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Testate amoebae as functionally significant bioindicators in forest-to-bog restoration

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ABSTRACT

In north-west Europe, large areas of open peatland have been drained and planted with spatially homogenous stands of non-native conifers. The detrimental impact of afforestation on peatland carbon and biodiversity have led to large-scale attempts to restore these landscapes back to their open, tree-less form. The responses of dominant microbial consumers - testate amoebae - to peatland forest-to-bog restoration are largely unexplored. We studied changes in testate amoebae with forest-to-bog restoration in the largest expanse of blanket bog in the UK and compared testate amoeba communities in relatively undisturbed open bog with forested and forest-to-bog restoration sites. Forested areas contained testate amoeba communities which were functionally different from open bog, characterised by a lack of mixotrophic taxa known to contribute to primary production. Seventeen years after restoration management, the microbial communities in the forest-to-bog sites remained more similar to forested areas than to the open bog community. Our results suggest that afforestation has reduced the trophic level of testate amoeba communities, which are only beginning to recover post-restoration in the wettest areas where *Sphagnum* has re-colonized. This study also highlights the need to consider a wide-range of reference sites to encompass the natural variability within ombrotrophic blanket bog. We conclude that testate amoebae have the potential to act as functionally-significant bio-indicators in peatlands undergoing forest-to-bog restoration.

Keywords: peatland, blanket bog, afforestation, restoration, protist, testate amoebae

Introduction

Afforestation is one of the most widespread vegetation changes currently occurring in peatlands worldwide (Lachance et al., 2005). While large areas of the world's peatlands are naturally forested, many areas of naturally tree-less peatlands are being drained for forestry. In north-west Europe much of this afforested peatland is blanket bog. Blanket bogs are predominantly tree-less peatland ecosystems mostly occurring in temperate, hyperoceanic regions (Gallego-Sala and Prentice, 2012; Lindsay et al., 1988; Moore 2002). These geographically restricted ecosystems store globally-significant quantities of carbon (Gorham 1991) and are important for biodiversity by providing habitat for unique assemblages of species adapted to the wet and acidic conditions (Bonn et al., 2016). Like many peatlands, blanket bogs have been widely exploited for fuel, agriculture and forestry threatening biodiversity and carbon storage (IUCN, 2014).

The British Isles hold a significant proportion (~20%) of the global blanket bog resource (Tallis, 1998). Between the 1940s and 1980s almost 20% of UK blanket bogs were planted with non-native conifers

(Stroud et al., 1987). Afforestation involved drainage by ploughing and ditch-cutting and planting with the conifers Lodgepole pine (*Pinus contorta)* and Sitka spruce (*Picea sitchenisis)* (Anderson et al., 2000). This afforestation of deep peat was essentially prohibited in the 1990s primarily due to concerns about impacts on birds (Stroud et al., 1987). For instance, losses of key conservation-priority species such as the European Golden Plover (*Pluvialis apricaria*) have been associated with edge effects surrounding forestry plantations (Wilson et al., 2014). More recently the impact of drainage on peatland carbon stock has also become an increasing concern. It is likely that water table drawdown following afforestation may promote peat mineralisation leading to carbon losses to the atmosphere and watercourses (Lindsay, 2010).

It is now widely recognised that peatlands in relatively good condition deliver a range of benefits to society, including: climate mitigation, flood prevention, provision of fresh water, support of biodiversity, as historic archives and for recreation opportunities (IUCN 2014). Land use changes such as the afforestation of blanket bogs have the potential to erode these benefits, with significant costs to society (Bonn et al., 2014). Large investments are now being made in peatland restoration with some of the most intensive schemes focused on the restoration of afforested blanket bog back to a more natural tree-less state. Forest-to-bog restoration typically involves tree-felling and ditch-blocking to raise the water table, with more recent trials also including whole tree harvest and furrow blocking.

Impacts of peatland afforestation on macroscopic organisms are relatively well-known with birds and plants particularly well-studied (Stroud et al., 1997; 1998; Lachance et al., 2005; Wilson et al., 2014). However impacts below-ground have seldom been explored. Many studies emphasize the need to integrate microbial communities in the evaluation of restoration in peatlands yet, our understanding of responses to these often large and rapid land-use changes is still fragmentary (Andersen et al., 2013a; Andersen et al., 2013b; ; Elliott et al., 2015; Swindles et al., 2016; Nwaishi et al., 2016). This is surprising given that peatland restoration is often justified in terms of carbon storage and biodiversity. Microorganisms constitute both the vast majority of species in peatlands and play crucial roles in carbon and nutrient cycling. Reduction of carbon losses can only be achieved if the imbalance between higher net primary productivity (NPP) by plants and lower decomposition by microbial communities can be restored (Andersen et al., 2013).

Testate amoebae form a polyphyletic group of unicellular eukaryotes exhibiting a shell (test), traditionally placed in the phylum Rhizopoda (Margulis and Chapman, 2009) now split between three major unrelated groups (Kosakyan et al., 2016). As microbial consumers considered to feed on bacteria, fungi, microalgae, ciliates, rotifers and nematodes (Yeates and Foissner, 1995; Gilbert et al., 1998; Jassey et al., 2013a) they are a key element in the functioning of peatland ecosystems playing an important role in carbon and nutrient cycling (Wilkinson and Mitchell, 2010; Rydin and Jeglum, 2013; Jassey et al., 2013b). These protists are widely-used as sensitive indicators of peatland surface moisture conditions and have been the focus of contemporary (Koenig et al., 2015; Sullivan and Booth, 2011; Lamentowicz and Mitchell, 2005; Lamentowicz and Obremska, 2010) and palaeoenvironmental studies (Booth 2002; Payne et al., 2006; Charman et al., 2007). While testate amoebae have been used as peatland restoration indicators in several previous studies (Buttler et al., 1996; Jauhiainen 2002; Davis and Wilkinson, 2004; Vickery 2006; Laggoun-Défarge et al., 2008; Valentine et al., 2012), we are aware of only one study which evaluates the use of testate amoebae as bio-indicators following blanket bog restoration (Swindles et al., 2016), and none which consider forest-to-bog restoration.

Functional traits (FT's) in testate amoebae have potential as restoration indicators (Fournier et al., 2012) and indicators of past environmental change in peatlands (Fournier et al., 2015). Recently, traits such as mixotrophy and aperture position/size have been reported to be potentially useful proxies of disturbance in *Sphagnum* peatlands (Marcisz et al., 2016). Recent research investigating FT's in naturally forested peatlands suggests afforestation could lower the trophic level of testate amoeba communities and reduce the contribution of mixotrophic taxa to primary production (Payne et al., 2016). Jassey et al, (2015d) highlight the potential magnitude of such effects on peatland carbon dynamics. However the functional response of testate amoebae to afforestation is not known, nor whether these changes are reversible.

The aims of this study were 1) to evaluate how testate amoebae respond to forest-to-bog restoration and 2) to assess whether they could be used as microbial indicators of disturbance and recovery. We used a multi-site approach to encompass the natural variability in blanket bog at the landscape scale, and within the blanket bog between different micro-topographic features. The following hypotheses were tested: **[H1].** There are distinct testate amoeba communities associated with afforested bog, open-bog and forest-to-bog restoration sites. **[H2].** Peatland restoration has shifted testate amoeba community composition back towards that of undisturbed open-bog habitat. **[H3]**. Testate amoebae are sufficiently specific (i.e. differ between open and afforested) and responsive (i.e. affected by disturbance and restoration) to be used as a bio-indicators in peatlands undergoing forest-to-bog restoration.

Methods

Study sites.

The study was undertaken in the blanket peatlands of Caithness and Sutherland, northern Scotland (the 'Flow Country') (Fig 1). This region comprises the largest single area of blanket bog in the UK (~27% total) and is perhaps the most important expanse of intact blanket bog worldwide (Holden 2005). We selected several sites ranging from 90 – 210 m elevation above sea level across the central area of the Flow Country (58^0 25' N, 3^0 34' W). The mean annual precipitation of this region is around 1196 mm with a minimum annual temperature of 3.6°C and a maximum annual temperature of 11.7°C (Turner et al., 2016).

Four paired afforested and un-afforested ('open') sites were selected along a West-East gradient: Cross Lochs ('cross'), Catanach ('cata'), Braehour ('brae'), and Bad a' Cheo ('bad') (Fig 1). All forested sites were drained, ploughed and planted with Sitka spruce *Picea sitchenisis* and Lodgepole pine *Pinus contorta* from the 1960s to the 1980's, usually at 2 metre spacings (Anderson et al., 2000). In addition to these plantations, we also sampled the forest-to-bog restoration site Talaheel ('tala'). At the restoration site trees were felled, rolled into furrows to slow drainage and main drains were blocked in 1997-1998. This is the oldest forest-to-bog restoration site in our study region and therefore provides a good opportunity to assess long-term restoration trajectories.

In all sites, there was strong micro-topographical variability. In open bog, the vegetation of hummocks consisted of *Sphagnum capillifolium*, lawns of *S. papiliosum* (*S. palustre* at Braehour), and hollows/pools with *S. cuspidatum* (*S. fallax* at Braehour). In the forested and forest-to-bog sites, regularly spaced microforms were created by the planting process: ploughing at a depth of ca 1m created linear “furrows”, the “plough throw” which created ridges on either sides of the furrows, leaving an untouched strip in the middle where the trees were planted, referred to as the ‘original surface’. Forested areas were generally covered with needle litter and non-*Sphagnum* bryophytes such as *Plagiothecium undulatum* and *Hylocomnium splendens*. At the forest-to-bog site the original surface consisted of sparse cushions of *S. capillifolium, Calluna vulgaris* and *Eriophorum sp.* with *S. cuspidatum* in the wetter furrows. The plough throw had *Polytrichum* spp. and *Cladonia* spp. colonising the bare peat along with *Calluna vulgaris, Deschampsia flexuosa* and *Eriophorum spp.*

***Field sampling and laboratory analyses*.**

Samples for testate amoeba and micro-environmental analyses (n = 108) were collected in October 2013. Sampling encompassed the natural variability between sites (n=4) and within sites, i.e. hummocks, lawns and pools in the open sites and plough throw, original surface and furrows in the forested and forest-to-bog sites. In the four paired open/forested sites (Cross, Cata, Brae, Bad) a quadrat (1 x 1 m) was randomly placed within each of the three microforms (hummock/plough throw, lawn/original surface, hollow/furrow) and three subsamples were taken at random within the quadrat. Open sites were sampled at least 100 metres from the forest edge to eliminate unwanted edge effects. We took four replicate sets of samples in the forest-to-bog site (tala), which we considered independent as they were at least 50m apart and separated by a series of ridges, furrows and/or rides (i.e. wider unplanted areas separating forestry blocks). At each sampling location, surface samples of approximately 5 x 5 x 10 cm^3 were collected for testate amoeba analysis and frozen prior to laboratory preparation. Samples for measurement of micro-environmental variables (bulk density, moisture content, loss on ignition, pH and conductivity) were extracted from the same locations as the testate amoeba samples and taken back to the laboratory.

Sample preparation.

Testate amoebae were prepared using a modified version of the method of Booth et al., (2010). For each sample the upper (photosynthetic portion) of each moss was removed and the top 3 – 5 cm was used for preparation (see Mitchell and Gilbert, 2004). To extract testate amoebae the sample was soaked and disaggregated in 50 ml de-ionised water and stirred occasionally. The material was washed through a 250 µm sieve to remove plant and coarse particulate matter and centrifuged at 3000 rpm for three minutes. Amoeba tests were identified and counted at 200 x and 400 x magnification using an Olympus CK2 inverted microscope. Search effort was restricted to 100 individuals per sample following Payne and Mitchell (2009). Morphological identification of testate amoebae was based on the guides of Charman et al., (2000), Clarke, (2003), Ogden and Hedley, (1980) and Mazei and Tsyganov, (2006). We did not differentiate between dead and alive individuals.

Samples for measurement of micro-environmental variables were prepared according to standard methods (Chambers et al., 2010). Bulk density samples were carefully extracted by cutting into the surface vegetation/peat using an open cylinder of known volume (400 ml). Samples were weighed, oven-dried at 105°C and reweighed to determine bulk density and moisture content. Organic matter content was determined by loss on ignition (LOI). Measurements for pH and conductivity were conducted using a Hanna HI991300 multiparameter probe.

***Statistical analysis*.**

We analysed the data for differences between open, forested and forest-to-bog sites using both multivariate community data and a number of indices. To investigate diversity patterns, taxon richness and the Shannon Diversity Index (SDI) were calculated for each sample (Shannon, 1948). In order to detect changes in community functioning two functional indices were calculated: mixotrophy ratio and aperture size. Mixotrophy was determined as the proportion of mixotrophic species within a community (Fournier et al., 2015). Aperture sizes were classified into three size categories: 1 = < 20 µm, 2 = < 40 µm and 3 = > 41µm following Payne et al. (2016). These data were log-transformed to homogenise variance prior to analysis. ANOVA was used to test for differences in diversity and functional indices between open, forested and forest-to-bog sites. We used the Kruskal-Wallis test for data which did not satisfy the assumptions of ANOVA.

Multivariate techniques were used to investigate controls on testate amoeba communities and differences between open bog, forested and forest-to-bog sites. Taxa present in less than ten samples were eliminated from the testate amoeba community dataset in order to reduce the influence of rare taxa. The species abundance data were Hellinger transformed prior to all multivariate analyses (Legendre and Gallagher, 2001). Nonmetric Multidimensional Scaling (NMDS; function *''isoMDS'*) was used to compare testate amoeba assemblages between the three treatments using the Bray-Curtis dissimilarity index. Nested Permutational Multivariate Analysis of Variance (PERMANOVA, function '*adonis'*) was used to determine the significance of treatments, sites and microforms in determining testate amoeba community composition.

In order to test the relationship between testate amoeba communities and environmental variables we used redundancy analysis (RDA) with the testate amoeba dataset as the response variable. The constraining variables were selected based on available environmental variables: vegetation (presence/absence of key taxa: pH, electrical conductivity (EC)), moisture content, bulk density (BD) and organic matter content (OM). The significant association of explanatory variables to the testate amoeba community data was tested using ANOVA (by "axis").

The IndVal approach (Dufrene and Legendre 1997) was adopted to identify indicator species of open bog, forested and forest-to-bog sites. The clusters were categorised by type (open, forested, forest-to-bog). Indicator species for each cluster were identified using the 'indval' function in the package 'labdsv' (Roberts, 2016). For each community, taxa with a p-value = < 0.01 and IndVal > 0.30 were selected as potential indicator species. IndVal analysis was performed on the full testate amoeba dataset.

Statistical analyses were performed with R (R Core Team, 2013) using the packages "vegan" (Oksanen et al., 2012), "MASS" (Venables and Ripley, 2002) and "labdsv" (Roberts, 2016).

Results

The overall testate amoeba community showed high diversity with 66 taxa (supplementary material) identified from 11,599 individuals in the 108 samples. The most abundant taxa in decreasing order of abundance were: *Trinema lineare* (14.2% of all tests), *Corythion dubium* (12.0%), *Archerella flavum* (11.9%), *Nebela tincta* (10.0%), *Assulina muscorum* (6.4*%), Cryptodifflugia oviformis* (4.3%), *Euglypha rotunda/laevis* (4.3%), *Euglypha ciliata* (3.0%), *Euglypha strigosa* (2.4%), *Euglypha tuberculata* type (2.2%), *Hyalosphenia elegans* (2.2%) and *Hyalosphenia papilio* (1.9%). These taxa accounted for a relatively high proportion (> 70%) of the overall assemblage (Figs 2 a – l).

We found significant differences (Kruskal-Wallis test, p = < 0.05) in relative abundance (%) between open, forested and forest-to-bog sites for all taxa with the exception of *C. oviformis* and *E. strigosa*. Certain taxa were present only in certain treatments, sites or microforms. For instance, *Archerella flavum* was completely absent in forested sites and *Bullinaria indica* was only observed in forest-to-bog habitats. We observed *Hyalosphenia elegans* and *Hyalosphenia papillo* in open sites, notably, taxa from this genus were also completely absent in both the forested and forest-to-bog sites.

Taxon richness was significantly higher in open sites than forested and forest-to-bog sites (F = 3.393, p = 0.037). In contrast, diversity (Shannon's index) was significantly lower in open sites (F = 9.304, p = < 0.001) and was highest in forest-to-bog sites (Figs 3a and b). Diversity was influenced by peatland microtopography, for instance, taxon richness was significantly greater in open pools than open hummocks (p = 0.002) and also different between the plough throw and furrows in forest-to-bog sites (p = 0.01). There were no significant differences in mean taxon richness in forested areas (p > 0.05).

The NMDS suggested a clear distinction between forested and undisturbed open sites at the community level (Fig 4). Testate amoeba communities in the forest-to-bog sites appeared intermediate but tended to display more similarity with forested sites compared to open bog. Furrows in forest-to-bog sites clustered closer to drier open bog microhabitats. PERMANOVA showed that community composition was significantly different between open bog, forested and forest-to-bog sites (F=13.227, p = 0.001). The testate amoeba community also differed significantly with microtopography (F=3.746, p = 0.001) and between sites (F=7.820, p = 0.001).

In the redundancy analysis environmental factors accounted for 34% of the variation in the testate amoeba community data. The first axis explained 8.4% of the variation (p = 0.001) and indicated a moisture-acidity gradient with the wetter, least acidic end associated with open bog and the drier, more acidic end mostly associated with the forested and forest-to-bog sites (Fig 5). The second axis explained 2.6% of the variation (p = 0.001) and indicated a bulk density-moisture gradient with higher bulk density mostly associated with the drier forested sites. The environmental variables measured were shown to be significant controls on the testate amoeba community: vegetation (presence/absence of key taxa: *Sphagnum*/non-*Sphagnum* bryophytes) (F = 7.43, p = 0.001), pH (F = 3.51, p = 0.003), conductivity (F = 2.15, p = 0.02), moisture (F = 3.03, p = 0.004) and bulk density (F = 2.22, p = 0.02). No significant association was found for organic matter and all sites were shown to be high in organic matter (Table 1).

Indicator species analysis revealed twelve significant indicator species (Table 2). The highest indicator values (IndVal) were found for *A. flavum* (0.79), *N. tincta* (0.51) and *T. lineare* (0.51). The best indicators of forested areas were *N. tincta, T. lineare* and *Trachelocorythion pulchellum.* In contrast, the best indicators of open bog were *Archerella flavum* (0.79), *Amphitrema wrightianum* (0.44), *Assulina seminulum* (0.33) and *Hyalosphenia elegans* (0.33). The third group indicative of forest-to-bog sites had the largest proportion of indicator species (41.6%) with *C. dubium, E. ciliata, N. tincta f. galeata, N. militaris* and *N. collaris* identified as significant indicators. Although not a significant indicator at our cut-off point (IndVal > 0.30), notably, *Arcella discoides* was shown to be a significant indicator of forested sites (IndVal 0.17, p = 0.02).

The functional trait analysis suggested some functional impacts of the differences in testate amoeba community composition between open bog, forested and forest-to-bog sites (Figs 6a and b). Kruskal-

Wallis test showed mixotrophs were a significantly larger proportion of the community in open undisturbed habitats (χ^2 (2) = 35.233, *p* = < 0.001) compared with forested and forest-to-bog sites. Open bog sites also supported taxa with significantly larger aperture sizes (χ^2 (2) = 26.665, *p* = < 0.001. Mixotrophy ratios and aperture sizes in forested and forest-to-bog sites were not significantly different (χ^2 (2) = 622.0, *p* = 0.770; χ^2 (2) = 38.500, *p* = 0.270).

Discussion

The impact of afforestation.

Our results demonstrate significant effects of commercial forestry practices on testate amoeba communities. Multivariate analysis revealed that whilst there was some variability between individual sampling points, we could accept our first hypothesis, there was a clear distinction in testate amoeba communities between relatively undisturbed open-bog, forested and forest-to-bog habitats.

Functional trait analysis revealed striking differences in testate amoeba communities between forested and undisturbed open bog. Taxa typifying the forested sites were mostly smaller and bacterivorous (e.g. *Trinema lineare; Corythion dubium*) and displayed smaller aperture sizes whereas the open bog microforms contained taxa with significantly larger aperture sizes and frequent mixotrophic taxa (e.g. *Archerella flavum* and *Hyalosphenia papilio*). These mixotrophs were completely absent in the forested sites. This parallels the loss of mixotrophs observed along transects across natural open-to-forested bog ecotones in Russian peatlands (Payne et al., 2016). Our findings suggest that afforestation of blanket bog has led to a total loss of mixotrophic taxa in forested sites with a corresponding loss of mixotrophic primary production. Constraining the magnitude of this loss will require further studies.

It has been known for some time that mixotrophic testate amoebae respond to light conditions (Heal 1964; Schönborn 1965) and it is clear that dense plantations will reduce ground-level light levels. Therefore, it is perhaps not surprising that mixotrophic taxa such as *A. flavum* and *H. papilio* were only found in undisturbed open bog as they cannot survive without photosymbionts and require light to survive. In contrast, *T. lineare* and *N. tincta* were the most dominant taxa in the forested sites and to our knowledge there are no records of photosynthetic endosymbionts in these taxa. Other studies have found taxa such as *T. lineare* and *N. tincta* able to thrive in shaded habitats (e.g. Marcisz et al., 2014). These findings could suggest mixotrophs are less competitive against strict heterotrophs in forested areas with extremely low light levels. In contrast, mixotrophs have the competitive advantage of its algal symbionts in *Sphagnum*-rich open bog when the density of its preferential prey is low (Jassey et al., 2013b). We believe light was the most important limiting factor for mixotrophs in this study, however, we recognise multiple disturbance mechanisms that may be important factors. The response of mixotrophs to drained and drained forested communities may provide further insights into the limiting factors affecting mixotrophic abundance and distribution in disturbed peatlands.

Moisture content is reported to be a significant control on testate amoebae in forested peatlands (Charman and Warner 1992). Our results indicated a moisture-acidity gradient with the wetter, least acidic end associated with open bog and the drier, more acidic end mostly associated with the forested and forest-to-bog sites. We considered moisture content a more appropriate measure of surface wetness than water table depth as the water table in forested areas was > 1 metre below the surface in most locations and was therefore considered independent of surface moisture. Swindles et al. (2016) show that water table was not a significant control on testate amoebae in blanket peatlands under restoration, reporting a poor relationship between water table and surface moisture. Whilst water table depth is widely known to be a significant control on testate amoeba communities in *Sphagnum*-rich open bog (Lamentowicz and Mitchell, 2005; Marcisz et al., 2014), measurements of surface moisture may be more useful in biomonitoring peatlands undergoing forest-to-bog restoration.

We considered the impacts of commercial forestry practises on peatland testate amoebae. These practices include multiple different disturbance mechanisms which encompass: 1) shading by the forest canopy; 2) fertilisation during forest planting; 3) water table drawdown; 4) physical disturbance in ploughing; 5) input of large quantities of conifer needle litter and 6) changes in microbial communities which may interact with testate amoebae, particularly the introduction of conifer root specific mycorrhizas. There may be further secondary impacts (e.g. change in pH) which will arise as a result of these direct impacts or their interactions. As these processes are complex and involve many components of the ecosystem which we have not directly studied we cannot identify the exact processes but believe they are likely to involve more than one mechanism.

The impact of restoration.

Whilst restoration has shifted the testate amoeba community, communities in forest-to-bog sites still more closely resembled forested sites, therefore we rejected our second hypothesis. This suggests that the seventeen year period between restoration and sampling has only seen relatively modest recovery in testate amoeba communities. Studies of microbial community change following peatland forest-to-bog restoration are rare and there has been no previous study of testate amoebae in this context. A study investigating the restoration of microbial processes following restoration of raised bog suggests timescales in excess of ten years are needed for microbial processes to be fully-re-established and resemble near-natural conditions (Andersen et al., 2013). Other studies from degraded blanket bare peat report timescales in excess of twenty-five years for restoration to deliver the desired outcome (Elliott et al., 2015).

There was a clear differentiation in the ecology of indicator species in open bog and forested sites. This perhaps reflected the contrasting abiotic and biotic controls, for instance, moisture conditions and the size and availability of prey (Jassey et al., 2013b). The ecology of forest-to-bog indicators nevertheless showed more similarity with forested indicators with small, bacterivorous generalists such as *Trinema lineare* and *Corythion dubium* identified as powerful indicators of afforestation and restoration sites. Certain taxa from the genus *Nebela* were also prominent indicators of afforestation (e.g. *N. tincta*) and could perhaps reflect the feeding behaviour and availability of food sources. For instance, fungal spores and mycelia have been shown to be important food sources for *Nebela tincta* (Jassey et al., 2013c). We speculate that afforestation and restoration has created a legacy of environmental conditions which has altered the availability of food sources and hence microbial feeding strategies. Other studies have assessed the links between peatland restoration of cutover bogs and *Sphagnum* presence (Buttler et al., 1996; Grosvernier et al., 1997). We argue that one of the main barriers to recovery depends on creating conditions conducive to *Sphagnum* re-establishment, which in turn, will shift the microbial food web towards pre-disturbance conditions.

Recovery of testate amoeba communities will be influenced by the availability of food sources, which, in turn, will be strongly influenced by vegetation composition and density (Sullivan and Booth, 2011; Jassey et al., 2013a). The partial recovery of testate amoebae in this study follows a similar trend to that visible in the vegetation community in Talaheel, which has still not fully recovered to be comparable to nearby reference sites. This is particularly the case on plough throws where conditions are dry (Hancock, M. personal communication). Similarly, it was evident from the multivariate analysis that testate amoeba communities in drier microforms in forest-to-bog sites resembled the forested sites. In contrast, testate amoeba in furrows where *Sphagnum* had established were beginning to show some resemblance to pool microforms in undisturbed open bog, with the exception of the driest site, Braehour. These findings highlight the need for using a wide range of reference sites to encompass natural variability in blanket bog.

Restoration methods in our study region have moved on from felling-to-waste, and more recent technical development mean that the whole tree can be harvested. In some sites including Talaheel, further management is also being undertaken where furrows are being blocked, leading to a step-change in the recovery of the water table which could potentially accelerate re-colonisation by *Sphagnum sp.* Given the range of management practices being trialled, there are now interesting opportunities to further test the potential of testate amoebae as indicator species, and use them in combination with hydrological conditions and vegetation assemblages to assess trajectories of recovery and inform management decisions.

Testate amoebae as bio-indicators in forest-to-bog restoration.

This is the second study to highlight the potential of testate amoebae as bioindicators in restoration of blanket bog and the first in the context of forest-to-bog restoration. Based on the variability between sampling points, we recommend that future sampling designs encompass microtopography. The importance of microtopography was demonstrated in the indicator species analysis which showed *Arcella discoides* to be a potential indicator in forested sites. We observed this taxon most abundantly in wetter furrows compared with open bog microhabitats and in forested sites it was frequently found together with dry indicators *Trachelocorythion pulchellum* and *Trinema lineare*. *A. discoides* is considered a key wet indicator in contemporary (Swindles et al., 2016) and palaeohydrological reconstructions of testate amoebae (Charman et al., 2007). Lamentowicz et al., (2008) propose that samples with a high abundance of both *A. discoides* and taxa indicative of dry conditions, as in the present study, should be interpreted with caution as they indicate highly variable, fluctuating water tables (e.g. temporary flooding of dry peat surfaces), resulting in the mixing of testate amoebae with contrasting wetness requirements.

Compared with groups of macroscopic organisms such as plants and animals, microorganisms have seldom been explored in afforested peatlands or those undergoing forest-to-bog restoration. Other studies recently highlight the potential of testate amoebae in biomonitoring peatlands (Koenig et al., 2015; Swindles et al., 2016). Koenig et al, (2015) demonstrated how ten easily identifiable testate amoeba taxa could be useful in biomonitoring of peatlands. We found all reported 'easily identifiable' taxa in our indicator species analysis with the exception of *Heleopera rosea*, Our findings thus support the idea that testate amoebae could be a good bio-indicator for the Flow Country because key species could be easily identified by practitioners and/or those with little taxonomic expertise in monitoring forest-to-bog restoration. It is promising that our study revealed

90% of the same indicator species published by Koenig et al., (2015), especially as we look to provide peatlands managers with sound tools for following restoration processes.

As well as species themselves, we show that functional traits in testate amoeba are specific and responsive enough to act as bioindicators in peatlands undergoing forest-to-bog restoration. Perhaps the most striking evidence was the complete loss of mixotrophic taxa observed in drier afforested sites compared with the wetter, oligotrophic conditions in open bog sites. Mixotrophy is an important foraging strategy in nutrient poor open bog habitats. Mixotrophs feed on preys through phagocytosis (heterotrophy) and also use their algal symbionts to acquire food and energy through photosynthetic activity (Jassey et al., 2013a; Jassey et al., 2013b; Fournier et al., 2015). Endosymbiotic algae are considered to provide mixotrophs with a competitive advantage over heterotrophic testate amoeba when density of preferential prey is low (Jassey et al., 2013b) and are suggested to influence the competitive hierarchy amongst species through a decrease of the predation pressure of testate amoebae on lower trophic levels (Fournier et al., 2015). In other words, mixotrophs can potentially modify the functioning of the microbial food web by influencing the imbalance between primary productivity and lower decomposition by microbial communities. Thus, mixotrophs may have potential to indicate changes at lower trophic levels (Payne 2013) and as such could be useful bioindicators.

Conclusion

We show that whilst afforestation has significantly changed testate amoeba communities in blanket bog, recovery is at least partially evident seventeen years after restoration management. We suggest that the “threshold” conditions which will lead to the recovery of the testate amoeba community are strongly dependent on the recovery of *Sphagnum*, which in turn, relies on a hydrological regime conducive to saturated conditions. Therefore, in order to recover microbial ecosystem function in blanket bog, it is essential to ensure that conditions are suitable for *Sphagnum* to regenerate, which in turn, may also trigger changes in the microbial communities, such as the recovery of mixotrophic taxa. Modern restoration techniques will provide new and interesting opportunities for future studies investigating the effects of forest-to-bog restoration on different groups of microorganisms. An indicator species approach using species traits may be particularly useful with potential for application in bio-monitoring peatlands under restoration.

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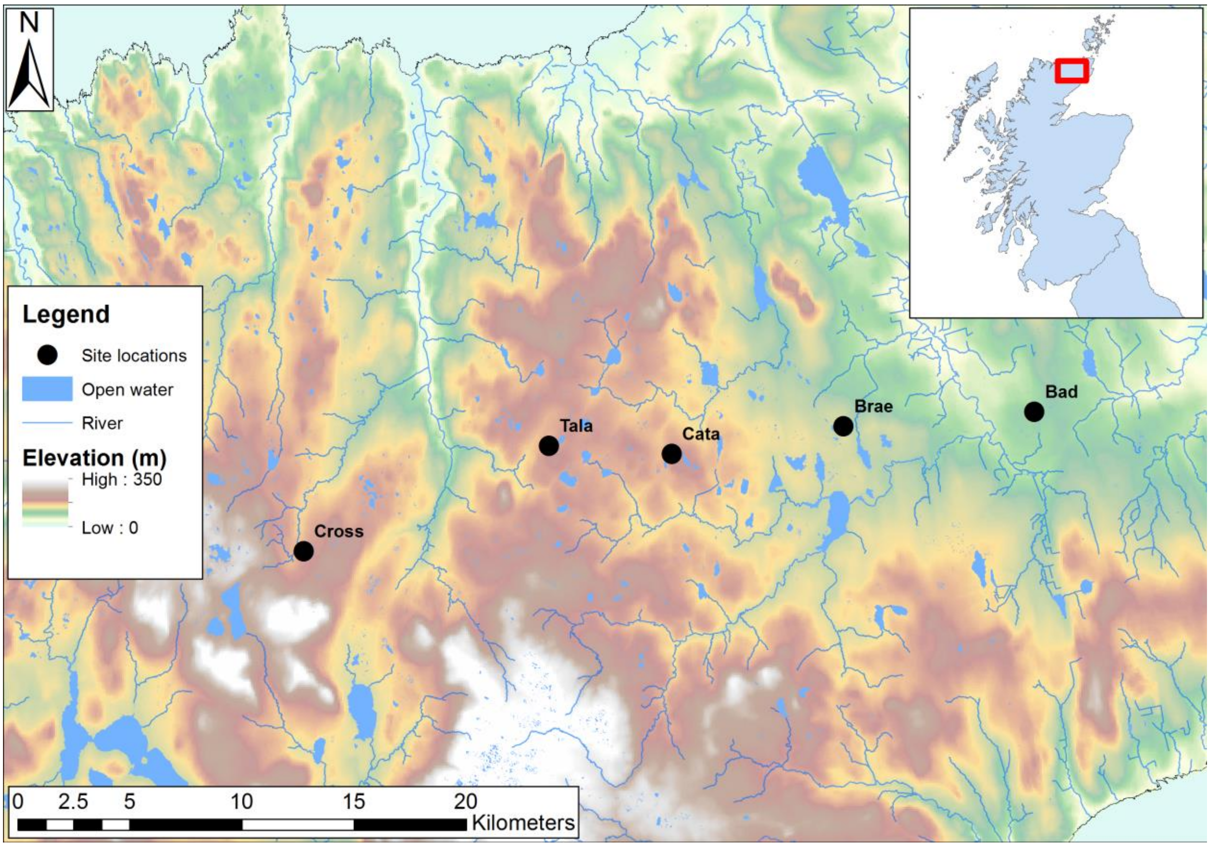
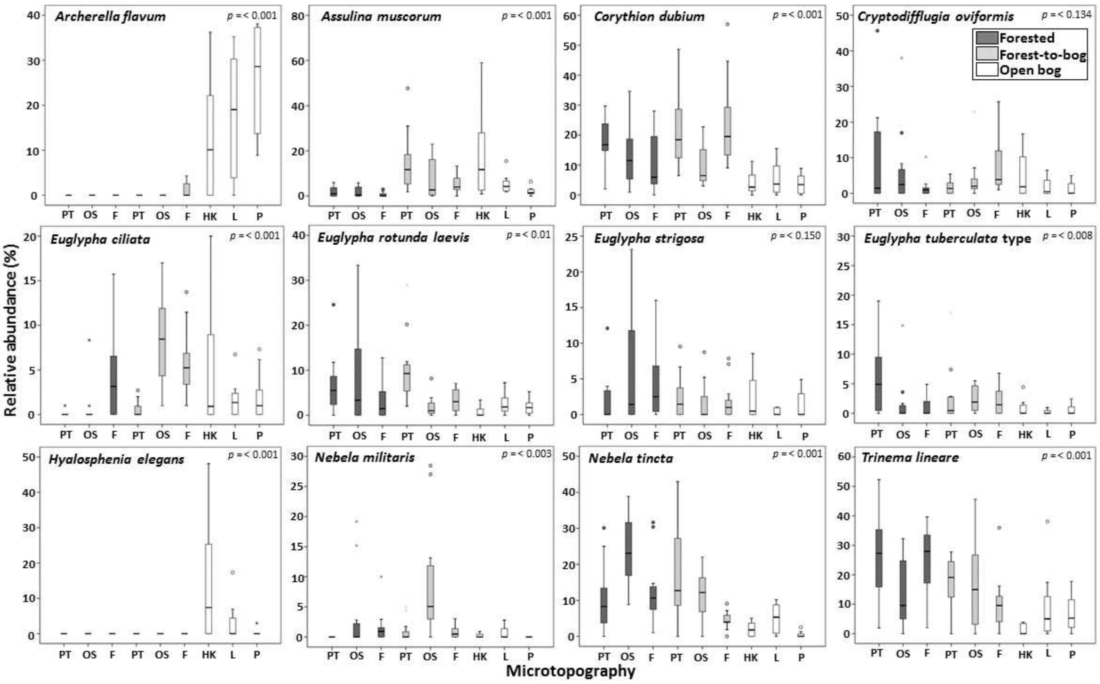
Figure and table captions

Figure 1. Location of sampling sites in this study.



Figures 2 a-l. Boxplots of the relative abundance (%) of dominant testate amoebae taxa in forested, forest-to-bog and open bog (site categories) between different microtopographies (PT = plough throw, OS = original surface, F = furrow, HK = hummock, L = lawn, P = pool). The lower boundary of the box indicates the 25th percentile, the line within the box marks the median and the upper boundary indicates the 75th percentile. The whiskers above and below the box indicates the 10th and 90th percentiles. Significant values represent differences between site categories (Kruskal-Wallis test).



Figures 3a and b. Taxon richness and Shannon Diversity Index between site categories and microtopography. Values are mean with standard errors. Significant values represent differences between sites categories (ANOVA test).

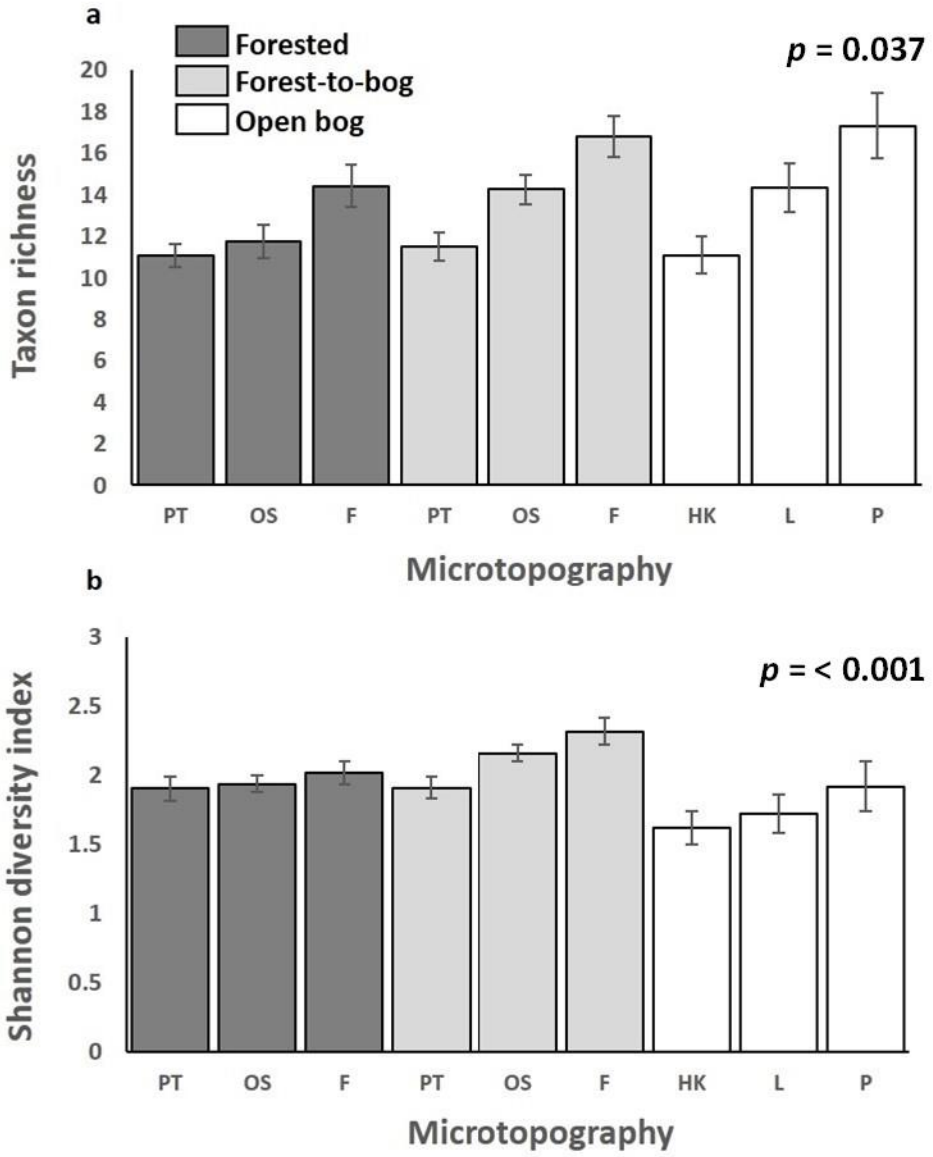
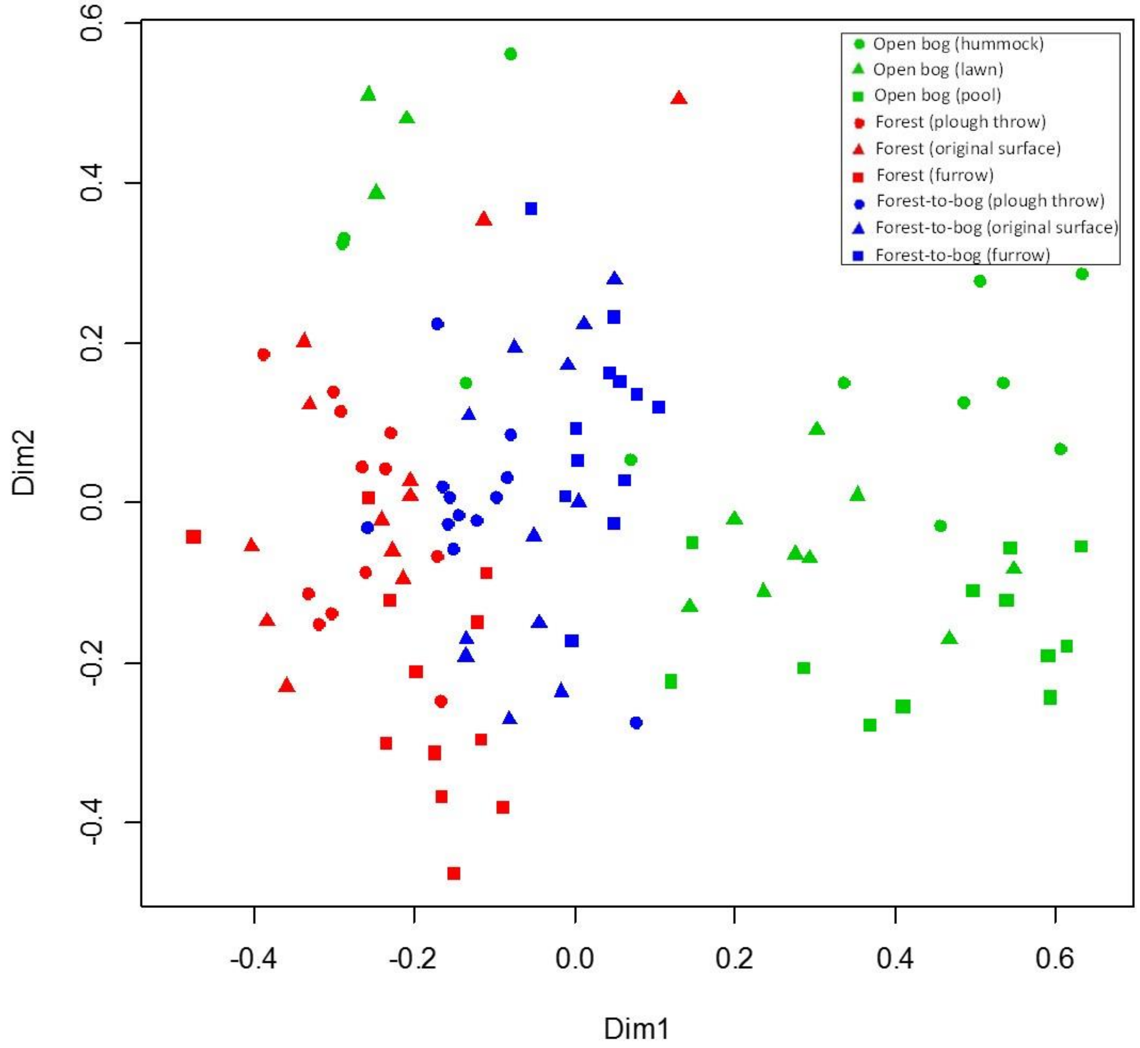
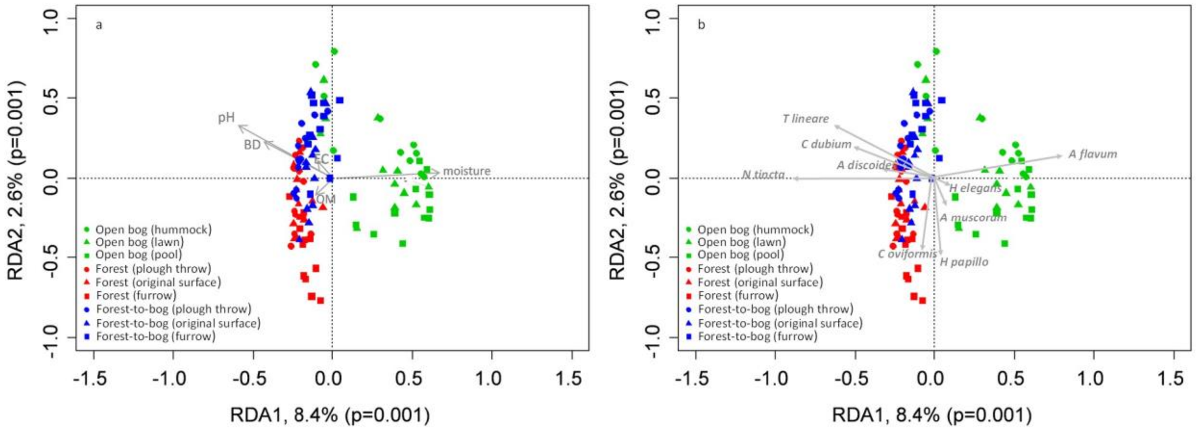


Figure 4. Non-Metric Multidimensional Scaling (NMDS) of Hellinger transformed testate amoeba community dataset.



Figures 5a and b. Redundancy analysis of testate amoeba dataset showing (a) environmental drivers of community change and (b) selected major species.



Figures 6a and b. Functional traits (a) mixotrophy ratio (%) and (b) aperture size class. Aperture sizes were classified into 3 size categories: 1 = < 20 µm, 2 = < 40 µm, 3 = > 41 µm. Values are means with standard errors. Significant values represent differences between site categories (Kruskal-Wallis test).

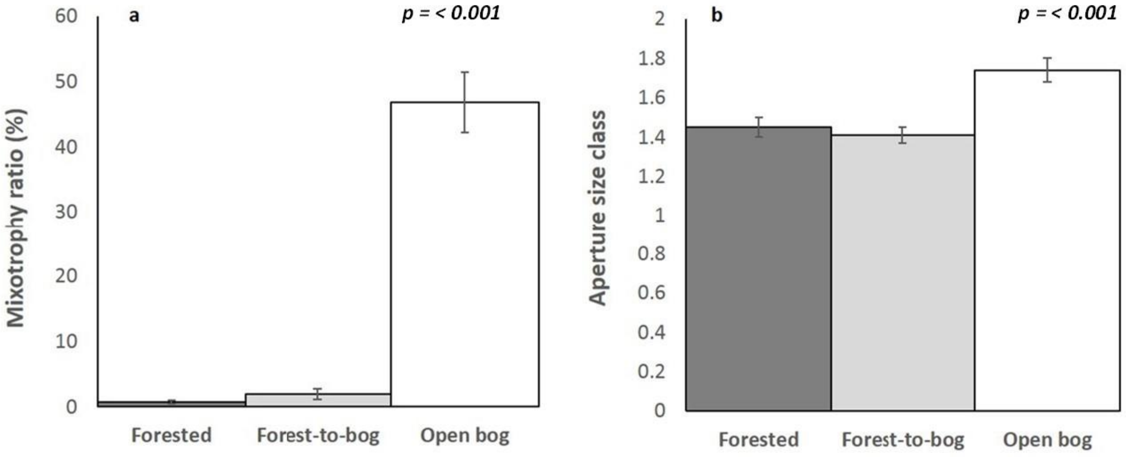
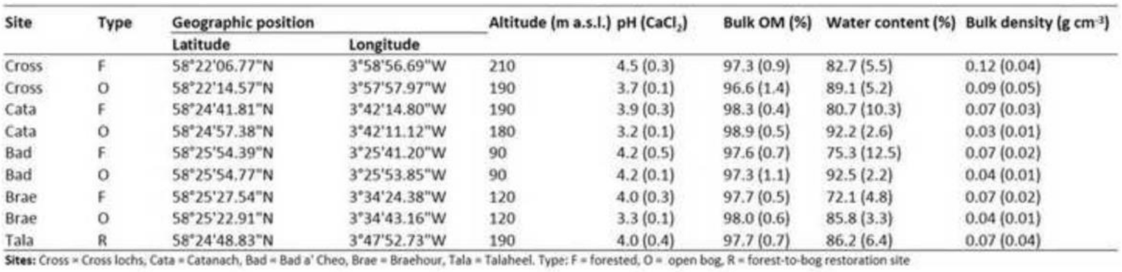


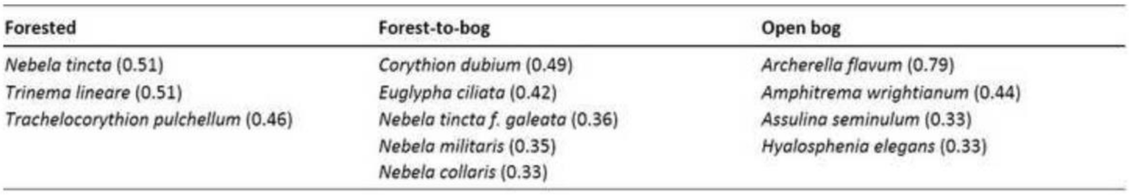
Table 1. Site characteristics, geographic positions and physico-chemical properties. Given are means and standard deviations.



Site	Type	Geographic position		Altitude (m a.s.l.)	pH ($CaCl_2$)	Bulk OM (%)	Water content (%)	Bulk density (g cm^{-3})
		Latitude	Longitude					
Cross	F	58°22'06.77"N	3°58'56.69"W	210	4.5 (0.3)	97.3 (0.9)	82.7 (5.5)	0.12 (0.04)
Cross	O	58°22'14.57"N	3°57'57.97"W	190	3.7 (0.1)	96.6 (1.4)	89.1 (5.2)	0.09 (0.05)
Cata	F	58°24'41.81"N	3°42'14.80"W	190	3.9 (0.3)	98.3 (0.4)	80.7 (10.3)	0.07 (0.03)
Cata	O	58°24'57.38"N	3°42'11.12"W	180	3.2 (0.1)	98.9 (0.5)	92.2 (2.6)	0.03 (0.01)
Bad	F	58°25'54.39"N	3°25'41.20"W	90	4.2 (0.5)	97.6 (0.7)	75.3 (12.5)	0.07 (0.02)
Bad	O	58°25'54.77"N	3°25'53.85"W	90	4.2 (0.1)	97.3 (1.1)	92.5 (2.2)	0.04 (0.01)
Brae	F	58°25'27.54"N	3°34'24.38"W	120	4.0 (0.3)	97.7 (0.5)	72.1 (4.8)	0.07 (0.02)
Brae	O	58°25'22.91"N	3°34'43.16"W	120	3.3 (0.1)	98.0 (0.6)	85.8 (3.3)	0.04 (0.01)
Tala	R	58°24'48.83"N	3°47'52.73"W	190	4.0 (0.4)	97.7 (0.7)	86.2 (6.4)	0.07 (0.04)

Sites: Cross = Cross lochs, Cata = Catanach, Bad = Bad a' Cheo, Brae = Braehour, Tala = Talaheel. Type: F = forested, O = open bog, R = forest-to-bog restoration site

Table 2. Indicator species for 3 clusters characterised by the categorical variables site categories and environment showing taxa with Indval > 0.30, significant at p < 0.01.



Forested	Forest-to-bog	Open bog
Nebela tincta (0.51)	*Corythion dubium* (0.49)	*Archerella flavum* (0.79)
Trinema lineare (0.51)	*Euglypha ciliata* (0.42)	*Amphitrema wrightianum* (0.44)
Trachelocorythion pulchellum (0.46)	*Nebela tincta f. galeata* (0.36)	*Assulina seminulum* (0.33)
	Nebela militaris (0.35)	*Hyalosphenia elegans* (0.33)
	Nebela collaris (0.33)	