

1 **The impact of simulated sulfate deposition on peatland testate amoebae.**

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ABSTRACT

Peatlands subjected to sulfate deposition have been shown to produce less methane, believed to be due to competitive exclusion of methanogenic archaea by sulfate reducing bacteria. Here we address whether sulfate deposition produces impacts on a higher microbial group, the testate amoebae. Sodium sulfate was applied to experimental plots on a Scottish peatland and samples extracted after a period of more than ten years. Impacts on testate amoebae were tested using redundancy analysis and Mann-Whitney tests. Results showed statistically significant impacts on amoebae communities particularly noted by decreased abundance of *Trinema lineare*, *Corythion dubium* and *Euglypha rotunda*. As the species most severely impacted are all small bacterivores we suggest that our results support the hypothesis of a shift in dominant prokaryotes, although other explanations are possible. Our results demonstrate the sensitivity of peatland microbial communities to sulfate deposition and suggest sulfate may be a potentially important secondary control on testate amoebae.

KEYWORDS: Mires, wetlands, volcanic impacts, acid deposition, methanogens, sulfate reducing bacteria.

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2 INTRODUCTION

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4 Peatlands are exposed to sulfate deposition from both anthropogenic sources,
5 primarily fossil fuel burning, and natural sources, primarily volcanoes. Recent studies
6 have shown that deposition of sulfate on peatlands leads to a reduction in methane
7 production [31, 46] and emission [9, 11]. This suppression of methane emission may be a
8 highly important process in terms of global climate. Sulfate emissions currently reduce
9 wetland CH₄ flux by around 8% and could contribute to a 50% reduction in the northern
10 wetland CH₄ flux following a large Icelandic eruption [10, 12]. The cause of this methane
11 suppression is believed to be the competitive exclusion of methanogenic archaea (MA)
12 by sulfate reducing bacteria (SRBs). An increase in sulfate reduction simultaneous with
13 inhibition of methane efflux has been demonstrated, supporting this hypothesis [8].
14 However, to date, no studies have directly investigated the impact of sulfate deposition on
15 peatland microbial communities. Here we explore whether sulfate deposition might
16 produce impacts on a higher microbial group, potentially relating to the inferred
17 ecological shift in methanogenic archaea and sulfate reducing bacteria communities. This
18 study focuses on testate amoebae, a polyphyletic group of protists, which constitute a
19 large proportion of microbial biomass in *Sphagnum* peatlands (Gilbert et al. [14] estimate
20 14%, Mitchell et al. [27] estimate up to 30%). Testate amoebae are a particularly suitable
21 object for study due to the presence of a solid shell (the test) which allows taxa to be
22 identified to species level without resorting to molecular techniques. The decay-resistant
23 test also allows testate amoebae to be identified after death, enabling longer-term
24 processes to be studied. Some peatland palaeoecological records show testate amoebae
25 community changes coincident with volcanic tephra deposition [7, 36]. One hypothesis
26 for these changes is that they are related to volcanogenic sulfate deposition. Testate
27 amoebae include both taxa that are directly bacterivorous and taxa which predate other
28 microorganisms as well as consuming fungi and particulate organic matter; some taxa are
29 mixotrophic [15]. The testate amoebae community response is therefore likely to be
30 complex. In this study we use an experimental approach to test the impact of sulfate
31 deposition on testate amoebae communities of a natural peatland.

1

2 SITE and METHODS

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4 Experiments were conducted on Moidach More, an ombrotrophic peatland in
5 Morayshire, northeast Scotland (UK grid reference NJ0241, 57° 27'N, 3° 36'W, 275m
6 asl). Vegetation of the site includes *Sphagnum* species (*S. magellanicum*, *S. recurvum*, *S.*
7 *capillifolium*), *Trichophorum cespitosum*, *Erica tetralix* and *Calluna vulgaris* [9]. The
8 site receives little ambient sulfate deposition (c.5 kg ha⁻¹ yr⁻¹ SO₄²⁻). Experiments were
9 conducted on an uncut area towards the west of the site. Twenty, 2 x 2 m plots were
10 established in three adjacent blocks. Sodium sulfate was applied at three concentrations
11 over a period of 18 months, commencing in June 1997. Measurements of methane flux
12 and related environmental data were carried out at regular intervals until December 1998
13 and then occasionally until late 2003 [11]. Experimental set-up is described in detail by
14 Gauci et al. [9]. Samples for the present study were extracted from control plots and plots
15 subjected to the heaviest sulfate treatment (95 kg ha⁻¹ yr⁻¹ SO₄²⁻) in April 2008. This level
16 of deposition is equivalent to the upper end of the range of anthropogenic deposition or
17 what might be expected in northern peatland areas following a large Icelandic volcanic
18 eruption. A high sampling intensity was used to account for fine-scale spatial variability
19 in testate amoebae communities [26]. Twenty-five samples were extracted from each of
20 three pairs of treatment plots and control, yielding a total of 150 samples. Plots are
21 referred to by their block (1, 2 or 3) and their treatment: control (A) or treated (B).

22 Samples approximately 30 x 30 x 50mm depth were extracted from randomly
23 selected positions covering the surface area of each plot. To minimize influence of
24 vegetation structure on testate amoebae communities, samples were extracted from a
25 single moss species, *Sphagnum magellanicum*. A variety of environmental data were
26 collected to allow evaluation of any differences between plots that are unrelated to the
27 experimental treatments. The main environmental controls on testate amoebae
28 communities are wetness, acidity and nutrient status [1, 33, 42]. Data relevant to all these
29 parameters was collected. The pH of the samples was determined by suspending 2cm³ of
30 surface peat in 50ml of deionised water and measuring pH using a Jenway 3320 pH meter
31 after one hour. Loss on ignition (LOI), which may be a proxy for nutrient status [34], was

1 determined by drying peat samples at 105° C, weighing, incinerating at 550° C and then
2 re-weighing. Depth to water table (DWT) was measured by making a small hole adjacent
3 to the sampling point and measuring the depth to the water table after leaving for at least
4 two hours to equilibrate.

5 Testate amoebae preparation used a slightly modified version of the method of
6 Hendon & Charman [19]. The upper 50mm of 10 stems of *Sphagnum magellanicum* were
7 separated from other bryophytes and used in testate amoebae sample preparation. The
8 volume of the sample was measured by displacement in water. Samples were boiled for
9 10 minutes to disaggregate and a *Lycopodium* innoculum added to allow calculation of
10 test concentration [39, 45]. The sample was filtered at 300µm with the fine fraction
11 retained. Back-filtering with a finer sieve was not used as this is liable to lead to the loss
12 of some smaller tests (e.g. *Cryptodiffugia oviformis*, *Trinema lineare*) and amoebae
13 concentrations were high. Samples were stained to allow differentiation of living from
14 dead amoebae. Samples were centrifuged to concentrate and then stored in water. Slides
15 were prepared by mixing a drop of the preparation with glycerol. A count of 150 tests
16 was aimed for (mean=163), higher than the total advocated by Payne & Mitchell [35] as
17 changes in amoebae community due to the experimental additions may be subtle.
18 Taxonomy generally followed the scheme of Charman et al. [4] with a few minor
19 exceptions such as splitting of the *Corythion-Trinema* type. Species abundances were
20 converted to biomass using the approach outlined by Gilbert et al. [13]. Biovolumes were
21 approximated by assuming geometrical shapes [24] based on dimensions in the published
22 literature or estimates under the microscope and converted to carbon biomass using the
23 conversion factor $1 \mu\text{m}^3 = 1.1 \times 10^{-7} \mu\text{gC}$ [48].

24 The data were collated and six multivariate datasets calculated: 1) Relative
25 abundances of taxa as a percentage of total number of tests. 2) Relative abundances of
26 taxa considering only living individuals. 3) Abundance of taxa as concentrations of all
27 tests. 4) Abundance of taxa as concentration considering live individuals only. 5)
28 Estimated biomass based on all individuals. 6) Estimated biomass based on living
29 individuals. In addition, five univariate datasets were also calculated: 7) Overall test
30 concentration. 8) Concentration of living amoebae. 9) Live individuals as a percentage of
31 total tests. 10) Species richness. 11) Total estimated biomass based on all individuals. 12)

1 Total estimated biomass based on live individuals. The impact of the treatments in the
2 univariate data was tested using Mann-Whitney tests in PAST ver. 1.84 [17]. The
3 multivariate data structure was investigated using principal components analysis (PCA)
4 and the impact of the treatments in the multivariate data was tested using redundancy
5 analysis (RDA). A series of RDAs were used to test the impact of a nominal variable for
6 experimental treatment both on its own and with various combinations of the
7 environmental data (pH, DWT, LOI) introduced as co-variables. Significance was
8 assessed using Monte Carlo permutation tests (999 permutations restricted for
9 experimental design). Species data were Hellinger transformed [23, 37]. All ordination
10 analyses were carried out in CANOCO ver. 4.53 [40].

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13 RESULTS

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15 A total of 31 taxa were encountered in the 150 samples. The most abundant taxa
16 were *Archerella flavum* (30.5% of total count), *Corythion dubium* (10.2% of total),
17 *Euglypha strigosa* (9.6% of total) and *Nebela tinctoria* type (7.8% of total). Some
18 differences between the treatments and controls are apparent in the total abundance of
19 taxa within plots (Table 1). Higher abundances of *Euglypha strigosa*, *Placocista spinosa*
20 type and *Hyalosphenia papilio* are apparent in the treated plots (although the later is
21 absent in area 2). Consistently lower abundances of *Euglypha rotunda* type and *Trinema*
22 *lineare* are apparent in the treated plots, although abundance of the former taxon is very
23 low. Differences between the treated and untreated samples are apparent but are not
24 particularly marked in the PCA plot (Fig. 2). For mid-values of axis one, treated samples
25 generally have higher scores than untreated samples on axis two, there are more treated
26 than untreated samples at the highest values on axis one.

27 Analysis of univariate data showed significant difference between treated and
28 untreated plots for proportion of living tests and concentration of live amoebae ($P < 0.05$)
29 but not for total test concentration, number of species and testate amoebae biomass based
30 on live and all individuals (in the later case the relationship is only marginally
31 insignificant, $P = 0.06$).

1 The redundancy analysis results show that the experimental treatment explains a
2 significant proportion of the variance with all but one of the multivariate datasets (Table
3 2). pH and LOI did not explain a significant proportion of the variance independent of the
4 other variables (probably due to limited range) and were therefore excluded from
5 analyses. Most variance is explained when considering all tests (either as concentration or
6 percentage); 3.1% of variance is explained by the treatment variable and this is slightly
7 reduced to 2.8% when DWT is partialled out. The weakest relationships are produced
8 when using the estimated biomass data, perhaps due to the inevitable approximations in
9 these calculations [2] or the comparatively small size of some of the most sensitive taxa.
10 The relationship between the treatment and the species data is not significant when
11 calculating biomass on the basis of live individuals alone.

12 Fig. 3 shows the ordination plot with percentage data based on all tests; plots
13 based on other data-sets are similar and are not presented. Taxa known to be
14 hydrophilous (*Archerella flavum*, *Amphitrema wrightianum*) are negatively correlated
15 with DWT while taxa such as *Heleopera petricola* *Assulina muscorum* and *Euglypha*
16 *cristata* are positively correlated, indicating they are more xerophilous (although the
17 overall water table range is quite limited). The treatment variable is positively correlated
18 with *Hyalosphenia papilio*, *Arcella arenaria* type and to a lesser extent *Cryptodifflugia*
19 *oviformis*, and negatively correlated with *Trinema lineare*, *Euglypha rotunda* type and
20 less distinctly *Corythion dubium* and *Trinema complanatum*. It is notable that these latter
21 taxa are similar, all small Euglyphid species. Post-hoc Mann-Whitney tests showed
22 significant differences ($P < 0.05$) in relative abundance of all these taxa between treated
23 and untreated samples.

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25 DISCUSSION

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27 The results demonstrate a significant impact of sulfate deposition on testate
28 amoebae communities. The univariate data analysis shows the experimental treatments
29 reduce the concentration of live amoebae and percentage of live tests, suggesting a less
30 active amoebae community. This has parallels with studies of the impact of nutrient
31 enrichment on peatland testate amoebae. Mitchell [24] and Gilbert et al. [13, 14] found

1 nutrient enrichment (with N&P, N and P,K,Ca & N,P,K,Ca) and CO₂ enrichment [27]
2 reduced the contribution of testate amoebae to microbial biomass. Although there was no
3 measurable impact on estimated biomass here, we attribute this to the large errors
4 involved in biomass estimates based on taxon assemblage data and the small size of many
5 of the most sensitive taxa. The significant changes in proportion of living individuals
6 supports the value of this simple index in testate amoebae-based biomonitoring [43, 44].

7 3.1% of variance is explained by the treatment variable with the percentage data
8 and this relationship is highly significant (P=0.001). Although this seems a small
9 proportion, in the context of inherently noisy testate amoebae data this is far from
10 irrelevant. By comparison, DWT, the strongest environmental control, explains 7.6% of
11 variance with the other environmental data partialled out (P=0.001). This result shows a
12 distinct impact of sulfate application on amoebae community structure. The impact of
13 treatment on amoebae emerges equally strongly in the RDA when using data based on
14 concentration or percentages, showing that there are absolute changes in the abundance of
15 amoebae taxa, not simply relative changes in abundance.

16 The relationships are stronger when considering all individuals than considering
17 only living individuals. The number of live individuals counted in some samples is very
18 low (as few as three amoebae), possibly related to boiling in sample preparation. With
19 such low counts the amoebae community will be poorly characterized [35]. A further
20 factor contributing to the weaker relationships when only live individuals are considered
21 is likely to be the length of time which elapsed between experimental treatments and
22 sample extraction. It is quite possible that the amoebae community over the period of
23 several years represented by the full test community has been more affected by the
24 experimental additions than the testate amoebae community currently living at the site.
25 Nevertheless, the fact that the treatment variable is still highly significant even when just
26 considering living amoebae shows a long-lasting impact, consistent with the observations
27 of prolonged methane flux suppression [11].

28 Determining the relationship between the experimental treatments and the
29 amoebae community changes is complex. As a group testate amoebae have wide food
30 preferences including bacteria, particulate organic matter, microalgae, cyanobacteria,
31 plant cells, other protists, fungi and micro-metazoa [6, 15, 50]. Ecologically meaningful

1 interpretation of species changes is difficult as comparatively little is known of the
2 autecology of individual taxa. Gilbert et al. [15] located published information on feeding
3 preferences for only 33 species (out of perhaps 2000 described species [28]). The degree
4 of specificity in food source is also largely unknown. Gilbert et al. [16] showed *Nebela*
5 *collaris* (*sensu lato*) to feed on a wide variety of material ranging from diatoms to fungal
6 spores. Other taxa may have much more specific food requirements; in an aquatic system
7 Nishibe et al. [32] found that *Penardochlamys* sp. preyed exclusively on cyanobacteria of
8 the genus *Microcystis*. Furthermore, food preferences may well be seasonally variable
9 [e.g. 18].

10 The RDA plot shows a positive relationship between treatment and abundance of
11 *Hyalosphenia papilio*, *Arcella arenaria* and *Cryptodifflugia oviformis* and a negative
12 relationship with *Euglypha rotunda* type, *Corythion dubium* *Trinema complanatum* and
13 *Trinema lineare*. *T. lineare*, *T.complanatum* and *E. rotunda* are believed to be
14 bacterivorous and *C. dubium* to prey on bacteria and fungi [15]. *H.papilio* has been noted
15 to feed on fungi, microalgae, ciliates and metazoa [15]. We are not aware of any
16 information on the feeding habits of *C.oviformis* or *A. arenaria*, although another *Arcella*
17 species (*Arcella gibbosa*) has been noted to feed on bacteria, microalgae, fungi and
18 flagellates.

19 It is notable that the species which appear to be deleteriously impacted by sulfate
20 additions are among comparatively few testate amoebae species which are largely
21 bacterivorous. By contrast, taxa that respond positively have less specific feeding
22 preferences. This pattern is unlikely to be a coincidence. We are not aware of any
23 previous research specifically relating testate amoebae and methanogenic archaea or
24 sulfate reducing bacteria. As testate amoebae are most abundant in upper peats while
25 archaea are largely constricted to deeper layers of the peat [47] it is unlikely that testate
26 amoebae are major predators of methanogenic archaea. Previous research does however
27 indicate that other wetland protists predate sulfate reducing bacteria (and indeed
28 methanotrophs [29, 30]).

29 The lack of research on how testate amoebae fit into the microbial foodweb in
30 peatlands means that we cannot fully explain the mechanism which relates sulfate
31 addition to changes in testate amoebae communities observed in this study. However it is

1 certainly tempting to conclude a relationship between the decline in bacterivorous testate
2 amoebae and the putative decline in methanogens. The mechanism for this is unlikely to
3 be as simple as these species preferentially consuming archaea over bacteria, it is more
4 probable that the interaction is indirect through other organisms. It is even possible that
5 sulfate deposition somehow promotes the predation of these taxa. Methanogenic
6 endosymbionts have been widely reported from protists [e.g. 20, 41], including wetland
7 ciliates [38], although as far as we are aware there has been no record of methanogenic
8 symbionts in testate amoebae. It is interesting to speculate that some of the apparent
9 association between methane flux suppression and testate amoebae community change
10 could be related to predation of ciliates with methanogenic symbionts by testate amoebae.

11 An alternative mechanism to a change in methanogens/SRBs is that sulfate
12 deposition directly or indirectly modifies the chemical environment such that it becomes
13 more suitable for some testate amoebae taxa than for others. While we cannot exclude
14 this possibility we cannot see a clear mechanism whereby this might occur. A further
15 possibility is that impacts are due to the sodium applied with the sulfate. We think this is
16 unlikely as: 1. The quantity of Na applied is very small, 2. Na⁺ was not shown to be a
17 significant variable in a recent ecological study [33]. 3. Gauci et al. [11] showed no
18 methane suppression in control plots with NaCl applied, suggesting that there is at least
19 no impact on the microbial community involved with methanogenesis. We suggest that
20 our results provide some circumstantial support for the hypothesis of a shift from
21 methanogens to SRBs and that this produces consequent impacts throughout the
22 microbial foodweb.

23 These experimental results suggest that sulfate may be an important
24 environmental control on testate amoebae communities. Where sulfates have been
25 measured in ecological studies, sulfate is correlated with major testate amoebae species
26 gradients [e.g. 49]. Opravilova & Hajek [33] and Mitchell et al. [27] have shown sulfate
27 to be a small but statistically significant independent environmental control on amoebae
28 communities. A contrary result was found by Lamentowicz et al. [22] although this study
29 was focused on a single site and therefore has limited environmental gradients. Taken
30 together, our experimental results and the previous ecological survey results suggest that
31 sulfate may be underestimated as a control on amoebae communities. It would certainly

1 be useful to analyse sulfate more regularly in ecological studies of testate amoebae, and
2 particularly interesting to analyse testate amoebae in peatlands along a gradient of
3 anthropogenic sulfate deposition. It would be interesting to repeat this study with a
4 greater number of plots and to see if impacts are still detectable with lower levels of
5 sulfate application. Studies combining analyses of testate amoebae with analyses of other
6 microbial groups [e.g. 21] might help unravel the mechanism of impact. It is perhaps
7 worth noting that saltmarshes (which have significant sulfate input) have notably
8 different testate amoebae communities from ombrotrophic peatlands (which generally do
9 not) although clearly there are also many other differences in these ecosystems [5].

10 Testate amoebae are increasingly widely used in palaeoecological studies to
11 provide a proxy-record of hydrological change [3, 28]. Inherent in this work is the
12 assumption that testate amoebae community change is primarily driven by peatland
13 hydrological change, and therefore by climate. These results suggest that sulfate pollution
14 may also be an important (albeit much weaker) control. This might complicate
15 hydrological reconstruction in peatlands subject to sulfate deposition.

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24

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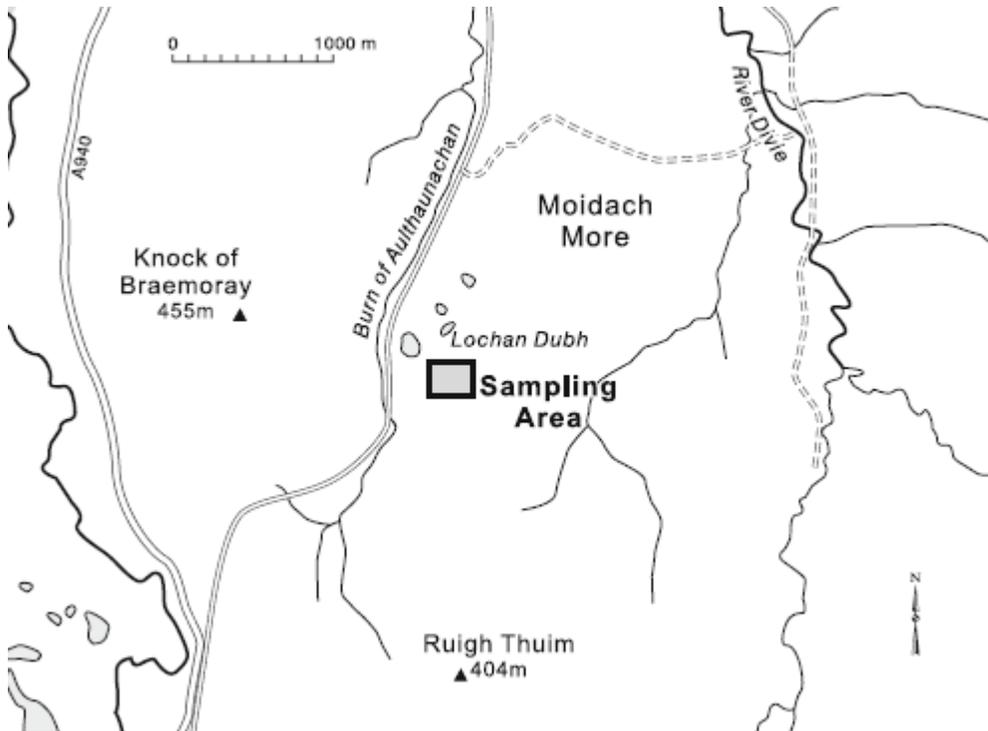
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2 FIGURES AND TABLES

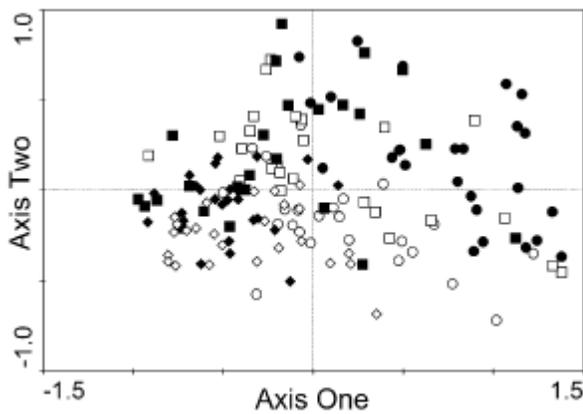
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4 Figure 1. Location map of Moidach More fieldsite.



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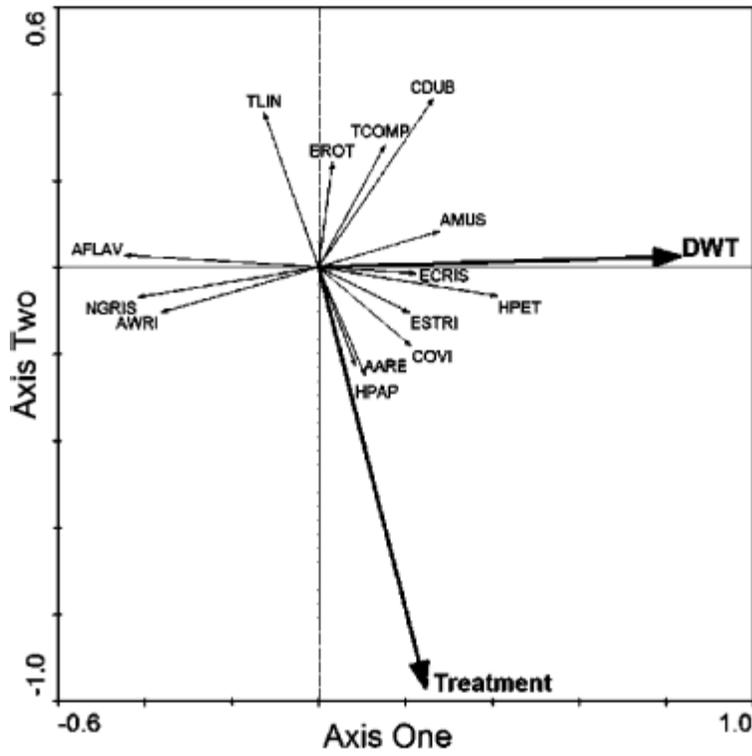
6 Figure 2. Principal components analysis of testate amoebae samples based on relative
7 abundance of all tests. Circles are block 1 samples, squares block 2 samples and
8 diamonds block 3 samples. Samples marked in white are from controls and samples in
9 black from treated plots.



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11 Figure 3. Redundancy analysis of testate amoebae data based on relative abundance of all
12 tests. Showing selected major species and significant environmental variables. Species

- 1 codes: AFLAV: *Archerella flavum*, TLIN: *Trinema lineare*, EROT: *Euglypha rotunda*
- 2 type, TCOMP: *Trinema complanatum*, CDUB: *Corythion dubium*, AMUS: *Assulina*
- 3 *muscorum*, ECRIS: *Euglypha cristata*, HPET: *Heleopera petricola*, ESTRI: *Euglypha*
- 4 *strigosa*, COVI: *Cryptodiffugia oviformis*, AARE: *Arcella arenaria* type, *Hyalosphenia*
- 5 *papilio*, AWRI: *Amphitrema wrightianum*, NGRIS: *Nebela griseola*.



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2 Table 1. Relative abundance of testate amoebae taxa (nearest whole %) in plots of this
 3 study showing major taxa (over 2% of overall total in at least one plot). Plot numbers
 4 reflect sampling area (1, 2 or 3) and whether the plot was treated (b) or control (a).

5

Taxon	Codes	Overall abundance (% total tests) in plot:					
		1a	1b	2a	2b	3a	3b
<i>Archerella flavum</i> Archer 1877	AFLAV	26	7	31	34	40	45
<i>Amphitrema wrightianum</i> Archer 1869	AWRI	1	0	0	2	3	2
<i>Arcella arenaria</i> Greef 1866 type	AARE	2	2	3	2	1	4
<i>Assulina muscorum</i> Greef 1888 type	AMUS	11	17	11	7	10	9
<i>Assulina seminulum</i> (Ehrenberg 1848)	ASEM	4	4	3	5	3	3
<i>Corythion dubium</i> Taraneck 1881	CDUB	14	14	12	4	10	7
<i>Euglypha ciliata</i> (Ehrenberg 1848)	ECIL	0	1	2	1	1	1
<i>Euglypha compressa</i> Carter 1864	ECOMP	0	0	0	1	2	2
<i>Euglypha rotunda</i> Wailes 1911 type	EROT	1	1	1	0	1	0
<i>Euglypha strigosa</i> (Ehrenberg 1872)	ESTRI	12	17	8	9	5	6
<i>Heleopera petricola</i> Leidy 1879	HPET	5	9	9	8	2	4
<i>Heleopera rosea</i> Penard 1890	HROS	2	1	1	1	0	0
<i>Hyalosphenia elegans</i> Leidy 1875	HELE	6	9	9	8	6	5
<i>Hyalosphenia papilio</i> Leidy 1875	HPAP	0	1	0	0	1	6
<i>Nebela griseola</i> Penard 1911	NGRIS	1	0	0	1	1	1
<i>Nebela tinctoria</i> (Leidy 1879) type	NTINC	6	12	8	11	7	4
<i>Placocista spinosa</i> (Carter 1865) type	PLSP	1	3	1	1	0	1
<i>Trinema lineare</i> Penard 1890	TLIN	6	2	1	0	2	1

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Table 2. Redundancy analysis of square-root transformed testate amoebae data showing percentage variance explained and P-values of these relationships assessed by Monte Carlo permutation tests (999 permutations restricted for split-plot design). ns= not significant at $P < 0.05$.

Dataset	Explanatory variable	Co-variable	% variance explained	P-value
All tests (%)	Treatment	-	3.1	0.001
	Treatment	DWT	2.8	0.001
All tests (concentration)	Treatment	-	3.1	0.001
	Treatment	DWT	2.8	0.001
Live amoebae (%)	Treatment	-	2.3	0.001
	Treatment	DWT	1.9	0.001
Live amoebae (concentration)	Treatment	-	2.3	0.001
	Treatment	DWT	1.9	0.001
Estimated amoebae biomass (based on all tests)	Treatment	-	2.4	0.007
	Treatment	DWT	2.3	0.008
Estimated amoebae biomass (live individuals only)	Treatment	-	1.1	ns
	Treatment	DWT	1.1	ns

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