The James Hutton Institute

SEPA Scottish Environment Protection Agency

Commissioned Report:

HP1108

Testing Soil Quality Indicators

Report submitted to:

Scottish Environment Protection Agency

Issue Date:

3 June 2013

COMMISSIONED REPORT

Commissioned Report No. HP1108

Testing Soil Quality Indicators

For further information on this report please contact:

Claudia Erber
SEPA Corporate Office
Erskine Court
Castle Business Park
STIRLING
FK9 4TR
Telephone: 01786-457700
E-mail: Claudia.erber@sepa.org.uk

This report should be quoted as:

Mitchell, R.J., Beesley, L., Briggs, R., Cuthbert, A. Dawson, J.J.C., Helliwell, R.C., Hewison, R.L., Kerr, C., Leith, I.D., Owen, I.J., Potts, J.M., Newman, G., Smith, D., Shephard, L.J., Sturgeon, F., Taylor, A.F.S., White, D., Williams, A., Williams, E, Black, H.I.J. .(2013). *Testing* soil quality indicators. *Scottish Environment Protection Agency Commissioned Report No.HP1108.*

COMMISSIONED REPORT

Summary

Testing Soil Quality Indicators

Commissioned Report No. HP1108
Contractor: The James Hutton Institute
Year of publication: 2013

Background

Potential soil indicators have already been identified through a literature review and expert evaluation in the SEPA project HP801 “To establish soil indicators to assess the impact of atmospheric deposition on environmentally sensitive areas” (Black *et al*., 2009). This project is the follow up from project HP801 to take the development of indicators to monitor the impact of a point source of nitrogen pollution on soils one step further forward. Firstly, the indicators have to be shown to be sensitive to nitrogen pollution using suitable laboratory techniques. Subsequently, knowledge of the level of variation and expected levels of change in the indicators is required to calculate the sampling intensity required for the sampling strategy at a given location (i.e. the number of samples per unit area). This project aimed to provide these different forms of information. We confined our work to organic rich, acidic soils as these represent a predominant soil type under semi-natural habitats and designated sites across Scotland.

The seven indicators tested in this project were as follows, with outline methods in parentheses:

- soil pH (water and $CaCl_2$),
- soil carbon to nitrogen ratio (C and N analyser),
- base cation to aluminium ratio (ICP-OES for (Ca+Mg+K)/Al),
- solution ammonium / nitrate (colourimetric NH_4-N/NO_3-N)
- fungal to bacterial ratio (PLFA),
- fungal molecular marker (cloning and TRFLP),
- phosphomonoesterase (acid phosphatase activity).

Main findings

- Using existing datasets available to the James Hutton Institute, we assessed variation in the seven soil indicators at a range of spatial scales. These results demonstrated that the indicators were highly variable at spatial scales ranging from “between site” (>20 km between sites), “within site” (an area of ca. 1 ha) and “within plot” (ca. 20 m x 20 m).

- Expected levels of change for each of the seven soil indicators under increased nitrogen (N) deposition loadings were identified from published literature.

- The literature suggests that a difference of 15 to 40 kg N $ha^{-1}yr^{-1}$ is required between low and high inputs of nitrogen for a significant difference to be found in the indicators.

- The seven indicators were tested in the field using soil samples taken from the Centre for Ecology and Hydrology's experimental site at Whim Moss, Edinburgh, UK. At this site, two nitrogen manipulation experiments have been running since 2002; (1) additions of nitrogen from wet deposition in two forms - NH_4 and NO_3, and (2) a dry deposition gradient of nitrogen as NH_3, with equivalent N inputs between the dry and wet deposition experiments.

- In the wet deposition experiment, only a few of the indicators showed statistically significant responses to increased N deposition: phosphomonoesterase changed significantly with wet NH_4 deposition, although with no consistent linear or quadratic response; soil pH and base cation/Al ratio increased with increasing NO_3 deposition and soil solution NH_4-N:NO_3-N showed a significant (quadratic) response to increased NO_3 deposition.

- More indicators responded significantly to the dry deposition gradient with soil pH, base cation/Al ratio and phosphomonoesterase all responding in a consistent manner by increasing with distance from the point source.

- The mean values of the indicators from both experiments were broadly consistent with expected values from other studies and from other data taken over the years at the experimental site. Although there was large variation in the data for all tested indicators, this variation was within the range of variation expected from existing data.

- The limited number of significant results in this trial does not mean that the indicators are useless and should be abandoned. There are outstanding questions to be addressed regarding the required sampling intensity and likely impacts of N deposition in organic soils in Scotland. In this study, a relatively low sample size was used for reasons of economy. Given the published literature, a greater sampling intensity would better inform on the sensitivity of the indicators to nitrogen. However the published literature reflects data from a range of sources. Site specific sampling intensity in this study will also have been influenced by responsiveness of the indicators in organic soils which have experienced a long history of N inputs from atmospheric deposition. These factors may also serve to explain why some of the indicators did not respond sufficiently or consistently or produce expected results. Thus further investigation is needed to examine the relative importance of these factors on the expected change in indicators under future additional N deposition, especially if these N inputs are relatively low compare to past inputs.

- Using existing data at the start of the project, we estimated the total number of samples required to detect a significant change in the indicators assuming two pollution loadings. These estimates were then revised using the data collected, to provide an improved estimate of the sample numbers required for organic soils in Scotland. This also demonstrated that the number of samples required differed depending on the type of N deposition.

- Guidance on how to set up site specific soil monitoring schemes is provided. The following are identified as essential information for developing a site specific soil monitoring scheme: vegetation map, soil map, N deposition footprint map.

- A laboratory manual of standard sampling and analytical techniques for the indicators studied was prepared as part of this project.

Acknowledgements

This report is the result of a partnership project between SEPA and The James Hutton Institute. The work was jointly funded by SEPA and the Scottish Government's Rural and Environment Research and Analysis Directorate. The Centre for Ecology and Hydrology (Edinburgh) kindly provided access to their long-term experimental site at Whim Moss.

Table of Contents **Page**

List of Figures **Page**

List of Tables **Page**

1 INTRODUCTION

1.1 Background

Nitrogen deposition, both diffuse and from point sources have been shown to impact on vegetation communities in terms of both community composition and physiological function (e.g. Aerts & Berendse, 1988; Pitcairn *et al.,* 1995 & 1998; Haines-Young *et al.,* 2000; Pearce & van der Wal, 2002; Pearce *et al.*, 2003; Smart *et al*., 2003; Mitchell *et al.,* 2004 & 2005; Hartley & Mitchell, 2005; Kirkby *et al*., 2005; Britton *et al*., 2008). Fewer studies have assessed below-ground impacts of N deposition, but those that have indicate that N deposition can have a significant effect on both soil chemistry and soil fauna (e.g. Phoenix *et al*., 2012). There is currently no consistent and standardized approach to assessing N pollution impacts on the soil. Responses of soil fauna and changes in soil chemistry to N deposition may vary depending on soil type and other environmental factors. Ideally a suite of indicators needs to be developed that can be used to assess N pollution impacts on soils across a range of habitats and conditions.

UK nature conservation agencies are required to assess and report on the condition of designated features on protected sites, such as Sites of Special Scientific Interest (SSSIs) and Special Areas of Conservation (SACs). Common UK standards for site condition monitoring were developed by JNCC. Although the current guidance for monitoring the condition of sites is not aimed at investigating air pollution impacts, many protected species and habitats are sensitive to elevated level of atmospheric deposition. Scottish Natural Heritage (SNH) have recommended that the Scottish Environment Protection Agency (SEPA) develop suitable soil indicators for assessing impact of N deposition. Habitat specific thresholds for these soil indicators may assist in identifying the impacts of atmospheric N deposition and provide support for the sustainable management of protected features as well as habitats and species in the wider countryside.

The UK conservation agencies are also statutory consultees under the Pollution Prevention and Control (PPC) Regulations 2000 and Conservation Regulations 1994 and provide advice on the effects on statutory nature conservation sites from new or existing industrial or agricultural installations. This same legislation places obligations on the pollution regulators for assessing impacts of air pollutants on sites. Soil monitoring is a potentially useful tool to inform this assessment.

Potential soil indicators have been assessed through a literature review and expert evaluation in the SEPA project HP801 "*To establish soil indicators to assess the impact of atmospheric deposition on environmentally sensitive areas*" (Black *et al.* 2009). This project reviewed soil indicators to assess the impact of atmospheric deposition from point sources on soil quality in habitats of conservation interest, with nitrogen as the primary pollutant of interest. At that time, given the published literature available, seven indicators were selected as the most suitable to assess the status of soil quality in habitats of conservation interest in Scotland with respect to atmospheric pollution, with an emphasis on N deposition. By providing information on a range of soil properties and processes, these indicators could inform on the maintenance and vulnerability of five soil functions[1] which are recognised within the Scottish Soil Framework (Scottish Government, 2009).

[1] Seven soil functions are identified by the Scottish Government (2009) of which five are relevant here: providing the basis for food, forestry and other biomass production; controlling and regulating environmental interactions; storing carbon; providing valued habitats and sustaining biodiversity and providing raw material.

1.2 Project aims

All designated sites receive some background atmospheric N deposition from diffuse sources with the amount received dependent on site location (RoTAP, 2012). This project aimed to assess the applicability of seven soil indicators in assessing impacts of N deposition loadings over-and-above the background N atmospheric deposition and primarily derived from localised N point sources e.g. farm livestock units. The project did not aim to assess the suitability of these indicators to assess the impacts of diffuse pollution or to detect impacts at low levels of increased N deposition e.g. 1% increase above critical loads linked to Pollution Prevention and Control (PPC) regulations.

The list of seven indicators which this work studied was modified slightly from that proposed by Black *et al.* (2009), the indicators in this study being:

- soil pH,
- soil carbon to nitrogen ratio,
- base cation to aluminium ratio,
- soil solution ammonium to nitrate ratio,
- fungal to bacterial ratio,
- fungal molecular markers,
- phosphomonoesterase.

The work aimed to:

1. Using existing data:
    - To assess "natural variation" in the 7 soil indicators at a range of spatial scales
    - To identify expected levels of change for each of the 7 soil indicators under increased N deposition loadings
    - To assess the number of samples that would be required to detect *significant changes* to the 7 soil indicators, given natural variance of each indicator and its expected level of change under increased N deposition loadings (Section 2).

2. Test the indicators along a gradient of N input (Section 3).

3. Develop standard methods for sampling and analysis (Section 3 and Annex B).

4. Evaluate the usefulness of the indicators for assessing N deposition impacts (Section 4).

5. Compare the results with published data on these indicators (Section 4).

6. Compare the spatial variability of the indicators with published data (Section 4).

7. Evaluate thresholds for enabling the detection of the impact of atmospheric N deposition (Section 4).

8. Provide guidance on how to set up an efficient site specific soil monitoring scheme to assess the impact of atmospheric deposition (Section 5 and Annex A).

2 SEVEN SOIL INDICATORS: NATURAL VARIATION, EXPECTED LEVELS OF CHANGES AND SAMPLE NUMBERS

The aim of this section is to review existing data on seven previously identified soil indicators: pH; C/N ratio; base cation/Al ratio; soil solution NH_4-N:NO_3-N ratio, phosphomonoesterase activity; fungal/bacterial ratio and fungal molecular identification.

The specific aims are:

- Assess "natural variation" in the 7 soil indicators at a range of spatial scales from existing data sets
- Identify expected levels of change for each of the 7 soil indicators under increased N deposition loadings
- Assess the number of samples that would be required to detect *significant changes* to the 7 soil indicators, given natural variance of each indicator and its expected level of change under increased N deposition loadings.

2.1 General principles

The first stage in designing a soil sampling strategy is to assess the "natural variation" of each soil indicator at a range of spatial scales using existing data sets. This natural variation will include variation due to background atmospheric pollution. Moreover, natural variation occurs across habitats, soil type and geographical location. To limit variability where possible, we predominantly assessed natural variation from studies incorporating heathland/bog/rough grassland habitats with acidic organic-rich soils as these habitats have most relevance to the ultimate aim of this project – the development of a sampling strategy for semi-natural habitats in Scotland. However, where data for the selected indicators was not available from these habitats then data from other habitats was be used. It should be noted that natural variation in the soil indicator values may be smaller or greater according to habitat. In addition to natural spatial variation there will also be natural temporal variation.

The second stage is to assess a likely level of change in soil indicator values under increasing N deposition loadings. Most studies tend to concentrate on individual sites (and habitats) but when comparing across studies, variability in the background N deposition, soil type and vegetation also influences soil indicator values and their response to changing N deposition. This variability and contrast in data highlights the difficulty in identifying absolute values of change for a given soil indicator. Again, to help limit this variability, we concentrated on heathland/bog/grassland habitats sites with acid organic-rich soils. However, for other broad habitats, such as alkaline grasslands, the absolute value of change under given N deposition loadings may be different (Phoenix *et al.*, 2012). Expected levels of change for other broad habitat types would need to be assessed using other appropriate existing datasets.

Once the natural variation and the expected level of change are identified for each soil indicator, the third stage is to estimate the number of samples required to identify the required level of change concomitant with natural variation. It should be noted, however, that differences in soil indicator values due to N deposition loadings are confounded with any inherent differences related to spatial location. Tests for significant differences can only conclude whether indicator values from areas are different, not whether this is due to N deposition.

2.2 Spatial variation

Here we assessed the natural variation (which includes any variability due to historical N deposition) of the 7 selected soil indicators at three spatial scales; within plot, within site variation and between site variation. Plots were defined as an area of *ca*. 20 x 20 m, sites as an area of *ca*. 1 km^2, with the distance between sites being *ca*. 20 km or more. We used data from multiple sites to estimate the variability that might be expected between plots within a single site. Data from 11 existing datasets were used (Table 2.1) including data from control plots in a range of experiments and data from surveys. Table 2.1 also details the experiments/sites and habitats and the spatial arrangement of the samples. Differences in data sets also reflect different soil sampling strategies and sampling at different depths e.g. some sample the top 15 cm irrespective of horizon, others sampled the top horizon – these differences are described in Table 2.1. Not all the datasets contained data on all the indicators used in this project (Table 2.2). The exact sizes/distances varied between the different datasets. Some of the data used from datasets 9, 10 and 11 might have been influenced by N deposition point sources but we were unable to assess this. Due to the scope of this project only a limited number of existing data sets could be assessed. The units of the data from each experiment were converted so they were the same in each dataset e.g. C/N ratios were calculated on a molar basis, pH was measured in water, and the base cation/Al ratio was calculated in milliequivalents using the following formula:

$$ratio = \frac{Ca + Mg + K}{Al}$$

To assess the variation within plot, between plots and between sites, each indicator for each data set was analysed using the Proc Mixed procedure in the SAS statistical software (SAS, 2008), which supports the analyses of different scales within the one procedure. Site and, where appropriate plot, were included as random effects. The model was run with no fixed-effects, and the covariance parameter estimates gave the variance for each spatial scale (within plot, plot and site). The variance components resulting from this analysis provides a measure of the variability of the data. The relative sizes of the variance components for plot and subplot can indicate whether it might be better to take several replicate samples per plot, or have more plots with fewer samples from each.

The results demonstrated that:
Soil pH was more variable between sites than within sites (between plots) in all the datasets analysed. The between plot variance in pH was less than 0.1 of a unit in all cases, as was the case for the subplot variance where available (Fig. 2.1).

Soil C/N ratio was highly variable (Fig. 2.2): for six of the data sets, the within site variability was less than the between site variability but for dataset 4 the within site variability was as great as the between site variability and for dataset 5 the within plot (subplot) variability was greater than the between site variability. The within site variability at either the plot or subplot scale ranged from 50 to less than 10 for soil C/N ratio.

Variance in the fungal/bacterial ratio was very different in dataset 4 from that in datasets 2 and 3 (Fig. 2.3). Datasets 2 and 3 both showed that the within site (plot) variability was greater than the between site variability; within site variance ranging from 0.0006 to 0.0014 (Fig. 2.3a). In dataset 4 the variances were much larger and the between site variance was much greater than the within site variance (Fig. 2.3b). This difference may relate to habitat differences in the structuring of the soil microbial community at scales ranging from 30 cm to ca. 6 m (Franklin and Mills, 2003).

Table 2.1. Details of the datasets used to assess spatial variation in the seven selected indicators. Where there are multiple samples at any given scale the number of samples is shown.

Data set	Habitat[1]	Reference	Multiple samples per plot	Multiple plots per site	Multiple sites	Soil depth
1	Moorland	Unpublished		24 plots per site, plots samples based on 5 samples per plot bulked	3	Top 5 cm of organic layer
2	Montane heath	Unpublished		10	15	Top 15 cm
3	Birch Woodland	Mitchell et al 2010b, 2012		9 plots per site, plot samples based on 5 cores per plot bulked	3	Usually top 15 cm, sometimes less
4	Moorland	Mitchell et al 2007, 2010b	4 samples per plot [2]Each of the 4 samples composed of 3 bulked samples	6	3	Top 15 cm but only the organic layer analysed
5	Moorland	Unpublished	up to 5 samples per plot	no	34	Top organic horizon
6	Bog	Unpublished	up to 5 samples per plot	no	24	Top organic horizon
7	Conifer woodland	Unpublished	up to 5 samples per plot	no	10	Top organic horizon
8	Rough grassland	Unpublished	up to 5 samples per plot	no	26	Top organic horizon
9	Moorland			4	no	Within organic horizon, collected every 3 weeks
10	Moorland			3	no	Within organic horizon, collected once a month
11	Moorland			4	no	Within organic horizon, collected once a month

[1]The background N deposition at each of these sites is unknown
[2]Dataset 4: no within plot sampling for PLFAs

Table 2.2. Soil indicators available from the datasets.

Data set	pH	C/N ratio	Base cation/Al ratio	Soil solution NH_4-N/NO_3-N	PLFA fungal/bacterial
1	X	X	X		
2	X	X			X
3	X	X	X		X
4	X	X	X		X
5	X	X			
6	X	X			
7	X	X			
8	X	X			
9				X	
10				X	
11				X	

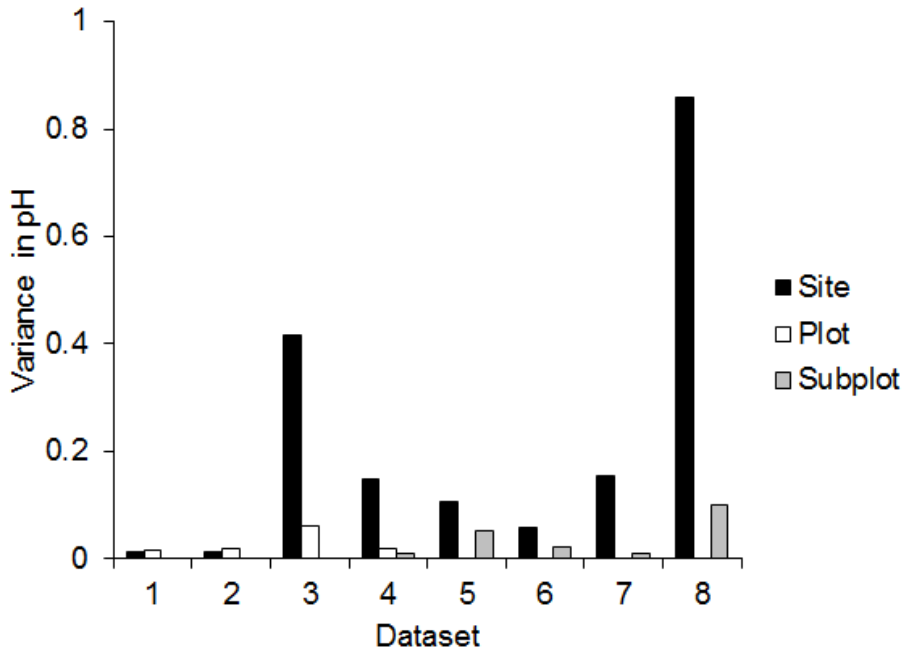


Figure 2.1. Spatial variance in soil pH for different data sets

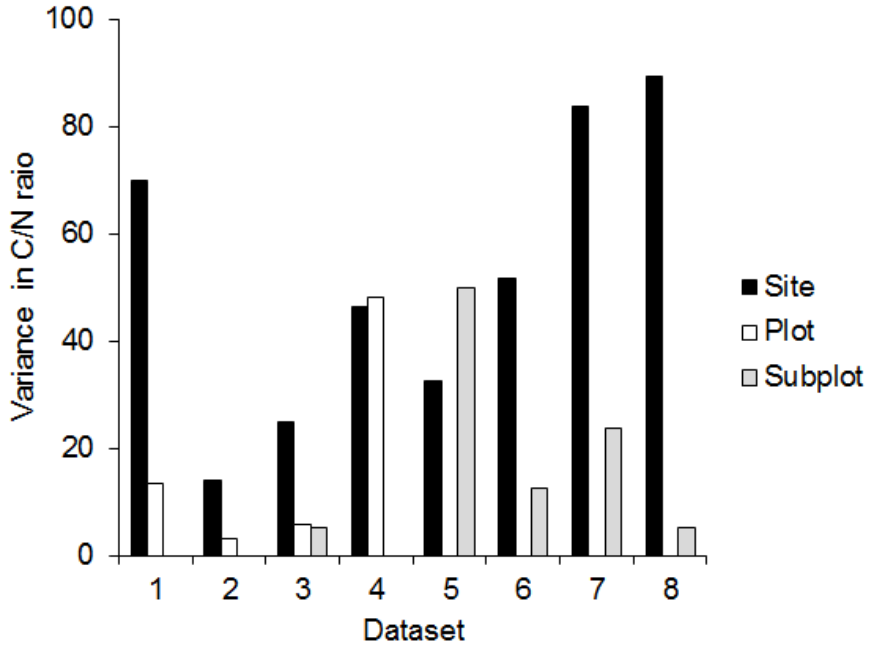


Figure 2.2. Spatial variance in soil C/N ratio for different data sets.

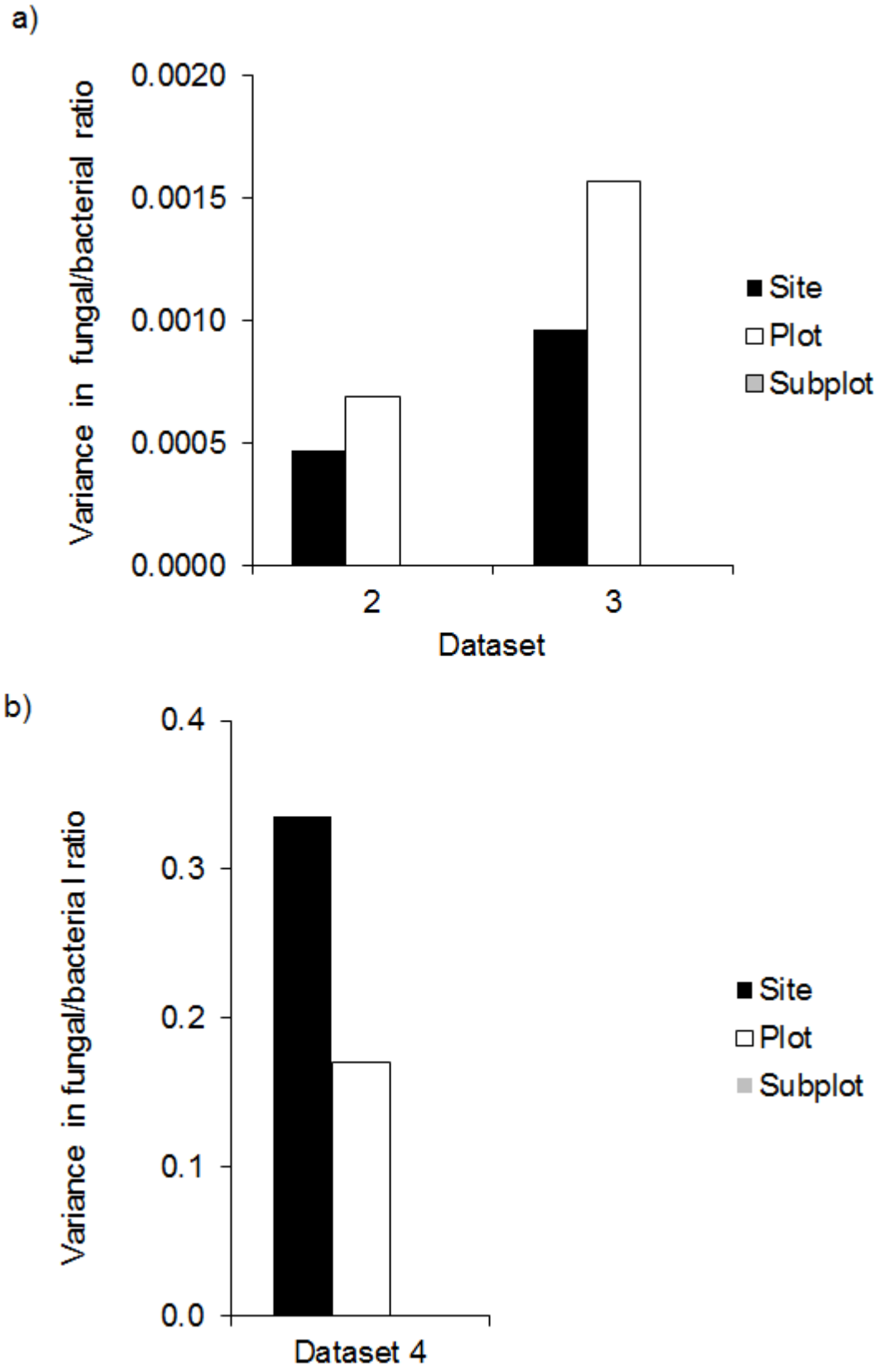


Figure 2.3. Spatial variation in PLFA fungal/bacterial ratio at three sites.

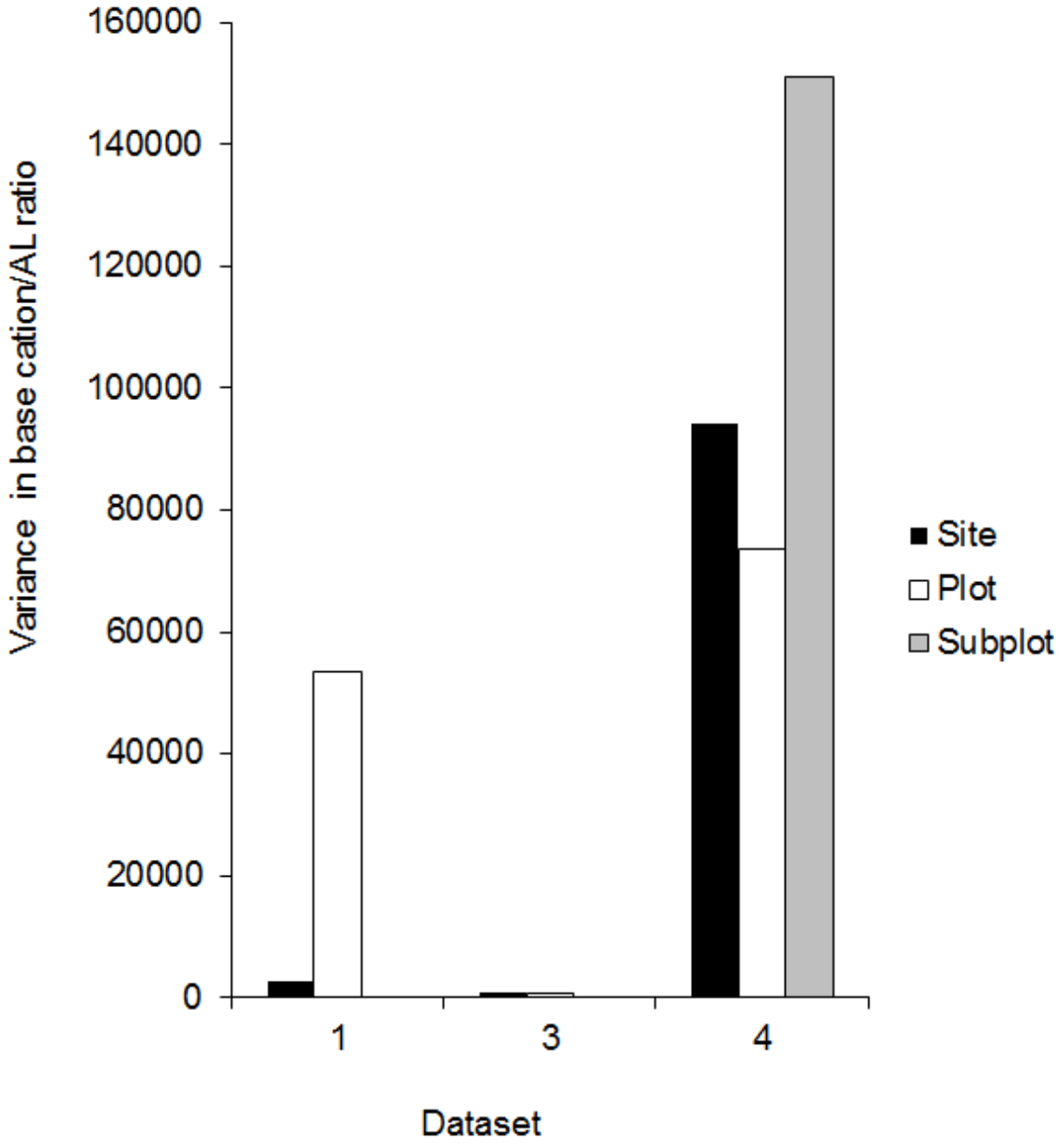


Figure 2.4. Spatial variation in base cation/Al ratio at three sites.

The variance in base cation/Al ratio was large in the two moorland datasets (datasets 1 and 4) but much smaller in the deciduous woodland dataset (dataset 3) (Fig 2.4). In both moorland datasets between plot variation was much larger than between site variation.

For soil solution NH_4-N/NO_3-N ratios one time point, April 2008, was chosen from the three sites available (Table 2.1). Analysis of the variance showed that the variance between sites was zero but the variance between plots was 7.01. Thus, there was large variation within a site for the NH_4-N/NO_3-N ratio.

Data on the spatial variability of fungal communities in the organic rich acidic soils on moorlands and bogs were not available. However, in grassland communities Mummey and Rillig (2008) demonstrated that arbuscular mycorrhizal fungi (AMF) diversity and abundance can be spatially structured at scales of <1m. Such small-scale heterogeneity in the soil has important implications for representative sampling of AMF communities in the field.

There were no data available on the spatial variation in phosphomonoesterase enzyme.

2.3 Temporal variation

Temporal variation is as important as spatial variation in the selected soil indicators. This is particularly so for the NH_4-N/NO_3-N soil solution ratios, which are known to be dynamic over short time scales. For three acidic organic moorland sites in north-eastern Scotland (Datasets 9, 10, 11 in Tables 2.1 and 2.2) we assessed temporal and spatial variation in NH_4-N/NO_3-N (Figs. 2.5-2.7). For both within sites and between sites, there was large variation in the NH_4-N/NO_3-N soil solution ratio. If the relative differences between samples within sites remain consistent over time then the temporal variation would be of less concern as the relative differences between samples could be compared. However, the relative differences between samples within sites did not remain consistent over time. Extreme weather events, (e.g. low temperatures, drought) have been shown to have a large influence on the average soil solution chemistry over a 4 week period (Helliwell *et al.*, 2010). Variability of a one off collection of soil solution taken over a few minutes would be expected to be even greater.

Soil pH, C:N and base cation/Al ratios are considered to be less temporally variable than NH_4-N/NO_3-N soil solution ratios. The activities and composition of rhizosphere fungal populations will also vary over short time periods; they may be related to root exudates and can follow the pattern of C release into the soil (Medeirosa *et al*., 2006) which will also fluctuate with the phenology of the plant community. However, soil moisture will also have a large impact on fungal communities, which may over-ride other temporal changes. Fungal molecular techniques will be more temporally stable than using fruiting bodies, the appearance of which is highly dependent upon weather conditions. In addition, the majority of the fungi present in the systems under investigation would not produce fruit bodies. The literature suggests that integrated ecological assessments appear to be more sensitive than chemical measures – due to large spatio-temporal variation in chemical measurements (Phoenix *et al*., 2012).

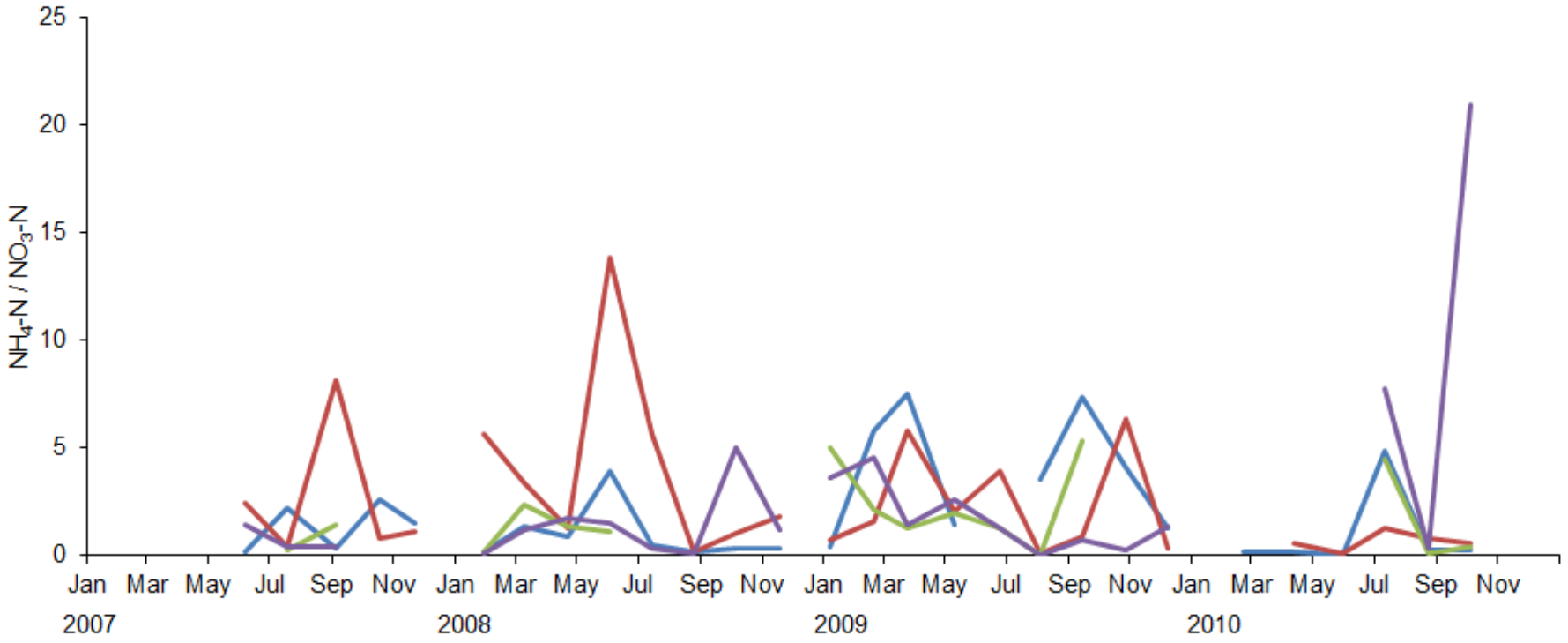


Figure 2.5. Temporal variation in soil water chemistry (NH_4-N/NO_3-N ratio) over 4 years (2007-2010). Datasets 9. The different lines show data from the four plots present at the site. Soil solution was collected monthly.

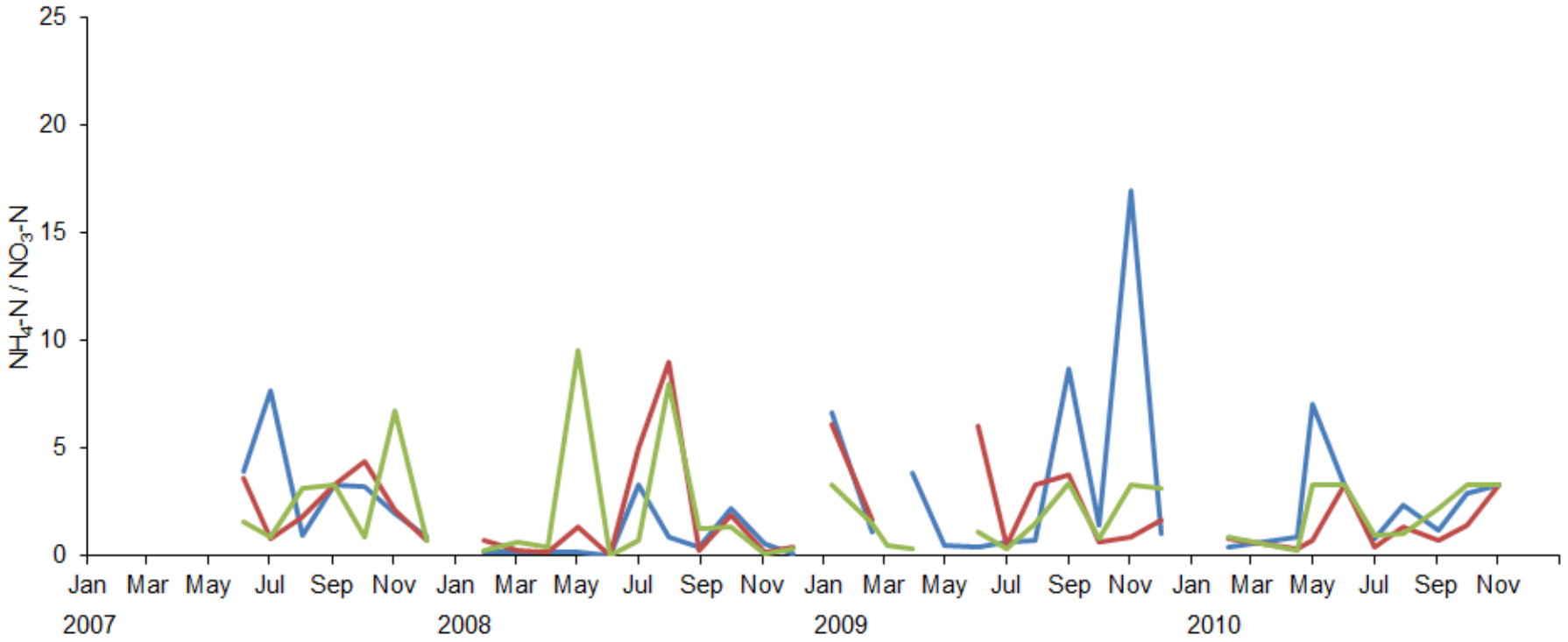


Figure 2.6. Temporal variation in soil water chemistry (NH_4-N/NO_3-N ratio) over 4 years (2007-2010). Datasets 10. The different lines show data from the three plots present at the site. Soil solution was collected monthly.

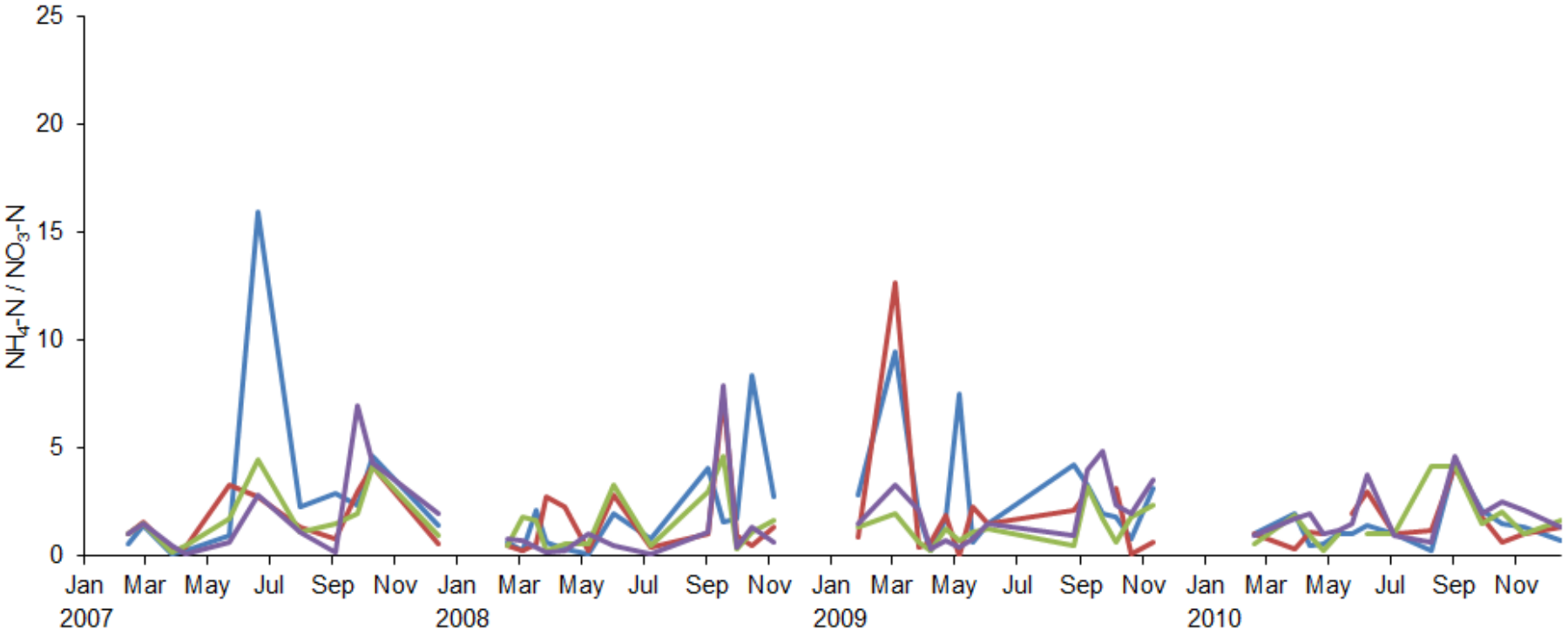


Figure 2.7. Temporal variation in soil water chemistry (NH4-N/NO3-N ratio) over 4 years (2007-2010). Datasets 10. The different lines show data from the four plots present at the site. Soil solution was collected monthly.

2.4 Identification of expected levels of change in the seven soil indicators under increased N deposition loadings

To assess how many samples are required it is important to not only know the "natural" spatial variation but also have some indication of the expected level of change in the indicators to detect response to a pressure. If spatial variation is low and level of change to be detected in a soil indicator is large then fewer samples will be required than if there is large spatial variation and the level of change to be detected in the soil indicators which is small.

Phoenix *et al.,* (2012) provide a literature review of the impacts of atmospheric N deposition across contrasting ecosystems in major long-term field experiments from England, Wales and Scotland. Using data from seven of these sites, we assessed by how much each soil indicator changed for significance to be detected and the minimal N deposition loadings required for significant change to be observed (Table 2.3). Significance will depend on the variability of the actual sample values and the sample size for each experiment, but we aimed to identify by how much the soil indicators were likely to change. All seven sites included acidic organic soils and NVC vegetation types applicable to this project e.g. acidic grasslands, bogs or heaths.

Table 2.3 provide information on a wide range of N loadings to illustrate when significant changes in the indicators are observed. It is acknowledged that the high and very high loadings in Table 2.3 are unlikely to be observed across designated sites but these data come from experimental plots where the aim was to assess at what N loading a change occurred.

Table 2.3 indicates an expected level of change for the selected soil indicators. Significant change values are absolute numbers, in the main from a single study (Pilkington *et al.,* 2005a). However, this first estimate gives an indication of the sensitivity and robustness of individual soil indicators required for detection of significant differences in soil/soil solution chemistry to N additions. However, values are derived from absolute differences from a set of "control" values and do not necessarily infer the same change will be significant with different controls, i.e. proportional change may need to be considered for estimating when significant change has occurred. It is important to note that the different soil horizons provided significant differences dependent on the assigned indicator, e.g. C/N ratio in litter layer or Ca/Al ratio in E_{ag}[2] horizon. An increase in N deposition and potential cascading effects e.g. increases in soil pH, will have different impacts depending on the starting composition of the soil, which will vary in chemical and microbiological composition according to the soil horizon.

Some of the data presented in Phoenix *et al.,* (2012) are not identical to the indicators we are assessing in this project. The indicator units or methods often differ in the literature making comparisons difficult. For base cation/Al measurements, Pilkington *et al.* (2005a), measured Ca, Mg, K, Na concentrations in $\mu Eq\ l^{-1}$ but where significant change is calculated only the Ca/Al (molar) ratio was used.

Table 2.3 suggests that between 15 and 40 kg N $ha^{-1}yr^{-1}$ difference between the low and high ends of the N inputs is required for a significant difference to be found in the indicators. These levels are typical of the levels emitted by intensive agricultural units (i.e. NH_3) but are lower than the values typically emitted by a single combustion sources (i.e. NO_x). This suggests that these indicators maybe suitable for assessing the impacts of point sources such as intensive pig or poultry farms.

[2] mineral soil horizon depleted of iron and/or aluminium with periodic water saturation.

Table 2.3. Indicative changes of assigned indicators to differential N loadings on acid organic-rich soils typical of upland moorland systems in Scotland

N Additional Loading[1] (kg N ha^{-1} yr^{-1})	Soil pH	Soil C/N ratio[2]	Base cation/Al ratio[3]	NH_4/NO_3-N ratio[4]	PME assay[5]	PLFA fungal/bacterial ratio	Fungal molecular technique
Control: 0							
Low: 0-15				✓			
Medium: >15-40	✓	✓	✓	✓	✓	✓	
High: >40-100	✓	✓	✓	✓	✓		
Very High: >100	✓	✓	✓	✓	✓		
Significant change	0.2 units[6]	%N = 0.2, C:N = 2.5[7]	Ca:Al = 0.1[8]	NO_3-N = 2.0[9]	6.5[10]	0.015	nd

✓Indicate a statistically significant effect within at least one of the seven heathland/bog/acid grassland relevant studies from Phoenix *et al*. (2012).
[1]Typical N loadings categorised from variable loadings in relevant studies described by Phoenix *et al*. (2012).
[2]Molar ratios; Data determined using a change in %N. Significant increase in N occurred with no significant change in C (Pilkington *et al*. 2005a).
[3]Molar ratios; Most studies use Ca/Al ratios, not base cation (Ca, Mg, K, Na)/Al ratios. Either can be used as an acid-base status change indicator.
[4]Data determined using a change in individual NO_3^- or NH_4 leachate (soil solution) concentrations.
[5]Enzyme used was phosphomonoesterase (PME).
[6]Data derived from the relevant studies in Phoenix *et al*. (2012); changes ranged from 0.1 to 0.5 pH units in response to various loadings of N.
[7]Data from N additions to upland moorland soils (litter layer) where change occurred at 80 kg N ha^{-1} yr^{-1} (Pilkington *et al*. 2005a).
[8]Data from N additions to upland moorland soils (E_{ag} horizon) where change occurred at 40 kg N ha^{-1} yr^{-1} (Pilkington *et al*. 2005a).
[9]Data from N additions to upland moorland soils (O_h horizon) where change occurred at 80 kg N ha^{-1} yr^{-1}(Pilkington *et al*. 2005a). Value expressed as NO_3-N flux only (kg N ha^{-1} yr^{-1}).
[10]Data from N additions to upland moorland soils (litter layer/Oh horizon) where change occurred at 80 kg N ha^{-1} yr^{-1}(Pilkington *et al*. 2005b). Value expressed as enzyme activity (nmol p-nitro-phenol g^{-1} dwt s^{-1}).

2.5 Example calculation of number of samples required.

Once the natural variance in soil indicator values is known (Section 2.2) and the size of the difference in values of the indicator to be detected between areas with low and high N deposition loadings (Section 2.4), we can then estimate the number of samples required to detect a significant change for each indicator. If the significance level of the test is α (generally 0.05) and the required probability of detecting a significant effect (the power, 1-β) is assumed to be 0.8 then the required sample size, assuming equal replication in the "low" and "high" areas is approximately given by:

$$\frac{4(z_{1-\beta} + z_{1-\alpha/2})^2 v}{d^2}$$

where *z* is the critical value of the standard normal distribution, *d* is the change to be detected and *v* is the variance. Using values of *z* obtained from statistical tables for the case where α=0.05 and 1-β=0.8 this simplifies to

$$\frac{31.4v}{d^2}$$

Alternatively, the sample size may be calculated more exactly for given values of *d* (change to be detected) and *v* (variance) using a statistical software package. The predicted sample size required calculated using this method and based on the variances calculated earlier are given in Table 2.4.

Table 2.4 indicates that between 18 and 212 samples maybe needed to detect an impact of the point source of pollution depending on the soil indicator and the level of variation in that indicator. However, these numbers are based on a large number of assumptions (see foot notes to Tables 2.3 and 2.4). For C/N ratio and the PLFA fungal/bacterial ratio examples of the number of samples required with both low and high variance are shown. This clearly indicates how the number of samples required increases as the variance (natural variation in samples) increases. The natural variation across a site to be sampled is unlikely to be known in advance.

It was not possible to calculate the number of samples required for the phosphomonoesterase as no data was available on the variance, although information was available on the expected level of change (Table 3). For the fungal molecular identification no information on the variance or expected level of change was available so sample size was not calculated. The data in Table 2.3 was soil solution was for NO_3-N flux only not for the NH_4/NO_3 ratio. The NO_3-N flux is known to be highly variable so this information was not considered suitable for use in determining sample size.

Table 2.4. Number of samples required to have an 80% chance of detecting the specified change as significant at the 5% level.

Indicator	Variance low/high example[1]	Variance value	Unit of change	Number of samples (total number across both low and high gradient)[2]
pH		0.02[3]	0.2[4]	18
C:N	low	4[5]	2.5[6]	24
C:N	high	50[7]	2.5[8]	254
PLFA fungal/bacterial	low	0.0007[9]	0.015[10]	100
PLFA fungal/bacterial	High	0.15[11]	0.15[12]	212
Ca/AL		2500[13]	50[14]	34

1Some of the datasets (Table 2.1) showed very different levels of spatial variance within any one indicator. When this occurred we have presented two examples, one with low variance and one with high variance. Footnotes 3, 5, 7, 9, 11, and 13 detail where the variance data was obtained from.

2This is the total number of samples required assuming two pollution loadings (low and high) so for example for pH 9 samples from a low pollution loading is required and 9 from a high, total 18.

3Based on plots and subplot variance from moorland datasets in Figure 2.1

4Taken from Table 2.3

5Plot variance Dataset 4 (moorland) Figure 2.1

6Taken from Table 2.3

7Plot variance Dataset 4 (montane moorland) Figure 2.1

8Taken from Table 2.3

9Plot variance from Dataset 2 (montane moorland) Figure 2.2

10Taken from Table 2.3

11Plot variance from Dataset 4 (moorland) Figure 2.2

12The PLFA fungal/bacterial ratio in Dataset 4 was an order of magnitude higher than that from Dataset 2. The expected level of change was therefore calculated based on proportional change taken from Table 2.3

13The base cation/Al ratio from datasets 1, 3, and 4 was recalculated as Ca/Al and converted to molar ratios to provide data in the same units as that in Table 2.3.

14The absolute value of change was recalculated from Table 2.3 based on based on proportional change, as the soil type for the value in Table 2.3 is very different from that in datasets 1, 2 and 4 and hence has a very different Ca/Al ratio.

3 TESTING THE SOIL INDICATORS

3.1 Introduction to the Whim Moss experiment

Whim Moss, an ombrotrophic peatland. The vegetation is dominated by the dwarf ericaceous shrub *Calluna*, the cyperaceae *Eriophorum vaginatum* and the hummock forming *Sphagnum capillifolium*, all species that are widespread in northern latitudes. The bog represents a transition between lowland and blanket bog, 282 m above sea level, on 3 to 6 m deep peat with an annual rainfall exceeding 900 mm.

The Whim bog experiment, which studies the impact of different forms and doses of nitrogen deposition on bogs, was established in 2002 and covers approximately 2 ha of Whim Moss (Fig. 3.1). The experiment is run by the Centre for Ecology and Hydrology, Edinburgh (CEH). Nitrogen treatments are supplied as wet deposition, in rainfall collected on site or as dry deposited ammonia gas. Wet treatments are supplied as either oxidized N, ($NaNO_3$) such as emitted by power stations and transport vehicles i.e. combustion or reduced N (NH_4Cl) such as emitted from agricultural sources. Three N doses are applied: 8, 24 and 56 kg N ha^{-1} y^{-1} to large approximately 13 m^2 replicated plots, (44 plots in total), using a highly realistic treatment scenario coupled to meteorology that provides deposition at low ionic strength and high frequency over the canopy. Dry deposited ammonia is released from a line source and provides a gradient (exponential) of ammonia concentrations (NH_3-N deposition), equivalent to the wet deposition doses. Control wet plots receive just the additional precipitation (<10 % annual). Further details of the experimental set up are in Sheppard *et al*., (2011) or at http://www.expeeronline.eu/index.php/list-of-sites/descriptions/133.

The wet deposition plots and the dry deposition gradient were sampled to test the suitability of the seven indicators (soil pH; C/N ratio; base cation/Al ratio; soil solution NH_4-N/NO_3-N ratio, phosphomonoesterase activity; fungal/bacterial PLFA ratio and fungal molecular identification) to assess N deposition impacts.

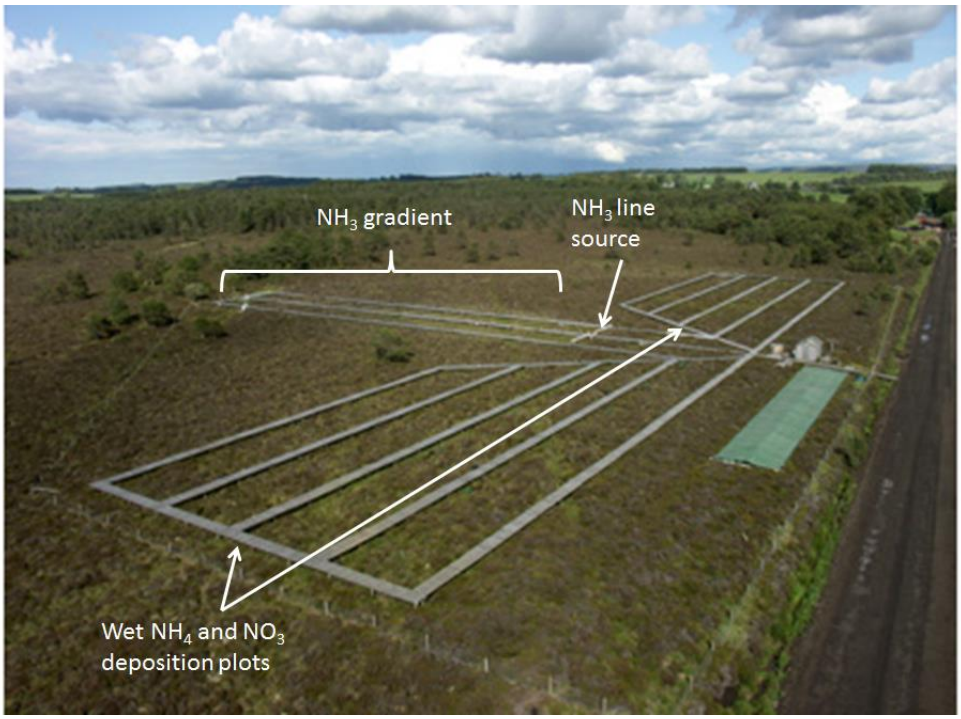


Figure 3.1. Overview of experimental layout at Whim Moss

3.2 Wet deposition plots

The wet deposition plots are laid out as a replicated block experiment (Fig. 3.2). Only the NH_4 and NO_3 treatments were sampled (Table 3.1); plots that also receive P and K were not included.). There are four blocks and each block has a control plot and three levels of N addition resulting in four levels of N deposition for each of NH_4 and NO_3. In total 28 plots were sampled from the wet deposition experiment, the sampling protocol is detailed in Section 3.4. Each plot is 12.8 m^2 (circular plots with a 2 m radius).

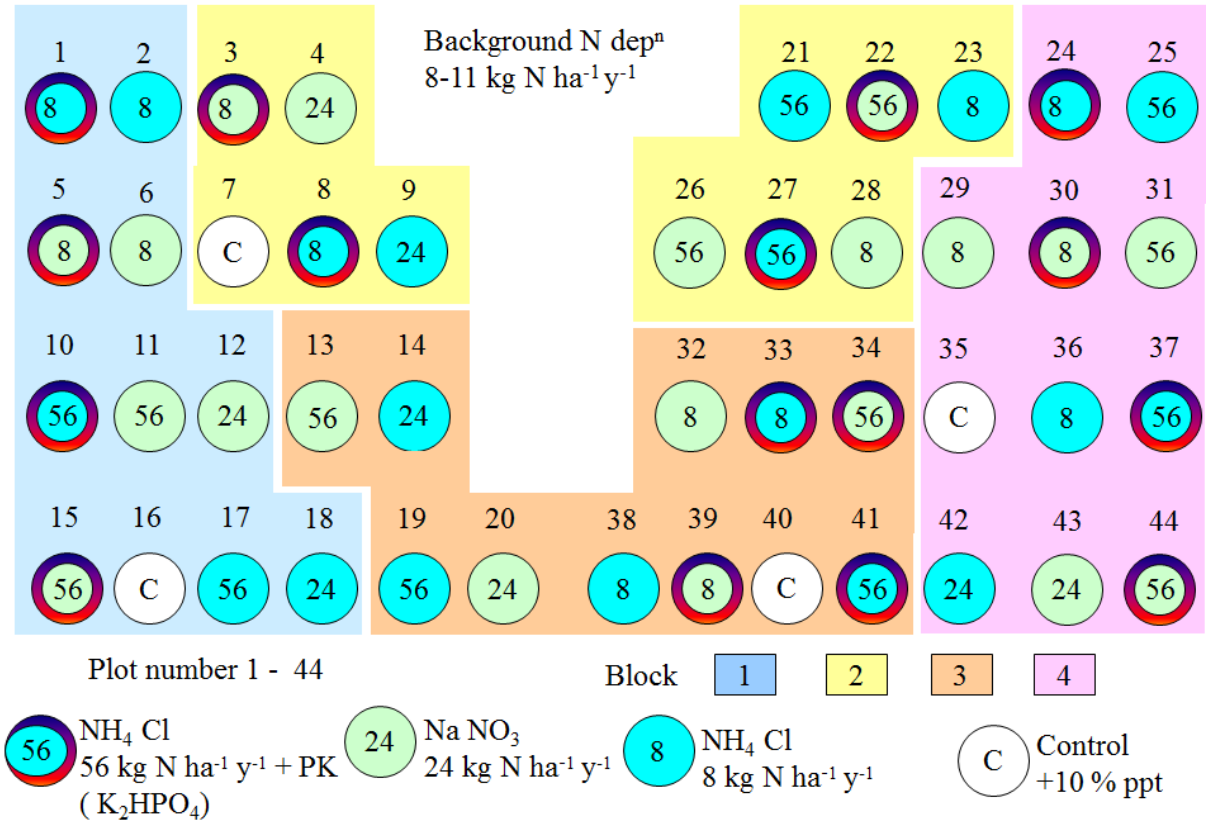


Figure 3.2. Schematic of the experimental lay out of wet deposition plots.

Table 3.1. Treatments sampled in the wet deposition experiment.

Treatment	Additional N added kg N ha^{-1} yr^{-1}	Total N including background kg N ha^{-1} yr^{-1}	Plot numbers
NH_4Cl	56	64	17, 21, 19, 25
	24	32	18, 9, 14, 42
	8	16	2, 23, 38, 36
$NaNO_3$	56	64	11, 26, 13, 31
	24	32	12, 4, 20, 43
	8	16	6, 28, 32, 29
Control	0	8	16, 7, 40, 35

3.3 Dry deposition

The dry deposition is emitted[3] from a point source (a 10 m line source at 1 m above the vegetation) giving an exponential gradient of 64 – 8 kg N ha^{-1} yr^{-1} including background deposition. The gradient is 64 m long. There are three board walks along the length of the gradient, one at each edge of the transect and one down the middle, giving two strips of habitat to sample. In each strip two transects were established, each 1.5 m away from the edge of the board walk, giving four transects in total (Fig. 3.3). Four sampling locations were done on each transect (Table 3.2) providing a total of 16 samples.

Table 3.2. Relationship between distance from point source and N deposition.

Distance from point source (m)	Annual NH_3-N deposition kg^{-1} ha^{-1*}
12	36-90
20	20-56
32	16-36
55	12-22

*data taken from Sheppard *et al*. 2011. Deposition range is taken from 2003-2009 and the variation in annual deposition reflects different meteorological conditions, e.g. wind direction, between years.

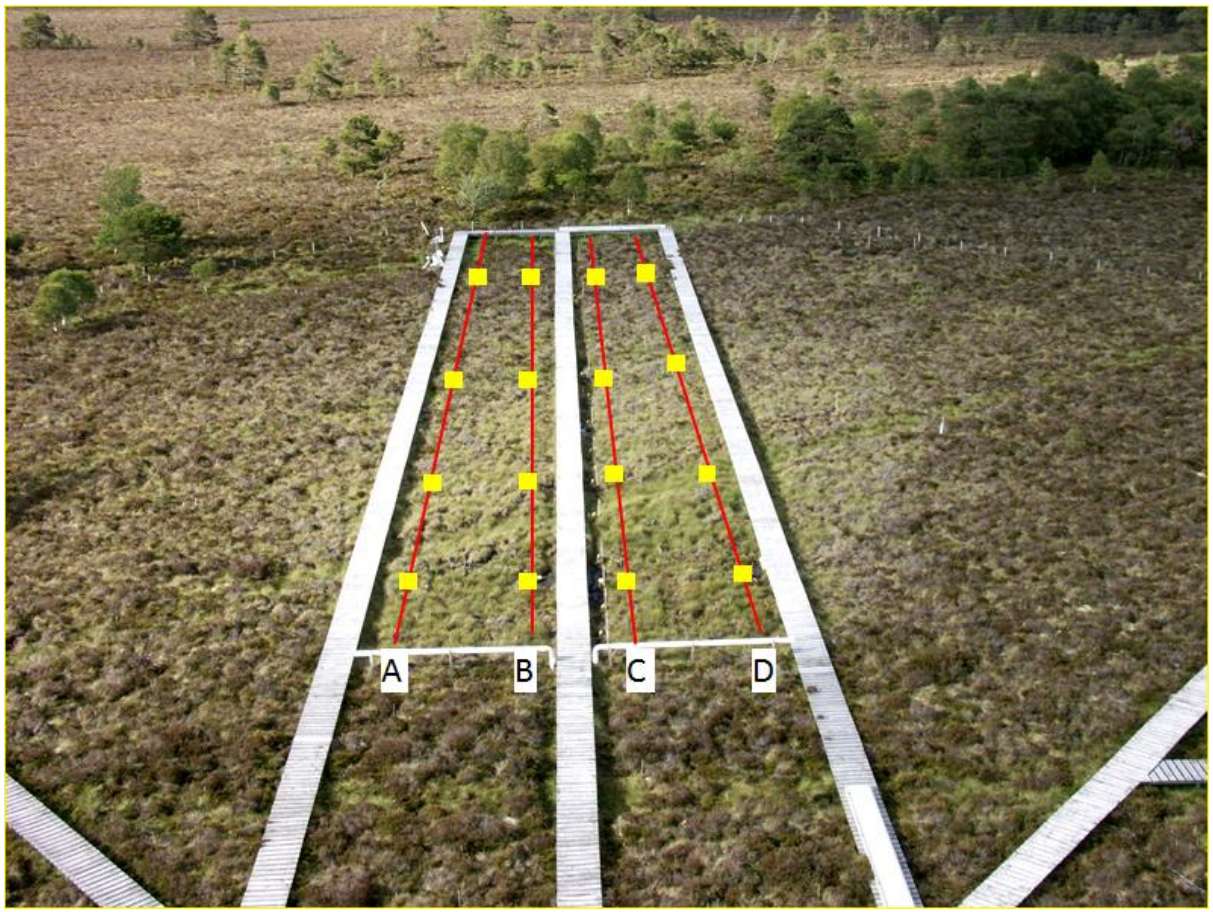


Figure 3.3. Arial photo of the dry deposition gradient. The red lines indicate the approximate position of the four transects, and yellow squares the approximate position of the sampling locations. A-D are the four transects.

[3] NH_3 is released only when the wind direction is between 180-215°, temperatures exceed freezing and when wind speeds exceed 2.5 m s^{-1} to ensure efficient mixing (Leith et al 2004).

3.4 Sampling strategy

At each sampling location (dry deposition) or plot (wet deposition) five cores (5 cm x 5 cm) to a depth of 15 cm were taken using a short peat corer (Fig. 3.4). One core was taken at the centre of the plot and four other cores taken 0.5 m away from this central core, one to each of the N, S, E, and W (Fig. 3.5). On the dry deposition transects the centre core was taken at the agreed distance from the point source and 1.5 m away from the board walk, and the other cores were taken 0.5 m away from this core in the same way as for the plots. Following extraction each core was cut to 15 cm and carefully wrapped in cling film and placed in a plastic bag. The cores were kept cold until they could be processed in the lab.

On return to the lab the cores were bulked (see section 3.5) to provide one result for each indicator per plot/sampling location. This gives 28 samples from the wet deposition experiment and 16 samples from the dry deposition. These sample numbers are lower than that calculated in Table 2.4 due to limited resources available for analytical work. The aim was that by bulking samples the variation within a plot would be sampled while still limiting the number of samples to be analysed.

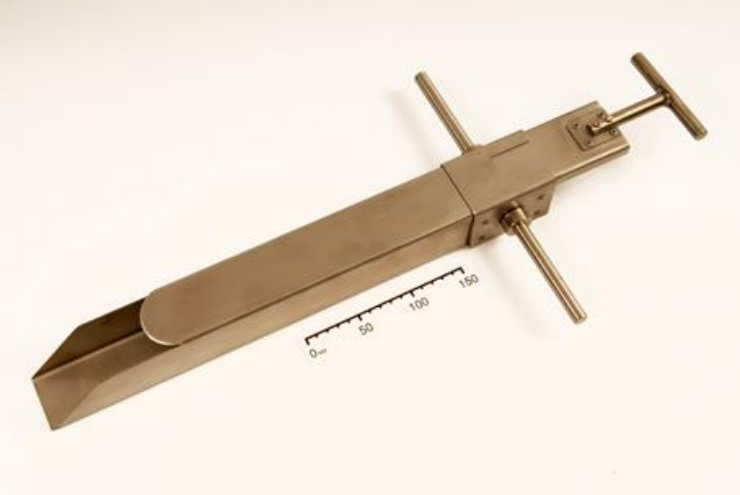


Figure 3.4. Picture of box corer used for sampling.

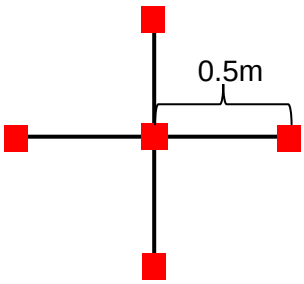


Figure 3.5. Diagram of location of cores (red squares = a core)

3.5 Analytical methods

On return to the laboratory the five cores from one plot or sampling location were laid out on the lab bench and split and bulked in the following way:

1. Each core was split length ways into quarters (to form four sub-cores 2.5 x 2.5 x 15 cm)
2. The top 5 cm from one sub-core from each core was cut off to form a 2.5 x 2.5 by 5 cm sub-core
3. The five sub-cores that were 5 cm in length (2 above) were bulked to form bulked sample 1.
4. Bulked sample 1 was used for biological indicators as biological indicators were expected to be most sensitive in the topmost part of the core.
5. One 15 cm length sub-core from each of the five cores were grouped to form three bulked samples: bulked samples 2-4.
6. Bulked sample 2 was used for moisture, total carbon and nitrogen, pH and exchangeable cations,
7. Bulked sample 3 was used to extract the soil water for NH_4 and NO_3
8. Bulked sample 4 was placed in storage.

Soil pH was measured on 7.5 g of soil in either water or $CaCl_2$ according to the method in Appendix 1. The C/N ratios were calculated on both a percentage and a molar basis, and the base cation/Al ratio was calculated in milliequivalents using the following formula:

$$ratio = \frac{Ca + Mg + K}{Al}$$

A full laboratory manual detailing the sample preparation and analytical techniques used on these cores is provided in Annex B.

3.6 Data analysis

The data analysis aimed to assess if the value of the indicators was significantly different under different N loadings. In statistical terms the data analysis aims to assess if there was a significant difference between plots or sampling locations receiving different N loadings. A significant difference is assessed as a 5% probability or less ($P<0.005$) that the value of the indicators is the same in all the plots or sampling locations irrespective of N loading.

The indicators pH, fungal/bacterial PLFA ratio, base cation/Al ratio, C/N ratio, NH_4-N/NO_3-N soil solution ratio, phosphomonoesterase provide univariate data; that is a single number or result per plot or sampling location. The fungal molecular data is multivariate: there are many numbers (results) per plot or sampling location – the presence/absence of each fungal species. These two types of data were analysed in two different ways.

Univariate data (pH, fungal/bacterial PLFA ratio, base cation/Al ratio, C/N ratio, NH_4-N/NO_3-N soil solution ratio, phosphomonoesterase) were all analysed using the Mixed model procedures in the SAS statistical software (SAS 2008). The Proc mixed procedure was used with block (wet deposition) or transect (dry deposition) as a random effect. The random effect takes into account that the four blocks or transects may differ from each other. For analysis of the wet deposition block and nitrogen (total N loading including background) were included as class variables. Class variables indicate that the numbers are not continuous, so in this experiment the N deposition was added at fixed amounts of 8, 24 and 56. Each indicator was analysed separately to assess if the value of the indicator was significantly

different with different N loadings. If a significant result was found then linear and quadratic equations were fitted to investigate the type of response present and the estimated change in the indicator that occurred with an increase in 1 kg N ha^{-1} yr^{-1} was calculated. For dry deposition, it was not possible to analyse the impact of N loading on the dry deposition as this varied annually (Table 3.2). The analysis therefore assessed if the indicator changed significantly with the distance from the point source. Transect was treated as a class variable and distance as a continuous variable. If a significant result was found then linear and quadratic equations were fitted to investigate the type of response present.

The fungal communities were assessed using two approaches (see Appendix 1). Terminal restriction fragment length polymorphism (T-RFLP) gives a low resolution overview of taxa present and cloning and sequencing which gives a high resolution but low coverage view of the most abundant taxa present. Terminal fragments length were determined using Genemapper (Applied Biosystems) and peaks<35 and >550 were discarded. The data was exported into T-REX (http://trex.biohpc.org/) where the clustering threshold was set to 0.99 and any TRF which occurred in less than 0.5% of samples were omitted. This clustering gave the final TRFs used in subsequent analyses. The potential effects of N loading on fungal taxon richness were analysed as for the other univariate analyses above. Community composition was investigated using multivariate analyses using PCORD 5.3 (McCune and Mefford, 2006) and EstimateS (Colwell, 2013). This was carried out separately for the dry and both wet deposition data sets. Patterns were sought in the community data using principal components analysis (PCA) and non metric multidimensional scaling (NMDS). The former assumes a linear model, while the latter makes no assumptions on the response model.

Identification of the sequences obtained from the cloning of the DNA was achieved using GenBank (www.ncbi.nlm.nih.gov/) and the UNITE database (http://unite.ut.ee/; Abarenkovet *al*., 2010). The sequences obtained (minimum lengths 350 bp; in most cases more than 400 bp) were blasted against both databases. A blast result that was considered to represent a positive identification was required to meet the following criteria. First, the match should be close to 100% (minimum 98%), and include the entire blasted sequence (e.g. not resulting in major gaps or only including the conservative 5.8S region). The relatively broad margin for variation of up to 2% was allowed to accommodate variation resulting from sequence ambiguities and editing errors. Mismatches at the beginning and end of the sequences, ambiguous base pairs in either of the sequences, repetitive sequence motives, single base pair mutations and small indels were tolerated. A considerable number of sequences could not be aligned with any known taxon and in these cases the highest reliable taxonomic resolution was assigned to the sequence. Due the paucity of reference sequence data in many fungal groups, in some cases this meant assigning 'Unknown fungus' as the best possible identification. Where sequences could only be assigned to a broad group, taxa were distinguished within these groups by aligning all sequences assigned to each broad group and then creating comparative similarity trees using MEGA5 (http://www.megasoftware.net/). Sequences assigned to the same branches could then be given a unique label (e.g. unknown ascomycete 1).

4 RESULTS FROM THE TESTING OF SOIL INDICATORS

4.1 Response of indicators to wet NH_4 deposition

Of the six soil indicators pH, fungal/bacterial PLFA ratio, base cation/Al ratio, C/N ratio, NH_4-N/NO_3-N soil solution ratio, phosphomonoesterase; phosphomonoesterase was the only indicator which showed a significant effect of N deposition ($F_{3,12}$, = 4.23[4],, $p<0.05$[5]). This means that the level of phophomonoesterase activity changes with different N loadings. We then tested to see if there was a linear or quadratic relationship between N loading and phosphomonoesterase, but neither model was significant, thus although there were significant differences between N treatments in phosphomonoesterase the indicator did not respond in a consistent (linear or quadratic) way to the change in N loading (Fig. 4.1). Fungal/bacterial PLFA ratio, pH, base cation/Al ratio, total C/N ratio, NH_4-N/NO_3-N soil solution ratio all showed large variability resulting in no significant patterns with increased N loading (Fig 4.1).

The cloning method for the fungal molecular markers obtained a total of 68 sequences. The sequences could be assigned to 35 different taxa of which only 11 (31.4%) could be identified to species level. Ascomycetes were the dominant taxonomic group with 20 taxa, basidiomycetes were next with 11, then zygomycetes with two and a single chytrid fungus was also identified. One sequence could be identified as being of fungal origin but could not be placed in any known taxonomic grouping and therefore represent a fungus with as yet unknown affinities. The distribution of taxa across samples was highly skewed. Of the 35 taxa distinguished 17 (48.5%) occurred as singletons (were only found in single samples). In total 30 taxa occurred in two or less samples. Only one taxon taxa occurred in more than four samples. This skewed and extremely heterogeneous distribution effectively means that the data are unsuitable for using multivariate analyses to examine potential links to N loading.

When the fungi were assessed using T-RFLP 231 separate TRFs were distinguished from the 9 samples from which data was obtained. Two samples failed to yield DNA even with repeated extraction attempts and one sample only yielded a small number of fragments and was excluded from further analysis after repeated attempts to obtained better usable TRF profiles. The average number of TRFs in a sample was 80.3 and the range was 65-102. Seventy-five TRFs occurred in single samples. As with the other indicators, there was considerable variation in the numbers of TRFs among the replicate samples (Table 4.1) resulting in no significant patterns in taxon richness with increased wet NH_4 deposition.

[4] This is the F value which is used in statistical testing together with the degrees of freedom and the residual degrees of freedom (the subscripts) to obtain the p value.

[5] In statistical significance testing the p-value is the probability of obtaining a result at least as extreme as the one that was actually observed, assuming that the null hypothesis is true. One often "rejects the null hypothesis" when the p-value is less than the predetermined significance level which is often 0.05. In this experiment the null hypothesis is that N deposition has no effect on the soil indicator.

Table 4.1. The number of terminal restriction fragments (TRFs) recovered from wet NH_4 deposition samples.

N kg ha^{-1} yr^{-1}	N	Mean	SD
8	3	71.0	8.9
16	3	77.0	18.3
32	4	80.0	5.5
64	2	86.0	22.6

Attempts to summarise the fungal community data using PCA and non-metric multidimensional scaling (NMDS) failed to retrieve any meaningful patterns relating to N loading in the wet NH_4 deposition dataset. The first major axis in the PCA analysis only explained 16.1% total variation in the data. Even the first three axes only explained a total of 39.4%.

The PCA analysis is best suited to datasets where there are few zero values and low skewness and in the TRF data there were ca. 75% zero values and many 'rare' taxa – nearly 40% of the TRFs occurred in 5 samples or less. NMDS is more robust in relation to the input data, but it also failed to find stable patterns within the dataset. The term stress is used in NMDS as a measure of the reliability of the resulting ordination with low values (usually < 10) being desirable. The level of stress in the NMDS analysis of the wet NH_4, deposition dataset was over 10, which means that there was no discernible pattern of N loading on the distribution or occurrence of the TRFs.

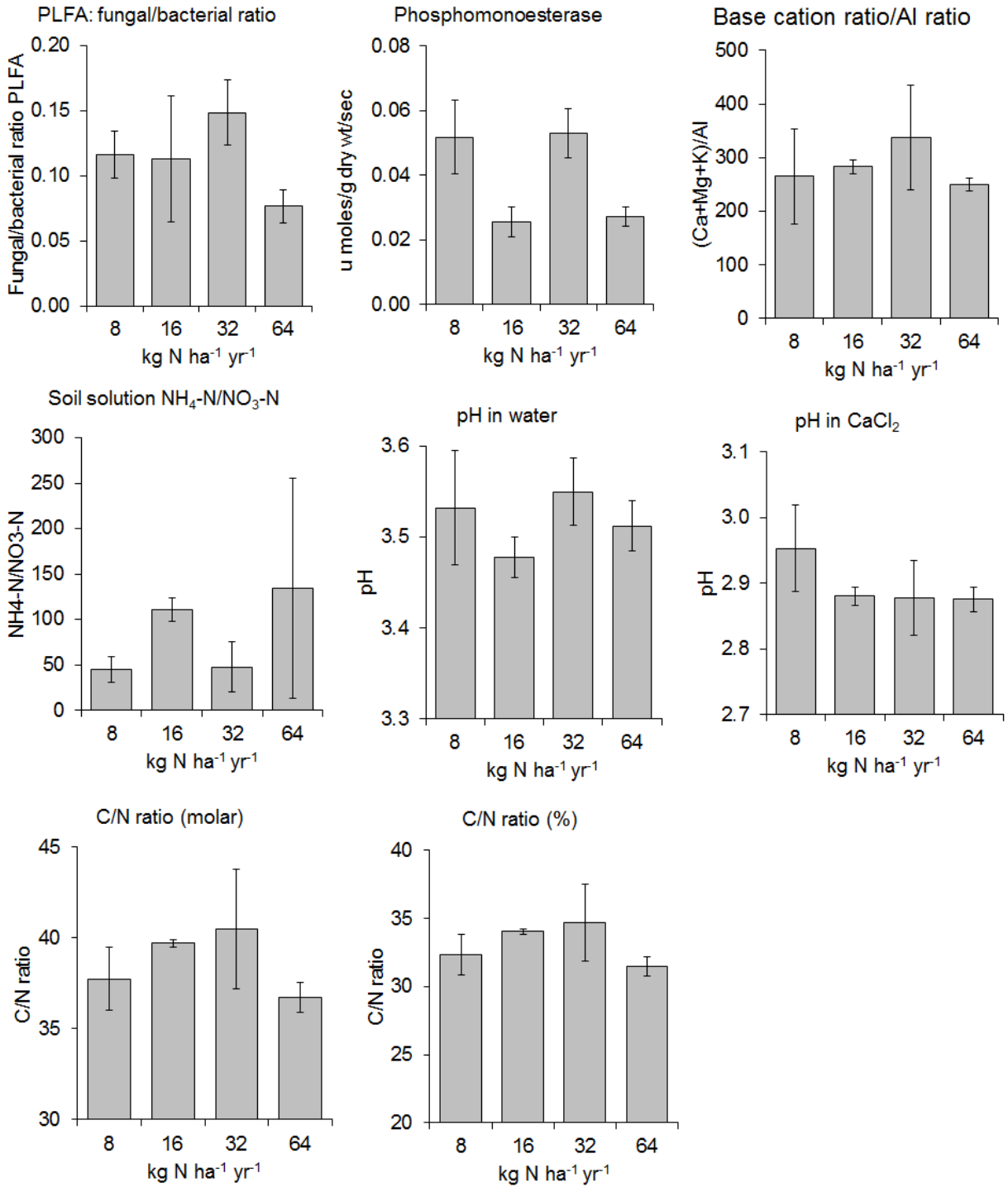


Figure 4.1. Changes in seven soil indicators receiving different loadings of wet NH_4 deposition. Means ± standard error are shown, n = 4.

4.2 Response of indicators to wet NO_3 deposition

More of the indicators were more sensitive to wet NO_3 deposition than wet NH_4 deposition with two indicators being significant and two marginally so (Fig. 4.2). Soil pH (measured in water) and the NH_4-N/NO_3-N soil solution ratio both increased in value as N deposition increased (soil pH: $F_{3,12}$, = 5.81 $p<0.05$; NH_4-N:NO_3-N: $F_{3,12}$, = 4.64, $p<0.05$). For each of these indicators we tested to see if there was a linear or quadratic relationship between N loading and the indicator. For pH the linear model was significant but the quadratic was not. An increase in 1 kg N ha^{-1} yr^{-1} is estimated to result in an increase of 0.004152 pH units. Thus a change from 8 to 64 kg N ha^{-1} yr^{-1} is predicted to result in an increase of 0.23 pH units. For soil solution a quadratic relationship was significant but a linear was not. The results showed that the NH_4-N/NO_3-N soil solution ratio increased as the N loading increased from 8 to 32 kg N ha^{-1} yr^{-1}, the ratio then declined as the N loading increased from 32 to 64 kg N ha^{-1} yr^{-1}. As the relationship is quadratic there is not a consistent rate of change in the ratio with a unit increase in nitrogen.

Soil pH when measured in $CaCl_2$ and the base cation/Al ratio were both marginally significant[6] (soil pH: $F_{3,12}$, = 3.86, $p=0.0502$; base cation ratio: $F_{3,12}$, = 3.80, $p=0.0519$). These variables were specifically tested for a linear or quadratic effect and a linear model was found to be significant for both of them (pH in $CaCl_2$: $F_{1,9}$, = 7.81, $p<0.05$; base cation ratio: $F_{1,9}$, = 8.83, $p<0.05$). An increase in 1 kg N ha^{-1} yr^{-1} is estimated to increase the soil pH (as measured in $CaCl_2$) by 0.002008 pH units. Therefore a change from 8 to 64 kg N ha^{-1} yr^{-1} is estimated to increase the pH by 0.11 units. An increase in 1 kg N ha^{-1} yr^{-1} is estimated to increase the base cation/Al ratio by 4.22, so a change from 8 to 64 kg N ha^{-1} yr^{-1} is predicted to result in an increase in the ratio of 236. Thus as N loading increases the total amount of Ca, Mg and K increases relative to the amount of Al.

The fungal molecular cloning method was not used for this part of the experiment, due to limited resources. The fungal community was assessed using T-RFLP. In total, 228 separate TRFs were distinguished from the 11 samples from which data was obtained. One sample failed to yield DNA despite repeated extraction attempts. The average number of TRFs in a sample was 62.5 and the range was 30 to 92. The high variation between samples masked any pattern in taxon richness related to increased wet NO_3 deposition (Table 4.2).

The proportion of singletons was again high 87 (38.2%). The high level of heterogeneity among replicate samples meant that the first major axis in the PCA analysis only explained 10.1% of the total variation in the data. The first three axes only explained a total of 27.6% of the variation in the dataset. NMDS also failed to resolve any patterns related to treatment with the stress value being 16.1

Table 4.2. The number of terminal restriction fragments (TRFs) recovered from wet NO_3 deposition samples.

N kg ha^{-1} yr^{-1}	N	Mean	SD
8	3	71.0	8.9
16	3	46.7	27.2
32	4	74.3	19.6
64	4	62.7	12.6

[6] Marginally significant means that the probably that the indicator was the same in all plots was slightly more than 5%.

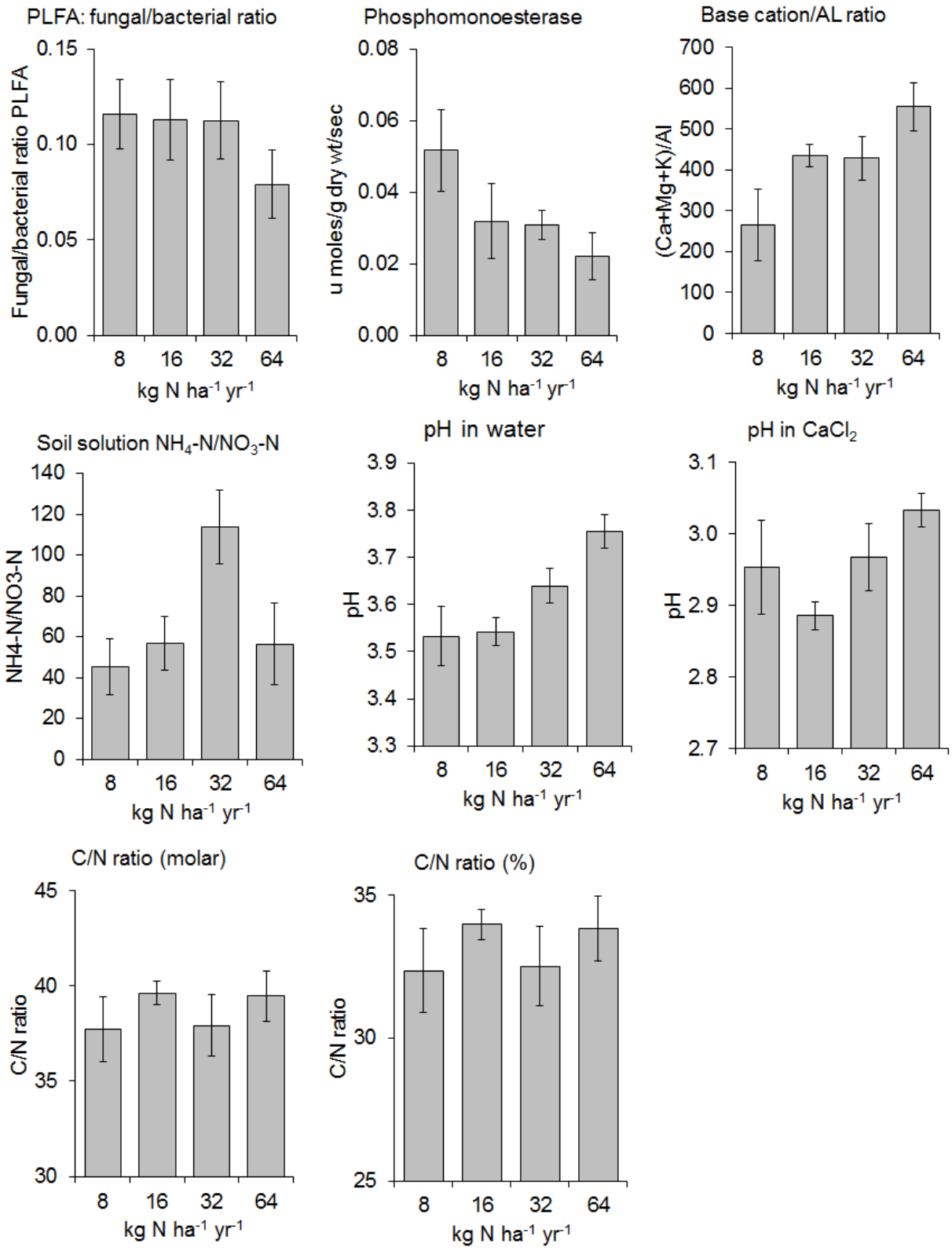


Figure 4.2. Changes in seven soil indicators receiving different loadings of wet NO_3 deposition. Means ± Standard Error are shown, n = 4.

4.3 Response of indicators to dry NH3 deposition

Three of the indicators changed significantly with distance from the dry deposition source. Soil pH in water increased with distance from the source ($F_{1,14}$ = 5.92; $p<0.05$). The base cation/Al ratio increased with distance from source ($F_{1,14}$ = 4.93; $p<0.05$) as did levels of phosphomonoesterase ($F_{1,14}$ = 6.93; $p<0.05$). For each of the indicators that were significant we tested to see if there was a linear or quadratic relationship between N loading and the indicator. In each case the linear model was significant but the quadratic was not.

When the fungal community composition was analysed using the cloning method a total of 79 sequences were obtained from the clone libraries. The sequences could be assigned to 37 different taxa of which only 11 (31.4%) could be identified to species level. Ascomycetes were the dominant taxonomic group with 24 taxa, basidiomycetes were next with 10, then zygomycetes with two and a single sequence could be identified as being of fungal origin but could not be placed in any known taxonomic grouping and therefore represent a fungus with as yet unknown affinities.

The distribution of taxa across samples was highly skewed. Of the 37 taxa distinguished 24 (64.8%) occurred as singletons (were only found in single samples). In total, 33 taxa occurred in two or less samples. Only two taxa occurred in more than four samples. Just as with the data from the wet NH_4 deposition samples, the skewed and extremely heterogeneous distribution means that the data are unsuitable for using multivariate analyses to examine potential links to N loading.

When analysed using T-RFLP 250 separate TRFs were distinguished from the 15 samples from which data was obtained. One sample failed to yield DNA. The average number of TRFs in a sample was 71.0 and the range was 54 to 92. The high variation between samples masked any pattern in taxon richness related to increased dry NH_3 deposition (Table 4.3). There were fewer singletons in these samples than in the other two treatments, only 65 (24%).

An analysis using PCA did not find any pattern relating to N deposition. The first axis only explained 16.1% of the total variation in the data and the first three axes cumulatively only explained a total of 27.9% of the variation. The level of stress in the NMDS analysis of the dry NH_3 deposition dataset was the highest of any of the treatments at 24.6 and essentially means that samples are placed at random in the ordination.

Table 4.3. The number of terminal restriction fragments (TRFs) recovered from dry NH_3 deposition samples.

Distance from source (m)	N	Mean	SD
12	4	71.7	8.4
20	3	69.0	7.5
32	4	69.7	18.9
55	4	71.0	8.9

.

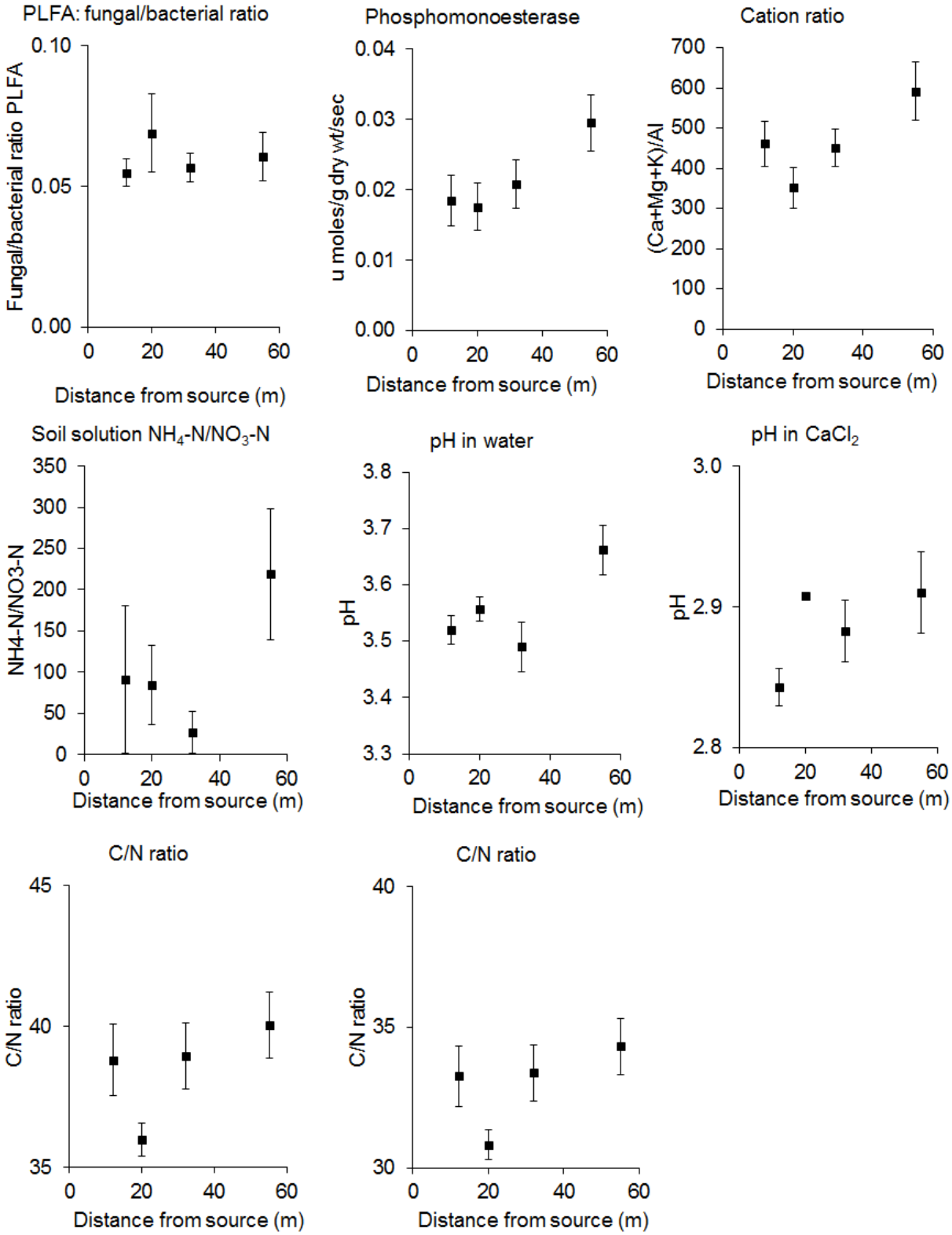


Figure 4.3. Changes in seven soil indicators receiving different loadings of dry deposition. Means ± standard error are shown, n = 4.

4.4 Spatial variability

Figs. 4.1, 4.2 and 4.3 show that for all the deposition types and all indicators there was large variability. Even taking account of block or transect within the analysis did not remove this variability. However the variance of the indicators from all three experiments (dry NH_3, wet NH_4, wet NO_3) at Whim Moss is similar to that from the other datasets studied in section 2 (Fig.4.4). Thus the predictions made about the variance of the indicators in section 2 are correct with the variance at Whim Moss not greater than that expected. It is possible that the lower sample size (only 4 per treatment) compared to that suggested in Table 2.4 may have resulted in a lack of significant results. This suggests that taking multiple samples (here 5) and bulking them to form one sample per a plot did not overcome the spatial variability. Sample numbers in this project was limited by the resources available for chemical analysis, however in future (if resources allowed) clearer results might be obtained if a great number of samples was taken and analysed, rather than bulking the samples.

The fungal data was highly variable both in terms of number of TRFs recovered from samples and with the taxon composition of communities. Even with 99 singletons removed from the data to decrease variation, the communities were still highly divergent. It is possible that increased sample numbers or repeated subsampling from bulked samples may reduce variation. The clone data also highlighted the highly variable nature of fungal communities. Increasing the numbers of clones analysed would certainly increase the usability of the data but given the potential richness of the communities this could mean several hundred clones per plot. There is a clear need here for use of high throughput sequencing approaches to deal with both the spatial and taxonomic variability.

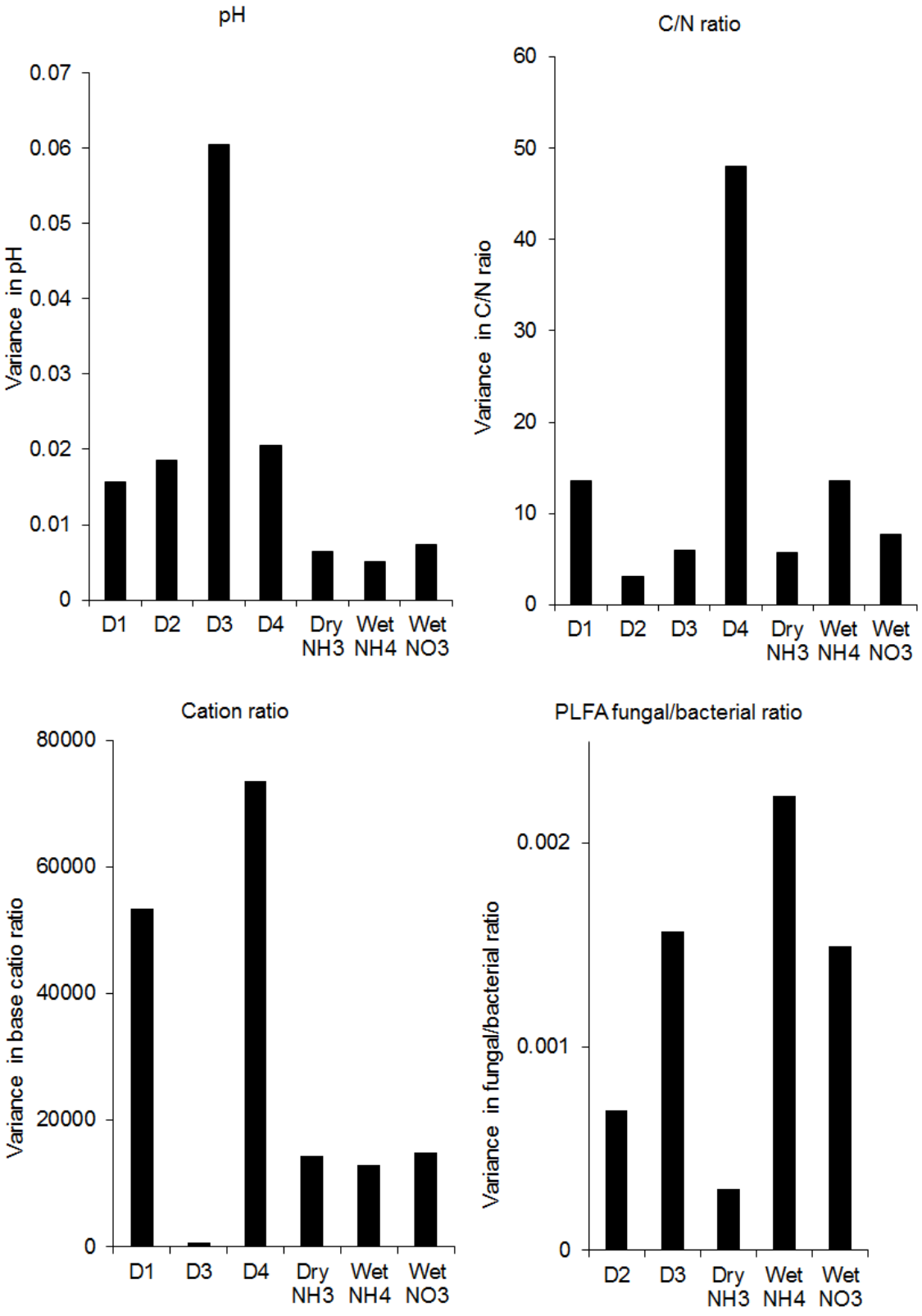


Figure 4.4. Comparison of the variability of the data from Whim Moss(dry NH_3, wet NH_4, wet NO_3) with the existing datasets (D1-4) used in Section 2. Only plot level variance from the datasets in Section 2 are shown together with the three datasets from Whim Moss.

4.5 Comparison of indicators and assessment of their suitability

As most of the indicators were not significant, it is impossible to compare their suitability, but the results do suggest that soil pH, base cation/Al ratio and phosphomonoesterase warrant further investigation in the first instance (section 4.8).

Across all the different N treatments, soil pH is the indicator that seems to respond most consistently to an increase in pH but the direction of change is not always as expected.

Many other studies have shown different responses or impacts depending on the form of N being released. The results from this study confirm that the soil indicators tested were more sensitive to changes driven by NO_3 than NH_4 and to dry deposition than wet deposition. This suggests that these indicators maybe more effective indicators for certain types of N pollution than others and could be considered as part of site condition monitoring activities where N impacts may be from a range of NOx sources such as combustion, transport etc. However with diffuse pollution where NH_3 is relatively more important, further work is needed to identify suitable indicators. One factor influencing the selection of all indicators will be a suitable baseline or benchmark for change. There are different options for this. An example would be comparable sites within the same geographical location but receiving relatively low and high loadings. Another maybe a broader baseline based on data from multiple comparable sites (e.g. values for soils of habitats in critical load exceedance versus those not in exceedance) where influences of other factors (vegetation, climate, historical N deposition) can be constrained.

In light of the results for fungal/bacterial ratio and molecular analysis of fungal communities, we may have to regard these as 'experimental' indicators in the short-medium term. These indicators may prove useful, but there is insufficient data at present to determine their usefulness. Future work should collect samples to establish the "natural variation" in these indicators and to increase our knowledge of the spatial and temporal impacts of N deposition on the soil.

Some groups of fungi are known to be highly sensitive to increases in soil mineral N. In particular, ectomycorrhizal (ECM) fungi have been shown to respond strongly to both N deposition and N fertilisation. The dominant mycorrhizal association at Whim Moss are ericoid associations and we know much less about the response of these fungi to N additions. It is possible that they are much less sensitive to N loading than ECM fungi. Mycorrhizal symbioses are based on nutrient exchange and it would be expected that changes in N availability would impact on the mycorrhizal fungi either directly or indirectly via redistribution of C allocation by the host plant. It is possible that the fungal community at Whim bog has already been altered by the elevated background N deposition resulting in the remaining community being more resilient to further increases in N.

In addition, the amount of fungal diversity in ecosystems such as Whim Moss is still largely unknown as there have been few studies in such systems. This makes it difficult to predict what may be expected both in terms of taxon richness and community composition.

The cloning data highlighted a major issue with the current state of reference databases, which is the lack of reference sequences from identified fungal taxa, particularly from Ascomycetes. Overall, only 23.8% of the sequences could be identified to species. Two of these turned out to be ectomycorrhizal fungi, *Tomentella ellisii* and *Tricholoma arvernense.* Interestingly, the latter species is the first record in the UK since 2004 and the fungus is only known from three sites – all Scots pine forest.

4.6 Sample sizes

Using the method described in Section 2.4 we recalculated the number of samples that would be required to have an 80% chance of detecting a specified change as significant at the 5% level. This was done for each of the three forms of nitrogen deposition using the levels of variance derived from the samples taken at Whim bog (Table 4.4). The unit of change was taken to be the mean of the highest N treatment or closest distance minus the mean of the lowest N treatment or furthest distance.

Table 4.4. The number of samples required to have an 80% chance of detecting a given unit of changed as significant at the 5% level.

	Unit of change	Variance	No of samples
Dry NH_3 deposition			
PLFA fungal/bacteria	-0.0056	0.0003	306
pH in water	-0.1425	0.00643	14
pH in $CaCl_2$	-0.0675	0.00178	16
Phosphomonoesterase	-0.0111	4.7E-05	16
Base cation ratio	-129.95	14385	30
Soil solution	-127.95	16819	36
C/N ratio	-1.2299	5.6737	120
Wet NH_4 deposition			
PLFA fungal/bacterial	-0.0394	0.00223	48
pH in water	-0.02	0.00502	398
pH in $CaCl_2$	-0.0775	0.00558	32
Phosphomonoesterase	-0.0247	0.00022	14
Base cation ratio	-14.779	12839	1848
Soil solution	88.9332	15715	66
C/N ratio	-1.0194	13.6104	414
Wet NO_3 deposition			
PLFA fungal/bacterial	-0.0368	0.00149	38
pH in water	0.2225	0.0074	8
pH in $CaCl_2$	0.08	0.0038	22
Phosphomonoesterase	-0.0297	0.00021	10
Base cation ratio	289.188	14852	10
Soil solution	11.221	823.78	208
C/N ratio	1.72753	7.6528	84

4.7 Next steps

The lack of significant results in this trial does not mean that the indicators are unsuitable; rather they may not be sufficiently sensitive at this sampling intensity or the levels of N inputs; sample size and soil conditions may explain why the indicators were not significant. The results of the seven indicators from Whim Moss show expected levels of variation when compared to published data (section 4.5) with values in the range identified from other experiments. Thus, the lack of significant results in this study is not due to the variation being greater than expected. We suggest that bulking the 5 soil cores per plot did not reduce the variation sufficiently and that a greater number of samples is required to detect significance. This is confirmed by Table 4.2. The next step would be to test this for a few indicators which show promise as indicators from this work.

For some indicators, the number of samples suggested in Table 4.2 is too large to warrant immediate further investigation but for other indicators such as pH, phosphomonoesterase and base cation/Al ratio the sample numbers are close to those already acquired by this work and the results suggest that these indicators are significant or nearly so. The sample numbers in Table 4.2 are the total number from low (8 kg N ha^{-1} yr^{-1}) and high (64 kg N ha^{-1} yr^{-1}) deposition plots, as this project already has 4 samples from each of these plots (8 in total) the additional number of samples required is reasonably small in some cases (Table 4.5).

For the indicators for which additional sampling is suggested the largest number of additional samples required is 24, 12 from the 8 kg N ha^{-1} yr^{-1} and 12 from the 64 kg N ha^{-1} yr^{-1} plots. As the 8 kg N ha^{-1} yr^{-1} treatment is the control plots, these only need to be sampled once for wet NO_3 and NH_4.

The following next steps are suggested for sampling in the wet deposition experiment:

- Take 3 cores from each from the 4 plots receiving 8 kg N ha^{-1} yr^{-1} (control plots)
- Take 3 cores from each from the 4 plots receiving 64 kg N ha^{-1} yr^{-1} as NH_4.
- Take 3 cores from each from the 4 plots receiving 64 kg N ha^{-1} yr^{-1} as NO_3.

The following next steps are suggested for sampling in the dry deposition experiment:

- Take 3 cores from each from the 4 transects at 12 m from source.
- Take 3 cores from each from the 4 transects at 55 m from source.

Analysis

- Analyse each core separately from the wet deposition control and NO_3 plots and dry NH_3 sampling locations for pH in $CaCl_2$, base cation/Al ratio and phosphomonoesterase
- Analysis each core separately from the wet deposition NH_4 plots for pH in $CaCl_2$ and phosphomonoesterase.

The above approach would give:

- 60 samples to be analysed for pH in $CaCl_2$ and phosphomonoesterase,
- 48 samples to be analysed for base cation/Al ratio.

This sampling approach would provide more material than needed for these analyses and the rest of the material could be stored for future analysis of the other indicators if required at a later date.

Table 4.5. Additional number of samples required split across low and high deposition plots.

	Total	Extra required	Resampling suggested
Dry NH_3 deposition			
PLFA fungal/bacterial	306	298	
pH in water	14	6	YES
pH in $CaCl_2$	16	8	YES
Phosphomonoesterase	16	8	YES
Base cation ratio	30	24	YES
Soil solution	36	28	
C/N ratio	120	112	
Wet NH_4 deposition			
PLFA fungal/bacterial	48	40	
pH in water	398	390	
pH in $CaCl_2$	32	24	YES
Phosphomonoesterase	14	6	YES
Base cation ratio	1848	1840	
Soil solution	66	58	
C/N ratio	414	406	
Wet NO_3 deposition			
PLFA fungal/bacterial	38	30	
pH in water	8	0	
pH in $CaCl_2$	22	14	YES
Phosphomonoesterase	10	2	YES
Base cation ratio	10	2	YES
Soil solution	208	200	
C/N ratio	84	64	

This would enable us to test if these indicators do change with changes in N loadings but if more samples are required. Results for phosphomonoesterase and base cation/Al ratio from the dry NH_3 deposition gradient have already been shown to be significant but this additional sampling will allow us to test if we can improve the level of significance of the result (decrease the P value) and decrease the variation by increasing the number of samples.

4.8 Discussion

This dataset is unique in that it tests many possible soil indicators simultaneously and the results can be set in the context of a long-term experiment and the associated data sets which allow us to check that the data obtained from a one off sample fits in with the results obtained with more regular sampling over the years.

Differences in the results here from those expected maybe due to a number of reasons (c.f. Phoenix *et al*. 2012) and could include: inability to detect signal of change due to limited numbers of samples collected and sampling depth, linked to the spatial heterogeneity of the site or lack of sensitivity of the indicator; lack of ecosystem response for a number of reasons (buffering, historical N loadings, interactions of the N in the very organic rich soils). Some of these differences could be tackled through more intensive and targeted sampling.

The lack of significant results shown by most of the indicators is probably, in part, due to the low sample size, rather than large variation as the variation was as expected. Our sample size was limited in part by the resources available for analytical work and in part by the experimental layout at Whim bog. Section 2 suggests that we needed 9 samples for pH, 12 for C/N ratio, 50 for PLFA fungal/bacterial ratio and 17 for base cation/Al ratio from each of a high and low N treatment. The sampling intensity could be increased through various routes, including multiple samples per replicate or gradient location with relevant statistical analyses.

Throughout the report from this experiment the levels of N deposition reported at each plot or sampling location is the total N (additional N plus background deposition) deposited on the surface, as per standard practice. It is not possible to state how the N moves through the soil or what levels of additional N are available at any given depth in the soil. Some of the nitrogen will be utilised by the plants and there may be a greater impact of N addition to the top layers of the soil than the lower layers. Sampling depth may therefore influence the results: if the N deposition only impacts the indicator in the first few cm of the core then this effect will be diluted if a deep core is sampled and the whole depth of the core mixed prior to analysis, as done in this work. The core depth was deliberately reduced (to 5 cm) for biological indicators in this work because of this potential issue. This was not done for all indicators due to the amount of soil required for analysis (See Annex B). For organic soils the dry weight of soil per a volume is low, thus in order to get enough material a core to 15 cm was taken. If a shallow core was taken for all indicators more cores would have to be taken in order to obtain enough material. Further work could investigate the effect of sampling depth on the suitability of the indicator and assess the depth to which an increase in N deposition has an impact on the indicator.

Lack of ecosystem response may occur for a number of reasons (buffering, historical N loadings, interactions of the N in the very organic rich soils). Most sites will have been affected to some degree by historical N deposition, thus the indicators may have already responded significantly with their current (lack of) sensitivity reflecting current status beyond “pristine” conditions. The relative importance of historical N loadings to the sensitivity of these indicators requires further attention.

In parallel, the significant results from certain indicators requires further analyses and interpretation since the responses did not always follow the expected responses as indicated from the literature. In the wet NO_3 deposition plots pH increases with increasing nitrogen, this is as expected and fits with the long-term results from CEH collected at this site (Sheppard *et al*., 2011). However In the wet deposition NH_4 plots almost no decrease in pH was found and may indicate low levels of nitrification in the organic soils while responses within dry deposition require further investigation. Phosphomonoesterase increased with distance from source along the dry gradient. Previous studies suggest that

phosphomonoesterase response can be highly variable, with both increased and decreased responses to N deposition. UK research on an upland moor detected an increase in phosphomonoesterase with increased N deposition. It is probable that soil phosphomonoesterase response is mediated by interactions between N availability and plant uptake. With wet NH_4 deposition the changes in phosphomonoesterase were not consistently related to the nitrogen loading which suggests that greater understanding is needed about phosphomonoesterase responsiveness to aid indicator development.

Soil fungal communities are typically taxonomically diverse and spatially highly variable (Bueé *et al*., 2009; Anderson and Cairney, 2004) and the results from this project support this view. High numbers of TRFs were recovered from the samples but nearly 30% were singletons – only occurring in 1 sample. Even with these removed the data were highly skewed with a further 40% occurring in five samples or less. One of the few studies which have examined fungal diversity in ecosystems like that at Whim is that by Bougoure *et al*. (2007). They examined fungi associated with the hair roots of ericaceous plants on a transect across a moorland in Scotland and found a high diversity of potentially ericoid mycorrhizal fungal taxa with ascomycetes were being more frequent than basidiomycetes; a very similar picture to that obtained in the present study. In addition Bougoure *et al*. found that the community of fungi associated with *Calluna vulgaris* hair roots was different for samples collected from the forest, open heathland and a transition zone between the two. This distinction was largely driven by the presence of ECM fungi in the transition zone and under the trees. However, within these zones, particularly the open heathland, there was great heterogeneity among samples, again a very similar situation to that found in the present project. It is clear that further studies are required, not only in moorland systems, but in a wide range of Scottish ecosystems to determine the fine scale spatial variation of fungal communities. The excellent studies carried out on ECM fungi at the Scots pine forest at Culbin provide the only example of such studies to date (see Genney *et al*., 2006).

The results from this project may differ from those expected due to the highly organic rich and nutrient poor soil on which the experiment was established. Nitrogen pollution can be both a nutrient and acidifying agent with a wide range of direct and indirect actions on soil (NEGTAP, 2001). Inputs of N to soil occur through deposition on litter, infiltration into the soil or through changes to the quality of plant litter and roots. Generally these inputs will alter the activity and structure of the soil biological community as the soil microbial biomass captures, uses and transforms the nitrogen, with consequences for the entire soil food web and in nutrient availability for plants. Changes to biologically driven nutrient cycling processes (e.g. mineralization and mobilisation of N) also alter the chemical form of N within the soil and produce an acidifying effect. The plant community will also utilise the increased availability of soil N, this results in changes in the plant community composition as the more competitive and faster growing species out compete other species. (Haines-Young *et al.,* 2000, Smart *et al.,* 2003, Kirkby *et al*., 2005). A feedback loop exists between soil and plants with eutrophication exacerbated as litter and soil C/N ratios lower through plant responses to N which in turn further alter soil N availability. In more acid soils and highly organic soils, a large proportion of NH_4 is immobilised by the soil microbial biomass and readily utilised by the plants. The lack of results from this experiment may in part be due to the fact that the plants are utilizing additional N and that the soil system is buffering the inputs of N through rapid microbial uptake and transfers. The exact impact of additional N on the soil chemistry and soil fauna will depend on the balance of all of these processes and how much N is already available within the soil.

5 SETTING UP A SITE-SPECIFIC SOIL MONITORING SCHEME

The aim of this section is to provide the background and discussion from which the sampling protocol in Annex A was produced. The protocol in Annex A is based on the best available evidence at the moment but needs to be tested and improved. This section documents why the protocol in Annex A is suggested.

The aim of the protocol is to provide a method of sampling soil indicators along a transect away from a point source of N deposition such as poultry or pig farms and to detect the impact of the point source over and above that of the back-ground N deposition. The method is not aimed at assessing the impact of N deposition from high towers, where the dispersion occurs over a much wider area, nor to assess the impact of back-ground levels of N deposition. The method is not designed to not detect the impact of small changes in N deposition such as the impact of a 1% increase above the critical load as required when making an assessment for a Pollution Prevention and Control permit (PPC).

There are four key pieces of information to consider when designing a sampling strategy:

- The spatial distribution on the N deposition and the type of N deposition that is occurring
- The vegetation
- The soil
- The number of samples
- The spatial arrangement of the samples.

5.1 N deposition

The basic approach is to sample along an N pollution gradient and to assess if the soil indicators change along this gradient. However a sampling approach with regular sampling along the gradient, such as Fig. 5.1a, is not recommended as a) there is no replication and b) the literature (Section 2) suggests that for many soil indicators a significant effect of N will only be observed if there is a difference of at least 15-40 kg N ha^{-1} yr^{-1} between sample locations along an N deposition gradient. Thus in Fig. 5.1a a difference may only be detected between the first and last sampling point. If possible the first task is therefore to assess what the N deposition is likely to be. If the difference between the highest and lowest N deposition at the site is less than 15 kg N ha^{-1} yr^{-1} then it is unlikely that the indicators will show a significant effect of N deposition. If the N deposition is gradient is 15-40 kg N ha^{-1} yr^{-1} then sampling at locations receiving the lowest and highest N inputs is recommended (Fig. 5.1b). If the N deposition gradient (difference between lowest and highest values) is 80 kg N ha^{-1} yr^{-1} or more then sampling 3 different N loadings with a difference of 40 kg N ha^{-1} yr^{-1} would be possible (Fig. 5.1c). Ideally an N deposition map of the site with N deposition contours would enable the areas within a site receiving particular N loadings to be located.

If a map of N deposition is not available then it has to be assumed that the prevailing wind direction will be the main gradient of N deposition, and that distance from source is related to N deposition. If no data is available on the N deposition received at the site it is recommended that some simple monitoring be put in place. This would involve rain water collection for wet deposition and ALHPA or DELTA sampling for dry deposition at both the closest and furthest points from the point source. Details of alpha and DELTA sampling can be found at http://pollutantdeposition.defra.gov.uk/ammonia_methodology.

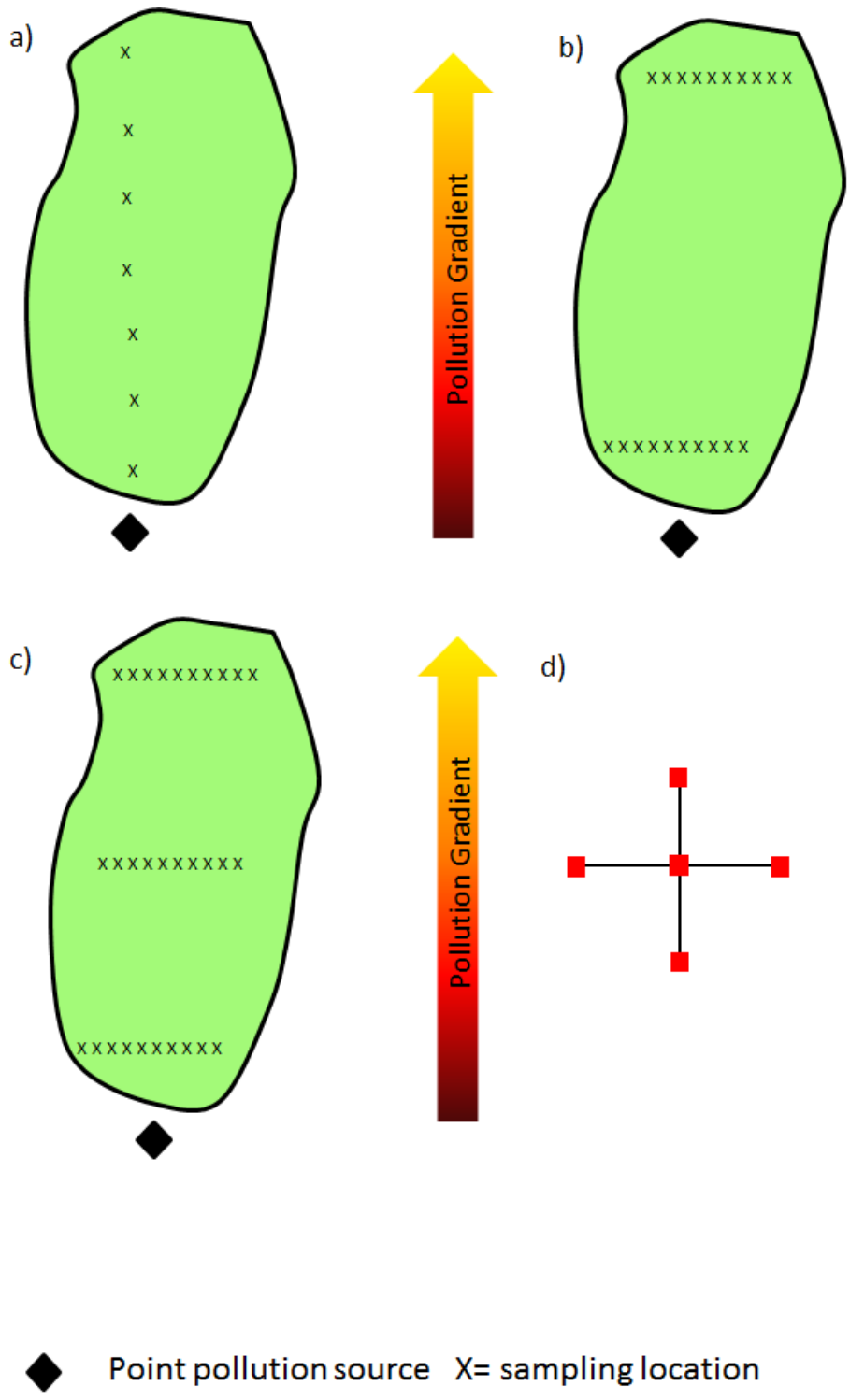


Figure 5.1. Examples of different sampling regimes along a N deposition gradient from a point source: a) single sampling locations at 7 different N deposition loadings from a point source – not recommended; b) multiple sampling at 2 N loading (high and low) recommended when the N gradient is between 15-40 kg N $ha^{-1}yr^{-1}$; c) multiple sampling at 3 N loading (high, medium and low) recommended when the N gradient is over 80 kg N $ha^{-1}yr^{-1}$. d) 5 cores (red squares) taken at one sampling location.

5.2 The vegetation

Simply sampling at locations with a known N loading or at a given distance from the N source may not show the true impact of the N point source due to the variability in soil indicator values caused by different vegetation/soil classes. For example, when birch trees colonise moorland, soil chemical properties and soil microbial community change considerably (Mitchell *et al.,* 2007). Thus, if sampling was undertaken anywhere at the required distance or anywhere on the required N contour loading different vegetation types might be sampled, for example moorland and birch woodland and the soil indicator values would potentially be significantly different due to differences in the vegetation rather than differences in N loadings. There are many other examples where vegetation differences and soil type would alter the seven selected soil indicators (Bargett & Wardle, 2010). However, over longer time periods, point source pollution may alter vegetation. For example, increased N deposition on moorland is likely to increase the cover of grasses and cause a decline in heather (Aerts & Berendse, 1988). This change in vegetation will subsequently drive changes in soil characteristics. Therefore, sampling should be confined to a single vegetation (habitat) class but the description of the habitat to be sampled should be broad enough to include possible changes in vegetation driven by the point source pollution but narrow enough to exclude changes to different broad habitat types.

A National Vegetation Classification (NVC) map of the site should be obtained and the different vegetation classes identified. The most wide spread vegetation type(s) should be identified and one class chosen for the sampling. If a range of vegetation classes are to be sampled these need to be replicated (i.e. if two vegetation classes are sampled twice the number of samples is required). It is important to define the vegetation classes for each site so a surveyor can make an “on the ground” assessment as to if the area if be sampled meets the requirements. This preliminary classification would have to be carried out for each site. It is therefore, necessary that vegetation maps are available prior to the sampling in order to aid the design of the sampling strategy. Such maps sometimes already exist for designated sites, with the local SNH office or the land owner being the first point of enquiry for such maps. Alternatively an experience botanical surveyor can provide NVC maps.

5.3 The soil

The soil type will influence the soil indicators (e.g. Bargett & Wardle, 2010), ideally only one soil type should be sampled as this will decrease variation and increase the chances of detecting a significant impact of the N deposition on the soil indicator. However this is rarely realistic as there are often a number of soil types within a habitat. Thus the sampling strategy and number of soil samples required should reflect the heterogeneity of soil types (c.f. Avery, 1990 and Soil Survey of Scotland, 1984). Ideally soil maps of the site should be used to identify the different soil types occurring on the site, with major soil group the initial class (Soil Survey of Scotland, 1984). The ultimate class level of soil type used will be dependant on a number of factors, including the heterogeneity of the habitat (soil and vegetation) and the availability of soil maps. The soil map should be overlaid with the vegetation map to produce combined vegetation/soil classes. The most wide spread vegetation/soil classes should be identified and one class chosen for the sampling. It is important to define the vegetation/soil classes for each site so a surveyor can make an “on the ground” assessment as to areas to obtain soil samples and move to a nearby location if the vegetation/soil is not as specified. Detailed soil maps (1:10,000) maybe not be easily available. If this is the case then sampling location will have to be based on the N deposition and vegetation maps alone, but notes taken on any major differences in soil type during sampling.

5.4 Combining N pollution maps, vegetation maps and soil maps.

Once the vegetation/soil class to be sampled has been defined the vegetation/soil map of the site should be overlain with a map of the N deposition footprint in order to identify areas of required vegetation/soil class receiving different levels of N loadings within the site boundaries. The key to the distribution of sample points is not the distance from the point source but the N deposition loadings present. Nitrogen deposition from localised N point sources such as intensive farms will have a very different N deposition foot print from other types of point sources, such as chimney stacks, that tend to provide more diffuse N deposition footprints. Therefore, it is important to establish the characteristics of the N deposition footprint (gradient) and the distance over which impact is to be assessed. The change in the N deposition loadings may not decline linearly with distance away from point source but may rather decline exponentially. In addition, the N-deposition loadings will be influenced by prevailing wind; soil 100 m downwind of a point source will receive a very different N deposition loading from soil 100 m upwind of a point source. The impact of the point source will be more accurately assessed if the samples can be taken from locations with known N deposition loadings rather than at set distances.

If the N deposition map is not available then the vegetation/soil map should be used to identify locations within the site where the same vegetation/soil class occurs at two different distances (close and far) from the point source.

In both cases a list of grid references of the sampling locations should be produced and given to the surveyor along with a description of the expected vegetation/soil class at these sites.

5.5 Sample numbers

Due to spatial variability, it is important to collect replicate soil samples for each N-deposition loading. This can be achieved by either taking “sub-samples” at each sampling location (Fig. 1d) or taking single point sampling on multiple gradients (Fig.5.1b&c). A combination of these two approaches is also possible. A balance should be struck between multiple samples at any one location (5.1d) and multiple locations (5.1b&c). If the limiting factor is analytical costs then bulking sub-samples from each sampling location may allow variation within the site to be accounted for (Fig. 5.1d) but reduce costs as was done in Section 3. However the results from this study (Section 3) suggest that such an approach should be used with caution and may not reduce the variation sufficiently to detect changes in indicators. Ideally more individual samples is better than fewer bulked samples (Section 3). Furthermore, the variability of each soil indicator will be different (Chapters 2 and 4); this may result in different numbers of samples being required, hence a further way of reducing analytical costs.

The exact number of samples to be taken to detect a significant impact of N deposition will depend on the site, its history of N deposition, the vegetation and the variability of the indicators at that site and the type of N deposition (NO_3, NH_4 or NH_3). Currently for an organic rich soil we suggest the numbers of samples listed in Table 5.1, this is the total number of samples and should be split equally between a low and high N deposition sampling locations.

Table 5.1. Suggested number of samples to be taken for different soil indicators. The total number of samples is shown, this should be split between a low and high N loading.

Indicator	Dry NH_3 deposition	Wet NH_4 deposition	Wet NO_3 deposition
PLFA fungal/bacterial	306	48	38
pH in water	20	400	8
pH in $CaCl_2$	20	30	22
Phosphomonoesterase	16	14	10
Base cation/Al ratio	30	1800	10
Soil solution NH_4-N/NO_3-N ratio	40	70	200
C/N ratio	120	400	80

5.6 Costing

Total costs will be dependent on sampling strategy and logistical complexity and scope of analyses required, however basic assumptions and requirements relevant to all projects can be discussed.

Table 5.2 Details of analyses undertaken within the project and associated information

Measurement	Standard methodology (BSI, ISO or equivalent)	Analyses available from commercial labs in Scotland	Commercial cost per sample [7]	Certified standard reference material available
Soil pH in water & $CaCl_2$	Y	Y	15.75	Y[8]
Soil carbon / nitrogen (C/N) ratio,	Y	Y	25.40	Y[9]
Base cation / aluminium ratio	Y	Y	52.45	N
Solution ammonium / nitrate (NH_4-N/NO_3-N)	N	N	47.30	N
Fungal to bacterial ratio (PLFA)	N	N	88.50	N
Fungal molecular analyses	N	N	n/a	N
Phosphomonoesterase	N	N	n/a	N

[7] Note: Costs are per sample and are full economic costs associated with the James Hutton Institute and time of publication. Actual costs charged by the James Hutton Institute reflect preferential rates attributed to the project and as a consequence are discounted from the above.

[8] Note: CRMs are available for the determination of soil pH. Care must be taken when determining soil pH in $CaCl_2$ to ensure correct methodology is maintained.

[9] Note: C/N ratio determined after total C & N analyses using Dumas combustion. CRMs are available for the determination of total C & N in a variety of matrices.

5.7 Laboratory selection

Laboratory selection is based on variety of parameters which ought to be considered prior to submission of samples.

- Appropriate standards and quality systems, e.g. ISO 17025, ISO 9001are held by the laboratory.
- Laboratory has proven record of soil analysis.
- Laboratory staff have experience in analysis of soil samples.
- Laboratory resources are sufficient to ensure analysis.
- Quality control materials are used which are appropriate to the analysis undertaken.
- Laboratory participates where applicable in appropriate inter laboratory trials.
- Laboratory has experience of undertaking specific soil analyses requested.

Table 5.3. Prevalence of laboratories routinely undertaking soil analyses in UK.

Analyses	Prevalence
Soil pH in water & CaCl2	Many commercial labs, standard methods applied
Soil carbon / nitrogen (C/N) ratio,	Many commercial labs, standard methods applied
Base cation / aluminium ratio	Many commercial labs, standard methods applied
Solution ammonium / nitrate (NH4-N/NO3-N)	Many commercial labs, method dependent
Fungal to bacterial ratio (PLFA)	Few commercial labs, non-standard methods
Fungal molecular analyses	Few commercial labs, non-standard methods

6 CONCLUSION

- Different indicators are likely to work better under different types of N deposition.

- The following maybe suitable indicators for current use under certain circumstances:
  - wet NH_4 deposition: phosphomonoesterase ,
  - wet NO_3 deposition: soil pH (measured in water or $CaCl_2$), NH_4-N:NO_3-N soil solution and base cation/Al ratio ;
  - dry NH_3 deposition: soil pH in water, cation/Al ratio and phosphomonoesterase .

- Further work to test the indicators that showed most promise (soil pH in $CaCl_2$, base cation/Al ratio and phosphomonoesterase) is suggested.

- The remaining indicators require more development before deployment.

- The levels of variability of the indicators in organic rich soils suggested by the literature appear to be realistic, thus use of this information to estimate the sample size required is valid, however further work on the expected value of change (difference between high and low loadings) would enable refinement of the number of sample sizes required.

- Bulking samples to account for spatial variation while limiting the number of samples analysed is not a substitute for more samples.

- A difference of at least 15-40 kg N ha^{-1} yr^{-1} between two N loadings is required to detect a change in the indicator.

- The number of samples required to detect a change in indicator will depend on the type of N deposition.

- Buffering, historical N loadings and interactions of the N in organic rich soils may all account for the lack of response in the indicators and require further investigation. Once the importance of these factors is known it may be possible to take them into account when predicting how indicators will respond to an increase in N loadings.

- These indicators maybe suitable for assessing the impacts of point sources from poultry farms etc. on the soil but will not detect small increases in N deposition, such as a 1% increase in critical loads.

- Changes in these indicators could be used to indicate a change in soil function and ecosystem services. However this linking needs further work.

- This study has concentrated on developing methods and assessing the appropriateness of the indicators for organic rich soils as a) these are the major soil type in the majority of designated sites in Scotland and b) most experimental work and hence available literature on N impacts on soil are conducted on organic rich soils. Further work should develop this approach for other soil types/habitats.

- When setting up a site specific soil monitoring schemes the following are required: vegetation map, soil map, N deposition footprint map.

- Ideally the chosen laboratory for soil analysis ought to be able to undertake the majority of the analyses ‘in-house’ with appropriate methodology and QC in place. Where sub-

contracting is required the laboratory ought to be able to provide organisational and sub-sampling services as appropriate to the analyses required.

7 REFERENCES

Abarenkov, K.; Nilsson, R.H.; Larsson, K-H.; Alexander, I.J.; Eberhardt, U.; Erland, S.; Hoiland, K.; Kjoller, R.; Larsson, E.; Pennanen, T.; Sen, R.; Taylor, A.F.S;Tedersoo, L.; Ursing, B.M.; Vralstad, T.; Liimatainen, K.; Peinter, U.; Koljalg, U. (2010). The UNITE database for molecular identification of fungi - recent updates and future perspectives. *New Phytologist,* 186, *281-285*

Aerts, R. & Berendse, F. (1988). The effect of increased nutrient availability on vegetation dynamics in wet heathlands. *Vegetatio*, 76, 63-69.

Anderson, I.C. & Cairney, J.W.G. (2004). Diversity and ecology of soil fungal communities: increased understanding through the application of molecular techniques. *Environmental Microbiology,* 6, 769-779

Avery. B.W. (1990) *Soils of the British Isles,* Wallingford: CAB International. 463 pp.

Bardett, R.D. & Wardle, D.A. (2010). *Aboveground-Belowground linkages*. *Biotic Interactios, Ecosystem Processes, and Global Change.* Oxford Series in ecology and evolution. Oxford University Press, Oxford.

Black, H.I.J.; Britton, A.J.; Helliwell, R.C.; Langan, S.J.; Taylor, A.F.S. & Booth P.D., (2009). To establish soil indicators to assess the impact of atmospheric deposition on environmentally sensitive areas. Consultancy Service, SEPA.

Bougoure D.S., Parkin, P.I., Cairney, J.W.G., Alexander, I.J., & Anderson, I.C. (2007). Diversity of fungi in hair roots of Ericaceae varies along a vegetation gradient. *Molecular Ecology,* 16, 4624–4636.

Britton, A.J.; Helliwell, R.C.; Fisher, J.M.; Gibbs, S. (2008). Interactive effects of nitrogen deposition and fire on plant and soil chemistry in alpine heathland. *Environmental Pollution*, 156, 409-416.

Buée M., Reich, M., Murat, C., Morin, E., Nilsson, R.H., Uroz, S. & Martin, F. (2009). 454 pyrosequencing analyses of forest soil reveal an unexpected high fungal diversity. *New Phytologist*, 184, 449-456

Colwell, R.K. (2013). *EstimateS: Statistical estimation of species richness and shared species from samples*. Version 9. Persistent URL <purl.oclc.org/estimates>.

Franklin, R.B. & Mills, A.L. (2003). Multi-scale variation in spatial heterogeneity for microbial community structure in an eastern Virginia agricultural field. FEMS *Microbiology Ecology,* 44, 335-346.

Genney, D.R., Anderson, I.C. & Alexander, I.J. (2006). Fine-scale distribution of pine ectomycorrhizas and their extramatrical mycelium. *New Phytologist,* 170, 381–390

Haines-Young, R.H., Barr, C.J., Black, H.I.J., Briggs, D.J., Bunce, R.G.H., Clarke, R.T., Cooper, A., Dawson, F.H., Firbank, L.G., Fuller, R.M., Furse, M.T., Gillespie, M.K., Hill, R., Hornung, M., Howard, D.C., McCann, T., Morecroft, M.D., Petit, S., Sier, A.R.J., Smart, S.M., Smith, G.M., Stott, A.P., Stuart, R.C. & Watkins, J.W. (2000). *Accounting for Nature: Assessing Habitats in the UK Countryside*. London: DETR

Hartley S.E. & Mitchell R.J. (2005). Manipulation of nutrients and grazing levels on heather moorland: Changes in *Calluna* dominance and consequences for community composition. *Journal of Ecology*, 93, 990-1004

Helliwell, R.C., Britton, A.J., Gibbs, S., Fisher, J.M., Potts J.M. (2010). Interactive Effects of N Deposition, Land Management and Weather Patterns on Soil Solution Chemistry in a Scottish Alpine Heath *Ecosystems,* 13, 696–711

Kirby, K.J., Smart, S.M., Black, H.I.J., Bunce, R.G.H., Corney, P.M. & Smithers, R.J. (2005). *Long term ecological change in British woodland (1971-2001).* English Nature Research Report 653. Peterborough: English Nature

Leith, I.D, Sheppard, L.J., Fowler, D., Cape, J.N., Jones, M., Crossley, A., Hargreaves, K.J., Tang, Y.S., Theobald M., Sutton., M.R. Quantifying dry NH_3 deposition to an ombrotrophic bog from an automated NH_3 field release system. *Water, Air, and Soil Pollution:* Focus 4, 207-218

McCune, B. & Mefford M.J. (2006). PC-ORD. *Multivariate Analysis of Ecological Data. Version 5.33, MjM Software*, Gleneden Beach, Oregon, U.S.A.

Medeirosa, P.M., Fernandesc, M.F., Dickc,R.P. & Simoneita B.R.T. (2006). Seasonal variations in sugar contents and microbial community in a ryegrass soil. *Chemosphere* 65, 832–839

Mitchell R.J, Campbell C.D., Chapman S.J., Osler G.H.R., Vanbergen A.J., Ross L.C., Cameron C.M., Cole L. (2007). The cascading effects of birch on heather moorland: a test for top-down control of an ecosystem engineer *Journal of Ecology*, 95, 540-554.

Mitchell, R.J. Campbell, C.D., Chapman, S.J., & Cameron, C.M. (2010a). The ecological engineering impact of a single tree species on the soil microbial community. *Journal of Ecology*, 98, 50-61.

Mitchell, R.J., Hester, A.J., Campbell, C.D., Chapman, S.J., Cameron, C.M., Hewison, R.L., Potts, J.M. (2012). Explaining the variation in the soil microbial community: do vegetation composition and soil chemistry explain the same or different parts of the microbial variation? *Plant and soil*, 351, 355-362

Mitchell, R.J., Hester, A.J., Campbell, C.D., Chapman, S.J., Cameron, C.M., Hewison, R.L., Potts, J.M. (2010b). Is vegetation composition or soil chemistry the best predictor of the soil microbial community? *Plant and Soil,* 333, 417-430.

Mitchell R.J., Truscott A-M, Leith I.D. , Cape J.N., Van Dijk N., Tang Y.S., Fowler, D. & Sutton M.A. (2005). A study of the epiphytic communities of Atlantic oak woods along an atmospheric nitrogen deposition gradient. *Journal of Ecology*, 93, 482-492

Mitchell, R.J., Sutton, M.A., Truscott, A-M, Leith, I.D., Cape, J.N., Pitcairn, C.E.R. & van Dijk, N. (2004). Growth and tissue nitrogen of epiphytic Atlantic bryophytes:effects of increased and decreased atmospheric N deposition. *Functional Ecology,* 18, 322-329.

Mummey, D.L. & Rillig, M.C. (2008). Spatial characterization of arbuscular mycorrhizal fungal molecular diversity at the submetre scale in a temperate grassland. FEMS *Microbiology Ecology,* 64, 260-270.

NEGTAP (National Expert Group on Transboundary Air Pollution) (2001). *Transboundary Air Pollution: Acidification, Eutrophication and Ground-Level Ozone in the UK*. ISBN 1 870393 61 9. Available online at http://www.nbu.ac.uk/negtap

Pearce, I.S.K. & van der Wal, R. (2002) Effects of nitrogen deposition on growth and survival of montane *Racomitrium lanuginosum* heath. *Biological Conservation* 104, 83–89.

Pearce, I.S.K., Woodin, S.J. & van der Wal, R. (2003). Physiological and growth responses of montane *Racomitrium lanuginosum* (Hedw.) Brid. to atmospheric nitrogen deposition. *New Phytologist,* 160, 145–155.

Phoenix, G.K., Emmett, B.A., Britton, A.J., Caporn, S.J.M., Dise, N.B., Helliwel, R., Jones, L., Leake, J.R., Leith, I.D., Sheppard, L.J., Sowerby, A., Pilkington, M.G., Rowe, e.D., Ashmore M.R. & Power, S.A. (2012). Impacts of atmospheric nitrogen deposition: responses of multiple plant and soil parameters across contrasting ecosystems in long-term field experiments. *Global Change Biology,* 18,1197-1215.

Pitcairn, C.E.R., Fowler, D. & Grace, J. (1995) Deposition of fixed atmospheric nitrogen and foliar nitrogen content of bryophytes and *Calluna vulgaris* (L.) Hull. *Environmental Pollution* 88, 193–205.

Pitcairn, C.E.R., Leith, I.D., Sheppard, L.J., Sutton, M.A., Fowler, D., Munro, R.C., Tang, S. & Wilson, D. (1998). The relationship between nitrogen deposition, species composition and foliar nitrogen concentrations in woodland flora in the vicinity of livestock farms. *Environmental Pollution,* 102, 41–48.

Pilkington, M.G. Caporn, S.J.M. Carroll, J.A. Cresswell, N. Lee, J.A. Ashenden, T.W. Brittain S.A., Reynolds, B. Emmett. B.A. (2005a). Effects of increased deposition of atmospheric nitrogen on an upland moor: leaching of N species and soil solution chemistry. *Environmental Pollution,* 135, 29-40.

Pilkington, M.G. Caporn, S.J.M. Carroll, J.A. Cresswell, N. Lee, J.A. Emmett, B.A. Johnson. D. (2005b). Effects of increased deposition of atmospheric nitrogen on an upland Calluna moor: N and P transformations. *Environmental Pollution,* 135, 469-480.

RoTAP (2012). *Reviwe of Transboundary Air Pollution; Acidification, Eutrophication, Ground Level Ozone and Heavy Metals in the UK*. Contract Report to the Department for Environment, Food and Rural Affairs. Centre for Ecology and Hydrology.

SAS (2008). *SAS Software* Version 9.2. SAS, Cary,NC,USA.

Scottish Government (2009). *The Scottish Soils Framework.* Scottish Government, Edinburgh.

Sheppard, L.J., Leith, I.D., Mizunuma, T., Cape, J.N., Crossley, A., Leeson, S., Sutton, M.A., van Dijk, N. & Fowler, D. (2011). Dry deposition of ammonia gas drives species change faster than wet deposition of ammonium ions; evidence from a long-term field manipulation. *Global Change Biology*, 17, 3589-3607.

Smart, S.M., Robertson, J.C., Shield, E.J. & van de Poll, H.M. (2003). Locating eutrophication effects across British vegetation between 1990 and 1998. *Global Change Biology,* 9, 1763-1774.

Soil Survey of Scotland. (1984). *Organisation and Methods. Handbook 8.* Macauley Institute for Soil Research

Annex A: Protocol for designing soil sampling strategy to assess the impact of a point source on soil indicators.

A1 PROTOCOL FOR DESIGNING SOIL SAMPLING STRATETGY

This Annex provides a provisional protocol for guidance on how to set up a soil sampling strategy. It is based on the results of this report and the sampling strategy used as part of this work. However, as highlighted within the report, further work is required to assess the best spatial arrangement of samples[10]. This protocol describes the method used for this report, which includes bulking of multiple soil samples, taken with a box corer. However, if resources allow better results may be obtained by analysing individual samples rather than bulking samples as described within Annexes A and B, but further work is required to test this.

Follow the numbered bullet points, where a question is asked go the relevant numbered bullet point according to your answer.

1	Identify point source		
2	Identify impacted site		
3	Is there a map of the N deposition from the point source over the site available?	Yes - go to 4	No - go to 6
4	Is the differences between the highest and lowest N deposition at the site at least 15-40 kg N ha-1 yr-1?	Yes - go to 9	No - go to 5
5	Indicators are unlikely to show a response to N deposition if the difference between the high and low N deposition is less than 15 -40 kg N ha^{-1} yr^{-1}. Consider if it is a good use of resources to sample the site.		
6	Identify prevailing wind direction.		
7	Assume pollution gradient is along prevailing wind direction and is at least 15 kg N $ha^{-1}yr^{-1}$ and therefore will be sufficient to detect a change between low and high ends of the gradient.		
8	Assume locations at similar distance from point source have same N deposition loadings and for rest of method treat N loading as distance from source, but note that it may be worth considering setting up some N deposition monitoring at the site to confirm that the gradient is as expected.		

[10] This maybe a start or W shape depending on the local variability and will need to be decided on the ground by a qualified soil surveyor.

9	Identify two pollution loadings/distances at opposite ends of the pollution gradient to sample. If the difference is more than 80 kg N ha-1 yr-1 between the low and high ends of the gradient consider sampling three different N loadings with a difference of at least 40 kg N ha-1 yr-1 between them.		
10	Is there an NVC map of the vegetation communities? Ideally 1:10,000 scale	Yes - go to 11	No - go to 17
11	Is there a soil map of the area? Ideally 1:10,000 scale	Yes - go to 12	No - go 15
12	Overlie the vegetation and soil maps to produce combined vegetation/soil classes		
13	Identify the vegetation/soil classes that occurs regularly along the N deposition gradient (this maybe the direction of the prevailing wind if no N deposition map is available), and decide on one vegetation/soil class to be sampled		
14	Identify the multiple sampling locations at the two (or more) N loadings/distances which have the same vegetation/soil class and record grid reference to be sampled. (See table below for number of samples required). Then go to 18.		
15	Identify the vegetation classes that occurs regularly along the N deposition gradient (this maybe the direction of the prevailing wind if no N deposition map is available), and decide on one vegetation class to be sampled.		
16	Identify the multiple sampling locations at the two (or more) N loadings/distances which have the same vegetation class and record grid reference to be sampled. (See table below for number of samples required). Then go to 18.		
17	Obtain vegetation map before proceeding.		
18	Gather necessary equipment - see list below (A2) and list of grid references to be sampled.		
19	Is a box corer to be used giving cores of dimensions 5 cm x 5cm by 15 cm to be used?	Yes - go to 22	No – go to 20

20 Calculate volume of soil that will be taken per a core

21 Calculate the number of cores required to provide a dry mass of approximately 180g per a sampling location (see Table A2).

22 Check that the mass of soil collected at each sampling location is sufficient for the analyses required (Table B1)

23 Go to grid reference location to be sampled

24 Is location the required vegetation/soil class? Yes - go to 22 No - go to 21

25 Move to nearby location that is of required vegetation/soil class

26 Take one undisturbed core at the centre of the location and 4 more cores to the N, E, S, W of the location, each 0.5 m away from the central core. If more than 5 cores are required (Step 21) then arrange them in a suitable star or W pattern depending on local variability as assessed by an experienced surveyor. The sample should remove the litter (L) but include the F and H horizons (see Section A4). Samples should be at least 15 cm deep. If necessary deeper cores maybe taken and chopped to the required depth in the lab (Annex B)

23 Wrap each core in cling film, bag and label. Labelling should include details of core orientation – top and bottom of core as well as sampling location.

24 Keep cores cool until reaching lab

25 Follow lab manual (Annex B) for instructions on processing cores.

A2 LIST OF SAMPLING EQUIPMENT REQUIRED:

- GPS
- List of grid references to be sampled
- Site map
- Soil corer (preferably box corer)[11]
- Ruler
- Cling film
- Bags
- Cool boxes
- Standard health and safety equipment (1st aid kit)
- Risk assessment

Note: Appropriate packaging and labelling ought to be used to ensure sample integrity is maintained during transportation to the laboratory and core orientation is labelled, i.e. top of core / bottom of core, if required.

[11] A soil screw auger or Dutch auger should not be used. If the soil is not consolidated enough to take intact soil cores then this protocol cannot be followed.

A3 NUMBER OF SAMPLES

The exact number of samples to be taken to detect a significant impact of N deposition will depend on the site, its history of N deposition, the vegetation and the variability of the indicators at that site and the type of N deposition (NO_3, NH_4 or NH_3). Currently for an organic rich soil we suggest the numbers of samples listed in Table A.1, this is the total number of samples and should be split between a low and high N deposition sampling location. For any additional distances sampled add half of the sampling number.

Table A3.1. Suggested number of samples to be taken for different soil indicators. The total number of samples is shown, this should be split between a low and high N loading.

Indicator	Dry deposition NH_3	Wet deposition NH_4	Wet deposition NO_3
PLFA fungal/bacterial	306	48	38
pH in water	20	400	8
pH in $CaCl_2$	20	30	22
Phosphomonoesterase	16	14	10
Base cation ratio	30	1800	10
Soil solution	40	70	200
C:N ratio	120	400	80

A4 VOLUME FIELD SAMPLING

The total volume of sample required to be removed from the field is dependent on the mass of sample required for each analyses and the type of sample, e.g. organic samples will typically have a greater proportion of water than mineral soil samples.

Table A4.1. Approximate mass of material obtained from suggested sampling protocol.

Sample Type	Volume[12] (cm3)	Assumptions	Mass[13] (g)
Organic	1875	90% water, 0% stones, wet bulk density 1 g/cm^3	187.5
Mineral	1875	35% water, 50% stones, dry bulk density 1 g/cm^3	230

Sampling protocols for determining sample volume ought to take into account both the required quantity of soil for each analyses and the soil type to be sampled.

Note: Analyses may require field moist, before or after sieving to specific sizes.

[12] Note: Assuming 5 x cores (5 x 5 x 15 cm) taken per sample point.
[13] Note: Final mass is assuming all sample is dried and sieved and constitutes dry mass (oven dry 105 °C).

A5 SOIL SURVEYORS

Based on sampling decisions made ensure soil surveyors of appropriate experience are used to undertake field sampling protocols, e.g. ensure criteria to distinguish between living and dead moss or litter layer and humus are agreed prior to sampling. It is recommended that the litter layer (L) be removed but that the sample should include the F and H horizons. It is important that these horizons are determined consistently when sampling.

Annex B – Laboratory Manual

B1 INTRODUCTION

This document outlines the methods and instrumentation used to prepare and analyse samples for the determinants analysed.

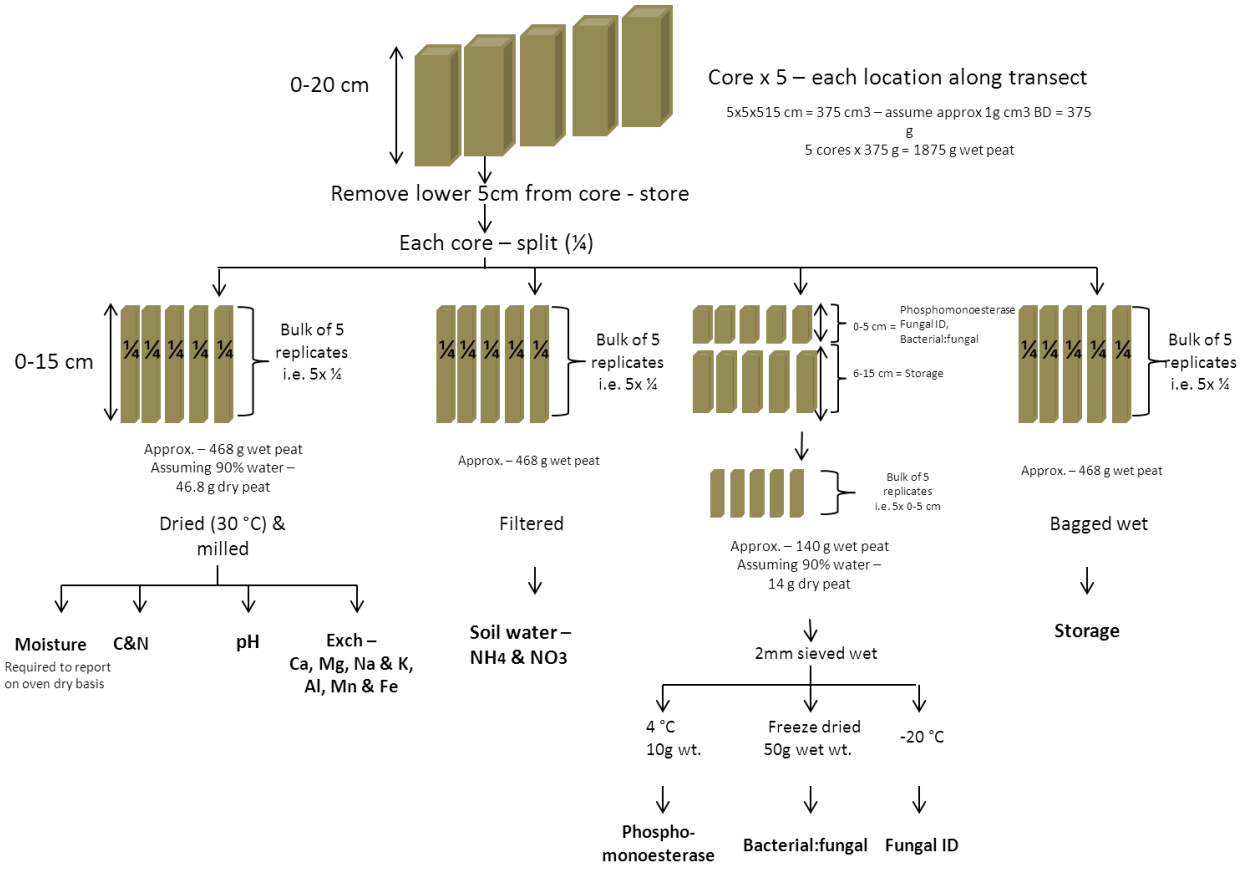
The sample preparation technique described (Section B2) is only appropriate for samples taken with a soil corer that produces intact soil cores. The method is not suitable for samples taken with a soil screw auger. The analytical techniques described are suitable for samples taken with either a soil corer or a soil screw auger, except for the method for soil water NH_4 and NO_3 (section B7) which is only suitable for samples taken with a soil corer. At present no suitable similar method exists to sample soil water using a screw auger.

All pertinent health and safety procedures must be discussed with appropriate personnel and adhered to prior to undertaking any work within your laboratory

B2 SAMPLE PREPARATION

If the soil (either organic or mineral) can be sampled intact using a corer then the samples maybe subsampled as described below. The protocol for sample preparation is based on the assumption that a box corer is used. If the soil is sampled using a soil screw auger then this sample preparation protocol cannot be followed.

Samples should be returned to the laboratory as individual soil cores (not bulked). On return to the laboratory the samples should be kept in cold room until processed as described below (Figure B1).



Note: After each soil core is split (¼) the sub-samples are bulked together and homogenised prior to preparation

Figure B1.1. Preparation of soil cores prior to analysis. (Diagram assumes all samples taken with a box corer providing cores of 5 cm x 5 cm x 20 cm.)

The cores should be processed one replicate at a time; one replicate constitutes all the cores taken at one sampling location (Step 26 of Annex A). If a box corer is used with dimensions of 5 cm x 5cm (as recommend) then one replicate constitutes 5 cores, but if smaller corers are used this number maybe greater (Step 21 Annex A). All cores should be cut to a depth of 15 cm and any material below this removed (in Figure B1 it is assumed that the cores are 20 cm long). The material below 15 cm maybe kept in storage, no analysis is undertaken on this material. The cores should be cut longitudinally, down the long axis, to split the cores into four quarters, i.e. to give four subsamples, of 15 cm long, by 2.5 x 2.5 cm. Three subsamples were labelled for a specific range of analyses. One sub sample was placed in storage.

The sub sample used to determine phosphomonoesterase, fungal/bacterial ratio and fungal ID had a further 10 cm removed from the bottom of the core, and placed in storage. The top 0-5 cm was used for analysis. Each sample for analysis constituted a sub sample of each replicate bulked together.

B2.1 Sample Weight

The sample weight required to undertake each analyses is dependent on the analyses (Table B2.1). Where samples are organic in nature as compared to mineral, sample weight may be lower, e.g. soil pH requires 15 g of air dry (30 °C) soil sieved to pass a 2 mm sieve if mineral in nature, but less if organic.

Table B2.1. Sample weight required to undertake analyses

Analyses	Sample Preparation	Mineral Soil Weight	Organic Soil Weight
Soil moisture (air dry)	<2mm air dried sieved	5 g	3 g
Soil pH (water & $CaCl^2$)	<2mm air dried sieved	15 g	5 g
Base cation / aluminium ratio	<2mm air dried sieved	10 g	10 g
Soil carbon / nitrogen (C/N) ratio,	Milled air dry soil	0.1 g	0.1 g
Solution ammonium/nitrate (NH_4-N/NO_3-N)	Wet soil	Total sample	Total sample
Fungal / bacterial ratio (PLFA)	Freeze dried 2 mm sieved wet soil	1.5 g	50 mg
Fungal analyses (TRFLP)	Frozen < 2mm sieved soil	1 g	1 g
Phosphomonoesterase	< 2mm sieved wet soil	10 g	10 g

B2.2 Sample Storage

Following sample preparation samples maybe stored as detailed in Table B2.2

Table B2.2. Sample stability following different storage conditions.

Sample	Storage Conditions	Analyses	Stability
Air dry soil (30 °C)	Stable temperature Sealed container <2mm sieved	Soil moisture (dry) pH, Base cation / aluminium ratio	>50 years
Milled air dry soil (30 °C)	Stable temperature Sealed container	Soil carbon / nitrogen (C/N) ratio,	>50 years
Freeze dried soil	Stable temperature Sealed container	Fungal to bacterial ratio (PLFA)	>10 yrs
Field moist soil	4 °C	Phosphomonoesterase, Solution ammonium / nitrate (NH_4-N/NO_3-N)	<2 weeks
Frozen soil	Frozen (-20 °C)	Fungal molecular analyses	>10 yrs

B3 MOISTURE

Source: Gardner, W. H. (1965). Physical and mineralogical properties, including statistics of measurement and sampling (eds Black, C. A.) *Methods of soil analysis Part 1* American Society of Agronomy, Madison p 83-96.

B3.1 Principle

Material is weighed out in silica/porcelain crucibles and dried in an oven (105 °C) to a constant weight.

B3.2 Reference Material

Bulk soil or herbage material may be used for in house quality control for determining loss of water.

B3.3 Equipment

- Silica or porcelain crucibles
- 4 place analytical balance
- Desiccator
- Oven maintained at 105°C
- Forceps (to take crucible out of oven and placing in desiccator when hot

B3.4 Performance check

Ensure all instrumentation and equipment is working within recognised parameters.

B3.5 Sample analysis

1 Weigh the sample (typically 0.5 – 8 g) accurately to four decimal places into dry silica/porcelain crucibles.

2 Place the crucibles in a preheated oven (105 °C). Dry the samples until a stable weight is achieved, typically samples are dried overnight.

3 Note the temperature of the oven in the laboratory workbook.

4 Remove the sample and place in a desiccator over silica gel desiccant. Allow to cool and reweigh.

B3.6 Calculation of results

The basic formulae for the calculation of results are:

$$\%\,\text{moisture} = \frac{(w_2 - w_3)}{(w_2 - w_1)} \times 100$$

or

$$\%\,\text{Dry matter} = \frac{(w_3 - w_1)}{(w_2 - w_1)} \times 100$$

where: w_1 = weight of the crucible alone
w_2 = weight of the crucible plus sample before ovenover-drying
w_3 = weight of the crucible plus sample after oven-drying

B3.7 Quality control

Quality control is maintained by analysing either in-house reference soil characterised against certified reference materials or certified reference materials. Where certified reference materials are not available comparison of data obtained for in house reference soil against accumulated historical data can be used. Analyse three reference soil samples with each set of samples.

B4 TOTAL C & N

Source: Pella, E. and Colombo, B. (1973) Study of carbon, hydrogen and nitrogen by combustion gas-chromatography. *Mikrochim Acta* 1973 697-719.

B4.1 Principle

Total C & N present on samples are determined using after Dumas combustion. Samples contained in a tin capsule are dropped into a combustion reactor maintained at 900^0C. The container melts and the tin promotes a 'flash reaction' in a helium atmosphere temporarily enriched with pure oxygen. This momentarily raises the temperature in the reactor to ~2000^0C combusting the sample. The combustion products are carried by a constant flow of helium through an oxidation catalyst, copper oxide and platinised Alumina. CO_2, N_2, NO_x and H_2O then flow into a reduction reactor containing copper wires held at 680^0C, where excess oxygen is removed and any nitrogen oxides are converted into nitrogen gas. Water is then absorbed by magnesium perchlorate. A chromatographic column held at 40^0C then separates the CO_2 and N_2 into defined peaks, and the relative amounts determined using a thermal conductivity detector.

B4.2 Reference Materials

A range of primary and secondary reference materials with certified C and N contents, are used to calibrate the instrument and as QC Check materials in this method.

B4.3 Reagents

- Tin cups
- Copper oxide and Copper wire
- Magnesium perchlorate
- Oxidation Catalyst (CuO & Platinised alumina)

Note : The above reagents are produced at purity suitable for use in micro-elemental analysis by specialised suppliers.

- Research Grade Oxygen gas
- Research Grade Helium gas

B4.4 Equipment

- Elemental Analyser from a suitable manufacturer
- Microbalance from a suitable manufacture
- Mirrored tile
- Forceps
- Oven
- Spatulas
- Glass vials
- Small brush

B4.5 Sampling and Sample Preparation

A subsample of air-dried, 2 mm sieved soil should be ground as fine as possible to ensure complete uniformity; this is usually achieved by using a ball mill. Sample results are generally expressed on a dry weight basis, before weighing, the samples are dried overnight at approximately 50°C in an oven. Once dried the samples are stored in a desiccator until ready for weighing.

B4.6 Instrument Performance Check

An instrument performance check is conducted. An instrument performance check is completed prior to any analyses been conducted.

B4.7 Reference Materials

Reference materials may be in house reference materials characterised against certified reference materials or certified reference materials.

B4.8 Analytical Procedure

1 As soon as possible after completing a satisfactory performance check continue with the analysis of samples.

2 Typically samples with a weight between 5 and 20 mg are weighed into tin cups. The elemental analyser is calibrated.

3 Calibration is based on the analysis of a reference material of known nitrogen and carbon values across a range of analyses weights. This ought to give a calibration graph consistent with samples under consideration and the limitations of the instrument used.

B4.9 Calibration and quality control

Quality control is achieved by analysing characterised materials similar (but not identical) to the references as unknowns and comparing the elemental composition with established data sets.

B5 PH

Source: McLean, E.O. (1982) *Methods of Soil Analysis Part 2 - Chemical and Microbiological Properties* Page, A.L, Miller, R.H and Keeney, D.R. (eds) 2nd Edition SSSA, Madison pp199 - 209

B5.1 Principle

The pH electrode forms a liquid junction of stable conductivity due to the presence of a saturated KCl solution. Upon immersion of the electrode in a sample solution, diffusion of the sample and the internal KCl solution alters the conductivity of the internal KCl solution. By comparison of the electro motive force obtained in the sample with that obtained in solutions of known pH values, the pH of the sample is estimated.

B5.2 Reference Material

Bulk soil samples characterised for pH are used as quality control.

B5.3 Reagents

The grade of reagents should be SLR or better.

- Calcium chloride for preparation of 0.10M Calcium chloride solution or alternatively use commercial grade 0.10M Calcium chloride
- Deionised water
- pH 4, 7 and 10 commercial reference solutions from two manufactures. One manufactures reference solutions will be used to calibrate the instrument, the other set of reference solutions will be used as an instrument performance check.

B5.4 Solution preparation

0.10M Calcium chloride

- Oven dry (1050C), for at least 2 hours, approximately 20 g of calcium chloride dihydrate (CaCl2.2H2O) and allow to cool in a dessicator with silica gel desiccant.
- Dissolve 11.10 g of oven-dried calcium chloride dihydrate in approximately 800 ml of deionised water in a 1 litre volumetric flask.
- Make up to the mark with deionised water.

B5.5 Equipment

- Timer
- 125 ml bottles
- 0.1M CaCl2 dispenser
- Two place balance
- Deionised water bottle
- Roller
- pH meter and electrode

B5.6 pH Meter maintenance and calibration

Ensure pH meter and electrode are maintained as per manufacturer's instructions, e.g. electrode is full of correct fill solution.

pH Meter Calibration: The pH meter is calibrated on each day of use, using pH 4 & 7, or if applicable pH 10, reference solutions, from one manufacturer. Calibrate in accordance with manufactures operating instructions.

Performance Check: Measure the pH of the reference solutions from the second manufacturer. Ensure the readings agree with acceptable variation of the method.

B5.7 Sample preparation

Samples should be air-dried (30 °C) and sieved to pass through a 2 mm mesh.

B5.8 Analytical procedure – Water and 0.01M Calcium Chloride Matrix

1 Weigh 15 g ±0.1 g of air-dry soil on a 2 place balance into a 125 ml bottle. Where the soil is organic only 2.5, 5 or 7.5g of soil may be used. Note the sample weight in the laboratory workbook.

2 Add 45 ml of deionised water. Mix the soil water slurry thoroughly on a roller for 2 hours, and allow to stand overnight.

3 Immerse pH electrode in the clear supernatant liquid. Start the timer. Take a reading after 5 minutes and note in the laboratory workbook.

4 Add 5 ml of 0.10M CaCl2 solution to each sample, roll for 30 minutes and allow to stand for 2 hours.

5 Immerse electrode in the clear supernatant liquid. Start the timer. Take a reading after 5 minutes and note in the laboratory workbook.

Note: The pH electrode is thoroughly washed with deionised water between each pH reading.

B5.9 Quality control

Quality control is maintained by analysing either in-house reference soil characterised against certified reference materials or certified reference materials. Analyse three QC soils with each set of samples.

B6 EXCHANGEABLE CATIONS

Source: Thomas, G.W., 1982 Methods of Soil Analysis Part 2 - Chemical and Microbiological Properties. Page, A.L, Miller, R.H and Keeney, D.R. (eds) 2nd Edition SSSA, Madison pp159 - 165

B6.1 Principle

The exchangeable cations in a soil are removed in solution by exchanging them with NH_4 from a 1M ammonium acetate solution (pH 7). The concentration of cations thus exchanged are estimated using inductively coupled plasma-optical emission spectroscopy (ICP-OES), atomic absorption spectroscopy (AAS) or another suitable technique.

B6.2 Reagents

The grade of reagents should be SLR or better.

- Acetic acid
- Ammonia solution
- Deionised (UHP) water

B6.3 Solution preparation

1M ammonium acetate:

1 Add 580 ±5 ml glacial acetic acid to 5 litres of deionised (UHP) water, allow to cool, in a fume cupboard.
2 Add 580±5 ml concentrated ammonia solution, allow to cool, in a fume cupboard.
3 Allow to stand overnight and make up to 10 litres with deionised (UHP) water.
4 Adjust to pH 6.98-7.02 by the addition of acetic acid or ammonia solution.

This solution is stable for 3 months.

B6.4 Equipment

- 100 ml beakers
- Glass stirring rods
- Whatman No 40 filter paper (15 cm)
- Funnels
- 250 ml volumetric flasks
- 1M ammonium acetate wash bottle
- pH Meter
- 4 place analytical balance

B6.5 Sample preparation

Samples should be air-dried (30 °C) and sieved to pass through a 2 mm mesh.

B6.6 Analytical procedure

1 Check the pH of 1M ammonium acetate before using, adjust to pH 7 if necessary.

2 Weigh approximately 10 g of soil accurately, on a 4 place analytical balance, into a 100 ml beaker.

3 Make up to the 50 ml mark with 1M ammonium acetate and thoroughly mix with a glass-rod.

4 Allow to equilibrate overnight.

5 Filter the soil slurry through Whatman No 40 filter paper into a 250 ml volumetric flask, ensuring the soil slurry is quantitatively transferred to the filter paper. Leach the soil slurry with approximately 100 ml of 1 M ammonium acetate and leave overnight.

6 Leach the soil slurry with 1 M ammonium acetate up to the mark on the 250 ml volumetric flask.

7 Transfer samples to polythene bottles and analyse for cations using inductively coupled plasma-optical emission spectroscopy (ICP-OES).

8 Ensure blank analyses are extracted with each set of analyses.

B6.7 Quality control

Quality control is maintained by analysing either in-house reference soil characterised against certified reference materials or certified reference materials. Where certified reference materials are not available comparison of data obtained for in house reference soil against accumulated historical data can be used. Analyse three reference soil samples with each set of samples.

B6.8 Moisture Content

Data are reported on an oven dry (105 °C) basis, after determination of the moisture content of the sample.

B6.9 Calculation of results

Data are expressed as milliequivalents per 100 g dry soil.

$$meq\ 100\ g\ oven\ dry\ soil = \left(X \times \left(\frac{250}{wt_{(g)}}\right)\right) \times \frac{1}{Y} \times 0.1$$

Where:

X= concentration of element as mg l^{-1}
Y = Elemental weight/charge

e.g.

- Ca = 20.04
- Na = 22.99
- K = 39.10
- Mg = 12.15

Moisture factor = 100/(100-moisture content)

Base cation to aluminium ratio expressed as:

$$ratio = \frac{Ca + Mg + K}{Al}$$

B7 SOIL WATER NH_4 AND NO_3

Source: James Hutton Institute in-house method.

B7.1 Principle

Soil water is extracted using deionised water prior to filtration and concentration of ammonium and nitrate ascertained colourimetrically.

B7.2 Reagents

- Deionised (UHP) water

B7.3 Equipment

- Whatman No 542 filter paper (15 cm)
- Funnels
- 125 ml polyethylene bottles
- place analytical balance

B7.4 Sample preparation

Store the samples at 4±3 °C prior to analysis. Homogenise the sample prior to analysis by manually mixing.

B7.5 Analytical procedure

1 Weigh approximately 10 g of soil accurately, on a 4 place analytical balance, into a 125 ml polyethylene bottles.

2 Add 30 ml of deionised water.

3 Place on an end over end shaker for two hours, prior to filtering through Whatman No 540 filter papers into polyethylene bottles.

4 Note: pre wash the filter papers with deionised water repeatedly and allow draining prior to use.

5 Analyse the filtrate for ammonium and nitrate concentration colourimetrically.

B7.6 Quality control

Ensure blank analyses extracted with each set of analyses.

B7.6 Moisture Content

Determine the moisture content of the sample (see Section b3).
This data is used to calculate the soil water in the original sample.

B7.7 Calculation of results

Data are expressed as mg l^{-1} soil water.
Determine the soil water in the original sample.

$$\text{Soil water(g)} = \frac{\text{Sample(g)}}{100} \text{ x Moisture \%}$$

$$\text{Concentration (mg l}^{-1}\text{)} = \frac{X}{100} \text{ x } \left(\left(\left(\frac{\theta_m}{100} \right) \text{ x M} \right) + 30 \right)$$

Where:

θ_m = mass of sample (g)
M = water content of sample (%)
X = instrumental concentration (mg/l)

NH_4-N : NO_3-N ratio expressed as:

$$ratio = \frac{NH_4 - N}{NO_3 - N}$$

B8 PHOSPHOMONOESTERASE ACTIVITY

Source: Tabatabai, M.A. and Bremner, J.M (1969) Use of *p*-Nitrophenyl phosphate for assay of soil phosphatase activity. *Soil Biology & Biochemistry* 1 (4) 301-307

B8.1 Principle of assay

Phosphatases are a group of enzymes which catalyse the hydrolysis of monophosphate esters. They are widely distributed in nature and can be classified on the basis of their activity under acidic (acid phosphatase activity) or alkaline (alkaline phosphatase activity) conditions. In soil, these enzymes allow soil microorganisms to access organically bound phosphate nutrients. The measurement of their activity in soil gives information of the demand for phosphates in soil.

They can be assayed by using a suitable substrate such as *para*-nitrophenyl phosphate which is converted to the chromogenic *para*-nitrophenol:

$$p\text{NPP} + H_2O \rightarrow p\text{NP} + P_i$$

The production of *para*-nitrophenol is measured spectrophotometrically at 405 nm and is dependent upon assay pH which should reflect the pH of the soil under test.

This protocol is used for the measurement of soil acid phosphatase.

B8.2 Reagents

- p-nitrophenyl phosphate (pNPP)
- p-nitrophenol (pNP)
- Sodium hydroxide
- Deionised water
- Sodium citrate
- Citric acid

B8.3 Equipment

- Automatic pipettes: to 1000 µl and 5000 µl
- Beakers
- Shaking water bath or shaking incubator
- Spatula
- Spectrophotometer
- 20 ml bottles
- place analytical balance
- Pasteur pipettes
- Timer
- Centrifuge
- 1.5 ml centrifuge tubes
- Volumetric flasks
- Pipette tips,
- Spectrophotometric cuvettes
- Measuring cylinder

- Sieve (mesh 2 mm)

Optional

- Boiling water bath
- 200 µl x 1 channel automatic pipettor
- 1 ml x 8 channel automatic pipettor
- 20-200 µl x 8 channel automatic pipettor
- 96 well (flat bottomed) plates
- 96 deep well plates
- Spectrophotometer capable of reading 96 well plates

B8.4 Preparation of Buffers

Citric acid buffer is prepared by preparing separate solutions of 0.1 M citric acid and 0.1 M sodium citrate, mixing together in the appropriate proportions to provide a solution of the desired pH (Table 4.1.). The assay pH should be the same as the soil pH.

To prepare a 100 ml buffer solution, first read off the values of both solutions for the required pH. Measure the quantity of 0.1M citric acid into a 200 ml beaker and then add the volume for the 0.1M sodium citrate. Whilst stirring add about 30 ml of deionised water and check pH. The pH should be near the required pH value. If the pH needs to be adjusted, add drop-wise small amounts (ca. 0.02 ml from Pasteur pipette) of ether citric acid (to decrease pH) or sodium citrate (to increase pH) until the correct pH is obtained. Transfer the solution to a 100 ml volumetric flask and make up to the mark.

Table B8.1. Volumes of citric acid solutions to prepare a citric acid buffer for 100 ml final volume of buffer.

pH	0.1 M citric acid	0.1 M sodium citrate
3.0	46.5	3.5
3.2	43.7	6.3
3.4	40.0	10.0
3.6	37.0	13.0
3.8	35.0	15.0
4.0	33.0	17.0
4.2	31.5	18.5
4.4	28.0	22.0
4.6	25.5	24.5
4.8	23.0	27.0
5.0	20.5	29.5
5.2	18.0	32.0
5.4	16.0	34.0
5.6	13.7	36.3
5.8	11.8	38.2
6.0	9.5	41.5
6.2	7.2	42.8

B8.5 Preparation of substrate

The enzyme substrate is a solution of *p*-nitrophenyl phosphate (*p*NPP) (5 mM) prepared in the citric acid buffer.

B8.6 Preparation of pNP standards

Stock solution of *p*-nitrophenol (1 mM) is prepared in the citric acid buffer. The stock solution is diluted in citric acid buffer to give a range of standards: 0.00, 0.06, 0.12, 0.18, 0.24, 0.30, 0.36, 0.44 mM. The volumes may be measured by weighing directly into volumetric flasks and making to the mark with buffer.

B8.7 Preparation of stop solution

The reaction stop solution is sodium hydroxide (2M) prepared in deionised water.

B8.8 Sample preparation

Wet samples should be passed through a 2 mm mesh sieve. Samples should be assayed as soon as possible after sieving, otherwise kept at 4°C.

B8.9 Analytical procedure

1 Wet soil samples (approx. 0.5 g) are weighed in triplicate (to 4 sig. figures) into 20 ml bottles and *p*NPP substrate solution (4 ml) is added to each sample.

2 These are then placed into a shaking water bath or a shaking incubator at 37°C for 30 min, noting the exact time they are incubated.

3 The samples are then removed from the water bath/incubator and triplicate 1 ml aliquots placed into a 1.5 ml centrifuge tube. This last step may be omitted if the soil solutions are very clear.

4 The tubes are then centrifuged (1 min at 15000 x g). This last step maybe omitted if the soil solutions are very clear.

5 To measure the amount of product an aliquot (500 ml) is removed of either the sample or a *p*NP standard and placed into a cuvette.

6 Sodium hydroxide (2 ml) is added and the solution mixed by pipetting up and down (at least 3 times). This stops the enzyme reaction and develops the colour.

7 The absorbance is measured in a spectrophotometer at 405 nm.

B8.10 Measurement of pNP standards

The whole range of standards are measured in triplicate at the same time as the samples. The range of pNP standards (standard curve) should be checked for linearity before analysis of the samples.

B8.11 Sample absorbance values falling outside the standard curve

If the *p*NP absorbance value of a sample falls beyond the range of the highest standard concentration a further more concentrated pNP solution would need to be prepared provided

the standard curve retains its linearity. Alternatively, the assay could be repeated at a shorter incubation time or with a reduced substrate concentration.

B8.12 Experimental blanks and controls

A non-soil sample (blank –substrate only) or a heated-treated (enzyme killed) soil sample should be included. The heated treated soil could be one of the samples. A quality control sample maybe also included.

B8.13 Moisture content

Moisture content is determined at 105°C.

B8.14 Construction of standard curve and calculation of results

The amount of *p*NP produced is determined from a graph of absorbance against amount of pNP (*x*moles). The line of the *p*NP standard solutions should be linear with an R^2 close to 1.

Calculation

The data are expressed as:

Moles pNP g^{-1} s^{-1} oven dry (105 °C) soil

For each of the samples replicates:

1 Average the OD405 replicates for each of the sample replicates

2 Calculate the amount of pNP (as xmoles pNP) produced from the standard curve. Multiply the slope of the line obtained from the standard curve with the OD405 from the samples.

3 Multiply any dilution factors. Using the amounts above multiply by 4 ml (the assay volume) /0.5 ml (aliquot volume removed to measure OD) i.e. x8.

4 Divide by the soil dry weight to give xmoles pNP/g soil dry wt

5 Divide by the incubation time to give xmoles pNP/g soil dry wt/ second

Average the sample replicates and subtract the average value of the blanks (use either the killed soil sample or the blank sample) to give the final value.

B9 PHOSPHOLIPID FATTY ACIDS (PLFA)

Source: Frostegård, A. Tunlid, A. and Bååth, E. (1991) Microbial biomass measured as total lipid phosphate in soils of different organic content. *Journal Microbiological Methods* 14: 151-163.

B9.1. Principle

The analytes determined by the method are phospholipid fatty acids (PLFA). The method can be applied to biological materials which include soil and microbial compounds. Range: 0.01 – 800 µg PLFA g^{-1} freeze dried sample.

Lipids are extracted from biological material using a chloroform:methanol:citrate buffer mixture (1:2:0.8 v/v). The lipids are separated using adsorption column chromatography. The phospholipids and/or neutral lipids are subjected to a mild acid methanolysis and the fatty acid methyl esters extracted into an organic solvent (iso-hexane). Fatty acid methyl esters determined by gas chromatography using a polar capillary column and a flame ionisation detector. Quantitation of the fatty acid methyl esters is achieved through the use of an internal standard. ().

B9.2 Reference Material

- No certified reference materials are available.
- Quality Control soils for the extraction are freeze-dried bulk sample of soils of known composition; a mineral soil QC and organic soil QC.
- A commercially available Fatty Acid Methyl Ester (FAME) standard – Mix C12-C20 is used as a quality control

B9.3 Extraction from soil

B9.3.1 Reagents for extraction from soil

- Chloroform
- Methanol
- Acetone (HPLG Grade)
- Iso-hexane
- Toluene
- Citric acid (anhydrous)
- Nondecanoic acid
- Buytlated hydroxyl toluene
- Sodium hydroxide
- Potassium chloride
- Acetic acid (Analar Grade)
- Decon 90
- Deionised water

B9.3.2 Equipment & Consumables

- SPE Columns - Silica 500mg/6ml
- Vial - Screw top fixed insert (300 µl), clear
- Cap – 9mm (blue) pre-fitted silicone/PTFE liner
- Pasteur Pipettes - short, 145mm
- Balance (5 decimal places)
- Dri-block heater with sample concentrator and stainless steel needles
- Vortex mixer
- Water bath
- pH meter
- 5ml dispenser(s)
- 1000 µl Pipettor and tips
- Centrifuge
- Sample Rotator
- Fridge/freezer
- Drying oven

B9.3.3 Sample Preparation and equipment care and preparation

Soils and other biological material are freeze-dried and milled.

All glassware is soaked in 10% Decon 90 and deionised rinsed, then muffled at 450^{o}C before use. Pasteur pipettes and vials are also muffled.

Dispensers are flushed with methanol (water first if contained buffer) and left to dry at the end of each day. At the end of the procedure, the 5ml Dispensers are taken apart, soaked overnight in warm water and dried thoroughly in the drying oven.

Taps are soaked in 10% Decon overnight, thoroughly rinsed with water, and dried in the drying oven. The taps are then soaked in methanol overnight before drying in the fume cupboard.

Sample concentrator needles are soaked in clean methanol.

B9.3.4 Reagent Preparation (amount for 40 samples)

1 Citrate Buffer (0.15M)
Weigh accurately to two decimal places 14.41 g anhydrous Citric acid dissolved in 500ml Milli-q water, and adjust pH to 4.0 ($\pm$0.02) with sodium hydroxide. Prepare fresh weekly.

2 Chloroform:Methanol:Citrate Buffer (1 : 2 : 0.8 v/v/v) [Bligh and Dyer]
Add 135 ml chloroform to 270 ml methanol to 108 ml citrate buffer. Prepare fresh weekly.

3 Methanol:Toluene (1 : 1 v/v)
Add 25 ml methanol to 25 ml toluene.

4 Potassium hydroxide (0.2M) in methanol

Weigh accurately to two decimal places, 0.56 g potassium hydroxide pellets (crushed) and dissolve in 50 ml. Prepare fresh on day of use.

5 Iso-hexane:Chloroform (4 : 1 v/v)
Add 160 ml iso-hexane to 40 ml chloroform.

6 Acetic Acid (1M)
Dilute 5.7 ml acetic acid with Milli-q water and make up to 100 ml.

7 C19:0 Internal Standard
Weigh accurately and record to 5 decimal places, approximately 6 mg of Nonadecanoic acid Methyl Ester ($C_{20}H_{40}O_2$) and dissolve in 250 ml methanol. Store at 3-5^{o}C, 12 month expiry.

8 Iso-hexane containing Butylated hydroxy toluene (0.001%)
Weigh accurately to 2 decimal places, 100 mg butylated hydroxy toluene and dissolve in 100 ml iso-hexane (0.1%) Dilute 1 ml in a volumetric flask with iso-hexane up to 100 ml (0.001%). Store at 3-5°C, 12 month expiry.

B9.3.5 Extraction of lipids

1 Weigh accurately to 4 decimal places freeze dried soil into a 120 mm x 20 mm borosilicate glass culture tube with a teflon-lined screw cap. Record the weight.

2 For every batch of soil samples, whether it's 1 – 40, there must be at least 3 replicates of an appropriate QC soil and 1 blank included in the PLFA extractions (equates to 33 - 36 samples per set of 40).

Table B9.1. QC soils for PLFA

Type Soil	Quality Control	Sample
Peaty/Organic	50 mg	50 mg
Mineral	500 mg	500 - 1500 mg

3 Add 9.2 ml Bligh & Dyer to each sample.
Mix the sample on a vortex mixer, then leave for 2 hours to extract, vortex mixing every 30 minutes. After the 2 hours, vortex the samples then centrifuge for 10 min at 1500 rpm at 20°C.

4 Using a clean glass Pasteur pipette for each sample, transfer the supernatant into a clean culture.

5 Add 2.5 ml Bligh & Dyer to the soil residue.

6 Vortex and centrifuge as before.

7 Again transfer supernatant to the culture tube using a clean Pasteur pipette.

8 To the supernatant add 3.1 ml $CHCl_3$ and 3.1 ml Citrate Buffer, and vortex.

9 Mix the samples on the sample rotator for 30 minutes and centrifuge as before. Both layers should be clear, especially the organic layer at the bottom, indicating that separation has been successful.

Note: If the layers have separated but are just cloudy, leave at room temperature for about 30 min or place in warm water, and allow the samples to warm up.

10 Using a clean Pasteur pipette, remove and discard the top aqueous layer.

11 Note: You will find that the mucky layer between the aqueous and organic layer will cling to the outer edge of the tube allowing 'clean' removal of the organic layer.

12 Using a clean Pasteur pipette, transfer all the lower organic phase to a clean scintillation vial.

13 Evaporate the sample to dryness under a stream of nitrogen on the dri-block heater set at 40°C.

14 Once the sample is completely dry add 1 ml methanol and evaporate to dryness under stream of nitrogen.

15 Add another 1 ml methanol and again evaporate to dryness under the nitrogen.

B9.3.6 Fractionation – Separation of lipid classes

Solid phase extraction uses silica columns with a sorbent mass of 500 mg and a reservoir volume of 6ml. One-way stopcocks (SPE) are fitted to each column.

- Each tap must be set so that not only is the drip-rate slow but is the same for all samples.
- Do not allow the column to dry out during fractionation.
- Allow C19-Standard to warm up to room temperature prior to use.
- For batches of 40, work in 2 x sets of 20.

1 Wash the column with 5 ml $CHCl_3$, and then close the taps.

2 Add 400 µL $CHCl_3$ to the sample, vortex twice and using a clean Pasteur pipette, transfer the sample to the column.

3 Wash the vial with 3 x 200 µL $CHCl_3$ and transfer the washings to the column.

4 Continue 2 and 3 until you have loaded all the columns.

5 Open each tap and allow the sample to load onto the column slowly.

6 Add 2 x 3 ml $CHCl_3$ and run to waste.

7 Add 2 x 3 ml Acetone and run to waste.

8 Once the acetone has passed through the column, add 4 ml and 2 x 3 mL Methanol and collect separately, the column is allowed to dry out. Once all the methanol has passed through the column (the phospholipids are eluted).

9 Evaporate the methanol eluate to dryness at 40^{o}C under stream of nitrogen on the dri-block heater.

10 Once the sample is completely dry add 50 µL Internal Standard and again evaporate to dryness under stream of nitrogen on the dri-block heater.

Note: At this stage the samples can be stored in the –20°C freezer.

B9.3.7 Mild Alkane Methanolysis

1 Prepare the 0.2M KOH in methanol for the amount required that day.

2 Add 1 ml Methanol:Toluene (1:1 v/v) to each sample and vortex.

3 Add 1 ml 0.2M KOH to each sample and vortex.

4 Place the samples in a rack and incubate at 37^{o}C (+/- 3^{o}C) in the water bath for 15 min.

5 After methanolysis, remove the samples from the water bath and add
- 2 ml Iso-hexane:$CHCl_3$ (4:1 v/v)
- 0.3 ml 1M Acetic Acid
- 2 ml Millipore water

6 Vortex and place on the sample rotator for 10 minutes.

7 Centrifuge for 10 min at 1500 rpm.

8 Using a clean Pasteur pipette transfer the upper organic phase to a clean scintillation vial, taking care not to take up any of the lower aqueous layer.

9 Add a further 2 ml Iso-hexane:$CHCl_3$ (4:1 v/v) to the culture tube containing the lower aqueous layer.

10 Vortex and centrifuge as before.

11 Again transfer the upper layer (using a clean Pasteur pipette) to the scintillation vial containing the first 'washing', taking care not to take up any of the lower aqueous layer.

12 Evaporate the sample to dryness under stream of nitrogen on the dri-block heater at 40°C.

B9.3.8 Preparation for GC

1 Add 3 x 150 µL iso-hexane to the sample and transfer to the appropriate GC vial using a clean glass Pasteur pipette.
2 Evaporate to dryness under nitrogen.
3 Add 50 µL iso-hexane (containing 0.001% butylated hydroxy toluene), and place the cap on the vial.
4 Submit for analysis along with details of sample weights and the C19 Standard concentration (see below).

B9.3.9 Calculation of Concentration of C19 Standard

Example. 6.11mg C19 methyl ester in 250 ml methanol

- 6110 μg = 6.11 mg
- 6110 / 250 = 24.44 μg ml^{-1}
- 24.44 μg ml^{-1} = 24.44 μg 1000μL^{-1}
- 24.44 / 20 = 1.222 μg 50 μL^{-1}.

B9.4 Estimation of Phospholipid Fatty Acid Content in Biological Materials by Gas Liquid

B9.4.1 Reagents

The grade of reagents should be SLR or Analar.
The grade of solvents should be HPLC.

- Iso-hexane
- Methanol
- FAME Mix C14-C22 (100mg ampoule) p/n 18917-1amp.
- FAME Mix Supelco 37 Component p/n 47885-U
- Dodecanoic Acid methyl ester C12:0
- Tetradecanoic Acid methyl ester C14:0
- Hexadecanoic Acid methyl ester C16:0
- Methyl nonackcanoate (Heptadecanoic (C19)) p/n N5377-5G

B9.4.2 Preparation of standards

Standards expire after 2 years if kept frozen.
Note: All glassware used should be grade B or better.
Weights must be obtained using a four place analytical balance.

Preparation of FAME QC standard

1 Measure 1 ml of the FAME mix Supelco 37 component (1ml ampoule, C12-C20, 15 compounds chosen as QC standard) to a 50 ml volumetric flask. Plus, add 1 ml of internal standard (A solution of C19 PLFA in iso-hexane is prepared at a concentration of approximately 0.5 mg/ml) stock solution to the flask. Make up to the mark with iso-hexane.

2 Dispense 1 ml aliquots of this solution into wide mouth, standard GC vials.

3 Leave the vials overnight in a fume cupboard to dry down then cap with snap caps and store in the – 80oC freezer in room 107 until required.

4 Prior to analysis on the GC, add 500μl of iso-hexane to each vial then cap with an aluminium crimp cap.

5 The absolute concentration of the 15 FAMEs in the QC standard mixture could be calculated (Table B9.2) from the certificated values (Supelco FAME Mix Con.) by diluting 25 times as above described.

Table B9.2. 15 FAMEs QC Standard concentration

	Certificated Supelco FAME Mix Con.(LOT No: LB-41303)	Absolute QC Standard Con.
Compounds	mg/ml	µg/ml
C12:0	0.399	15.96
C13:0	0.201	8.04
C14:0	0.399	15.96
C15:0	0.200	8.00
C16:1	0.200	8.00
C16:0	0.598	23.92
C17:1	0.200	8.00
C17:0	0.203	8.12
C18:2(9,12)	0.200	8.00
C18:1w9	0.399	15.96
C18:0	0.406	16.24
C20:4w(5,8,11,14)	0.201	8.04
C20:5w3	0.200	8.08
C20:1w9	0.200	8.00
C20:0	0.399	15.96

The internal standard (C19:0) was introduced into the 15 FAMEs solution as QC standard, so the relative response factor (RRF) can be calculated (as followed) for each compound of 15 FAMEs and the new QC limits will be set for RRF to monitor the bias.

$$RRF = \frac{\text{area of analyte}}{\text{area of internal standard}} \text{ x } \frac{\text{wt of internal standard}}{\text{weight of } \textit{analyte}}$$

Preparation of FAME RF standard

1 Transfer the entire contents of the FAME standard mix (100mg ampoule, C14-C22, 8 compounds) to a 50 ml volumetric flask, rinsing the vial thoroughly with iso-hexane and adding the rinsings to the volumetric flask.

2 Make up to the mark with iso-hexane.Pipette 2 ml of the above solution to a 100 ml volumetric flask and make up to the mark with iso-hexane.

3 Dispense 1 ml aliquots of this solution into wide mouth, standard GC vials, an accurate weight of the standard should be noted and the RF standards labelled and numbered.

4 Leave the vials overnight in a fume cupboard to dry down then cap with snap caps and store in the – 80oC freezer until required.

5 Prior to analysis on the GC, add 1 ml of iso-hexane to each vial then cap with an aluminium crimp cap.

Preparation of FAME Performance Check Standard

The following pure FAME standards are available commercially:

- Dodecanoic Acid methyl ester C12:0
- Tetradecanoic Acid methyl ester C14:0
- Hexadecanoic Acid methyl ester C16:0

1 Weigh out approx 0.1 g of the C16:0 into a small beaker, noting the weights. The C12:0 and C14:0 are liquid and usually purchased in 1.0ml volume.

2 Add approx 0.1 g of each compound to the beaker and note the weights, ensure the standards are dissolved in the methanol, then transfer to a 100ml volumetric flask.

3 Rinsing the beaker thoroughly with methanol and add the rinsings to the flask.

4 Make up to the mark with methanol. Dispense 1ml aliquots into GC vials, cap and store at approximately 4°C.

B9.4.3 Preparation of Linearity Check Standards

Linearity checks will be performed every three months.

A FAME standard – Mix C14-C22 ampoule is used to prepare the linearity standards.

Transfer the entire contents of the FAME standard ampoule (100mg) to a 50ml volumetric flask. Rinse the ampoule thoroughly with iso-hexane and add the rinsings to the flask. Make up to the mark with iso-hexane.

The following volumes of diluted FAME standard are weighed out into GC vials in triplicate: 25μl, 50μl, 100μl, 200μl and 300μl, to give three sets of replicates. The weights must be recorded.

A solution of C19 PLFA in iso-hexane (for use as an internal standard) is prepared at a concentration of approximately 25.8μg/ml. To each set of replicate FAME standards, internal standard is added and the weights noted. To replicate set 1, 75μl C19 is added; to replicate set 2, 150μl is added and to replicate set 3, 300μl is added; refer to Table B9.3.

Table B9.3. Volumes used for preparation of Linearity Checks

	Volume of FAME std added (μl)				Volume of C19 ISTD added (μl)
Replicate set 1	25	50	200	300	75
Replicate set 2	25	50	200	300	150
Replicate set 3	25	50	200	300	300

*Note: due to the volatility of the iso-hexane, the vial must be covered e.g. with a cap to obtain an accurate weight of standard. It is recommended that the following weights are noted: (i) vial + cap (empty), (ii) vial + cap+ standard and (iii) vial + cap + standard + C19. The weights of FAME standard and C19 ISTD can then be calculated by subtraction.

Allow the solvent in the vials to evaporate overnight in a fume cupboard and cap with snap caps. The standards can then be stored until required at approximately -80^{o}C.

Prior to analysis, 500µL of iso-hexane should be added to each vial and the vial capped with a crimp cap.

Two RF standards should be analysed with each batch of linearity checks.

Take two of the RF standards weighed as described for the FAME QC. Add 300 µL of C19 ISTD and note the weight. Allow the solvent to evaporate, cap with a snap cap and store in the freezer until required.

*Note: RF standards may be prepared in advance and stored frozen until required.

Add 1ml of iso-hexane to the RF standard prior to analysis.

B9.4.4 Equipment

- Gas chromatograph fitted with a flame ionisation detector, a split/splitless injector and autosampler
- Capillary gas liquid chromatography column: 50 m x 0.20 mm id x 0.33 µm film thickness, coated with 5%-Phenyl-methylpolysiloxane
- Hewlett Packard Chemstation software.

B9.4.5 Setting up the Gas Chromatographs

Gas chromatograms ought to be set up in accordance with manufacturer's instructions dependent on the analyses undertaken.

B9.4.6 Environmental control

Analyses are carried out in an air-conditioned laboratory. The temperature is monitored weekly and should remain between 22±5°C.

Standards requiring to be kept in a fridge are kept at approximately 4°C (± 3°C) the temperature of the fridge is recorded weekly with a thermometer.

B9.4.7 Analytical procedure

Ensure there has been a performance check analysed on the instrument and that the retention times and areas were within the stated limits. For data which falls out with these limits, analysis should be stopped until the cause of the failure is established.

Samples are received previously extracted and ready for analysis. If required, iso-hexane is added to each sample, as specified by the customer.

Set up a sequence for the new job, the method used is the one that has just been created specifically for that job. Set up the sequence in the following order:

FAME QC standard

Sample 1
Sample 2
Sample 3

Sample 10
FAME QC standard
Sample 11
Sample 12 etc.

i.e. a FAME QC standard after every 10 samples, finishing with a standard.

Place the samples in the autosampler carousel according to the newly created sequence. Ensure 500µl iso-hexane has been added to the FAME standards and place them in their appropriate positions in the autosampler carousel.

Ensure the solvent wash bottles are full (iso-hexane)-these may require re-filling during the course of a run, depending on the number of samples to be analysed.

Press START on the Chemstation Method and Run Control page to begin the analysis.

B9.4.8 Calculation of results

The basic formula for the calculation of each phospholipid is:

$$\text{Concentration} = \frac{\text{area of analyte}}{\text{area of internal standard}} \times \frac{\text{wt of internal standard}}{\text{weight of sample}}$$

The results are expressed in µg n-plfa/g sample.

B9.4.9 Quality Control

Quality Control Limits for the relative response factor (RRF) of the components contained in a FAME QC standard have been set by the repeated analysis of this standard.

For data which falls out with the QC limits, it is necessary to double check the FAME QC standard peaks (including peak area, retention time and peak shapes) when failure occurs. If all these are fine, it allows that the QC failure is up to 4 compounds of total 15 QC compounds in each QC sample.

The QC standard contains the following components:

Table B9.4. Components in QC Standard

Component	CAS No.	**Peak**
Methyl Laurate	111-82-0	C12:0
Methyl Tridecanoate	1731-88-0	C13:0
Methyl Myristate	124-10-7	C14:0
Methyl Pentadecanoate	7132-64-1	C15:0
Methyl Palmitoleate	1120-25-8	C16:1
Methyl Palmitate	112-39-0	C16:0
Cis-10-Heptadecenoic Acid Meth	75190-82-8	C17:1
Methyl Heptadecanoate	1731-92-6	C17:0
Methyl Linoleate	112-63-0	C18:2(9,12)
Cis-9-Oleic Methyl Ester	112-62-9	C18:1w9
Methyl Stearate	112-61-8	C18:0
Methyl-Cis-5,8,11,14-Eicosatet	2566-89-4	C20:4w (5,8,11,14)
Methyl Cis-5,8,11,14,17-Eicosa	2734-47-6	C20:5w3
Methyl Eicosenoate	2390-09-2	C20:1w9
Methyl Arachidate	1120-28-1	C20:0

B9.4.10 Linearity

Linearity checks should be carried out on every three months.

Standards are prepared as described in B9.4.3 Preparation of Linearity Check Standards'. Each batch of linearity standards includes 5 levels of PLFA and 3 levels of ISTD plus the two RF standards.

Samples are analysed using the most recent jobs method. Once analysed, the results are processed manually and the areas obtained for each peak are noted. The actual concentrations of PLFA's in the standard are compared to the expected values. Note: Since it is unclear which of the four components comprise C18:pk1 and C18:pk2, the linearity calculations for these peaks are performed based on the combined sum of their areas and the sum weight of all four components.

This method is empirical and the uncertainty quoted is based on long term quality control data.

The uncertainty associated with the measurement of methyl esters in FAME QC at the mean RRF determined for the QC are shown in Table B9.4, where the uncertainty is reported as an expanded uncertainty calculated using a coverage factor of 2 which gives a level of confidence of 95%.

Table B9.4 Uncertainty Measurements

Component	Relative response factor	Uncertainty measurement (± 2 S.D)
C12:0	0.7383	0.19
C13:0	0.8353	0.13
C14:0	0.9137	0.07
C15:0	0.9576	0.06
C16:1	0.9393	0.07
C16:0	0.9863	0.05
C17:1	0.9781	0.05
C17:0	0.9563	0.05
C18:2(9,12)	0.9408	0.08
C18:1w9	1.4913	0.06
C18:0	0.9969	0.03
C20:5w3	0.8174	0.11
C20:4w(6,10,14,18)	0.7858	0.13
C20:1w9	0.9363	0.10
C20:0	1.0102	0.04

B10 MOLECULAR ANALYSIS OF FUNGAL COMMUNITIES

Source: Kabir G. Peay, Peter G. Kennedy & Thomas D. Bruns (2008) Fungal Community Ecology: A Hybrid Beast with a Molecular Master. BioScience, 58, 799-810.

Berthelet, M., Whyte, L.G. & Greer, C.W. (1996). Rapid, Direct Extraction of DNA from Soils for PCR Analysis Using Polyvinylpolypyrrolidone Spin Columns. FEMS Microbiology Letters, 138, 17-22.

Gardes, M. & Bruns, T.D. (1993). ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhiza and rusts. Molecular Ecology, 2, 113–118.

B10.1 Reagents

- Sodium phosphate buffer pH 8.0
- 0.5 M Tris HCl pH 8.0,
- 1M NaCl
- 1% SDS (lysing buffer)
- Ammonium acetate (7.5M)
- FastDNA spin Kit for Soil, MP Biomedicals (Cambridge, UK)
- Binding matrix
- SEWS-M - salt/ethanol wash solution, DNase-free
- DES - (DNA Elution Solution-Ultra Pure Water)
- 2 mM MgCl2
- dATP, dCTP, dGTP and dTTP
- Bovine serum albumin (BSA)
- High Fidelity Taq polymerase (Bioline)
- Primers: ITS1F, ITS4, ITS1F labelled with the fluorescent dye 6-FAM

B10.2 Equipment

- Centrifuge (capable 12,000 g)
- Vortex mixer
- 96 well plates
- Dyad DNA engine thermal cycler (MJ Research)
- QIAquick PCR purification Kit (Qiagen)
- Agarose
- Ethidium bromide
- pGEM-T Easy Vector system (Promega)
- Genemapper (software)

B10.3 Sample preparation

1 Individual frozen samples were thawed then thoroughly mixed and 1 cm^3 was filled into a lysing matrix tube.

2 DNA was extracted using components from the MP Biomedicals (Cambridge, UK) FastDNA spin Kit for Soil (cat. 116560200) but with different buffers (Berthlett et al., 1996).

3 100 mM sodium phosphate buffer pH 8.0 (500 µl) and lysing buffer (250 µl) (containing 0.5 M Tris HCl pH 8.0, 0.1 M NaCl and 1% SDS) were added to the soil samples in the lysing matrix tubes.

4 Samples were processed in a Precellys24 at 6500 rpm for 2x15s.

5 The samples were then centrifuged at 12,000g for 3 min and the supernatants transferred to a clean 1.5 ml tube.

6 Ammonium acetate (7.5M) 0.4 vol was added, the samples were vortexed and placed on ice for 10 min to precipitate proteins.

7 The samples were then centrifuged 12,000g for 3min and the supernatant transferred into fresh 2 ml tubes.

8 Binding matrix (1 ml) was added and the tubes inverted for 2 min and then left to stand for 5 min.

9 The top 500 µl of the binding matrix was removed and discarded with the remainder of the binding matrix re-suspended by gently flicking the side of the tube.

10 In two aliquots, this was then passed through a Spin Filter and centrifuged at 12,000g for 1 min.

11 When all of the suspension had passed through the filter SEWS-M (500 µl) was added and the filter re-spun as before to wash the DNA.

12 The tubes were re-spun at 12,000g for 2 min to remove residual SEWS-M and dry the filter.

13 After air drying (5 min), the DNA was eluted into a clean tube by adding 3 x 50 µl DES and centrifuging (14,000 g, 1 min).

14 The DNA samples were transferred into 96 well plates.

B10.4 Amplification of the ITS

Fungal internal transcribed spacer (ITS) regions were amplified in 50-µL reaction volumes containing approximately 50 ng of template DNA; 20 pmol of ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al*. 1990) primers; 2 mM $MgCl_2$; 250 µm of each of dATP, dCTP, dGTP and dTTP; 10× reaction buffer; 1 µL bovine serum albumin (BSA) and 0.75 µL of High Fidelity *Taq* polymerase (Bioline). Amplifications were performed on a Dyad DNA engine thermal cycler (MJ Research) with an initial 5-min denaturation at 95 °C followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. 5ul of the PCR products were electrophoresed in 1% (w/v) agarose gels, stained with ethidium bromide and visualized under UV light.

B10.5 Cloning procedure and sequencing of clones

PCR products were purified using the QIAquick PCR purification Kit (Qiagen). and purified products were quantified. A clone library was generated for each sample using the pGEM-T Easy Vector system (Promega) according to the manufacturer's protocol, with ca. 50ng of purified PCR product used for each ligation reaction. Ten colonies from each clone library

were selected at random. Transformed ITS products from each individual clone were re-amplified as for ITS above using the M13 forward and reverse primer pair.

PCR products were sent to Macrogen Europe (http://www.macrogen.com/eng/index/) for purification and sequencing.

B10.6 T_RFLP generation

To generate products for T-RFLP analysis, fungal ITS regions were amplified as described above but using the ITS1F labelled with the fluorescent dye 6-FAM and ITS4. PCR products were purified using QIAquick PCR purification Kit (Qiagen). Ca. 200ng of purified PCR products were digested in 10ul reaction containing 10 units of Hha I (New England Biolabs) at 37C for 3 hours. 2ul of each digest was submitted for T-RFLP analysis. T-RFLP patterns were visualised using Genemapper.

B10.7 Quality Control

Quality control measures ought to be adopted relevant to the analytical technique undertaken. Exact quality control criteria used to assess sample data are influenced by the technique and instrumentation used to obtain data.

Typically for any Quality Control sample analysis result which falls outside the Fixed Limits based on ±3SD established for that method action should be taken and a record of this kept. If there are 2 consecutive breaches of the ±2SD based fixed limit the same procedure as above is followed.