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Long-term acidification of pH neutral grasslands affects soil biodiversity, fertility and function in a heathland restoration

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Long-term acidification of pH neutral grasslands affects soil biodiversity, fertility and function in a heathland restoration

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ABSTRACT

In the wider context of heathland restoration, we investigated how field scale experimental acidification with sulphur (sulfur) affected soil biodiversity, fertility and function over a period of 17 years. A field experiment was conducted in the Isle of Purbeck, England, using ferrous sulphate and elemental sulphur as acidifying agents. We tested the effects of acidification on soil fertility, plant communities, litter decomposition, microbiology (including fungi bacteria and actinomycetes), arbuscular and ericoid mycorrhizal colonisation, and soil fauna (including earthworms, nematodes, rotifers and tardigrades). We found that elemental sulphur had a considerable and persistent effect on soil pH, lowering it to levels found in the surrounding reference acid grassland and heathland sites. A newly adapted heathland restoration index based on soil chemistry, found that elemental sulphur was by far the most successful treatment leading to soil conditions similar to the heathlands. Overall, acidification caused a loss of base cations and an increase in toxic aluminium compounds. Consequently the more mesotrophic components of soil biology were reduced by acidification during the course of the experiment. This transformed the soil biological system into one typical of acid grasslands and heathlands. Concomitant litter decomposition was similarly inhibited by acidification, with the microbiota more strongly hindered in acidified soil than the macroscopic fauna. Acidification resulted in a reduction in nematode and rotifer abundance and earthworm biomass. The vegetation community was also strongly modified by the elemental sulphur treatments and, where grazing was restricted, soil acidification allowed a restored heathland community to endure. Arbuscular mycorrhizal colonisation of grasses was reduced where heather plants were established, while ericoid mycorrhizas had developed sufficient populations in the acidified pastures to match the colonisation rate in the native heathlands.

1. Introduction

Soil is a habitat for a huge variety of living organisms contributing enormously to global biodiversity (Orgiazzi et al., 2016a) yet is subject to several threats due to human interventions. A decline in soil biodiversity is the reduction of forms of life inhabiting soil (both in terms of quantity and variety), and this can cause a subsequent deterioration or loss of one or more soil functions. Soil biodiversity does not, however, decline independently of other factors and is usually related to an abiotic deterioration in soil quality, resulting in a reduction in the condition and/or number of biological habitats in the soil that support soil biodiversity.

In general and geographical terms, the state of soil biodiversity has been well described in atlases of soil biodiversity (Jeffery et al., 2010; Orgiazzi et al., 2016b). These atlases attempt to address a fundamental problem with assessing soil biodiversity: if we do not know what is out there, how do we know if it is in decline? Even with these atlases it is challenging to gauge soil biodiversity at national, European and global scales. At some local levels it is clear that soil biodiversity is in decline. For example, soil sealing causes the death of soil biota by cutting off water and carbon and nutrient inputs (Turbé et al., 2010). In this extreme case, most biodiversity is lost. In other cases, soil biodiversity decline has been linked to soil erosion, organic matter depletion, salinization, contamination and compaction (Gardi et al., 2013;

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Montanarella, 2015).

Wherever soil biodiversity decline occurs, it can significantly affect the soils' ability to function normally (e.g. nitrification or litter comminution) and respond to perturbations (Nielsen et al., 2015), such as a drought. The soil biota has an innate capacity to resist change, and has a certain capacity to recover from disturbances. However, a loss of biodiversity reduces the functional opportunities for the biotic community and can lead to a soil with lower resistance to perturbation and reduced capacity to recover subsequently (Allison and Martiny, 2008; Downing et al., 2012).

Soil biodiversity and its functions are influenced by complex interactions of abiotic and biotic factors, on which land-use change and degradation may have a negative effect. Along with other physical and chemical variables, changes in soil pH can have a strong influence on soil communities. Griffiths et al. (2011) analysed > 1000 soil cores across the UK using molecular bacterial community profiling techniques, and found a positive correlation of increasing pH with α-diversity, while β-diversity increased at lower pH. On a larger scale, on a transect across Europe encompassing a range of land-uses as part of the Eco-FINDERS project (da Silva et al., 2016), both in terms of organism and geography, soil pH was the main parameter influencing collembolan richness and communities. Soil pH also differentiated the structure of microbial communities in post-glacial (Tripathi et al., 2018) and arable soils (Rousk et al., 2010a). Such interesting observations on the effect of soil pH on soil biodiversity are surprisingly rare and few papers deal with the impacts of pH changes on soil biodiversity directly. In a search in the Core Collection of Web of Science in February 2018 only one paper on record was returned on a search of soil* & biodiversity & pH in the title field, this was Oldén et al. (2016). While pH is often related to the populations of soil organisms, evidence of its direct effect on soil biodiversity through contrived experimentation is less prevalent. Hence the effect of direct acidification as a threat to soil biodiversity is not well documented despite pH being such a vital controlling (master) variable.

Soil acidification in Europe has been used as a common technique in cases of heathland and acidic grassland restoration (Dunsford et al., 1998; Owen et al., 1999; Owen and Marrs, 2000; Lawson et al., 2004; Tibbett and Diaz, 2005; Diaz et al., 2008; van der Bij et al., 2018). Both habitats develop only on acid soils and have specialised soil biota and ericaceous plant communities. The extent of European lowland heath and acid grasslands has seen a dramatic decline since the 1750's due to the abandonment of traditional agricultural practice, driven by shifting economic circumstance and, more recently, the development of heathland for housing, roads, golf courses and the like. The reversion of improved agricultural land to an acidic grassland and heathland mix has become a conservation priority since the 1980's in the UK, to protect the rare plants and animals it supports. However, the management required to maintain the heathland plagioclimax (i.e. preventing succession to woodland) is often neglected where heathland is present as small fragments, resulting in the continued loss of heathland and biodiversity in the landscapes that remain (Diaz et al., 2013). The remaining fragments of heathland and acid grassland can exhibit an extinction debt (future extinction as a result of fragmentation) for some of the flora and fauna present, as population sizes are already too small (Piessens and Hermy, 2006; Gibson et al., 2013).

The restoration of modern agricultural land to heathland is a far more challenging task than the management of existing heathland fragments. Twentieth century agricultural improvement of podzolic soils often required aggressive physical and chemical interventions; such as liming and fertilizer application, thus transforming a heterogeneous, nutrient poor acidic system into a uniform nutrient enriched circum-neutral soilscape. To restore such land back into nutrient poor acid systems, countermanding and equally aggressive measures are required such as top soil removal or acidification by elemental sulphur (Diaz et al., 2008). Top soil removal can be effective (Allison and Ausden, 2004), but disposal of the removed soil can be costly if adopted for conversion of large areas and archaeological remains can be damaged during removal. Soil pH in experimental plots have previously been shown to respond to sulphur treatment, particularly for elemental sulphur treatment six years after application (Diaz et al., 2008). However, previous analysis of soils from our field sites have considered only superficial (0–4 cm) effects soon after application (Tibbett and Diaz, 2005) or 15 cm depths in 2006 (Diaz et al., 2008) and have been entirely chemical in nature. In such improved pasture soils, acidification has unknown consequences for soil biodiversity and function which require consideration.

Given the paucity of knowledge on the direct effects of soil acidification on soil biodiversity we investigated how experimental acidification using two different measures, of high and low acidification potential, has affected soil biodiversity, fertility and function over a period of up to 17 years. The experimental measures were imposed on improved agricultural pasture land on the Isle of Purbeck in England, with a view to provide treatment options for agricultural reversion to traditional acid grassland and heathland systems. We report here on measurements made and experiments conducted between 2008 and 2017. Changes to the chemical environment for soil organisms and the abundance of a broad range of key biotic groups were assessed. Monitoring response across a wide variety of sizes of soil organisms (from microbes to earthworms) can provide an important indication of the effects of soil acidification on soil biodiversity. We tested the contribution made by different components of the soil biota using biotic-size-partitioning litterbag experiments and examined changes in soil biology at a range of scales including key components of the macro and microbiota and their respective activities. Other variables that may be affected by changes in soil biodiversity and function were also assessed including the effect on soil nutrient availability, mycorrhizas and plant community composition. We investigated the longer term effects of acidification treatments in the context of heathland and acid grassland restoration, specifically testing how the treatments have differentially affected (i) soil chemistry, (ii) vegetation composition, (iii) soil microbiology and litter decomposition, (iv) mycorrhizas and, (v) soil fauna.

2. Materials and methods

2.1. Case study area and monitoring sites

The study site is located near Wareham, Dorset, UK (2040 W, 50390 N) on the Isle of Purbeck, not a true island but a peninsula of ~200 km^2 on the south coast of England. It forms part of the Dorset Area of Outstanding Natural Beauty and directly abuts the neighbouring Hartland Moor National Nature Reserve and Middlebere Heath. It has a mild temperate Atlantic climate with mean annual rainfall of around 777 $mm \cdot y^{-1}$ and an average temperature of around 11 °C. Purbeck is a complex multifunctional, multi-land-use landscape with a range of competing pressures from arable farming, high and low intensity livestock grazing, touristic land use, with quarrying and military areas all sharing the lands' ecosystem services.

The geology of the Isle of Purbeck comprise complex geological deposits, including Tertiary sand, Jurassic limestone and clay. Glacial drift over Mesozoic and Tertiary clay and loam constitute almost 60% of the study area. The predominant soil type is a Tertiary deep sand (Sollon Series, Association 641b; FAO Endogleyic Albic Carbic Podzols) which are generally stone free and naturally acidic. These are humose sandy soils with a bleached subsurface horizon typically affected by groundwater and comprise more than a quarter of the Purbeck Peninsula by land area (NSRI, 2001). The other less common soil type present is similar (Isleham Series, Association 861a: FAO Arenic Mollic Gleysols) which are typically seasonally wet, deep sandy soils with a humose or peaty surface horizon. These gleysols have complex soil patterns with hummock and hollow micro-relief and can be at risk of winter flooding and wind erosion (NSRI, 2001). See Supplemental Material 1 for further details.

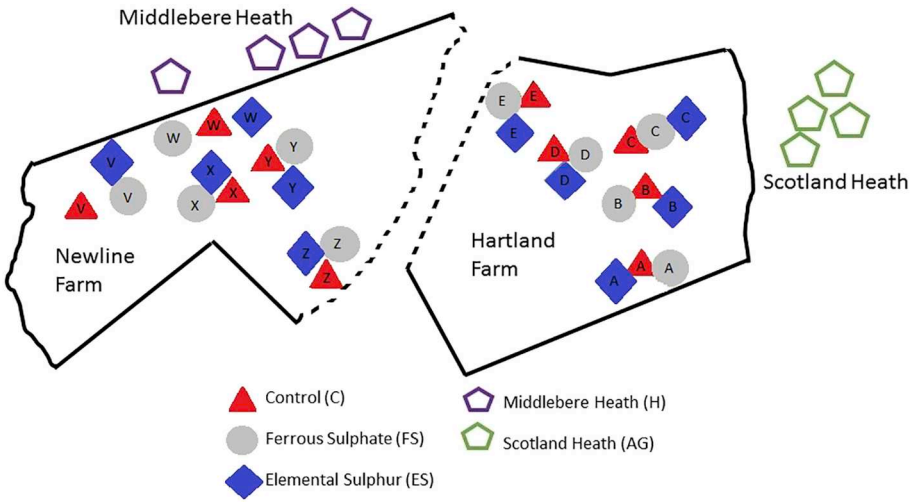

Fig. 1. Schematic diagram of the plot layout across two contiguous farms on the Isle of Purbeck, UK.

2.2. Experimental design and treatments

Experimental plots were established in 1999 to test the effects of pH management on soil and plant variables (Fig. 1). The study area was established on agricultural pasture on two contiguous farms (Newline and Hartland Farms) owned by the National Trust and run for many decades as one entity. The farms were created during the 1950s and 1960s by the gradual improvement of the podzolic heathland soil through the addition of rock phosphate, manure and chalk marl. This increased the pH and nutrient levels of the soil sufficiently to allow the growth of mesotrophic grassland for cattle grazing (Tibbett and Diaz, 2005; Diaz et al., 2008). Across this contiguous farmland, 30 representative plots (50 × 50 m) were selected to be amended with ten replicates of each treatment of either: ferrous sulphate (FS) as Wet Copperas 50™ (19% Fe and 13% S) or elemental sulphur (ES) as Brimestone 90 (90% S), alongside a control plot (C) with no amendment added, organised in block formation. Treatments were applied (surface dressed) at a rate of 2000 kg ha^{-1} in May 2000 with an additional 1600 kg ha^{-1} applied the following year. In order to determine whether amended or control plots move towards the native heathland and acid grassland systems in terms of fertility, biodiversity and function, adjacent reference sites for heathland (H) and acid grassland (AG) were included with four replicates representative of heathland and acid grassland obtained from Middlebere and Scotland Heath (Fig. 1). *Calluna vulgaris* clippings from the adjacent Middlebere Heath, were sown in 2001 and 2003 across all plots but were generally unsuccessful on ferrous sulphate and control plots. For a detailed site description and field experimental methodology, see Green et al. (2007) or Diaz et al. (2008).

2.3. Study overview

A number of studies took place at the field site between 2008 and 2017 that are reported on here. Chronologically, these studies considered: litter decomposition in 2008–2009; soil microbiology in 2009; vegetation and soil chemistry in 2014; earthworm biomass in 2016; and other soil fauna and mycorrhizal colonisation in 2017.

2.4. Soil sampling and chemical analysis (2014)

Soil samples were collected using a gouge auger in November 2014 from 0 to 5, 5–10 and 10–15 cm depths (removing any litter layer if present). Twenty-five soil samples were collected from each plot, following a standard 'W' spatial sampling pattern, and mixed into one composite sample representative of each plot. For each experimental treatment (FS, S, C) ten replicate samples were taken (30 samples), plus 4 from acid grassland and 4 heathland reference sites (38 samples in total).

Composite soil samples were sieved to 2 mm and were air-dried for chemical analysis. Soil pH was measured by 2.5:1 water-soil slurry after shaking for 15 min at 120 rpm (Rowell, 1994). Exchangeable Al^{3+}, Ca^{2+}, Na^{+}, Mg^{2+}, K^{+} and Mn^{2+} and extractable Fe were determined by 1 M ammonium nitrate (NH_4NO_3) (Stuanes et al., 1984), in a 10:1 extractant to soil ratio. The final centrifuged supernatant was filtered and run with Agilent Technologies 5100 inductively coupled plasma optical emission spectrometry (ICP-OES). Soil available P was extracted by a 0.1 M H_2SO_4 solution (Sørensen and Bülow-Olsen, 1994). The sample was centrifuged and the supernatant was analysed by flow injection analysis (FIA). Total C and N in the soils were determined by an elemental analyser (Thermo Fisher Flash EA 1112).

2.5. Vegetation assessment (2014)

In September 2014, elemental sulphur, ferrous sulphate and control plot sward composition was assessed with a 1 × 1 m quadrat. In each 50 m × 50 m experimental plot 12 quadrats were recorded in sets of three randomly selected locations in each quarter in order to assess a representative sample population. Plants species were visually assessed and classified as the mean percentage cover of five functional groups: grasses, forbs, legumes, shrubs and heather.

2.6. Litter decomposition study (2008–2009)

An experimental decomposition study was installed during August 2008 and assessed during August 2009, alongside the microbiological studies below. A subset of plots were selected from the full trial to represent four acidified heather-dominated elemental sulphur plots, four control plots, four native heathland and four acid grassland plots. The criteria for selecting the elemental sulphur plots was having the lowest soil pH, of the 10 available elemental sulphur plots, and best heather establishment consistent with native heathland communities. The control, heathland and acid grassland plots were randomly selected.

Nylon litterbags of 9 cm × 8.5 cm with mesh sizes of 100 µm, 2 mm and 4.7 mm were obtained from a commercial supplier (Northern Mesh and Technical Fabrics, Oldham, UK). The smallest mesh (100 µm) allowed only the soil micro-fauna and flora to access the litter, the medium mesh (2 mm) included the meso-fauna, and the largest mesh size (4.7 mm) also permitted the macro-fauna to access the litter (Bradford et al., 2002). Thus, this method provides an insight into the contribution made by different components of the soil biota. Each litter bag was filled with 0.9–1.1 g of barley straw and sealed. Six replicates of each mesh size were inserted into the soil at random locations of each plot at a depth of 3–5 cm in 2008. The 288 litterbags (16 plots [4 heathland, acid

grassland, elemental sulphur and control treatments] × 3 mesh sizes × 6 litterbag replicates) were retrieved one year later. The litter remaining in each bag was carefully washed to removed adhered soils and roots, dried at 30 °C and then re-weighed to determine percent mass loss.

2.7. Soil microbial community and activity (2009)

Soils from the same elemental sulphur, control, heathland and acid grassland plots, used for the litter bag study, were sampled for microbiological and chemical analysis in June 2009. Twenty-five soil samples (0–15 cm) were taken following the same 'W' pattern as used for chemical analysis (above) for each plot before being bulked providing 16 composite samples. Samples were briefly stored at 4 °C prior to microbial analysis. Remaining soil samples were then air-dried for chemical analysis.

The number of colony forming units (CFUs) of bacteria, Actinomycetes and fungi were determined by a selective viable count procedure. Microorganisms were extracted from 0.2 g of fresh soil by shaking in 20 ml of sterile H_2O for 10 min. Extracts were serially diluted and plated onto selective media. Selective media for bacterial and Actinomycete cultures were prepared according to Yang and Yang (2001). Fungi were cultured on rose Bengal chloramphenicol agar (Oxoid Ltd., Basingstoke, UK). Microorganisms were cultured at 25 °C for 6 days before the number of colonies were counted. Determinations were repeated four times for each plot (four independent measurements) and results expressed as CFU g^{-1} air dried soil.

Soil microbial activity was determined through the hydrolysis of fluorescein diacetate (FDA) to fluorescein using the method described by Adam and Duncan (2001). Briefly, 2 g of fresh soil was added to a flask containing 15 ml of 60 mM potassium phosphate buffer (pH 7.6) and 0.2 ml of 1000 μg ml^{-1} FDA was added. Flasks were incubated at 30 °C in a shaker/incubator for 20 min. Following incubation, hydrolysis of FDA was stopped by the addition of 15 ml chloroform/methanol (2:1 *V*/V). Samples were then centrifuged and filtered to remove soil particles prior to fluorescein release being quantified by measuring absorbance at 490 nm (Varian Cary 50 spectrophotometer). Two blanks were analysed for each soil sample as previously described, but with the omission of FDA solution. Absorbance of the blanks was removed from the samples to control for humic substances remaining after centrifugation/filtering. Results were then calculated as μg fluorescein released g^{-1} soil h^{-1}.

In addition, soil chemical properties were measured including pH in 1:2.5 soil:water (Rowell, 1994), gravimetric soil moisture at 105 °C (Rowell, 1994), total C and N (Thermo Fisher Flash EA 1112) and organic matter through loss on ignition at 450 °C (Rowell, 1994). Available Al, Ca, Mg and P were determined by ICP-OES (Varain Vista Pro) after extraction by 0.01 M $CaCl_2$ (Houba et al., 1996).

2.8. Mycorrhiza sampling (2017)

2.8.1. Arbuscular mycorrhizal colonisation in grass roots

Roots of the perennial grass *Holcus lanatus* L. (Yorkshire Fog) were sampled in June 2017 from plants identified in all experimental plots. Single 10 g root samples (one per plot) were washed thoroughly, from which 4 cm root lengths were randomly sub-sampled. Sub-samples were cleared in KOH solution (10% *w/v*) at 50 °C overnight, and then stained in a 5% (v/v) black ink vinegar solution for 1 h before being washed and transferred to a solution of lactoglycerol (Walker, 2005). Colonisation was scored by the line intercept method, in which the presence of either hyphae, arbuscule or vesicule was considered evidence of mycorrhizal colonisation (Giovannetti and Mosse, 1980).

2.8.2. Ericoid mycorrhizal colonisation in heather roots

Roots of *Calluna vulgaris* and *Erica* spp. were sampled in June 2017 from the heathland and acid grassland plots, which all had heather present, along with elemental sulphur plots where heather plants were present. Staining took place as described and mycorrhizal colonisation enumerated by the appearance of densely stained hyphal coils within the cells.

2.9. Earthworm sampling (2016)

Samples were collected in November 2016. For each plot, one cube of soil 20 × 20 × 20 cm (8000 cm^3) was excavated using a flat shovel and placed in trays in the field for hand sorting. Worms were carefully removed, counted and placed in a subsample of soil to be transported back to the lab for characterisation. Specimens were rinsed, blotted dry and weighed individually and recorded as juvenile or adult.

2.10. Nematode, rotifer and tardigrade sampling (2017)

Soil samples were collected in June 2017 from all experimental plots and heathland and acid grassland reference sites using a gauge auger (2.5 cm diameter, ~0–15 cm deep), with 25 cores taken following the same 'W' pattern as used for chemical analysis and microbiological studies (above). These were combined into single composite samples per plot. Soil samples were stored at 4 °C for processing. Nematodes, rotifers and tardigrades were extracted from ~100 g fresh soil with a modified Baermann funnel technique by substituting extraction trays for funnels, with samples collected after 24 and 72 h. Nematodes, rotifers and tardigrades in the extracts were counted while alive on a Leitz Wilovert inverted microscope at 4× magnification. The two sampling times (24 and 72 h) were counted separately and then combined. Abundance was expressed as the number of individuals per 100 g soil dry weight equivalent (Yeates, 2003; Yeates et al., 1993).

2.11. Statistical analysis

2.11.1. Plant and soil data (2014)

Statistical analyses were performed with the STATISTICA data analysis software system StatSoft, Inc., version 12, unless otherwise stated, with $P < 0.05$ used in all tests. Variables were preliminary checked in order to clarify if variables fulfilled the ANOVA assumptions of independency, normality and homogeneity of variance. The sample size in the control, ferrous sulphate and elemental sulphur plots was $N = 10$ while in acid grassland and heathland plots it was $N = 4$. Variables were checked for normality with the normal probability plot of the residuals and the Shapiro-Wilk W test for normality. Homogeneity of variance was analysed, in order to check if the variances in the different groups were not significantly different, with the Levene and Barlett tests.

When independence, normality and homogeneity of variance were not rejected, a parametric one-way ANOVA was performed where Farm was selected as the random effect and treatment was selected as the fixed effect. A Fisher's LSD post-hoc test was then performed in cases where significant differences were found among treatments.

For variables where there was no independence, a parametric unbalanced one-way ANOVA was performed. When the assumptions of normality and/or homogeneity of variance were not met, a Box-Cox transformation was performed to achieve normality and homogeneity of variances that were no longer significantly different, followed by a parametric one-way ANOVA, as described above. For variables where the assumptions for normality and/or homogeneity of variance were still rejected after the Box-Cox transformation, a non-parametric Kruskal-Wallis ANOVA by ranks test was performed.

2.11.2. Plant data

Acidification treatment effects were analysed in vegetation cover data for grasses, legumes and forbs with the parametric balanced one-way ANOVA and sample size was balanced, $N = 10$. Heather and shrubs variables were analysed with the non-parametric Kruskal-Wallis ANOVA by ranks test. Principal component analysis (PCA) was performed on soil vegetation data.

2.11.3. Soil data

Treatment effect was analysed for soil chemical properties using parametric unbalanced one-way ANOVA, and the sample size was

unbalanced, $N = 4$ and $N = 10$. Depth effect was analysed for the soil chemical properties for the control, ferrous sulphate and elemental sulphur plots with the unbalanced one-way ANOVA mixed model and the sample size was balanced, N = 10. Data obtained from acid grassland and heathland plots was analysed with a parametric one-way ANOVA, with no Farm effect and equal sample size, $N = 4$. Fisher's LSD post-hoc test ($P < 0.05$) was performed afterwards when significant differences were found among depths.

Principal component analysis (PCA) was performed soil data from the plots, based on nine soil chemical properties (pH, Al, Ca, Fe, K, Mg, Mn, Na and P) sampled at three depths (0–5, 5–10 and 10–15 cm). The soil chemical PCA was, in turn, used in the creation of a Heathland Restoration Index (see below).

2.12. Heathland restoration index (2014)

A Heathland Restoration Index (HRI) was generated for each treatment, using chemical data collected in 2014 (described above), using a similar technique to the Soil Quality Index (SQI) outlined by Andrews et al. (2002) and Romaniuk et al. (2011). Creating this single value was thought to be beneficial for communication of the results to local stakeholders, rather than presenting a complicated multivariate dataset. Briefly this used a linear scoring method to compare control, elemental sulphur and ferrous sulphate plots to acid grassland and heathland reference sites for each variable. However, rather than using all 27 variables available (9 chemical properties at 3 depths, see above) this method uses a PCA to generate a minimum data set of indicators (MDS) for the HRI. These MDS indicators were then used in the linear scoring model to generate an HRI. Results of the PCA can be found in the supplementary material (Table S1). Each variable was initially subjected to Kruskal-Wallis one-way ANOVA on ranks. Only variables that showed statistically significant differences between treatments at $p < 0.05$ were then further analysed by PCA (the full list of variables used can be found in supplementary material, Table S1). Principal components (PCs) with eigen values > 1 were included in the generation of the MDS, following established procedure (Andrews et al., 2002 and Romaniuk et al., 2011). PC1–3, accounted for a cumulative variation of 74.9%. For each of these PCs the 'highly weighted factors' were retained for the MDS. These are defined as the variable with the highest absolute eigen vector, and any other within 10% of the highest absolute eigen vector (see supplementary material, Table S1). When there were two or more 'highly weighted factors' retained from a single PC, a Pearson´s product product moment correlation coefficient was computed, to ensure one does not influence the other. If $r^2 > 0.70$, only the variable with the highest Eigen vector was retained for the MDS; if $r^2 < 0.70$, both of them were retained for the MDS. Each variable

Table 1
The effects of sulphurous amendments on soil chemical properties. Mean (SE). Means with different lowercase letters (a, b, c, d) represent a significant difference between treatments (within the same depth) based on a mixed-model ANOVA ($n = 4$ for heathland and acid grassland and $n = 10$ for others). Means with different uppercase letters (A, B, C) represent a significant difference among depths (within the same treatment). Omission of letters meaning no significant difference between treatments or depths.

Soil properties	Depth (cm)	Treatment control	Ferrous sulphate	Elemental sulphur	Acid grassland	Heathland
pH	0–5	5.6 (0.1)a	5.5 (0.1)ab	5.1 (0.2)c	5.1 (0.1)bc	3.9 (0.1)d B
	5–10	5.5 (0.1)a	5.4 (0.1)a	4.7 (0.2)b	5.0 (0.2)ab	4.3 (0.2)b AB
	10–15	5.5 (0.1)a	5.5 (0.1)a	4.8 (0.2)b	5.1 (< 0.1)ab	4.7 (0.1)b A
Al^{3+} (mmol(+) kg^{-1})	0–5	0.4 (0.1)c	0.4 (0.1)c	1.9 (1.0)b	1.0 (0.3)abc	5.6 (0.8)a
	5–10	0.6 (0.2)b	0.4 (0.1)b	3.0 (1.0)a	1.0 (0.4)ab	3.8 (0.9)a
	10–15	1.1 (0.7)b	0.3 (0.1)b	3.6 (0.9)a	1.2 (0.5)ab	3.7 (0.8)a
P (mg kg^{-1})	0–5	8.9 (2.0)bc	30.7 (8.5)a	14.0 (2.8)ab	6.6 (1.6)abc A	3.3 (0.8)c A
	5–10	7.2 (1.7)bc	23.4 (6.5)a	20.0 (6.6)ab	2.8 (0.2)abc B	1.4 (0.1)c B
	10–15	11.1 (4.8)a	19.6 (6.2)a	19.9 (5.9)a	2.0 (0.2)ab B	0.9 (0.1)b B
Ca^{2+} (mmol(+) kg^{-1})	0–5	63.3 (4.9)a	65.6 (9.7)a	32.5 (6.3)b	31.1 (7.8)ab	30.0 (2.6)b A
	5–10	55.3 (4.0)a	63.9 (6.8)a	31.1 (6.1)b	30.1 (5.8)ab	16.3 (4.5)b B
	10–15	56.0 (4.4)a	62.0 (3.6)a	34.8 (6.4)b	25.0 (3.5)a	9.5 (2.9)b B
Mg^{2+} (mmol(+) kg^{-1})	0–5	10.7 (1.2)bc A	11.8 (0.7)b A	8.6 (0.6)c A	12.6 (1.0)b A	25.3 (1.9)a A
	5–10	6.9 (0.7)bc B	8.2 (0.6)b B	6.0 (0.6)c B	10.9 (0.4)ab A	15.4 (2.7)a B
	10–15	5.5 (0.5)b B	5.8 (0.4)b C	4.7 (0.5)b B	7.9 (0.5)ab B	10.6 (1.6)a B
Ca^{2+}: Mg^{2+}	0–5	6.5 (0.8)a C	5.6 (0.7)a C	4.0 (0.9)b B	2.4 (0.4)ab	1.2 (< 0.1)b
	5–10	8.6 (0.9)a B	8.3 (1.2)a B	6.0 (1.6)b AB	2.7 (0.4)abc	1.0 (0.1)c
	10–15	10.8 (1.1)a A	11.1 (1.0)a A	8.1 (1.7)b A	3.2 (0.5)abc	0.8 (0.1)c
K^{+} (mmol(+) kg^{-1})	0–5	1.0 (0.1)b A	1.1 (0.1)b A	0.9 (0.1)b A	1.0 (0.1)b A	2.7 (0.3)a A
	5–10	0.3 (0.1) B	0.3 (0.1) B	0.4 (0.1) B	0.5 (0.1) B	0.6 (0.1) B
	10–15	0.1 (< 0.1) C	0.1 (< 0.1) C	0.3 (0.1) B	0.2 (0.1) B	0.2 (0.1) C
Fe (mg kg^{-1})	0–5	1.4 (0.2)b	2.1 (0.3)a A	3.4 (1.0)a	1.9 (0.4)ab	3.4 (0.6)a
	5–10	1.4 (0.1)b	1.5 (0.2)b AB	4.4 (1.0)a	2.1 (0.8)ab	1.9 (0.4)ab
	10–15	1.5 (0.2)b	1.3 (0.1)b B	4.1 (1.0)a	2.4 (0.9)ab	2.2 (0.9)ab
Mn^{2+} (mmol(+) kg^{-1})	0–5	0.10 (0.02) A	0.13 (0.02) A	0.10 (0.01) A	0.05 (0.01) A	0.05 (< 0.01) A
	5–10	0.04 (0.01)b B	0.06 (0.01)a B	0.04 (0.01)b B	0.02 (< 0.01)b B	0.01 (< 0.01)b B
	10–15	0.04 (0.01)a B	0.05 (0.01)a B	0.05 (0.01)a B	0.01 (< 0.01)b B	0.01 (< 0.01)b B
Total C (%)	0–5	4.1 (0.3)b	4.4 (0.4)b	4.0 (0.3)b	4.2 (0.5)b	10.1 (2.0)a
Total N (%)	0–5	0.3 (< 0.1)a	0.3 (< 0.1)a	0.2 (< 0.1)a	0.2 (< 0.1)a	0.3 (0.1)a
C:N	0–5	16.1 (0.7)bc	15.8 (1.6)c	19.0 (1.9)b	18.3 (0.7)bc	30.3 (1.1)a

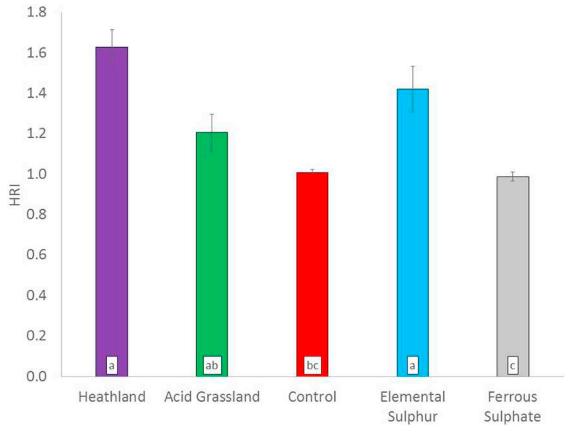

Fig. 2. The effect of sulphurous amendments on Heathland Restoration Index (HRI). Error bars represent the SE of the means. Means with different letters (a,b,c) represent a significant difference between treatments based on one-way ANOVA and Tukey's HSD post hoc test ($p < 0.05$).

selected for the MDS was then transformed using a linear scoring method. For "less is better" variables (native heathland soils had lower values than the control plots) the lowest observed value, that was not an outlier in computed boxplots, was divided by each observation (e.g. the lowest observed value received a score of 1) and for "more is better" variables (native heathland soils had higher values than control plots), each observation was divided by the highest observed value, that was not an outlier (e.g. the highest observed value received a score of 1).

Once scored, each of the PCs with eigen values > 1 (in our case PC 1–3) were weighted as follows (see Supplementary material, Table S1):

$$\textit{Weighted factor} = \frac{\%\text{of the variation explained by PC}}{\text{cumulative \% of the variation explained by all PCs with eigen vectors} > 1}$$

Finally, the scored indicators were used to calculate the HRI as follows. Where S is the score of the indicator variable, and W the weighted factor derived from the PCA:

$$HRI = \sum_{i=1}^{n} W_i S_i$$

2.12.1. Mycorrhizal and faunal analysis

Arbuscular and ericoid mycorrhizal, nematode, rotifer tardigrade and earthworm data were treated in the following manner. When assumptions of homogeneity of variances were rejected, (unbalanced) one-way ANOVA was conducted using Welch's F ratio. The significance of differences between the means of each treatment were assessed using Tukey's HSD post hoc test. Correlations between variables were evaluated using Pearson's product moment correlation coefficient. Where data presented insufficient replication, descriptive statistics were used.

3. Results

3.1. Soil chemistry (2014)

Of the two measures tested, only elemental sulphur was successful in decreasing the pH below the control plots in all three depths, thirteen years after application ceased. This acidification in the elemental

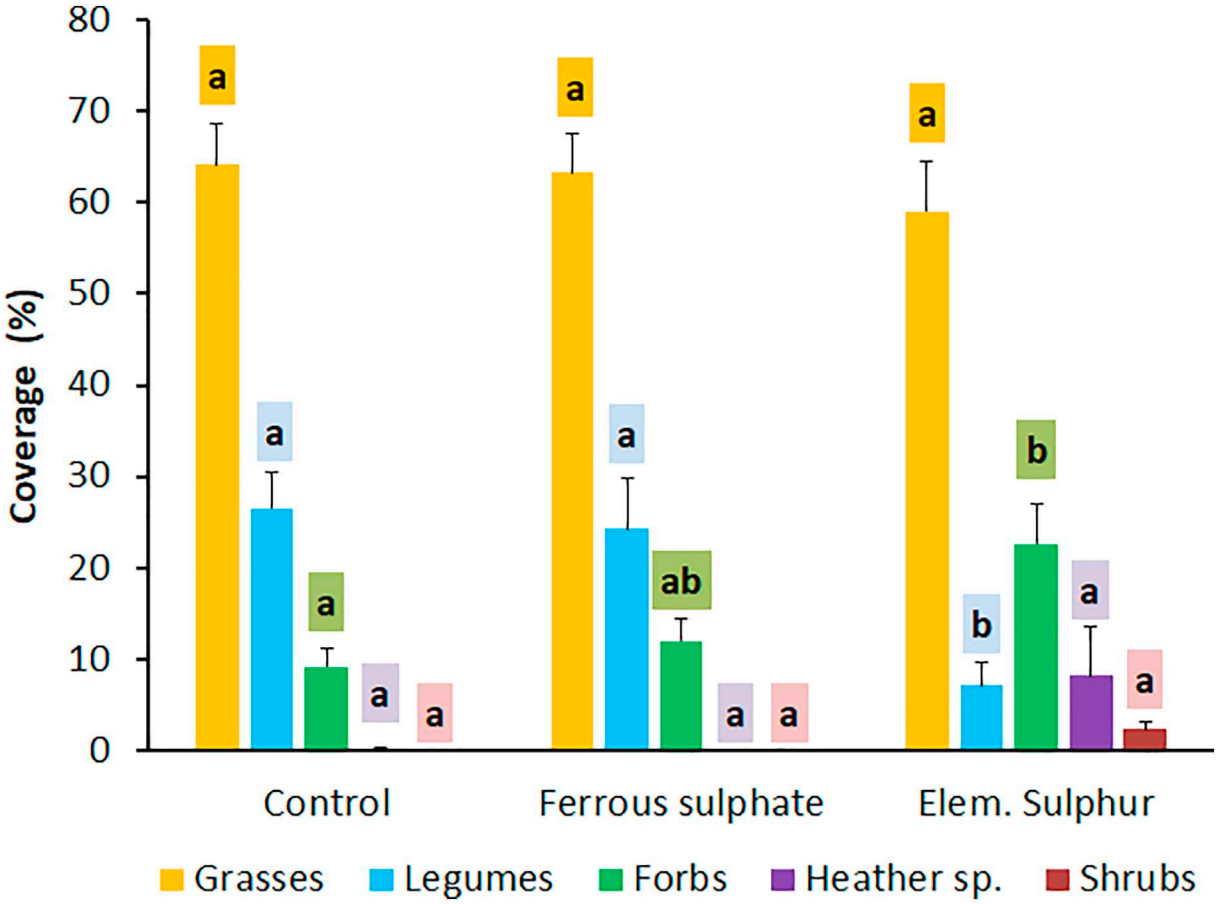

Fig. 3. The effects of sulphurous amendments on vegetation coverage classified by functional groups. Error bars represent the SE of the means. Means with different letters (a, b) represent a significant difference between treatments based an unbalanced one-way mixed model and Fisher's LSD post-hoc test ($P < 0.05$) for grasses, legumes and forbs. Non-parametric Kruskal-Wallis analysis and Bonferroni test ($P < 0.05$) for heather and shrubs.

sulphur plots was sufficient to result in pH that did not differ significantly to native acid grassland at all depths, and native heathland at 5–10 and 10–15 cm (Table 1).

Principal components analysis for the HRI revealed that the chemical characteristics with the highest factor loadings, aside from pH, were available Al, P and Fe (see Supplementary material Table S1, Fig. S1). So assessment here will focus on these three elements. These characteristics, in turn, were used as the MDS for the calculation of the HRI (see below).

In order to determine whether unamended control plots move towards the native heathland and acid grassland systems in terms of soil chemistry, we first considered whether heathland and acid grassland are significantly different to control plots for each variable. With the exception of 0–5 cm pH, no other variable, at any depth, differed significantly between acid grassland reference sites and the control plots (Table 1). Therefore the sections to follow discussing soil chemistry will focus on comparisons between control, elemental sulphur, ferrous sulphate plots and heathland reference sites.

3.1.1. Available Al

Available Al was significantly higher in heathland reference sites compared to control plots at all depths (Table 1). There was also a significant difference for available aluminium concentrations (referred to from hereafter to as Al^{3+}) between elemental sulphur and control plots. In fact, the elemental sulphur application elevated Al^{3+} to reach concentrations that were not significantly different to heathland references at the two lower depths. As with pH, there was no significant difference between ferrous sulphate and control plots (Table 1).

3.1.2. Available P

Although available P was lower in heathland sites, compared to the control, this difference was not significant at 0–5 cm or 5–10 cm (Table 1). Application of ferrous sulphate, however, resulted in significantly higher concentrations of available P than the control at 0–5 cm and 5–10 cm (Table 1). Despite there being no significant difference between ferrous sulphate and elemental sulphur, elevated concentrations of available P in the elemental sulphur treatment were not significantly higher than the control. Application of both sulphur treatments, however, resulted in a significant increase in available P at all three depths, when compared to the native heathland site (Table 1).

3.1.3. Available Fe

The only significant difference between heathland reference sites and controls in available Fe was observed at 0-5 cm (Table 1), where heathland (3.4 mg Fe kg^{-1}) was significantly higher compared to control (1.4 mg Fe kg^{-1}). Available Fe concentrations were significantly lower in the control plots, compared to elemental sulphur plots, at all depths (Table 1). Although a significant difference between the control and the ferrous sulphate treatments (2.1 mg Fe kg^{-1}) was seen in 0–5 cm, this was not seen deeper in the profile (5–10 or 10–15 cm). However, both the elemental sulphur and ferrous sulphate treatment, at all depths, resulted in Fe concentrations that were not significantly different to heathland sites (Table 1).

3.1.4. Base cations (Ca, Mg, K)

Available Ca was significantly lower in the heathland sites compared to the control, with the exception of 0–5 cm depth (Table 1). Concentrations of Ca in the elemental sulphur treated plots were significantly reduced compared to the controls, at all depths, resulting in concentrations that did not differ significantly to native heathland (Table 1). Application of ferrous sulphate had no appreciable effect on Ca.

Concentrations of Mg were significantly higher in the native heathland, when compared to all other treatments, at all depths (Table 1). Application of the two sulphurous amendments was not able to increase concentrations of Mg to match those seen in the native heathland. In fact, application of elemental sulphur showed a significant reduction in Mg when compared to application of ferrous sulphate.

Available K was significantly higher in the heathland sites than the controls at 0–5 cm only (2.7 and 1.0 mg K kg^{-1} respectively, Table 1). However concentrations did not differ significantly between the control, elemental sulphur and ferrous sulphate plots at any depth.

3.1.5. Total C and N

Total C, (only measured at 0–5 cm), was significantly higher in the native heathland (c. 10%, Table 1) when compared to controls (c. 4%).

Plate 1. Heather community (*Calluna vulgaris* and *Erica* spp. dominated) successfully established 14 years after elemental sulphur application on a 50 × 50 m plot

However, there was not significant difference in total N between heathland and controls. Application of either elemental sulphur or ferrous sulphate did not differ significantly in concentration of C or N when compared to control plots.

3.1.6. Heathland restoration index (HRI)

The variables singled out as the MDS, used in the scoring system for the HRI were as follows: 0–5 cm pH, Fe and P; 5–10 cm pH, Al and P; 10–15 cm Fe (see supplementary material, Table S1). Therefore, taking into account the weighted factors presented in the supplementary material (Table S1), the highest possible HRI (i.e. if the treatment scored the maximum of 1 for each of these variables) is 1.87. Native heathland sites resulted in a mean HRI of 1.63 (Fig. 2), which was significantly higher than the control (HRI = 1.01, Fig. 2). Only elemental sulphur application resulted in an HRI significantly higher (HRI = 1.42) than the control plots (Fig. 2). The elemental sulphur treatment HRI was elevated to such a level to have no significant difference to native heathland sites in terms of the HRI based on soil chemistry. Ferrous sulphate, did not differ significantly to the control treatment.

3.2. Soil chemistry (2009)

These chemistry data are from samples taken in tandem with the litter decomposition microbiological studies (below). The general patterns in this limited data set reflect those of the later 2014 sampling (above). Nine years after application started, elemental sulphur amendment depressed soil pH compared to the control plots and pH levels in the plots restored using elemental sulphur showed a soil pH indistinguishable from that of the native heathland plots. However, whilst soil organic matter, total C, total N and the C:N ratio were significantly different among the sites, values for the elemental sulphur plots remained very close to those of the controls. Indeed, total N levels in elemental sulphur treated plots were ~50% lower than native heathland, whilst organic matter levels (loss on ignition) and total carbon were ~ 70% lower (see supplementary material, Table S2).

3.3. Vegetation cover (2014 assessment)

Grasses were dominant in all treatments with a mean coverage of around 60%, while heather and other shrub species were only registered in elemental sulphur plots, with 8% and 2% coverage respectively (Fig. 3). However, these generic analyses conceal a wide range of vegetation responses with some elemental sulphur plots having successfully reverted to heather domination (Plate 1). Almost no heather was present (< 1% cover) in the control and ferrous sulphate treated plots.

Coverage of grasses ($F = 0.61$, $P = 0.551$), heather ($H = 3.58$, $P = 0.167$) and shrubs ($H = 12.01$, $P = 0.003$, but post-hoc test $P = 0.054$) between elemental sulphur and ferrous sulphate plots were not significantly different among treatments at the $p < 0.05$ level.

A significant difference among treatments was only found in legumes ($F = 4.89$, $P = 0.017$) and forbs ($F = 4.93$, $P = 0.016$) communities. Control and ferrous sulphate plots, with 25% coverage by legumes, were found to be significantly different to elemental sulphur plots, where legumes covered only 7% of the surface. In comparison, forb communities were significantly different between control and elemental sulphur plots, in this case, with the highest 23% of forb coverage registered in the elemental sulphur plots. Coverage of forbs in the ferrous sulphate plots, however, does not differ significantly from either the control or elemental sulphur plots.

Two important clusters were differentiated in the PCA ordination representing different vegetation communities (Fig. 4). The first group was formed by control and ferrous sulphate plots, both plots

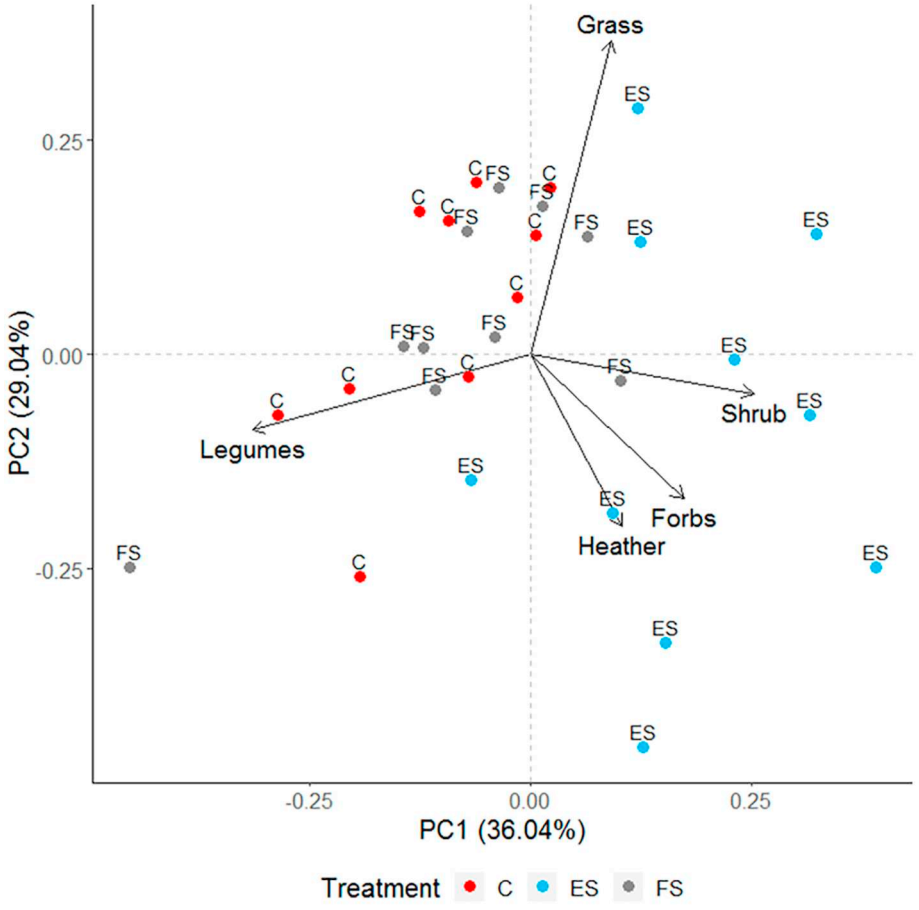

Fig. 4. PCA biplot on vegetation coverage classified by functional groups in the plots sampled: C = Control, FS = Ferrous sulphate, ES = Elemental sulphur. Labels in italic represent the variables calculated, the five different vegetation groups. Vectors represent their direction and magnitude. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

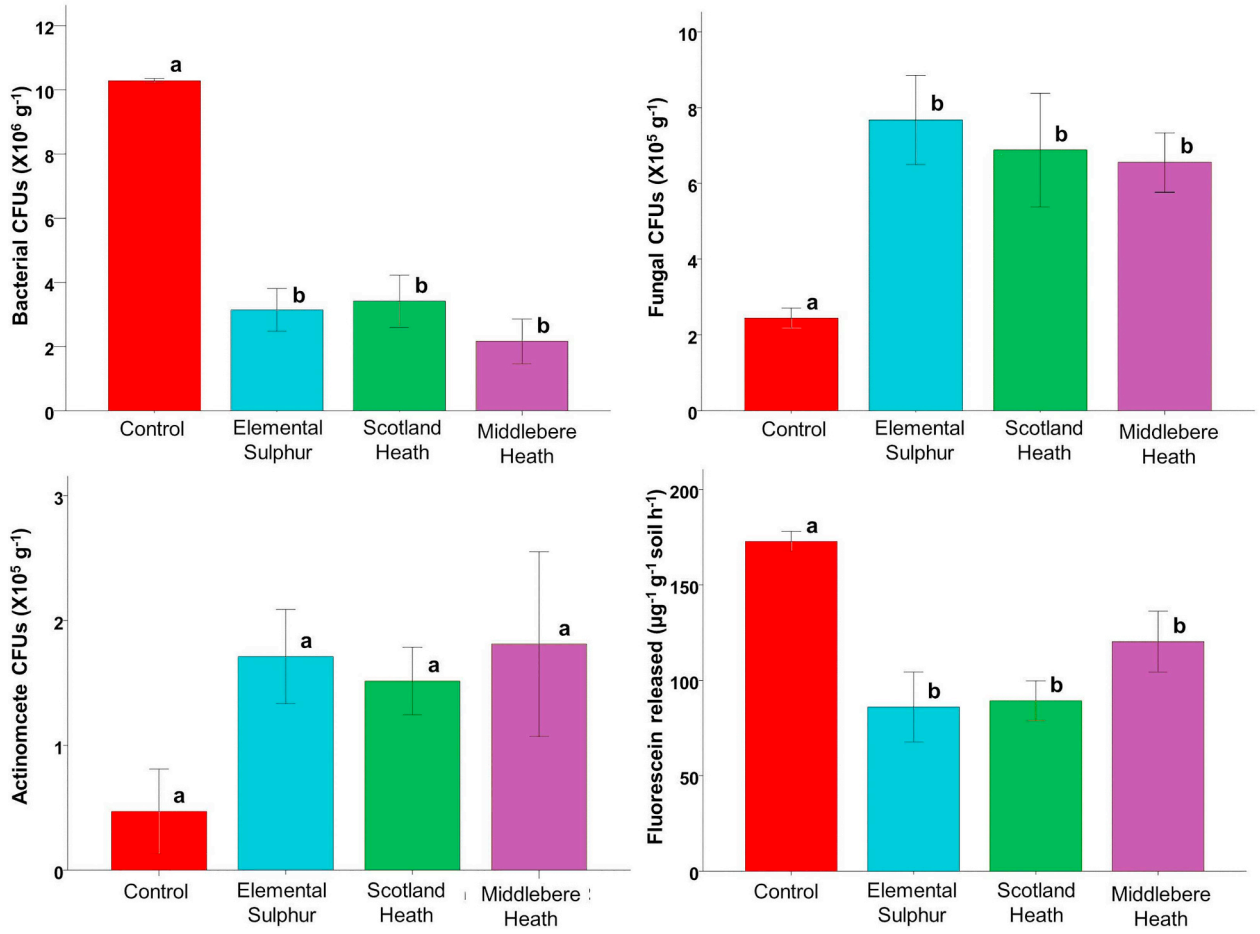

Fig. 5. The effects of sulphurous amendments on soil microbiology i) bacterial colony forming units (CFUs) ii) fungal CFUs iii) actinomycete CFUs iv) soil microbial activity determined through the hydrolysis of fluorescein diacetate (FDA). Error bars represent the SE of the means. Means with different letters (a, b) represent a significant difference between treatments based on one-way ANOVA using Welch's F ratio followed by Tukey's HSD post hoc test ($p < 0.05$).

characterised by high percentages of grasses and legumes species coverage. The second cluster was formed by the elemental sulphur plots, where forbs, heather and shrub species were more dominant.

3.4. Soil microbial community, activity and litter decomposition (2009)

Microbial parameters for the soils showed significant differences among the sites, with differences primarily occurring between control (untreated) pasture plots and the other acidic or acidified plots (Fig. 5). The numbers of bacterial CFUs were significantly higher in the control plots (ca. 10×10^6 CFU g^{-1} soil), whilst CFUs for the sulphur treated plots did not differ significantly from the heathland sites (ca. 3×10^6 CFU g^{-1} soil, $F(3,12) = 35$, $P < 0.001$). The number of fungal CFUs also differed significantly but with enumeration reversed: fungal CFUs were higher in the soils from native heathland and sulphur treated plots (ca. 2.2×10^5 CFU g^{-1} soil for controls and ca. 7×10^5 CFU g^{-1} soil for the other plots, $F(3,12) = 5.1$, $P = 0.017$). Again, levels of fungal CFUs were indistinguishable between elemental sulphur treated and native heathland plots. The low count of actinomycete CFUs showed a similar pattern to those of fungi, but differences were not significant ($F(3,12) = 1.74$, $P = 0.21$). The level of microbial activity in the soil as measured by the amount of fluorescein released from FDA, was significantly higher in the control plots. In all four microbial parameters (bacterial, fungal, actinomycete CFUs; and fluorescein released) elemental sulphur and heathland plots did not differ significantly from each other.

Microbial activity in the soil was very strongly and negatively correlated with fungal abundance and to a lesser extent, actinomycete abundance (Table 2). A very strong positive correlation was found between microbial activity and bacterial abundance. The relationship between fungal and bacterial abundance showed a very strong negative correlation with each other.

Bacterial abundance in the soil was also very strongly positively correlated with soil pH (Table 2) and negatively correlated with the C:N

Table 2
Correlations coefficients between the number of CFU (g^{-1} soil) of soil microbes and selected parameters of the soil (*$P \leq 0.5$, **$P \leq 0.01$, ***$P \leq 0.001$, $n = 16$). Correlations between variables were evaluated using Pearson's product moment correlation.

	pH	Moisture (%)	OM (%)	Al (mg kg^{-1})	Total C (g kg^{-1})	Total N (g kg^{-1})	C:N
Bacterial CFUs	0.78***	−0.26	−0.46	−0.47	−0.54*	−0.44	−0.62*
Actinomycete CFUs	−0.23	0.25	0.33	0.08	0.32	0.25	0.33
Fungal CFUs	−0.57*	0.12	0.35	0.09	0.33	0.28	0.32

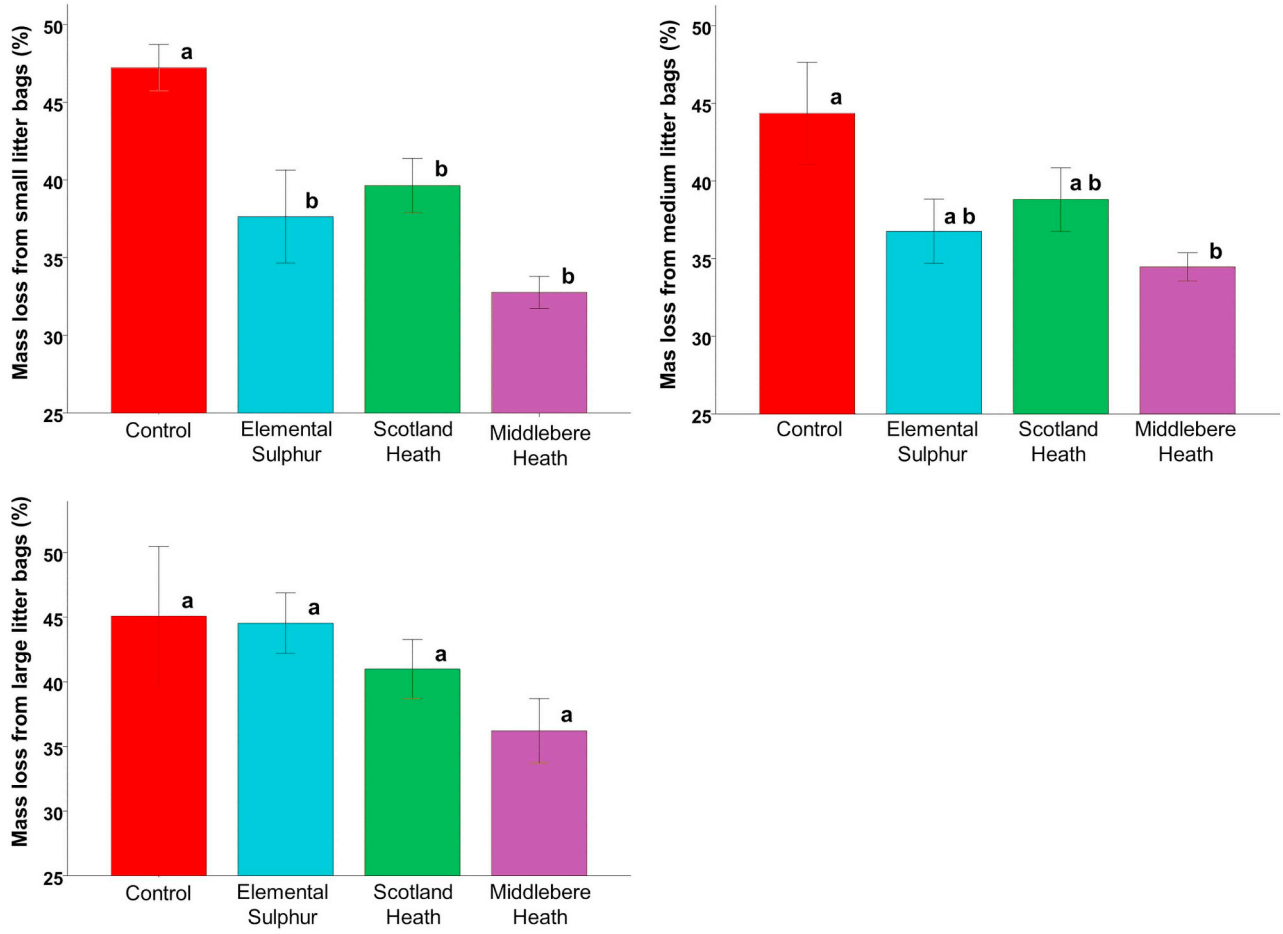

Fig. 6. The effects of sulphurous amendments on decomposition. Mass loss from i) small (100 μm) ii) medium (2 mm) and iii) large (4.7 mm) mesh size litter bags. Error bars represent the SE of the means. Means with different letters (a, b) represent a significant difference between treatments based on one-way ANOVA using Welch's F ratio and Tukey's HSD post hoc test ($p < 0.05$).

ratio and total C content of the soil. Actinomycete abundance showed no significant correlations with any of the soil parameters measured, reflecting a lack of significant difference in abundance between treatments. Fungal abundance showed a strong negative correlation with soil pH, but was not significantly correlated with any other soil parameters (Table 2).

The extent of litter mass loss resulting from only microbial decomposition (i.e. in the small mesh size litterbags) was significantly different between the control and elemental sulphur treatments (Fig. 6; $F(3,12) = 9.4$, $P = 0.002$). The highest level of decomposition occurred in the soil of the control pasture plots, whilst decomposition in the restored (elemental sulphur) and native heaths sites was significantly lower and did not differ significantly between them. The inclusion of meso-fauna in the decompositional process (medium sized mesh) had little effect on overall mass loss from the litter, but resulted in a noticeable change in the pattern of litter mass loss among the treatments. A significant difference was only found between the control (pasture) and Middlebere heath. The sulphur treated plots and Scotland heath did not show a significance difference from either the control plots or Middlebere heath. When the macro-fauna had access to the litter (large mesh size) differences in mass loss among the treatments were not significant with mean mass loss between 36% and 45% (Fig. 6, $F(3,12) = 1.45$, $P = 0.28$).

Mass loss from the litter bags with a small mesh size was strongly correlated with the microbiology of the soil (Table 3). Mass loss was most strongly correlated with bacterial abundance. The microbial activity in the soil as measured by the release of fluorescein from FDA also showed a strong positive correlation. By contrast, fungal abundance exhibited a strong negative correlation with litter mass loss. Mass loss from the litter in bags with the medium mesh indicated that the abundance of the soil microbial community made a weaker contribution to litter decomposition. Mass loss was still significantly correlated with bacterial and fungal abundance, but the strength of these relationships was reduced. Increasing the mesh size further decreased the importance of the abundance of the microbial community to litter decomposition, with mass loss from large mesh size bags showing no significant correlations with any of the microbial properties of the soil.

Table 3
Correlations coefficients between mass lost from litter bags with three different mesh sizes and the abundance of microbial groups (CFUs g^{-1} soil) and microbial activity (μg fluorescein released g^{-1} soil h^{-1}; n = 16). Correlations between variables were evaluated using Pearson's product moment correlation. Family wise error rate was not controlled.

	Bacterial CFUs	Actinomycete CFUs	Fungal CFUs	Microbial activity
Small mesh	0.73**	−0.48	−0.64**	0.61*
Medium mesh	0.63**	−0.30	−0.61*	0.48
Large mesh	0.27	−0.50	−0.29	0.24
Bacterial CFUs	–	−0.41	−0.65**	0.68**
Actinomycete CFUs	−0.41	–	0.63**	−0.66**
Fungal CFUs	−0.65**	0.63**	–	−0.82***

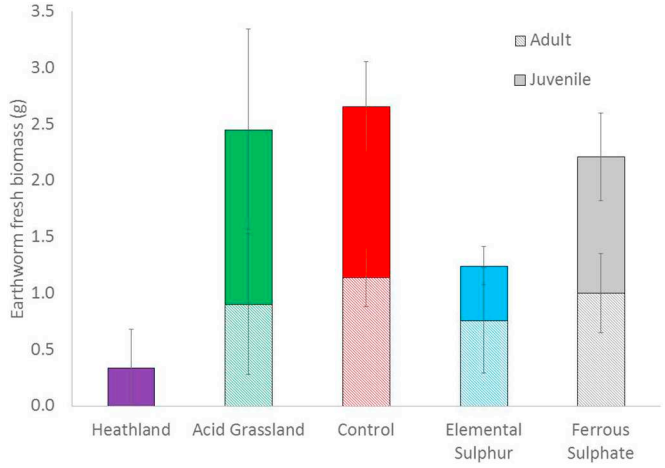

Fig. 7. The effects of sulphurous amendments on juvenile and adult earthworm biomass. Error bars represent the SE of the means: n = 10 for Control, Ferrous Sulphate and Elemental Sulphur and n = 4 the Acid Grassland and Heathland.

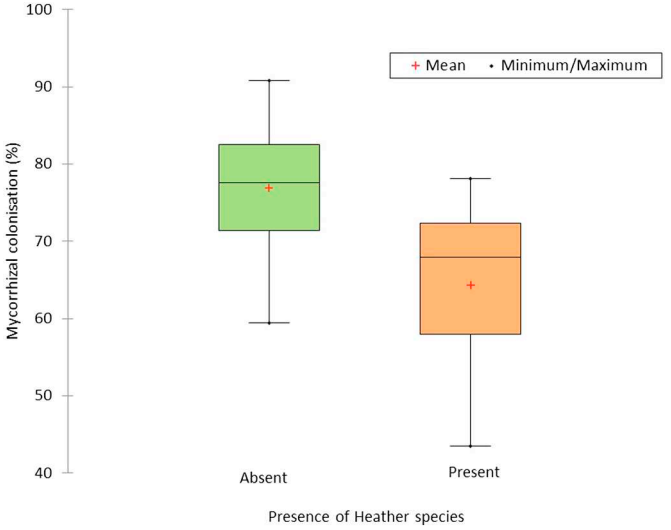

Fig. 8. Box and whisker plots, with arithmetic mean, of mycorrhizal colonisation of *Holcus lanatus* in plots where heather species were present or absent. Means are significantly different ($P < 0.05$) based on an unbalanced one-way ANOVA using Welch's F ratio and Tukey's HSD post hoc test ($p < 0.05$).

3.5. Ericoid and arbuscular mycorrhizal colonisation (2017)

Mean ericoid mycorrhizal colonisation in heather roots was not significantly different in the heathland (66.81%; $n = 7$), acid grassland (77.33%; $n = 8$), or the elemental sulphur (69.74%; $n = 3$) plots. Ferrous sulphate and control plots were not included as heather was effectively absent on these plots (Fig. 3).

There was no significant difference in arbuscular mycorrhizal colonisation of *H. lanatus* roots between any treatments (data not shown $F = 0.373$, $P = 0.773$). Mean colonisation was relatively high (ca. 70%) throughout. A closer examination of arbuscular mycorrhizal colonisation of *H. lanatus* roots across all plots showed that in the plots where heather plants (*C. vulgaris* or *Erica* spp.) were established, due to successful restoration, the level of arbuscular mycorrhizal colonisation was significantly lower than in plots where heather plants were absent (Fig. 8). This was based on an unbalanced one-way ANOVA using Welch's F ratio and Tukey's HSD post hoc test.

3.6. Earthworm sampling (2016)

There were no significant differences in juvenile, adult or total earthworms between treatments (Fig. 7). However, we have outlined some brief observations below. Earthworm abundance was the lowest in the heathland and elemental sulphur plots (Fig. 7). There were no adult earthworms present in the 20 cm × 20 cm × 20 cm soil cubes that were surveyed in any of the four heathland plots. In the elemental sulphur plots the adult earthworms accounted for more biomass than the juveniles, whereas in the control, ferrous sulphate treatment and acid grassland the juvenile biomass accounted for a larger proportion.

3.7. Nematode, rotifer and tardigrade sampling (2017)

The mean number of rotifers present in 100 g of soil was < 9 individuals, with no significant effect of treatments compared to the control (Fig. 9a). The abundance of nematodes was significantly higher in the control plots (768 nematodes $100\,g^{-1}$, Fig. 9b) than acid grassland plots (447 nematodes $100\,g^{-1}$). However, there was no significant difference between the control treatment and heathland reference sites (479 nematodes $100\,g^{-}1$, Fig. 9b). The application of elemental sulphur, however, resulted in significantly lower number of nematodes than the control plots (293 nematodes $100\,g^{-1}$, Fig. 9b). The number of nematodes in the ferrous sulphate plots was not significantly different from any of the other treatments. Tardigrades were absent in the vast majority of samples so data are not included.

4. Discussion

4.1. Long-term changes in soil chemistry

Soil pH in experimental plots have previously been shown to respond to sulphur treatment (Owen et al., 1999; Owen and Marrs, 2000; Lawson et al., 2004; Tibbett and Diaz, 2005; van der Bij et al., 2018), particularly for elemental sulphur treatment six years after application on our study plots (Diaz et al., 2008). The elemental sulphur treatment has remained effective in reducing pH significantly compared to the control 14 years after application started (Table 1). This demonstrates the longer-term effectiveness of sulphur treatment that may be sustained if heather plants establish and provide acidic litterfall into the soil-plant system (Grubb et al., 1969). The application of ferrous sulphate, however, was fairly ineffective in reducing pH over 14 years.

Previous analysis of soils from our field sites have considered only surficial (0–4 cm) effects soon after application (Tibbett and Diaz, 2005) or 15 cm depths in 2006 (Diaz et al., 2008). Here (2014 sampling) we have considered the soil in our experimental plots and the adjacent acid grassland and heathland in 5 cm increments to 15 cm. The acid grassland plots have a pH ca.5 regardless of depth while the heathland plots are far more acidic with a distinct and significant change with depth, pH 3.9 at the surficial increment (0–5 cm) and pH 4.7 at the deeper increment (10–15 cm). This is in contrast to previous studies where little difference with depth was reported (Pywell and Webb, 1994). For heathlands, this strongly supports the tenet that acidic litterfall from *Calluna* and *Erica* species act as an acidifying agent in the soil from the top down (Grubb et al., 1969; Price, 2003). After 14 years the elemental sulphur treated plots were not significantly different to the acid grassland or heathland, except for the upper layer of the heathland soil which remains significantly lower in soil pH. Therefore it is likely that application of elemental sulphur will have a greater influence on vegetation assemblage and indicators of soil biodiversity on the experimental plots, when compared to the ferrous sulphate and control plots.

The removal of base cations from the original pasture soils, and their subsequent replacement by acidic cations is a significant step towards the recreation of acidic systems (Tibbett and Diaz, 2005). Ca^{2+} in the elemental sulphur plots is particularly closely matched to the acid grassland and heathland soils in addition to being significantly different from the ferrous sulphate and control plots. In contrast the available P

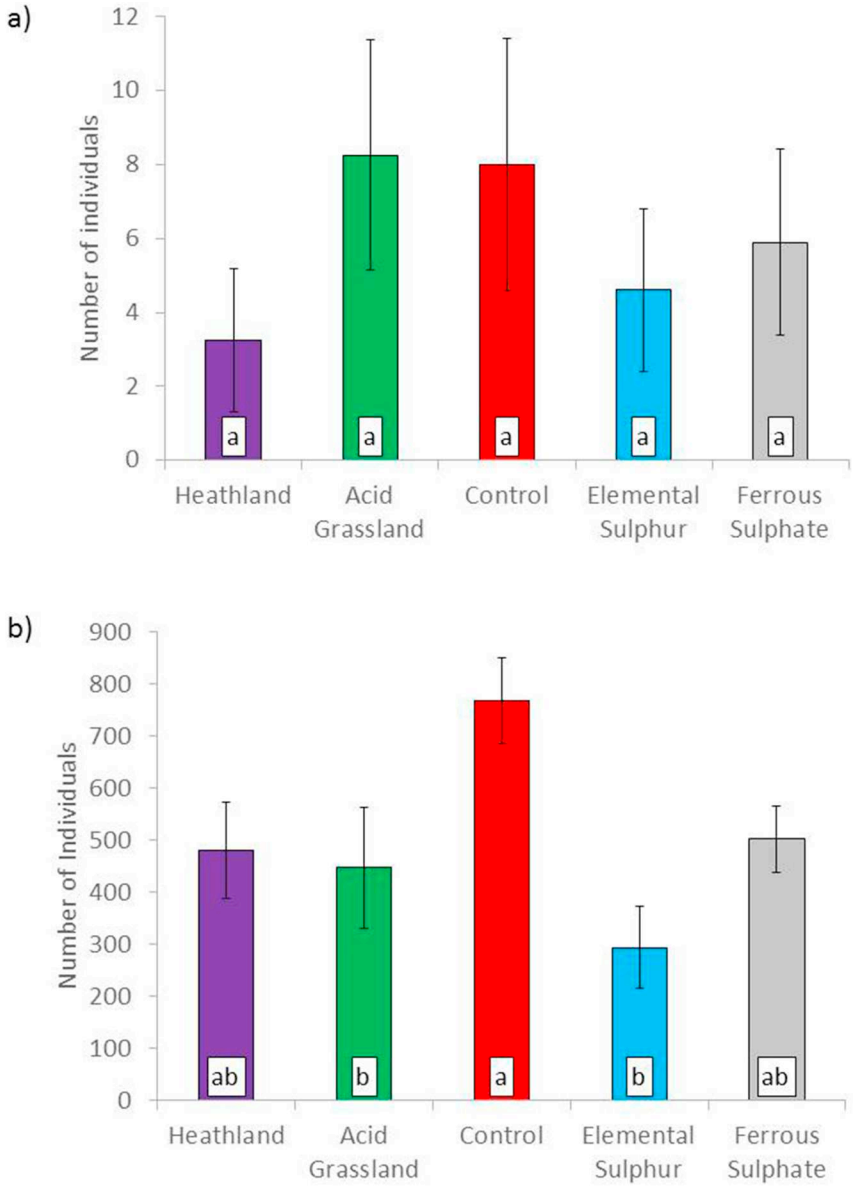

Fig. 9. The effects of sulphurous amendments on a) rotifer and b) nematode abundance based on 100 g soil dry weight equivalent. Error bars represent the SE of the means. Means with different letters (a, b) represent a significant difference between treatments, based on one-way ANOVA using Welch's F ratio and Tukey's HSD post hoc test ($p < 0.05$).

in the soil increased under sulphur treatments. This may be due to acidification causing a release of P from historical rock phosphate amendments (see Tibbett and Diaz, 2005). Antecedent P amendment to pasture soil has previously been shown to be accumulated in sandy surface soils (Ryan et al., 2017), and this seems to have occurred here due to PO_4^- release by acidification from rock phosphate stores. Notably, however, ferrous sulphate application has increased available phosphate not only soon after application (Tibbett and Diaz, 2005) but also after 14 year into this experiment (Table 1). The precise mechanism behind this response is unknown.

The significant difference in available aluminium, likely present as Al^{3+}, is a key driver in plant community change in heathland (De Graaf et al., 1997) and, in turn, most likely to affect soil biodiversity as a toxic element. The parity of Al concentrations in elemental sulphur plots compared with our reference heathland sites and the significant differences to control and ferrous sulphate plots after 14 years indicates a clear toxicity driver caused by soil pH change after sulphur application (see Tibbett and Diaz, 2005).

While some available element concentrations described above can change due to sulphur induced acidification, the total N and C concentration remained the same. There was no effect of treatment after 14 years but there was a large and important difference in percentage of total C in heathland soils, which were over 10% C compared with a little over 4% in all other soils, including the acid grassland. This difference represents centuries of OM accumulation in the system where acid litters produce a recalcitrant organic matter layer (mor) (Grubb et al., 1969; Jalal and Read, 1983) not likely to be replicated for many decades under restoration programmes. This lack of soil carbon accumulation may be exacerbated in heathland restoration schemes where soil stripping and soil inversion has been employed.

Overall, application of elemental sulphur has influenced soil pH and chemistry to such a degree that soil conditions, as described by the Heathland Restoration Index, was comparable to those in the native heathland and acid grassland sites. The application of ferrous sulphate was unable to achieve these conditions. The index shows notable parity with individual soil conditions.

4.2. Long-term changes to vegetation community

The shifts in plant community assembly in 2006 reported previously (Diaz et al., 2008) are essentially retained in our survey of 2014, 14 years after initial treatment applications. The elemental sulphur treatment had a long-term impact on the plant community composition with an increase in forbs and heather species, and a decrease in legumes, separating the elemental sulphur community from the control and ferrous sulphate treatments. There was a mixed retention of heathland vegetation communities that were well established in 2006, which were anecdotally related to grazing management and access of cattle to the plots.

Almost no (< 1% cover) heather species were observed in the control or ferrous sulphate plots despite the application of ericaceous clippings from the adjacent Middlebere Heath, that were sown in 2001 and 2003 across all plots. The lack of regeneration of *C. vulgaris* in these plots is probably due to the retention of nutrient-rich competitive species (Lawson et al., 2004) due to the high fertility still found in these soils (Helsper et al., 1983). Where clippings were applied, establishment of heather was successful in all elemental sulphur plots initially (see Diaz et al., 2008 for further details); however, anecdotal evidence suggests that on many plots unmanaged grazing pressure eradicated many of the establishing seedlings over the following years.

4.3. Changes in soil microbial community, activity and litter decomposition

Different components of the soil microbial community are known to be strongly related to pH (Bååth and Anderson, 2003). Soil bacterial populations are favoured at higher soil pH where fungi are generally more dominant in acidic conditions (Kooijman et al., 2018; Rousk et al., 2010b). In this section of work we tested the change in the soil microbial community in four restored elemental sulphur amended plots against their target heathland soils, and the control soils. We were particularly interested to investigate whether predicted bacterial dominance at high pH was replaced by fungal dominance at low pH.

The number of CFUs for bacteria and fungi almost directly mirrored each other supporting the established tenet on soil pH and microbial community dominance (Bååth and Anderson, 2003; Kooijman et al., 2018; Rousk et al., 2010b). There were more than double the CFUs for bacteria in the control plots with the higher pH profiles than the elemental sulphur and heathland plots. For fungal CFUs these were the reverse, with significantly fewer CFUs in the control plots than in the more acidic elemental sulphur and heathland plots. Indeed bacterial CFUs were strongly positively correlated with pH and fungal CFUs were negatively correlated with pH in concordance with the findings of Rousk et al. (2010a). It is an important part of the reversion of the whole plant-soil system that the microbiology of the elemental sulphur plots is in close parity with the target heathland systems. Whether the plant community change led to the change in the microbiota or the soil microbiology facilitated change in vegetation is an outstanding question for restoration ecologists (Harris, 2009). Although van der Bij et al. (2018) have recently provided evidence that the latter may apply.

The function of the soil microbial community also appeared to have been largely restored, at least in terms of litter breakdown and microbial activity. FDA hydrolysis is thought to reflect overall soil microbiological activity (Nannipieri et al., 2003), and as such microbial activity was positively correlated with bacterial abundance and negatively correlated with fungal abundance, indicating a decline in microbial activity in fungal dominated acidified soils. Reduced FDA hydrolysis has been shown to be correlated with reduced soil respiration suggesting an overall diminution of the capacity to cycle carbon and other nutrients (Bååth et al., 1980; Schnürer and Rosswall, 1982).

Mass loss in the litter bags with small mesh size (allowing only microbes access to the litter) showed significant correlation to the bacterial abundance in the soil and microbial activity. Soil acidification, mediated by elemental sulphur application, degraded fertility, suppressed bacterial abundance, microbial activity and reduced microbial decomposition of litter. These are all characteristics of the soils of the native heaths and comply with our measurements of microbial activity (Price, 2003; Walker et al., 2004).

As litterfall becomes dominated by ericaceous species, known to be resistant to decomposition (Price, 2003), the reduction in litter decomposition rate could potentially increase soil organic matter over a longer period. Fourteen years after the beginning of the experiment the concentration of carbon remained similar among all grassland sites (control, ferrous sulphate, elemental sulphur and acid grasslands). Only the heathland soils contained high concentration of carbon (10%, as opposed 4% for grassland sites), demonstrating that stable belowground restoration of all ecosystem properties still has some way to go. Higher organic matter could be facilitated by increased fungal biomass and low bacterial abundance, contributing to typical O horizon of mor humus podzolic soil that develops from litter decomposition dominated by fungi in acidic conditions (Ponge, 2013). Acidification of soil has been shown to reduce the availability of C and N to the microorganisms, facilitating mor humus formation (Persson et al., 1989). The successful re-establishment of ericaceous dwarf shrubs should support this process and the N poor, acidic and polyphenolic litter of these plants is also a requirement of mor humus formation (Grubb et al., 1969).

The present study demonstrated that barley straw decomposition was not significantly different among the sites when the soil *meso* and macro fauna had access to the litter. *Calluna* litter will behave differently to barley straw and there was significantly greater soil organic matter in the soil from heaths, despite the same level of decomposition as the controls. Moreover, litter loss from bags with larger mesh sizes does not equal organic matter loss from the soil, i.e. meso/macrofauna feeding activity may shred the litter leading to loss from the bag and eaten litter may be exported from the litterbag in the animals, but not necessarily from soil. Observational evidence suggests that *Calluna* litter is building up on the soil surface, but it remains to be seen if this will reduce available N levels.

4.4. Changes in mycorrhizal colonisation

Mycorrhizas have a critical role in most terrestrial ecosystems and are recognised as having a key role in habitat restoration (Kariman et al., 2018). Arbuscular mycorrhizal (AM) colonisation, communities and functions can be strongly affected by soil pH (Coughlan et al., 2000; Hepper, 1984; Tipton et al., 2018). Acidic conditions cannot only suppress the beneficial effect of AM but also suppress the uptake of P even when freely available (Graw, 1979). In our field site P availability in soil was enhanced under acidification almost certainly due to dissolution of antecedent rock phosphate application (Tibbett and Diaz, 2005). In acidic heathland soils ericoid mycorrhizas dominate the soil-plant system, not only as absorptive organs but also as active participants in nutrient mobilisation far beyond the capability of AM fungi (Read et al., 2004). The reduced colonisation of AM in *H. lanatus* roots where ericaceous plants are present is therefore not surprising, although mean colonisation levels remain high in almost all samples, regardless of treatment or the presence of heather. It should be noted that important aspects of the ecology of mycorrhizas at this site, such as their community composition and spatial structuring, remain to be elucidated (e.g. Prober et al., 2014). Arbuscular mycorrhizal communities in acid grasslands, heathlands and experimental plots may be quite different and spatially distinctive, which would have implication for belowground restoration targets if clarified.

The potential importance of ericoid mycorrhizas (ERM) in heathland restoration is commonly acknowledged but rarely investigated (e.g. van der Bij et al., 2018). In our previous work (Diaz et al., 2006; Diaz et al., 2008) we showed that juvenile heather plants (2–4 years after establishment) in the elemental sulphur treated plots were mainly uncolonised by ERM, and when they were colonised the rates were ca.10–20% of the root length. In contrast we found that heather plants

in the native heaths and acid grassland were nearly all colonised at levels exceeding 70% (statistically different). Here we report very different findings 13–15 years after heather sowing. All plants examined in the elemental sulphur treated plots were colonised with a mean of 70% of root length which was no longer significantly different to that found in the heathland and acid grassland plants. This demonstrates clearly that given sufficient time, perhaps a decade or more, a natural population of ERM can support a heather-based plant community. This is an important step towards ecosystem restoration in terms of plant-soil interaction that has not previously been shown.

4.5. Changes in soil fauna

It has recently been recognised how the role of soil fauna has been overlooked and undervalued in ecological restoration assessments (Cross et al., 2019). In the current study we have uniquely reported on changes in a range of soil faunal abundance associated with acidification and the restoration of heathlands.

The artificial acidification caused by sulphurous amendments resulted in a reduction in nematode abundance and earthworm biomass. Although a significant reduction was only seen in the elemental sulphur treatment for the abundance of nematodes, the trend was similar for the rotifers and earthworms. This is in keeping with other published studies (Hyvönen and Persson, 1990; Curry, 1998). Acidification of the soil can increase H^+, Al^{3+} and NH_4^+ ions, resulting in toxic effects on plants and soil organisms (Kuperman and Edwards, 1997). In a two year study on soil acidification, Chen et al. (2013) reported a larger effect on the nematode and microbial communities than the aboveground plant communities. However, monitoring changes over a longer time frame may result in the changes to soil biodiversity and soil chemistry having a feedback on the plant communities (and vice versa). In our long-term study comparing artificial acidification to semi-natural heathland and acid grassland, it is interesting to note the elemental sulphur plots had lower abundances of the measured soil fauna than the acid grassland, despite having similar pH values. The disturbance caused by the application of elemental sulphur on other soil chemical properties and plants may have been a driving factor in the inability of the system to recover even after 17 years. Lavelle et al. (1995) reported that soil fauna have a limited capability to adjust to soil pH, resulting in soil acidification having a negative effect on soil biodiversity. At a soil pH of < 4, earthworms and Coleoptera were reduced compared to pH 4–5, while termites actually showed an increase in the number of individual at pH < 4–5 but above pH 5 there was a large reduction (Lavelle et al., 1995). Although the abundance and biomass of organisms does not give detailed information on the functions or diversity of the system, it does support other studies that have found a reduction in soil faunal abundance and diversity under land-use intensification (Tsiafouli et al., 2015).

5. Conclusions

Elemental sulphur treatment has proven to be an effective method of acidifying the soil in improved pasture systems for various ecological aspects 8 to 17 years after application. The soil chemistry and the derived HRI showed a shift of plots subject to sulphur treatment strongly in the direction of the heathland soils. The ecology and biodiversity of the acidified sites has changed considerably with different ecologies developed above and belowground. The shift in soil biological communities such as the microbiology, nematodes, earthworms and mycorrhizas, along with functional changes in litter decomposition, demonstrated the profound effects the acidification has had on biological form and function. These components of the soil biota interact upon one another (e.g. earthworms, mycorrhiza and nematodes) and pH but the precise nature of these effects remain to be clearly elucidated e.g. (Muchane et al., 2019; Räty and Huhta, 2003). Overall, the reduced microbial decomposition shows the importance of microbes in the functioning of the soil's ecology which is most sensitive under acidification. Thirteen to fifteen years after heather sowing, we found that ericoid mycorrhizal plants in acidified soils were colonised to levels equivalent to the surrounding natural heathlands demonstrating a capacity for this critical symbiosis to develop in a manner that can support an emerging heathland community.

Elemental sulphur is clearly a useful restoration tool for acid grasslands and heathlands, although further work is needed on application rates and timing as well as integration with grazing management.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.catena.2019.03.013.

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