Evaluating heathland restoration belowground using different quality indices of soil chemical and biological properties

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Evaluating Heathland Restoration Belowground Using Different Quality Indices of Soil Chemical and Biological Properties

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Abstract: Reversion of agricultural land to heathland and acid grassland is a priority for the conservation of these rare habitats. Restoration processes require interventions to reverse the effects of fertilization and acidity amelioration undertaken during decades of agricultural production. Belowground assessments of restoration success are few, and we have examined the utility of soil indices as a rationalized tool for land managers and restoration practitioners to assess the efficacy of restoration practice. To achieve this, we assessed a large number of variables, many of which might be near redundant, that could be optimized for such indices. We used a 14-year field experiment contrasting acidified pasture (treated with elemental sulphur), control (untreated) pasture, and adjacent native heathland and acid grassland sites. Based on biotic and abiotic parameters, several 'heathland restoration indices' (resembling soil quality indices) were generated using a minimum dataset identified through principal component analysis and a linear scoring system. For comparison we also conducted alternative analyses of all parameters, using nonmetric multidimensional scaling plots and analyses of similarity (ANOSIM). Use of heathland restoration indices showed that elemental sulphur application had changed the soil chemical conditions, along with the vegetation assemblage, to be comparable to that of native acid grassland, but not the belowground biology. ANOSIM on full datasets confirmed this finding. An index based on key variables, rather than an analysis of all biotic and abiotic parameters, can be valuable to land managers and stakeholders in acid grassland and heathland restoration.

Keywords: Restoration ecology; heathland restoration index; soil biology; heathland; acid grassland; biological indicators

1. Introduction

Lowland heathland and acid grasslands are both listed as priority habitats in the European Commission Habitats Directive [1] and the UK Biodiversity Action Plan [2,3]. These landscapes are of particular importance as they now provide a last refuge for many rare and endangered fauna, but have seen rapid decline with agricultural intensification since the beginning of the 19th century. In England, for example, 72% of heathland was lost between 1830 and 1980 [4], largely as a result of improvement of these systems for agricultural production. The remaining ~58,000 ha of lowland heathland in the UK represents approximately 20% of the total global area of this habitat [5]. Therefore, particular interest has been paid for decades to methods that revert improved agricultural land back to heathland or acid grassland systems [2].

Soil conditions required for heathland or acid grassland ecosystems to establish include low pH and low nutrient availability [6–8]. Improved agricultural land has typically undergone a long-term regime of heavy fertilization and acidity amelioration in order to maximize yield for modern agricultural production [9]. These activities can cause long-lasting changes to soil chemical characteristics that leave soils unsuitable for the growth of heathland flora [10].

The re-establishment of heathland and acid grassland plant communities, therefore, needs to begin belowground through the modification of soil chemistry toward conditions found in existing heaths and grasslands [11]. One such restoration technique involves chemical amendment of improved agricultural land through the application of elemental sulphur as an acidifying agent [9,12]. The reduction of soil pH to <5 is usually a prerequisite to re-establishing acid grassland and heathland communities [10]. Therefore, successful establishment of heathland and acid grassland requires a reversal of the changes imposed on the soil by decades of fertilization and liming [13–15]. Consequently, heathlands and acid grasslands would be considered poor quality in the context of agricultural production. Therefore, restoration activities on agricultural land would require a perceived reduction in conventional soil quality (at least in terms of chemical fertility) presuming a good quality soil is intended for optimized agricultural productivity.

Statistically based soil quality indices, which use principal component analysis (PCA) to create a minimum dataset for linear scoring functions, are commonly used in the quantification of soil quality. Soil quality indices (SQIs), calculated in this manner, have been used to assess the effects of management on soil quality, such as land use change [16] and application of soil amendments [17,18], and agricultural systems, such as rice paddy [19] and vegetable production [20]. Variables that have been included in these indices include soil physico-chemical variables (e.g., pH, soil organic matter content, extractable nutrients, particle size distribution) and biological (e.g., microbial biomass, respiration, enzyme activity). Consolidation of multivariate datasets into a single index makes results of complicated datasets more accessible and easily communicated to practitioners and stakeholders. The use of statistical tools to generate a minimum dataset not only avoids data redundancy (i.e., lots of variables may be correlated or confound with one another, rendering the use of all of them unnecessary), but also reduces the opportunity for bias, compared to methods of soil quality assessment that use expert opinion [21].

Soil conditions required for a heathland ecosystem to establish include a low pH and low nutrient availability. Therefore, the variables that are considered to be 'less is better' or 'more is better' in an SQI scoring system to assess agricultural land would be reversed when applied to an SQI developed for heathland restoration. In response, we have replaced the term Soil Quality Index (SQI) with Heathland Restoration Index (HRI) to reflect reversal of soil conditions from those suitable for agricultural purposes toward those that are beneficial for heathland restoration (i.e., a soil with a high HRI may score poorly in an SQI for agricultural land).

Based on a field experiment on the Isle of Purbeck (Dorset, UK), a study developed an HRI to evaluate the efficacy of long-term chemical acidification treatments for the reversion of agricultural soil to heathland and acid grassland systems using a field experiment [22]. The work concluded that the application of elemental sulphur to acidify pasture was successful in creating an edaphic environment that was comparable with nearby native heathland [22]. A long-term impact on the plant community composition was also observed, with an increase in forbs and heather species in the

acidified pasture, compared to the control [22,23]. As the restoration technique was the application of chemical amendments, only soil chemical parameters, such as extractable nutrients and pH, were used in the development of this HRI. An important limitation of an HRI based entirely on chemical characteristics is that it excludes consideration of the influence of, and interaction with, soil biota. Our work will build on this original assessment, on the same field site, to encompass biological variables, as well as chemical variables.

Soil biota, such as 'decomposers' and 'nutrient transformers' (e.g., microorganisms), 'biocontrollers' (e.g., nematodes), and 'ecosystem engineers' (e.g., earthworms), are an important indicator of heathland habitat quality [24,25]. Moreover, management activities and perturbations may promote a response in soil biological properties more rapidly than chemical and physical attributes [26]. Therefore, if the biological characteristics of the chemically treated soils were also to be considered when developing an HRI, it is likely that a different HRI would be observed. If so, an index based solely on chemical variables could be inadequate as a tool to provide an early indication of restoration success belowground. Alternatively, if the inclusion of biological variables in the development of an HRI has no impact on the overall structure of the resulting HRI, then this will also provide valuable insights concerning how the soil system should be assessed during reversion to heathland and acid grassland. Biological indicators often require more resources to measure than chemical indicators. So, if an HRI based on chemical variables alone is sufficient to inform restoration managers of the success of reversion to heathland, this outcome would have clear financial and logistical benefits. Such a finding would also result in an HRI that satisfies the criteria, laid out by Menta [27], that indicators of soil health or quality should: (1) Be sensitive to variations of soil management, (2) have good correlation with the beneficial soil functions, (3) be helpful in revealing ecosystem processes, (4) have comprehensibility and utility for land managers, and (5) be cheap and easy to measure.

The current paper extends the development of an HRI to incorporate biological parameters, including micro- and macrofauna abundance and microbial respiration, in order to assess soil quality in a heathland-restoration context. The objectives of this study were: (1) To establish whether the acidification treatments that alter soil chemistry also affect the aboveground (autotrophic) community, (2) to investigate whether soil chemistry (or an HRI based on chemical variables) also leads to changes in belowground communities (or an HRI based on biological variables), (3) to ascertain whether an HRI based on biological variables can distinguish between treatments more accurately than an HRI based only on chemical variables, and (4) to determine whether any HRI based on a minimum dataset and a linear scoring system identifies acidification treatment effects in the same manner as multivariate techniques such as PCA or analysis of similarity. Our results encompassed numerous chemical and biological variables, which we did not consider as univariate data as this was beyond the scope of our objectives. Here we will focus on the separation of acidifying treatments in terms of multivariate analyses and heathland restoration indices. Detailed discussion of individual chemical (e.g., pH, extractable elements) and biological variables (microbial community, decomposition, earthworm biomass, nematode abundance, etc.) can be found in Tibbett et al. [22]. Comparisons of different soil quality index evaluation methods, such as expert opinion versus statistically modeled minimum datasets and linear versus nonlinear scoring have taken place elsewhere [17,20,21,28]. However, our research was interested in the effects of the initial variables included in the PCA on the resultant index, with particular reference to biological versus chemical variables in a heathland restoration context.

2. Materials and Methods

2.1. Site Description

We compared four environments: (1) Chemically acidified pasture, (2) control pasture, (3) native heathland, and (4) native acid grassland. A detailed description of the field experiment utilized can be found in Diaz et al. [23], Green el al. [6], and Tibbett et al. [9,22]. Briefly, experimental plots treated with different acidifying agents were established in 1999 on the Isle of Purbeck, Dorset, UK

(50.657425° N, −2.067085° W). The plots were on agricultural pasture of two contiguous farms, farmed as one consistent enterprise since the 1950s. During the 1950s and 1960s an aggressive policy of agricultural improvement of heathland through the addition of rock phosphate, manure, and chalk marl altered the soil conditions considerably. Compared to the surrounding (unimproved) acidic grassland and heath, soil pH had been increased from 3.6 to 6.6. In addition, rock phosphate had been applied over this period to elevate total phosphorus (Olsen P) levels by one-third, from 1000 mg/kg in the surrounding landscape to 1550 mg/kg in the experimental field (see Tibbett et al. [9] for more details). At the start of the experiment in the vegetation was an improved pasture in which *Lolium perenne* and *Agrostis capillaris* were the most constant and abundantly occurring grasses (see Diaz et al. [23] for a more detailed vegetation assessment).

The chemically *acidified pasture* treatment consisted of 10 replicate 50- × 50-m plots of improved agricultural pasture that were treated with 2000 kg ha^{-1} of elemental sulphur in May 2000 as an acidifying agent, and an additional 1600 kg ha^{-1} in 2001. Clippings of *Calluna vulgaris* were sown in the control and acidified pasture one year after the final application of elemental sulphur. The *control pasture* treatment consisted of 10 replicates of 50- × 50-m plots that received no chemical amendments. In addition, four replicate sites, representative of *native heathland* and *native acid grassland*, were selected in the nearby Middlebere Heath and Scotland Heath, respectively, in order to put the acidified pasture into context.

2.2. Soil Sampling

Fourteen years after initial application of elemental sulphur amendments, soil samples were collected using a gauge auger at 0–5-cm, 5–10-cm, and 10–15-cm depths (after removal of the litter layer). Twenty-five composite soil samples were taken from each plot following a standard 'W' sampling pattern, as outlined in ISO 18400-104 [29] and homogenized, resulting in three samples for each plot, one for each depth. All soils were sieved to 2 mm. The 0–5-cm depth layer was then split into two subsamples, the first for microbial was stored at 4 °C until analysis (described below). The second subsample was air-dried for chemical analysis. Chemical analysis was also conducted on the 2-mm sieved, air-dried, 5-10-cm, and 10-15-cm layer.

2.3. Vegetation Survey

Each plot was surveyed 15 years after initial application of elemental sulphur amendments on the control pasture and acidified pasture treatments. The percentage cover of all plant species was recorded in 10 randomly located 2-m × 2-m quadrats in each plot. Plant species were then classified as either: (1) Characteristic of acid/calcifugous grassland and heathlands (*AH*), (2) characteristic of mesotrophic grassland (*M*), or (3) intermediate species which can occur in both mesotrophic and acid grasslands (*In*). Classifications were according to the National Vegetation Classification [30,31].

2.4. Chemical Variables

Soil pH of 2.5:1 water-soil slurry was measured after shaking for 15 min at 120 rpm [32]. Exchangeable Al^{3+}, Ca^{2+}, Na^{+}, Mg^{2+}, K^{+} and Mn^{2+} and extractable Fe were determined in a 10:1 soil to 1 M ammonium nitrate (NH_4NO_3) extraction [33]. Soil available P was extracted with a 0.1 M H_2SO_4 solution [34]. Results and methods of chemical analysis are presented in Tibbett et al. [22].

2.5. Biological Variables

Biological variables encompassed both microbial analysis and soil fauna. Soil microbes react to changes in their environment by adjusting their (1) activity, (2) biomass, and (3) community structure [35]. Consequently, microbial analysis of the soil included microbial biomass determination of C, N, and P; microbial respiration; and microbial community composition. Microbial techniques were applied only to the top 0–5-cm soil depth.

Faunal groups selected as indicators needed to: (1) form a dominant group and occur in all soil types, (2) have a high abundance and high biodiversity, and (3) play an important role in soil

functioning, e.g., in food webs [35]. These factors, combined with ease of collection and identification, led to our selection of the following soil fauna variables: Abundance and biomass of juvenile and adult epigeic, endogeic, and anecic earthworms; abundance and feeding group of nematodes; abundance of rotifers; and abundance of tardigrades. Methods for each of these variables are described below.

2.5.1. Soil Microbial Biomass

Microbial biomass was determined by the fumigation–extraction method. Two sets of field-fresh soils were bottled with a mass equivalent to 12.5 g for C and N extraction, and 5 g for P extraction of oven-dried sample. One set of the samples was fumigated with ethanol-free chloroform ($CHCl_3$) for 24 h ± 1 h, for comparison.

Microbial biomass C and N were extracted in fumigated and nonfumigated samples with 50 mL of 0.5 M potassium sulphate (K_2SO_4) solution [36]. Microbial biomass P was extracted in fumigated and nonfumigated samples with 100 mL of sodium hydrogen carbonate ($NaHCO_3$) reagent [37]. Briefly, samples were shaken for 30 min and then filtered and stored at −15 °C until analysis. The determination of microbial biomass C and N consisted of the removal of inorganic C in the form of CO_2 through potassium persulphate ($K_2S_2O_8$) acidification and ultraviolet (UV) light irradiation. The solution was measured at 550 nm in a spectrophotometer.

For the determination of microbial biomass P, 5 mL soil extract, 1 mL 1.5 M H_2SO_4, 20 mL 0.15% m/v ammonium molybdate reagent, and 5 mL ascorbic acid ($C_6H_8O_6$) were mixed and left for 30 min for color development. The absorbance was measured at 880 nm in the spectrophotometer and microbial P, in mg L^{-1}, determined from a standard graph.

Final microbial biomass C, N, and P were calculated with the conversion coefficients for carbon (K_{EC} = 0.45), nitrogen (K_{EN} = 0.45), and phosphorus (K_{EP} = 0.40) [38–41].

2.5.2. Community-Level Physiological Profiles

Microbial respiration for different carbon sources was determined using the multiple substrate-induced respiration (MSIR) MicroResp™ method [42,43] as a convenient, sensitive, and rapid analysis for detection of community-level physiological profiles (CLPP). The MicroResp™ colorimetric method [44] is based on the measurement of the rate of CO_2 generated by soil microorganisms in response to addition of different carbon substrates. The resultant profiles reflect the functional ability of the soil community to degrade the added substrates. After incubation of fresh soils (3–5 days at 25 °C), 0.25–0.48 g of soil was added to a 96-deep-well microtiter plate. Each soil was prepared in 32 wells (4 replicates).

The carbon sources selected were: Two amino acids, γ-Aminobutyric acid (GABA) and L-Alanine (ALA); one carbohydrate, α-D-Glucose (Glu); three carboxylic acids, L-Malic acid (MalA), Citric acid (CitA), and α-Ketoglutaric acid (aKG); and water. Carbon sources were added to soils in deep-well plates and then sealed with the detection plates containing cresol red.

The detection plates were read at 570-nm absorbance at the beginning of the incubation (time 0) and immediately after 6-h incubation (time 6). Carbon dioxide development, expressed as μg C g^{-1} h^{-1}, was assessed using SoftMax Pro 6 Software.

2.5.3. Phospholipid Fatty Acid Analysis

Fatty acid profiles, indicative of microbial community phenotypic profile, were attained by phospholipid fatty acid (PLFA) analysis. The lipid extraction was accomplished by the modified Bligh and Dyer method [45] with a water replacement by citrate buffer [46] in a final 0.8:1:2 (v/v/v) citrate buffer:chloroform:methanol reagent solution. The reagent was added to 5 g of ground, freeze-dried soil. The resultant upper organic layer was mixed with 4 mL of chloroform and 4 mL of 0.15 M citrate buffer. Under a stream of N_2 at <37 °C, the lower organic layer was dried. Solid phase extraction, or silicic acid column chromatography, was used as the lipid fractionation method [47]. Phospholipid fractions were eluted with 8 mL of methanol and dried under a stream of N_2 at <37 °C. The

phospholipid fraction was reconstituted and 0.2 M methanolic potassium hydroxide (KOH/MeOH) in order to hydrolyze the lipids. The solution was incubated for 30 min and 1 M acetic acid was added to stop the reaction. Finally, 5 mL of 4:1 (v/v) hexane:chloroform and 3 mL of deionized water were added. The top layer was filtered through sodium sulphate (Na_2SO_4) and rinsed with hexane. The solution was dried under a stream of N_2 at room temperature. The dried samples were reconstituted with hexane, peak separated, and detected gas chromatography flame ionization detector (GC-FID) with an Agilent Technologies 6890N. The software used for analysis was Agilent G2070 ChemStation for GC systems and results were expressed as mol%.

Signature PLFAs were classified as biomarkers in five microbial groups: Gram-positive bacteria, gram-negative bacteria, arbuscular mycorrhizal fungi, ectomycorrhizal fungi, and actinobacteria. Twelve of the 33 fatty acids analyzed have been reported as biomarkers of specific microorganisms to differentiate the bacterial or fungal dominance in the soils (Table 1). The PLFA 18:1*ω*9*c* was not included in any group for the total biomarkers' calculation as it has been found in both fungi [48–50] and bacteria [51–53].

Table 1. Phospholipid fatty acids (PLFAs) or biomarkers in specific microbial groups.

Microbial Group	Signature PLFAs or Biomarkers	References
Total bacteria	15:0, 15:0*i*, 15:0*ai*, 16:0*i*, 17:0*i*, 17:0*ai*, 16:1 *ω*7*c*, 17:0*c*, 19:0*c*	[47,54,55]
Gram-positive bacteria	15:0*i*, 15:0*ai*, 16:0*i*, 17:0*i*, 17:0*ai*	[56,57]
Gram-negative bacteria	16:1*ω*7*c*, 17:0*c*, 19:0*c*	[51,58]
Arbuscular mycorrhizal fungi	16:1ω5	[59]
Ectomycorrhizal fungi	18:2ω6,9	[46,59–63]
Actinobacteria	18:0 (10Me)	[64,65]

2.5.4. Soil Fauna

Earthworms, nematodes, rotifers, and tardigrades were collected in each of the plots 16 years after initial application of elemental sulphur amendments. For each plot, a 20-cm × 20-cm × 20-cm soil block was removed using a flat shovel and placed in trays in the field for hand sorting. Earthworms were carefully removed, counted, and placed in a subsample of soil to be transported back to the lab for classification. Specimens were rinsed, blotted dry, and weighed individually (fresh weight). Earthworms were also recorded as juvenile or adult, and allocated to ecological groups: Epigeic, endogeic, or anecic, following Sherlock [66].

Soil from the block was homogenized prior to collecting ~100 g subsamples which were sieved to 3.35 mm and stored at 4 °C. Nematode, tardigrade, and rotifer extractions were conducted on these subsamples using a modified Baermann funnel technique, substituting extraction trays for funnels (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 4x magnification. The two sampling times (after 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. Gravimetric soil moisture content was determined by drying at 105 °C [32]. In addition, 100 nematodes per sample were also identified to feeding-group level (bacterial feeder, fungal feeder, plant parasite, omnivore, or predator) as described by Yeates et al. [67,68].

2.6. Heathland Restoration Index (HRI)

Five different HRIs were created in this study (Table 2): (1) An HRI based on chemical variables only (HRI_{chem}), (2) an HRI based on biological variables only (HRI_{bio}), and (3) a combined HRI encompassing both chemical and biological indicators (HRI_{comb}). Biological indicators were then partitioned to create a biological HRI for: (4) Microbes (HRI_{mic}) and (5) soil micro- and macrofauna (HRI_{fau}) (Table 2).

Calculations of the HRIs were based on a soil quality index (SQI) described by Andrews et al. [20] and Tibbett et al. [22]. 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 analyzed by PCA. Variables were analyzed using PCA in order to generate a minimum dataset based on the so-called 'highly weighted factors'. Highly weighted factors are defined as the variable with the highest eigenvector in each principal component (PC) that has an eigenvalue >1, plus any variables with eigenvectors within 10% of the highest eigenvector, or >0.4 in PCs with an eigenvalue >1. A Pearson's product moment correlation coefficient (r^2) was conducted pair-wise on variables highlighted to be highly weighted factors. When $r^2 > 0.60$, only the variable with the highest eigenvector was retained for the minimum dataset; when $r^2 < 0.60$, both variables were retained for the minimum dataset. Variables within the minimum dataset were then subjected to a linear scoring system based on whether 'less is better' (native heathland sites are lower than the control plots) or 'more is better' (native heathland sites are higher than the control plots). The higher the HRI, the closer the sample resembles a heathland environment. For 'less is better' variables, the lowest observed value was divided by each observation (e.g., the lowest observed value received a score of 1) and for 'more is better' indicators, each observation was divided by the highest observed value (e.g., the highest observed value received a score of 1).

The *HRI* was then calculated as follows:

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

where *S* is the score of the indicator variable and W the weighted factor derived from the PCA:

$$W = \frac{V\%}{CV\%}$$

where *V* is the proportion variance (%) explained by the principal component in which the variable has the highest eigenvalue (or is within 10% of the highest eigenvector, or above >0.4). *CV* is the cumulative variance (%) explained by all principal components with eigenvalue >1. The variables input into the PCA depended on the HRI in question (Table 2). The weighed factors, based on the PCA output, means that each HRI will have a different value for the highest possible HRI that can be assigned to a sample. We elected not to standardize the different HRIs to one another because, at this stage, we were interested in treatment effects within the different HRIs (chemical only, biological only, etc.).

2.7. Statistical Analysis

The effect of treatments (chemically acidified pasture, control pasture, native heathland, and native acid grassland) on a single characteristic, such as HRI or pH, was assessed with the use of a one-way ANOVA and Tukey's post hoc testing. This analysis used Minitab Version 19, after being tested for homogeneity of variances with a Levene's test. Multivariate analysis, including PCA, nonmetric multidimensional scaling (nMDS), cluster analysis, and analysis of similarity (ANOSIM), was conducted in Primer Version 6. An nMDS of plant community was conducted using a Bray Curtis resemblance matrix based on the square root of plant species' abundance, with 1000 restarts and one-way ANOSIM, with treatment as a factor (1000 permutations). Soil chemical and biological variables were analyzed using PCA, cluster analysis, and ANOSIM (based on Euclidean distance resemblance matrices) to examine multivariate differences between treatments. Data were normalized prior to Euclidean distance resemblance matrices and were generated using Primer Version 6.

Table 2. Variables included in principal component analysis in order to generate a minimum dataset for five different variations of Heathland Restoration Index: chemical variables only (HRI_{chem}); soil micro- and macrofauna variables only (HRI_{fau}); soil microbial variables only (HRI_{mic}); soil biological variables (microbes an fauna) combined (HRI_{bio}); and all variables (chemical and biological) combined (HRI_{comb}).

Chemical HRI (HRI_{chem})	**Soil Micro- and Macrofauna HRI (HRI_{fau})**	**Soil Microbes HRI (HRI_{mic})**	**Biological HRI (HRI_{bio})**	**Combined HRI (HRI_{comb})**
0-5 cm: Al; **Ca**; **Fe**; K;	*Earthworm Abundance and Mass:*	Total Bacteria Abundance	All variables listed in HRI_{fau} and HRI_{mic}	All variables listed in HRI_{chem} and HRI_{bio}
Mg; Mn; Na;	Total (juvenile and adult; all ecological groups)	G^+:G^-	together	together
P; **pH**	Adult (all ecological groups)	**Total Actinobacteria Abundance**		
	Juvenile (all ecological groups)	Total Fungi Abundance	34 Variables	58 variables
5-10 cm: Al; Ca; **Fe**; K;	Total epigeic (juvenile and adult)	**Total Arbuscular Mycorrhizal Fungi Abundance**		
Mg; Mn; Na;	Adult epigeic	Bacteria:Fungi	7 Variables in minimum dataset:	15 variables in the minimum dataset
P; pH	Juvenile epigeic	Microbial Biomass C, N and P		
	Total endogeic (juvenile and adult)		**Gram positive bacteria: Gram negative bacteria ratio (G^+:G^-)**	**G^+:G**
10-15 cm: Al; Ca; Fe;	Adult endogeic	*Microbial Respiration (MicroResp™)*	**Total Actinobacteria abundance**	**Total Actinobacteria abundance**
K; **Mg**; **Mn**;	**Juvenile endogeic**	α-Ketoglutaric acid	**Total Fungi Abundance**	**Bacteria:Fungi**
Na; P; pH	Total anecic (juvenile and adult)	L-Alanine	**Total Arbuscular Mycorrhizal Fungi**	**Microresp α-D-Glucose**
	Adult anecic	**Citric acid**	**Microresp Citric Acid**	**Microbial Biomass N**
	Juvenile anecic	ɣ-Aminobutyric acid	**Juvenile Endogeic Abundance**	**Juvenile Endogeic Mass**
24 Variables	*Nematode Abundance:*	α-D-Glucose	**Total Tardigrade Abundance**	**Total Tardigrade Abundance**
	Total (all functional groups)	L-Malic acid		**0–5 cm pH**
7* Variables in	Plant Parasite	Water		**0–5 cm Ca**

minimum dataset	Bacterial Feeder		**0–5 cm Fe**
	Fungal Feeder	14 Variables	**5–10 cm Mg**
	Predator		**5–10 cm P**
	Omnivore	3* Variables in minimum dataset	**10–15 cm Al**
	Total Rotifer Abundance		**10–15 cm Mn**
	Total Tardigrade Abundance		**10–15 cm Na**
	24 Variables		
	2* Variables in minimum dataset		

* Variables that are part of the minimum dataset are in bold underline.

3. Results

3.1. Soil pH

Fourteen years after the first application of the acidifying agent, the acidified pasture maintained a significantly lower soil pH than the control pasture in all depth increments (Figure 1). This reduced soil pH in the acidified pasture was comparable with the neighboring acid grassland environment at all depths, and heathland from 5 cm downwards (Figure 1).

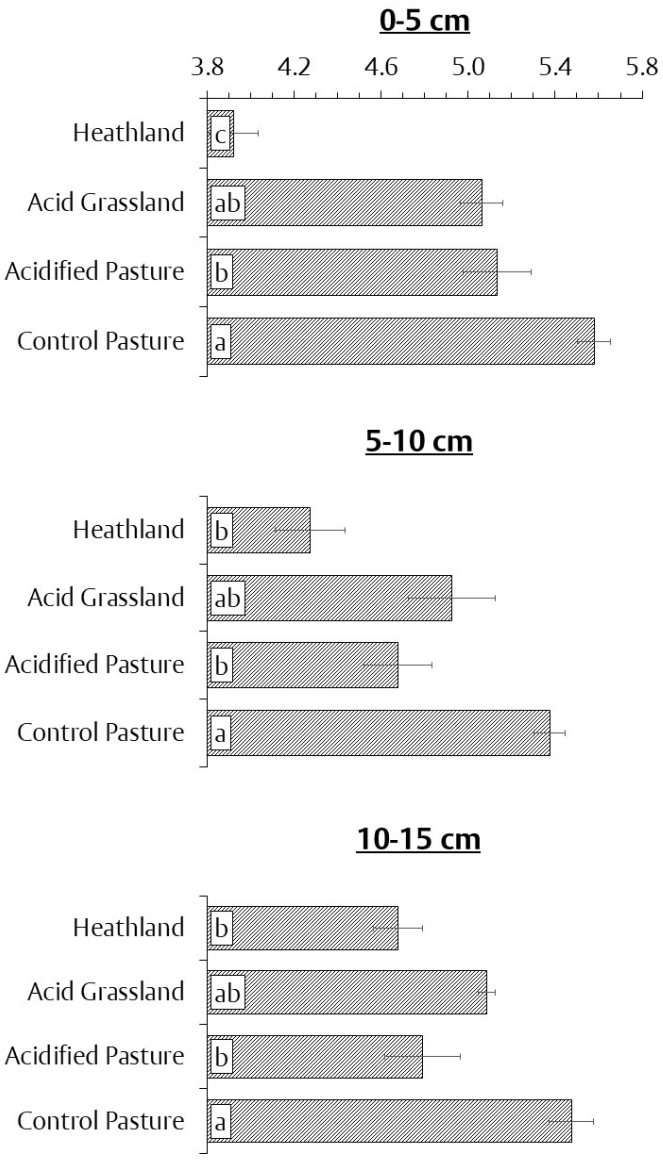

Figure 1. Soil pH at 0–5-cm, 5–10-cm, and 10–15-cm depths. Measured in 2.5:1 water–soil slurry. Error bars represent standard error and the same lowercase letters in bars denote treatments that are not significantly different, within each depth, according to one-way ANOVA and Tukey's post hoc testing ($p > 0.05$); n = 10 for acidified pasture and control pasture; n = 4 for heathland and acid grassland.

3.2. Plant Community

Nonmetric multidimensional scaling of the vegetation assemblage of the acidified pasture and the control pasture showed that acidified pasture resulted in a plant community that is significantly different to the control pasture (Figure 2). This difference is significant according to ANOSIM ($p < 0.05$). Vectors representing heathland and acid grassland species, such as *Calluna vulgaris* (labeled as AH 3 in Figure 2), *Erica cinereal* (AH 4), *Erica tetralix* (AH 5), and *Agrostis capillaris* (AH 1), resulted in acidified pasture sites starting to cluster above the control pasture, which is dominated by more mesotrophic grassland species.

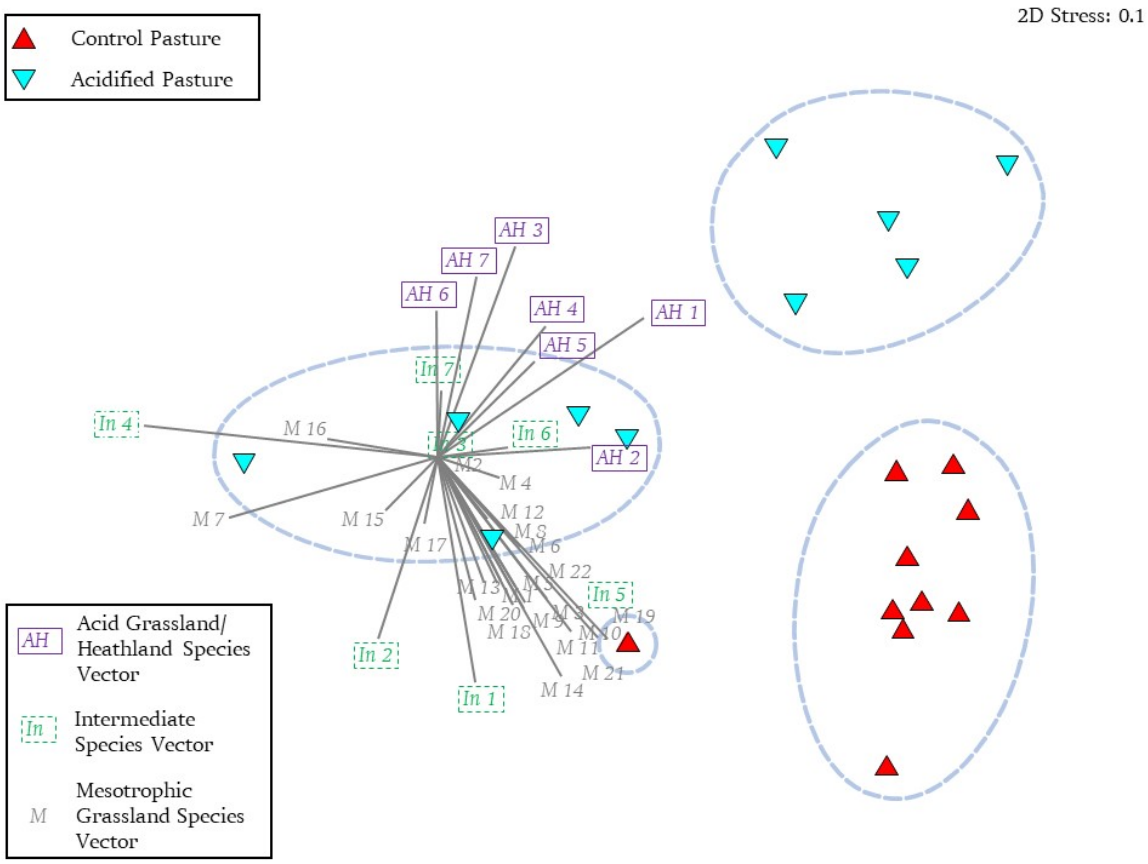

Figure 2. Nonmetric multidimensional scaling of plant community on acidified and control pasture. Based on Bray-Curtis resemblance matrix, dashed-line clusters encircle plots ≥ 50% similarity. Species vectors' key: AH 1—*Agrostis capillaris*; AH 2—*Anthoxanthum odoratum*; AH 3—*Calluna vulgaris*; AH 4—*Erica cinereal*; AH 5—*Erica tetralix*; AH 6—*Molinia caerulea*; AH 7—*Ulex europaeus*; In 1—*Cynosurus cristatus*; In 2—*Holcus lanatus*; In 3—*Juncus articulates*; In 4—*Juncus effuses*; In 5—*Lotus corniculatus*; In 6—*Luzula campestris/multiflora*; In 7—*Rumex acetosella*; M 1—*Achillea millefolium*; M 2—*Agrostis stolonifera*; M 3—*Bellis perennis*; M 4—*Centaurium erythraea*; M 5—*Cerastium fontanum*; M 6—*Cirsium arvense*; M 7—*Cirsium palustre*; M 8—*Crepis capillaris*; M 9—*Elymus repens*; M 10—*Festuca rubra*; M 11—*Hypochoeris radicata*; M 12—*Leontodon autumnalis*; M 13—*Lolium perenne*; M 14—*Plantago lanceolate*; M 15—*Ranunculus repens*; M 16—*Rubus fructicosus*; M 17—*Senecio jacobaea*; M 18—*Taraxacum officinale agg.*; M 19—*Trifolium dubium*; M 20—*Trifolium pratense*; M 21—*Trifolium repens*; M22—*Vicia sativa*. Groupings of species (AH, In, or M) according to National Vegetation Classification [30,31].

3.3. Chemical Heathland Restoration Index (HRI_{chem})

The soil chemical conditions in the acidified pasture moved in a trajectory that resulted in an HRI_{chem} that is significantly higher than the control pasture and statistically comparable to acid grassland (Figure 3a). The chemical environment in the acidified pasture has not, however, matched the native heathland in terms of the $HRI_{chem.}$

Principal component analysis put forward seven variables for the minimum dataset of the $HRI_{chem.}$ These vectors are labelled in Figure 3b. In PC1, the acidified pasture showed a chemical composition closer to acid grassland and between the control pasture and heathland soils. In PC2, the acidified pasture deviated from the other treatments (Figure 3b).

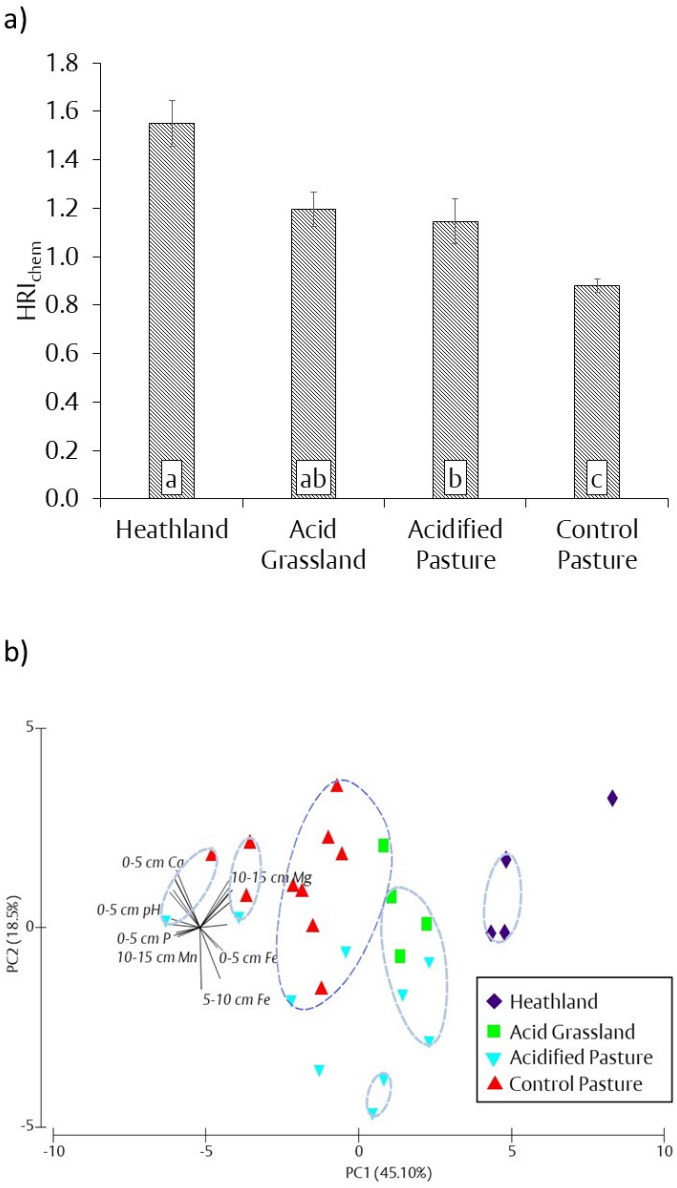

Figure 3. Chemical variables (**a**) Heathland Restoration Index (HRI_{chem}). Error bars represent standard error and the same lowercase letters in bars denote treatments that are not significantly different ($p > 0.05$) according to one-way ANOVA and Tukey's post hoc testing. The highest possible value for HRI_{chem} was 1.92 (i.e., if a sample scored 1 for every variable). (**b**) Principal component analysis for HRI_{chem}. Vectors of minimum dataset variables used in the HRI_{chem} labeled. See Table 2 for list of variables used. Dashed-line clusters surround samples with a Euclidean distance ≤4.5.

The trends observed in the HRI_{chem} were in concordance with $ANOSIM_{chem}$ (Table 3). The acidified pasture was significantly different from the control pasture and heathland, but comparable with the acid grassland (Table 3).

Table 3. Analysis of similarity matrix based on chemical variables ($ANOSIM_{chem}$). Pair-wise tests of Euclidean distance of treatment resemblance based on chemical variables (see Table 2 for list of variables used). Treatments that are significantly different from one another have a *p*-value <0.05 and are shown in bold.

	ANOSIM Pair-wise Test *p*-value		
	Heathland	**Acid Grassland**	**Acidified Pasture**
Heathland	-	-	-
Acid Grassland	**0.029**	-	-
Acidified Pasture	**0.004**	0.378	-
Control Pasture	**0.002**	**0.021**	**0.002**

3.4. Microbial Heathland Restoration Index (HRI_{mic})

Contrary to the HRI_{chem}, the HRI_{mic} was not significantly different for the acidified pasture when compared to the control (Figure 4a). That said, native acid grassland was also comparable to the control pasture. In terms of HRI_{mic}, only the heathland sites had a significantly different HRI_{mic} than the other treatments (Figure 4a). The PCA revealed that, although the heathland is more dissimilar to the other treatments due to clear separation along PC1 (Figure 4b), the acidified pasture is separated from the control pasture on PC2, but it seems to have become more dissimilar to the acid grassland and heathland environment (Figure 4b). These observations are reiterated in the $ANOSIM_{mic}$ (Table 4), where there is a significant difference between the acidified pasture and all other treatments. The native heathland and the acid grassland environments are the only two treatments that are not significantly different to one another, according to $ANOSIM_{mic}$.

Table 4. Analysis of similarity matrix based on microbial variables ($ANOSIM_{mic}$). Pair-wise tests of Euclidean distance of treatment resemblance based on microbial variables (see Table 2 for list of variables used). Treatments that are significantly different from one another have a *p*-value < 0.05 and are shown in bold.

	ANOSIM Pair-wise Test *p*-value		
	Heathland	**Acid Grassland**	**Acidified Pasture**
Heathland	-	-	-
Acid Grassland	0.057	-	-
Acidified Pasture	**0.003**	**0.013**	-
Control Pasture	**0.001**	**0.004**	**0.002**

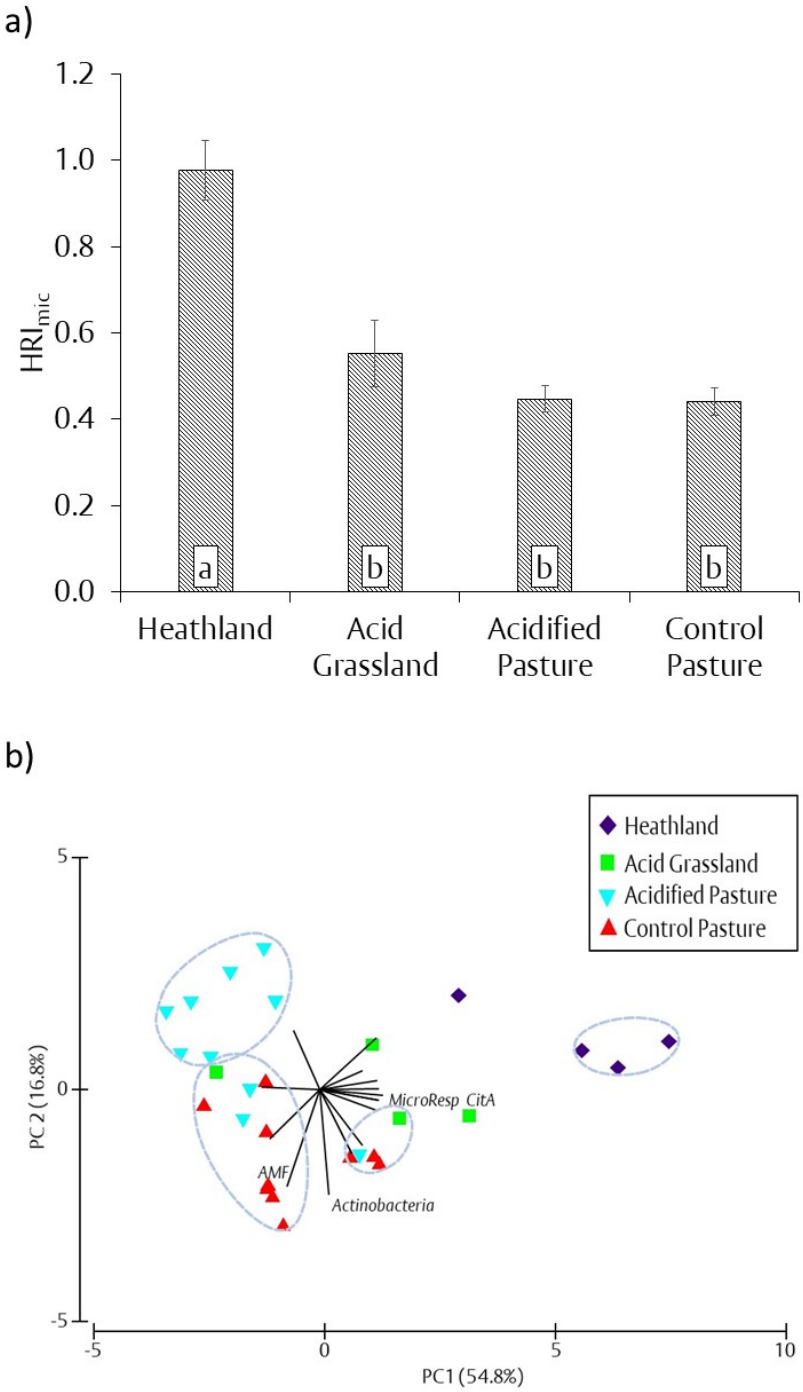

Figure 4. Microbial variables (**a**) Heathland Restoration Index (HRI_{mic}). Error bars represent standard error and the same lowercase letters in bars denote treatments that are not significantly different ($p > 0.05$) according to one-way ANOVA and Tukey's post hoc testing. The highest possible value for HRI_{mic} is 1.24 (i.e., if a sample scored 1 for every variable). (**b**) Principal component analysis for HRI_{mic}. Vectors of minimum dataset variables used in the HRI_{mic} labeled. See Table 2 for list of variables used. Dashed-line clusters surround samples with a Euclidean distance ≤4.

3.5. Soil Fauna Heathland Restoration Index (HRI_{fau})

The acidified pasture had a greater HRI_{fau}, compared to the control pasture, but not to a degree that resulted in a significant difference (Figure 5a). However, the acidified pasture HRI_{fau} was not significantly different to the heathland, suggesting an upward trajectory of the acidified pasture treatment toward the native heathland environment, in terms of HRI_{fau} (Figure 5a). Using multivariate analysis (Figure 5b), the acidified pasture is more similar to the heathland resulting in there being no significant difference between them, according to $ANOSIM_{fau}$ (Table 5). However, unlike HRI_{fau}, there was no significant difference between the heathland and the control pasture according to $ANOSIM_{fau}$.

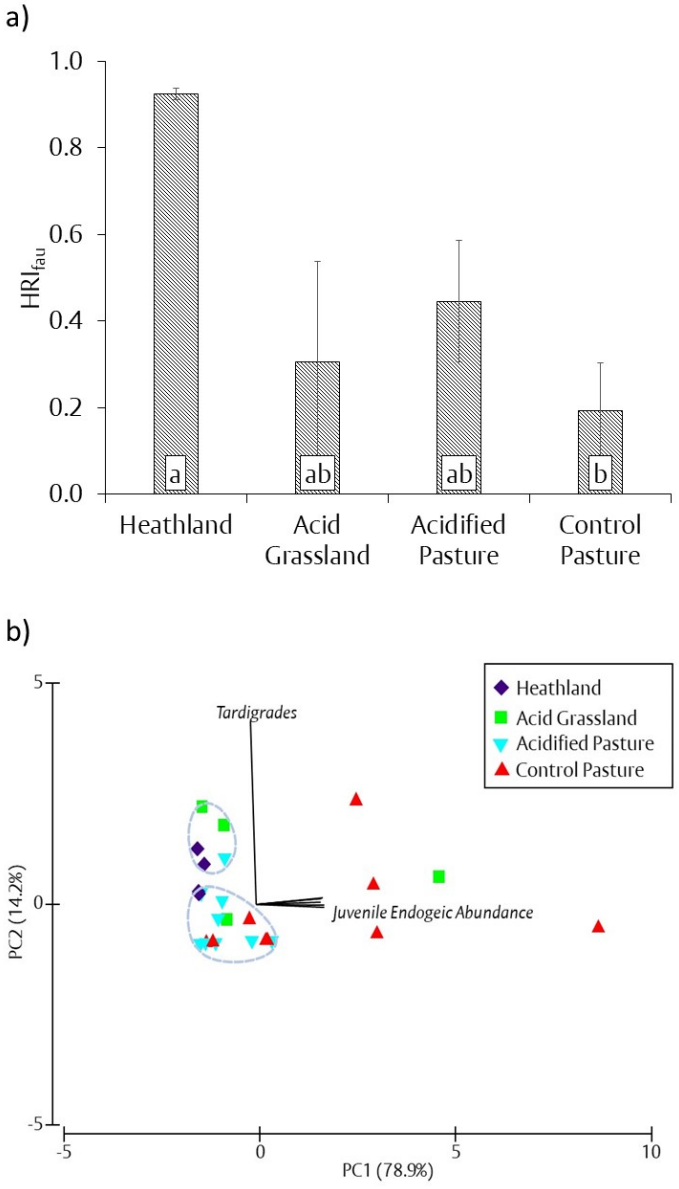

Figure 5. Soil fauna variables (**a**) Heathland Restoration Index (HRI_{fau}). Error bars represent standard error and the same lowercase letters in bars denote treatments that are not significantly different ($p > 0.05$) according to one-way ANOVA and Tukey's post hoc testing. The highest possible value for HRI_{fau} is 1.00 (i.e., if a sample scored 1 for every variable). (**b**) Principal component analysis for HRI_{fau}. Vectors of minimum dataset variables used in the HRI_{fau} labeled. See Table 2 for list of variables used. Dashed-line clusters surround samples with a Euclidean distance ≤2.

Table 5. Analysis of similarity matrix based on soil fauna variables (ANOSIM$_{fau}$). Pair-wise tests of Euclidean distance of treatment resemblance based on soil fauna variables (see Table 2 for list of variables used). Treatments that are significantly different from one another have *p*-value < 0.05 and are shown in bold.

	ANOSIM Pair-wise Test *p*-value		
	Heathland	**Acid Grassland**	**Acidified Pasture**
Heathland	-	-	-
Acid Grassland	0.086	-	-
Acidified Pasture	0.068	**0.016**	-
Control Pasture	0.243	0.229	**0.017**

3.6. Biological Heathland Restoration Index (HRI$_{bio}$)

When microbial and soil faunal parameters were combined, the resulting HRI$_{bio}$ mirrored the trends seen in HRI$_{mic,}$ i.e., there were no significant differences between the control pasture, acidified pasture, and acid grassland. However, the heathland HRI$_{bio}$ is significantly higher than all other treatments (Figure 6a). This is also apparent in the separation in the clustering of treatments in the PCA (Figure 6b) since the acidified pasture is dissimilar from the control pasture on PC2, resulting in a significant difference according to ANOSIM$_{bio}$ (Table 6). However, the acidified pasture treatments are still separated from the acid grassland or heathland treatments on PC1, reflected in Table 6 with acidified pasture being significantly different to all other treatments according to ANOSIM$_{bio}$.

Table 6. Analysis of similarity matrix based on biological variables, microbial, and soil fauna variables combined (ANOSIM$_{bio}$). Pair-wise tests of Euclidean distance of treatment resemblance based on biological variables (see Table 2 for list of variables used). Treatments that are significantly different from one another have *p*-value < 0.05 and are shown in bold.

	ANOSIM Pair-wise Test *p*-value		
	Heathland	**Acid Grassland**	**Acidified Pasture**
Heathland	-	-	-
Acid Grassland	0.057	-	-
Acidified Pasture	**0.001**	**0.006**	-
Control Pasture	**0.003**	0.084	**0.001**

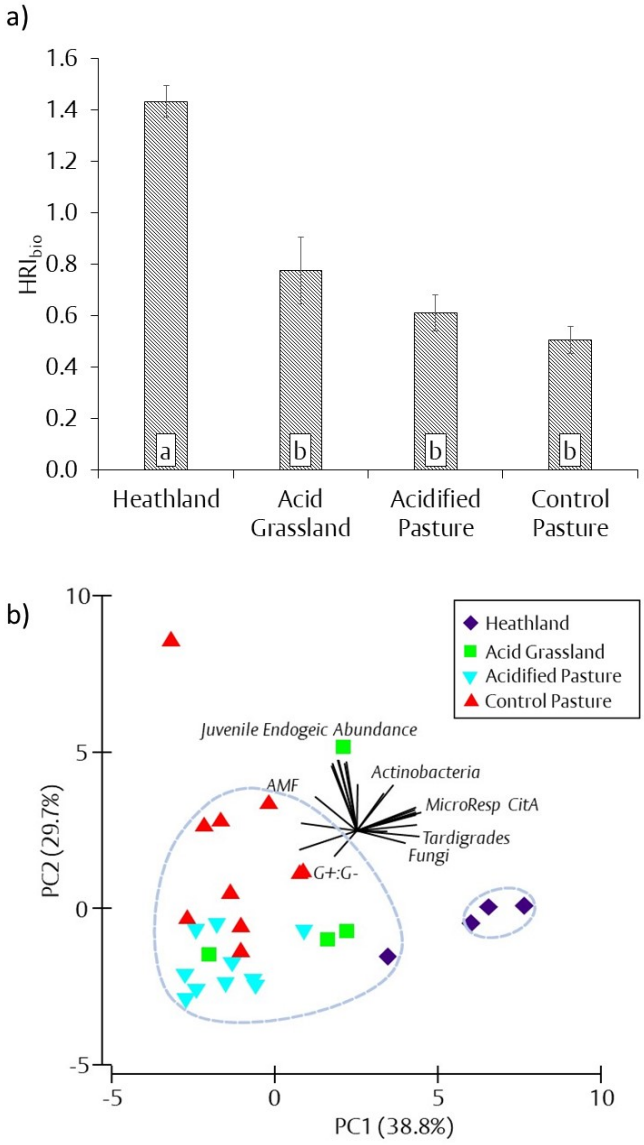

Figure 6. Biological variables, microbial, and soil fauna variables combined (**a**) Heathland Restoration Index (HRI_{bio}). Error bars represent standard error and the same lowercase letter in bars denote treatments that are not significantly different ($p > 0.05$) according to one-way ANOVA and Tukey's post hoc testing. The highest possible value for HRI_{bio} is 1.63 (i.e., if a sample scored 1 for every variable). (**b**) Principal component analysis for HRI_{bio}. Vectors of minimum dataset variables used in the HRI_{bio} labeled. See Table 2 for list of variables used. Dashed-line clusters surround samples with a Euclidean distance ≤ 6.

3.7. Combined Chemical and Biological Heathland Restoration Index (HRI_{comb})

When all chemical, microbial, and soil fauna variables were included to generate a combined HRI (HRI_{comb}), the acidified pasture was not significantly different from the control pasture treatment (Figure 7a). However, it was a little higher than the control treatment, so that it was also not significantly different from the acid grassland environment, suggesting an upwards trajectory. Multivariate analysis ($ANOSIM_{comb}$, Table 7) indicates that the acidified pasture is significantly different from all other treatments. However, closer examination of Figure 7b indicates that, although the acidified pasture treatment is separated from the control pasture on PC2, it is still separated from the heathland and acid grassland environments on PC1.

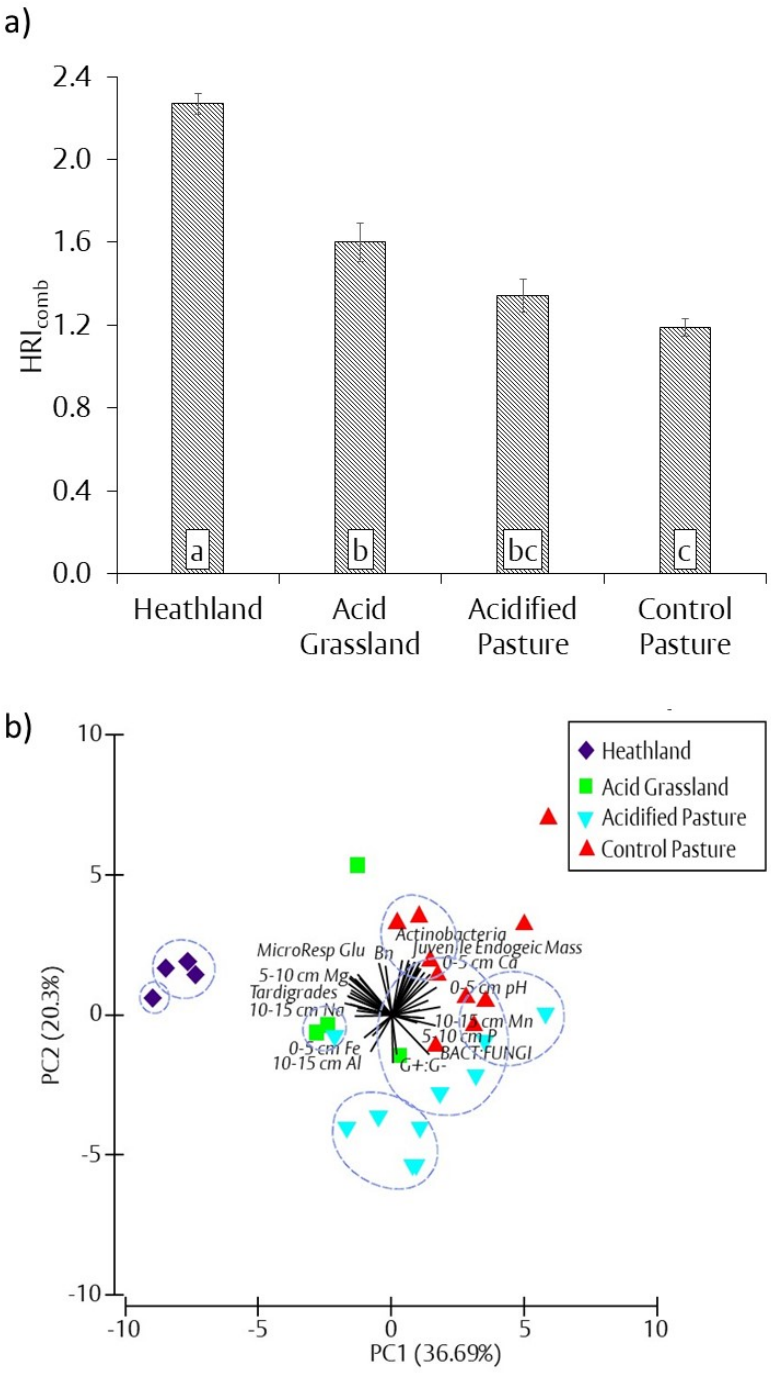

Figure 7. All available variables combined (**a**) Heathland Restoration Index (HRI_{comb}). Error bars represent standard error and the same lowercase letter in bars denote treatments that are not significantly different ($p > 0.05$) according to one-way ANOVA and Tukey's post hoc testing. The highest possible value for HRI_{comb} is 3.02 (i.e., if a sample scored 1 for every variable). (**b**) Principal component analysis for HRI_{comb}. Vectors of minimum dataset variables used in the HRI_{comb} labeled. See Table 2 for list of variables used. Dashed-line clusters surround samples with a Euclidean distance ≤ 6.

Table 7. Analysis of similarity matrix based on all available variables combined ($ANOSIM_{comb}$). Pair-wise tests of Euclidean distance of treatment resemblance based on all variables (see Table 2 for list of

variables used.). Treatments that are significantly different from one another have p-value <0.05 and are shown in bold.

	ANOSIM Pair-wise Test p-value		
	Heathland	**Acid Grassland**	**Acidified Pasture**
Heathland	-	-	-
Acid Grassland	**0.029**	-	-
Acidified Pasture	**0.001**	**0.048**	-
Control Pasture	**0.001**	0.060	**0.001**

3.8. Data Summary

Table 8 summarizes the significant differences observed between the control pasture and the acidified pasture, and the acidified pasture and heathland. According to ANOSIM, regardless of the variables used (chemical, microbial faunal, or a combination), the acidified pasture treatment was significantly different to the control pasture. Using an HRI, however, only resulted in a significant difference between the acidified pasture and the control pasture when the index was based on chemical variables alone (HRI_{chem}). With the exception of soil fauna, there was a significant difference between the acidified pasture and the heathland in both HRI and ANOSIM (Table 8).

Table 8. Summary of pair-wise comparisons of acidified pasture compared to control pasture and heathland, according to Heathland Restoration Index and analysis of similarity. *Heathland restoration indices marked with a ✓ resulted in a significant difference in treatments according to one-way ANOVA and Tukey's post hoc testing ($p < 0.05$). See part a of Figures 3–7 for full results. **Analysis of similarity pair-wise comparisons marked with a ✓ resulted in a significant difference in treatments based on Euclidean matrix of similarity ($p < 0.05$).

Significant Difference in Pair-wise Comparison? (✓ if $p < 0.05$)				
	Heathland Restoration Index*		Analysis of Similarity**	
Acidified Pasture Vs Control Pasture	HRI_{chem}	✓	$ANOSIM_{chem}$	✓
	HRI_{mic}		$ANOSIM_{mic}$	✓
	HRI_{fau}		$ANOSIM_{fau}$	✓
	HRI_{bio}		$ANOSIM_{bio}$	✓
	HRI_{comb}		$ANOSIM_{comb}$	✓
Acidified Pasture Vs Heathland	HRI_{chem}	✓	$ANOSIM_{chem}$	✓
	HRI_{mic}	✓	$ANOSIM_{mic}$	✓
	HRI_{fau}		$ANOSIM_{fau}$	
	HRI_{bio}	✓	$ANOSIM_{bio}$	✓
	HRI_{comb}	✓	$ANOSIM_{comb}$	✓

4. Discussion

4.1. Aboveground Response to Changes in Soil Chemical Properties

Application of elemental sulphur has resulted in the chemical conditions in the acidified pasture being significantly different to the control pasture, both in terms of multivariate analysis of similarity ($ANOSIM_{chem}$) and Heathland Restoration Index (HRI_{chem}). Soil pH and phosphorus have been found to drive aboveground floral community composition in heathland and grassland systems [69–71] and low pH and P concentrations are characteristic of the native heathland in this study [22].

Consequently, the plant community in the acidified pasture is starting to shift toward one containing more ericaceous species and other species typical of UK heathlands, such as *Agrostis capillaris* (common bent) [72]. As a result, the vegetational assemblage of the acidified pasture is significantly different to the control pasture according to ANOSIM. The influence of acidity on aboveground plant communities in heathlands has been well documented in other locations [71,73] and this study is in concordance with those findings.

For surficial soil pH (0–5 cm), the acidified pasture is equivalent to that observed in the acid grassland; however, the pH has not reduced enough to be comparable with the heathland. For soil chemistry, using $ANOSIM_{chem}$ or HRI_{chem}, there is no significant difference between the acidified pasture and the acid grassland, but we do observe a significant difference between the acidified pasture and the heathland. It is clear that the elemental sulphur amendments have reduced the soil pH so that it now reflects an acid grassland environment. However, the soil pH of the acidified pasture, 14 years after application of elemental sulphur, is more than one pH unit higher than it was just three years after application, when it would have been comparable to nearby heathland [23]. This suggests that we have passed the peak of influence of elemental sulphur application and the acidification process is beginning to reverse in the acidified pasture.

4.2. Belowground Response to Changes in Soil Chemical Properties

Management activities and perturbations are thought to induce a response in soil biological properties more rapidly than a response in soil chemical or physical properties [26]. However, no HRI that included biological variables (HRI_{mic}, HRI_{fau}, $HRI_{bio,}$ or HRI_{comb}) resulted in a significant difference between the acidified pasture and the control pasture. This finding suggests that either (1) belowground biology, in this system, is slower to respond to adjusted soil chemistry than the aboveground plant community, (2) belowground biology is less affected by changes in soil chemistry compared to aboveground vegetation, or (3) belowground biology rebounded more rapidly if the acidified pasture soils were beyond the peak effects of the elemental sulphur application (discussed above), i.e., biological parameters respond more rapidly to perturbation (i.e., acidifying elemental sulphur application), but also revert more rapidly after the perturbation.

Whether changes in plant community composition lead to changes in belowground soil microbial communities or whether the belowground microbial communities facilitate a change in vegetation is a quandary for restoration ecologists [74]. The data we present on the shifts in plant community composition and the HRI_{mic} suggest that the former is the case in heathland reversion from improved pasture. Vegetation structure was found by Vogels et al. [75] to play an important role in determining density and species richness of the aboveground faunal groups in heathlands; so, perhaps the same is true for belowground soil fauna and microorganisms. However, clippings of *Calluna vulgaris* were sown in the acidified pasture one year after the final application of elemental sulphur [23], facilitating aboveground community change. Early-stage presence, or inoculation, of heathland belowground communities can reinforce the development of a heathland system through the interactions they form [76]. No such introduction of microbial communities was undertaken in our study.

Return of species in restoration does not only depend on the habitat quality, e.g., the appropriate soil conditions, but also on the capacity of the species to recolonize the habitat [77]. It has been suggested that plant communities condition the soil through rhizodeposition and exudates, which modify the physical, chemical, and biological environment in soil, thereby impacting on the microbial community [78]. However, some studies had contrasting results and found that plants have no impact on shaping the microbial community in restoration projects [79,80]. Alteration in the aboveground community does not automatically initiate a response in the belowground community, and the presence of aboveground or belowground heathland species alone does not necessarily lead to restored plant–soil interactions [81,82]

Soil amended chemically with elemental sulphur, although similar in pH, will differ from a soil that has acidified naturally as a result of build-up of ericaceous plant litter. Tibbett et al. [22] observed that soil carbon in acidified pasture was not significantly different to the control pasture, and was

much lower than carbon held in heathland soils (data taken the same year as our soil chemical data). This similarity between acidified pasture and control pasture would have implications for the soil biology and may provide a reason why differences in biologically based HRIs have not been observed between the acidified pasture and the control pasture.

Some studies have shown that only the presence of both above- (vegetation) and belowground (microbiota and fauna) heathland species leads to a fast assembly toward the target ecosystem. [81,83]. Therefore, in terms of assessing the efficacy of application of elemental sulphur to acidify pasture, sole reliance on chemical indicators to quantify the success of a restoration may lead to misleading conclusions.

4.3. HRI Based on a Minimum Dataset and Linear Scoring System Compared to Analysis of Similarity

The use of an HRI based on a minimum dataset, as opposed to multivariate analysis of a large dataset, will have obvious advantages to practitioners. Specifically, resources required for analysis of soil samples will be reduced with a minimum dataset. The findings using HRIs and analysis of similarities based on belowground biology concurred, showing that acidified pasture was not yet comparable with heathland and acid grassland systems. However, the HRIs failed to reflect differences in belowground biology between the control and acidified pasture, whereas multivariate analysis of similarity identified there were significant differences. On closer inspection of the multivariate analysis, we noted that rather than being similar to an acid grassland or a heathland (which would have resulted in a similar HRI), the acidified pasture shifted toward a different state entirely in terms of belowground biology. From this we might ask: *Is the multivariate dissimilarity between the control pasture and the acidified pasture of consequence to land managers?* Aboveground ericaceous species are being supported in the acidified pasture; so, depending on the motivations of the restoration project, belowground biology may not concern land managers if heathland plant species are dominant. Therefore, in the interest of directness, simplicity, and practicality for land managers and stakeholders, the use of an index may be more appropriate than comprehensive multivariate outputs, and will at least bring belowground processes into consideration. Further research will be needed to explore the motivations of conservation projects, and whether plants growing on acidified pasture can support the rare and endangered aboveground fauna that are the likely drivers for conservation efforts. To scientists, however, the new state of acidified pasture will be of interest, particularly with regard to the sustainability of the heathland vegetation in the absence of a comparable heathland soil biological community. This will require a more detailed analysis of variables that are contributing to the differences between acidified pasture, heathland, and acid grassland systems, to allow a more functional understanding of the processes and mechanisms in play.

5. Conclusions

Lowland heathland and acid grasslands are both listed as priority habitats in conservation efforts in the UK. Reversion of agricultural land back to heathland and acid grassland environments requires intervention to reverse the effects of the heavy fertilization and acidity amelioration undertaken during agricultural conversion. Application of elemental sulphur to acidify pasture resulted in chemical conditions in the acidified pasture being comparable to acid grassland. This, in turn, impacted aboveground plant communities toward more ericaceous species and other species typical of UK acid grassland and heathlands. However, heathland restoration indices that included belowground biological variables were not significantly different between acidified pasture and control pasture. When assessing the efficacy of heathland restoration, biological variables provide different insights compared to abiotic variables into soil responses to exogenous chemical modification. Multivariate analysis of similarity and heathland restoration indices concurred, showing that belowground biology in acidified pasture was not yet comparable to that of heathland or acid grassland. We, therefore, propose that the use of a simplified heathland restoration index,

rather than an analysis that includes all variables, can be valuable to stakeholders in heathland restoration.

Author Contributions: Conceptualization, M.T., A.D., and S.D.; methodology, all authors; formal analysis, all authors; resources, M.T. and K.R.R.; data curation, M.T., A.D., S.D., and T.F.; writing—original draft preparation, S.D.; writing—review and editing, all authors; visualization, S.D.; supervision, M.T.; project administration, M.T.; funding acquisition, M.T. All authors have read and agreed to the published version of the manuscript

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Conflicts of Interest: The authors declare no conflict of interest.

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