 9 exception of ‘COR-TRI’ which shows a *Corythion-Trinema* type (following Charman
 10 et al. 2000 but probably only representing *C. dubium* here) and ‘HPESY’ which
 11 shows a grouped *Heleopera petricola*- *Heleopera sylvatica* type.

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TABLES

Table 1. Plant species of the three experimental plots at the time of sampling.

Table 2. Relative abundance of major taxa¹ (over 1% total tests) in three plots: MAC11 (sulphuric acid treated), MAC2 (control) and MAC30 (additional control), see text for full details of experimental set-up. Also showing relative abundance of living individuals by taxon (in parentheses) and taxon abbreviations used in Figs. 3 and 4. Data for living individuals is based on small counts and should be treated with caution.

Table 3. Redundancy analysis of testate amoeba data showing percentage variance explained and P-values of these relationships assessed by Monte Carlo permutation tests (999 permutations).

1 Table 4. Results of cluster analysis (Paired Group Method using a Bray-Curtis
2 distance measure) on % total tests data showing groups identified at the third level of
3 division.
4
5

1 Table 1. Plant species of the three experimental plots at the time of sampling.

2

Plot	No. Samples	Plant species present
MAC11	20	<i>Calluna vulgaris</i> , <i>Eriophorum vaginatum</i> , <i>Aulacomnium palustre</i> , <i>Hypnum cupressiforme</i> , <i>Cladonia portentosa</i> , <i>Carex</i> (undiff.).
MAC2	10	<i>Calluna vulgaris</i> , <i>Eriophorum vaginatum</i> , <i>Aulacomnium palustre</i> , <i>Hypnum cupressiforme</i> , <i>Cladonia portentosa</i> , <i>Carex</i> (undiff.), <i>Sphagnum</i> (undiff.), <i>Odontoschisma sphagni</i> .
MAC30	20	<i>Calluna vulgaris</i> , <i>Eriophorum vaginatum</i> , <i>Aulacomnium palustre</i> , <i>Hypnum cupressiforme</i> , <i>Cladonia portentosa</i> , <i>Sphagnum</i> (undiff.), <i>Odontoschisma sphagni</i> .

3

1 Table 2. Relative abundance of major taxa¹ (over 1% total tests) in three plots:
 2 MAC11 (sulphuric acid treated), MAC2 (control) and MAC30 (additional control),
 3 see text for full details of experimental set-up. Also showing relative abundance of
 4 living individuals by taxon (in parentheses) and taxon abbreviations used in Figs. 3
 5 and 4. Data for living individuals is based on small counts and should be treated with
 6 caution.

7

Taxon	Abbreviation	Relative abundance all tests in plot (relative abundance living individuals):		
		MAC11 (%)	MAC2 (%)	MAC30 (%)
<i>Assulina muscorum</i> Greef 1888	AMUSC	19.2 (10.1)	18.8 (3.9)	26.5 (10.8)
<i>Assulina seminulum</i> (Ehrenberg 1848)	ASEM	3.8 (0.0)	0.7 (1.0)	1.4 (0.4)
<i>Centropyxis aerophila</i> Deflandre 1929 type	CAERO	4.6 (1.3)	3.4 (2.5)	4.4 (1.3)
<i>Corythion dubium</i> Taranek 1881	CDUB	3.3 (3.1)	17.4 (13.6)	11.3 (9.6)
<i>Diffflugia pristis</i> Penard 1902 type	DPRIS	10.1 (17.3)	1.4 (0.0)	0.3 (0.0)
<i>Euglypha rotunda</i> Wailes 1911 type	EROT	0.6 (0.0)	1.2 (1.8)	1.8 (1.1)
<i>Euglypha strigosa</i> (Ehrenberg 1872)	ESTRI	4.5 (3.2)	9.6 (10.0)	5.9 (8.3)
<i>Heleopera petricola</i> Leidy 1879	HPETR	8.8 (7.3)	4.0 (7.5)	4.8 (3.2)
<i>Heleopera rosea</i> Penard 1890	HROS	0.4 (0.5)	3.4 (2.8)	0.9 (1.6)
<i>Hyalosphenia subflava</i> Cash and Hopkinson 1909	HSUBF	4.9 (6.6)	0.7 (1.1)	0.3 (0.0)
<i>Nebela militaris</i> Penard 1890	NMILI	3.2 (4.7)	3.5 (5.2)	1.6 (0.9)
<i>Nebela tincta</i> (Leidy 1879) type	NTINC	18.0 (30.0)	16.8 (34.5)	26.5 (57.2)
<i>Phryganella acropodia</i> (Hertwig & Lesser 1874) type	PACRO	9.3 (6.7)	11.7 (0.7)	7.3 (0.9)
<i>Pseudodiffflugia fulva</i> Penard 1901 type	PFUL	2.7 (5.2)	0.5 (0.0)	0.5 (0.8)
<i>Trigonopyxis arcula</i> (Leidy 1879)	TARC	4.0 (2.8)	0.8 (0.0)	1.0 (0.0)

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¹Minor taxa are: *Bullinularia indica*, *Centropyxis aculeata*, *Cryptodiffflugia oviformis*, *Diffflugia minutissima* type, *Euglypha ciliata*, *Euglypha cristata*, *Hyalosphenia papilio*, *Nebela flabellum*, *Nebela tubulosa*, *Placocista spinosa*, *Sphenoderia fissirostris*, *Trinema complanatum*, and *Trinema lineare*, plus *Habrotrochoa angusticollis*.

1 Table 3. Redundancy analysis of testate amoeba data showing percentage variance
2 explained and P-values of these relationships assessed by Monte Carlo permutation
3 tests (999 permutations).

4

Dataset	Explanatory variable	Co-variable	% variance explained	P-value
All tests (%)	Treatment	-	17.9	0.001
	Treatment	pH	4.8	0.001
All tests (concentration)	Treatment	-	18.4	0.001
	Treatment	pH	6.7	0.001
Live amoebae (%)	Treatment	-	14	0.001
	Treatment	pH	5.2	0.001
Live amoebae (concentration)	Treatment	-	13.3	0.001
	Treatment	pH	5.3	0.001

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3 Table 4. Results of cluster analysis (Paired Group Method using a Bray-Curtis
4 distance measure) on % total tests data showing groups identified at the third level of
5 division.

6

Group	Samples
1	MAC11 (1 sample)
2	MAC11 (1 sample)
3	MAC11 (13 samples), MAC2 (1 sample)
4	MAC30 (20 samples), MAC2 (9 samples), MAC11 (5 samples)

7