Payne R, Thompson AM, Standen V, Field CD & Caporn SJM (2012) Impact of simulated nitrogen pollution on heathland microfauna, mesofauna and plants, *European Journal of Soil Biology*, 49, pp. 73-79.

This is the peer reviewed version of this article

Impact of simulated nitrogen pollution on heathland microfauna, mesofauna and plants.

Richard J. Payne[1,2*], Astrid M. Thompson[3], Valerie Standen[3], Christopher D. Field[1], Simon J.M. Caporn[1]

[1]School of Science and the Environment, Manchester Metropolitan University, Chester Street, Manchester, M1 5GD, UK.

[2]Geography, School of Environment and Development, University of Manchester, Oxford Road, Manchester M13 9PL, UK.

[3]School of Biological and Biomedical Sciences, Durham University, South Road, Durham, DH1 3LE, UK.

*To whom correspondence should be addressed, e-mail r.payne@mmu.ac.uk

ABSTRACTS

Deposition of reactive nitrogen derived from intensive agriculture and industrial processes is a major threat to biodiversity and ecosystem services around the world; however our knowledge of the impacts of nitrogen is restricted to a very limited range of organisms. Here we examine the response of groups of microfauna (testate amoebae), mesofauna (enchytraeid worms) and plants to ammonium nitrate application in the Ruabon heathland long-term experiment. Plant data showed significant differences between treatments, particularly characterised by a loss of bryophytes in nitrogen-treated plots, by contrast enchytraeids showed a non-significant increase in abundance in response to treatment. Testate amoebae showed no significant changes in abundance or inferred biomass but significant changes in community structure with a reduced abundance of *Corythion dubium*, interpreted as a response to the loss of bryophytes. Our results suggest that simple indices of plant community may have value for bioindication while the bioindication value of testate amoebae and enchytraeids is not clearly demonstrated.

KEYWORDS: Pollution, Reactive Nitrogen, Enchytraeids, Testate amoebae, Bioindication, Heathlands

1. INTRODUCTION

Since the first commercial application of the Haber-Bosch process in 1913 human production of reactive nitrogen (N_r) has grown rapidly, with an increase of over 120% since 1970 [1]. N_r deposition in the absence of human activity is generally less than around 0.5 kg N ha^{-1} yr^{-1}, while in the United Kingdom some areas currently receive deposition in excess of 40 kg N ha^{-1} yr^{-1}. These levels of nitrogen deposition are sufficient to lead to a significant reduction in biodiversity [2,3] and damage to ecosystem services. Species-loss from ecosystems is driven by both eutrophication and acidification with the relative contributions of these processes varying by habitat type.

Heathlands are a UK Biodiversity Action Plan priority habitat, covering over 2,000,000 ha of upland Britain but in England and Wales their cover declined by an estimated 27% between 1947 and 1980 [6]. A critical load range of 10-20 kg N ha^{-1} yr^{-1} is exceeded in many heathland areas of the British Isles with N deposition shown to reduce plant biodiversity, particularly marked by a loss of lichens and bryophytes [7]. Large-scale ecological surveillance data shows a reduction in plant species richness along the N deposition gradient even when accounting for other drivers [4]. Impacts of nitrogen on groups of heathland organisms other than plants are however poorly documented. Here we examine the response of plants and major groups of eukaryotic microorganisms and mesofauna in the same ecological experiment and consider the possible inter-relations between these groups. Our study aims to provide a broader understanding of the ecosystem-wide consequences of nitrogen pollution in heathlands and to identify possible bioindication approaches.

1.1 *The studied groups and their inter-relations*

Testate amoebae are a group of eukaryotic microorganisms characterised by a solid shell (test) which can constitute a very large proportion of microbial biomass in organic soils [8] and are likely to have an important role in nutrient cycling [9,10]. Testate amoebae have been shown to respond to soil environmental changes to which other groups are insensitive [11] and have broad feeding preferences making them good synthesisers of overall microbial community change. Previous studies have demonstrated testate amoeba sensitivity to nutrient enrichment [12, 13, 14] and have suggested impacts from NO_2 exposure [15].

The enchytraeidae are a group of detritovorous, bacterivorous and fungivorous annelid worms, typically 3-30mm in length. Enchytraeids constitute a large proportion of mesofaunal biomass in many temperate soils (c. 75%: [16]) and may fill a keystone role in heathlands [17]. Enchytraeid abundance has been shown to respond to application of nitrogen fertilizer [18]. It seems possible that enchytraeids

might predate testate amoebae given their size and observations of predation by other groups of worms [19 cited in 10]. Bacteria feeding on enchytraeid faeces are likely to provide a food supply for some testate amoebae and enchytraeid burrowing may aerate soil, modifying the amoeba's habitat and translocating individuals [cf. 20]. Enchytraeids may compete with testate amoeba species for food, for instance with members of the Centropyxidae for fungi [21, 22, 23].

Testate amoeba and enchytraeid communities are both intricately linked to plant communities with plants shaping the organism's physical, chemical and biotic environment. Precise mechanisms are difficult to pin-down but it is probable that for instance amoebae are affected by the chemical quality of plant litter [24], are closely linked to mycorrhizas [25] and are affected by changes in root exudation [e.g. 26]. As decomposers enchytraeids are highly sensitive to the quality of plant litter and experimental removal of different plant species has been shown to differentially modify enchytraeid abundance [27]. Both enchytraeids and testate amoebae are likely to be involved in nutrient mineralisation and thereby influence plant nutrition [28].

2. SITE and METHODS

Experiments were first established on wet upland heath near Ruabon, Clwyd, North Wales (53° 02'N, 3°08'W; 470m asl) in 1989 and have been extensively discussed in previous publications [29, 30, 31, 32, 33]. The climate of the site is cool and oceanic: average annual air temperature is 9.8°C (2008-9 data), average annual soil temperature 6.9°C (2008-9 data) and average annual precipitation 1053mm (2007-2009 data). Vegetation of the site is dominated by *Calluna vulgaris* with subordinate bryophytes and scattered *Vaccinium myrtillus*. The site is representative of the *Calluna*-dominated heaths (NVC type H12: *C. vulgaris*-*V. myrtillus* heath [34]) which cover large areas of upland Britain. Soil is silty clay loam with pH around 4.4 and depth of around 50cm. Ambient nitrogen deposition is around 19.9 kg N ha^{-1} yr^{-1} (UK Air Pollution Information System (APIS) www.apis.ac.uk), at the upper limit of the critical load range (10-20 kg N ha^{-1} yr^{-1}). The original experiments consisted of 1x1m plots which were established in May 1989, subsequent experiments with 2x2m plots were established in 1998. Nitrogen as ammonium nitrate is applied ten times a year to plots at concentrations of 0, 40, 80 and 120 kg N_r ha^{-1} yr^{-1} in the 1989- ('old') plots and 0, 10, 20, 40 and 120 kg N_r ha^{-1} yr^{-1} in the 1998- ('new') plots with four replicates for each concentration. The old plots were burned in 2000 in keeping with normal management practise [35].

For testate amoeba analysis samples were extracted from the control and heaviest treated (120 kg N ha^{-1} yr^{-1}, hereafter termed 120N) of the older (1989) plots in November 2009, more than 20 years after the onset of the treatment. Approximately 5cm^3 of surface soil with any overlying litter and bryophytes were removed with a knife, sealed in plastic bags and refrigerated. In the laboratory testate amoebae were extracted using a method based on the standard methodology [36]. Sub-sample volume was measured by displacement in deionised water, samples were soaked for c.2 hours and stirred to disaggregate. The majority of recent testate amoeba studies have been based on relative abundance data (for ease of application to the palaeoecological record) however this approach may lead to loss of information [37]. Here we analyse both percentage and concentration data; an exotic *Lycopodium clavatum* innoculum of counted spores was added to samples to allow calculation of concentrations [38]. Suspensions were sieved at 300µm but were not back-sieved to avoid loss of small taxa [39]. Samples were mounted in glycerol and a count of 100 individuals aimed for [40]. A variety of taxonomic guides were used [41, 42, 43]; the *Euglypha rotunda*, *Centropyxis aerophila* (=*Centropyxis cassis*) and *Difflugis pristis* types follow [41]. Tests with visible cytoplasm (termed 'live individuals') were recorded separately from empty shells (although it was not possible to distinguish living from simply undecayed individuals). Taxon-specific biovolumes were calculated based on assumed geometric shapes and published biometric data and converted to estimated biomass [8, 14].

For enchytraeid analysis soil cores (50mm diameter, 50mm depth) were extracted from the 0, 20, 40 and 120 N_r treatments of the newer (1998) plots between May 2002 and September 2003. Three replicate cores were taken from each plot at six intervals over this period (May, July and September in 2002 and 2003) giving a total of 216 samples. Enchytraeids were extracted using the wet funnel technique [44] and identified following Nielsen and Christensen [45].

Changes in plant communities of these plots have been extensively considered over more than 20 years (Table 1). Here we focus solely on vascular plant species with bryophytes and lichens identified to functional types, a simple approach which may have considerable potential as a quick and effective bioindication strategy [*cf*. 46]. Our analysis updates the previous results of Carroll et al. [30] more than a decade after that study. A 15-point pin quadrat was placed in the centre of each of the old plots (4 replicates of 3 treatments + control) in summer 2005, recording all touches in four categories (*Calluna vulgaris, Vaccinium myrtillus,* bryophytes and lichens). Lichens were too rare for meaningful data analysis. *Calluna* canopy height was also measured at each pin point.

2.1 *Data analysis*

For the testate amoeba data Shannon (H) and Simpson (D) diversity indices, and related equitability measures (E_H, E_D) were calculated. A sequence of nested-ANOVAs were used to identify significant differences between treated and untreated plots for species richness, diversity and equitability, proportion of occupied tests (a measure of general community health) and amoeba concentration and biomass based on both all tests and only live individuals. For the enchytraeid count data a repeated measures ANOVA (RM-ANOVA) was used to compare plot mean data over the experimental period. For the plant data separate nested-ANOVAs were conducted for total pin touches and covers (a 1-15 scale counting each pin as one point) of each plant type and for *Calluna* canopy height. All data satisfied the requirements of ANOVA.

To examine nitrogen-induced differences in testate amoeba community structure we use a non-parametric approach based on Bray-Curtis dissimilarity [47], which has been shown to be a useful and robust similarity coefficient for many ecological datasets [48, 49]. We use a non-metric multi-dimensional scaling (NMDS) ordination to visualise the data and then apply a sequence of one-way analyses of similarity (ANOSIM [50]) to test for similarity between treated and untreated samples. Significance testing used permutation tests with 10,000 permutations. To identify the taxa principally responsible for the differences between groups we follow ANOSIM with a Similarity Percentage (SIMPER) analysis, a simple Bray-Curtis based approach to identify the taxa contributing to observed community difference [50]. Six sets of multivariate data analyses were conducted using: 1) Percentages of all tests, 2) Concentrations of all tests, 3) Estimated biomass based on all tests, 4) Percentages of live amoebae, 5) Concentrations of live amoebae, and 6) Estimated biomass based on only living individuals. Multivariate data analyses were carried out using PAST ver. 1.84 [51] and univariate analyses with SPSS ver. 18.

3. RESULTS

The amoeba community of these plots was predominantly composed of generalist taxa which are very abundant in soils with heavy dominance by *Corythion dubium* (36% all tests); other major taxa included *Assulina muscorum* (12%), *Cryptodifflugia oviformis* (8%) and *Nebela tincta* type (8%)(Table 2). While most common genera were represented to some extent there was a particular predominance of small taxa with filopodia. There was a significant difference in both Shannon (H) and Simpson (D) diversity between samples from treated and untreated plots (nested-ANOVA $F_{1,32}$=9.1, P=0.02 for H;

147 $F_{1,32}$=13.8, P=0.01 for D), driven by increased equitability in treated plots ($F_{1,32}$=11.5, P=0.02 for E_H;
148 $F_{1,32}$=10.1, P=0.02 for E_D) rather than species richness, which did not significantly differ between plots
149 (P>0.05). This increased equitability is driven by a higher relative abundance of *Corythion dubium* (Fig. 1)
150 in the control plots; if this taxon is removed there is no significant difference between treatments
151 (P>0.05 for H, D, E_H & E_D). There was no difference between treatment and control in concentration of
152 total tests, concentration of live amoebae, proportion of occupied tests, estimated biomass based on all
153 tests or estimated biomass based only on living amoebae (P>0.05).

154 An NMDS ordination shows the relation of the two sets of samples with a tendency for treated
155 samples to have higher x-coordinates than untreated samples but considerable overlap (Fig. 2, it should
156 be noted that the stress value is relatively high so it would be unwise to read too much into the fine
157 details of sample positioning). Initial analyses of similarity found no evidence for differences between
158 plots with the same treatment so simple one-way analyses of similarity were used in subsequent tests.
159 There was a significant difference between treated and control samples for amoeba community based
160 on the relative abundance of all tests but not for data based on concentrations, biomass or live
161 individuals only (P>0.05). Differences were relatively small but highly significant (R_{ANOSIM}=0.12, P=0.002).
162 SIMPER identifies the greatest contributors as *Corythion dubium*, *Cryptodifflugia oviformis* and *Assulina*
163 *muscorum*. If *Corythion dubium* is removed from the relative abundance data the analysis loses
164 significance. If differences in abundance of the major taxa are tested individually there are significant
165 differences in relative abundance for only two taxa: *C. dubium* and *A. muscorum*, and no significant
166 differences in concentration for any taxa (Table 2).

167 The community composition of enchytraeids showed little diversity; over 90% of the individuals
168 identified to species level were *Cognettia sphagnetorum*, with *Mesenchytraeus sanguineous* the most
169 abundant subordinate species. Given this heavy dominance by a single species only abundance of *C.*
170 *sphagnetorum* was used in data analysis. Number of individuals per core varied from 1 to 191
171 (mean=59). Numbers were highly variable both within cores from the same plots and between plots
172 with the same treatment. There was considerable change over time with populations of all plots
173 crashing in the summer of 2003. While there was a general trend of higher enchytraeid numbers in the
174 most heavily N-treated plots (Fig. 3), there was no significant treatment, or time*treatment effect
175 (P>0.05), although the difference between control and 120N treatment (as considered by the testate
176 amoeba analyses) approached significance in post-hoc testing (Fishers LSD, P=0.06).

The plant data showed significant differences between treatments for bryophyte total touches (nested-ANOVA $F_{3,280}$=7.0 P=0.003) and cover ($F_{3,280}$=11.5 P<0.001). In all treated plots bryophytes were significantly less abundant than in control plots (P<0.001 in post-hoc testing with Tukey's HSD; Fig. 4), individual treatments were significantly different from each other (P<0.01) with the exception of the 40N and 120N treatments which could not be distinguished (P>0.05). There were significant differences between treatments for *Calluna* touches ($F_{3,280}$=4.2 P=0.02) with more touches in the 20N and 120N plots (P<0.01) than the controls, but no difference between controls and 40N plots (P>0.05) and no overall trend within the treated plots. There were no differences between treatments for *Calluna* cover or for *Vaccinium* cover and touches (P>0.05). There were differences between treatments for *Calluna* height ($F_{3,280}$=5.4 P=0.009), with taller *Calluna* in all treated plots (Tukey's HSD P<0.001; Fig. 5) than controls.

4. DISCUSSION

4.1 *Testate amoeba response*

The testate amoeba results from plots treated with high levels of nitrogen for 20 years show evidence for changed community structure but not for changed abundance or biomass, in contrast to the combined effects of N and P [14]. That significant differences are only found when using relative abundance data may reflect the inter-dependence of taxon values amplifying real abundance differences. The low counts of live individuals, exotic marker technique used to derive concentrations, and the biovolume and carbon content conversions used to estimate biomass will inevitably introduce some errors into these data. Biovolumes estimated using the geometric shapes approach have been shown to deviate substantially from direct instrumental measurements [52] and given that an amoeba may not occupy the full shell volume are likely to over-estimate values. The *Lycopodium* innoculum technique has not been formally tested for testate amoebae and differential loss in sample preparation is not unlikely given the potentially large differences in morphology and density.

The most distinct change in community composition is a reduced abundance of *Corythion dubium* in the control plots. *C. dubium* is a widely dispersed and locally highly-abundant taxon which predates bacteria and heterotrophic flagellates [22] and is particularly abundant in mosses [53]. Three explanations for the decline of *C. dubium* can be proposed. Firstly that *C. dubium* is directly affected by chemical changes due to the nitrogen additions. Previous studies have demonstrated increased concentrations of ammonium and nitrate in leachate, and modest increases in soil acidity and

Aluminium concentrations in treated plots [32]. It is possible that *C. dubium* is being affected by these changes, however there is no particular reason to suspect greater sensitivity in this taxon and there is no evidence for change towards a more acidophilic community composition. A second hypothesis is that *C. dubium* declines because of a reduced food supply due to a decline in abundance in lower microbial groups. While microbial biomass has been shown to decline following N addition in some ecosystems, in this heathland the available evidence suggests an increased bacterial and overall microbial biomass [54]. While *C. dubium* might exhibit selective predation among prokaryotes and small protists it seems more probable that the decline of *C. dubium* is not directly mediated by availability of prey organisms. A final possibility is that the decline of this species is related to changes in the amoeba's environment through changed plant communities (discussed below). Given how intimately linked plant and testate amoeba communities are (section 1.1) it can be expected that significant plant community change would be manifested in changed testate amoeba communities [14]. The known preference of *C. dubium* for bryophytes and the demonstrable decline in bryophytes in these plots therefore strongly suggests that testate amoebae are responding to the changed plant communities. Although the testate amoeba samples were extracted four years after the plant data discussed below the changes demonstrated were still highly apparent in 2009 with little bryophytes in any of the treated plots.

As significant changes in testate amoebae communities are shown by our results it is possible that testate amoebae may have value for bioindication of nitrogen deposition in heathlands. Such an approach would have some advantages. Generation times of testate amoebae can be very short (several generations per week in laboratory conditions [21]) so testate amoebae could potentially be a highly responsive bioindicator group allowing real-time monitoring of changing impacts. Furthermore, the analysis of empty tests alongside live amoebae allows simultaneous determination of the amoeba community at both a single moment in time and integrated over a period of perhaps several years. This multiple time-period approach would be a rather unique advantage of testate amoebae for bioindication. However our results also point to two important potential drawbacks in the use of testate amoebae as bioindicators of nitrogen. Firstly, the response is characterised by a reduced abundance of *Corythion dubium*, a change which could conceivably be caused by independent environmental changes such as climatic warming/drying [e.g. 55]. Secondly, it appears probable that the testate amoeba response is mediated by plant community change, specifically the loss of bryophytes. If this deduction is correct then it implies that the testate amoeba response to nitrogen is likely to be indirect and therefore their use as indicators may add little to the direct use of plant communities for bioindication which would be vastly quicker and simpler.

4.2 *Enchytraeid response*

The enchytraeid data from plots treated for four years showed a general trend towards higher abundance in treated plots but this was not statistically significant. The lack of a significant difference between treatments may be largely explained by the very high spatial and temporal variability in numbers (Fig. 3). Particularly low numbers were found in the summer of 2003, probably due to the severe drought of that year perhaps with vertical migration of enchytraeids to below the sampling zone [56]. It is possible that with more replication a significant effect might have been identified but this was not feasible without undue disturbance to the plots. The non-significant trend towards higher enchytraeid abundance in N-treated plots contrasts with severe reductions in some N-addition experiments in other ecosystems [18, 57, 58]. The lack of a significant change in enchytraeid abundance here does however parallel that of Prendergast-Miller et al. [59] who found no significant change in enchytraeids in response to ammonia fumigation. Although we find no strong evidence for impacts of nitrogen deposition on enchytraeids our results do not rule out such impacts, it is possible that with longer treatment periods chemical changes in above-ground plant material would increasingly manifest themselves in changed enchytraeid food quality and therefore changed enchytraeid abundance [59]. Our results do however add to other recent studies in questioning whether enchytraeids could provide a viable bioindication approach given their primary control by soil moisture conditions and extremely patchy distribution [59].

4.3 *Plant response*

Our data show a very marked nitrogen-induced decline in the bryophytes of these plots. This decline is particularly apparent in the heaviest treated 120N plots where no bryophyte pin-touches were recorded. The 120N treatment is very high; however even in the 20N plots, representing a more-frequently encountered pollution level, a decline in bryophyte cover is apparent and statistically significant and *Calluna* height is increased.

Our results largely match those of a number of earlier studies from these plots (Table 1) showing increased vigour of *Calluna* and decreased vigour of bryophytes, although a more complex picture emerges when considering low N doses and interactions with P [60]. Loss of bryophytes has been widely

found in experimental and gradient studies of nitrogen in a number of habitats [7, 31], including a decline in *Hypnum jutlandicum,* the overwhelmingly dominant bryophyte of these plots in response to ammonia exposure [61].

That distinct changes can be identified at relatively realistic doses supports previous research in suggesting the potential of plant community-based indices for bioindication of nitrogen pollution. On the basis of our experiments it seems that even a taxonomically-crude *Calluna* : bryophyte ratio might perform well for bioindication. Furthermore the fact that testate amoebae may respond to the plant community changes suggests that using plants as bioindicators may also reveal indirect impacts of nitrogen on other components of the ecosystem. A complicating factor is the extent to which heathlands are an anthropogenic ecosystem with their form and composition heavily dependent on human management. It is possible that the developmental stage of the *Calluna* (pioneer/building/mature/degenerate) will be a serious impediment to the use of plant community based indicators of nitrogen pollution. Addressing such issues will require larger-scale field data and will be discussed more in future publications.

5. CONCLUSIONS

Our results illustrate some of the less-considered consequences of nitrogen deposition in semi-natural ecosystems. For the first time we demonstrate that application of nitrogen alone has the potential to modify community structure in an abundant but little studied group of soil protists, the testate amoebae. By contrast our data do not provide evidence for the sensitivity of enchytraeid abundance to nitrogen. While this negative result may partly be explained by the sampling intensity and treatment period of this study it seems probable that other environmental controls are more important than nitrogen. Plant communities respond strongly to nitrogen deposition and these changes may be the cause of the testate amoebae changes. Plant community-based bioindication may therefore be both sensitive to nitrogen deposition and represent changes in the broader ecosystem. Future work could usefully examine the response of different groups of organisms and their bioindication potential in the same experimental setting, this is difficult in our study as samples represent differing treatment periods for different groups.

ACKNOWLEDGEMENTS

Funding for set-up and monitoring of the Ruabon experiment has been provided by the UK Natural Environment Research Council, the Department for Food, Environment and Rural Affairs (DEFRA) and Manchester Metropolitan University. RJP and SJMC were supported by NERC through the FP6 BiodivERsA Eranet project PEATBOG. VS and AMT were supported by NERC as part of the Global Atmospheric Nitrogen Enrichment (GANE) programme. Thanks to David Wilkinson and two anonymous reviewers for comments on a previous version of the paper.

REFERENCES

[1] J.N. Galloway, A.R. Townsend, J.W. Erisman, M. Bekunda, Z. Cai, J.R. Freney, L.A. Martinelli, S.P. Seitzinger, M.A. Sutton, Transformation of the nitrogen cycle: recent trends, questions, and potential solutions, Science 320 (2008) 889-892.

[2] C.J., Stevens, N.B. Dise, J.O. Mountford, D.J., Gowing, Impact of nitrogen deposition on the species richness of grasslands, Science 303 (2004) 1876-1879.

[3] C.J. Stevens, C. Dupre, E. Dorland, C. Gaudnik, , D.J. Gowing, A. Bleeker, M. Diekmann, D. Alard, R. Bobbink, D. Fowler, E. Corcket, J.O. Mountford, V. Vandvik, P.A. Aarrestad, S. Muller, N.B. Dise, Nitrogen deposition threatens species richness of grasslands across Europe, Environ. Pollut. 158 (2010) 2940–2945.

[4] L.C. Maskell, S.M. Smart, J.M. Bullock, K. Thompson, C.J. Stevens, Nitrogen deposition causes widespread loss of species richness in British habitats, Glob. Change Biol. 16 (2010) 671-679.

[5] C.J. Stevens, K. Thompson, J.P. Grime, C.J. Long, D.J. Gowing, Contribution of acidification and eutrophication to declines in species richness of calcifuges grasslands along a gradient of atmospheric nitrogen deposition, Funct. Ecol. 24 (2010) 478-484.

[6] A. Mattock (Ed.), UK Biodiversity Action Plan: Priority habitat descriptions, Joint Nature Conservation Committee, Peterborough, 2008.

[7] R. Bobbink, M. Hornung, J.G.M. Roelofs, The effects of air-borne nitrogen pollutants on species diversity in natural and semi-natural European vegetation, J. Ecol. 86 (1998) 717-738.

[8] D. Gilbert, C. Amblard, G. Bourdier, A. Francez, The microbial loop at the surface of a peatland: structure, functioning and impact of nutrients inputs, Microb. Ecol. 35 (1998) 83-93.

[9] D.M. Wilkinson, Testate amoebae and nutrient cycling: peering into the black box of soil ecology, Trends Ecol. Evol. 23 (2008) 596-599.

[10] D.M. Wilkinson, E.A.D. Mitchell, Testate amoebae and nutrient cycling with particular reference to soils, Geomicrobiol. J. 27 (2010) 520-533.

[11] W. Foissner, Protozoa as bioindicators in agroecosystems, with emphasis on farming practices, biocides and biodiversity, Agric. Ecosyst. Environ. 62 (1997) 93-103.

[12] H. Berger, W. Foissner, H. Adam, Field experiments on the effects of fertilizers and lime on the soil microfauna of an alpine pasture, Pedobiologia. 29 (1986) 261-272.

[13] E. Aescht, W. Foissner, Effects of mineral and organic fertilizers on the microfauna in a high-altitude reafforestation trial, Biol. Fert. Soils. 13 (2002) 17-24.

[14] E.A.D. Mitchell, Response of testate amoebae (Protozoa) to N and P fertilization in an Arctic wet sedge tundra, Arct. Antarct. Alp. Res. 36 (2004) 77-82.
[15] H. Nguyen-Viet, D. Gilbert, D. Bernard, E.A.D. Mitchell, P-M. Badot, Relationship between atmospheric pollution characterized by NO_2 concentrations and testate amoebae density and diversity, Acta Protozool. 43 (2004) 233-239.
[16] L. Cole, R.D. Bardgett, P. Ineson, P.J. Hobbs, Enchytraeid worm (Oligochaeta) influences on microbial community structure, nutrient dynamics and plant growth in blanket peat subjected to warming, Soil Biol. Biochem. 34 (2002) 83-92.
[17] J. Laakso, H. Setala, Sensitivity of primary production to changes in the architecture of below ground food webs, Oikos 87 (1999) 57-64.
[18] G. Abrahamsen, W.N. Thompson, Long-term study of the enchytraeid (Obgochaeta) fauna of a mixed coniferous forest and the effects of urea fertilization, Oikos. 32 (1979) 318-327.
[19] D. Chardez, Observation d'un annelé oligochète prédateur de Thécamobiens, Revue Verviétoise d'Histoire Naturelle (1992) 57-59.
[20] W.A.M. Didden, Ecology of terrestrial Enchytraeidae, Pedobiologia 37 (1993) 2-29.
[21] M.M. Coûteaux, Relationships between testate amoebae and fungi in humus microcosms, Soil Biol. Biochem. 17 (1985) 339-345.
[22] D. Gilbert, C. Amblard, G. Bourdier, A-J. Francez, E.A.D. Mitchell, Le régime alimentaire des thécamoebiens (Protista, Sarcodina). L'Année Biologique 39 (2000) 57-68.
[23] K. Hedlund, A. Augustsson, Effects of enchytraeid grazing on fungal growth and respiration, Soil Biol. Biochem. 27 (1995) 905-909.
[24] C.A. Sutton, D.M. Wilkinson, The effects of Rhododendron on testate amoebae communities in woodland soils in north west England, Acta. Protozool. 46 (2007) 333-338.
[25] M. Vohník, Z. Burdíková, J. Albrechtová, M. Vosátka, Testate amoebae (Arcellinda and Euglyphida) vs. ericoid mycorrhizal and DSE fungi: A possible novel interaction in the mycorrhizosphere of ericaceous plants? Microb. Ecol. 57 (2009) 203-214.
[26] E.A.D. Mitchell, D. Gilbert, A. Buttler, C. Amblard, P. Grosvernier, J.-M. Gobat, Structure of microbial communities in *Sphagnum* peatlands and effect of atmospheric carbon dioxide enrichment, Microb. Ecol. 46 (2003) 187-199.
[27] J. Mikola, G.W. Yeates, D.A. Wardle, G.M. Barker, K.I. Bonner, Response of soil food-web structure to defoliation of different plant species combinations in an experimental grassland community, Soil Biol. Biochem. 33 (2001) 205-214.

[28] R.D. Bardgett, K.F. Chan, Experimental evidence that soil fauna enhance nutrient mineralization and plant nutrient uptake in montane grassland ecosystems, Soil Biol. Biochem. 31 (1999) 1007-1014.
[29] S.J.M. Caporn, W. Song, D.J. Read, J.A. Lee, The effect of repeated nitrogen fertilization on mycorrhizal infection in heather [*Calluna vulgaris* (L.) Hull], New Phytol. 129 (1995) 605-609.
[30] J.A. Carroll, S.J.M. Caporn, L. Cawley, D.J. Read, C.A. Lee, The effect of increased deposition of atmospheric nitrogen on *Calluna vulgaris* in upland Britain, New Phytol. 141 (1999) 423-431.
[31] A. Cunha, S.A. Power, M.R. Ashmore, P.R.S. Green, B.J. Haworth, R. Bobbink, Whole ecosystem nitrogen manipulation: An updated review, JNCC Report No. 331, Joint Nature Conservancy Council, Peterborough, 2002.
[32] M.G. Pilkington, S.J.M. Caporn, J.A. Carroll, N. Cresswell, J.A. Lee, T.W. Ashden, S.A. Brittain, B. Reynolds, B.A. Emmett, Effects of increased deposition of atmospheric nitrogen on an upland moor: Leaching of N species and soil solution chemistry, Environ. Pollut.135 (2005) 29-40.
[33] RoTAP, Review of Transboundary Air Pollution, Department for Environment Food and Rural Affairs, London, in press.
[34] J. Rodwell, British Plant Communities Volume 2 - Mires and heaths, Cambridge University Press, 1991.
[35] M.G. Pilkington, S.J.M. Caporn, J.A. Carroll, N. Cresswell, G.K. Phoenix, J.A. Lee, B.A. Emmett, T. Sparks, Impacts of burning and increased nitrogen deposition on nitrogen pools and leaching in an upland moor, J. Ecol. 95 (2007) 1195-1207.
[36] D. Hendon, D.J. Charman, The preparation of testate amoebae (Protozoa: Rhizopoda) samples from peat, Holocene. 7 (1997) 199-205.
[37] K. Tolonen, B.G. Warner, H. Vasander, Ecology of testaceans (Protozoa: Rhizopoda) in mires in southern Finland: II. Multivariate analysis, Archiv. Protistenk. 144 (1994) 97-112.
[38] J. Stockmarr Tablets with spores used in absolute pollen analysis, Pollen et Spores 13 (1971) 615-621.
[39] R.J. Payne, The standard preparation method for testate amoebae leads to selective loss of the smallest shells, Quaternary Newsletter 119 (2009) 16-20.
[40] R.J. Payne, E.A.D. Mitchell, How many is enough? Determining optimal count totals for ecological and palaeoecological studies of testate amoebae, J. Paleolimnol. 42 (2009) 483-495.
[41] D.J. Charman, D. Hendon, W. Woodland, The identification of testate amoebae (Protozoa:Rhizopoda) in peats, Quaternary Research Association, Technical Guide Series, Cambridge, 2000.

[42] K. J. Clarke, *Guide to Identification of Soil Protozoa - Testate Amoebae*. Freshwater Biological Association, 2003.
[43] C.G. Ogden, R.H. Hedley, An atlas of freshwater testate amoebae. Oxford University Press, 1980.
[44] F. B. O'Connor, Extraction of enchytraeid worms from a coniferous forest soil, Nature 175 (1955) 815-816.
[45] C.O. Nielsen, B. Christensen, The Enchytraeidae. Critical revision and taxonomy of European species. Natura Jutlandica 8 (1959) 1-54.
[46] C.J. Stevens, L.C. Maskell, S.M. Smart, S.J.M. Caporn, N.B. Dise, D.J. Gowing, Identifying indicators of atmospheric nitrogen deposition impacts in acid grasslands, Biol. Conserv. 142 (2009) 2069-2075.
[47] J.R. Bray, J.T. Curtis, An ordination of the upland forest communities of southern Wisconsin, Ecol. Monogr. 27 (1957) 325-349.
[48] D.P. Faith, P. Minchin, L. Belbin, Compositional dissimilarity as a robust measure of ecological distance, Vegetatio 69 (1987) 57-68.
[49] K.R. Clarke, P.J. Somerfield, M.G. Chapman, On resemblance measures for ecological studies, including taxonomic dissimilarities and a zero-adjusted Bray-Curtis coefficient for denuded assemblages, J. Exp. Mar. Biol. Ecol. 330 (2006) 55-80.
[50] K.R. Clarke, Non-parametric multivariate analyses of changes in community structure, Aust. J. Ecol. 18 (1993) 117-143.
[51] Ø. Hammer, D.A.T. Harper, P.D. Ryan, PAST: Paleontological Statistics Software Package for Education and Data Analysis. Palaeontologia Electronica 4 (2001).
[52] F. Charriere, N. Pavillon, T. Colomb, C. Depeursinge, T.J. Heger, E.A.D. Mitchell, P. Marquet, B. Rappaz, Living specimen tomography by digital holographic microscopy: morphometry of testate amoeba. Opt. Express. 14 (2006) 7005-7013.
[53] H. Smith, The Signy Island terrestrial reference sites: III. Population ecology of *Corythion dubium* (Rhizopoda: Testacida) in Site 1, Brit. Antarct. Surv. B. 33&34 (1973) 123-135.
[54] D. Johnson, J.R. Leake, J.A. Lee, C.D. Campbell, Changes in soil microbial biomass and microbial activities in response to 7 years simulated pollutant nitrogen deposition on a heathland and two grasslands, Environ. Pollut. 103 (1998) 239-250.
[55] R.J. Payne, K. Kishaba, J.J. Blackford, E.A.D. Mitchell, The ecology of testate amoebae in southcentral Alaskan peatlands: Building transfer function models for palaeoenvironmental inference, Holocene. 16 (2006) 403-414.

[56] J.A., Springet, J. E. Brittain, B. P. Springet, Vertical movement of Enchytraeidae (Oligochaeta) in moorland soils, Oikos 21(1970) 16 - 21.
[57] V. Huhta, Response of *Cognettia sphagnetorum* (Enchytraeidae) to manipulation of pH and nutrient status in coniferous forest soil, Pedobiologia 27 (1984) 245-260.
[58] M. Prendergast-Miller, V. Standen, I.D. Leith, L.J. Sheppard, Response of enchytraeid worm populations to different forms of nitrogen (ammonia, ammonium, and nitrate) deposition, Soil Organisms 81 (2009) 225-236.
[59] M. Prendergast-Miller, L. Cole, V. Standen, R. Rees, J. Parker, I. Leith, L. Sheppard (2008) Are enchytraeid worms (Oligochaeta) sensitive indicators of ammonia-N impacts on an ombrotrophic bog? Eur J. Soil Biol. 44 (2008) 101-108.
[60] M.G. Pilkington, S.J.M. Caporn, J.A. Carroll, N. Cresswell, J.A. Lee, B.A. Emmett, R. Bagchi, Phosphorus supply influences heathland responses to atmospheric nitrogen deposition, Environ. Pollut. 148 (2007) 191-200.
[61] L.J. Sheppard, I.D. Leith, A. Crossley, N. van Dijk, J.N. Cape, D. Fowler, M.A. Sutton, Long-term cumulative exposure exacerbates the effects of atmospheric ammonia on an ombrotrophic bog: Implications for critical levels, in: M.A. Sutton, S. Reis, S.M.H. Baker (Eds.), Atmospheric Ammonia: Detecting emission changes and environmental impacts, Springer, 2009, pp. 49-50.
[62] J.A. Lee, S.J.M. Caporn, D.J. Read, Effects of increasing nitrogen deposition and acidification on heathlands, in: T. Schneider (Ed.) Acidification research, evaluation and policy applications, Elsevier, Amsterdam, 1992, pp. 97-106.
[63] J.A. Lee, S.J.M. Caporn, Ecological effects of atmospheric reactive nitrogen deposition on semi-natural terrestrial ecosystems, New Phytol. 139 (1998) 127-134.
[64] J.L. Edmondson, J.A. Carroll, E.A.C. Price, S.J.M. Caporn, Bio-indicators of nitrogen pollution in heather moorland, Sci. Total Environ. 480 (2010) 6202-6209.

Figures and Tables

Fig. 1. Box plot showing relative abundance of *Corythion dubium* in 120N (120 kg N ha^{-1} yr^{-1}) treated and control plots of Ruabon experiment. Box-plots show median (central line), first and third quartiles (grey box), tenth and ninetieth percentiles ('whiskers') and fifth and ninety-fifth percentiles (dots).

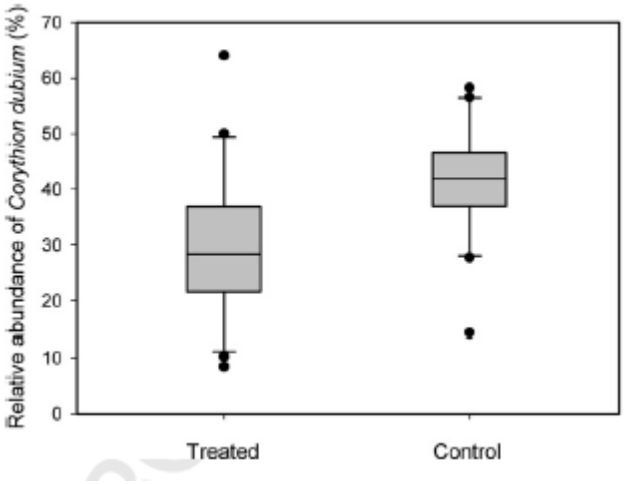


Fig. 2. NMDS ordination plot based on Bray-Curtis dissimilarity (stress=0.2) for testate amoeba relative abundance data from 120N (120 kg N ha^{-1} yr^{-1}) treated and control plots is autumn 2009.

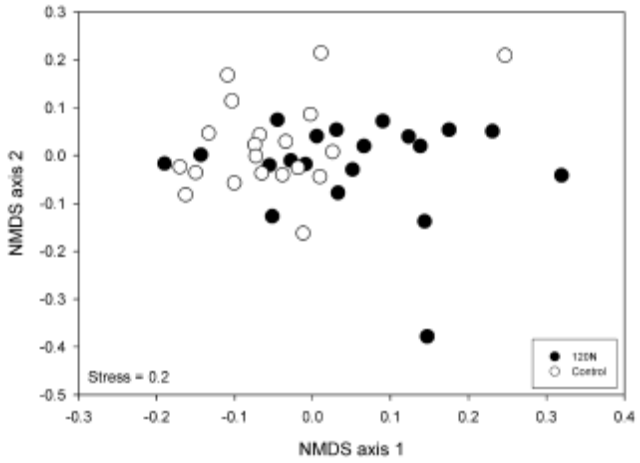
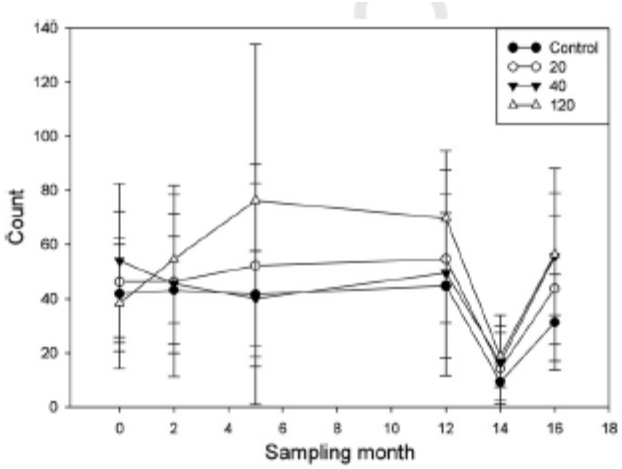
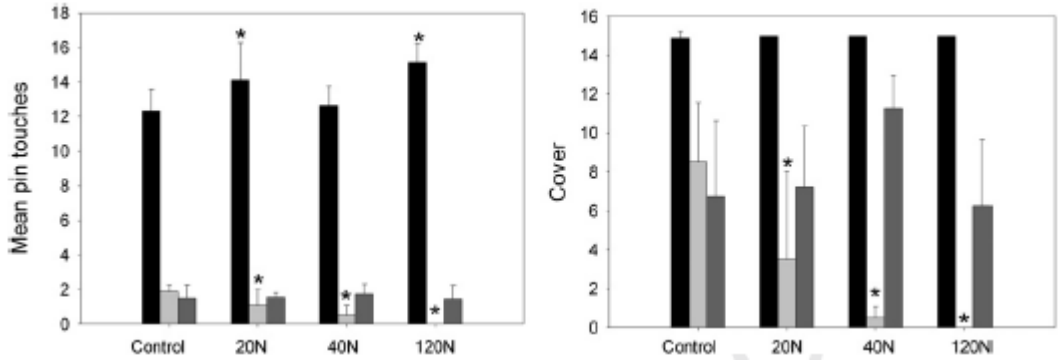


Fig. 3. Numbers of the enchytraeid *Cognettia sphagnetorum* from Ruabon experimental plots over a 16 month period between May 2002 and September 2003. Results shown as mean numbers per core (0.001m^2) and standard deviations.



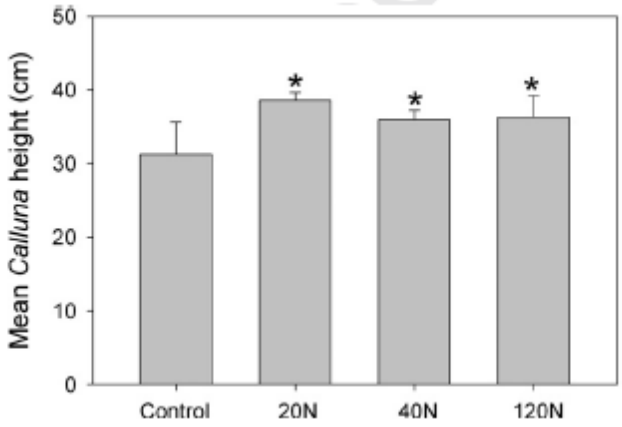
471

472 **Fig. 4.** Average pin touches and total cover values (1-15 scale) for *Calluna vulgaris* (black bar),
473 bryophytes (light grey bar) and *Vaccinium myrtillus* (dark grey bar) in Ruabon plots in summer 2005.
474 Results shown as plot means and standard deviations. Significant differences between treatments for
475 bryophyte touches (P=0.003) and cover (P<0.001), and *Calluna* touches (P=0.02) but not for *Calluna*
476 cover and *Vaccinium* cover or touches (P>0.05). Bars marked '*' show significant difference from
477 controls in post-hoc testing.



478

479 **Fig. 5.** Mean *Calluna* height for experimental plots in summer 2005 showing 1σ error bars of plot
480 means. Significant difference between treatments (P=0.009), bars marked '*' show significant difference
481 from controls in post-hoc testing.



482

483 **Table 1.** Previous studies of plant response in Ruabon experiments. Showing only properties considered
484 to have value for ecological indication with minimal resources (i.e. excluding properties requiring
485 repeated site visits and chemical and physiological parameters).

Reference	Period	Plots	Response
[29,62]	1992	Old	Increased canopy height.
[63]	1995	Old	Increased canopy height. Increased *C. vulgaris* cover. Reduced bryophyte and lichen cover.
[30]	1995-1996	Old	Increased canopy height. Increased *C. vulgaris* cover. Reduced bryophyte and lichen cover.
[60]	1998-2002	New	Increased bryophyte cover, non-significant decrease in lichen cover (with 20 kg N ha^{-1} yr^{-1}).
[64]	2005	New	Decreased bryophyte cover. Decreased bryophyte diversity (Shannon 'H).
This study	2005	Old	Decreased bryophyte cover. Increased canopy height.

486

487

Table 2. Testate amoeba community composition in control and ammonium nitrate treated plots from Ruabon, North Wales. Showing, mean concentration and relative abundance of all tests of major taxa (>5% total tests) in four replicates of treated and control plots. Standard deviations shown in parentheses. Differences between the treated and control plots tested using nested-ANOVA *P<0.05, **P<0.01.

Taxon	Control		Treated	
	Mean concentration total tests (tests cm^{-3})	Relative abundance total tests (%)	Mean concentration total tests (tests cm^{-3})	Relative abundance total tests (%)
Assulina muscorum	2462 (2202)	9.9 (5.7)	4247 (5713)	14.2 (7.9)*
Corythion dubium	11085 (8390)	41.8 (10.4)	7414 (4185)	30.3 (13.0)**
Cryptodifflugia oviformis	4057 (5680)	9.8 (8.7)	1870 (1760)	7.4 (5.0)
Cyclopyxis eurystoma	2188 (3350)	5.3 (4.6)	2001 (2240)	6.6 (3.6)
Euglypha rotunda type	1372 (1056)	5.9 (3.7)	2919 (4046)	9.7 (6.1)
Nebela tincta	2313 (1955)	8.0 (4.0)	2521 (2276)	8.4 (5.2)
Trinema lineare	1910 (2783)	4.8 (4.4)	1736 (2101)	5.3 (5.9)