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

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11 ABSTRACTS

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

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

25 1. INTRODUCTION

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

33            Heathlands are a UK Biodiversity Action Plan priority habitat, covering over 2,000,000 ha of  
34 upland Britain but in England and Wales their cover declined by an estimated 27% between 1947 and  
35 1980 [6]. A critical load range of 10-20 kg N ha<sup>-1</sup> yr<sup>-1</sup> is exceeded in many heathland areas of the British  
36 Isles with N deposition shown to reduce plant biodiversity, particularly marked by a loss of lichens and  
37 bryophytes [7]. Large-scale ecological surveillance data shows a reduction in plant species richness along  
38 the N deposition gradient even when accounting for other drivers [4]. Impacts of nitrogen on groups of  
39 heathland organisms other than plants are however poorly documented. Here we examine the response  
40 of plants and major groups of eukaryotic microorganisms and mesofauna in the same ecological  
41 experiment and consider the possible inter-relations between these groups. Our study aims to provide a  
42 broader understanding of the ecosystem-wide consequences of nitrogen pollution in heathlands and to  
43 identify possible bioindication approaches.

#### 44 1.1 *The studied groups and their inter-relations*

45            Testate amoebae are a group of eukaryotic microorganisms characterised by a solid shell (test)  
46 which can constitute a very large proportion of microbial biomass in organic soils [8] and are likely to  
47 have an important role in nutrient cycling [9,10]. Testate amoebae have been shown to respond to soil  
48 environmental changes to which other groups are insensitive [11] and have broad feeding preferences  
49 making them good synthesisers of overall microbial community change. Previous studies have  
50 demonstrated testate amoeba sensitivity to nutrient enrichment [12, 13, 14] and have suggested  
51 impacts from NO<sub>2</sub> exposure [15].

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

56 might predate testate amoebae given their size and observations of predation by other groups of worms  
57 [19 cited in 10]. Bacteria feeding on enchytraeid faeces are likely to provide a food supply for some  
58 testate amoebae and enchytraeid burrowing may aerate soil, modifying the amoeba's habitat and  
59 translocating individuals [cf. 20]. Enchytraeids may compete with testate amoeba species for food, for  
60 instance with members of the Centropyxidae for fungi [21, 22, 23].

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

## 69 2. SITE and METHODS

70 Experiments were first established on wet upland heath near Ruabon, Clwyd, North Wales (53°  
71 02'N, 3°08'W; 470m asl) in 1989 and have been extensively discussed in previous publications [29, 30,  
72 31, 32, 33]. The climate of the site is cool and oceanic: average annual air temperature is 9.8°C (2008-9  
73 data), average annual soil temperature 6.9°C (2008-9 data) and average annual precipitation 1053mm  
74 (2007-2009 data). Vegetation of the site is dominated by *Calluna vulgaris* with subordinate bryophytes  
75 and scattered *Vaccinium myrtillus*. The site is representative of the *Calluna*-dominated heaths (NVC type  
76 H12: *C. vulgaris*-*V. myrtillus* heath [34]) which cover large areas of upland Britain. Soil is silty clay loam  
77 with pH around 4.4 and depth of around 50cm. Ambient nitrogen deposition is around 19.9 kg N ha<sup>-1</sup> yr<sup>-1</sup>  
78 <sup>1</sup> (UK Air Pollution Information System (APIS) [www.apis.ac.uk](http://www.apis.ac.uk)), at the upper limit of the critical load  
79 range (10-20 kg N ha<sup>-1</sup> yr<sup>-1</sup>). The original experiments consisted of 1x1m plots which were established in  
80 May 1989, subsequent experiments with 2x2m plots were established in 1998. Nitrogen as ammonium  
81 nitrate is applied ten times a year to plots at concentrations of 0, 40, 80 and 120 kg N<sub>r</sub> ha<sup>-1</sup> yr<sup>-1</sup> in the  
82 1989- ('old') plots and 0, 10, 20, 40 and 120 kg N<sub>r</sub> ha<sup>-1</sup> yr<sup>-1</sup> in the 1998- ('new') plots with four replicates  
83 for each concentration. The old plots were burned in 2000 in keeping with normal management practise  
84 [35].

85 For testate amoeba analysis samples were extracted from the control and heaviest treated (120  
86 kg N ha<sup>-1</sup> yr<sup>-1</sup>, hereafter termed 120N) of the older (1989) plots in November 2009, more than 20 years  
87 after the onset of the treatment. Approximately 5cm<sup>3</sup> of surface soil with any overlying litter and  
88 bryophytes were removed with a knife, sealed in plastic bags and refrigerated. In the laboratory testate  
89 amoebae were extracted using a method based on the standard methodology [36]. Sub-sample volume  
90 was measured by displacement in deionised water, samples were soaked for c.2 hours and stirred to  
91 disaggregate. The majority of recent testate amoeba studies have been based on relative abundance  
92 data (for ease of application to the palaeoecological record) however this approach may lead to loss of  
93 information [37]. Here we analyse both percentage and concentration data; an exotic *Lycopodium*  
94 *clavatum* inoculum of counted spores was added to samples to allow calculation of concentrations  
95 [38]. Suspensions were sieved at 300µm but were not back-sieved to avoid loss of small taxa [39].  
96 Samples were mounted in glycerol and a count of 100 individuals aimed for [40]. A variety of taxonomic  
97 guides were used [41, 42, 43]; the *Euglypha rotunda*, *Centropyxis aerophila* (= *Centropyxis cassis*) and  
98 *Diffflugis pristis* types follow [41]. Tests with visible cytoplasm (termed 'live individuals') were recorded  
99 separately from empty shells (although it was not possible to distinguish living from simply undecayed  
100 individuals). Taxon-specific biovolumes were calculated based on assumed geometric shapes and  
101 published biometric data and converted to estimated biomass [8, 14].

102 For enchytraeid analysis soil cores (50mm diameter, 50mm depth) were extracted from the 0,  
103 20, 40 and 120 N<sub>r</sub> treatments of the newer (1998) plots between May 2002 and September 2003. Three  
104 replicate cores were taken from each plot at six intervals over this period (May, July and September in  
105 2002 and 2003) giving a total of 216 samples. Enchytraeids were extracted using the wet funnel  
106 technique [44] and identified following Nielsen and Christensen [45].

107  
108 Changes in plant communities of these plots have been extensively considered over more than  
109 20 years (Table 1). Here we focus solely on vascular plant species with bryophytes and lichens identified  
110 to functional types, a simple approach which may have considerable potential as a quick and effective  
111 bioindication strategy [cf. 46]. Our analysis updates the previous results of Carroll et al. [30] more than  
112 a decade after that study. A 15-point pin quadrat was placed in the centre of each of the old plots (4  
113 replicates of 3 treatments + control) in summer 2005, recording all touches in four categories (*Calluna*  
114 *vulgaris*, *Vaccinium myrtillus*, bryophytes and lichens). Lichens were too rare for meaningful data  
115 analysis. *Calluna* canopy height was also measured at each pin point.

116

## 117 2.1 Data analysis

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

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

## 140 3. RESULTS

141 The amoeba community of these plots was predominantly composed of generalist taxa which  
142 are very abundant in soils with heavy dominance by *Corythion dubium* (36% all tests); other major taxa  
143 included *Assulina muscorum* (12%), *Cryptodifflugia oviformis* (8%) and *Nebela tincta* type (8%)(Table 2).  
144 While most common genera were represented to some extent there was a particular predominance of  
145 small taxa with filopodia. There was a significant difference in both Shannon (H) and Simpson (D)  
146 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*, *Cryptodiffugia 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 sanguineus* 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,  $P=0.06$ ).

177 The plant data showed significant differences between treatments for bryophyte total touches  
178 (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  
179 significantly less abundant than in control plots ( $P<0.001$  in post-hoc testing with Tukey's HSD; Fig. 4),  
180 individual treatments were significantly different from each other ( $P<0.01$ ) with the exception of the  
181 40N and 120N treatments which could not be distinguished ( $P>0.05$ ). There were significant differences  
182 between treatments for *Calluna* touches ( $F_{3,280}=4.2$   $P=0.02$ ) with more touches in the 20N and 120N  
183 plots ( $P<0.01$ ) than the controls, but no difference between controls and 40N plots ( $P>0.05$ ) and no  
184 overall trend within the treated plots. There were no differences between treatments for *Calluna* cover  
185 or for *Vaccinium* cover and touches ( $P>0.05$ ). There were differences between treatments for *Calluna*  
186 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  
187 controls.

188

## 189 4. DISCUSSION

### 190 4.1 *Testate amoeba response*

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

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



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

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

240

#### 241 4.2 *Enchytraeid response*

242           The enchytraeid data from plots treated for four years showed a general trend towards higher  
243 abundance in treated plots but this was not statistically significant. The lack of a significant difference  
244 between treatments may be largely explained by the very high spatial and temporal variability in  
245 numbers (Fig. 3). Particularly low numbers were found in the summer of 2003, probably due to the  
246 severe drought of that year perhaps with vertical migration of enchytraeids to below the sampling zone  
247 [56]. It is possible that with more replication a significant effect might have been identified but this was  
248 not feasible without undue disturbance to the plots. The non-significant trend towards higher  
249 enchytraeid abundance in N-treated plots contrasts with severe reductions in some N-addition  
250 experiments in other ecosystems [18, 57, 58]. The lack of a significant change in enchytraeid abundance  
251 here does however parallel that of Prendergast-Miller et al. [59] who found no significant change in  
252 enchytraeids in response to ammonia fumigation. Although we find no strong evidence for impacts of  
253 nitrogen deposition on enchytraeids our results do not rule out such impacts, it is possible that with  
254 longer treatment periods chemical changes in above-ground plant material would increasingly manifest  
255 themselves in changed enchytraeid food quality and therefore changed enchytraeid abundance [59].  
256 Our results do however add to other recent studies in questioning whether enchytraeids could provide a  
257 viable bioindication approach given their primary control by soil moisture conditions and extremely  
258 patchy distribution [59].

259

#### 260 4.3 *Plant response*

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

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

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

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

283

## 284 5. CONCLUSIONS

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

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308 REFERENCES

- 309 [1] J.N. Galloway, A.R. Townsend, J.W. Erisman, M. Bekunda, Z. Cai, J.R. Freney, L.A. Martinelli, S.P.  
310 Seitzinger, M.A. Sutton, Transformation of the nitrogen cycle: recent trends, questions, and potential  
311 solutions, *Science* 320 (2008) 889-892.
- 312 [2] C.J., Stevens, N.B. Dise, J.O. Mountford, D.J., Gowing, Impact of nitrogen deposition on the species  
313 richness of grasslands, *Science* 303 (2004) 1876-1879.
- 314 [3] C.J. Stevens, C. Dupre, E. Dorland, C. Gaudnik, D.J. Gowing, A. Bleeker, M. Diekmann, D. Alard, R.  
315 Bobbink, D. Fowler, E. Corcket, J.O. Mountford, V. Vandvik, P.A. Aarrestad, S. Muller, N.B. Dise, Nitrogen  
316 deposition threatens species richness of grasslands across Europe, *Environ. Pollut.* 158 (2010) 2940–  
317 2945.
- 318 [4] L.C. Maskell, S.M. Smart, J.M. Bullock, K. Thompson, C.J. Stevens, Nitrogen deposition causes  
319 widespread loss of species richness in British habitats, *Glob. Change Biol.* 16 (2010) 671-679.
- 320 [5] C.J. Stevens, K. Thompson, J.P. Grime, C.J. Long, D.J. Gowing, Contribution of acidification and  
321 eutrophication to declines in species richness of calcifuges grasslands along a gradient of atmospheric  
322 nitrogen deposition, *Funct. Ecol.* 24 (2010) 478-484.
- 323 [6] A. Mattock (Ed.), UK Biodiversity Action Plan: Priority habitat descriptions, Joint Nature Conservation  
324 Committee, Peterborough, 2008.
- 325 [7] R. Bobbink, M. Hornung, J.G.M. Roelofs, The effects of air-borne nitrogen pollutants on species  
326 diversity in natural and semi-natural European vegetation, *J. Ecol.* 86 (1998) 717-738.
- 327 [8] D. Gilbert, C. Amblard, G. Bourdier, A. Francez, The microbial loop at the surface of a peatland:  
328 structure, functioning and impact of nutrients inputs, *Microb. Ecol.* 35 (1998) 83-93.
- 329 [9] D.M. Wilkinson, Testate amoebae and nutrient cycling: peering into the black box of soil ecology,  
330 *Trends Ecol. Evol.* 23 (2008) 596-599.
- 331 [10] D.M. Wilkinson, E.A.D. Mitchell, Testate amoebae and nutrient cycling with particular reference to  
332 soils, *Geomicrobiol. J.* 27 (2010) 520-533.
- 333 [11] W. Foissner, Protozoa as bioindicators in agroecosystems, with emphasis on farming practices,  
334 biocides and biodiversity, *Agric. Ecosyst. Environ.* 62 (1997) 93-103.
- 335 [12] H. Berger, W. Foissner, H. Adam, Field experiments on the effects of fertilizers and lime on the soil  
336 microfauna of an alpine pasture, *Pedobiologia.* 29 (1986) 261-272.
- 337 [13] E. Aescht, W. Foissner, Effects of mineral and organic fertilizers on the microfauna in a high-altitude  
338 reforestation trial, *Biol. Fert. Soils.* 13 (2002) 17-24.

- 339 [14] E.A.D. Mitchell, Response of testate amoebae (Protozoa) to N and P fertilization in an Arctic wet  
340 sedge tundra, *Arct. Antarct. Alp. Res.* 36 (2004) 77-82.
- 341 [15] H. Nguyen-Viet, D. Gilbert, D. Bernard, E.A.D. Mitchell, P-M. Badot, Relationship between  
342 atmospheric pollution characterized by NO<sub>2</sub> concentrations and testate amoebae density and diversity,  
343 *Acta Protozool.* 43 (2004) 233-239.
- 344 [16] L. Cole, R.D. Bardgett, P. Ineson, P.J. Hobbs, Enchytraeid worm (Oligochaeta) influences on microbial  
345 community structure, nutrient dynamics and plant growth in blanket peat subjected to warming, *Soil*  
346 *Biol. Biochem.* 34 (2002) 83-92.
- 347 [17] J. Laakso, H. Setälä, Sensitivity of primary production to changes in the architecture of below  
348 ground food webs, *Oikos* 87 (1999) 57-64.
- 349 [18] G. Abrahamsen, W.N. Thompson, Long-term study of the enchytraeid (Oligochaeta) fauna of a  
350 mixed coniferous forest and the effects of urea fertilization, *Oikos*. 32 (1979) 318-327.
- 351 [19] D. Chardez, Observation d'un annelé oligochète prédateur de Thécamoebiens, *Revue Vervétoise*  
352 *d'Histoire Naturelle* (1992) 57-59.
- 353 [20] W.A.M. Didden, Ecology of terrestrial Enchytraeidae, *Pedobiologia* 37 (1993) 2-29.
- 354 [21] M.M. Coûteaux, Relationships between testate amoebae and fungi in humus microcosms, *Soil Biol.*  
355 *Biochem.* 17 (1985) 339-345.
- 356 [22] D. Gilbert, C. Amblard, G. Bourdier, A-J. Francez, E.A.D. Mitchell, Le régime alimentaire des  
357 thécamoebiens (Protista, Sarcodina). *L'Année Biologique* 39 (2000) 57-68.
- 358 [23] K. Hedlund, A. Augustsson, Effects of enchytraeid grazing on fungal growth and respiration, *Soil Biol.*  
359 *Biochem.* 27 (1995) 905-909.
- 360 [24] C.A. Sutton, D.M. Wilkinson, The effects of *Rhododendron* on testate amoebae communities in  
361 woodland soils in north west England, *Acta. Protozool.* 46 (2007) 333-338.
- 362 [25] M. Vohník, Z. Burdíková, J. Albrechtová, M. Vosátka, Testate amoebae (Arcellinda and Euglyphida)  
363 vs. ericoid mycorrhizal and DSE fungi: A possible novel interaction in the mycorrhizosphere of ericaceous  
364 plants? *Microb. Ecol.* 57 (2009) 203-214.
- 365 [26] E.A.D. Mitchell, D. Gilbert, A. Buttler, C. Amblard, P. Grosvernier, J.-M. Gobat, Structure of microbial  
366 communities in *Sphagnum* peatlands and effect of atmospheric carbon dioxide enrichment, *Microb.*  
367 *Ecol.* 46 (2003) 187-199.
- 368 [27] J. Mikola, G.W. Yeates, D.A. Wardle, G.M. Barker, K.I. Bonner, Response of soil food-web structure  
369 to defoliation of different plant species combinations in an experimental grassland community, *Soil Biol.*  
370 *Biochem.* 33 (2001) 205-214.

371 [28] R.D. Bardgett, K.F. Chan, Experimental evidence that soil fauna enhance nutrient mineralization and  
372 plant nutrient uptake in montane grassland ecosystems, *Soil Biol. Biochem.* 31 (1999) 1007-1014.

373 [29] S.J.M. Caporn, W. Song, D.J. Read, J.A. Lee, The effect of repeated nitrogen fertilization on  
374 mycorrhizal infection in heather [*Calluna vulgaris* (L.) Hull], *New Phytol.* 129 (1995) 605-609.

375 [30] J.A. Carroll, S.J.M. Caporn, L. Cawley, D.J. Read, C.A. Lee, The effect of increased deposition of  
376 atmospheric nitrogen on *Calluna vulgaris* in upland Britain, *New Phytol.* 141 (1999) 423-431.

377 [31] A. Cunha, S.A. Power, M.R. Ashmore, P.R.S. Green, B.J. Haworth, R. Bobbink, Whole ecosystem  
378 nitrogen manipulation: An updated review, JNCC Report No. 331, Joint Nature Conservancy Council,  
379 Peterborough, 2002.

380 [32] M.G. Pilkington, S.J.M. Caporn, J.A. Carroll, N. Cresswell, J.A. Lee, T.W. Ashden, S.A. Brittain, B.  
381 Reynolds, B.A. Emmett, Effects of increased deposition of atmospheric nitrogen on an upland moor:  
382 Leaching of N species and soil solution chemistry, *Environ. Pollut.* 135 (2005) 29-40.

383 [33] RoTAP, Review of Transboundary Air Pollution, Department for Environment Food and Rural Affairs,  
384 London, in press.

385 [34] J. Rodwell, *British Plant Communities Volume 2 - Mires and heaths*, Cambridge University Press,  
386 1991.

387 [35] M.G. Pilkington, S.J.M. Caporn, J.A. Carroll, N. Cresswell, G.K. Phoenix, J.A. Lee, B.A. Emmett, T.  
388 Sparks, Impacts of burning and increased nitrogen deposition on nitrogen pools and leaching in an  
389 upland moor, *J. Ecol.* 95 (2007) 1195-1207.

390 [36] D. Hendon, D.J. Charman, The preparation of testate amoebae (Protozoa: Rhizopoda) samples from  
391 peat, *Holocene.* 7 (1997) 199-205.

392 [37] K. Tolonen, B.G. Warner, H. Vasander, Ecology of testaceans (Protozoa: Rhizopoda) in mires in  
393 southern Finland: II. Multivariate analysis, *Archiv. Protistenk.* 144 (1994) 97-112.

394 [38] J. Stockmarr Tablets with spores used in absolute pollen analysis, *Pollen et Spores* 13 (1971) 615-  
395 621.

396 [39] R.J. Payne, The standard preparation method for testate amoebae leads to selective loss of the  
397 smallest shells, *Quaternary Newsletter* 119 (2009) 16-20.

398 [40] R.J. Payne, E.A.D. Mitchell, How many is enough? Determining optimal count totals for ecological  
399 and palaeoecological studies of testate amoebae, *J. Paleolimnol.* 42 (2009) 483-495.

400 [41] D.J. Charman, D. Hendon, W. Woodland, The identification of testate amoebae  
401 (Protozoa:Rhizopoda) in peats, Quaternary Research Association, Technical Guide Series, Cambridge,  
402 2000.

403 [42] K. J. Clarke, *Guide to Identification of Soil Protozoa - Testate Amoebae*. Freshwater Biological  
404 Association, 2003.

405 [43] C.G. Ogden, R.H. Hedley, *An atlas of freshwater testate amoebae*. Oxford University Press, 1980.

406 [44] F. B. O'Connor, Extraction of enchytraeid worms from a coniferous forest soil, *Nature* 175 (1955)  
407 815-816.

408 [45] C.O. Nielsen, B. Christensen, The Enchytraeidae. Critical revision and taxonomy of European species.  
409 *Natura Jutlandica* 8 (1959) 1-54.

410 [46] C.J. Stevens, L.C. Maskell, S.M. Smart, S.J.M. Caporn, N.B. Dise, D.J. Gowing, Identifying indicators of  
411 atmospheric nitrogen deposition impacts in acid grasslands, *Biol. Conserv.* 142 (2009) 2069-2075.

412 [47] J.R. Bray, J.T. Curtis, An ordination of the upland forest communities of southern Wisconsin, *Ecol.*  
413 *Monogr.* 27 (1957) 325-349.

414 [48] D.P. Faith, P. Minchin, L. Belbin, Compositional dissimilarity as a robust measure of ecological  
415 distance, *Vegetatio* 69 (1987) 57-68.

416 [49] K.R. Clarke, P.J. Somerfield, M.G. Chapman, On resemblance measures for ecological studies,  
417 including taxonomic dissimilarities and a zero-adjusted Bray-Curtis coefficient for denuded assemblages,  
418 *J. Exp. Mar. Biol. Ecol.* 330 (2006) 55-80.

419 [50] K.R. Clarke, Non-parametric multivariate analyses of changes in community structure, *Aust. J. Ecol.*  
420 18 (1993) 117-143.

421 [51] Ø. Hammer, D.A.T. Harper, P.D. Ryan, PAST: Paleontological Statistics Software Package for  
422 Education and Data Analysis. *Palaeontologia Electronica* 4 (2001).

423 [52] F. Charriere, N. Pavillon, T. Colomb, C. Depeursinge, T.J. Heger, E.A.D. Mitchell, P. Marquet, B.  
424 Rappaz, Living specimen tomography by digital holographic microscopy: morphometry of testate  
425 amoeba. *Opt. Express.* 14 (2006) 7005-7013.

426 [53] H. Smith, The Signy Island terrestrial reference sites: III. Population ecology of *Corythion dubium*  
427 (Rhizopoda: Testacida) in Site 1, *Brit. Antarct. Surv. B.* 33&34 (1973) 123-135.

428 [54] D. Johnson, J.R. Leake, J.A. Lee, C.D. Campbell, Changes in soil microbial biomass and microbial  
429 activities in response to 7 years simulated pollutant nitrogen deposition on a heathland and two  
430 grasslands, *Environ. Pollut.* 103 (1998) 239-250.

431 [55] R.J. Payne, K. Kishaba, J.J. Blackford, E.A.D. Mitchell, The ecology of testate amoebae in  
432 southcentral Alaskan peatlands: Building transfer function models for palaeoenvironmental inference,  
433 *Holocene.* 16 (2006) 403-414.



434 [56] J.A., Springet, J. E. Brittain, B. P. Springet, Vertical movement of Enchytraeidae (Oligochaeta) in  
435 moorland soils, *Oikos* 21(1970) 16 - 21.

436 [57] V. Huhta, Response of *Cognettia sphagnetorum* (Enchytraeidae) to manipulation of pH and nutrient  
437 status in coniferous forest soil, *Pedobiologia* 27 (1984) 245-260.

438 [58] M. Prendergast-Miller, V. Standen, I.D. Leith, L.J. Sheppard, Response of enchytraeid worm  
439 populations to different forms of nitrogen (ammonia, ammonium, and nitrate) deposition, *Soil*  
440 *Organisms* 81 (2009) 225-236.

441 [59] M. Prendergast-Miller, L. Cole, V. Standen, R. Rees, J. Parker, I. Leith, L. Sheppard (2008) Are  
442 enchytraeid worms (Oligochaeta) sensitive indicators of ammonia-N impacts on an ombrotrophic bog?  
443 *Eur J. Soil Biol.* 44 (2008) 101-108.

444 [60] M.G. Pilkington, S.J.M. Caporn, J.A. Carroll, N. Cresswell, J.A. Lee, B.A. Emmett, R. Bagchi,  
445 Phosphorus supply influences heathland responses to atmospheric nitrogen deposition, *Environ. Pollut.*  
446 148 (2007) 191-200.

447 [61] L.J. Sheppard, I.D. Leith, A. Crossley, N. van Dijk, J.N. Cape, D. Fowler, M.A. Sutton, Long-term  
448 cumulative exposure exacerbates the effects of atmospheric ammonia on an ombrotrophic bog:  
449 Implications for critical levels, in: M.A. Sutton, S. Reis, S.M.H. Baker (Eds.), *Atmospheric Ammonia:*  
450 *Detecting emission changes and environmental impacts*, Springer, 2009, pp. 49-50.

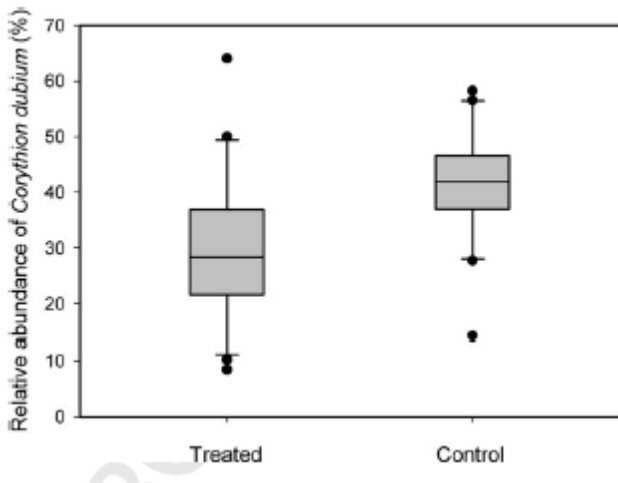
451 [62] J.A. Lee, S.J.M. Caporn, D.J. Read, Effects of increasing nitrogen deposition and acidification on  
452 heathlands, in: T. Schneider (Ed.) *Acidification research, evaluation and policy applications*, Elsevier,  
453 Amsterdam, 1992, pp. 97-106.

454 [63] J.A. Lee, S.J.M. Caporn, Ecological effects of atmospheric reactive nitrogen deposition on semi-  
455 natural terrestrial ecosystems, *New Phytol.* 139 (1998) 127-134.

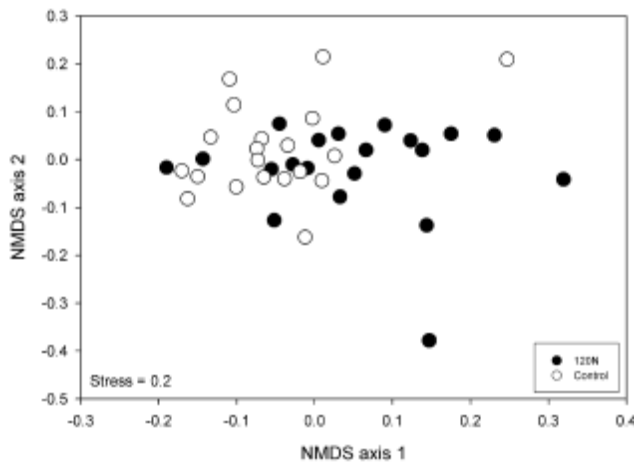
456 [64] J.L. Edmondson, J.A. Carroll, E.A.C. Price, S.J.M. Caporn, Bio-indicators of nitrogen pollution in  
457 heather moorland, *Sci. Total Environ.* 480 (2010) 6202-6209.

458

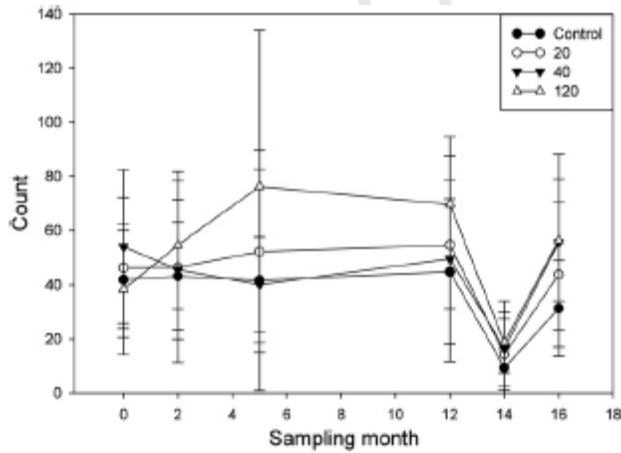
460 **Fig. 1.** Box plot showing relative abundance of *Corythion dubium* in 120N (120 kg N ha<sup>-1</sup> yr<sup>-1</sup>) treated and  
461 control plots of Ruabon experiment. Box-plots show median (central line), first and third quartiles (grey  
462 box), tenth and ninetieth percentiles ('whiskers')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



463  
464 **Fig. 2.** NMDS ordination plot based on Bray-Curtis dissimilarity (stress=0.2) for testate amoeba relative  
465 abundance data from 120N (120 kg N ha<sup>-1</sup> yr<sup>-1</sup>) treated and control plots is autumn 2009.

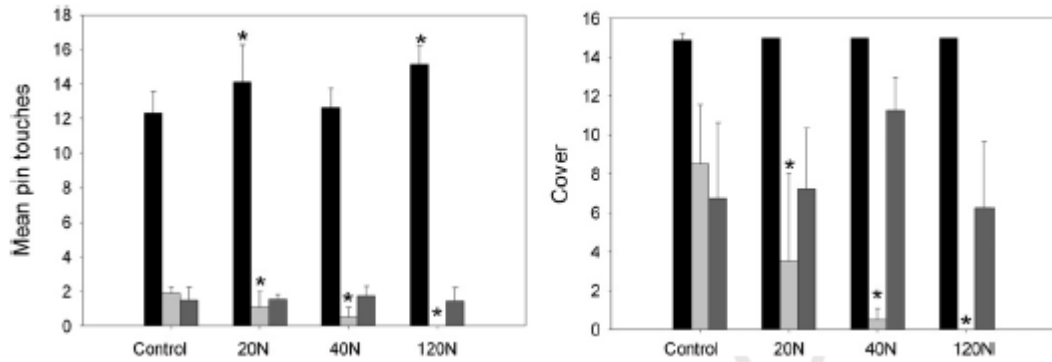


466  
467 **Fig. 3.** Numbers of the enchytraeid *Cognettia sphagnetorum* from Ruabon experimental plots over a 16  
468 month period between May 2002 and September 2003. Results shown as mean numbers per core  
469 (0.001m<sup>2</sup>) and standard deviations.



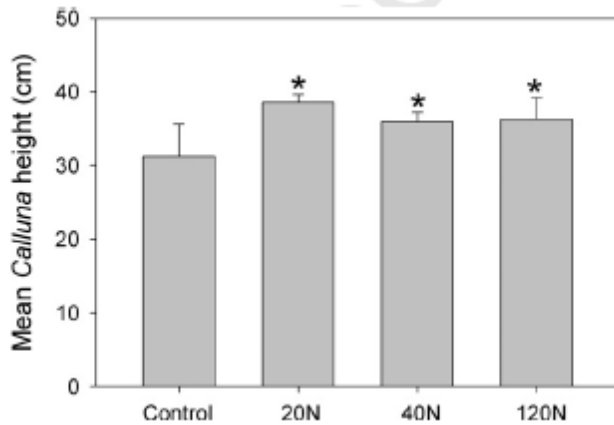
471

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  $1\sigma$  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 <i>C. vulgaris</i> cover. Reduced bryophyte and lichen cover.
[30]	1995- 1996	Old	Increased canopy height. Increased <i>C. vulgaris</i> cover. Reduced bryophyte and lichen cover.
[60]	1998- 2002	New	Increased bryophyte cover, non-significant decrease in lichen cover (with 20 kg N ha <sup>-1</sup> yr <sup>-1</sup> ).
[64]	2005	New	Decreased bryophyte cover. Decreased bryophyte diversity (Shannon 'H).
This study	2005	Old	Decreased bryophyte cover. Increased canopy height.

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489 **Table 2.** Testate amoeba community composition in control and ammonium nitrate treated plots from  
 490 Ruabon, North Wales. Showing, mean concentration and relative abundance of all tests of major taxa  
 491 (>5% total tests) in four replicates of treated and control plots. Standard deviations shown in  
 492 parentheses. Differences between the treated and control plots tested using nested-ANOVA \*P<0.05,  
 493 \*\*P<0.01.

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

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