SCOPING BIOLOGICAL INDICATORS OF SOIL QUALITY

PHASE II

DEFRA PROJECT SP0534

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Executive summary

This report presents results from a field assessment of a limited suite of potential biological indicators of soil quality to investigate their suitability for national-scale soil monitoring. The methods included;

- Terminal restriction fragment length polymorphism (TRFLP) to characterize genetic structure of soil bacterial, fungal and archaeal communities [GENOTYPIC]
- Phospholipid fatty acid analysis (PLFA) to profile soil microbial community structure [PHENOTYPIC]
- Dry extractions of soils to characterize microarthopods and other invertebrates [PHENOTYPIC]
- Wet extraction of soils to characterize nematode community structure [PHENOTYPIC]
- Microplate fluorometric assay to profile potential enzyme activities in soil [FUNCTIONAL]
- Multiple substrate induced respiration (MSIR) by GC or MicroResp™ to profile soil respiration responses and carbon dynamics [FUNCTIONAL]

Each of these methods can produce a number of soil biological measures with potential for use as biological indicators of soil quality. The project adapted and progressed standard operating procedures for these methods to establish a set of method suitable for large scale surveys and monitoring. The method highlighted the need to establish suitable soil biological reference materials for quality control in monitoring.

The methods were tested against three well known pressures to UK soils at three long-term sites to determine whether the methods would produce soil biological measures that would be sensitive enough to respond to the pressures against their inherent temporal or spatial variability. At each site, there was a gradient of intensity for one of the pressures; nitrogen deposition (ADAS Pwllpeiran, Wales), heavy metals from sludge applications to land (Hartwood Farm, Scotland) and habitat restoration after mining (Sutton Courtenay, England). All three sites were sampled at bi-monthly intervals over 12 months with soil samples subsequently analysed using the suite of soil biological methods. The results demonstrated that the majority of soil biological measures displayed significant levels of temporal variability, corresponding to seasonal dynamics. However in certain measures, variability was not significant and responses to pressures were clearly detected; MSIR, PLFA, TRFLP fungi and TRFLP archaea (restoration at Sutton Courtenay), PLFA, microarthropods and TRFLP bacteria (sludge metals at Hartwood) and microarthropods and MSIR (N deposition at Pwllpeiran). Overall, the results indicate that there is no universal indicator (measure) or method that will provide sensitivity to a range of constrasting pressures. A suite of soil biological methods would be a more informative approach to monitoring changes in soil biological status where multiple pressures are at play, or where the pressures influencing soil are unknown. From the sensitivity results, this suite would include, as a minumum: PLFAs, TRFLP (for fungi, bacteria and archaea), MSIR and microarthropods.

The methods were further tested to determine the power of individual, multiple and integrated soil biological measures to discriminate between nine constrasting land uses; crops+weeds, fertile grassland, infertile acid grassland, infertile calcareous grassland, lowland deciduous wooded, upland deciduous wooded, moorland grass mosaic and heath/bog. The sampling locations for these nine land uses were selected from Countryside Survey with 101 locations sampled during the 2007 survey. All soil biological methods were applied to all soil samples. The results clearly demonstrated that all methods could be used to discriminate between different land uses to a greater or lesser extent. Numerous individual, ratios/indices and multivariate measures of genotypic, phenotypic and functional traits could be used to establish baselines or target values in soil biological status for individual land uses. These differences could be used to monitor and interpret status and changes in soil biological quality in much the same way that shifts in community structure have been used to develop approaches to good ecological status for habitats and water quality.

Overall, the discrimination results complement the results from the sensitivity trial in that they suggest that a suite of soil biological methods would be an informative approach to monitoring the biological status of soils, as opposed to relying on a single method or a single measure. From the discrimination results, this suite would include: PLFAs, TRFLP (for fungi and archaea), MSIR and multi-enzymes. These methods produced the most significant and interpretable land use discrimination patterns from statistical analyses of univariate, multiple and integrated measures. Furthermore, the results suggest that there would be a clear rationale for selecting methods which would provide information on the three characteristics of soil biology, namely genotypic, phenotypic and functional traits.

The results suggest that the use of multiple measures from these methods could be used to define characteristic baselines of soil biological status for different land uses. Further work is required to build up a comprehensive dataset for a broader range of land uses across UK and to investigate the influence of management or pressures on soil biological status within these land uses. These data could be generated in different ways, either through extensive survey or through targeted sampling of key land uses. This work should complement the determination of the sensitivity of soil biological measures to different pressures. The primary issue must be to ensure that any data collected are entirely compatible with existing and future data through the use of common SOPs, reliable reference materials and complementary statistical approaches.

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1 Introduction

The principal aim of this project (SP0534 Scoping biological indicators of soil quality SQID phase II) was to field trial a candidate suite of biological indicators of soil quality to investigate their suitability for national-scale soil monitoring. A suite of candidate biological indicators, using six methods, was prioritised from a semi-objective assessment in the preceding SQID Phase I project (SP0529). The candidate indicators demonstrated particular relevance to the support of three soil functions (food and fibre production, environmental interactions and habitats and biodiversity) and the associated methods demonstrated technical suitability to large-scale soil monitoring schemes.

Doran and Zeiss (2000) recommended that an indicator of soil quality should be well correlated with soil function. In accordance with this, the suite of candidate indicators chosen in this project demonstrated clear correspondence to ecological processes and properties which support the three soil functions in question. This correspondence is described in some detail in the preceding $SQID_I$ project report (Black *et al*., 2005) and associated journal publication (Ritz *et al*., 2009). For example, fungal/bacterial ratios derived from PLFAs are known to have correspondence to soil N and, to a lesser extent, C cycling pathways and have shown consistent responses with regard to grassland management (Smith *et al*., 2005, 2008). Table 1-1 lists the candidate indicators, their associated methods and the information that would be generated by the methods. The methods selected proved the most promising in relation to various scientific and technical criteria relevant to national soil monitoring. The purpose of this phase was to rigorously test these methods under relevant field conditions and to evaluate their suitability for national soil monitoring alongside the usefulness of the indicator measures.

A series of generic issues, regarding the deployment of biological indicators in a national-scale soil monitoring, were identified from Phase I of the SQID project which were;

- Process-based measurements for carbon cycling were considered essential for soil monitoring by the policy and researchers consulted during Phase I. The favourable technique MSIR by GC was considered impracticable for large-scale surveys so alternative methods needed to be assessed (e.g. MicroResp^{TM}).

- Robust standard operating procedures (SOPs) are required, including appropriate quality-control standards, to ensure reproducibility and consistency of analyses and compatibility between different laboratories.

- Many biological indicators under consideration show discrimination between habitats and soils however, data are required from a comprehensive large sample to establish discrimination or sensitivity against background temporal and spatial variability.

- Analysis of large datasets (large sample N across a wide range of environmental factors) is required to determine which primary measurements, indices or multivariate approaches would be most useful for national-scale soil monitoring.

- The degree of surrogacy between biological indicators is unknown. Surrogacy

would open up scope for increasing cost-effectiveness in large-scale deployment.

- Careful consideration of logistical issues is required since the implementation of biological indicators will require relatively fast turnover of soil samples for laboratory analyses.

This phase of the project was designed to address these issues with the focus on establishing the most appropriate biological measures, and associated methods, with which to assess soil quality in a monitoring scheme i.e. the potential for application and not the actual application. As successfully shown by the New Zealand SINDI system (Sparling and Schipper, 2002) and the Dutch Soil Quality Network (Mulder *et al*., 2004; Bruere *et al*., 2004), setting standards or expected values for soil quality indicators, including biological, will be reliant upon obtaining sufficient information from a range of soils and land uses relevant to the purpose of the indicators. An objective of $SQID_{II}$ was to generate extensive data that could be used to inform the discussion and debate on the setting of such standards or values with respect to the ultimate purpose of a monitoring scheme in the UK.

Table 1-1 Candidate biological indicators of soil quality

Indicator	Indicator method	Indicator method descriptor
Soil respiration rates, community level physiological profiling	Multiple substrate induced respiration (MSIR) by GC or MicroResp™	Activity capability profile of soil community for soil carbon cycling
Potential enzyme activities	Microplate fluorometric assay	Enzyme activity for a range of soil biogeochemical cycles e.g. C, S, P, N
Nematode community structure	Nematode Baermann extraction procedure	Diversity and size of soil nematode community
Soil microbial community structure and biomass	PLFA (phospholipid fatty acid) profiles	Composition of specific groups in soil microbial community and soil microbial biomass
Microarthropod community structure	Tullgren dry extraction	Diversity and size of soil invertebrate community
Genetic structure of the soil microbial community structure	TRFLP (terminal restriction fragment length polymorphism)	Genetic profiling of the bacterial, fungal and archaeal components of the soil microbial community
Functional structure of the microbial community*	TRFLP	Genetic profiling of soil microbial functional groups important for carbon and nitrogen cycling

* Only applied to a limited number of samples in the discrimination trial due to cost and status of method development at that time.

2 Objectives

The principal aim of $SQID_{II}$ was to field trial a candidate set of biological indicators of soil quality for the purposes of national-scale soil monitoring.

Thus, the **specific objectives** of SQID Phase II were;

1. To establish a set of standard operating procedures (SOPs) for the candidate indicators that optimise inter-laboratory comparability and reproducibility of results and that are transferable to large-scale soil monitoring schemes.

2. To evaluate and aim to bring the MicroResp™ method for multiple substrate-induced respiration (MSIR) to a deployable status.

3. To pilot the candidate suite of biological indicators in contrasting field experiments to assess whether they are sensitive enough to detect change against the background of inherent spatial and temporal variability (SENSITIVITY trial).

4. To pilot the candidate suite of biological indicators in a national survey to assess whether they provide consistent and reproducible results across a representative range of UK soil:land use combinations (DISCRIMINATION trial).

5. To determine the degree of surrogacy between biological indicators from data obtained in the field trails.

6. To provide an assessment of the logistical issues for national-scale soil monitoring with biological indicators of soil quality.

7. To prioritise the candidate suite of biological indicators for national-scale soil monitoring.

3 Standard operating procedures for the candidate indicators

3.1 Methods

Table 3-1 lists the methods used in this project for the determination of the candidate indicators along with a summary of the information produced by each method. Each method can produce a range of individual measures which can be used to generate ratios, indices and multivariate measures. It is important to recognize that it is these measures which could be used as biological indicators of soil quality, and not the method as is frequently reported in the literature.

The following sections summarise the main characteristics of each method and highlight issues with the methods which were identified from SQID Phase 1. The detailed SOP for each method is provided in Appendix A.

3.1.1 Multiple enzyme fluorometric assay to profile the activity of soil enzymes

Measurement of the activity of soil enzymes provides information about the functional repertoire and activity of soil organisms. There are many enzymes that can be profiled which can be selected to relate to almost any soil biochemical transformation (Burns and Dick, 2002). The multiple enzyme fluorometric assay approach was selected in this instance since this assay can provide information on more than one biochemical process. In addition, an increasingly wide range of fluorescently-labelled substrates are available. The microtitre multiple enzyme assay enables sensitive measurements to be made on small quantities of soil, permitting high-throughput systems on user-prescribed suites of enzymes (Marx *et al*., 2001). This method is suited primarily to enzymes involved in C-cycling, since the majority of fluorescently labelled substrates available target C-transforming enzymes. However, fluorescently labelled substrates that relate to phosphatase and sulphatase are also commercially available, and others may enter the market over time. SQID phase 1 identified certain issues with the potential routine and extensive application of the multiple enzyme assay. These were:

- There were no published data on the reproducibility of the method or the inter-laboratory comparability of results.
- There was no information available on standards or reference materials to quality-control this assay. In principle, prescribed purified enzymes, or mixtures thereof, could be utilised for this purpose, for example based on stipulation of number of International Enzyme Units (IU). This concept may be appropriate to explore if the assay were to be applied in a full-scale monitoring programme.

Table 3-1 List of the methods used for the SQID Phase II project with a summary of the measures obtained from each method.

Method	**Indicators**	**Single measures**	**Indices or ratios of measures**	**multivariate measures**
Microplate fluorometric multi-enzyme assay	Activity of multiple enzymes which reflect the activity of a range of soil biogeochemical cycles e.g. C, S, P, N	response rates of individual enzymes	n/a	PC axes
Multiple substrate induced respiration by MicroResp™	Respiration responses from carbon substrates which reflect an activity capability profile of the soil microbial community for carbon cycling	respiration rates from individual substrates	n/a	PC axes
Multiplex TRFLP	Genetic profiling of the bacterial, fungal and archaeal components of the soil microbial community and certain functional groups	relative abundance of terminal restriction fragments (TRFs). *These are not used individually*	diversity indices e.g. Shannon H'and E (evenness).	PC axes
PLFA profiles by GC analysis	Soil microbial community structure and relative abundance, and soil microbial biomass	abundance of microbial groups (bacteria, fungi, gram positive bacteria, gram negative bacteria, actinomycetes etc); total microbial biomass (PLFA abundance)	fungal / bacterial ratio; gram +/ gram - ratio; diversity indices	PC axes
Tullgren funnel dry extractions	Size and composition of the soil invertebrate community, primarily microarthropods (mites and collembola)	mites and collembola taxonomic groups, diversity indices for invertebrates	mite / collembola ratio; diversity indices	PC axes
Modified Baermann funnel wet extractions	Size and composition of the soil nematode community	feeding groups (plant, omnivores, fungal, predators), total nematode abundance	fungal feeding / bacterial feeding ratio	PC axes

3.1.2 Multiple substrate induced respiration using MicroResp™

The concept underlying the multiple substrate-induced respiration (MSIR) approach, also referred to as community level physiological profiling, is to characterise how a soil community responds and catabolises a range of carbon substrates of differing chemical status (Degens and Harris, 1997). The principle is to add a range of substrates, separately but simultaneously, to aliquots of a soil sample and measure the short-term respiratory responses that ensue. The resultant physiological profiles reflect the ability of the extant soil microbial community to utilise the substrates as an energy source, and provide a measure of the functional diversity of the soil microbial community. The respiratory responses of soils can be measured by a variety of techniques. MicroResp™ (Campbell *et al*., 2003) ranked highly in SQID phase 1 since it was a suited to high-throughput processing of soil samples and did not require specialist equipment beyond a 96-well microplate reader. Respiration determination by use of gas chromatograph (GC; Degens and Harris, 1997) has been more widely used to determine MSIR profiles but it is far less suitable for high throughput analyses without further method development. SQID phase 1 identified a few issues with the potential routine and extensive application of MSIR by MicroResp™. These were:

- The MicroResp™ system was considered more practical for high throughput analyses in soil monitoring than MSIR by GC. However it was untested for extensive or large-scale sampling while the literature is dominated by respiration results using the MSIR by GC method. A comparative assessment of the two methods was considered sensible to determine the responsiveness of MicroResp™ compared to the more widely used MSIR GC method, and to examine reproducibility between laboratories.

3.1.3 Multiplex TRFLP to profile soil microbial community structure

In SQID phase I, several nucleic acid methods scored highly in relation to measuring soil microbial community structure. Primarily these methods are advantageous since they allow characterisation of non-culturable as well as culturable microbes. They also have practical advantages in that soil can be stored frozen for later analysis and high throughput analysis is possible using certain methods. Although there be can some loss of information in using high throughput methods, this is in part compensated for by ease and cost-effectiveness of analysis when dealing with a high number of samples.

Multiplex TRFLP (terminal restriction fragment length polymorphism) was considered the most appropriate method for this study since it can be used to characterise fungal, bacterial and archaeal communities and, potentially, functional microbial groups. TRFLP is one of several methods for DNA/RNA fingerprinting and provides profiles that are representative of the genetic structure of the community, as defined by the availability and selection of appropriate genetic primers. The multiplex component is a reproducible and robust molecular tool for simultaneous investigation of multiple taxa, which allows more complete and higher resolution of microbial communities to be obtained more rapidly and economically (Singh *et al*., 2006). SQID phase 1 identified a few issues with the potential routine and extensive application of TRFLP. These were:

- Despite routine use in many laboratories, standard Operating Procedures (SOP) would be required that detailed the steps used in extraction, PCR, restriction/incubation conditions and fingerprint analysis.
- Work is required to identify the most suitable primers and optimise the PCR, restriction and fingerprinting steps, especially for actinomycetes, methanogens, methanotrophs and denitrifiers.
- TRFLP, including different primers, had not been applied to a wide range of soil types and there was no systematic understanding of discrimination and sensitivity potential of the method and its primers. Consequently it would sensible to pilot applicability across a range of representative UK soils.

3.1.4 Phospholipid fatty acid analysis to profile soil microbial community structure and biomass

The use of phospholipid fatty acids (PLFAs) as signature lipid biomarkers of soil organisms has become widely used to study soil microbial communities (*c.f.* Zelles, 1999). Like DNA/RNA based methods, PLFAs can be determined from soil extractions and do not rely upon culturing soil microbes. The total amount of PLFAs measured is indicative of total soil microbial biomass while individual fatty acids or suites of fatty acids can be related to the relative abundance of different taxa or structural characteristics of the soil microbial community e.g. fungi, bacteria, Gram negative bacteria, actinomycetes, fungal/bacterial ratios, etc. There are significant advantages to the PLFA method when considering large-scale soil sampling. The method is a semi-quantitative and can be applied to soil extracts which can be stored for a long-time prior to analysis. This single method will yield information on bacteria, fungi, actinomycetes and other eukaryotes, as well as soil microbial biomass, all in one analysis. There is extensive literature that demonstrates that PLFAs from soil can be highly discriminatory of land use, soil type, management and pollution. There appear to be some trends across studies which show that ratios of bacteria to fungi change in predictable ways e.g. extensification of grassland and heavy metal pollution. SQID phase 1 identified certain issues with the potential routine and extensive application of PLFAs. These were:

- There are several PLFA methods in common use. It would be beneficial to have a standard operating procedure with QC reference soils which could be be tested in an inter-laboratory trial to be fully confident of reproducibility of results between laboratories, studies and time.
- PLFA analysis has been widely used but as with most methods there has been no systematic study of an extensive range of soil types or land uses that might be covered by a soil monitoring exercise. A study of a systematic set of samples could look at the number of PLFAs required to optimise discrimination and sensitivity compared to the level of effort required since variable numbers of PLFA peaks can be identified depending on the rigour and time available to a analytical laboratory.

3.1.5 Modified Baermann funnel wet extraction to determine the size and structure of the soil nematode community

Nematodes are among the most abundant multi-cellular soil organisms and their potential as biological indicators of soil quality is widely acknowledged (Mulder *et al*., 2004) with changes in nematode community structure corresponding to changes in soil nutrient cycling, plant growth and plant species composition. The most widely appreciated indicator is the Maturity Index (MI) which reflects the distribution of nematodes across functional groups (Bongers, 1990). More amenable indicators are currently the total number of nematode taxa and abundance of individual functional groups which are proving reliable in discriminating between different management practices within the Dutch Soil Quality Network (Mulder *et al*., 2004). An important consideration for all indicators is the sampling period since community structure alters throughout the year with respect to seasonality.

Nematodes can be passively extracted over a short-time period from soil samples of a known weight or volume into water, with the soil gently heated from overhead lights to encourage the nematodes to move out of the soil. The efficiency of the extraction varies with soil type and the exact methodology. The Baermann method has been widely used for many decades and has proved reliable in obtaining estimates of nematode populations. It is also relatively cost-effective method to set-up and run. The principal effort comes after extraction in the enumeration and identification of the individual nematode taxa. Other methods can extract more of the nematode community but these are often more labour intensive. The Baermann extraction was considered a simple and effective method for general assessments of the nematode community structure and for handling large numbers of soil samples. SQID phase 1 identified certain issues with the potential routine and extensive application of the Baermann method. These were:

- Most laboratories use their own variations of the Baermann extraction technique with in-house constructed equipments. Therefore a standard operating procedure is required to establish consistency between survey periods and laboratories.
- Further analysis is required to identify which metrics show the greatest discrimination and sensitivity to environmental pressures and drivers for UK soils.
- Identification to functional group and species relies heavily on highly trained experts. Nucleic acid techniques have potential to help ease the reliance on a dwindling reserve of taxonomists and also offer the potential for consistent identification and rapid through-put.

3.1.6 Tullgren funnel dry extraction of soil invertebrates, in particular soil microarthopods

Tullgren dry extractions support the passive extraction of invertebrates from soil or litter samples of known weight or volume into a preservative through the application of heat over a set period of time, typically several days. The Tullgren extraction is relatively cost-

effective and easy to use with much of the effort going into the identification and enumeration of the invertebrates post-extraction. It is one of the most widely used extraction methods for characterizing size and structure of the soil invertebrate community and has practical benefits for large scale soil sampling. For example, once the invertebrates are extracted into a preservative, the samples can be stored for a long period prior to further identification and the samples are amenable to long-term archiving. The method is particular useful for extracting soil microarthropods e.g. acari (*mites*) and collembola (*springtails*). These two groups are amongst the most numerous and widespread soil invertebrates in British soils and are important in litter decomposition, in regulating the soil microbial community and as a food sources, especially for birds. Both the acari and collembola have been proposed as reliable biological indicators and have been used in a number of soil quality monitoring projects. With both groups, the enumeration from Tullgren dry extraction is fairly straight-forward although higher levels of identification requires expert skills and reliable keys for identification. There is currently no published key for UK soil mites. Quality control is mainly through checking the efficiency of individual personnel with reference specimens.

- The original Tullgren extraction method has been modified over the years with many different adaptations in current use. This hampers comparisons and compatibility since reproducibility of the method has rarely been addressed. Standardisation could be introduced via equipment specification, length of extraction period used and testing extraction efficiencies.

- Identification to functional group and species level relies heavily on trained staff and expert taxonomists. With a rapidly declining pool of taxonomic experts, there is pressing need to investigate the potential to use molecular techniques and/or digital recognition for consistent identification and rapid through-put.

- Further consideration is required to determine which metrics show the greatest discrimination between soil:land use combinations and sensitivity to environmental pressures and drivers for UK soils.

3.2 Modifications and future developments to methods

3.2.1 Multiple enzyme fluorometric assay to profile the activity of soil enzymes

Method Modifications. Initially fluorescence was measured as a time series following 0, 30, 60, 120 and 180 minutes of plate incubation. This always resulted in a straight line graph of fluorescence vs. time. The enzymes activity rate was calculated from the gradient of this line. As the time series was always linear, fluorescence was subsequently only measured at t=3hrs. A blank was prepared (x2) for each enzyme on each plate and subtracted from the three hour reading to account for baseline variation. Initially the whole procedure was performed using sterile water, equipment and reagents where possible. Purchase of sterile plates was expensive, and initial observations of results suggested that this was not necessary. The revised SOP does not require sterile conditions.

Future Developments.

Incubation/soil moisture. Soil enzyme activities are likely to vary with differing soil moisture properties. A potential problem with the current incubation method is that soil moisture is determined at day one of the incubation period. The soil is then left in a bag with a "wick" to allow the soil to breath for 7 to 14 days. During this incubation period it is likely that the moisture content will change. No attempt is made to ensure that the moisture content of the soil is the same on day one as it is on the last day of incubation. A better method of incubation would maintain the moisture content at a constant predetermined state, and also to standardise the moisture content of soils for all samples (e.g. maintain at 50% water holding capacity). Thereby soil microbiology would not have bias that would be associated with variable moistures.

Enzyme extraction method. Various methods of enzyme extraction are available, including sonication and shaking. Research should be conducted to determine the preferred/best extraction method. The extraction method employed may well liberate different portions of cellular enzymes i.e. intracellular vs. extracellular enzymes.

Soils extract acquisition. In the current method the soil sample (0.5g) is added to water (50ml) and the sample stirred continuously to obtain a homogenous soil/extractant mix. 50µl of this solution is removed by pipette. The reasoning for this is that some extracellular enzymes may be adsorbed onto the surface of soil particles, and thereby the soil particles should be included in the enzyme reaction. The problem with this is that it is very difficult to obtain a homogenous soil/extractant mixture using the current SOP. This may lead to sampling bias. Additionally it is often very difficult to accurately withdraw the 50µl of soil suspension as organic matter often clogs up the pipette, thereby adding uncertainty as to whether the full 50µl has actually been withdrawn. An alternative would be to filter/centrifuge the sample and omit the soil particles in the reaction, but this would then remove enzymes that have been adsorbed onto the soil particles surface.

Variety of enzymes. The current method analyses the activity rate of 8 enzymes, all based on the fluorescence of 4-methylumelliferone. In addition to the 8 substrates used, there are many other enzymes that could be studied using the current SOP. Additionally the method could be modified to utilise substrates bound to 7-amino-4-methyl coumarin e.g. L-leucine-AMC.

Standard Soil. The standard soil of choice used throughout the SQID project had low enzyme activities when compared to other soils. Indeed the average enzyme activity of the standard soil throughout the discrimination trial was actually less than the Limit of Detection for 6 out of the 8 enzymes. Subsequently results are likely to be variable. In future studies, the standard soil should have higher activity for all enzymes and the use of more than one standard soil may be more useful.

Conclusions. The hydrolytic enzymes method is relatively cost-effective and easy to perform. However the method would be more useful if it was optimised for greater

efficiency which may reduce the inherent variability. The above suggests some ways in which the method could be optimised.

3.2.2 Multiple substrate induced respiration using MicroResp™

Method Modifications: To take into account abiotic CO_2 release from the addition of carbon substrates to calcareous soils, a 30 minute delay was introduced prior to placing the seal and detection plate onto the deepwell plate.

Future Developments:

- For large experiments, soil can be added and pre-incubated in the deepwell plates prior to the addition of carbon substrates, thus pre-incubation can be reduced to 3-5 days (MicroResp™ Manual v 2.1, Macaulay Scientific Consulting Ltd., Aberdeen).
- A reference soil could be introduced for Inter-laboratory trials, large experiments or temporal experiments to support comparison of absolute values for respiration rates between sites, dates or locations.
- Carry out a comprehensive calibration to obtain a large range of CO_2 values. This can be done either by using large numbers of varying soil types and/or high response carbon substrates (e.g. alpha ketoglutaric acid) or by using a range of carbon dioxide standards rather than soil.
- The starting Absorbance values used in the analyses should be equivalent to that used in the calibration to be able to quantify respiration and to allow data comparisons between different MicroResp™ analyses (e.g. sampling times, laboratories, sites). Further work is needed to define the precision required in preparing the detection plates to constrain the Absorbance values within a narrow range to support extended reproducibility.

3.2.3 Multiplex TRFLP to profile soil microbial community structure

Method modifications. These included the following from the initial to the final SOP;

- The addition of introductory and explanatory paragraphs at the beginning and throughout the document.
- The required reagents section was expanded and an equipment list was added.
- Section on sample storage and preparation added.
- Ethanol precipitation was step moved from post-PCR to post-extraction step.
    - Good lab technique is critical to successful completion of this protocol. Ethanol precipitations and restriction enzyme digests are the most important steps to carry out correctly. During precipitation, the supernatant must be removed by brief, gentle centrifugation of the inverted sample plate. Failure to do this results in loss of sample. When adding the restriction enzyme mix to samples, it is important to add the mix to the wells before adding the sample. Failure to do this correctly may lead to undigested product in the final TRFLP profile.
- PCR cleanup kit step introduced (replaces ethanol precipitation).
- TRFLP section expanded and explained in more detail.

- Section on use of Applied Biosystems Genemapper software for preliminary visualisation/analysis and export of data added.
- Standards. Fresh frozen soils were used as the internal standards which were sampled from well characterised soils [Countesswells soil – Mineral and Glensaugh soil – Organic].

Future Developments.

- Soils with an extremely high organic content can be problematic and may require alternate extraction methods. Extraction of extremely high organic content soils gave very poor TRFLP profiles, especially for ITS. Soils with a medium to high organic content can benefit greatly from an ethanol precipitation treatment after DNA extraction. Samples treated in this manner yield satisfactory TRFLP profiles. Different DNA extraction techniques or PCR enhances could be used to improved TRFLP profiles.
- The successful use of freeze-dried soils in DNA extraction could make sampling ans storage of soil more flexible.

3.2.4 Phospholipid fatty acid analysis to profile soil microbial community structure and biomass

Method Modifications.

The amount of soil to be used in the extraction was amended to reflect the soil organic matter content of the soil, where known.

Future Developments.

It was apparent with the organic soil samples that improvements could be made in the analysis of these soils. Due to the limitations of the silica columns used to separate the extract into the lipid fractions, the amount of soil used is very small (50 mg). This in turn means that the resolution of the peaks on the GC is low which compromises on the identification and quantification of the smaller peaks present in the PLFA profile. It has been suggested that decreasing the amount of internal standard from 200 µl to 50 µl, and concentrating the sample for the GC to 50 µl (using tapered inserts in the GC vials) could produce better resolution of the peaks.

It is also important to establish quality control (QC) limits for the fatty acids with a standard set of reference soils (Quality Control Soil) to ensure the quality of the PLFA extraction itself. This can be achieved by different mechanisms.

- The extraction of reference soils in sufficient quantity prior to an experiment to establish the QC limits
- Inclusion of the reference soil with analytical batches of samples
- Continual monitoring of the reference soil with routine adjustments to the QC limits where extractions are carried out on a routine basis.

3.2.5 Modified Baermann funnel wet extraction to determine the size and structure of the soil nematode community

Method Modifications. None were required.

Future Developments.

- There has been little/no standardisation of extraction procedures amongst laboratories. Development of an inter-laboratory standard technique is still required. There maybe lessons to be learnt from how inter-lab and repeated ecological assessments of freshwaters using invertebrates are standardised.
- The amount of soil sample used here (~100g) was relatively low compared to other studies, particularly in agricultural systems. This may be reflected in the relatively low numbers of nematodes obtained. Extraction of a greater mass of soil (e.g. 200 g) may remedy this problem.
- The identification phase is the most time limiting stage and is dependant on having sufficient skilled staff to process the samples within a relatively short time frame. An increase in the availability of skilled and, ideally, suitably qualified people to carry out identifications and/or DNA identification of soil organisms would greatly advance the capacity to characterise community structure in soil nematodes.
- Further testing to identify the best/optimal extraction method for soil nematodes for large-scale soil sample. This should compare the modified Baermann method using a greater mass of soil with alternative methods, with an assessment of the relative cost-effectiveness of the methods.

3.2.6 Tullgren funnel dry extraction of soil invertebrates, in particular soil microarthopods

Modifications to SOP. None were required since the SOP had been trialled previously for large-scale soil processing.

Future Developments.

- There has been little/no standardisation of extraction procedures amongst laboratories. Development of an inter-laboratory standard technique is still required. There maybe lessons to be learnt from how ecological assessments of freshwaters is standardised.
- The identification phase is the most time limiting stage and is dependant on having sufficient skilled staff to process the samples within a relatively short time frame. An increase in the availability of skilled and, ideally, suitably qualified people to carry out identifications and/or DNA identification of soil organisms would greatly advance the capacity to characterise community structure in soil invertebrates. DNA identification of soil organisms would greatly advance the capacity to characterise community structure in soil invertebrates (collembola, mites and other groups) but this should be supported by publication of further and update keys for UK soil invertebrates, in particular as mites.

3.3 Summary

- All methods proved amenable for the purpose of analyzing large numbers of soil samples.
- There are clear requirements for the development of suitable standards and calibration approaches for all methods to improve or establish data compatibility and reproducibility.
- The extraction method for nematodes could be improved or changed to obtain higher extraction numbers, and therefore reduce variability between samples.
- Availability of skilled people for taxonomic identification of mites, collembola and nematodes can constrain the processing of these samples. There would be clear benefits to the development of molecular identification for soil faunal groups in UK soils equivalent to that for soil microbes.

4 Evaluate and aim to bring MicroResp™ method for multiple substrate-induced respiration (MSIR) to a deployable status

Multiple substrate-induced respiration (MSIR) was identified as a high-ranking potential indicator in SQID Phase 1. MSIR provides an assessment of the activity of the soil microbial community by measuring the release of CO_2 (soil respiration) from soil after the addition of various carbon substrates. This is often termed community level physiological profiles (CLPP). The majority of published studies have used gas chromatography (MSIR-GC) to measure soil respiration rates. However, this method is not well suited to high throughput assessments for a large number of samples in a short time-frame. An alternative microplate based system (MicroResp™) offered the potential for a high-throughput assay (Chapman *et al.*, 2007) as it can be used to analyse respiration from a number of soil samples simultaneously. However, this assay had not been deployed in large-scale surveys and two issues were identified to evaluate the suitability of MicroResp™ for this purpose. The first was whether this assay would produce equivalent results to the more widely used GC-based MSIR method in a comparative trial. The second issue was how reproducible the assay would be, when analysed in different laboratories. It should be noted that reproducibility is not an issue specific to MicroResp™, but there was less published information available to review this issue for this assay, compared to other methods.

To examine these two issues, three contrasting land uses were sampled, viz. arable, grassland and woodland, in each of three geographical regions within Great Britain to obtain a total of nine bulked soil samples. The sampling sites were prescribed to provide a range of soil physical and chemical characteristics (Table 4-1) and to ensure a range and contrast in the soil microbiological properties being considered in this study. The three partner organizations were each responsible for identifying, collecting, preparing and distributing soil samples from a suitable arable, grassland and woodland site. Sampling was conducted in March 2006, when five randomly distributed soil sub-samples (0–10 cm depth) were collected from each site. At each laboratory, soil samples were bulked within site, passed through a 2-mm sieve and stored at 4°C prior to distribution to other partners and subsequent use in the method trials. The methods for MSIR by GC and MicroResp™ are provided in Appendix A.

Table 4-1 Summary of origins, land-use and principal characteristics for soils used in the MSIR and multi-enzyme assay trials

Grid Ref[a]	Land-use	Code	Texture	pH_{H20}	SOM (%)	WHC (%)	Carbonate present[b]
TL082356	Arable	1	Clay loam	7.1	6.4	43.6	N
SD498399	Arable	2	Sandy silt loam	6.5	5.8	40.2	N
NJ183626	Arable	3	Loamy sand	6.6	3.1	31.4	Y
TL335510	Grassland	4	Sandy silt loam	7.5	13.0	49.3	Y
SD349457	Grassland	5	Sandy silt loam	6.1	9.3	44.4	N
NO665785	Grassland	6	Silt loam	6.9	15.2	44.2	N
TL082356	Woodland	7	Sandy loam	6.7	2.2	35.6	N
SD435795	Woodland	8	Silt loam	6.9	25.3	59.6	N
NO652802	Woodland	9	Organic	4.5	44.4	49.9	N

[a]UK OS National Grid [b]HCl effervescence test (Hodgson, 1997)

4.1 Benchmarking of MicroResp™ against MSIR by GC

Degens and Harris (1997) developed a multiple carbon-source, substrate induced respiration method (MSIR) that measures the respiration response of the soil microbial community from soil samples as opposed to soil extracts, which are used in the original CLPP "Biolog" method (Garland and Mills, 1991). The Degans and Harris approach is a progression from a widely used SIR (substrate induced respiration) approach to measuring soil microbial biomass (West and Sparling, 1986). Carbon dioxide production is measured by GC, infra-red spectroscopy or some other suitable assay from soil samples incubated in small glass bottles or vials. This method is widely used to investigate the responsiveness of soil respiration to pressures such as contamination, climate change, plant diversity etc. However, the applicability or practicality of this method has not been tested in studies where sample numbers exceed 100's or even 1000's. The practical considerations of using MSIR within a large-scale survey were considered a limitation to using this method within soil monitoring; large numbers of soil samples, restricted quantity of soil, analyses in a relatively short time-frame, etc (Black *et al*., 2005).

MicroResp™ (Campbell *et al*. 2003) was designed to be a 'whole soil' method, which uses the practical convenience of a 96 well microtitre plate format. Soils can be incubated within deep-wells to which, solutions of carbon substrates can be added. The technique uses a detection plate which contains a gel-based indicator dye that will respond to carbon dioxide evolved from the soil. Colour changes in the gel can be read on a standard laboratory microplate reader to provide CO_2 levels. The number and types of carbon substrates can be varied to suit the purpose of the study. The method has been proposed as suitable for processing large numbers of samples in a cost-effective manner.

A simple trial was carried out to establish whether MicroResp™ could produce comparable results to those obtained by MSIR by GC. The calibration suite of 9 soils (Table 4-1) was used to test MSIR-GC in parallel with MicroResp™ using the same seven substrates plus

water in each method. The substrates included: L-arginine, L-malic acid, citric acid, γ-amino butyric acid (GABA), N-acetyl glucosamine (NAGA), D(+) glucose and α-ketoglutarate (AKGA). These substrates provide a spectrum of compounds, from acids, basic sugars and proteins, which are known to produce differences in soil respiration. The detailed methodologies for this trial are provided in Appendix A. For consistency, the trial was carried out by one organization (CEH) as an independent laboratory with established expertise in deploying soil respiration methods. All statistical tests were performed with STATISTICA v8.0 (Statsoft, 2008).

Figure 4-1 presents mean soil respiration data from all substrates for all nine soils by each method. These illustrate how similar the trends are in soil respiration across the nine soils though, in general, MicroResp™ was more sensitive to lower levels of soil respiration from arginine, NAGA, GABA and glucose than MSIR-GC. Soil respiration was generally far higher from the MSIR-GC method compared to MicroResp™, which reflects the use of more soil in the former (4 g compared to 400 µl volume). This may also account for lower sensitivity in MSIR-GC. Figure 4-2 further demonstrates the close correspondence in soil respiration between the two methods for all substrates. Principal components analysis (PCA) was used to integrate soil respiration data from all substrates. Figure 4-3 illustrates that both methods produced similar separation of the individual test soils (1 to 9). The PCA highlights that the first PC axis (PC1) accounted for MicroResp™ 89% of the variation between the soil samples, compared to 64% for MSIR-GC i.e. the former method provided better discrimination between the test soils. Overall the results suggest that MicroResp™ provides complementary results to MSIR-GC and may in fact provide a more sensitive and improved discriminating method than the MSIR-GC method.

Figure 4-1 Comparison of soil respiration (CO_2-C μg g^{-1} h^{-1}) from a range of carbon substrates using the MSIR-GC and MicroResp™ techniques using soils from nine contrasting sites in the UK.

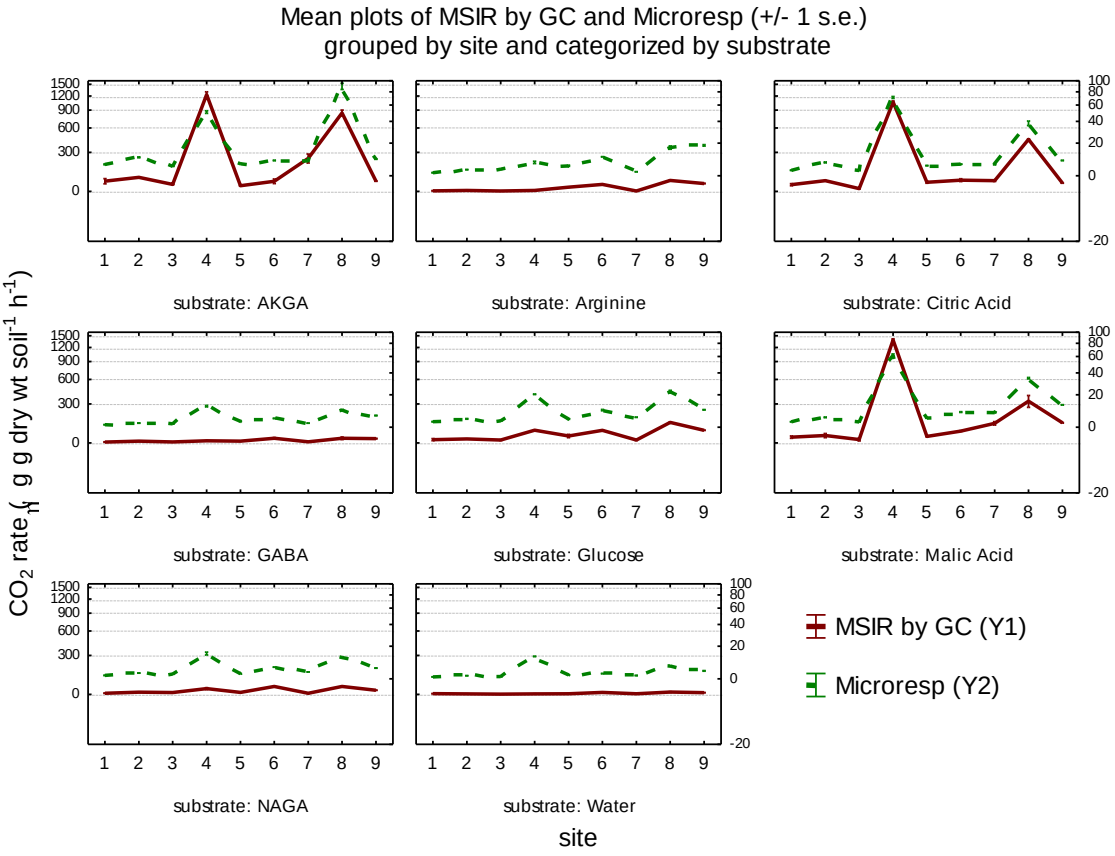


Figure 4-2 Scatterplot comparison of soil respiration (CO_2-C μg g^{-1} h^{-1}) using multiple substrates for the two methods: MSIR-GC and MicroResp™

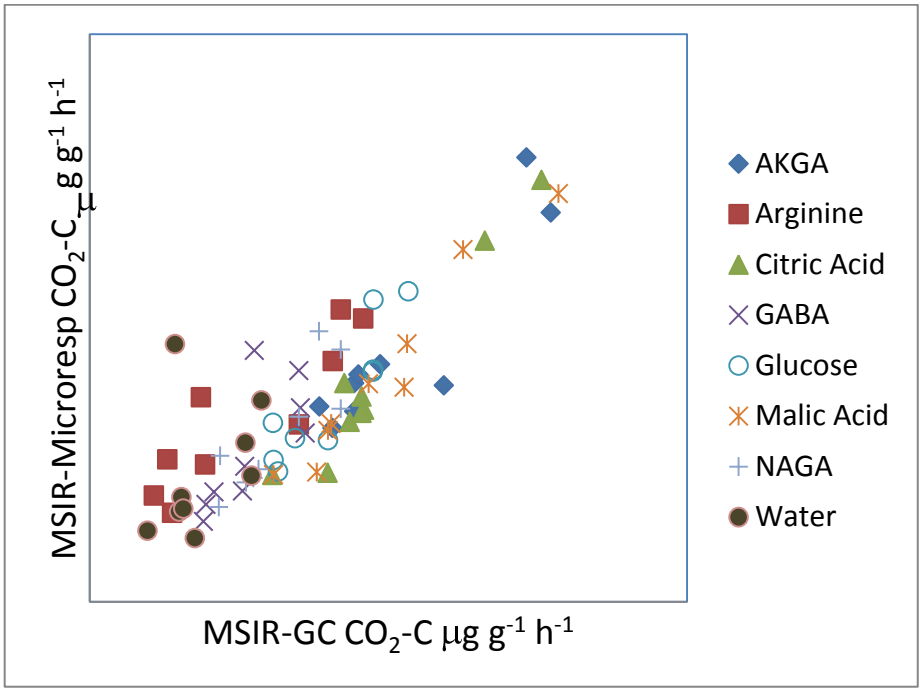
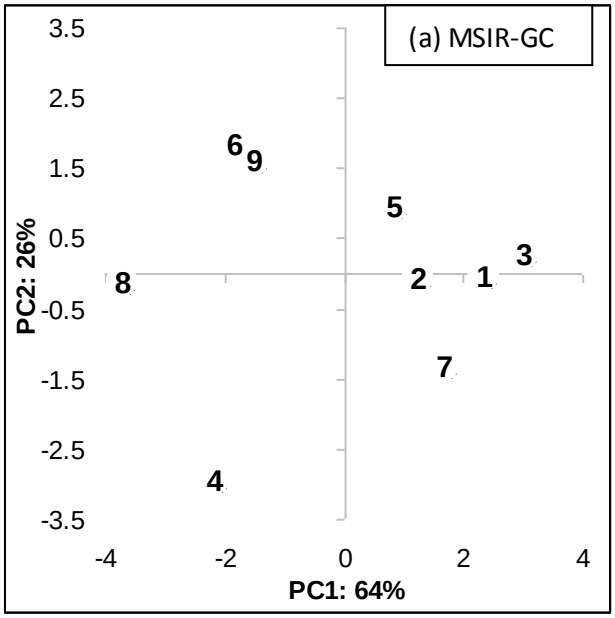
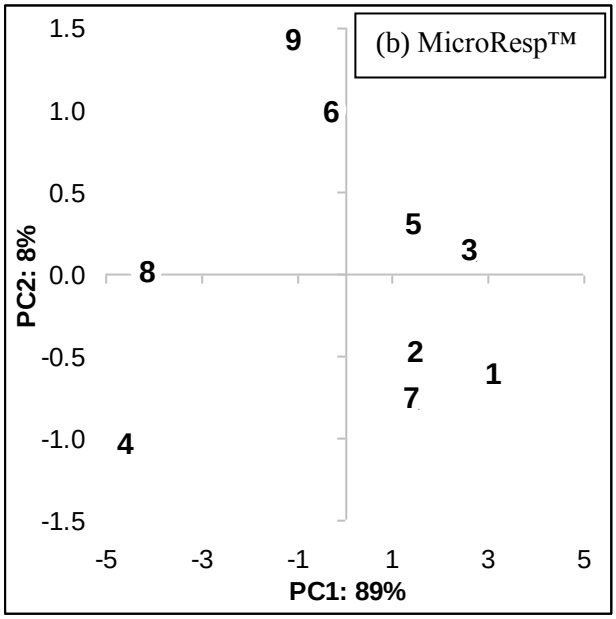


Figure 4-3 2D plots for the separation of sites (1-9) using the first 2 axes from principle components analyses of the multiple substrates respiration data from MSIR-GC and MicroResp™. Percentage variation accounted for by PC1 and PC2 shown in parentheses. GLM ANOVA results for discrimination between soils were significant for each axis, P<0.005. All data were log transformed prior to statistical analyses. See Table 4-1 for details on the sites.





4.2 Inter-laboratory trial to assess reproducibility of MSIR by MicroResp™

The calibration suite of 9 soils (Table 4-1) was used to test the reproducibility of MSIR using the MicroResp™ method by carrying out a trial across three laboratories. Results from this trial were published by Creamer *et al*. (2009). This report summarises the salient points from this paper with additional information on method reproducibility.

The three partner organizations carried out the inter-laboratory trial using sub-samples of the same soils using the same protocols at the same time with the same seven carbon substrates. For consistency, the substrates were all purchased by one laboratory, sub-sampled from the same source and batch (confirmed via batch number) and then distributed amongst all three laboratories. The substrates included: L-arginine, L-malic acid, citric acid, γ-amino butyric acid (GABA), N-acetyl glucosamine (NAGA), D(+) glucose and α-ketoglutarate (AKGA). These substrates provide a spectrum of compounds, from acids, basic sugars and proteins, which are known to produce differences in soil respiration. The detailed protocols for this trial are provided in Appendix A. The respiration rate data (μg CO_2–C g^{-1} h^{-1}) produced after 6 hours incubation at 25°C were statistically analysed to investigate the reproducibility of the absolute values for respiration and how inter-laboratory differences in the data produced could influence the overall interpretation of the results.

The respiration data produced by each laboratory were collated and statistically analysed by one organisation (Cranfield). Data were tested for normality using the Anderson–Darling test (significance level of $p<0.05$). Respiration data showed a non-normal distribution and transformations did not improve the dataset. Therefore, statistical tests were applied which did not require a normal distribution. The reproducibility of the MicroResp™ method across the three laboratories was assessed by two approaches. The first was pair-wise regression of the seven C substrates and water using geometric mean regression (GMR) (Sokal and Rohlf, 1981). The slope of the regression line for each pair-wise comparison between two laboratories was compared to unity (1) to test the hypothesis that there was no significant difference between the absolute values for respiration rates from the two laboratories. R^2 (the proportion of variance explained by the linear relationship) was then used to quantify the degree of agreement between the laboratories. Where absolute values did not agree this resulted in a high R^2 value demonstrating that one laboratory consistently measured higher respiration compared to the other laboratory. In the second approach, multivariate analyses of all respiration data (all seven substrates and water) for the nine sites from all three laboratories was carried out using principal components analysis (PCA). This analysis was used to determine the reproducibility of site discrimination patterns amongst the three laboratories using all available data, and to examine whether similar substrates were contributing to the discrimination patterns displayed by the respiration data from each laboratory. All statistical tests were performed with STATISTICA v8.0 (Statsoft, 2008).

The results from each laboratory for individual substrates are summarized in Table 4-2 and

the statistical analyses of these results using GMR are presented in Table 4-3. The pairwise GMR analyses highlighted significant differences in the majority of absolute amounts of CO_2-C produced by the individual substrates across the three laboratories. In general, laboratory 2 generally producing higher values of CO_2-C compared to laboratories 1 and 3. However, the results also indicate that there were broadly similar patterns of responses from all substrates for the nine soils amongst the three laboratories, as illustrated in Figure 4-4 for AKGA. This comparability in responses was further demonstrated by the multivariate PCA analyses of all substrate data. Results from all three laboratories are illustrated in Figure 4-5. These demonstrate that the MSIR data from each laboratory produced similar ordination patterns amongst the nine soils. However, there was not a direct 1-to-1 correspondence in the ordination patterns due to the differences in the absolute values of CO_2-C. Figure 4-6 further serves to show that these ordination patterns were produced by similar loadings from the individual substrates. The high percentage of variation accounted for in PC1 (77 to 86%) indicates that this limited set of substrates was good at discriminating between the nine soils from different geographical regions and land uses.

These results demonstrate that MSIR is a consistent and effective method for identifying differences between soil samples from different origins. However, there are practical constraints to be considered in the application of the method in large-scale studies which would require multiple laboratories and/or comparisons of data between different studies across space or time. Absolute values of CO_2-C would not be directly comparable between different laboratories without further development of inter-laboratory comparability. It would not be advisable to compare or integrate MSIR data produced by multiple laboratories without prior determination of a suitable inter-laboratory calibration procedure, including a suitable quality control.

Table 4-2 Results for individual MSIR substrates (CO_2-C μg g^{-1} h^{-1}) from soil samples analysed by three laboratories using MicroResp™.

	substrate	AKGA		Arginine		Citric Acid		GABA		Glucose		Malic Acid		NAGA		Water	
Lab	soil	Means	s.e.	Means	s.e.	Means	s.e.	Means	s.e.	Means	s.e.	Means	s.e.	Means	s.e.	Means	s.e.
1	1	2.05	0.10	0.71	0.02	1.10	0.14	0.49	0.04	1.19	0.08	1.30	0.03	0.76	0.04	0.44	0.04
	2	7.61	0.11	1.76	0.14	5.48	0.23	1.12	0.05	2.94	0.05	2.74	0.40	1.71	0.04	0.64	0.04
	3	2.35	0.04	1.55	0.06	1.07	0.02	0.79	0.02	1.55	0.04	1.63	0.07	1.10	0.05	0.43	0.01
	4	19.47	0.32	4.94	0.72	21.35	0.31	5.94	0.27	10.07	0.34	21.38	0.53	7.24	0.35	5.77	0.55
	5	3.47	0.15	2.91	0.17	2.89	0.12	1.80	0.04	3.02	0.06	3.02	0.12	1.75	0.05	1.05	0.13
	6	4.54	0.20	3.73	0.11	3.44	0.23	2.70	0.03	5.28	0.08	4.87	0.10	3.98	0.03	1.27	0.03
	7	4.50	0.15	1.16	0.12	3.68	0.13	1.15	0.03	2.90	0.04	3.74	0.29	1.95	0.05	0.79	0.03
	8	21.16	0.51	7.22	0.46	16.14	0.20	5.85	0.31	11.65	0.13	13.82	0.32	6.46	0.09	2.95	0.18
	9	5.02	0.40	5.06	0.61	4.19	0.11	3.49	0.11	6.46	0.22	7.67	0.69	3.48	0.08	1.79	0.10
2	1	5.89	0.35	1.47	0.11	2.70	0.14	1.26	0.06	2.75	0.13	2.72	0.08	1.61	0.05	0.91	0.04
	2	10.10	0.60	3.02	0.13	7.00	0.38	2.07	0.05	4.26	0.29	5.09	0.13	2.83	0.12	1.49	0.04
	3	4.83	0.14	3.23	0.10	2.62	0.05	1.68	0.06	3.21	0.07	2.62	0.14	2.35	0.05	1.05	0.03
	4	50.74	5.54	7.01	0.89	71.09	3.13	11.77	0.48	20.36	0.71	61.69	5.52	14.60	1.27	12.64	0.96
	5	6.23	0.12	5.05	0.25	5.20	0.14	2.94	0.07	4.16	0.12	4.68	0.10	2.51	0.07	1.88	0.04
	6	8.16	0.29	10.49	0.27	6.00	0.12	4.52	0.12	9.31	0.35	8.13	0.24	6.07	0.16	2.58	0.08
	7	7.97	0.37	1.93	0.13	5.79	0.27	2.05	0.06	5.14	0.22	7.80	0.30	3.40	0.10	1.57	0.04
	8	89.66	15.34	16.75	1.40	38.03	4.01	9.40	0.38	22.36	1.29	34.50	1.34	11.89	0.60	6.70	0.24
	9	9.01	0.26	18.34	0.95	8.16	0.33	6.13	0.25	9.52	0.21	12.68	0.34	5.51	0.18	4.01	0.07
3	1	3.58	0.36	1.79	0.08	2.18	0.05	1.14	0.02	2.13	0.04	2.01	0.13	1.45	0.04	0.93	0.02
	2	9.56	0.55	5.36	0.14	7.49	0.64	2.60	0.17	4.87	0.12	5.85	0.13	3.31	0.13	1.81	0.03
	3	4.45	0.05	3.29	0.08	2.16	0.06	1.77	0.05	3.17	0.09	2.84	0.16	2.52	0.05	1.34	0.09
	4	24.59	0.54	10.73	0.79	25.85	0.42	10.41	0.42	15.63	0.50	26.40	0.43	12.79	0.76	12.27	0.55
	5	5.55	0.18	5.01	0.22	3.69	0.32	2.87	0.17	4.21	0.18	5.12	0.15	2.94	0.09	2.18	0.10
	6	6.87	0.70	7.65	0.80	5.94	0.27	4.98	0.15	9.08	0.16	8.89	0.61	7.00	0.12	2.99	0.13
	7	5.86	0.21	3.02	0.24	5.18	0.26	2.46	0.04	4.28	0.06	6.29	0.13	3.34	0.06	2.03	0.08
	8	28.37	0.90	14.26	0.32	18.60	1.95	9.09	0.45	17.13	0.34	21.09	0.28	10.82	0.36	7.75	0.40
	9	10.93	2.32	12.35	1.70	8.04	0.15	7.24	0.38	12.46	1.61	14.56	3.36	7.34	0.30	7.68	1.79

Table 4-3 Correlation coefficients (R^2) and results from geometric mean regression (GMR) of CO_2-C respiration rates for individual MSIR substrates in a pairwise comparison between laboratories. P indicates where statistical results are significant; ns, not significant.

Substrate	Laboratory comparisons	R^2	P	GMR	P
H_2O	1 vs 2	0.9903	<0.005	1.20 ± 0.09	ns
	1 vs 3	0.8501	<0.005	1.70 ± 0.52	<0.005
	2 vs3	0.8654	<0.005	1.42 ± 0.21	<0.001
Arginine	1 vs 2	0.6728	<0.001	3.78 ± 0.88	<0.005
	1 vs 3	0.9296	<0.005	2.67 ± 0.27	<0.005
	2 vs3	0.9299	<0.005	0.70 ± 0.08	<0.05
Malic acid	1 vs 2	0.9596	<0.005	1.66 ± 0.21	<0.005
	1 vs 3	0.9773	<0.005	1.25 ± 0.07	<0.05
	2 vs3	0.9470	<0.005	0.75 ± 0.05	<0.05
(GABA) γ-Aminobutyric acid	1 vs 2	0.9757	<0.005	1.79 ± 0.11	<0.005
	1 vs 3	0.9741	<0.005	1.62 ± 0.10	<0.005
	2 vs3	0.9725	<0.005	0.91 ± 0.06	ns
NAGA (n-Acetyl glucosamine)	1 vs 2	0.9769	<0.005	1.97 ± 0.11	<0.005
	1 vs 3	0.9862	<0.005	0.87 ± 0.08	ns
	2 vs3	0.9587	<0.005	0.87 ± 0.07	ns
Glucose	1 vs 2	0.9777	<0.005	1.99 ± 0.11	<0.005
	1 vs 3	0.9857	<0.005	1.49 ± 0.07	<0.001
	2 vs3	0.9515	<0.005	0.75 ± 0.06	<0.05
AKGA (α-Ketoglutaric acid)	1 vs 2	0.8549	<0.005	0.28 ± 0.17	<0.005
	1 vs 3	0.9918	<0.005	1.29 ± 0.04	<0.05
	2 vs3	0.8303	<0.005	4.54 ± 0.22	<0.005
Citric acid	1 vs 2	0.8392	<0.005	0.39 ± 0.26	<0.005
	1 vs 3	0.9886	<0.005	1.25 ± 0.05	<0.05
	2 vs3	0.9893	<0.005	3.24 ± 0.07	<0.005

Figure 4-4 Results for AKGA from nine soils determined by three laboratories using MicroResp™ (CO_2-C μg g^{-1} h^{-1} mean +/- 1.s.e)

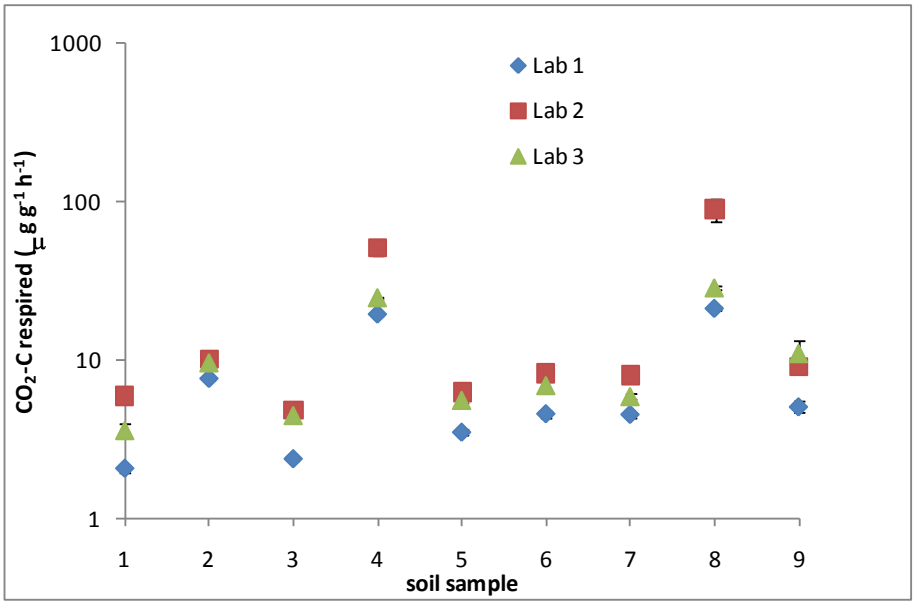
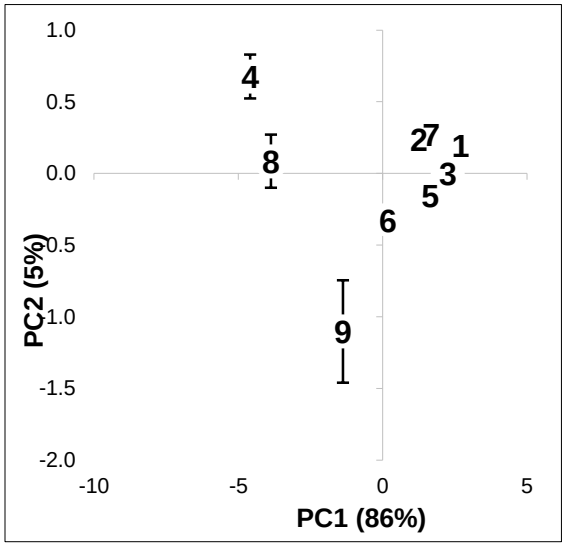
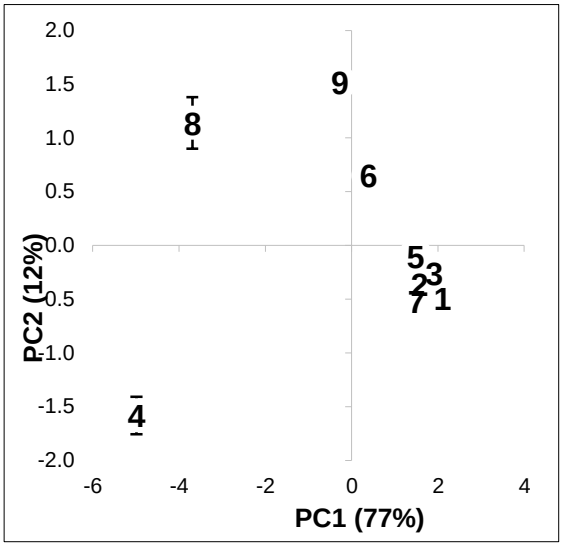


Figure 4-5. 2D plots showing the separation of sites (1-9) using the first two axes from the principal components analyses of the MSIR results for all substrates from each laboratory. Variation accounted for by each PC is shown in parentheses. Bars indicate standard errors.

(a) Laboratory 1



(b) Laboratory 2



(c) Laboratory 3

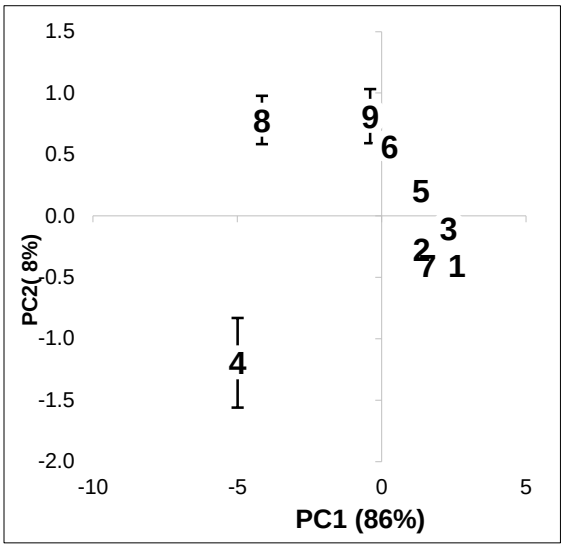
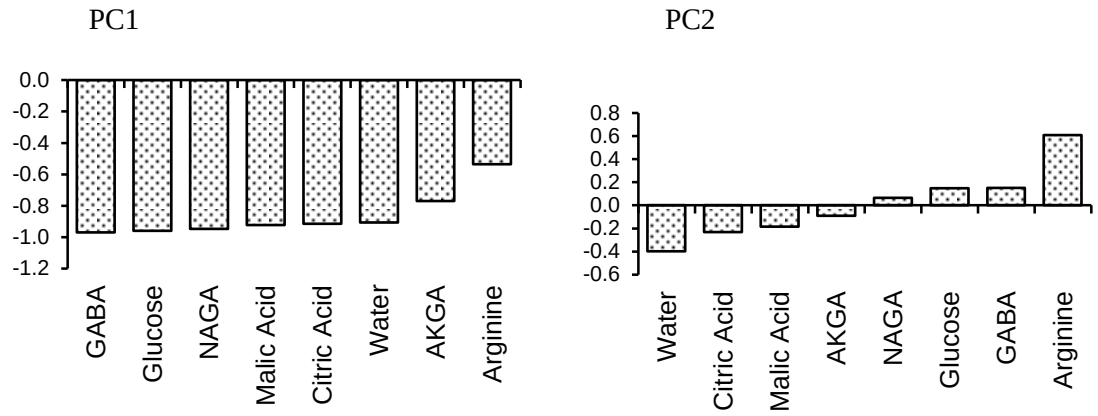
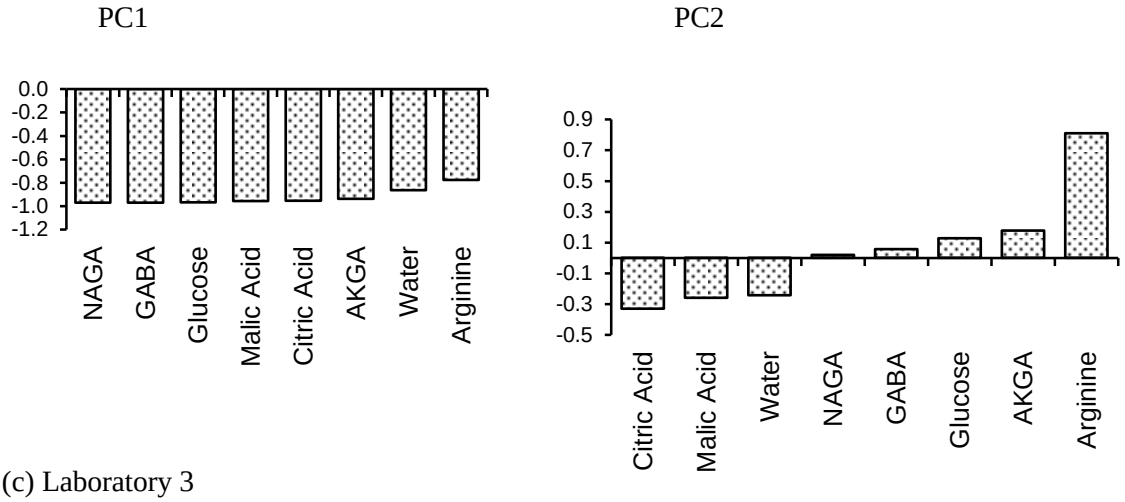


Figure 4-6 Loadings for the individual substrates associated with the PC1 and PC2 axes of the principal components analyses of the MSIR results from each laboratory.

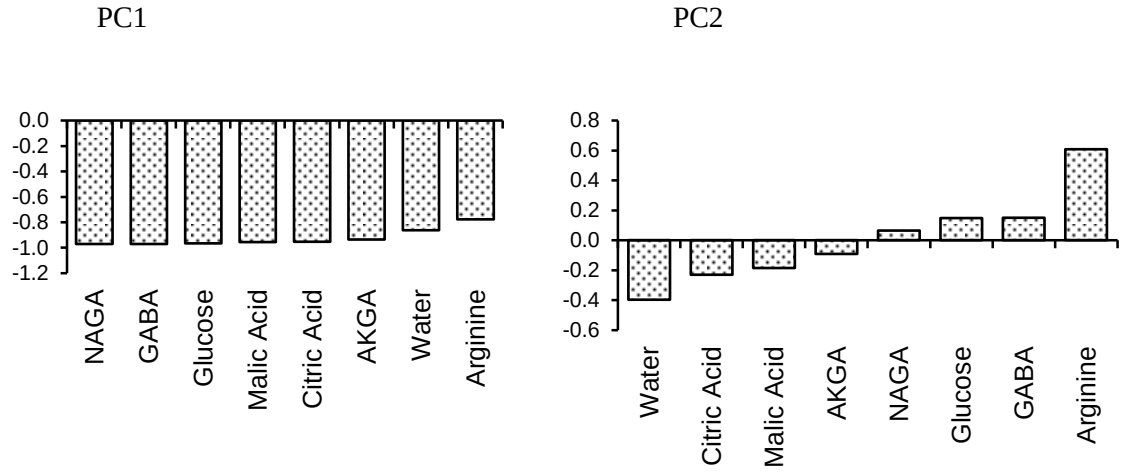
(a) Laboratory 1



(b) Laboratory 2



(c) Laboratory 3



5 Test the candidate biological indicators for their sensitivity to distinct environmental pressures

The objective was to evaluate the sensitivity of the indicators to distinct environmental pressures against the temporal variability and spatial heterogeneity of each indicator under typical field conditions. Soil biological properties and processes are typified by their dynamic nature, which often reflects diurnal and seasonal changes in above and below-ground conditions e.g. plant growth, soil moisture content and temperature. In parallel, the spatial distribution of soil biological properties and processes is heterogeneous from micro- to macro-scales. These intrinsic characteristics can be viewed as the background noise against which the signal of an indicator has to be sufficiently powerful enough to be registered and significant e.g. signal-to-noise ratio. This sensitivity test was carried out to determine the relative differences in and, importance of, the signal-to-noise ratio across the candidate indicators under seasonal field conditions. It should not be considered as a comprehensive assessment of spatial and temporal variability for each indicator but rather a suitable test to determine whether the indicators are sufficiently robust enough to demonstrate sensitivity to a pressure over and above its own inherent variability and heterogeneity.

Three field sites were selected to provide contrasting pressures relating to the three soil functions identified in SQID Phase I (*viz*. food/fibre, environmental interactions, habitat/biodiversity). The pressures selected were sewage sludge applications to agricultural land, simulated atmospheric nitrogen deposition on upland grassland habitats and restoration of open-cast mine sites to grassland habitat. Each of these reflects a relatively widespread and common pressure on UK soils and a pressure which has documented influence on soil biological properties and processes.

The re-cycling of sewage sludge to land is a widespread practice on UK grassland and arable soils and can result in considerable ecological and agricultural benefits. However, a build-up of heavy metals can reduce the size and activity of the microbial biomass and reduce the numbers of effective N-fixing *Rhizobium*. Several field experiments that address both the addition of sludge (low in metals) as well as sludges with inhibitory concentrations of metals were available from the field experiments affiliated to the UK Sewage Sludge Network. Sites within this network have recorded changes in soil microbial community structure and biomass under elevated zinc and copper sludge treatments (MacDonald *et al*., 2007; Defra, 2007).

Many UK soils are considered to have exceeded the Critical Load for nitrogen due to historical and continued deposition of nitrogen from atmospheric pollution, which has also been linked to UK water quality issues and widespread changes to plant community structure across the UK (NEGTAP, 2001). Soil biological properties and processes are intimately linked to a changing nitrogen status of soils, a release of nitrogen to water and an increased availability of nitrogen for plant growth and establishment. Several long-term field experiments that address the addition of nitrogen, in different forms and in varying concentrations, to various land uses are available via the Defra / NERC network for UK Research on The Eutrophication and Acidification of Terrestrial Ecosystems

(http://ukreate.defra.gov.uk/).

Habitat creation and restoration are significant issues within the UK. Continued expansion and renovation of urban and industrial land necessitates a greater emphasis on planned restoration to achieve healthy living environments, sustainable drainage systems, recreational areas, etc. In parallel, UK conservation bodies are working to improve the ecological status of our native and semi-natural habitats, many of which are in a degraded state due to historical land use, management, pollution, etc. Re-establishment of an active, healthy soil biological community, with its associated biological processes, is fundamental to successful habitat restoration, including associated ecosystem services (Harris, 2009).

5.1 Methodology for the sensitivity trial

5.1.1 Field sites

Three field sites were selected within the UK from long-term experimental, monitoring and disturbance sites known to the project team. The sites were selected to be representative of individual pressures (nitrogen deposition, sewage sludge metals or restoration) based on the longevity of treatments, prior knowledge of impacts on soil properties and processes, accessibility of site and permission to sample. The objective was to select sites where there was a good likelihood of impacts to the indicators and hence an opportunity to test the influence of sampling period on the sensitivity of the indicators.

Hartwood Sludge Metals site. This site is located on fertile grassland and heavy, poorly drained gleys at the Hartwood Research Station in North Lanarkshire, Scotland (http://www.hutton.ac.uk/about/facilities/hartwood). The trial is part of the UK Long Term Sewage Sludge Experiment which commenced in 1994 to examine the interaction between sewage sludge and metal concentrations on soil fertility and agricultural productivity. The experimental treatments included; three naturally contaminated metal-rich sludge cakes, with relatively high zinc, copper or cadmium concentrations relative to other metals; long-term build-up treatments where the same sludge cakes were added at a low rate over several years to gradually increase the metal concentrations in soil; and no sludge and uncontaminated sludge cakes as control treatments. Each treatment was applied to four replicated plots (6 m x 8 m). This project sampled the high zinc (Zn450; 450 mg / kg soil target) and the long-term zinc (LTZn, 116 kg Zn ha^{-1} y^{-1}) treatments along with the no sludge and uncontaminated digested sludge plots (blank). The soil mean (s.e.) concentrations of Zn at the time of sampling were the following for each treatment (MLURI, unpublished data); control 81.04 (3.43), digested sludge 103.55 (4.95), Zn450 473.58 (35.92), LTZn 161.18 (4.98). The Zn450 levels exceed current statutory limits for UK agricultural soils (MAFF, 1989). Permission to sample this site was granted by the UK Sewage Sludge Steering Group.

Pwllpeiran Tir Emrys Nitrogen Additions experiment. This nitrogen addition experimental site is located in mid-Wales on species-poor upland acid grassland with peaty podzols. The experiment was initiated in 1995 across 'light' grazed and 'heavy' grazed paddocks to determine the interaction between grazing pressure and nitrogen deposition on the re-establishment of dwarf shrubs and plant species richness. Research has been carried

out over several years on nitrogen fluxes and critical chemical values in soils, vegetation and waters, which are indicative of changes in plant species performance. This experiment involved fortnightly additions of ammonium sulphate (10 and 20 kg N ha^{-1} yr^{-1}; AS10 and AS20 respectively) and sodium nitrate (20 N ha^{-1} yr^{-1}; SN20) to replicated plots (3 per treatment; 3 x 3 m) with water only controls (C). Permission to sample the experimental plots was granted by the NERC.

Sutton Courtenay restoration gradient. This site is located adjacent to Didcot power station, near to Sutton Courtenay, in Oxfordshire, England. It has been used for mineral extraction for several decades with subsequent use as a landfill site and associated land reclamation activities as the site has been re-filled. As a disturbed site, there is a heterogeneous mix of man-made soils. A grassland gradient of restoration ages (4 y, 13 y, +20 y) was identified at the site with undisturbed benchmark grasslands (B) adjacent to the site. Four sampling locations were identified along transects aligned to the restoration gradient. Permission to sample the site was granted from the site contractor.

5.1.2 Field sampling and initial processing

Soils were sampled from experimental or transect plots bi-monthly over 12 months. Each partner in the consortium was responsible for field sampling at their specified site and for the distribution of soil samples to the relevant partners for laboratory analyses; Hartwood (Macaulay), Pwllpeiran (CEH) and Sutton Courtenay (Cranfield University).

A standard sampling protocol was followed at each site (the full protocol is available in Appendix A). In summary the following were taken from each treatment plot; two cores (4 cm diam by 8 cm depth) for nematode and microarthropod analyses and one ca. 250 g bulk soil sample for microbial and enzyme analyses. All samples were transported in cold boxes and stored at 4^{o}C prior to distribution and processing. On arrival at each laboratory, core samples were couriered in cold boxes to CEH Lancaster for extraction. Each laboratory sieved (<2 mm) the individual bulk soil samples and determined soil moisture on all samples. Sieved soils were then divided into ¼ of the sample for Macaulay (minimum 25g) and ¾ for Cranfield (minimum 150g). These soils were then couriered to Cranfield and Macaulay respectively.

5.1.3 Laboratory analyses

Individual partners were responsible for specified analyses on all soil samples. Detailed protocols for each method are provided in Appendix A. Table 5-1 lists the relevant Appendix for each method and the laboratory associated with carrying out each method. A fluorometric multi-enzyme assay and MSIR by MicroResp™ were applied to determine sensitivity of soil biological processes. Eight enzymes were used in the enzyme assay to reflect the potential mineralization of organic and inorganic substrates. Seven substrates and a water control were used in the MicroResp™ method to assess the respiration of carbohydrates, amides and acids.

PLFA and TRFLP were applied to characterise soil microbial community structure including individual analyses of bacterial, fungal and archaeal communities. Up to 47 individual PLFAs were used to characterise and quantify bacterial and fungal components

of the soil microbial community. 16S, ITS, & 16S rRNA primers were used in the TRFLP method to identify bacteria, fungi and archaea, respectively. Wet and dry extractions of soil samples, followed by microscope identification, were applied to enumerate and characterise soil microarthropods and nematodes. Mites and collembola were identified to order and family level respectively while nematodes were identified to functional group level.

The process-based methods were completed first followed by the microbial methods and finally the invertebrate methods. In each instance, the methods were primarily carried out by one experienced scientist with good laboratory skills and prior experience of the individual methods. The difference in completion time reflects a faster capacity to analyse the process and microbial methods through a greater reliance on automation plus a necessity to complete process measures rapidly after field sampling. The identification component of the invertebrate protocols requires relatively more staff time. DNA based identification methods for invertebrates could reduce this time to at least equivalent to that required for microbial DNA based methods. Soil DNA extraction offers the potential to incorporate identification of invertebrate and microbial groups into a single method. Quantification would need to be resolved to make this equivalent to current microscopic approaches which quantify as well as identify. Q-PCR (quantified PCR) has recently been developed for soil nematodes in UK (Neilson *et al.*, 2009) but there is currently no equivalent for other important UK groups including mites, collembola or earthworms.

Table 5-1 Laboratory responsibilities and appendix source for laboratory methods used within the sensitivity trial

Methods	Laboratory	Appendix
Multi-enzyme assay	CRANFIELD	A1
MicroResp™	CRANFIELD	A2
TRFLP	MLURI	A3
PLFA	MLURI	A4
Dry extract - microarthropods	CEH	A5
Wet extract - nematodes	CEH	A6

The data outputs from each method were analysed to produce a range of measures for each method. Table 5-2 summaries these into three groups for each method; individual measures, indices or ratios from two or more individual measures and multivariate measures generated by combining several measures.

Table 5-2 Measures derived from the individual laboratory methods within the sensitivity trial. PCA = principal components analysis.

Indicator Methods	**Individual measures (univariate)**	**Indices or ratios of measures (univariate)**	**multivariate measures**
Microplate fluorometric multi-enzyme assay	response rates of individual enzymes	n/a	PCA with mean values for PC axes
Multiple substrate induced respiration by MicroResp™	respiration rates from individual substrates	n/a	PCA with mean values for PC axes
Multiplex TRFLP for bacteria (16S), fungi (ITS) and archaea (16S rna)	Relative abundance of terminal restriction fragments (TRFs) for bacteria, fungi and archaea. *These are not used individually*.	diversity indices*	PCA with mean values for PC axes for bacteria, fungi and archaea
PLFA profiles by GC analysis	abundance and relative abundance of microbial groups (bacteria, fungi, gram positive bacteria, gram negative bacteria, actinomycetes etc); total microbial biomass (PLFA abundance)	fungal / bacterial ratio; gram +/ gram - ratio; diversity indices*	PCA with mean values for PC axes
Tullgren funnel dry extractions	abundance and relative abundance of invertebrate, mites and collembola taxonomic groups	mite / collembola ratios, diversity indices*	PCA with mean values for PC axes
Modified Baermann funnel wet extractions	abundance and relative abundance of feeding groups (plant, omnivores, fungal, predators), total nematode abundance	fungal / bacterial feeding ratio	PCA with mean values for PC axes

* Shannon Weiner H', Shannon Weiner Eveness, MacIntosh Evenness. These indices are not widely applied with TRF data since their ecological significance is largely unknown in this instance. They were applied here to support comparison with multivariate analyses and between ratio/index measures across methods.

5.1.4 Data management and statistical analyses

A standard procedure for data management was established for the project and followed by each laboratory. Final datasets, with relevant metadata, were compiled by each laboratory to a common format within a MS Excel file which was subsequently submitted to the project data manager. Final data and metadata were entered into the project database which was developed in MS Access. These data were then used for statistical analyses and production of graphs in a unified approach. Repeated measures analysis of variance (RMANOVA) was carried out to determine the effects of the pressures, sampling period and the interaction between pressures and sampling period on the measures derived from the methods at each site. Data transformations were carried out where required to achieve normality. Typically this included log transformation (loge+1 or ln+1) for abundance data. Certain measures, notably for microarthropods, were excluded from statistical analyses where they did not conform to normality, even under data transformation. Data analyses were carried out in STATISTICA v8.0 (Statsoft, 2008). A standardised worksheet-based approach was developed in MS Excel to capture and summarise the statistical results from all measures from each of the methods at each site in a directly comparable manner. There is one file for each method for each site with a separate worksheet for each measure. This worksheet captures the RMANOVA analysis, the summary statistics for the measure and graphs of means + standard errors for the pressure (treatment or TMT), sampling period (TIME) and the interaction between the two (TMTxTIME). Figure 5-1 presents an example of a worksheet layout using the results for the enzyme sulphatase at the Sutton Courtenay restoration site.

Figure 5-1 An example of a worksheet layout which captures the statistical analyses of individual measures for each indicator method. This example shows the results for the enzyme sulphatase at the Sutton Courtenay restoration site.

Sutton Courtney

PROPERTY: Sulphatase Units: nmol MUB g-1 soil h-1

FROM STA:

	TMT	TIME	MEAN	SE
1	BCH	S1_T1	120	6
2	BCH	S1_T2	79	14
3	BCH	S1_T3	119	8
4	BCH	S1_T4	125	11
5	BCH	S1_T5	136	28
6	BCH	S1_T6	139	11
7	4Y	S1_T1	76	6
8	4Y	S1_T2	67	14
9	4Y	S1_T3	88	8
10	4Y	S1_T4	89	11
11	4Y	S1_T5	123	28
12	4Y	S1_T6	83	11
13	13Y	S1_T1	92	6
14	13Y	S1_T2	63	14
15	13Y	S1_T3	69	8
16	13Y	S1_T4	111	11
17	13Y	S1_T5	97	28
18	13Y	S1_T6	184	11
19	20Y	S1_T1	74	6
20	20Y	S1_T2	56	14
21	20Y	S1_T3	64	8
22	20Y	S1_T4	92	11
23	20Y	S1_T5	115	28
24	20Y	S1_T6	119	11

FROM STA:

TMT	MEAN	SE
BCH	120	6
4Y	88	6
13Y	103	6
20Y	87	6

FROM STA:

	MEAN	SE
06_07	91	3
06_09	66	7
06_11	85	4
07_01	104	5
07_03	118	14
07_05	131	5

FROM STA:

	SS	gr. of Freed	MS	F	p
Intercept	708253	1	708253	1193.7174	0.000000
TMT	12820	3	4273	7.2023	0.011610
Error	4747	8	593		
TIME	32928	5	6586	9.7771	0.000004
TIME*TMT	18547	15	1236	1.8357	0.063370
Error	26943	40	674		

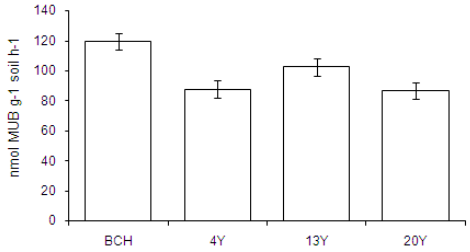
MEAN	06_07	06_09	06_11	07_01	07_03	07_05
BCH	120	79	119	125	136	139
4Y	76	67	88	89	123	139
13Y	92	63	69	111	97	184
20Y	74	56	64	92	115	119
SE						
BCH	6	14	8	11	28	11
4Y	6	14	8	11	28	11
13Y	6	14	8	11	28	11
20Y	6	14	8	11	28	11
TMT	MEAN	SE		TxT SE	15.0	
BCH	120	6				
4Y	88	6		G_MN	99	
13Y	103	6		CV	26	
20Y	87	6				

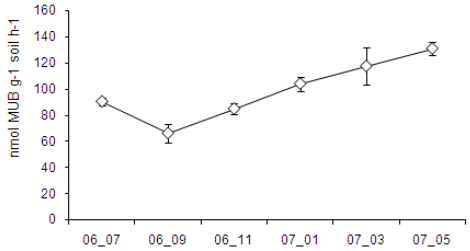
Sutton Courtney

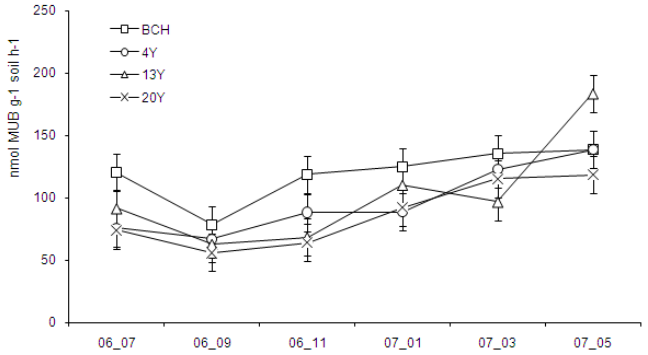
Sulphatase

Range: 128 = 129% CV: 26%

TMT 0.0116 *
TIME 0.0000 ***
TIME x TMT 0.0634







5.2 Results from the sensitivity trial

The following summarises the statistical results for each indicator method and then brings the results from all methods together. P-values are presented in a series of Tables as a summary of the statistical analyses for each measure from each method at each site. Where shown in red and bold, the P-values indicate where there were significant effects on the measure of the pressure (treatment), sampling period (time) or the interaction between the two i.e. where the effect of treatment alters with sampling period.

A hierarchy of simple principles has been adopted in the interpretation of these results.

(i) A measure can be considered a sensitive pressure indicator where there is a significant treatment effect and no effect of either time or treatment x time interaction. Inherent variability does not affect sensitivity to the pressure. Reliability of the indicator would then be dependent on an interpretable response to the pressure.

(ii) A measure can be considered a sensitive pressure indicator where there is a significant treatment effect and a significant time effect, *as long as there is not a significant interaction effect*. Inherent variability does not alter sensitivity to the pressure. Reliability of the indicator would be dependent on an interpretable response to the pressure.

(iii) A measure cannot be considered a reliable indicator of a pressure if there is no statistically significant treatment effect even if there is a significant interaction effect, or where there is only a significant interaction effect. The measure is either not responsive to the pressure or the inherent variability masks sensitivity to the pressure.

5.2.1 Multi-enzyme assay

P-values for the statistical analyses of the enzyme assay at each site are presented in Table 5-3. These results demonstrate that both individual and combined PCA enzyme measures are significantly and overwhelming influenced by sampling period irrespective of pressure. In all but one instance, there was a highly significant effect ($P<0.005$) of sampling period on the enzyme measures. The individual enzymes did not demonstrate any sensitivity to nitrogen deposition at the Pwllpeiran site. The multivariate PC analysis of the enzymes did not produce any significant pressure effects.

There were only three instances where individual enzymes demonstrated a significant response to the pressures. Glucosaminidase was sensitive to the pressures at the sewage sludge metal site (Hartwood). Figure 5-2 illustrates the response of glucosaminidase to treatment, sampling period and their interactions at this site. The mean treatment responses for this enzyme (Figure 5-2a) demonstrate that the enzyme response was lowered where sludge was applied, with or without metal additions.

Therefore, there was no clear response of glucosaminidase to elevated metal concentrations in soil.

Sulphatase was responsible for the two remaining significant pressure effects. These occurred under the sewage sludge metal treatments and restoration treatments. Sulphatase responses were elevated under the Zn450 (high soil zinc concentrations), Figure 5-2a, and consistently lower than the benchmark in the restoration treatments, Figure 5-3a. Sulphatase also demonstrated significant variability over the sampling period in these instances (Figure 5-3b and Figure 5-4b). Although the interaction between treatment and time was not statistically significant, Figure 5-3c and Figure 5-4c clearly demonstrate that the time of sampling would influence the interpretation of any response. In this instance, and for the majority of the other enzymes, the clearest distinction between treatments occurred at the last sampling in May.

Summary of sensitivity in the multi-enzyme assay.

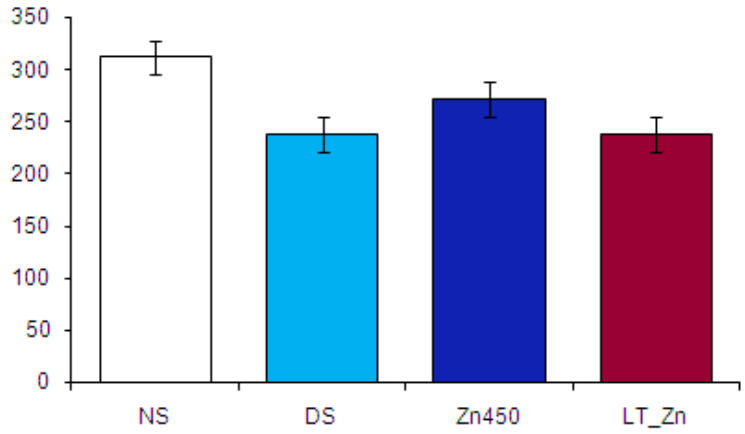
- Sampling period was a significant factor for all eight enzymes and the multivariate analyses of the combined enzymes. As a consequence it was impossible, in most instances, to determine whether or not the enzymes were sensitive to treatments but this was masked by the variability.
- Sulphatase and glucosimindase were the only two enzymes sensitive to any of the pressures.
- In this instance, the use of a multi-enzyme assay did not add value to the results since too few enzymes were sensitive to the pressures.
- Results across the sampling period suggest that Spring may be the optimal sampling window to achieve the greatest sensitivity in the multiple enzyme assay.

Table 5-3 MULTI-ENZYME ASSAY. P-value results from repeated measures analysis of variance to test for the significance of treatment, sampling period and interactions between treatment and sampling period on individual indicator measures. P values in red and bold indicate a significant effect.

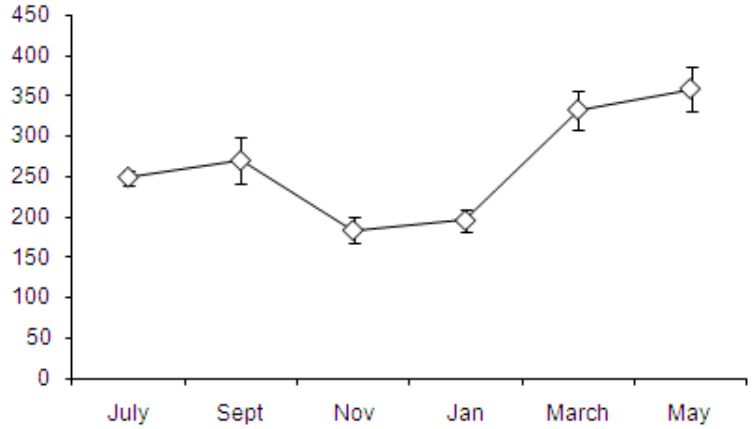
Site	Restoration (Sutton Courtenay)			Sludge metals (Hartwood)			N deposition (Pwllpeiran)			
Measure / Effect	Treatment	Sampling	T X S	Treatment	Sampling	T X S	Treatment	Sampling	T X S	unit
Cellobiohydrolase	0.766	**0.011**	0.056	0.051	**0.000**	0.916	0.423	**0.021**	0.742	nmol MUB g^{-1} soil h^{-1}
Glucosaminidase	0.204	**0.000**	0.132	**0.042**	**0.000**	0.606	0.834	**0.000**	0.815	nmol MUB g^{-1} soil h^{-1}
Glucosidase	0.610	**0.000**	0.218	0.584	**0.000**	0.708	0.762	**0.010**	0.735	nmol MUB g^{-1} soil h^{-1}
Acid phosphatase	0.372	**0.000**	0.204	0.230	**0.000**	0.633	0.656	**0.000**	0.570	nmol MUB g^{-1} soil h^{-1}
Galactosaminidase	0.440	**0.000**	0.142	**0.042**	**0.000**	0.351	0.609	**0.000**	0.486	nmol MUB g^{-1} soil h^{-1}
Xylosidase	0.562	**0.000**	0.457	0.295	**0.000**	0.238	0.722	**0.000**	0.357	nmol MUB g^{-1} soil h^{-1}
Galactosidase	0.723	**0.000**	**0.027**	0.063	**0.016**	0.687	0.492	0.169	0.637	nmol MUB g^{-1} soil h^{-1}
Sulphatase	**0.012**	**0.000**	0.063	**0.037**	**0.000**	0.926	0.695	**0.044**	0.808	nmol MUB g^{-1} soil h^{-1}
ENZ_PC1	0.523	**0.000**	0.117	0.358	**0.000**	0.700	0.812	**0.001**	0.608	n/a
ENZ_PC2	0.343	**0.000**	0.058	0.520	**0.000**	0.568	0.997	**0.000**	0.821	n/a

Figure 5-2 Results for glucosaminidase (nmol MUB g^{-1} soil h^{-1}) from the multi-enzyme assay of soils from the Hartwood sewage sludge metal site. NS = no sludge control; DS = digested sludge only; ZN450 = single Zn treatment to achieve a target soil concentration exceeding current limits; LTZN = repeated intermediate Zn additions to soil.

(a) Treatment means (+/- 1 s.e.)



(b) Time means (+/- 1 s.e.)



(c) Treatment x time interactions (+/- 1 s.e.)

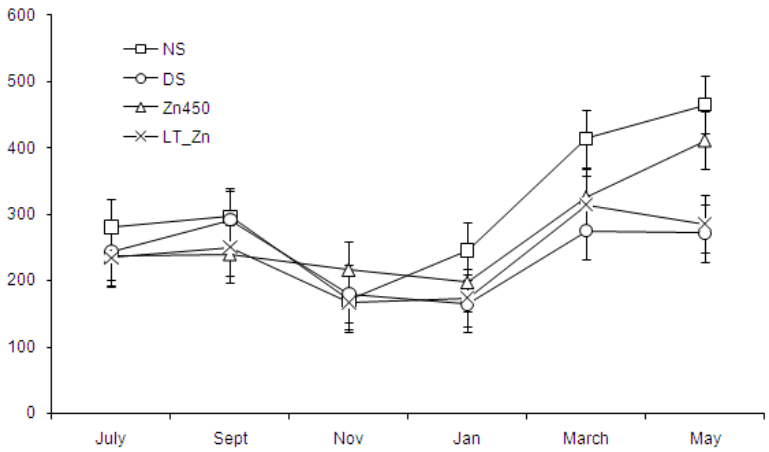
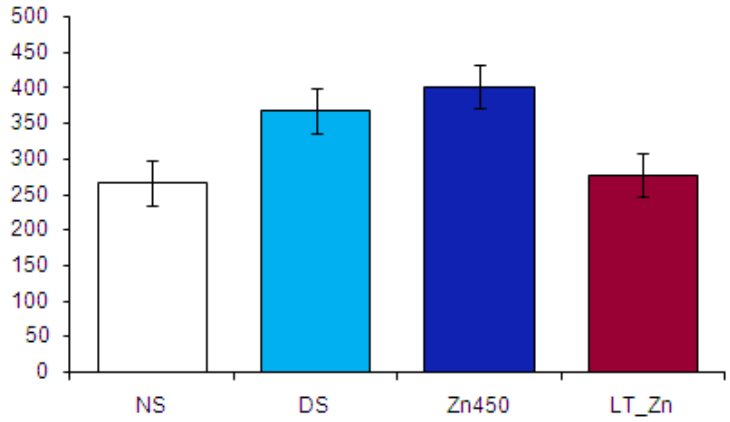
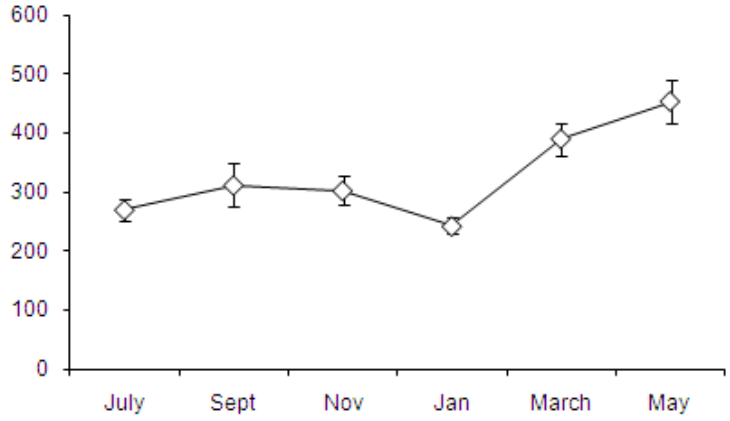


Figure 5-3 Results for sulphatase (nmol MUB g^{-1} soil h^{-1}) from the multi-enzyme assay of soils from the Hartwood sewage sludge metal site. NS = no sludge control; DS = digested sludge only; ZN450 = single Zn treatment to achieve a target soil concentration exceeding current limits; LTZN = repeated intermediate Zn additions to soil.

(a) Treatment means (+/- 1 s.e.)



(b) Time means (+/- 1 s.e.)



(c) Treatment x time interactions (+/- 1 s.e.)

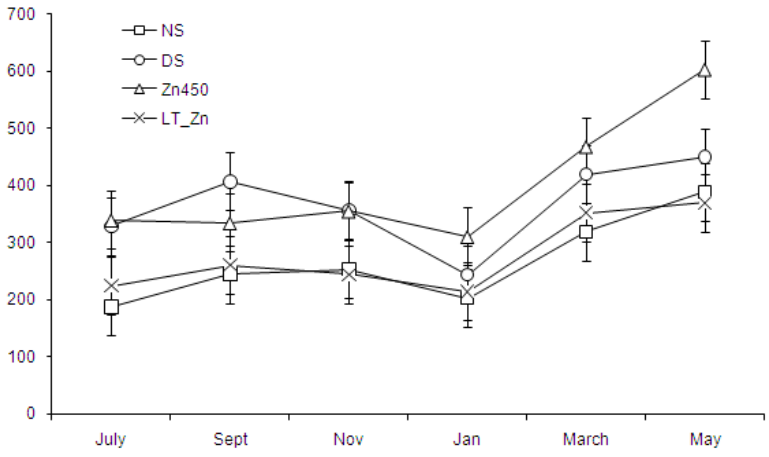
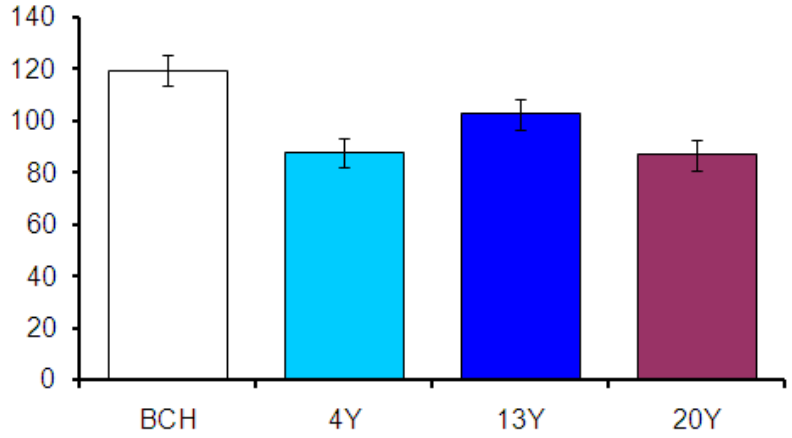
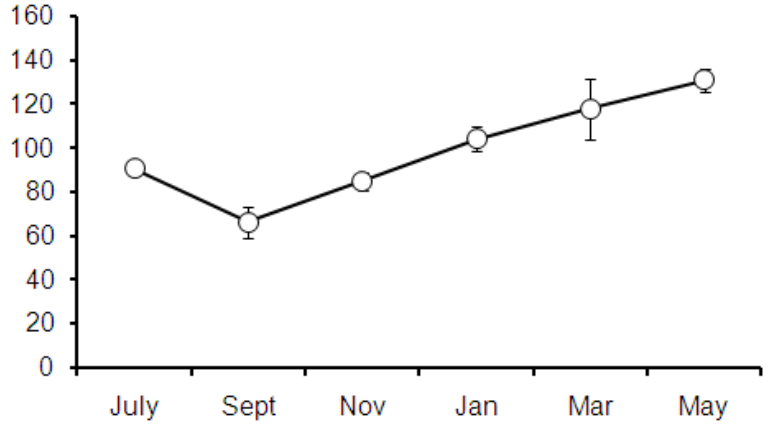


Figure 5-4 Results for sulphatase (nmol MUB g^{-1} soil h^{-1}) from the multi-enzyme assay of soils from the Sutton Courtenay restoration site. BCH = adjacent grassland benchmark site; 4Y, 13Y, 20Y = years after restoration to grassland.

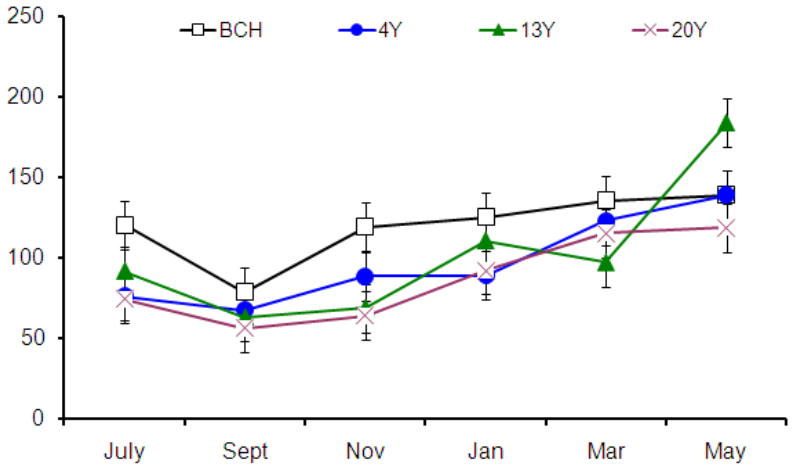
(a) Treatment means (+/- 1 s.e.)



(b) Time means (+/- 1 s.e.)



(c) Treatment x time interactions (+/- 1 s.e.)



5.2.2 Multiple substrate induced respiration (MSIR)

P-values for the statistical analyses of MSIR at each site are presented in Table 5-4. These results demonstrate that both individual and combined MSIR substrates were significantly influenced by sampling period. In all but one instance (AKGA), there was a significant effect ($P<0.005$) of sampling period on the substrates. There was a consistent pattern to the sampling period variability, which reflects a seasonal transition, with a lowering of substrate respiration from summer (July) through autumn (Sept) to lowest respiration in winter (Nov) followed by an increase in respiration at spring (March) with highest activity in May, late Spring.

The individual substrates did not demonstrate any sensitivity to sewage sludge or sludge plus metals at the Hartwood site. At the restoration site, there were significant pressure effects for all of the individual substrates, irrespective of sampling period. In most instances, respiration was lower in the plots under restoration compared to the benchmark. In general, respiration differed in the 4 year and +20 year compared to the 13 year restoration plots. These results are illustrated in Figure 5-5.

At the N deposition site, there were significant pressure effects for all but one substrate (arginine), irrespective of sampling period. Each substrate demonstrated a similar response to the pressures with lower respiration under the nitrogen addition treatments compared to the control, as illustrated in Figure 5-6.

The multivariate PC analysis of the substrates, unlike the enzymes, did produce significant pressure effects for PC1 at the N deposition and restoration sites. In these instances, the effects followed the same responses as those demonstrated for the individual substrates, as illustrated in Figure 5-7 and Figure 5-8. There were no significant pressure effects for the other PC axes.

There were five instances where there was a significant treatment x time interaction and therefore where sampling period would clearly influence any respiration responses. In this instance, the significant pressure responses of basal respiration (water) and glucose derived respiration under restoration were detrimentally influenced by the time x treatment interaction. Examination of the remaining measures demonstrates a similar trend with clearest distinction of pressure effects in the late spring (May) sampling.

Summary of sensitivity in multiple substrate induced respiration:

- Sampling period was a significant factor for all eight carbon substrates and the multivariate analyses of the combined substrates. As a consequence it was impossible to determine whether or not the substrates were sensitive to treatments at the sludge metal sites since variability may have masked any response. However, the variability did not influence the sensitivity of several substrates in the restoration and N deposition sites.

- All carbon substrates and multivariate principal components were sensitive to the restoration treatments and the N deposition treatments, excepting arginine

at the N deposition site. The results were interpretable with respect to the treatments or type of pressures in most cases.

- Variability, temporal (across the sampling dates) and spatial (within the sampling dates), was a significant issue with the water control and glucose substrate at the restoration site and this compromised the observed sensitivity to the treatments.

- The use of multiple substrates did not add obvious value to the results with respect to the individual substrates, in this instance. However, reliance on a single substrate could be risky given the high variability of all substrates. This is particularly relevant to glucose since this substrate is widely used to determine soil basal respiration in a single assay. This trial only examined three sites and the benefits from using multiple substrates may be more obvious under a wider range of soil:land use circumstances.

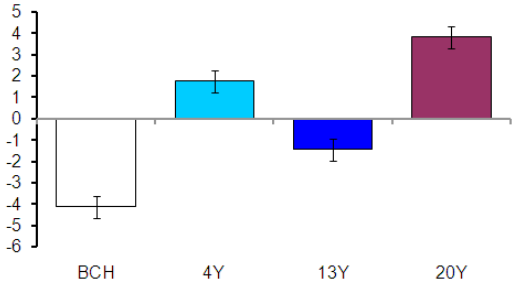
- Results across the sampling period suggest that Spring may be the optimal sampling window.

Table 5-4 MSIR. P-value results from repeated measures analysis of variance to test for the significance of treatment, sampling period and interactions between treatment and sampling period on individual indicator measures. P values in red and bold indicate a significant effect.

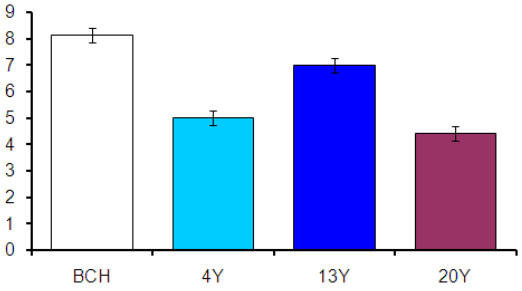
Site	Restoration (Sutton Courtenay)			Sludge metals (Hartwood)			N deposition (Pwllpeiran)			
Measure / Effect	Treatment	Sampling	T X S	Treatment	Sampling	T X S	Treatment	Sampling	T X S	unit
Water	**0.001**	**0.000**	**0.000**	0.197	**0.000**	0.569	**0.003**	**0.000**	0.407	µg CO_2 g^{-1} h^{-1}
Glucose	**0.002**	**0.000**	**0.045**	0.082	**0.000**	0.498	**0.024**	**0.000**	0.588	µg CO_2 g^{-1} h^{-1}
AKGA	**0.000**	0.073	0.945	0.258	**0.000**	0.086	**0.022**	**0.000**	0.558	µg CO_2 g^{-1} h^{-1}
Arginine	**0.002**	**0.000**	0.766	0.677	**0.000**	**0.004**	0.074	**0.000**	0.387	µg CO_2 g^{-1} h^{-1}
Citric acid	**0.000**	**0.016**	0.199	0.325	**0.000**	0.328	**0.017**	**0.000**	0.530	µg CO_2 g^{-1} h^{-1}
GABA	**0.004**	**0.000**	0.065	0.082	**0.000**	0.498	**0.018**	**0.000**	0.639	µg CO_2 g^{-1} h^{-1}
Malic acid	**0.000**	**0.009**	0.742	0.533	**0.000**	**0.027**	**0.013**	**0.000**	0.312	µg CO_2 g^{-1} h^{-1}
NAGA	**0.007**	**0.000**	0.207	0.278	**0.000**	0.437	**0.023**	**0.000**	0.411	µg CO_2 g^{-1} h^{-1}
MSIR_PC1	**0.000**	**0.007**	0.594	0.300	**0.000**	0.074	**0.011**	**0.000**	0.489	n/a
MSIR_PC2	0.362	**0.000**	0.837	0.185	**0.000**	**0.036**	0.815	**0.000**	0.105	n/a

Figure 5-5 Treatment means (µg CO2 g^{-1} h^{-1} +/- 1 s.e.) for individual carbon substrates from MicroResp™ analyses of soils from the Sutton Courtenay restoration site. BCH = adjacent grassland benchmark site; 4Y, 13Y, 20Y = years after restoration.

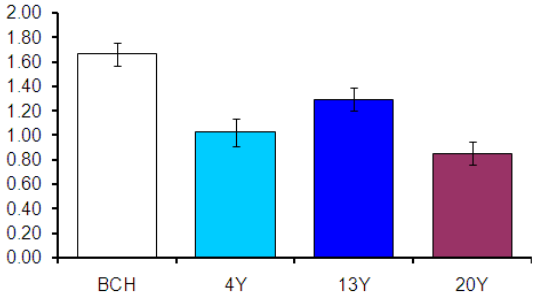
(a) Arginine



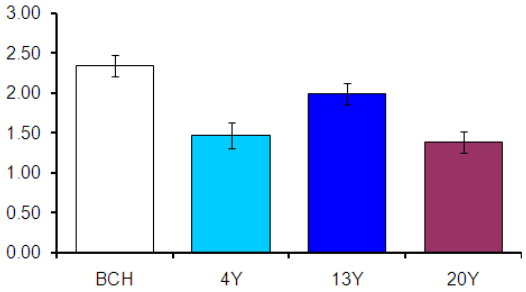
(b) Malic acid



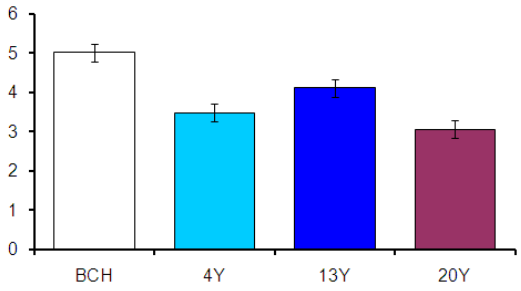
(c) GABA



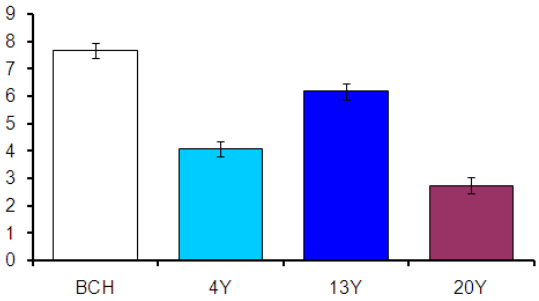
(d) NAGA



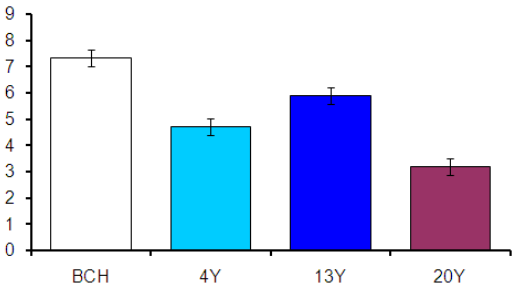
(e) Glucose



(f) a-ketoglutarate



(g) Citric acid



(h) Water

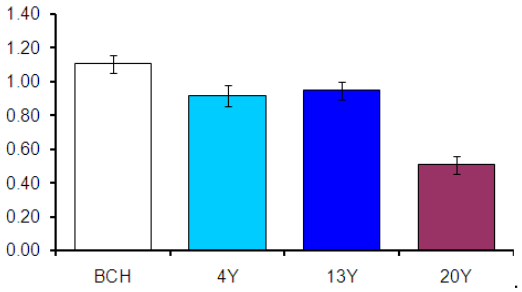
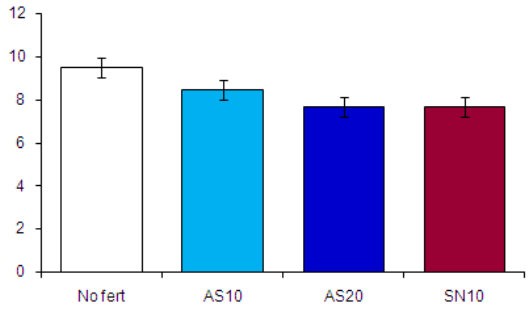
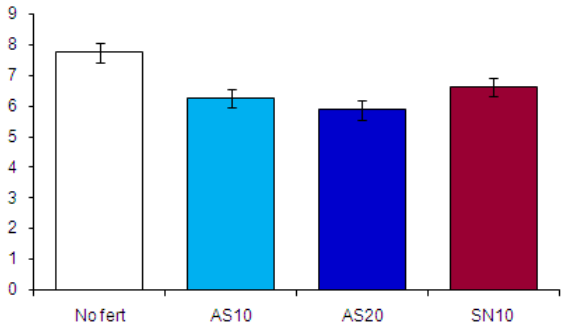


Figure 5-6 Treatment means (µg CO2 g^{-1} h^{-1} +/- 1 s.e.) for individual carbon substrates from MicroResp™ analyses of soils from the Pwllpeiran N deposition site. No fert = water only; AS10 = ammonium sulphate 10 kg ha yr^{-1}; AS20 = ammonium sulphate 20 kg ha yr^{-1}, SN10 = sodium nitrate 10 kg ha yr^{-1}.

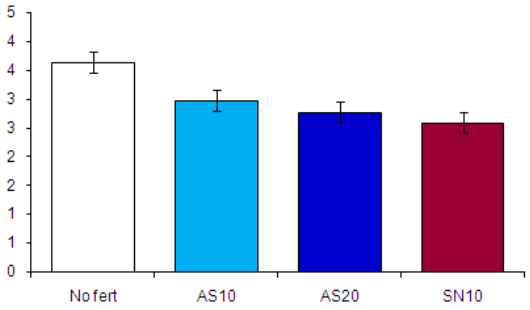
(a) Arginine



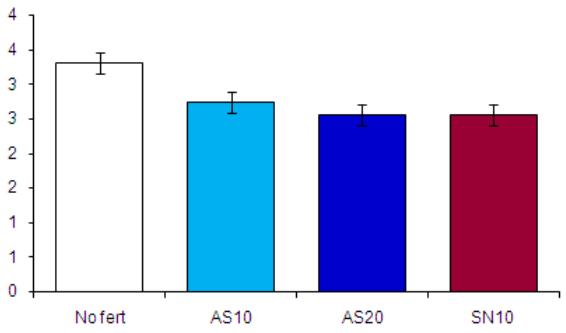
(b) Malic acid



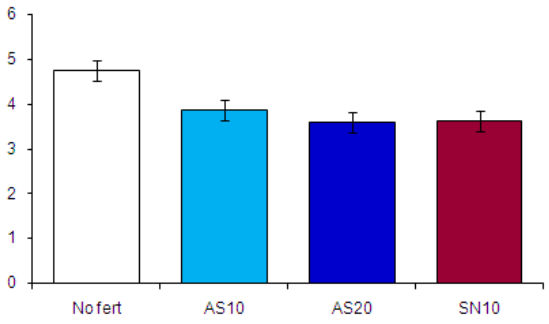
(c) GABA



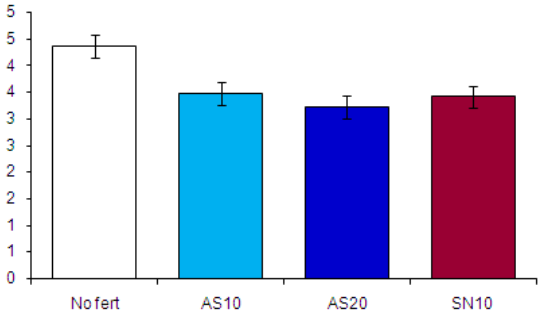
(d) NAGA



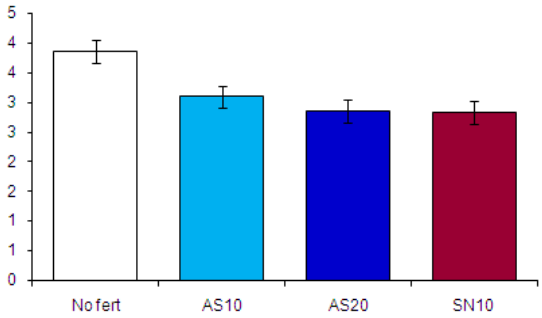
(e) Glucose



(f) AKGA



(g) Citric acid



(h) Water

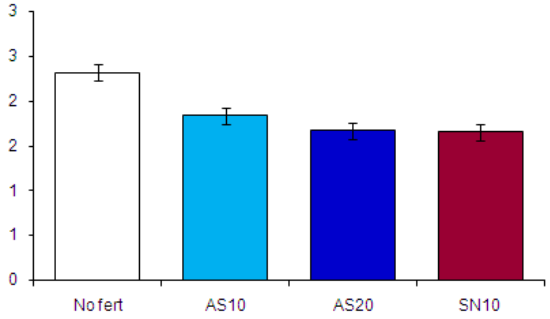
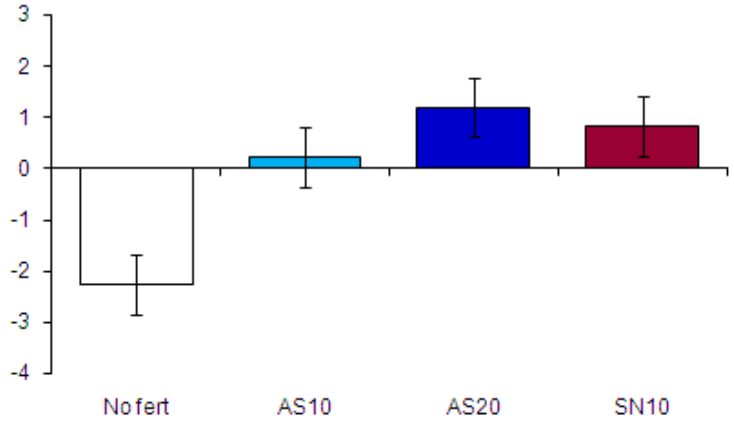
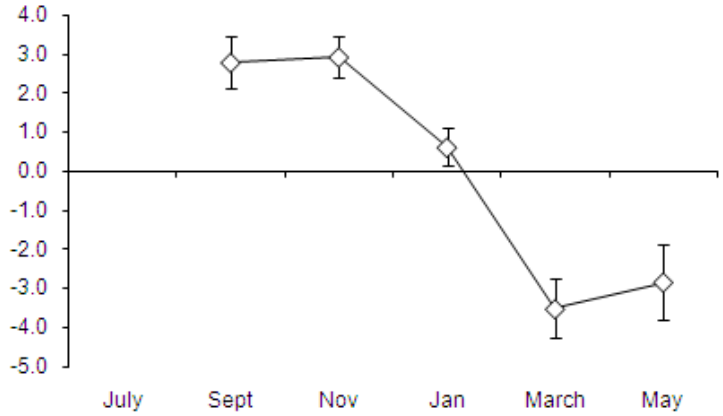


Figure 5-7 Results for multivariate analyses of MSIR for soils from the Pwllpeiran N deposition site. No fert = water only; AS10 = ammonium sulphate 10 kg ha yr^{-1}; AS20 = ammonium sulphate 20 kg ha yr^{-1}, SN10 = sodium nitrate 10 kg ha yr^{-1}.

(a) Treatment means for PC1 axis of PCA (+/- 1 s.e.)



(b) Time means for PC1 axis of PCA (+/- 1 s.e.)



(c) Treatment x time interactions for PC1 axis of PCA (+/- 1 s.e.)

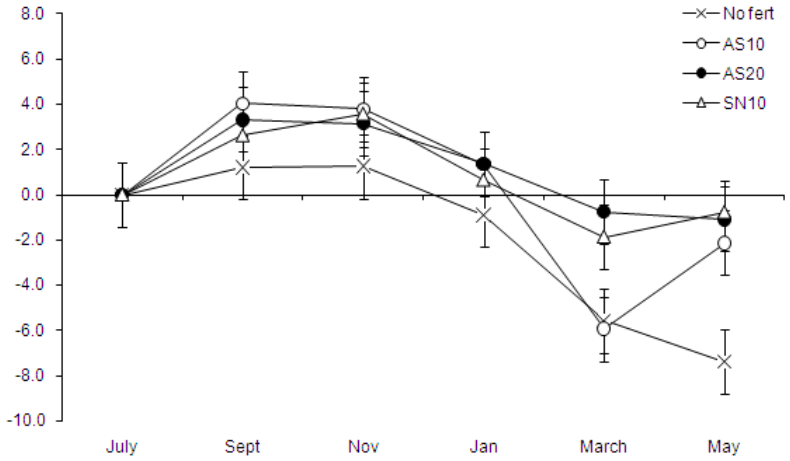
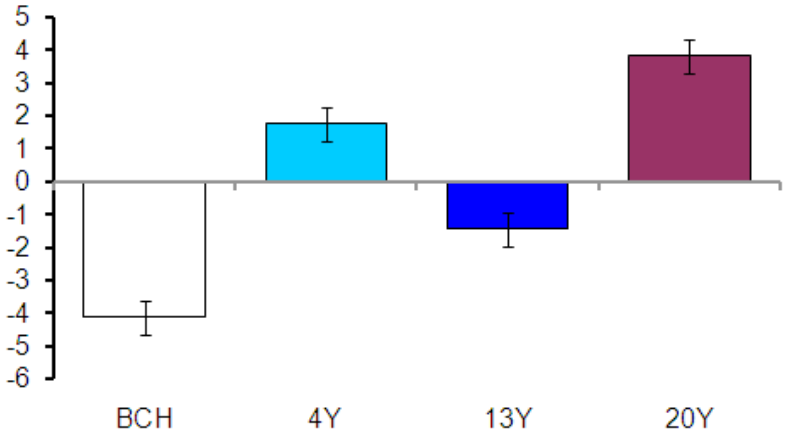
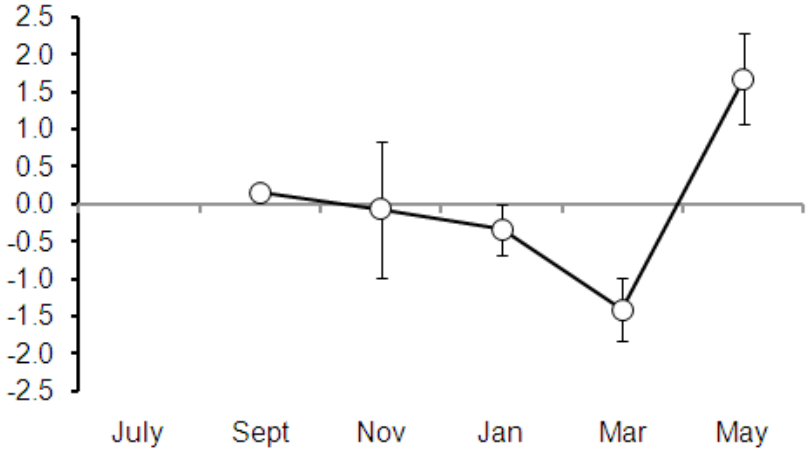


Figure 5-8 Results for multivariate analyses of MSIR for soils from the Sutton Courtenay restoration site. BCH = adjacent grassland benchmark site; 4Y, 13Y, 20Y = years after restoration to grassland.

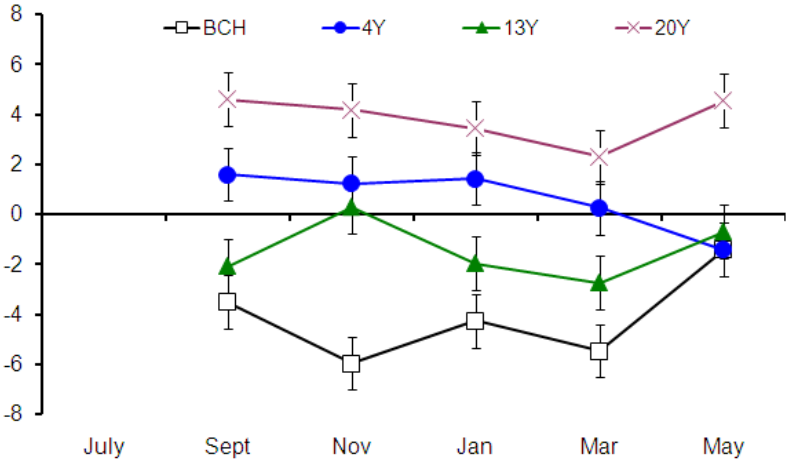
(a) Treatment means for PC1 axis of PCA (+/- 1 s.e.)



(b) Time means for PC1 axis of PCA (+/- 1 s.e.)



(c) Treatment x time interactions for PC1 axis of PCA (+/- 1 s.e.)



5.2.3 TRFLP

P-values for the statistical analyses of bacterial, fungal and archaeal TRFs at each site are presented in Table 5-5. These results demonstrate that both individual and multivariate combinations of microbial TRFs are not greatly influenced by sampling period, although trends are demonstrated in the community structure of bacterial, fungal and archaeal groups with sampling period. There were only 10 instances (<20% from 56 analyses) where the sampling period produced a significant effect. There was no consistency in the significance of sampling period between measures across sites or with microbial group.

The microbial measures did not demonstrate sensitivity to N deposition treatments at the Pwllpeiran site. At the restoration site, there were significant pressure effects for archaeal diversity indices and fungal multivariate analyses, irrespective of sampling period. None of these results were compromised by a treatment x time interaction. In all instances, the measures demonstrate differences in archaeal and fungal diversity between the restoration plots and the benchmark site. These results are illustrated for archaeal diversity and fungal diversity in Figure 5-9 and Figure 5-10, respectively. These figures also demonstrate that sampling period could influence the interpretation of the results.

At the sewage sludge site, there were significant pressure effects for bacterial, archaeal and fungal measures; 5 in total. Two archaeal measures with significant pressure effects (Shannon E and McIntosh E) demonstrated highest archaeal diversity in Zn metal treatments, Figure 5-11. Elevated diversity is often considered an indicator of stress in a system but these effects were compromised by a significant treatment x time interaction, as illustrated in Figure 5-11c and d. Diversity indices were lower during the July (Summer) and following May (late Spring) sampling which may reflect seasonal dynamics in archaeal community structure. The bacterial 16 Shannon E diversity index measure (Figure 5-12) and fungal multivariate TRFLP PC1 (Figure 5-13) demonstrated that microbial community structure was significantly altered by sludge applications, irrespective of metal additions.

Summary of sensitivity in soil microbial community structure from TRFLP

- Fungal index and bacterial index and multivariate measures were fairly unresponsive to the pressures across all three sites. One bacterial diversity measure was sensitive to the pressures at the sludge metals site.
- No individual, indices or multivariate measure of microbial community structure demonstrated sensitivity to the pressures at the nitrogen deposition site.
- Fungal multivariate and archaeal diversity measures were sensitive to the pressures at the restoration and sludge metal sites.
- There was little significant effect of sampling period on the measures.

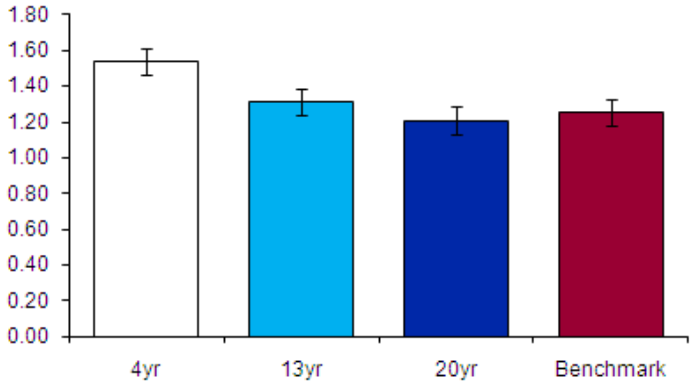
- However further consideration would be required of appropriate sampling periods since seasonal dynamics were demonstrated in the majority of the microbial measures. The July and following May sampling periods generally demonstrated different responses relative to the intervening Autumn and Winter sampling periods.

Table 5-5 TRFLP. P-value results from repeated measures analysis of variance to test for the significance of treatment, sampling period and interactions between treatment and sampling period on individual indicator measures. P values in red and bold indicate a significant effect. TRF = terminal restriction fragment.

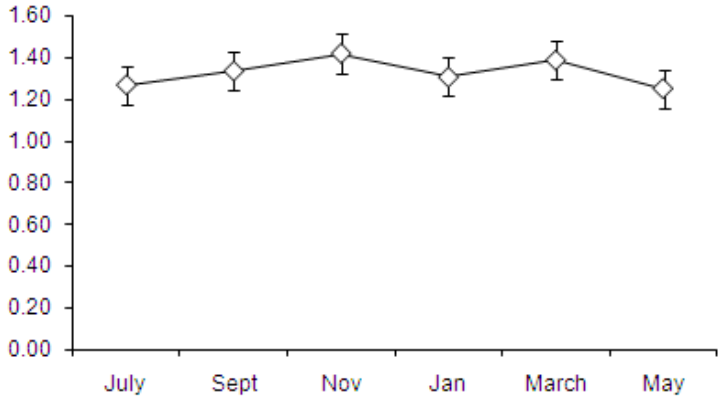
Site	Restoration (Sutton Courtenay)			Sludge metals (Hartwood)			N deposition (Pwllpeiran)			
Measure / Effect	Treatment	Sampling	T X S	Treatment	Sampling	T X S	Treatment	Sampling	T X S	unit
Bacterial 16S Richness	0.129	**0.033**	0.931	0.433	0.443	0.444	0.378	0.106	0.296	TRF
Bacterial 16S Shannon H	0.397	0.076	0.366	0.433	0.142	0.354	0.494	**0.008**	**0.016**	na
Bacterial 16S Shannon E	0.430	0.211	0.120	**0.049**	0.064	0.067	0.971	0.171	0.584	na
Bacterial 16S McIntosh E	0.305	0.202	0.139	0.337	0.057	0.077	0.913	0.168	0.562	na
Fungal ITS Richness	0.249	0.227	0.554	0.591	0.308	0.200	0.771	0.693	0.923	TRF
Fungal ITS Shannon H	0.612	0.458	0.578	0.532	0.869	0.209	0.807	0.705	0.995	na
Fungal ITS Shannon E	0.854	0.783	0.237	0.464	0.511	0.362	0.314	0.952	0.363	na
Fungal ITS McIntosh E	0.836	0.954	0.341	0.246	0.482	0.147	0.529	0.842	0.511	na
Archaeal Richness	**0.015**	0.961	0.615	0.864	0.638	0.224	0.997	0.379	0.982	TRF
Archaeal Shannon H	**0.030**	0.774	0.875	0.870	**0.014**	0.096	0.881	0.142	0.669	na
Archaeal Shannon E	**0.000**	0.567	0.947	**0.004**	0.063	**0.002**	0.347	0.387	0.366	na
Archaeal McIntosh E	**0.000**	0.748	0.960	**0.001**	**0.010**	**0.000**	0.378	0.440	0.351	na
Bacterial 16S PC1	0.309	0.198	0.245	0.960	0.645	0.620	0.324	0.936	0.960	PC axis
Bacterial 16S PC2	0.569	**0.027**	0.951	**0.002**	**0.042**	0.687	0.625	0.106	0.230	PC axis
Fungal ITS PC1	**0.048**	**0.019**	0.551	0.055	0.082	0.104	0.936	0.469	0.694	PC axis
Fungal ITS PC2	**0.010**	0.248	0.913	**0.003**	**0.018**	0.529	0.626	0.461	0.641	PC axis
Archaeal PC1	0.627	**0.001**	0.795	-	-	-	0.310	0.358	0.812	PC axis
Archaeal PC2	0.490	0.943	0.969	-	-	-	0.757	**0.025**	0.648	PC axis

Figure 5-9 Results for analyses of archaeal diversity for soils from the Sutton Courtenay restoration site. BCH = adjacent grassland benchmark site; 4Y, 13Y, 20Y = years after restoration to grassland.

(a) Treatment means for Archaeal Shannon H index (+/- 1 s.e.)



(b) Time means for Archaeal Shannon H index (+/- 1 s.e.)



(c) Treatment x time interactions for Archaeal Shannon H index (+/- 1 s.e.)

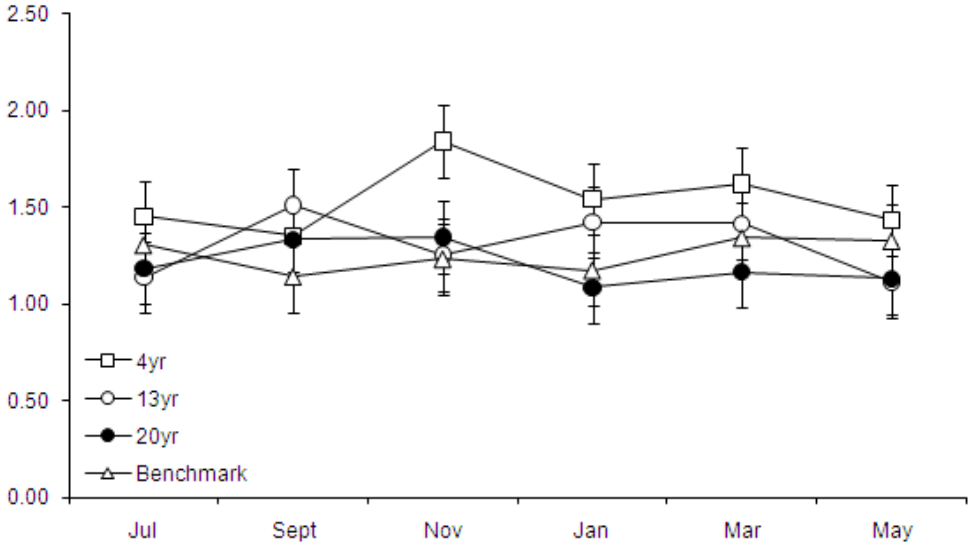
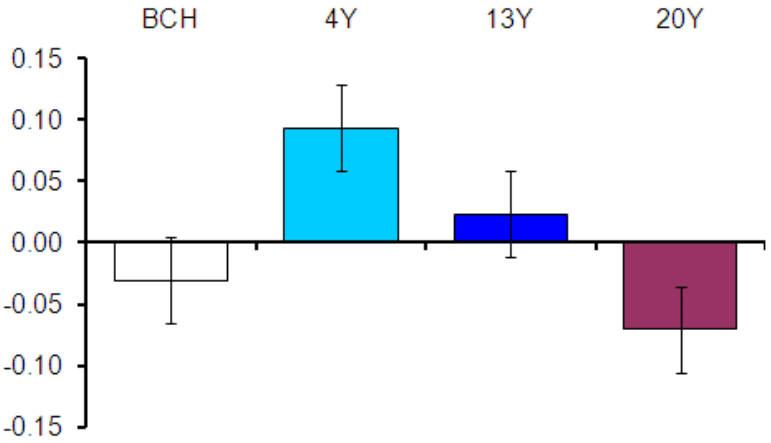
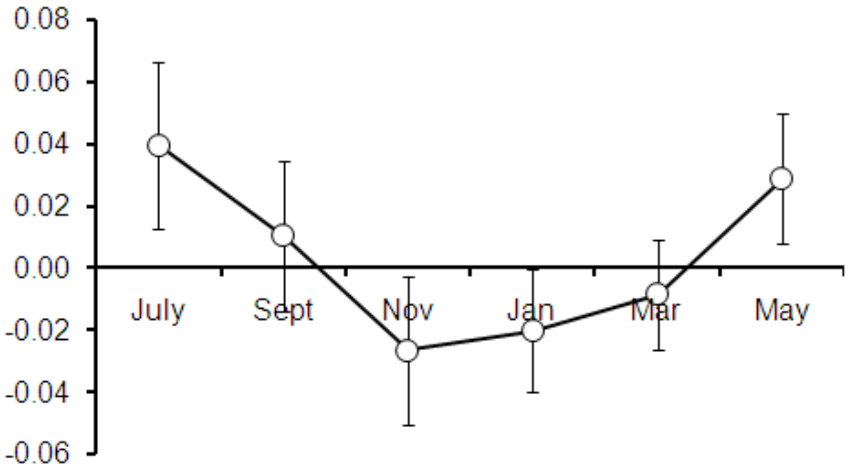


Figure 5-10 Results for multivariate analyses of fungal TRFs for soils from the Sutton Courtenay restoration site. BCH = adjacent grassland benchmark site; 4Y, 13Y, 20Y = years after restoration to grassland.

(a) Treatment means for PC1 axis of PCA (+/- 1 s.e.)



(b) Time means for PC1 axis of PCA (+/- 1 s.e.)



(c) Treatment x time interactions for PC1 axis of PCA (+/- 1 s.e.)

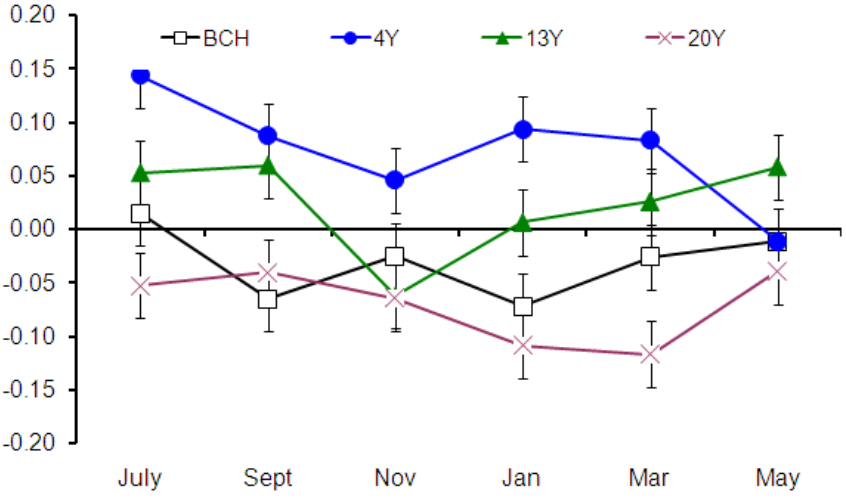
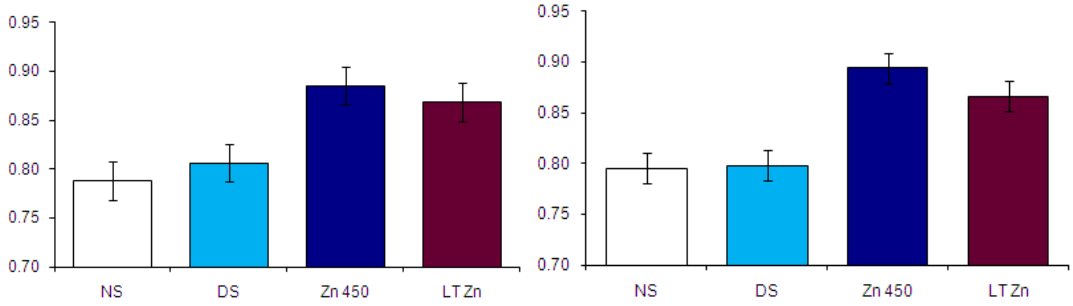
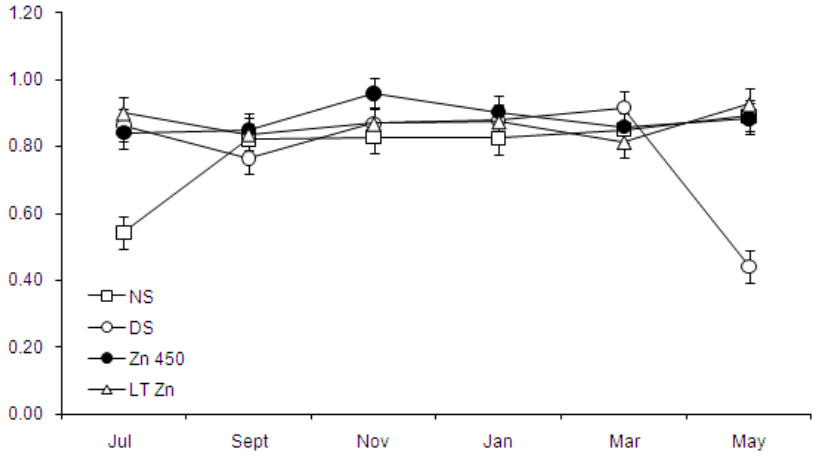


Figure 5-11 Results for analyses of archaeal diversity indices for soils from the Hartwood sewage sludge metal site. NS = no sludge control; DS = digested sludge only; ZN450 = single Zn treatment to achieve a target soil concentration exceeding current limits; LTZN = repeated intermediate Zn additions to soil.

(a) Treatment means for Shannon E (b) Treatment means for McIntosh E



(c) Interaction means for Shannon E



(d) Interaction means for McIntosh E

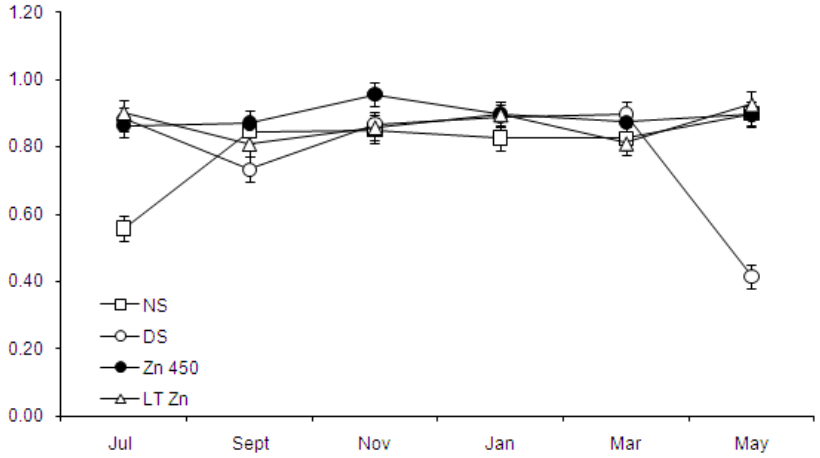
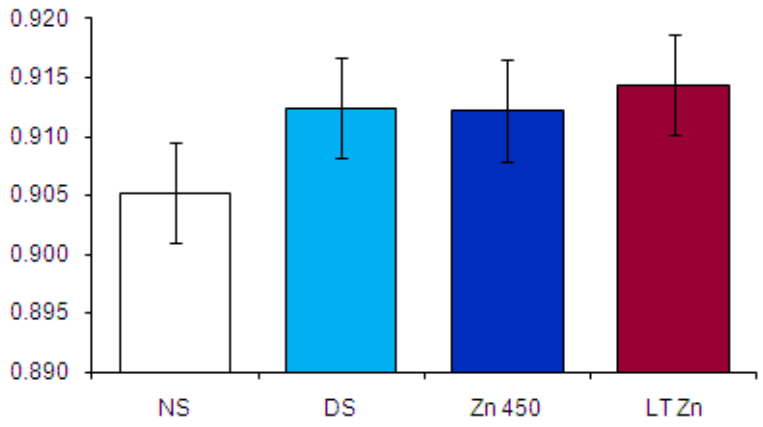
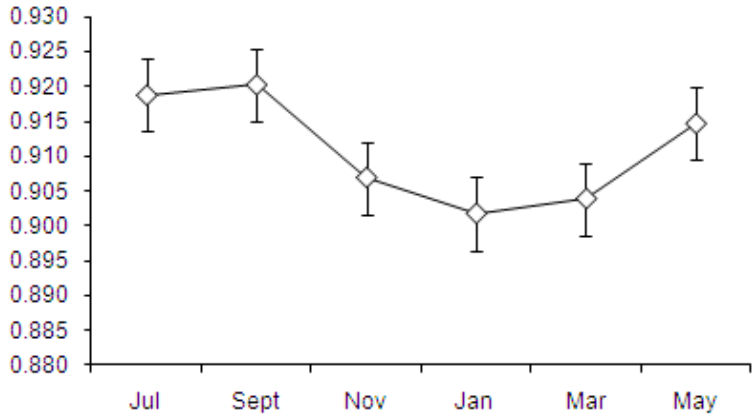


Figure 5-12 Results for bacterial 16S Shannon E diversity index for soils from the Hartwood sewage sludge metal site. NS = no sludge control; DS = digested sludge only; ZN450 = single Zn treatment to achieve a target soil concentration exceeding current limits; LTZN = repeated intermediate Zn additions to soil.

(a) Treatment means (+/- 1 s.e.)



(b) Time means (+/- 1 s.e.)



(c) Treatment x time interactions (+/- 1 s.e.)

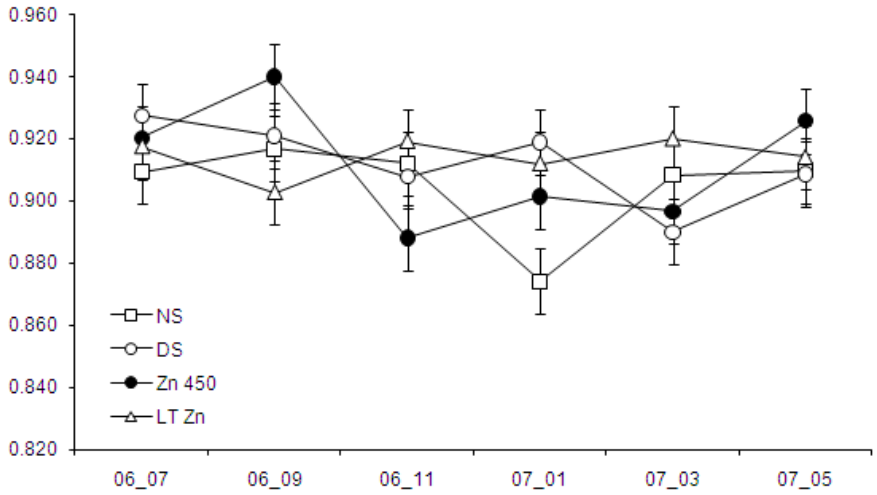
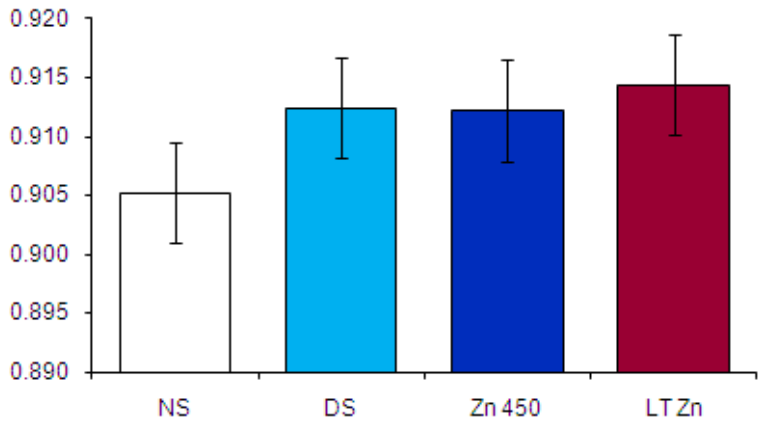
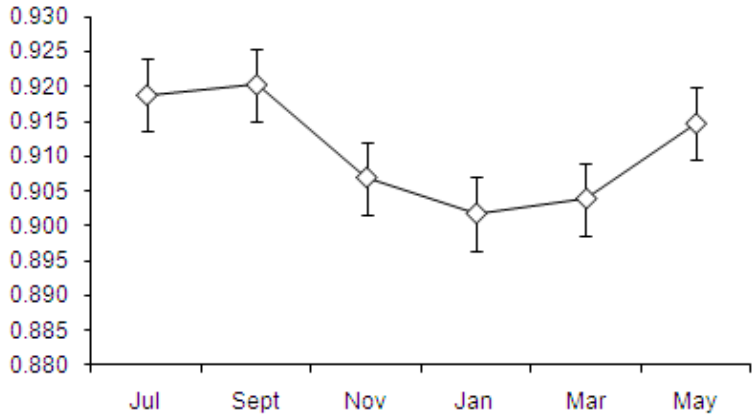


Figure 5-13 Results for fungal multivariate PC1 from the TRFLP analyses of soils from the Hartwood sewage sludge metal site. NS = no sludge control; DS = digested sludge only; ZN450 = single Zn treatment to achieve a target soil concentration exceeding current limits; LTZN = repeated intermediate Zn additions to soil.

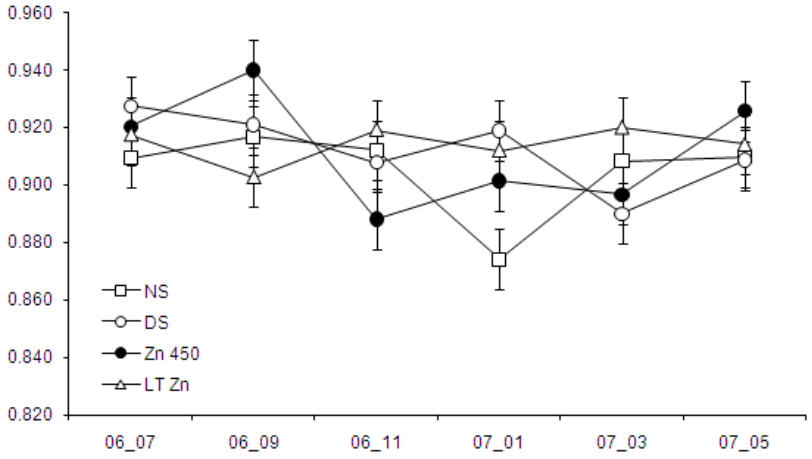
(a) Treatment means (+/- 1 s.e.)



(b) Time means (+/- 1 s.e.)



(c) Treatment x time interactions (+/- 1 s.e.)



5.2.4 PLFAs

P-values for the statistical analyses of microbial PLFAs at each site are presented in Table 5-6. These results demonstrate that total PLFA, which can be used as an estimate of soil microbial biomass, and total bacterial PLFAs were not influenced by any of the pressures. None of the PLFA measures demonstrate significant sensitivity to N deposition treatments at the Pwllpeiran site.

At the restoration site, there were significant pressure effects for fungal/bacterial ratios, actinomycetes, Gram positive/Gram negative bacterial ratios and for the PC analyses of all PLFAs combined. The treatment results for all the measures demonstrate differences in microbial community structure between the restoration plots and the benchmark site. In all but one measure (p/n ratio), microbial community structure differed markedly between the benchmark, and +20Y with the 4Y site. These results are illustrated for Figure 5-14. Sampling period was a significant effect in several measures. Although there were significant effects of sampling period, none of these results were compromised by a treatment x time interaction. Figure 5-16a demonstrates this for fungal/bacterial ratios at the restoration site.

At the sewage sludge site, there were similar significant pressure effects for fungal/bacterial ratios, Gram positive/Gram negative bacterial ratios and the PC analyses of all PLFAs combined, along with additional significant effects for fungal and gram negative PLFAs. The treatment results for all the measures demonstrate differences in microbial community structure between the sludge only, the sludge metal treatments and the no sludge control. These results are illustrated for Figure 5-15. Sampling period was a significant effect in several measures. Although there were significant effects of sampling period, the results were generally not compromised by a treatment x time interaction. Figure 5-16b demonstrates this for fungal/bacterial ratios at the sludge metal site.

Summary of sensitivity in soil microbial community structure from PLFA

- None of the PLFA based microbial community measures demonstrated sensitivity to the pressures at the nitrogen deposition site.
- Total microbial and total bacterial PLFAs measures were unresponsive to the pressures across all three sites.
- Ratios of gram negative/gram positive bacterial PLFAs were sensitive to the pressures at the sludge metals site and restoration site, largely reflecting an effect on gram negative bacteria.
- Fungal/bacterial ratios and PC analyses of all PLFAs were sensitive to the pressures at the restoration and sludge metal sites.
- The significant effects for all of the measures could be interpreted as generally consistent responses to the pressures at the restoration and sludge metal sites.

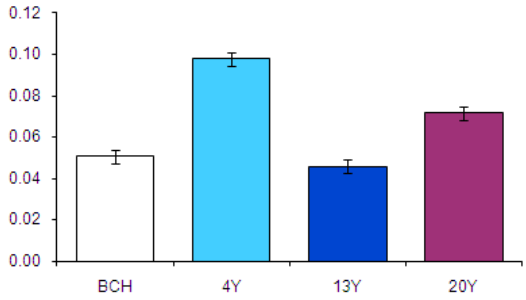
- There were consistent significant effects of sampling period on the measures which demonstrated a typical seasonal dynamic. The latest sampling in May, and often the previous July sampling, demonstrated different responses relative to the intervening Autumn, Winter and early Spring sampling periods. This dynamic did not have a significant influence on the pressure effects and further examination of the interactions means for the measures suggests that restriction of the sampling period may be less of an issue with PLFAs than for other measures.

Table 5-6 PLFA. P-value results from repeated measures analysis of variance to test for the significance of treatment, sampling period and interactions between treatment and sampling period on individual indicator measures. P values in red and bold indicate a significant effect.

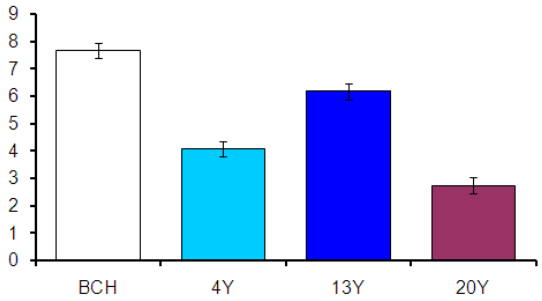
Site	Restoration (Sutton Courtenay)			Sludge metals (Hartwood)			N deposition (Pwllpeiran)			
Measure / Effect	Treatment	Sampling	T X S	Treatment	Sampling	T X S	Treatment	Sampling	T X S	unit
Total PLFA	0.142	0.183	0.272	0.074	0.153	0.895	0.357	0.098	0.647	nmol g^{-1}
Bacteria PLFA (B)	0.106	0.304	0.232	0.086	0.217	0.728	0.315	0.080	0.578	nmol g^{-1}
Fungal PLFA (F)	0.061	**0.037**	0.606	**0.037**	**0.000**	0.901	0.234	**0.011**	0.460	nmol g^{-1}
FB ratio	**0.000**	0.089	0.156	**0.004**	**0.000**	0.508	0.165	**0.009**	0.837	n/a
Actinomycetes	**0.002**	**0.008**	0.126	0.296	**0.002**	0.077	0.545	0.230	0.913	nmol g^{-1}
Gram positive bacteria (P)	0.098	0.324	0.482	0.116	0.317	0.676	0.385	**0.033**	0.370	nmol g^{-1}
Gram negative bacteria (N)	0.108	0.382	0.245	**0.025**	0.097	0.745	0.282	0.134	0.618	nmol g^{-1}
PN ratio	**0.004**	**0.023**	0.313	**0.001**	0.424	0.386	0.847	**0.027**	0.058	n/a
All PLFAs_PC1	**0.001**	0.246	0.215	**0.001**	0.475	0.857	0.618	0.076	0.146	PC axis
All PLFAs_PC2	**0.001**	**0.000**	0.434	**0.009**	0.466	0.333	0.336	0.211	0.662	PC axis
All PLFAs_PC3	**0.026**	**0.001**	0.930	**0.019**	**0.007**	0.098	0.717	0.497	0.984	PC axis

Figure 5-14 Treatment means (nmol g^{-1} +/- 1 s.e.) for individual microbial groups, measure ratios and PCA multivariate analyses of soils from the Sutton Courtenay restoration site. BCH = grassland benchmark site; 4Y, 13Y, 20Y = years after restoration.

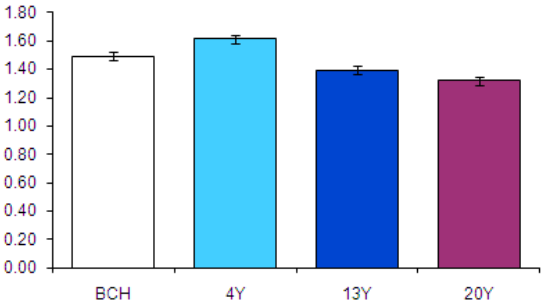
(a) fungal / bacterial ratio



(b) actinomycetes



(c) p/n ratio



(d) PC1

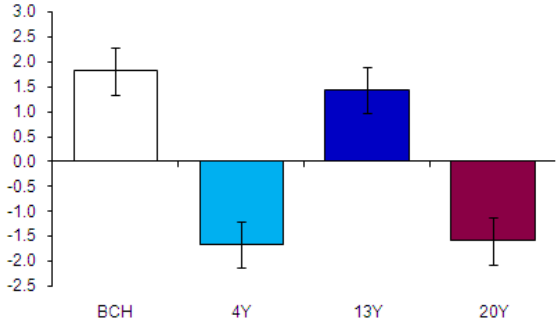
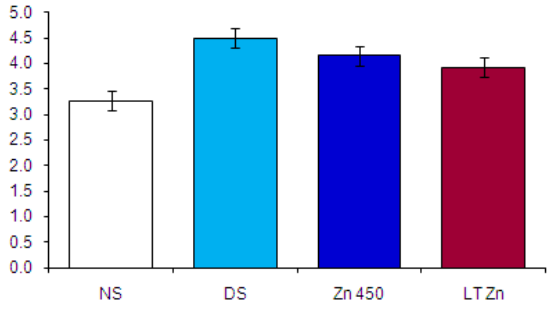
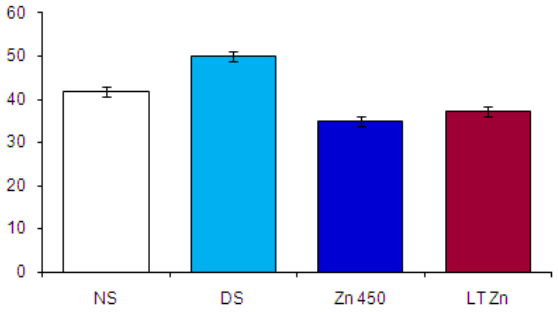


Figure 5-15 Treatment means (nmol g^{-1} +/- 1 s.e.) for individual microbial groups, ratios and PCA multivariate analyses of soil PLFAs from the Hartwood sludge metal site. NS = no sludge control; DS = digested sludge only; ZN450 = single Zn treatment to achieve a target soil concentration exceeding current limits; LTZN = repeated intermediate Zn additions to soil.

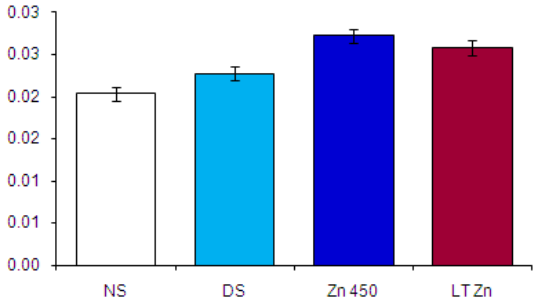
(a) fungal PLFA



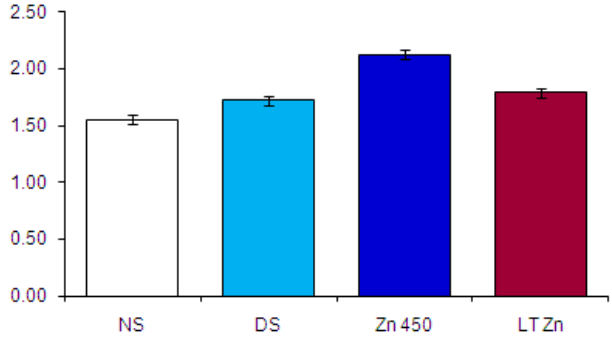
(b) gram negative bacteria



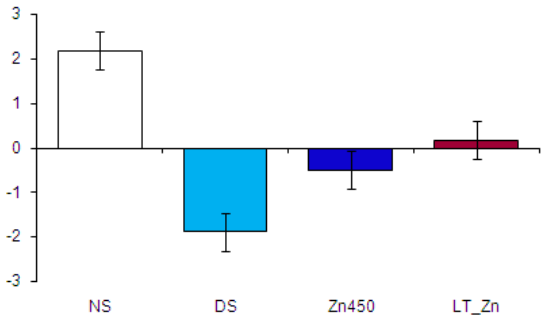
(c) fungal / bacterial ratio



(d) p/n ratio



(e) PC1



(f) PC2

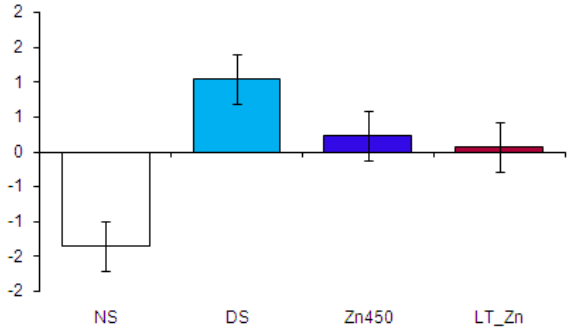
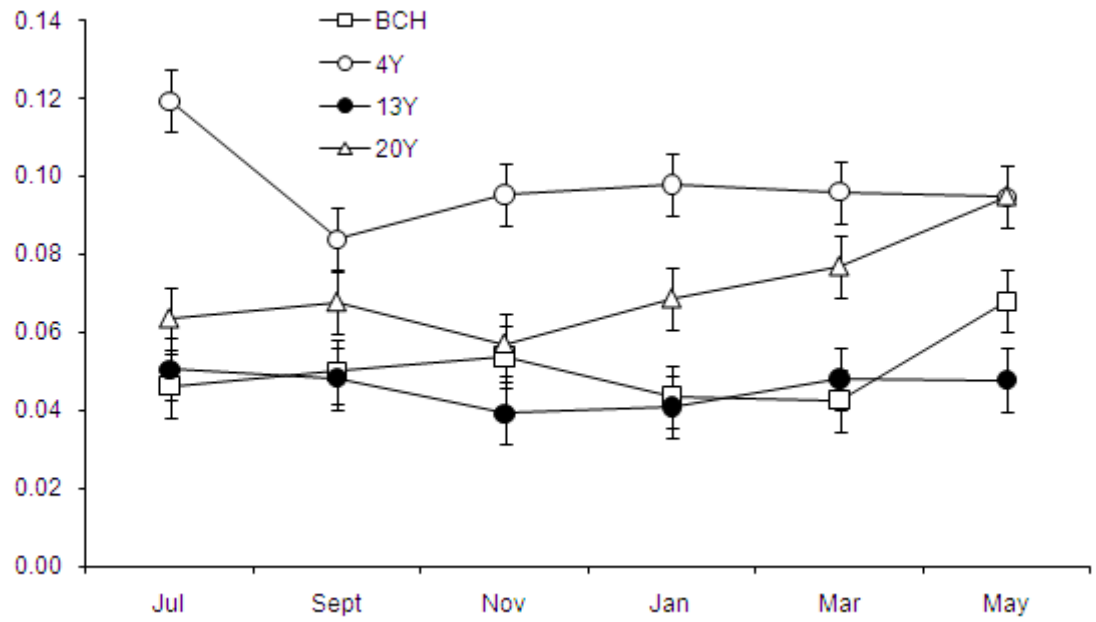
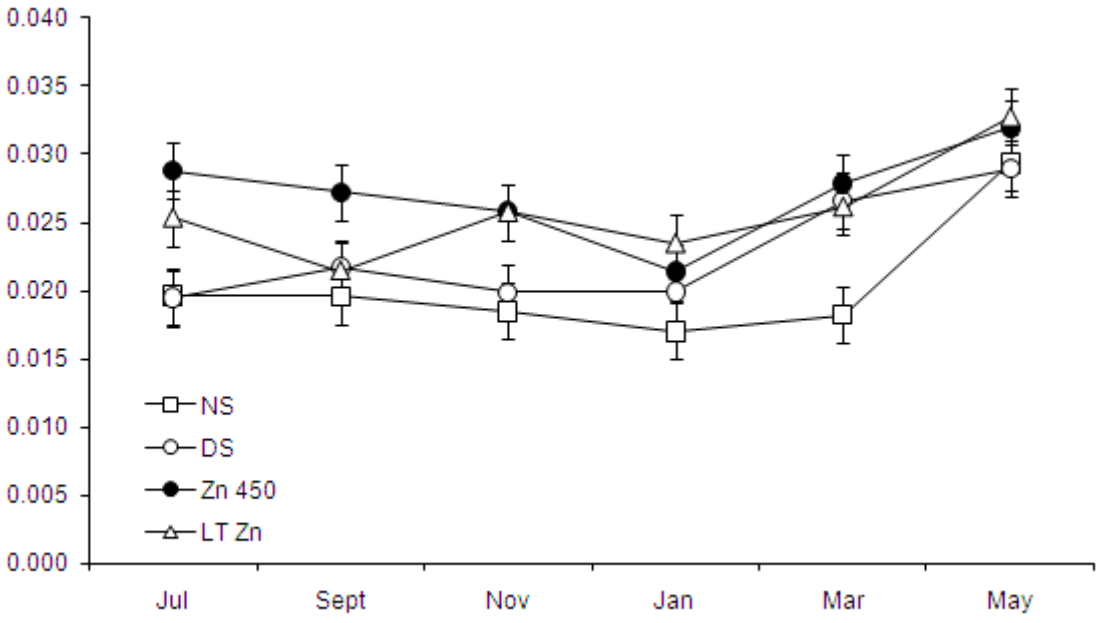


Figure 5-16 Time x treatment interaction means (nmol g^{-1} +/- 1 s.e.) for soil fungal / bacterial ratios from the Sutton Courtenay restoration site and the Hartwood sludge metal site. BCH = grassland benchmark site; 4Y, 13Y, 20Y = years after restoration. NS = no sludge control; DS = digested sludge only; ZN450 = single Zn treatment to achieve a target soil concentration exceeding current limits; LTZN = repeated intermediate Zn additions to soil.

(a) Mean interaction F/B ratios for the Sutton Courtenay restoration site



(b) Mean interaction F/B ratios for the Hartwood sludge metal site



5.2.5 Nematodes

P-values for the statistical analyses of nematode community structure at each site are presented in Table 5-7. There are far fewer statistical results for nematodes compared to the microbial and process measures. Several of the nematode measures could not be adequately transformed to support parametric statistical analyses. In general, relative abundance measures (%) were slightly more amenable to statistical analyses than absolute abundance (numbers m^{-2}). PC analysis was not appropriate in this instance since there were too few measures to warrant multivariate analyses.

The results show that none of the nematode measures demonstrated significant sensitivity to the N deposition treatments at the Pwllpeiran site or to the sludge metals at the Hartwood site. Sampling period was significant for several of the measures, as illustrated in Figure 5-17 for total nematode abundance and the ratio of fungal/ bacterial feeding nematodes. Overall the sampling period results illustrate a seasonal dynamic. However for fungal/bacterial feeding nematode ratios, this dynamic is not consistent and resulted in a significant interaction effect, as shown in Figure 5-17c.

At the restoration site, there were was only one significant pressure effect, which was for total nematode abundance. The treatment means for total nematodes (Figure 5-18) demonstrate differences between the 4Y and 20Y restoration plots and the benchmark site. However, this result was not consistent across the sampling period since there was a significant interaction effect, as illustrated in Figure 5-18c, with the 4Y and 20Y plots more dynamic than the benchmark and 13Y plots.

Summary of sensitivity in soil nematode community structure

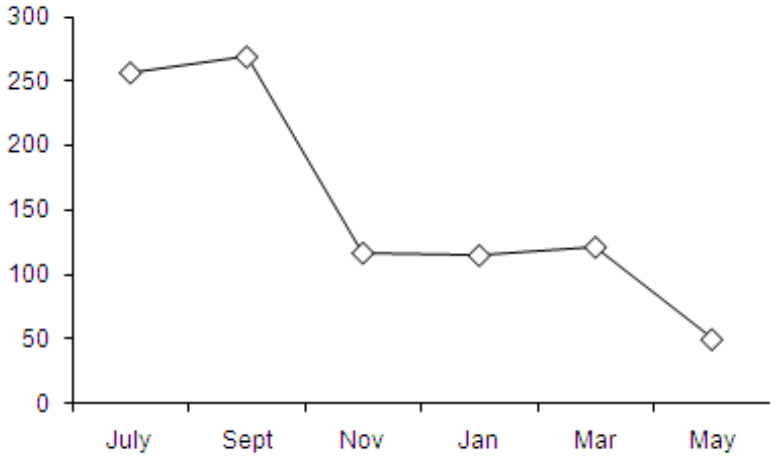
- Only one of the nematode measures (total nematode abundance) demonstrated sensitivity to only one pressure, viz restoration.
- Seasonal dynamics was a major influence on nematode community structure and this influenced the effect of the pressures at all sites.
- Many of the nematode measures were not suitable for parametric statistical analyses due to high variability and high numbers of zero counts. This suggests that the sampling and/or extraction approaches were not adequate for effectively characterizing nematodes at these sites.

Table 5-7 NEMATODES. P-value results from repeated measures analysis of variance to test for the significance of treatment, sampling period and interactions between treatment and sampling period on individual indicator measures. P values in red and bold indicate a significant effect.

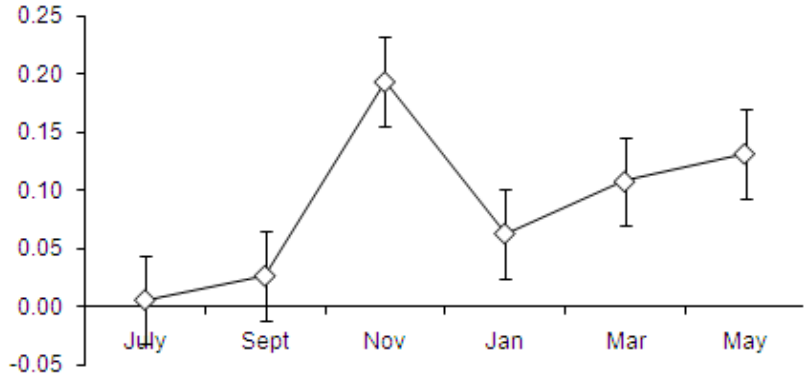
Site	Restoration (Sutton Courtenay)			Sludge metals (Hartwood)			N deposition (Pwllpeiran)			
Measure / Effect	Treatment	Sampling	T X S	Treatment	Sampling	T X S	Treatment	Sampling	T X S	unit
bacterial feeders	0.109	**0.000**	**0.003**	0.688	**0.000**	0.760	0.361	0.195	0.730	N m^{-2}
fungal feeders	-	-	-	-	-	-	-	-	-	N m^{-2}
omnivores	-	-	-	-	-	-	-	-	-	N m^{-2}
predator	-	-	-	-	-	-	-	-	-	N m^{-2}
plant feeders	-	-	-	0.291	**0.000**	**0.041**	0.357	**0.004**	0.260	N m^{-2}
total	**0.011**	**0.000**	**0.007**	0.701	**0.000**	0.427	0.266	**0.049**	0.648	N m^{-2}
%bacterial feeders	0.849	0.697	0.209	0.779	**0.000**	0.850	0.857	**0.009**	0.467	%
%fungal feeders	-	-	-	-	-	-	-	-	-	%
%omnivores	0.267	**0.002**	**0.036**	-	-	-	0.279	**0.033**	0.840	%
%plant feeders	-	-	-	0.783	**0.000**	0.299	0.854	**0.003**	0.282	%
%predator	0.965	0.797	0.258	-	-	-	-	-	-	%
fungal/bacterial	0.593	0.133	0.547	0.575	**0.002**	0.068	0.076	**0.012**	**0.037**	n/a

Figure 5-17 Mean abundance and ratios (+/- 1 s.e.) for nematodes at the Pwllpeiran N deposition site. No fert = water only; AS10 = ammonium sulphate 10 kg ha yr^{-1}; AS20 = ammonium sulphate 20 kg ha yr^{-1}, SN10 = sodium nitrate 10 kg ha yr^{-1}.

(a) Sampling time means for total nematodes (n $100g^{-1}$)



(b) Sampling time means for the ratio of fungal / bacterial feeding nematodes



(c) Treatment x time interactions for the ratio of fungal / bacterial feeding nematodes

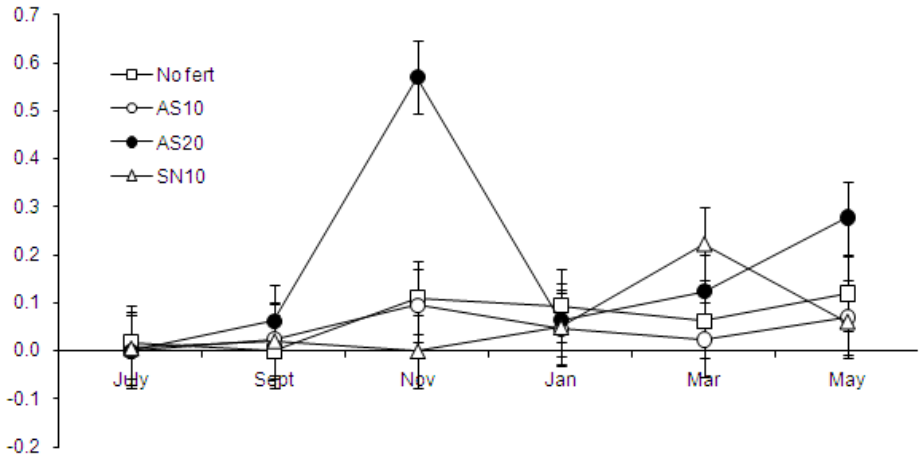
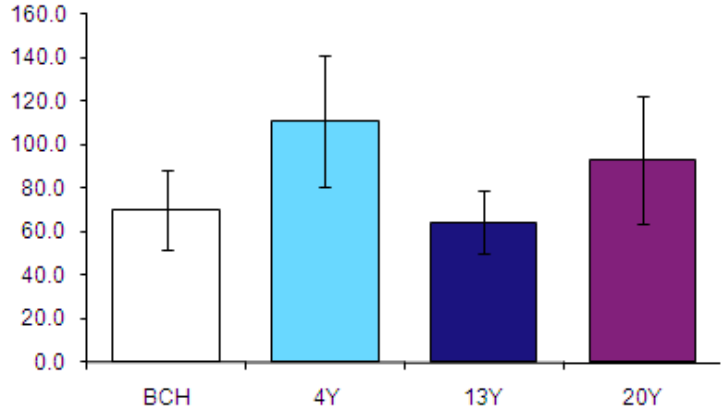
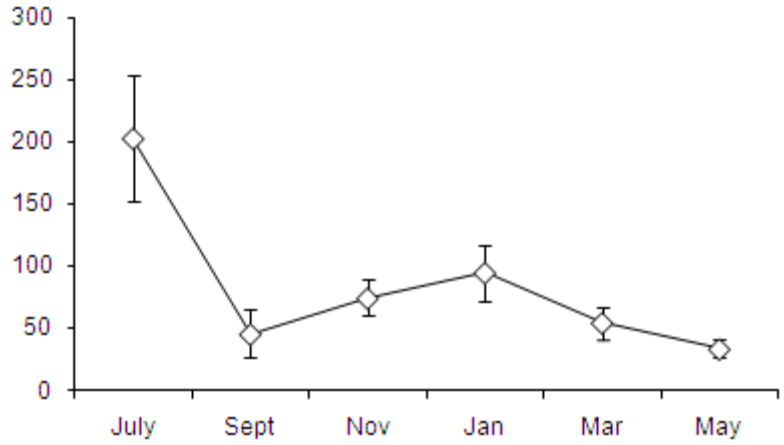


Figure 5-18 Mean abundance (n $100g^{-1}$+/- 1 s.e.) of total nematodes at the Sutton Courtenay restoration site. BCH = grassland benchmark site; 4Y, 13Y, 20Y = years after restoration.

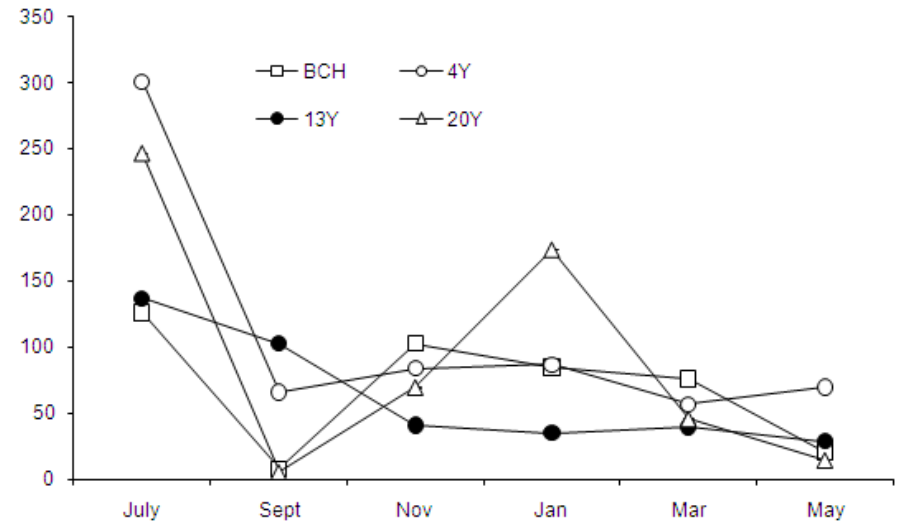
(a) Treatment means



(b) Sampling time means



(c) Treatment x time interaction means



5.2.6 Microarthropods

P-values for the statistical analyses of microarthropod measures at each site are presented in Table 5-8. There are a reduced number of statistical results for microarthopods compared to the microbial and process measures. Similar to the nematodes, several of the microarthropod measures could not be adequately transformed to support parametric statistical analyses. This was a particular issue with data from the restoration site. In general, relative abundance measures (%) were slightly more amenable to statistical analyses than absolute abundance (numbers m^{-2}). PC analysis was not appropriate in this instance since there were too few measures to warrant multivariate analyses.

Several of the microarthropod measures demonstrated significant sensitivity to the N deposition treatments at the Pwllpeiran site. These results are presented in Figure 5-19, which shows the measures were, in general, most responsive to the higher nitrogen (AS20) treatment. The results for the relative abundance (%) of mites and collembola were both calculated as % of total number of invertebrates collected from the dry extractions. These produced similar, mirror image results, since these two groups dominate the invertebrates extracted using this method. Therefore, only one of these ratios is required. The lowest level of taxonomic identification i.e. Subclass ("Acari") or Order ("Collembola") produced similar pressures responses as more detailed taxonomic identifications. However, comparison of the detailed identification does provide contrasting information on the sensitivity to the pressures. This is an insight into which groups may decline, while others may increase and, as a consequence, what causes the changes in microarthropod numbers and community structure. This is clearly demonstrated for %oribatid and %prostigmatid mites (both % of total mite N). Oribatids increased their dominance of the mite community under the AS20 and SN10 treatments while prostigmatid significantly increased its contribution to the mite community in AS10, relative to the control and the AS20 and SN10 treatments. The more detailed taxonomic measures for mites were also significantly influenced by sampling period. Figure 5-20 illustrates this significant effect for %Oribatid and %Prostigmatid mites. There were no significant effects of treatment x time interactions. However, the interaction means for %Oribatid and %Prostigmatid mites. Figure 5-20c and d, illustrate that sampling during the winter period (Nov/Jan) could reduce the sensitivity of the measures. For the Sutton Courtenay restoration site, only four measures could be statistically analysed using RMANOVA; % mesostigmatid mites, % oribatid mites, % mites and % collembola. The only significant result was a treatment effect for % oribatids. In this instance, %oribatids was lower in 4Y and 20Y plots compare to the 13Y and benchmark site. These results are illustrated in Figure 5-21. There was no significant effect of sampling period. Examination of the interaction means in Figure 5-21c shows that there is some temporal variability but it is not significant enough to influence the treatment effects. This variability could however, influence the interpretability of %oribatid responses at individual sampling times.

It was possible to statistically analyse a wider range of microarthropod measures from

the Hartwood sludge metal site since data were more amenable to transformation. There were two significant treatment effects; %poduroidae as a proportion of total collembola numbers and total numbers of collembola. Both demonstrate similar sensitivity with increased numbers of collembola and a higher proportion of poduroidae in the highest sludge metal treatment (Zn450). These results are illustrated in Figure 5-22. The significant increase in total collembola is partly a response to an increase in poduroidae abundance, which was not statistically significant in itself. There were several significant sampling period effects for both mites and collembola, which relate to characteristic seasonal population dynamics, as illustrated for total collembola in Figure 5-22b. Although there were no significant interactions between treatment and time, the results suggest that further consideration of the optimal sampling period is required since temporal variability would influence the interpretability of the measures.

Summary of sensitivity in soil microarthopod community structure:

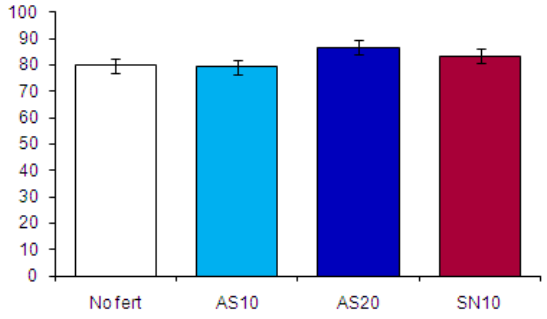
- There were few significant responses of microarthropod measures to the three distinct pressures.
- The results demonstrated that there were no consistent responses between the pressures. Collembola were more sensitive to sludge metal treatments while both mites and collembola were sensitive to N deposition. Only oribatid mites were sensitive to restoration, which may partly reflect an inability to carry out parametric analyses of other measures.
- Seasonal dynamics was a major influence on microarthropod community structure. This variability had little influence on the overall pressure responses but it could influence the interpretability of the pressures at any single sampling time. An optimal sampling window was not obvious from the results obtained since there was little consistency in the measure responses across the sampling period.
- Several of the microarthropod measures were not suitable for parametric statistical analyses due to high variability and high numbers of zero counts. This was a particular issue for the restoration site. This suggests that the sampling and/or extraction approaches were not adequate for effectively characterizing microarthropods at the sites.

Table 5-8 MICROARTHROPODS. P-value results from repeated measures analysis of variance to test for the significance of treatment, sampling period and interactions between treatment and sampling period on individual indicator measures. P values in red + bold indicate a significant effect.

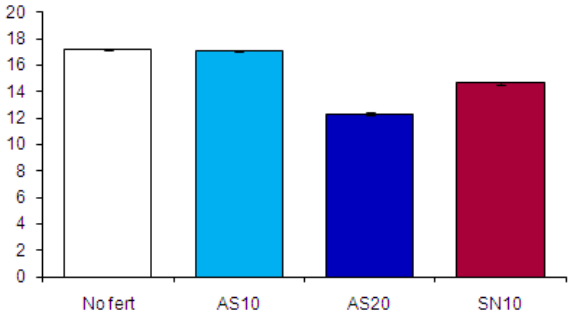
Site	Restoration (Sutton Courtenay)			Sludge metals (Hartwood)			N deposition (Pwllpeiran)			
Measure / Effect	Treatment	Sampling	T X S	Treatment	Sampling	T X S	Treatment	Sampling	T X S	unit
% mesostigmatid mites	0.156	0.640	0.649	0.086	0.063	0.809	-	-	-	%
% oribatid mites	**0.018**	0.881	0.090	0.083	**0.039**	0.991	**0.010**	**0.004**	0.375	%
% prostigmatid mites	-	-	-	0.937	**0.005**	0.252	**0.029**	**0.000**	0.152	%
% entomybroidae – collembola	-	-	-	0.124	**0.005**	0.489	**0.012**	**0.000**	0.300	%
% poduroidae – collembola	-	-	-	**0.029**	0.394	0.851	-	-	-	%
mites / collembola ratio	-	-	-	0.795	0.474	0.468	**0.027**	0.699	0.121	n/a
mites / (mites+collembola) ratio	-	-	-	0.157	**0.038**	0.860	-	-	-	n/a
mesostigmatid mites	-	-	-	0.051	**0.015**	0.758	-	-	-	m^{-2}
oribatid mites	-	-	-	0.412	0.276	0.996	0.360	**0.000**	0.564	m^{-2}
prostigmatid mites	-	-	-	0.751	**0.000**	0.130				m^{-2}
total mites	-	-	-	0.482	**0.001**	0.435	0.518	**0.000**	0.880	m^{-2}
entomybroidae - collembola	-	-	-	0.575	**0.002**	0.068	-	-	-	m^{-2}
poduroidae – collembola	-	-	-	0.117	**0.031**	0.819	**0.001**	**0.037**	0.430	m^{-2}
total collembola	-	-	-	**0.020**	**0.000**	0.622	0.190	**0.005**	0.261	m^{-2}
% mites	0.961	0.714	0.249	0.821	0.646	0.958	**0.005**	0.736	0.336	%
% collembola	0.961	0.714	0.249	0.118	**0.021**	0.777	**0.012**	0.737	0.135	%

Figure 5-19 Treatment means (+/- 1 s.e.) for microarthropod measures at the Pwllpeiran N deposition site. No fert = water only; AS10 = ammonium sulphate 10 kg ha yr^{-1}; AS20 = ammonium sulphate 20 kg ha yr^{-1}, SN10 = sodium nitrate 10 kg ha yr^{-1}.

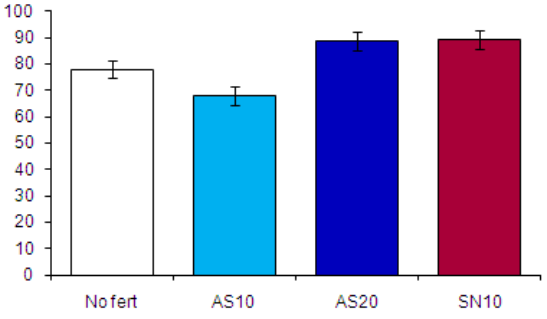
(a) %mites



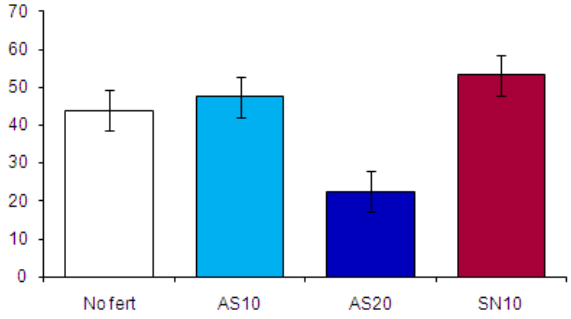
(b) %collembola



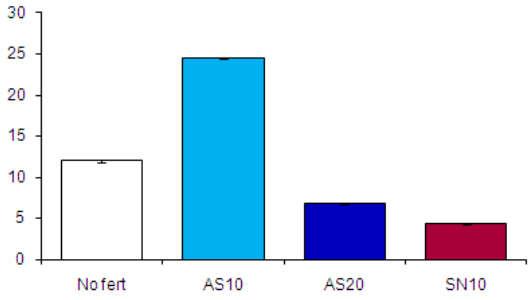
(c) %oribatid mites



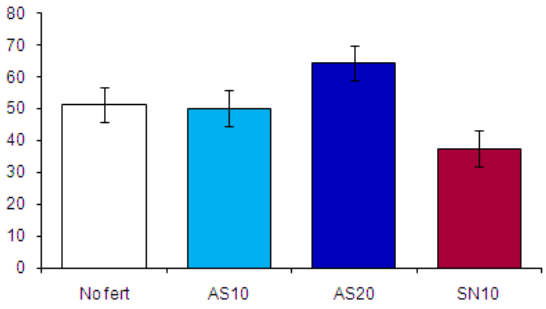
(d) % entomybroidae - collembola



(e) %prostigmatid mites



(f) poduroidae – collembola



(g) mites / collembola ratio

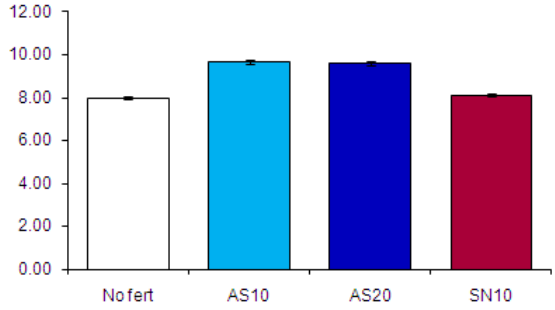
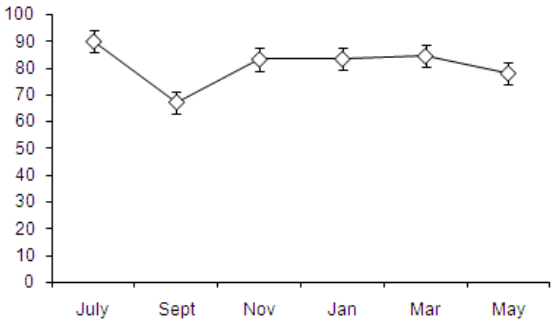
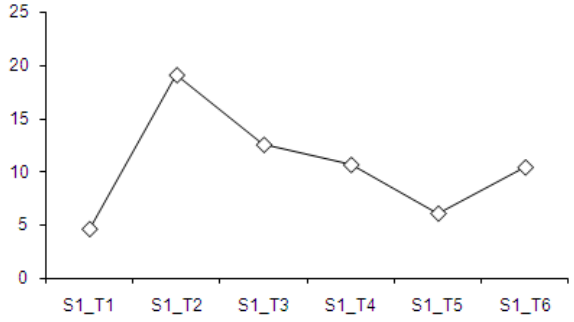


Figure 5-20 Mean relative abundance (% +/- 1 s.e.) for Oribatid and Prostigmatid mites at the Pwllpeiran N deposition site. No fert = water only; AS10 = ammonium sulphate 10 kg ha yr^{-1}; AS20 = ammonium sulphate 20 kg ha yr^{-1}, SN10 = sodium nitrate 10 kg ha yr^{-1}.

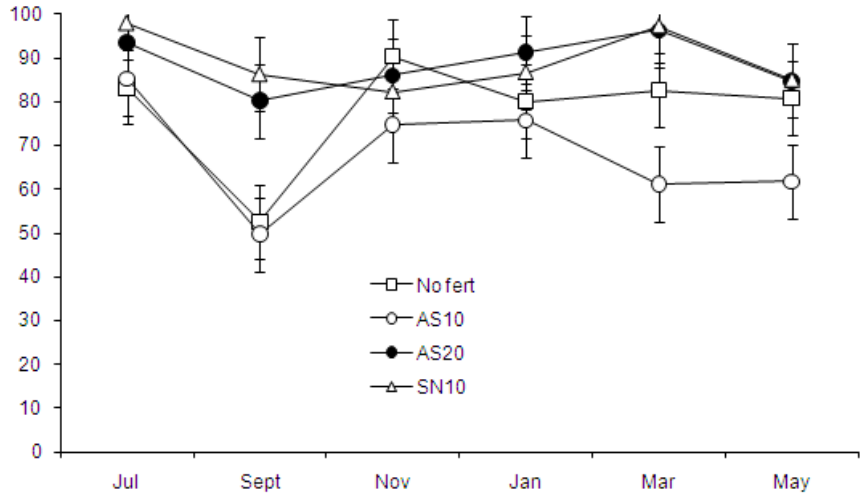
(a) Time means for % Oribatids



(b) Time means for % Prostigmatids



(c) Treatment x time interactions for % Oribatids



(d) Treatment x time interactions for % Prostigmatids

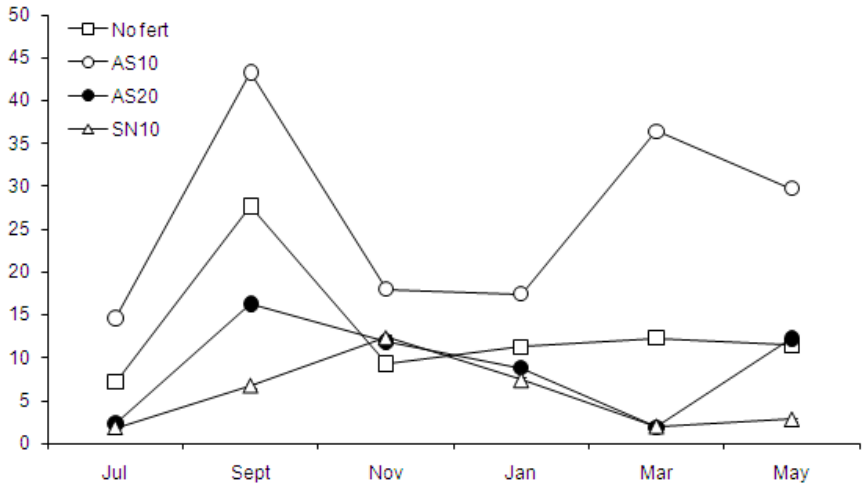
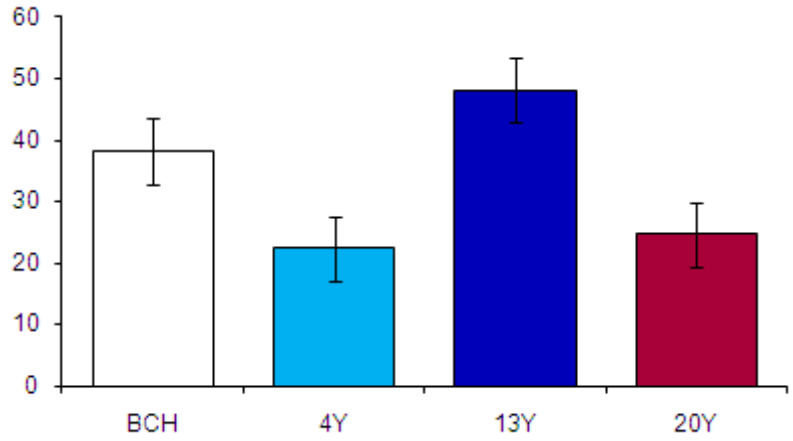
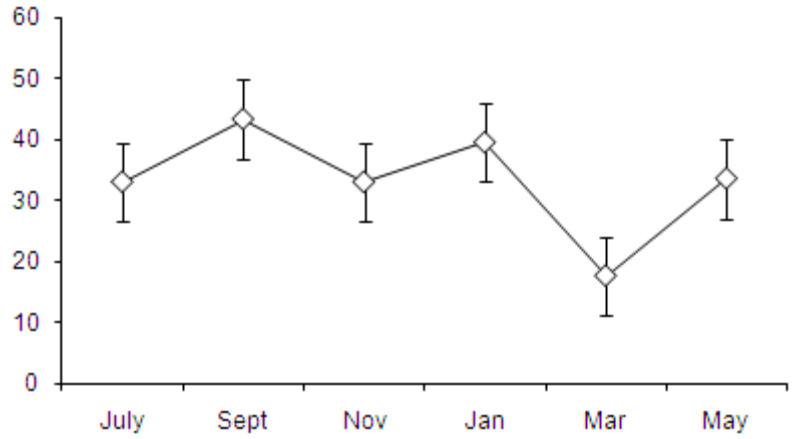


Figure 5-21 Mean relative abundance (% +/- 1 s.e.) of Oribatid mites at the Sutton Courtenay restoration site. BCH = grassland benchmark site; 4Y, 13Y, 20Y = years after restoration.

(a) Treatment means



(b) Sampling time means



(c) Treatment x time interaction means

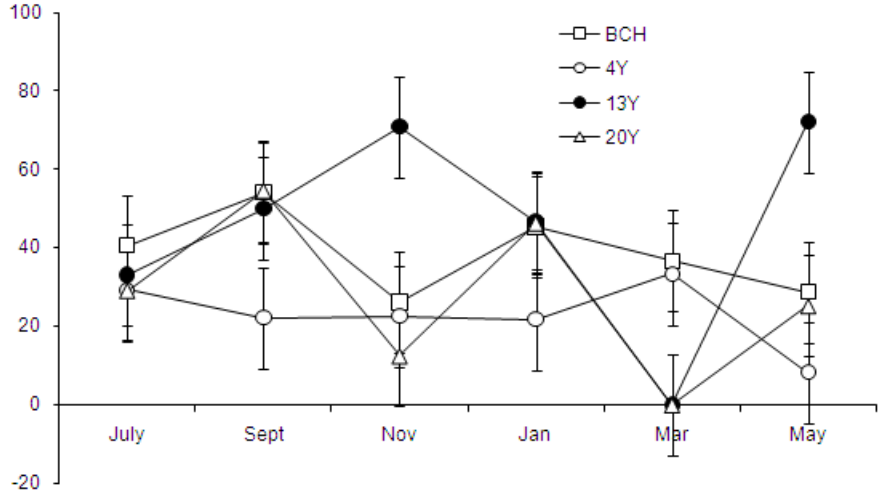
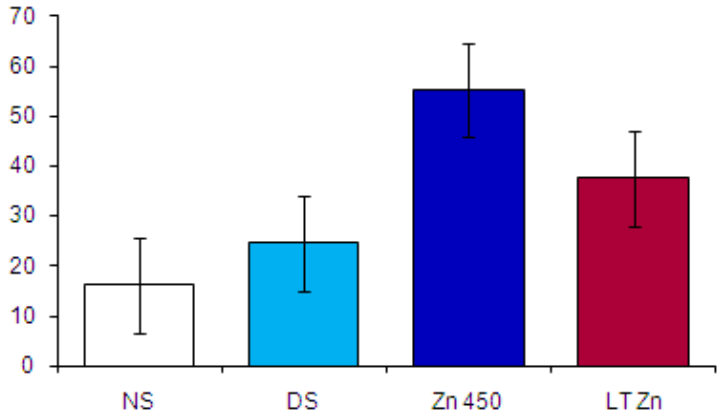
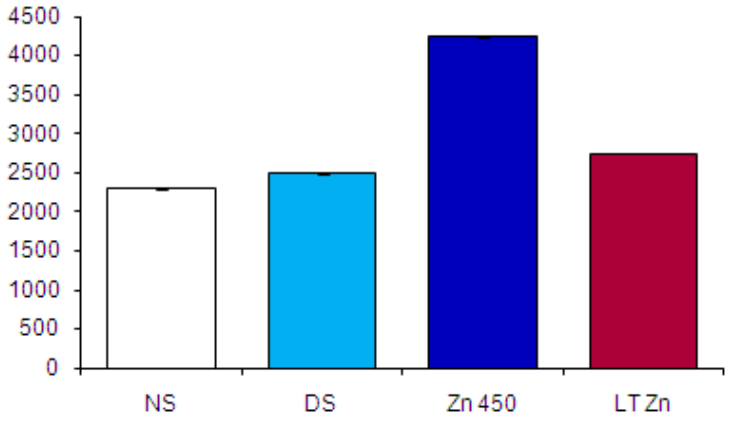


Figure 5-22 Results for Collembola measures from the Hartwood sewage sludge metal site. NS = no sludge control; DS = digested sludge only; ZN450 = single Zn treatment to achieve a target soil concentration exceeding current limits; LTZN = repeated intermediate Zn additions to soil.

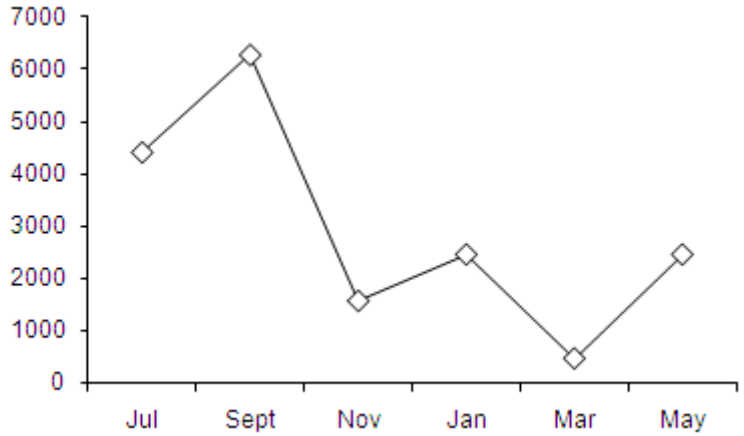
(a) Treatment means (%+/- 1 s.e.) for %poduroidae as a proportion of total collembola



(b) Treatment means (m^{-2} +/- 1 s.e.) for total abundance of collembola



(c) Time means for total collembola (m^{-2} +/- 1 s.e.)



5.3 Outcomes from the sensitivity trial

Table 5-9 summarises the significant P-values for the statistical analyses of all measures across the three sites. This table is split into three groups; group A for significant treatment effects only; group B for significant effects of sampling time only and group C significant effects for the interaction between treatments and time. Following the simple principles set out at the start of the research, group A represents the most reliable indicators, followed by group B, while Group C cannot be considered reliable indicators until issues of temporal variability are addressed.

Table 5-9 demonstrates that no single measure and no single method were consistently sensitive to all three pressures. All methods produced measures which were significantly influenced by the pressures although some methods were more sensitive than others. At the Sutton Courtenay restoration sites, the most sensitive measures were obtained primarily from MSIR, TRFLP and PLFAs. At the Hartwood sludge metals site sensitive measures were obtained primarily from PLFAs, TRFLPs and microarthropods. At the Pwllpeiran N deposition site, sensitive measures were obtained only from microarthropods and MSIR. The colour coding summarises this further to illustrate that functional, phenotypic and genotypic methods produced significant treatment effects at both the Sutton Courtenay restoration site and Hartwood sludge metal site, while only functional and phenotypic methods produced significant results at the Pwllpeiran site. Given the results obtained so far the most sensitive methods with least variability issues across the three pressures were PLFAs, TRFLP, MSIR and microarthropods.

The influence of sampling period was significant in most measures. However, this only had a significant statistical effect on the sensitivity of measures in a few cases, as demonstrated by the interactions effect. Despite this, closer examination of the results for the individual measures suggests that limiting sampling to single or a few sampling periods could alter the interpretation of the influence of a pressure. For several measures, an optimal sampling period may be during the spring and early summer months. In parallel, the sampling strategy could be improved to reduce variability at any one point in time. This could be accomplished in different ways depending on the objective. Increasing the total number of samples across and increasing the within site sub-sampling can both be used to reduce variability. In monitoring or surveying where large numbers of sites are generally involved there will be an optimal compromise between these two approaches based on the statistical robustness of results to be obtained and overall cost-effectiveness.

These results suggest that a suite of soil biological methods would be a less risky and more informative approach to monitoring changes in soil biological status where multiple pressures are at play, or where the pressures influencing soil are unknown. From the results here, this suite would include: PLFAs, TRFLP, MSIR and microarthropods.

Interpretation of changes to soil biological status from a soil monitoring scheme will need further information on the expected responsiveness of individual measures to individual pressures. This could mirror the approaches adopted in vegetation science where drivers of change in plant communities can be interpreted through knowledge of the stress responses of individual plants to specific pressures e.g. nitrogen or shade.

The results obtained raise a question regarding what responses are to be expected from soil biological indicators. In this study, pressure responses could be compared to a zero treatment control or a benchmark target which gives some guidance in the interpretation of the results. However, we are still remarkably ignorant of the responses to expect from soil biological indicators (e.g. trajectories of change), and at what point changes are moving beyond unacceptable limits or boundaries.

Table 5-9 Significant P-value results from RMANOVA for measures by site. P values in red and bold indicate a significant effect. Methods are colour coded accordingly; function (blue), genotype (yellow) and phenotype (brown). TMT = treatment; INT = interactions between TMT and TIME; NEMA = nematodes, MICA = microarthropods.

	Site	**Restoration (Sutton Courtenay)**				**Sludge metals (Hartwood)**					**N deposition (Pwllpeiran)**				
	Method	Measure	TMT	TIME	INT	Method	Measure	TMT	TME	INT	Method	Measure	TMT	TIME	INT
A. Significant treatment effects only	**MSIR**	AKGA	0.000	ns	ns	**PLFA**	PN ratio	0.001	ns	ns	**MICA**	%mites	0.005	ns	ns
	TRF	Archaea McIntosh E	0.000	ns	ns	**PLFA**	PLFA_PC1	0.001	ns	ns	**MICA**	%collembola	0.012	ns	ns
	PLFA	FB ratio	0.000	ns	ns	**PLFA**	PLFA_PC2	0.009	ns	ns	**MICA**	mites/collembola	0.027	ns	ns
	TRF	Archaea Shannon E	0.000	ns	ns	**PLFA**	Gram negative	0.025	ns	ns					
	PLFA	PLFA_PC1	0.001	ns	ns	**MICA**	%poduroidae	0.029	ns	ns					
	TRF	Fungal PC2	0.010	ns	ns	**TRF**	Bacteria Shannon E	0.049	ns	ns					
	TRF	Archaea Richness	0.015	ns	ns										
	MICA	% oribatids	0.018	ns	ns										
	TRF	Archaea Shannon H	0.030	ns	ns										
B Significant treatment and time effects	**MSIR**	MSIR_PC1	0.000	0.007	ns	**TRF**	Bacteria PC2	0.002	0.042	ns	**MICA**	poduroidae	0.001	0.037	ns
	MSIR	Malic acid	0.000	0.009	ns	**TRF**	Fungal PC2	0.003	0.018	ns	**MSIR**	Water	0.003	0.000	ns
	MSIR	Citric acid	0.000	0.016	ns	**PLFA**	FB ratio	0.004	0.000	ns	**MICA**	% oribatids	0.010	0.004	ns
	PLFA	PLFA_PC2	0.001	0.000	ns	**PLFA**	PLFA_PC3	0.019	0.007	ns	**MSIR**	MSIR_PC1	0.011	0.000	ns
	PLFA	Actinomycetes	0.002	0.008	ns	**MICA**	total collembola	0.020	0.000	ns	**MICA**	%entomybroidae	0.012	0.000	ns
	MSIR	Arginine	0.002	0.000	ns	**ENZE**	Sulphatase	0.037	0.000	ns	**MSIR**	Malic acid	0.013	0.000	ns
	PLFA	PN ratio	0.004	0.023	ns	**PLFA**	Fungal	0.037	0.000	ns	**MSIR**	Citric acid	0.017	0.000	ns
	MSIR	GABA	0.004	0.000	ns	**ENZE**	Galactosaminidase	0.042	0.000	ns	**MSIR**	GABA	0.018	0.000	ns
	MSIR	NAGA	0.007	0.000	ns	**ENZE**	Glucosaminidase	0.042	0.000	ns	**MSIR**	AKGA	0.022	0.000	ns
	ENZE	Sulphatase	0.012	0.000	ns						**MSIR**	NAGA	0.023	0.000	ns
	PLFA	PLFA PC3	0.026	0.001	ns						**MSIR**	Glucose	0.024	0.000	ns
	TRF	Fungal PC1	0.048	0.019	ns						**MICA**	%prostigmatids	0.029	0.000	ns
C all effects	**MSIR**	Water	0.001	0.000	0.000	**TRF**	Archaea Shannon E	0.004	ns	0.002					
	MSIR	Glucose	0.002	0.000	0.045	**TRF**	Archaea McIntosh E	0.001	0.010	0.000					
	NEM	total	0.011	0.000	0.007										

6 Test the candidate biological indicators for their ability to discriminate between a diverse range of different land use:soil combinations

The aim of this objective was to evaluate the discriminatory power of the candidate indicators with respect to the typical range of soil:land use combinations in the UK. The trial was aimed to address whether there would be characteristic responses of the biological indicators both within and between land uses. This information is the first step in defining a typical status of soil biological properties and processes for distinct land uses and what might be expected in terms of change when moving from one land use to another, or within a land use under new or increasing pressures.

The discrimination trial was designed to test the robustness of the indicators under a wide range of conditions likely to be encountered during extensive monitoring across the UK. The Countryside Survey (Carey *et al*., 2008) provided a rare opportunity to obtain a set of soil samples from locations across Great Britain where there would be detailed past and current habitat and soils information to enable the investigation of discrimination amongst the candidate biological indicators. The Countryside Survey is a detailed audit of the UK's natural environment which includes a field survey of more than 590 1 km squares located in England, Wales and Scotland. There have been five field surveys since 1978 with habitat information collected in all instances and soils data obtained from three field surveys, including 2007 (Emmett, *et al*., 2010). This information was used to select sampling locations where land use would have been relatively constant since 1978, and which covered the typical range of soil organic matter and soil pH for UK soils. Consistent land use was desired to examine whether the candidate indicators would display characteristic values and ranges for individual land uses with minimum interference from land use change or from contrasting vegetation types within the same land use.

6.1 Methodology

6.1.1 Selection of the sampling locations from the Countryside Survey field survey

The primary objective in the selection of the sampling locations was to obtain sites which demonstrated similar vegetation composition over the history of the survey. The assumption would be that this consistency in vegetation composition would demonstrate consistent land use over this period.

Prior to the field survey in 2007, 126 locations were identified from the Countryside Survey for the collection of soil samples for analyses of the functional, phenotypic and genotypic candidate indicators. These locations were targeted at the Countryside Survey "X plots" which are fixed 200 m x 200 vegetation quadrats within 1 km survey squares. Previous soil sampling, along with detailed vegetation surveys, had

been located near to the central 2 m x 2m point of these quadrats (Black *et al*., 2003). Vegetation composition data from these quadrats has been used to define vegetation classes from the Countryside Vegetation System (CVS) using multivariate analyses (Bunce *et al*., 1999), see Figure 6-1. The information on the vegetation classes from previous surveys was used to identify sampling locations for this study. Nine distinct land use types were identified from CVS vegetation and aggregate vegetation classes (AVC). These included intensive arable agriculture, fertile and infertile grasslands, lowland and upland woods, moorland/grass mosaic and heath/bog. Infertile grassland was further expanded to include infertile grassland on acid soils and infertile calcareous grasslands. Upland wooded was also expanded to include deciduous wooded on neutral/acid soils and coniferous wooded on acid soils. The sampling locations were identified by sifting data from previous Countryside Surveys using the following criteria.

1. Selection of specific vegetation classes within the Countryside Vegetation Scheme to match aggregate classes of interest. Where more than one vegetation class is required to obtain the number of sampling sites required for the AVC then the classes are selected from those closest in the ordination space demonstrated in Bunce *et al*., 1999. This ordination is illustrated in Figure 6-1.
2. Selection of sampling locations where there was consistency in location of the X plots from 1978 to 1998.
3. Selection of sampling locations where there was consistency of vegetation class/habitat from 1978 to 1998.
4. Select where soils data was available from 1998.
5. Select by planned 2007 survey month to constrain sampling window to May, June and July as much as possible.
6. Select, where possible, across the range of environmental zones.
7. Ca. 25% extra locations identified to allow for losses.

Table 6-1 summarises the number of sampling locations allocated to the nine land uses, as defined by the CVS aggregate vegetation class and the number of suitable locations sampled during Countryside Survey 2007. Of the 126 locations selected, 101 locations provided soil samples for analyses. Samples were lost for a variety of reasons from inability to sample in the field due to foot and mouth disease restrictions to a few samples being lost in the post. The locations of the sampling sites are illustrated in Figure 6-2. It is important to remember that the aggregate vegetation class descriptions are used throughout the report to define the land uses (e.g. crops+weeds, fertile grassland, etc). However the land uses are more specifically defined by the dominant vegetation. For example, sampling of crops+weeds was specifically targetted at almost weed free wheat/other crops, infertile grassland was specifically targeted at rye-grass/yorkshire fog grassland while heath/bog sampling was focussed on saturated bog. The results obtained are therefore typical of these vegetation classes within the broader land use.

Table 6-1 Allocation of soil sampling locations to CVS aggregate classes for sampling during Countryside Survey 2007.

aggregate classcode	aggregate class description	locations selected	samples obtained	dominant vegetation
1	crops and weeds	15	12	almost weed free wheat / other crops
3	fertile grassland	15	13	fertile mixed grassland
4	infertile grassland (A)	16	13	rye-grass / Yorkshire fog grassland
4	infertile grassland (B)	8	8	calcareous grassland
5	lowland wooded	13	10	deciduous woodland
6	upland wooded (A)	14	11	predominately deciduous woodland
6	upland wooded (B)	16	11	predominately coniferous woodland
7	moorland grass/mosaic	14	11	moorland grass / heath on podzolic soils
8	heath/bog	15	12	saturated bog
	Total	126	101	

Figure 6-1 Distribution of the 100 CVS vegetation classes, grouped by aggregate classes, on the first two axes of the CVS decorana ordination carried out in the ECOFACT2 project. Axis 1 is correlated with a gradient from fertile to infertile soils, and axis 2 with a light gradient and indirectly with disturbance. The numbers within each polygon refer to each CVS vegetation class. Taken from Bunce *et al.*, 1999.

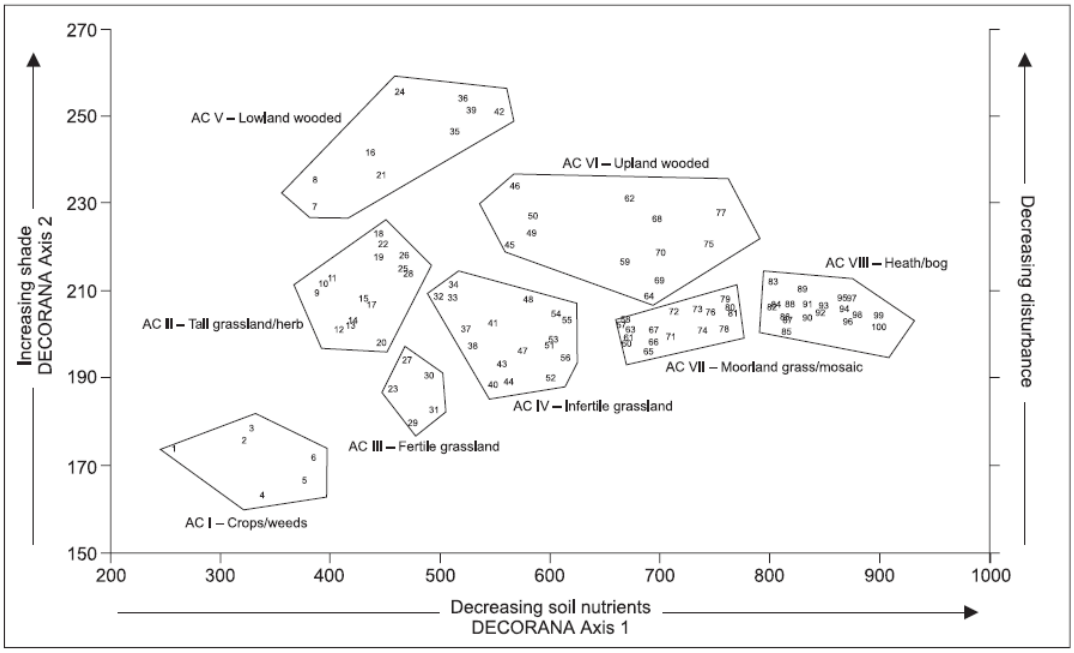
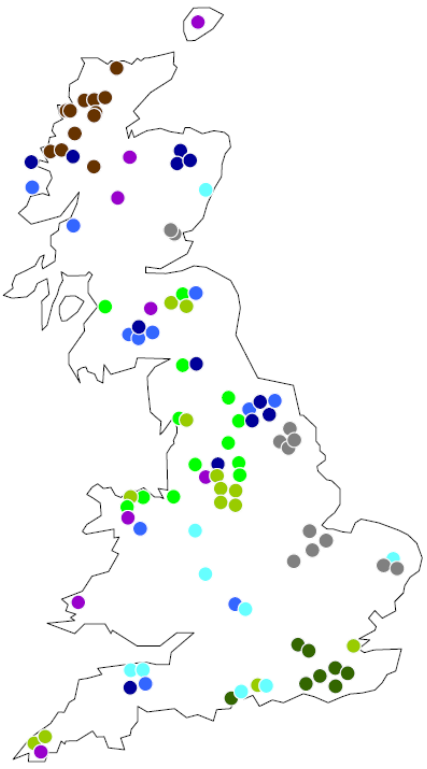


Figure 6-2 Location of soil sampling for 101 sites across the UK mainland, based upon a subset of the Countryside Survey 2007. Sites were representative of nine habitats, equating to Aggregated Classes (AC) of the Countryside Vegetation System. n = number of samples per class.

Habitat	n
Arable crops	12
Fertile grassland	13
Infertile grassland (acid/neutral)	13
Infertile grassland (calcareous)	8
Lowland wood	10
Upland deciduous wood	11
Upland coniferous wood	11
Moorland grass	11
Heath / bog	12



6.1.2 Field sampling and initial processing

The sampling and processing was included in the main soil sampling campaign of Countryside Survey. Full details for sampling and processing are provided in Emmett *et al*., 2008. The protocols relevant to this study are included in the Appendix A. To summarise, for this study, two soil samples were taken from 101 1 km squares identified as sampling locations for this study. The first sample was a small core (dimensions 4 cm diam. x 8 cm depth) which was taken at the south corner of a central 2m x 2m quadrat of the relevant X plot within the 1 km square. This sample was used to extract for soil microarthropods. The second sample was a bag sample which consisted of a composite of 8 to 10 sub-samples of soil taken around the edge of a 5m x 5m quadrat surrounding the central quadrat using a small trowel graded to 15cm. This sample was used for the process, microbial and nematode measures. These soil samples were stored in cool boxes before dispatch by post to a central processing laboratory at CEH Lancaster. Dispatch was usually within 24hrs of field sampling. On arrival at CEH Lancaster, all soil samples were logged and placed in a cold room prior to further processing. Tullgren extraction was carried out on the white core samples as soon as possible after arrival. The bag samples were processed within 48 hrs of arrival at CEH Lancaster. Each sample was split into three portions for distribution to the respective laboratories for analyses of PLFA and TRFLP (Macaulay), enzymes and MSIR (Cranfield) and nematodes (CEH). The full protocol for sampling processing is included in Appendix A.

6.1.3 Laboratory analyses

The laboratory analyses followed the protocols from the sensitivity trial. The full method protocol for each analysis is available in the Appendix A. Table 6-2 summarises the methods used and whether further modifications to the method were required for the discrimination trial.

Table 6-2 Summary of the methods used in the discrimination trial

Candidate indicator	Group	Method	Method modifications for the discrimination trial	Lab.	Appendix for SOP
MSIR	Functional	MicroResp™	Fizz test for carbonate content	Cranfield	A2
Enzymes	Functional	Fluorometric multi-enzyme assay	None	Cranfield	A1
Microbial community structure	Phenotypic	PLFA	Weight of soil sample extracted related to loss-on-ignition values	Macaulay	A4
Microbial community structure	Genotypic	Multiplex TRFLP for bacteria, archaea and fungi	None	Macaulay	A3
Nematodes	Phenotypic	Modified Baermann wet extraction	Extraction carried out on 100 g soil sample instead of white core soil sample	CEH	A6
Microarthropods	Phenotypic	Tullgren funnels dry extraction	None	CEH	A5

6.1.4 Data management and data analyses

A standard procedure for data management was established for the project and followed by each laboratory. Final datasets, with relevant metadata, were compiled by each laboratory into a template of standard worksheets developed in a MS Excel file which was submitted to the project data manager. Final data and metadata were entered into the project database which was developed in MS Access. These data were then used for statistical analyses and production of graphs in a unified approach.

The methods employed produce multivariate profiles which reflect the different characteristics of the soil communities they represent. Each profile was analysed separately using principal component (PC) analysis, and a second PC analysis was then performed on a combination of all profiles from all methods. We elected to use PC analysis for three reasons: (i) there is no *a priori* allowance taken for the origin of the samples, so the approach is particularly rigorous in testing for consistency or discrimination between samples; (ii) since a consistent analysis is applied the results can be compared directly, and aggregated, in a coherent manner and; (iii) it is appropriate to apply to all nine methods, with many precedents for such application. Raw data were transformed as appropriate to ensure normality. The first three PCs relating to each of the nine habitats were analysed by one-way analysis of variance (ANOVA) to determine the effects of the land use and the resultant land use means for each PC axis were used to produce X-Y graphs and radar plots.

Data transformations were carried out where required to achieve normality. This included log transformation (loge+1 or $\log_{10}$ n+1) for abundance data and sqrt(n+0.5) for relative abundance data. Certain measures, notably for microarthropods, were excluded from statistical analyses where they did not conform to normality even under data transformation. Data analyses were carried out in STATISTICA v8.0 (StatSoft, 2008) while PRIMER v6 was used to calculate diversity indices (Clarke and Gorley, 2006).

A common worksheet-based approach was developed in MS Excel to capture and summarise the statistical results from the PCA results from each method in a comparable way. There is one file for each method. This file captures the PCA loading values for PC1, PC2 and PC3, eigenvalues for the PC axes, 2D and 3D graphical presentations for these axes and land use means and standard errors for PC1, PC2 and PC3 with summary results from ANOVA GLM analyses of these means.

6.2 Results

The results are presented in two sections. First section presents the results from the application of each indicator method. For each method, there is a summary of stastitics for the individual measures obtained from each method and results from statistical analyses of the individual measures for effects of land use and sampling month, where appropriate. A series of graphs are provided to illustrate the discrimination of land use by the individual measures and finally the presentation of results from the multivariate (PCA) analyses of the measures from each method. In

the second section, the different indicator methods are brought together to investigate how multiple indicators discriminate amongst land uses. Three approaches have been adopted for this section using data from (1) the individual measures (univariate data), (2) the multivariate results from all methods (multivariate data) and (3) a fully integrated multivariate analyses of the different methods and associated measures (integrated multivariate analyses).

6.2.1 Multi-enzyme assay

The multi-enzyme assay was applied in the same way as for the sensitivity trial with no modifications (Appendix A). A total of eight enzymes were used in the assay. Table 6-3 presents summary results for the individual enzymes by the nine land use classes. All enzyme data required transformation (ln n+1) prior to statistical analysis.

Analysis of variance showed that there were significant effects of sampling month and enzyme on the overall enzyme responses but no interaction between the two (Table 6-4). Enzyme responses were lower in the month of July compared to the other months (Figure 6-3). Univariate analysis of variance indicates that there were significant effects of land use on 5 out of the 8 enzymes (Table 6-5). The response patterns (log data) for these five enzymes are illustrated in Figure 6-4. This shows that there were similar patterns of enzyme responses across all nine land uses with highest activity in grasslands (mainly fertile and infertile acid) and heath/bog and relatively little difference in enzyme rates between the remaining land uses (crops+weeds, woodlands and moorland grass mosaic). The statistics in Table 6-3 and Figure 6-5 further demonstrate that the overlapping ranges in standard errors and confidence intervals would make it difficult to establish typical values for semi-natural habitats, except heath/bog. However the results suggest it would be possible to establish typical ranges for enzyme responses within agricultural land uses (crops+weeds and grasslands).

Principle components analysis utilized data from all enzymes. The 3D plot of the results (Figure 6-6) illustrates that the multi-variate discrimination amongst the nine land uses is distinct. Analysis of variance showed significant effects of land use on the first three principle components axes. The first axis (PC1) explained 74% of the variance associated with this discrimination while PC2 and PC3 subsequently explained 15% of the variation; a grand total of 89%. Summary results for the PC axes illustrate the effects of each axis on the multiple enzyme responses by land use (Figure 6-7). The results show separation of the agricultural land uses (crops+weeds and grasslands) from the semi- natural land uses, mainly along PC3. PC1 and PC2 produced separation of crops+weeds from grasslands, and heath/bog from woodlands and moorland-grass mosaic along, with PC2 producing further separation of lowland and upland (coniferous) wooded from upland deciduous wooded and moorland-grass-mosaic. The loading values for the individual enzymes illustrate that the land use patterns in PC1 were produced by a combination of all enzymes while PC2 and PC3 reflect the responses primarily from four or five enzymes.

Table 6-3 Summary statistics for individual enzyme responses by land use (ηmol MUB g^{-1} soil h^{-1})

Enzyme		Cellobiohydrolase		Glucosaminidase		Glucosidase		Acid phosphatase		Galactos-aminidase		Xylosidase		Galactosidase		Sulphatase	
Code / Land Use		Means	s.e	Means	s.e	Means	s.e	Means	s.e	Means	s.e	Means	s.e	Means	s.e	Means	s.e
1	crops and weeds	56.1	13.0	153.4	24.5	437.7	48.1	437.6	54.4	42.8	8.4	50.3	10.7	34.1	11.8	80.3	11.4
3	fertile grassland	137.9	25.9	298.0	63.2	1002.2	145.5	1511.3	205.2	165.6	26.8	347.4	93.8	228.2	44.0	736.3	100.8
4A	infertile grassland (A)	108.8	21.1	294.4	48.8	1056.4	199.0	1509.9	179.9	169.4	29.9	312.2	43.4	217.6	48.0	831.4	110.1
4B	infertile grassland (B)	152.6	58.1	728.0	271.8	1530.1	328.6	2159.5	336.2	311.8	91.3	177.5	48.3	139.9	43.8	232.3	33.4
5	lowland wooded	113.4	33.5	253.8	71.8	829.1	193.3	943.0	335.4	139.8	39.2	305.3	113.3	187.1	71.8	115.8	45.8
6A	upland wooded (A)	118.6	47.0	486.6	199.6	876.3	297.4	1332.3	506.1	176.7	72.8	354.5	133.0	192.4	68.3	604.1	284.4
6B	upland wooded (B)	540.0	252.1	656.6	260.5	2038.3	765.1	1688.2	468.7	871.6	676.9	692.7	226.3	373.5	135.1	169.0	81.6
7	moorland grass/mosaic	181.4	67.5	301.8	75.6	1012.8	309.7	2052.6	691.4	226.0	76.3	676.8	252.5	205.5	92.0	547.0	241.0
8	heath/bog	146.1	23.4	462.1	145.2	1684.6	563.1	6701.2	1173.7	419.6	138.6	897.1	202.0	567.6	240.0	544.8	107.5
overall		170.6	31.6	389.5	48.7	1148.1	128.8	2054.0	250.4	275.4	77.4	429.5	54.2	242.5	37.8	449.7	53.3

Table 6-4 Results from ANOVA to test for the significance of effects of sampling month and enzymes on enzyme responses.

Enzyme	SS	df	MS	F	p
Intercept	16281	1	16281	6015	0.000
Month sampled	324.13	5	64.83	24	0.000
enzyme	537.96	7	76.85	29	0.000
Month *enzyme	66.16	35	1.89	0.7	0.905
Error	2057	760	2.71		

Table 6-5 Results from ANOVA to test for the significance of land use effects on individual enzyme responses.

Enzyme	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
Cellobiohydrolase	14.67	8	1.83	300.65	92	3.27	0.56	0.807
Glucosaminidase	20.84	8	2.60	241.05	92	2.62	0.99	0.446
Glucosidase	20.85	8	2.61	129.37	92	1.41	1.85	0.077
Acid phosphatase	54.67	8	6.83	80.48	92	0.87	7.81	**0.000**
Galactosaminidase	55.71	8	6.96	298.24	92	3.24	2.15	**0.039**
Xylosidase	73.48	8	9.19	276.10	92	3.00	3.06	**0.004**
Galactosidase	99.40	8	12.43	309.81	92	3.37	3.69	**0.001**
Sulphatase	139.28	8	17.41	333.08	92	3.62	4.81	**0.000**

Figure 6-3 Mean enzyme responses (log ηmol MUB g^{-1} soil h^{-1}+1) by month sampled

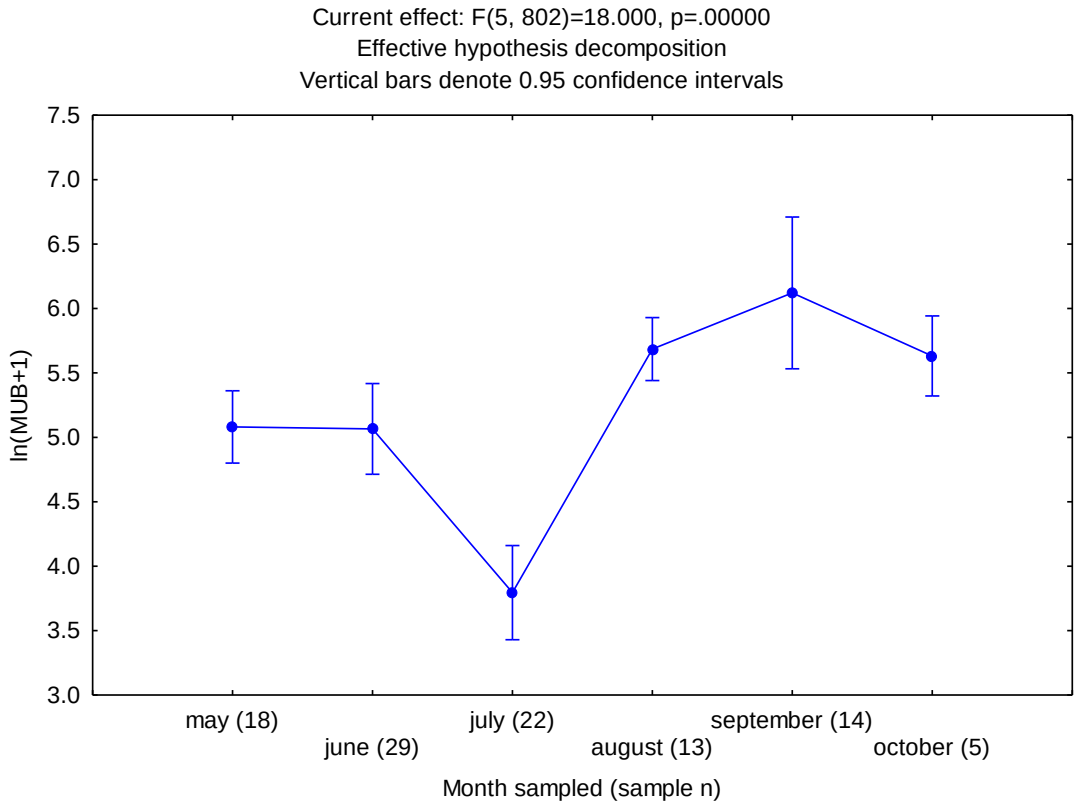


Figure 6-4 Mean enzyme responses (log ηmol MUB g^{-1} soil h^{-1}+1) by land use

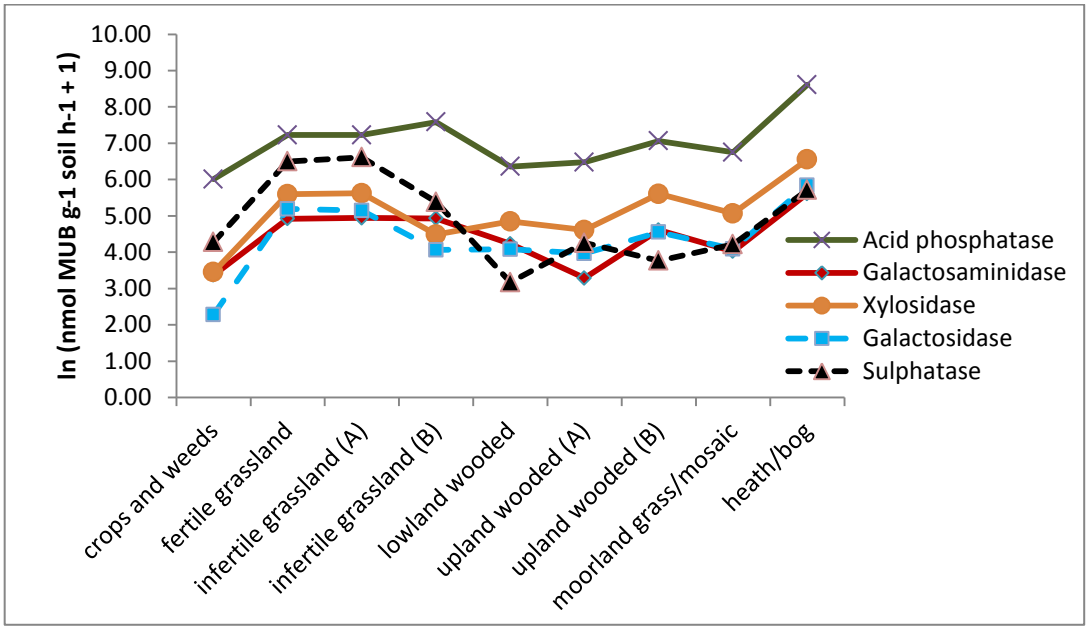


Figure 6-5 Box-whisker plots of enzyme responses (log ηmol MUB g^{-1} soil h^{-1}+1) by land use

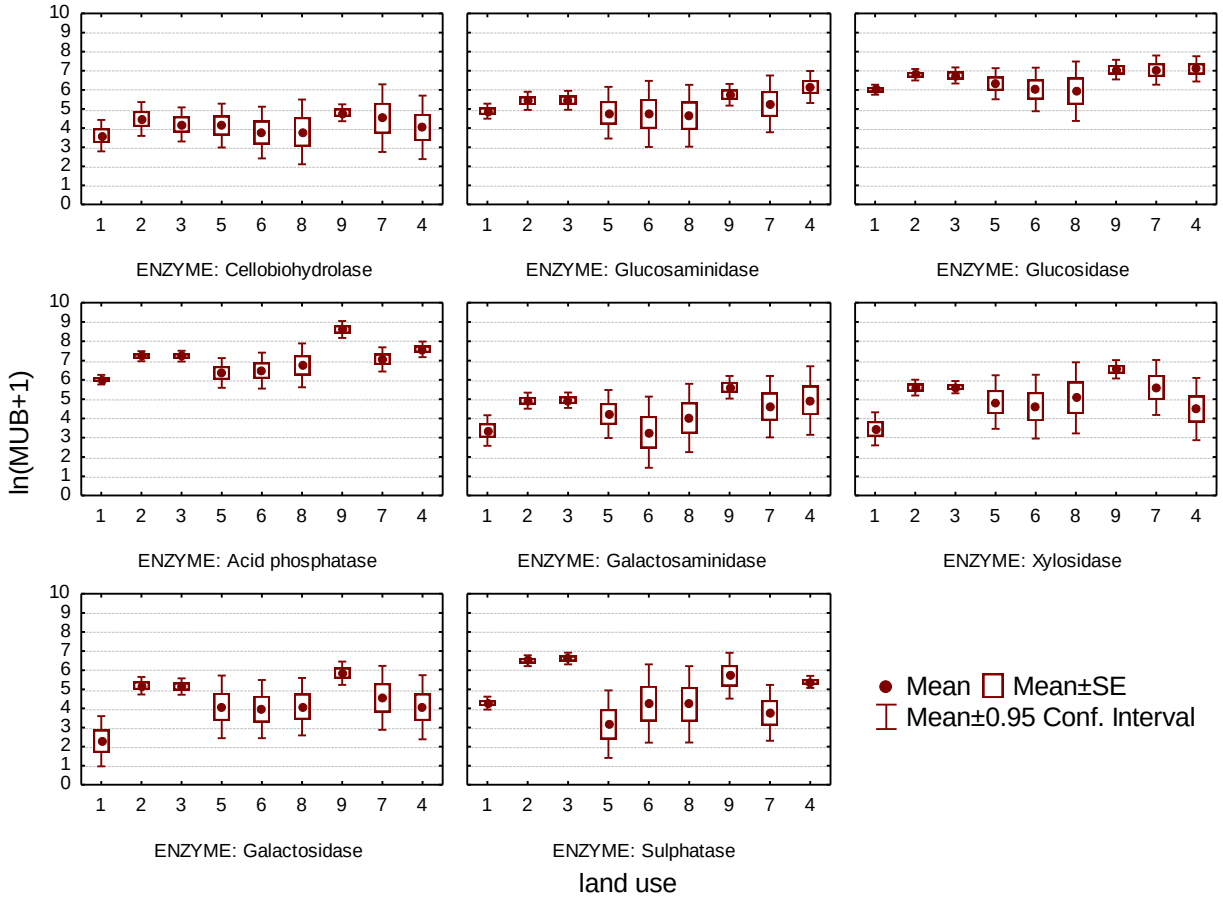
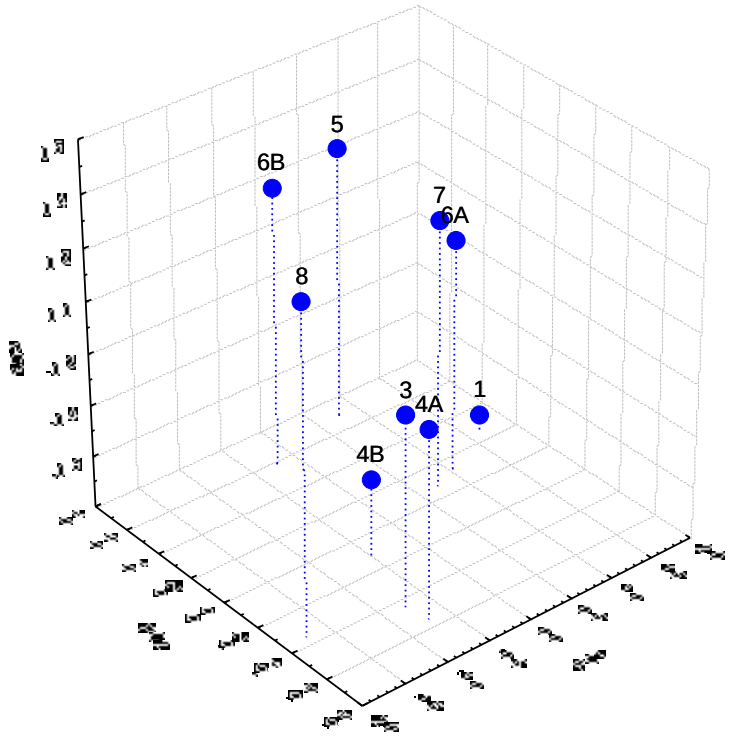


Figure 6-6 3D plot of land use results using the first three axes of PCA for the multi-enzyme assay

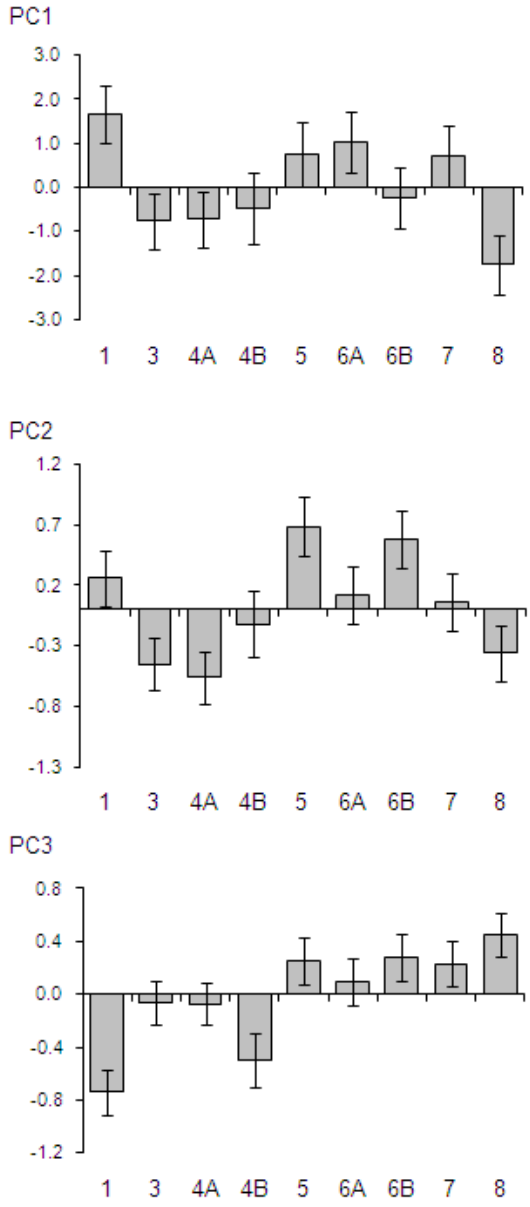
	PC1	PC2	PC3
%Eigenvalue	74	9	6
ANOVA Px	0.012	0.001	0.000



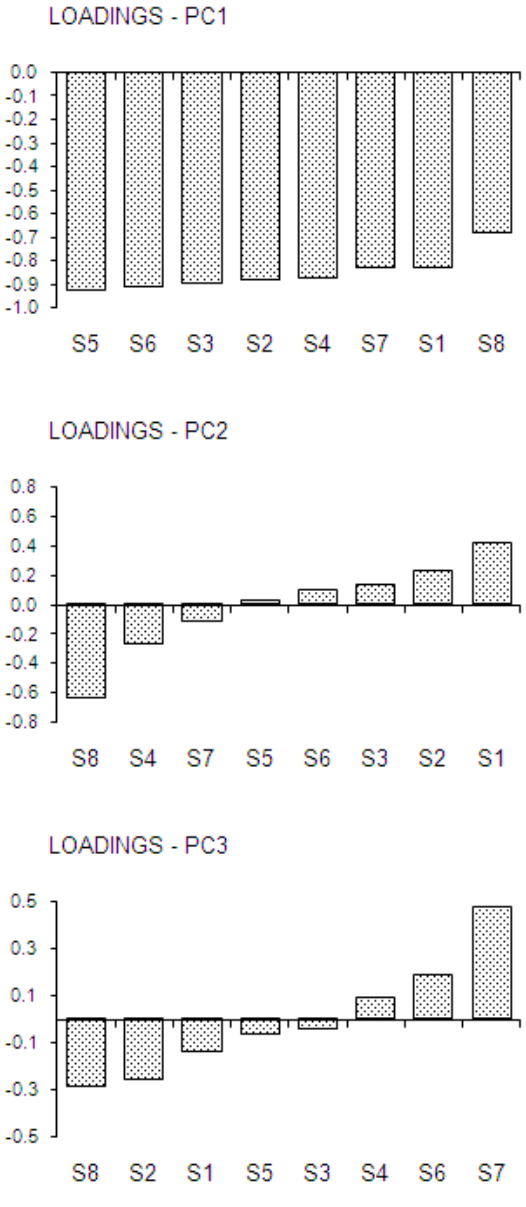
Key to land uses	
1	crops+weeds
3	fertile grass
4A	infertile acid grass
4B	infertile calcareous grass
5	lowland deciduous wooded
6A	upland deciduous wooded
6B	coniferous deciduous wooded
7	moorland grass mosaic
8	heath/bog

Figure 6-7 Summary results for PC1, PC2 and PC3 from PCA of the multi-enzyme assay

(a) Land use means +/- 1 s.e



(b) Loadings for individual enzymes



1	Crops+weeds
3	Fertile grassland
4A	Infertile grassland (acid)
4B	Infertile grassland (calcareous)
5	Lowland wooded
6A	Upland wooded (deciduous)
6B	Upland wooded (coniferous)
7	Moorland grass mosaic
8	Heath / bog

S1	Cellobiohydrolase
S2	Glucosaminidase
S3	Glucosidase
S4	Acid phosphatase
S5	Galactosaminidase
S6	Xylosidase
S7	Galactosidase
S8	Sulphatase

6.2.2 Multiple substrate induced respiration (MSIR)

The MSIR method was applied in the same way as for the sensitivity trial with no modifications (Appendix A). A total of seven carbon-based substrates, along with water, were used in the method. Table 6-6 presents summary results for CO_2-C respiration from the individual substrates and water (included as a "substrate" for ease of presentation) by the nine land use classes. All respiration data required transformation (ln n+1) prior to statistical analysis.

Analysis of variance showed that there were significant effects of sampling month and substrate on overall respiration responses with no interaction between the two (Table 6-7). Carbon substrate responses were lower in the month of May compared to the other months (Figure 6-8). Univariate analysis of variance indicates that there were significant effects of land use on all substrates (Table 6-8). The response patterns (log data) for these are illustrated in Figure 6-9. This shows that there were similar patterns of substrate responses across the semi-natural habitats (woodlands, moorland-grass mosaic and heath/bog) with highest respiration from moorland grass mosaic and heath/bog. Respiration across the substrates was less consistent. Respiration was lower in crops+weeds compared to grasslands for the majority of substrates while respiration responses across grasslands differed by substrate. The statistics in Table 6-6 and Figure 6-10 it would be possible to define typical ranges for carbon substrate responses which could be used to discriminate between crops+weeds and grasslands and between woodlands, moorland grass mosaic and heath/bog.

Multivariate analyses by PCA utilized all the MSIR data. The 3D plot of the MSIR results (Figure 6-11) illustrates that the multivariate discrimination amongst the nine land uses is distinct. Analysis of variance showed significant effects of land use on the first three principle components axes. The first axis (PC1) explained 86% of the variance associated with this discrimination while PC2 and PC3 subsequently explained 10% of the variation; a grand total of 96%. Summary results for the PC axes illustrate the effects of each axis on MSIR by land use (Figure 6-12). The results show separation along PC1 of crops+weeds from grasslands, woodlands, moorland-grass mosaic and heath bog, with the agricultural land uses (crops+weeds and grasslands) from the semi- natural land uses, mainly along PC3. PC1 and PC2 produced separation of crops+weeds and woodlands from grasslands, moorland-grass mosaic and heath/bog. PC2 produced separation of crops+weeds and infertile calcareous grassland from the other land uses. PC3 produced further separation of the agricultural land uses from moorland-grass mosaic and heath/bog.

The loading values for the individual substrate illustrate that the land use patterns in PC1 were produced by a combination of all substrates while PC2 and PC3 reflect the responses primarily from four or five substrates.

Table 6-6 Summary statistics for MSIR substrate responses (µg CO_2-C g^{-1} h^{-1})

AV code	MSIR substrate	Arginine		Citric acid		GABA		Glucose		AKGA		Malic acid		NAGA		Water	
	Land Use	Means	s.e	Means	s.e	Means	s.e	Means	s.e	Means	s.e	Means	s.e	Means	s.e	Means	s.e
1	crops and weeds	0.89	0.17	3.44	0.37	1.01	0.10	2.47	0.22	4.81	0.51	3.58	0.44	1.35	0.15	0.58	0.08
3	fertile grassland	4.19	0.88	3.78	0.71	3.09	0.86	6.80	1.72	5.96	0.77	6.23	1.56	5.61	2.16	1.45	0.41
4A	infertile grassland (A)	3.44	0.46	3.97	0.57	2.70	0.41	5.53	0.68	6.20	0.84	5.65	0.71	3.58	0.51	1.52	0.26
4B	infertile grassland (B)	2.70	0.47	9.34	1.25	3.17	0.45	6.67	0.90	11.13	1.98	9.61	1.32	3.78	0.52	2.49	0.37
5	lowland wooded	3.26	1.13	3.52	0.92	2.17	0.68	4.27	1.21	5.16	1.34	6.06	1.50	2.52	0.75	1.23	0.43
6A	upland wooded (A)	3.36	0.64	3.49	0.64	2.17	0.41	4.69	0.88	5.53	1.05	6.10	0.99	2.63	0.53	1.34	0.30
6B	upland wooded (B)	4.09	1.19	3.56	1.15	3.04	1.02	4.91	1.46	5.08	1.55	6.69	1.92	3.44	1.14	1.60	0.58
7	moorland grass/mosaic	5.19	0.77	4.61	1.04	3.55	0.76	6.62	1.61	6.23	1.31	10.88	2.06	3.33	0.70	1.79	0.33
8	heath/bog	10.72	1.63	8.94	1.52	6.70	1.40	12.13	1.74	12.23	1.51	21.90	2.09	6.43	1.30	4.00	1.04

Table 6-7 Results from ANOVA to test for the significance of effects of sampling month and substrate on MSIR.

MSIR	SS	df	MS	F	p
Intercept	1342.09	1	1342	3778.5	0.000
Month sampled	70.17	7	10.02	28.2	0.000
substrate	25.80	5	5.16	14.5	0.000
Month *substrate	3.52	35	0.10	0.28	0.999
Error	267.1	752	0.355		

Table 6-8 Results from ANOVA to test for the significance of land use effects on substrates used in the MSIR

Substrate	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
Arginine	20.25	8	2.53	21.08	92	0.23	11.05	**0.000**
Citric acid	10.79	8	1.35	25.47	92	0.28	4.87	**0.000**
GABA	9.60	8	1.20	25.73	92	0.28	4.29	**0.000**
Glucose	12.42	8	1.55	26.39	92	0.29	5.41	**0.000**
AKGA	10.10	8	1.26	28.11	92	0.31	4.13	**0.000**
Malic acid	21.30	8	2.66	21.80	92	0.24	11.24	**0.000**
NAGA	8.43	8	1.05	27.54	92	0.30	3.52	**0.001**
Water	6.45	8	0.81	22.16	92	0.24	3.35	**0.002**

Figure 6-8 Mean MSIR responses (ln µg CO_2-C g^{-1} h^{-1} +1) by month sampled

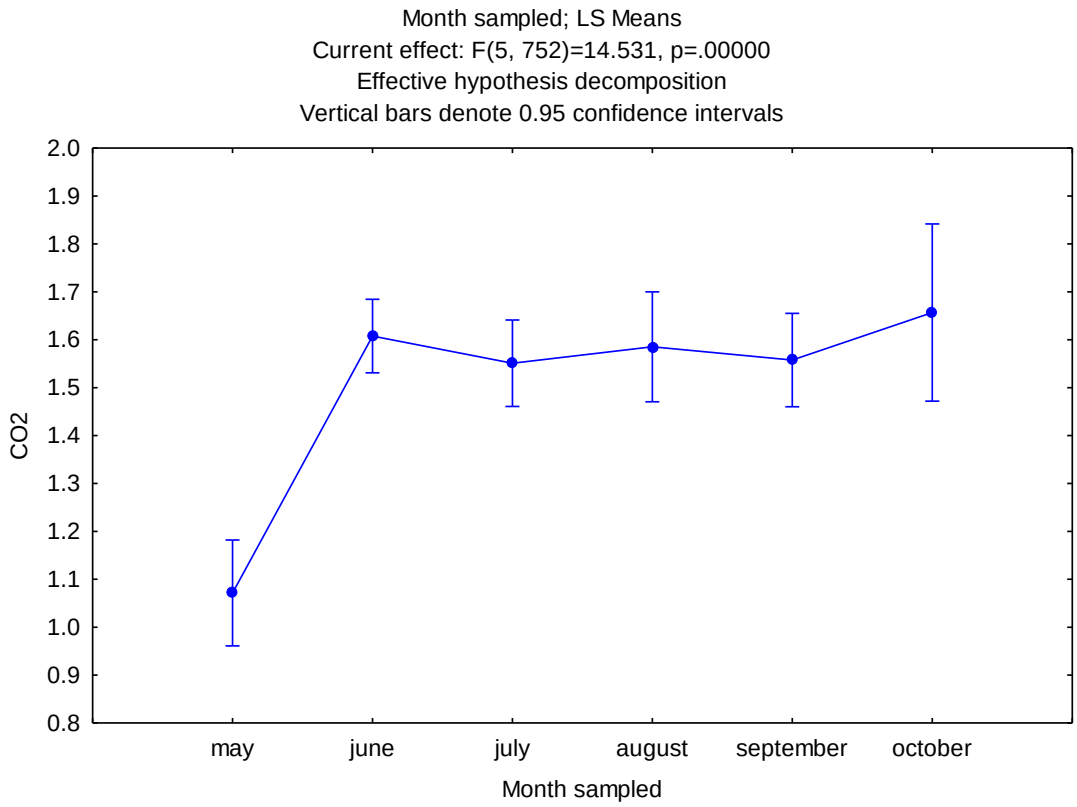


Figure 6-9 Mean substrate responses (ln µg CO_2-C g^{-1} h^{-1} +1) by land use

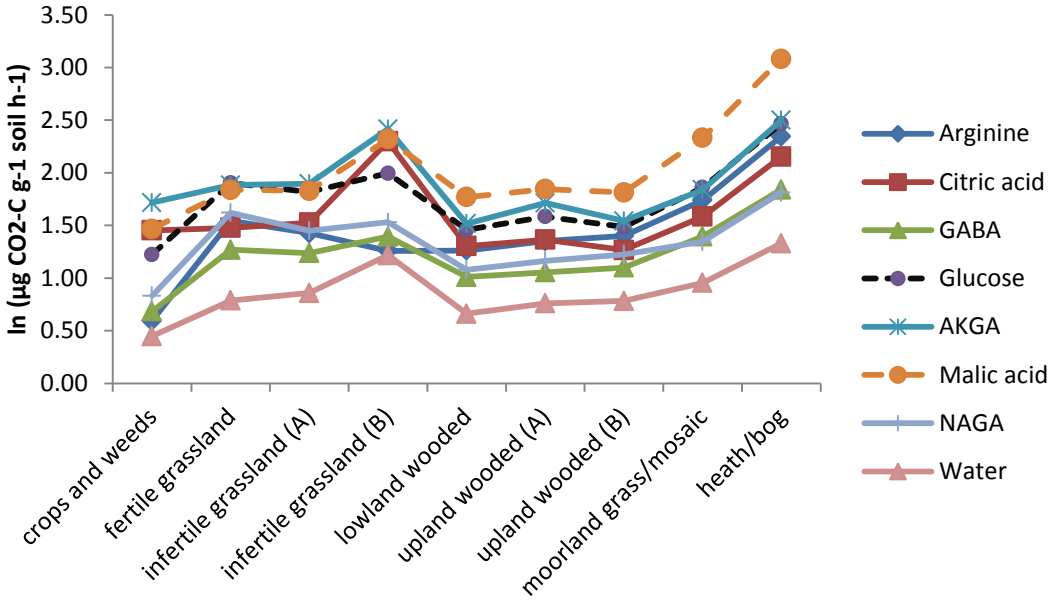


Figure 6-10 Box-whisker plots of carbon substrate responses (ln µg CO_2 g^{-1} h^{-1} +1) by land use

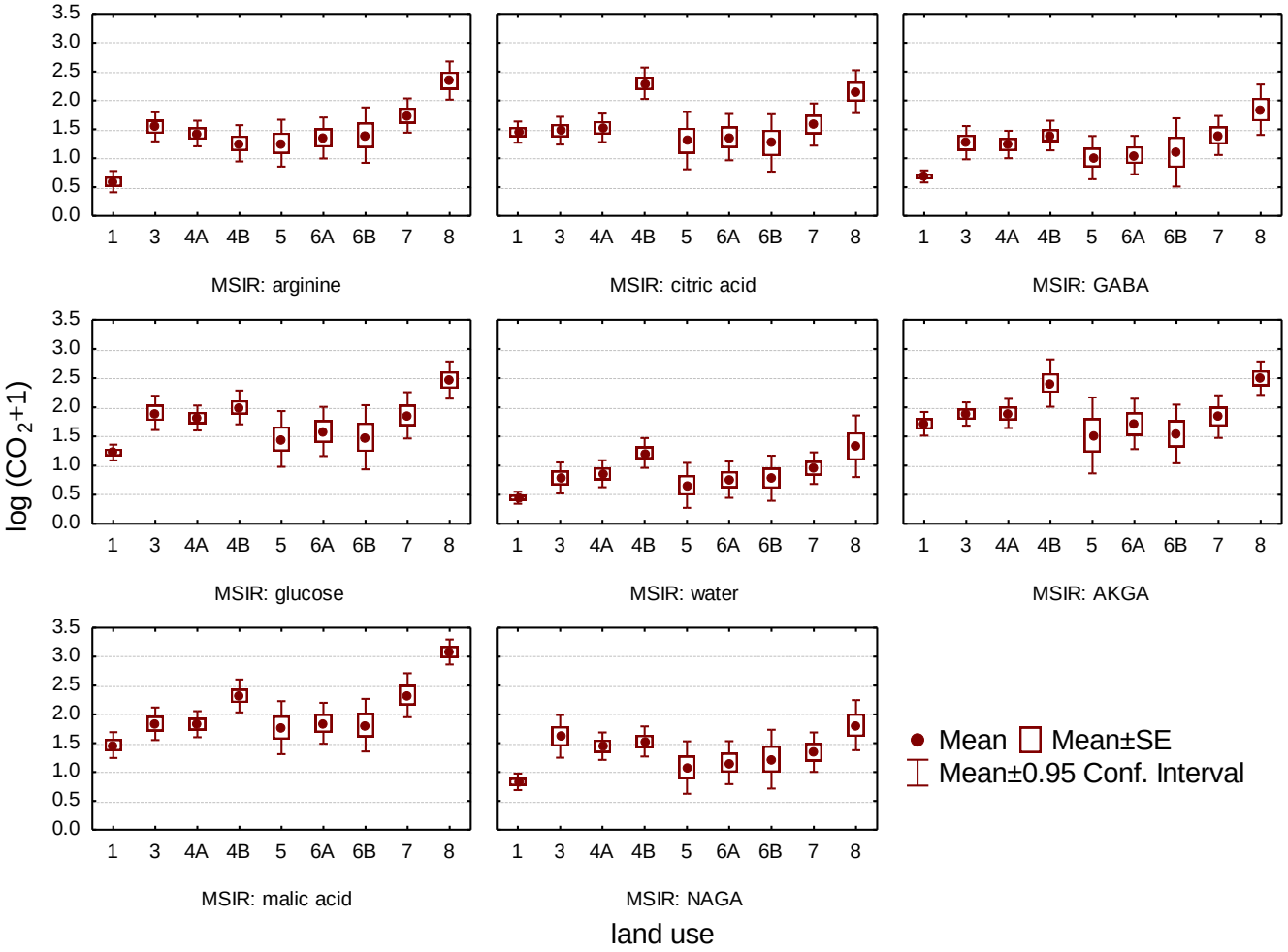
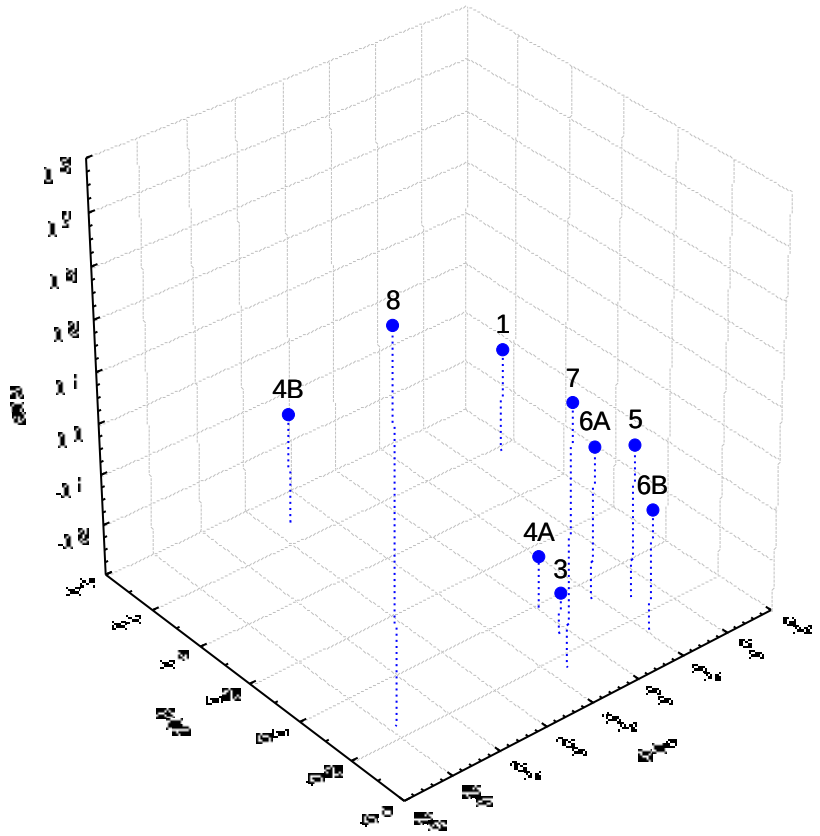


Figure 6-11 3D plot of land use results using the first three axes of PCA for MSIR

	PC1	PC2	PC3
%Eigenvalue	86	6	4
ANOVA Px	0.000	0.000	0.000

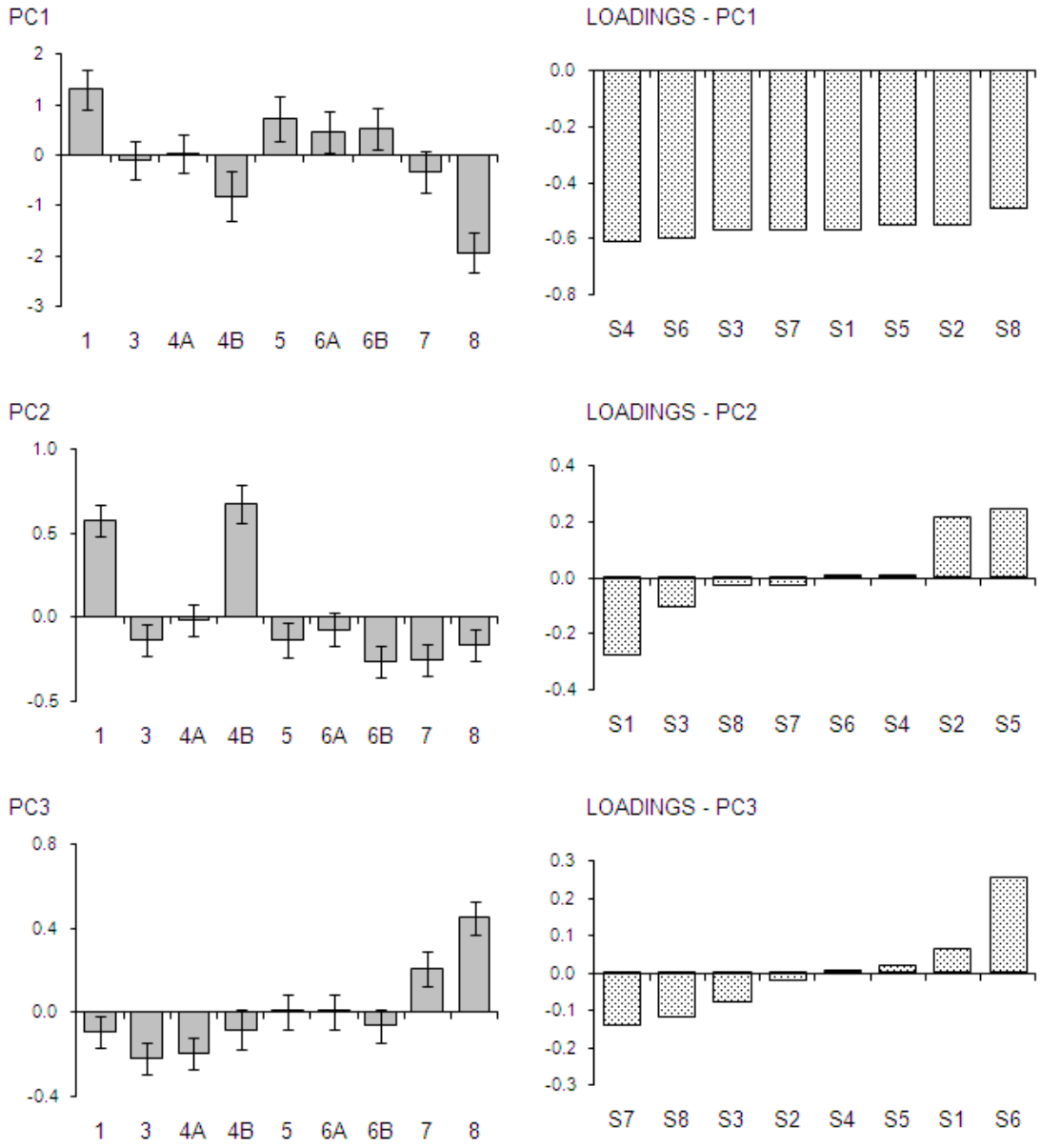


Key to land uses	
1	crops+weeds
3	fertile grass
4A	infertile acid grass
4B	infertile calcareous grass
5	lowland deciduous wooded
6A	upland deciduous wooded
6B	coniferous deciduous wooded
7	moorland grass mosaic
8	heath/bog

Figure 6-12 Summary results for PC1, PC2 and PC3 from PCA for MSIR

(a) Land use means +/- 1 s.e

(b) Loadings for individual substrates



1	Crops+weeds
3	Fertile grassland
4A	Infertile grassland (acid)
4B	Infertile grassland (calcareous)
5	Lowland wooded
6A	Upland wooded (deciduous)
6B	Upland wooded (coniferous)
7	Moorland grass mosaic
8	Heath / bog

1	Arginine
2	Citric acid
3	GABA
4	Glucose
5	AKGA
6	Malic acid
7	NAGA
8	Water

6.2.3 TRFLP

The multiplex TRFLP method was applied in the same way as for the sensitivity trial with no modifications (Appendix A). The genotypic structure of bacterial, fungal and archaeal microbial groups was assessed using 16S, ITS, & 16S rRNA primers, respectively. Raw TRF data required transformation (ln n+1) prior to statistical analysis. Diversity indices were calculated from the relative abundance of the TRFs in each microbial group. These indices did not require transformation. The following described the results for the three microbial groups individually.

6.2.3.1 Bacterial TRFs

A total of 148 individual TRFs were characterized from the multiplex method. The majority of TRFs were of low relative abundance (<1%) with only two TRFs exceeding 10% average relative abundance. Across all samples, only 19 TRFs exceeded 10% of the community. Thus the microbial communities are typified by a large number of relatively low abundance genotype units. Given the large number of low abundance results, it would be inappropriate to statistically analyse individual TRFs. Diversity indices were used to characterise bacterial community structure using TRFs for univariate analyses.

Table 6-9 presents summary results for diversity indices of the bacterial community by the nine land use classes. This illustrates that there was an average of 30 to 39 TRFs per land use class. Analysis of variance showed that there were no significant effects of sampling month (not shown) or land use on the bacterial diversity indices (Table 6-10). Representatibe patterns for these indices are shown in Figure 6-13. This shows that there were similar subtle patterns in diversity across the land uses with highest diversity in grasslands and lowest diversity in moorland grass mosaic and heath/bog.

Multivariate analyses by PCA utilized all the 16S bacterial TRF data. The 3D plot of the bacterial 16S TRF results (Figure 6-14) illustrates that the multivariate discrimination amongst the nine land uses is more distinct than that for the univariate results. However analysis of variance did not show significant effects of land use on either of the first three principle components axes. Summary results for the PC axes illustrate that there are trends for moorland-grass mosaic and heath/bog being different to the other land uses on PC1, and agricultural land uses to be different from semi-natural land uses in PC2 (Figure 6-15). The loading values for the individual TRFs illustrate that the majority of the TRFs were contributing to the (lack of) land use discrimination.

Further PCA was carried out on a reduced set of 16S bacterial TRFs which were identified by a process of including the most abundance TRFs which did not cross correlate with each other. The 3D plot of this reduced set (Figure 6-16) demonstrates a similar discrimination pattern to that of the full set of bacterial TRFs. In this instance analysis of variance showed significant effects of land use on the second principle components axis.

Summary results for the PC axes illustrate the effects of each axis on multiple TRFs by land use (Figure 6-17). The results show similar patterns to the full set of TRFs with trends for differences in moorland-grass mosaic and heath/bog compare to the other land uses on PC1, and agricultural land uses different to semi-natural land uses in PC2. Here the loading values for the individual substrate illustrate that the land use patterns in PC1 and PC2 were produced by a combination of the range of TRFs in the reduced set. This demonstrates that bacterial community structure could be characterized by a limited number of TRFs but further information and analyses is required to identify the distinctive characteristics of bacterial community structure for individual land uses.

Table 6-9 Summary statistics for 16S bacteria diversity indices

Diversity index		16S richness		16S Shannon H'		16S Shannon E		16S McIntosh E		Simpson J'	
code	Land Use	Means	s.e	Means	s.e	Means	s.e	Means	s.e	Means	s.e
1	crops and weeds	33.58	2.13	2.96	0.10	0.85	0.02	0.85	0.02	33.58	2.13
3	fertile grassland	39.23	1.73	3.18	0.08	0.87	0.01	0.87	0.01	39.23	1.73
4A	infertile grassland (A)	39.31	1.65	3.19	0.07	0.87	0.01	0.87	0.01	39.31	1.65
4B	infertile grassland (B)	37.13	3.82	3.13	0.16	0.87	0.02	0.87	0.02	37.13	3.82
5	lowland wooded	37.70	2.84	3.07	0.13	0.85	0.02	0.85	0.02	37.70	2.84
6A	upland wooded (A)	34.18	2.72	3.01	0.11	0.86	0.01	0.86	0.01	34.18	2.72
6B	upland wooded (B)	34.91	2.45	3.01	0.10	0.85	0.01	0.85	0.01	34.91	2.45
7	moorland grass/mosaic	33.82	2.24	2.94	0.10	0.84	0.01	0.84	0.01	33.82	2.24
8	heath/bog	30.08	1.61	2.81	0.07	0.83	0.01	0.83	0.01	30.08	1.61

Table 6-10 Results from ANOVA to test for the significance of land use effects on 16S bacteria diversity indices

Bacteria 16S	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	P
16S richness	888.88	8	111.11	5402.07	92	58.72	1.892	0.070
16S shannon H'	1.50	8	0.19	10.24	92	0.11	1.682	0.113
16S shannon E	0.02	8	0.00	0.20	92	0.00	1.247	0.281
16S McIntosh E	0.02	8	0.00	0.28	92	0.00	1.012	0.433

Figure 6-13 Mean diversity results for 16S bacterial TRFs by land use

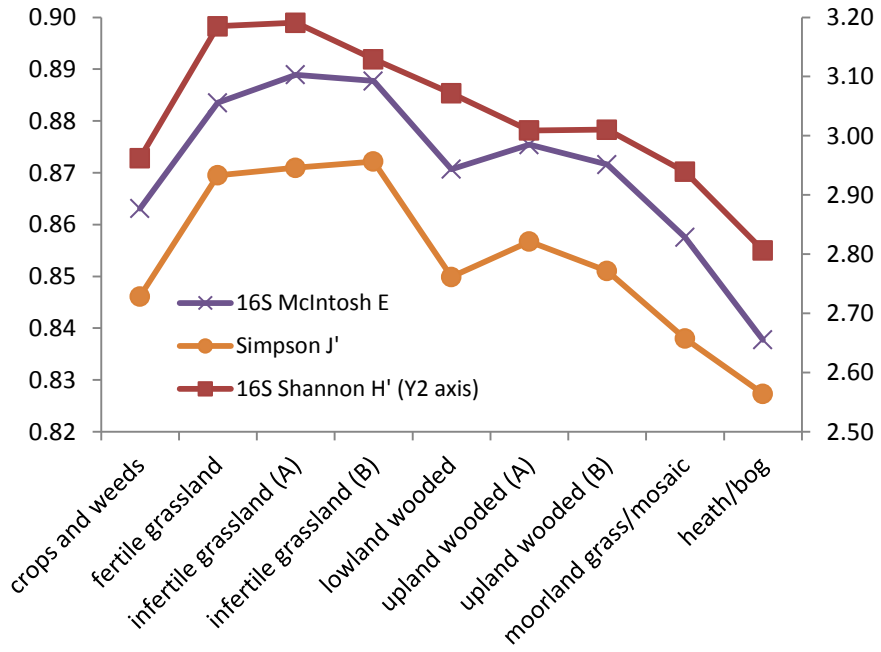
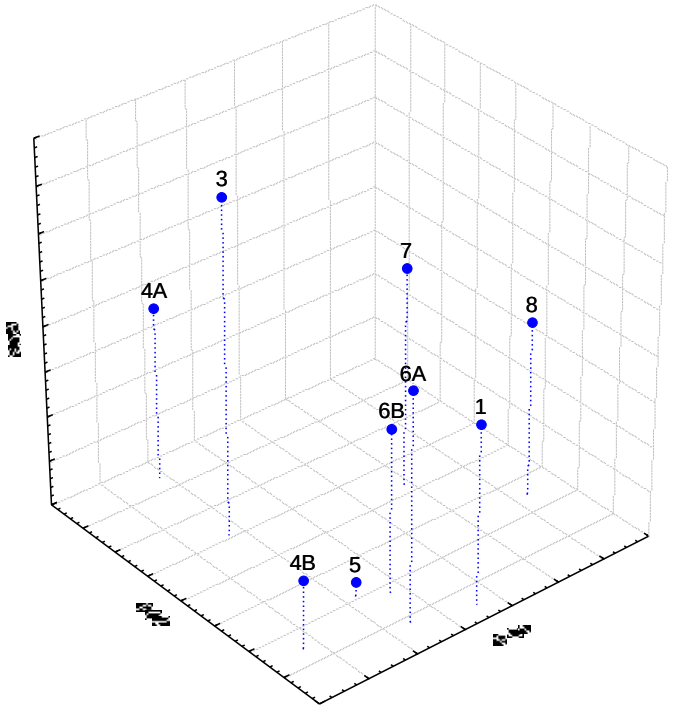


Figure 6-14 3D plot of land use results from the first three axes of PCA for 16S bacterial TRFs

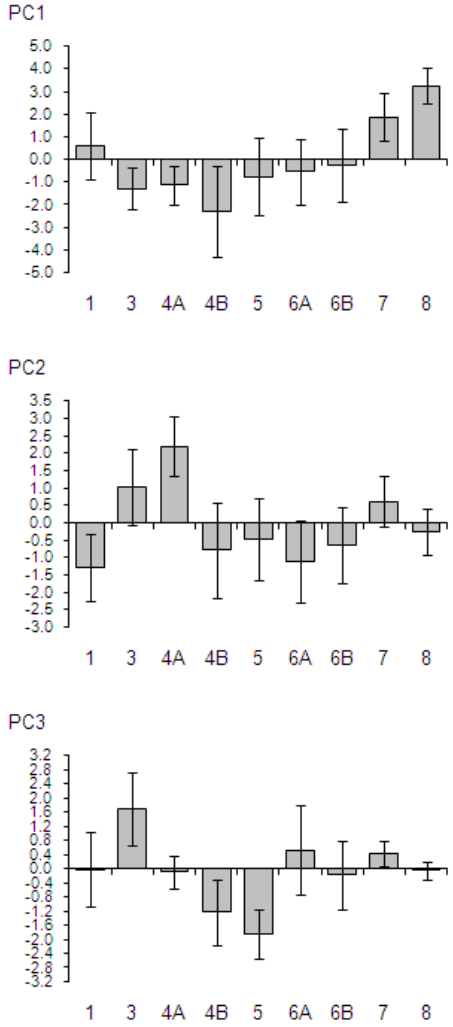
	PC1	PC2	PC3
%Eigenvalue	20.2	11.8	8.3
ANOVA Px	0.105	0.216	0.216



Key to land uses	
1	crops+weeds
3	fertile grass
4A	infertile acid grass
4B	infertile calcareous grass
5	lowland deciduous wooded
6A	upland deciduous wooded
6B	coniferous deciduous wooded
7	moorland grass mosaic
8	heath/bog

Figure 6-15 Summary results for PC1, PC2 and PC3 from PCA of 16S bacterial TRFs

(a) Land use means +/- 1 s.e



1	Crops+weeds
3	Fertile grassland
4A	Infertile grassland (acid)
4B	Infertile grassland (calcareous)
5	Lowland wooded
6A	Upland wooded (deciduous)
6B	Upland wooded (coniferous)
7	Moorland grass mosaic
8	Heath / bog

(b) Loadings for individual 16S TRFs

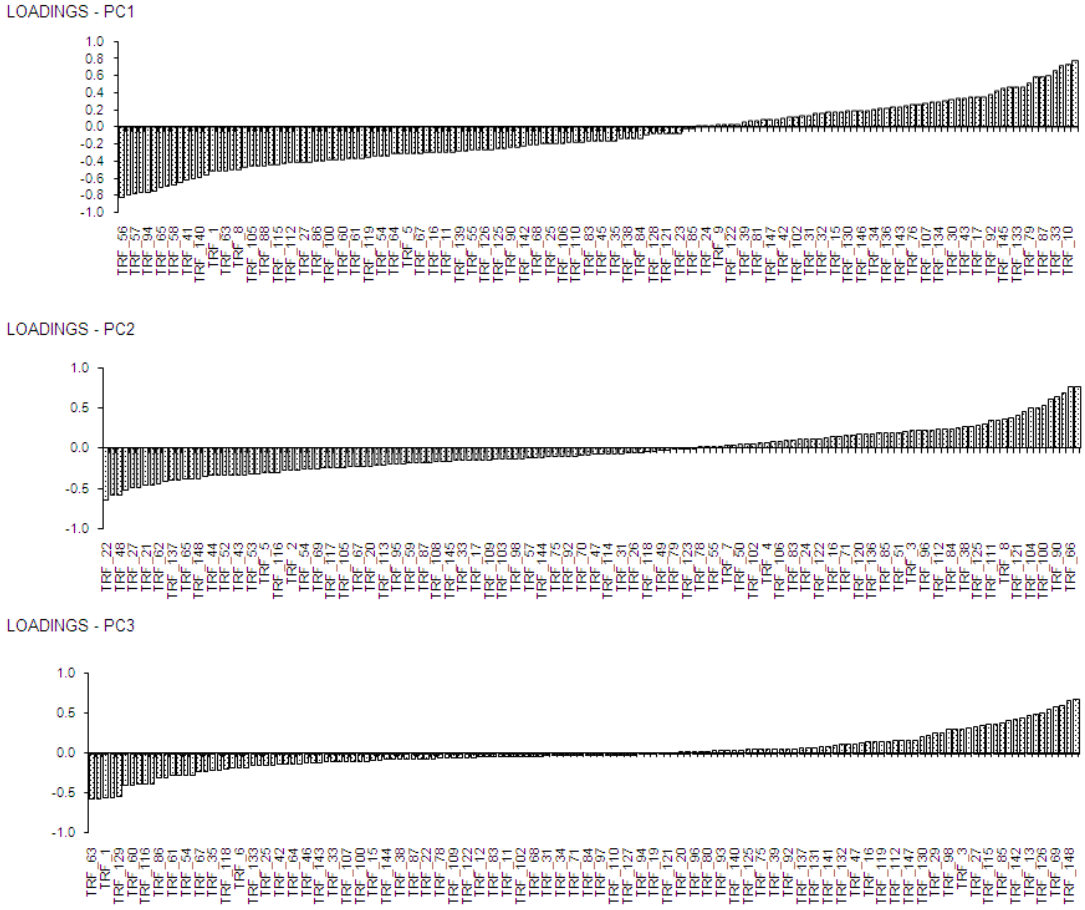
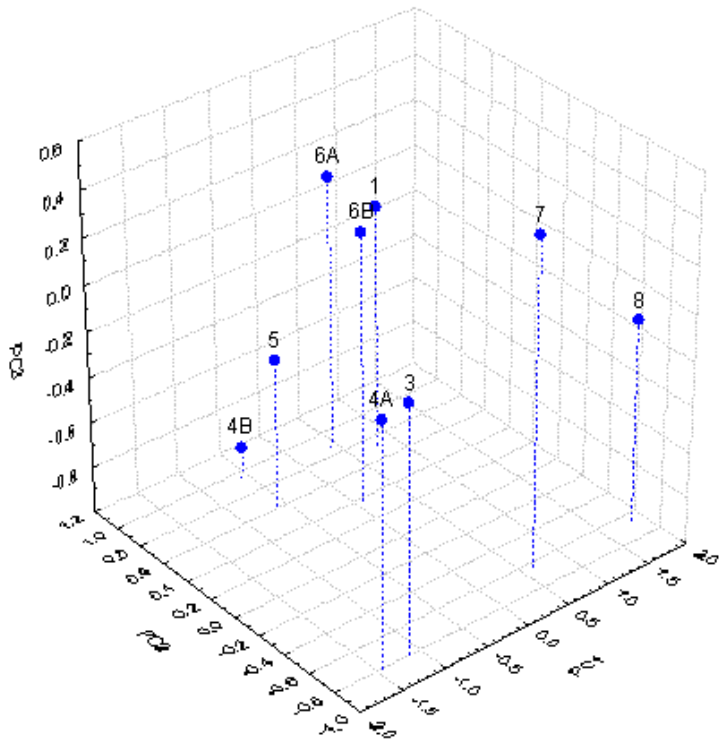


Figure 6-16 3D plot of land use results from the first three axes of PCA for reduced set of bacterial TRFs

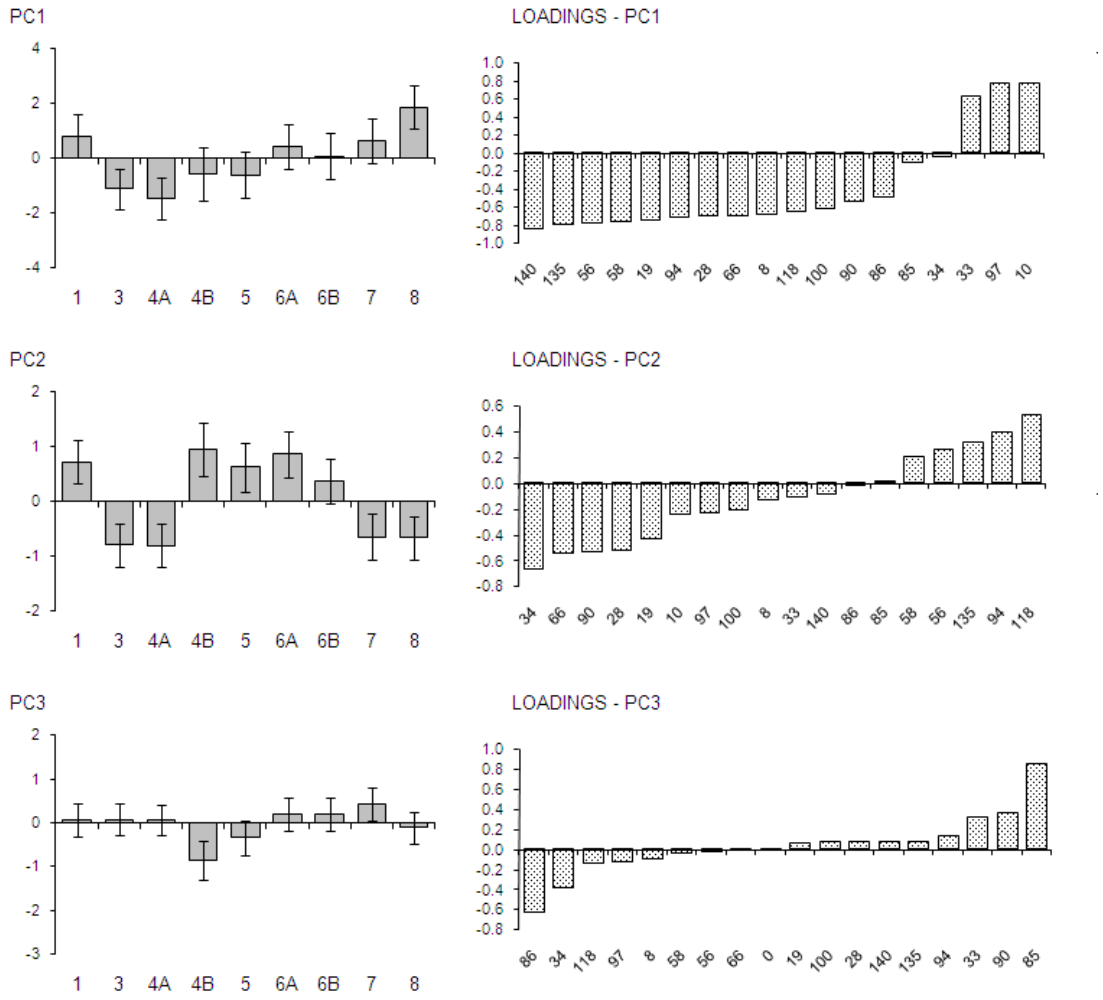
	PC1	PC2	PC3
%Eigenvalue	44.16	12.80	8.84
ANOVA Px	0.090	0.001	0.608



Key to land uses	
1	crops+weeds
3	fertile grass
4A	infertile acid grass
4B	infertile calcareous grass
5	lowland deciduous wooded
6A	upland deciduous wooded
6B	coniferous deciduous wooded
7	moorland grass mosaic
8	heath/bog

Figure 6-17 Summary results for PC1, PC2 and PC3 from PCA for reduced set of bacterial TRFs

(a) Land use means +/- 1 s.e (b) Loadings for individual TRFs



1	Crops +weeds
3	Fertile grassland
4A	Infertile grassland (acid)
4B	Infertile grassland (calcareous)
5	Lowland wooded
6A	Upland wooded (deciduous)
6B	Upland wooded (coniferous)
7	Moorland grass mosaic
8	Heath / bog

6.2.3.2 Archaeal TRFs

A total of 42 individual archaeal TRFs were characterized from the multiplex method. The majority of TRFs were of low relative abundance (<1%) with only two TRFs exceeding 10% average relative abundance. Across all samples, 26 TRFs exceeded 10% of the community. Given the large number of low abundance results, it would be inappropriate to statistically analyse individual TRFs. Diversity indices were used to characterise archaeal community structure using TRFs for univariate analyses.

Table 6-11 presents summary results for diversity indices of the archaeal community by the nine land use classes. This illustrates that the average number of archaeal TRFs (richness) ranged from 4 to 10 per land use class. Analysis of variance showed that there were no significant effects of sampling month on the archaeal diversity indices (Table 6-12). The patterns for three of these diversity indices are shown in Figure 6-18. This shows that there were similar subtle patterns in diversity across the land uses with archaeal richness and Shannon H', with highest diversity in heath/bog There were no land use patterns for the diversity evenness indices (Shannon E and McIntosh E).

Multivariate analyses by PCA utilized all the archaeal TRF data. The 3D plot of the archaeal TRF results (Figure 6-19) illustrates that the multivariate discrimination amongst the nine land uses is distinct. Analysis of variance showed significant effects of land use on the first two principle components axes. The first axis (PC1) explained 7.78% of the variance associated with this discrimination while PC2 explained a further 7.39% of the variation; a grand total of 15.17% between the two axes. Summary results for the PC axes illustrate the effects of each axis on archaeal TRFs by land use (Figure 6-20a). The results show separation along PC1 of crops+weeds and infertile grasslands, from upland (coniferous), woodland moorland-grass mosaic and heath bog, with the remaining land uses between these two groups while PC2 produced separation of woodlands from grasslands. The loading values for the individual TRFs (Figure 6-20b) illustrate that the majority of the TRFs were contributing to land use discrimination.

Further PCA was carried out on a reduced set of archaeal TRFs (8 in total) which were identified by a process of including the most abundance TRFs which did not cross correlate with each other. The 3D plot of this much reduced set of archaeal TRFs (Figure 6-21) demonstrated discrimination of the land uses in a more consistent and interpretable pattern to that of the full set of archaeal TRFs. In this instance analysis of variance also showed significant effects of land use on the first and second principle components axis. The first axis (PC1) explained 27% of the variance associated with this discrimination while PC2 explained a further 21% of the variation; a grand total of 48% between the two axes. Summary results for the PC axes illustrate the effects of each axis on multiple TRFs by land use (Figure 6-22). The results show different patterns to the full set of archaeal TRFs. Separation of agricultural land uses from semi-natural land uses is demonstrated on PC1, with further separation of lowland and upland deciduous wooded from the other semi-natural land uses. Fertile and infertile acid grasslands separate from other land uses on

PC2. The loading values for the individual TRFs (Figure 6-22) illustrate that the land use patterns in PC1 and PC2 were produced by a combination of the range of TRFs in the reduced set. This demonstrates that archaeal community structure could be characterized by a very limited number of TRFs. However further information and analyses would be required to identify the truly distinctive characteristics of archaeal community structure for individual land uses.

Table 6-11 Summary statistics for archaeal TRFs diversity indices

Diversity index		Archaeal shannon H'		Archaeal shannon E		Archaeal McIntosh E		Archaeal richness	
		Mean		Mean		Mean		Mean	
code	Land Use	s	s.e	s	s.e	s	s.e	s	s.e
1	crops and weeds	0.97	0.06	0.59	0.04	0.54	0.04	6.67	1.08
3	fertile grassland	1.26	0.13	0.72	0.07	0.67	0.07	6.08	0.94
4A	infertile grassland (A)	1.09	0.16	0.72	0.10	0.69	0.10	4.62	0.73
4B	infertile grassland (B)	1.00	0.08	0.66	0.06	0.60	0.07	5.13	0.74
5	lowland wooded	1.01	0.12	0.60	0.07	0.55	0.08	6.00	0.98
6A	upland wooded (A)	1.18	0.10	0.75	0.05	0.72	0.05	6.36	1.36
6B	upland wooded (B)	1.26	0.12	0.63	0.07	0.61	0.07	8.45	0.79
7	moorland grass/mosaic	1.38	0.10	0.73	0.04	0.71	0.04	7.55	1.04
8	heath/bog	1.54	0.06	0.69	0.04	0.68	0.05	10.50	1.12

Table 6-12 Results from ANOVA to test for the significance of land use effects on archaeal TRFs diversity indices

Archaea	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
Arc shannon H'	3.34	8	0.418	13.4	92	0.145	2.879	0.007
Arc shannon E	0.33	8	0.041	4.3	92	0.046	0.889	0.529
Arc McIntosh E	0.42	8	0.053	4.6	92	0.050	1.050	0.405
Arc richness	300.23	8	37.529	1050.5	92	11.419	3.287	0.002

Figure 6-18 Mean diversity results by land use for archaeal TRFs diversity indices

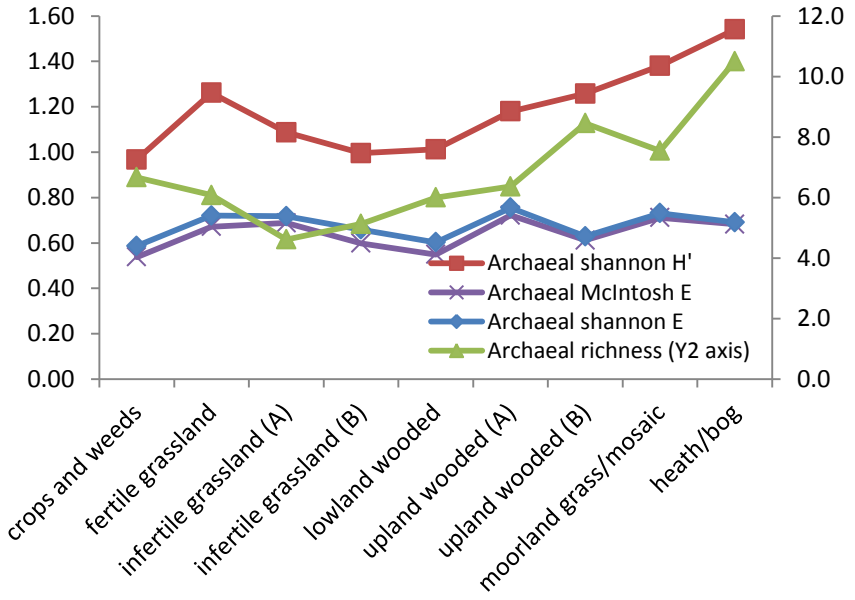
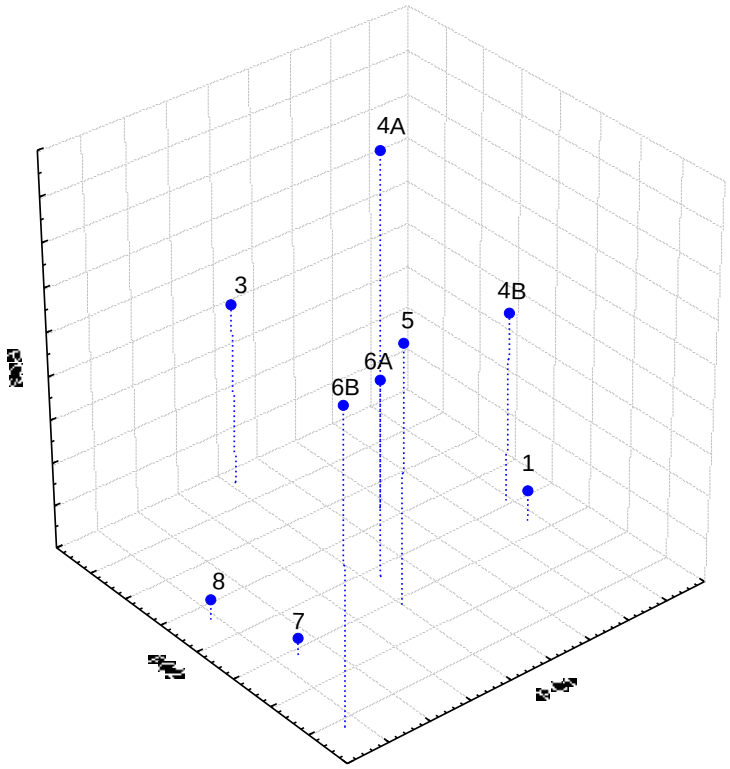


Figure 6-19 3D plot of land use results from the first three axes of PCA for archaeal TRFs

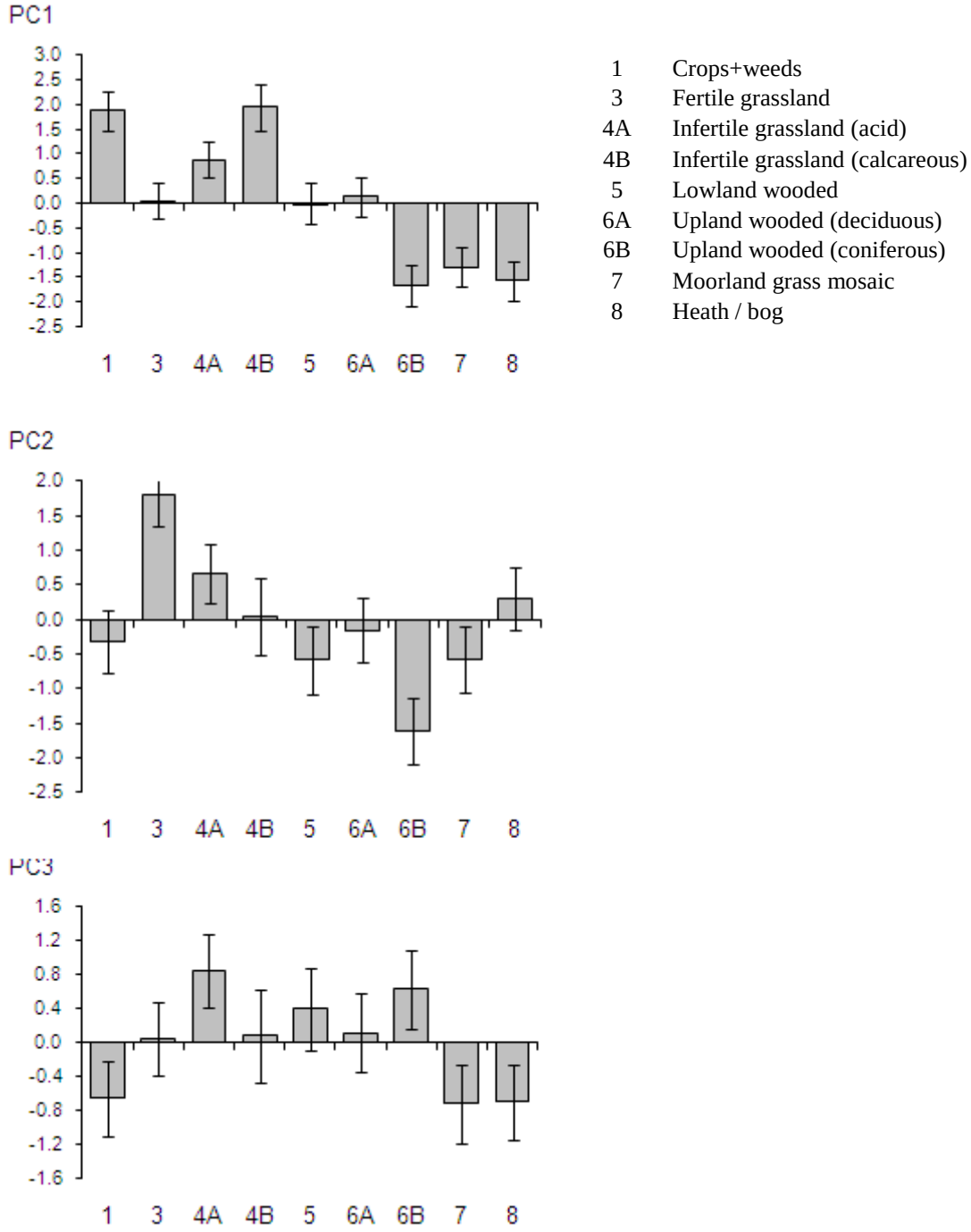
	PC1	PC2	PC3
%Eigenvalue	7.78	7.39	5.98
ANOVA Px	0.000	0.000	0.167



Key to land uses	
1	crops+weeds
3	fertile grass
4A	infertile acid grass
4B	infertile calcareous grass
5	lowland deciduous wooded
6A	upland deciduous wooded
6B	coniferous deciduous wooded
7	moorland grass mosaic
8	heath/bog

Figure 6-20 Summary results for PC1, PC2 and PC3 from PCA of archaeal TRFs

(a) Land use means +/- 1 s.e



1	Crops+weeds
3	Fertile grassland
4A	Infertile grassland (acid)
4B	Infertile grassland (calcareous)
5	Lowland wooded
6A	Upland wooded (deciduous)
6B	Upland wooded (coniferous)
7	Moorland grass mosaic
8	Heath / bog

(b) Loadings for individual Archaeal TRFs

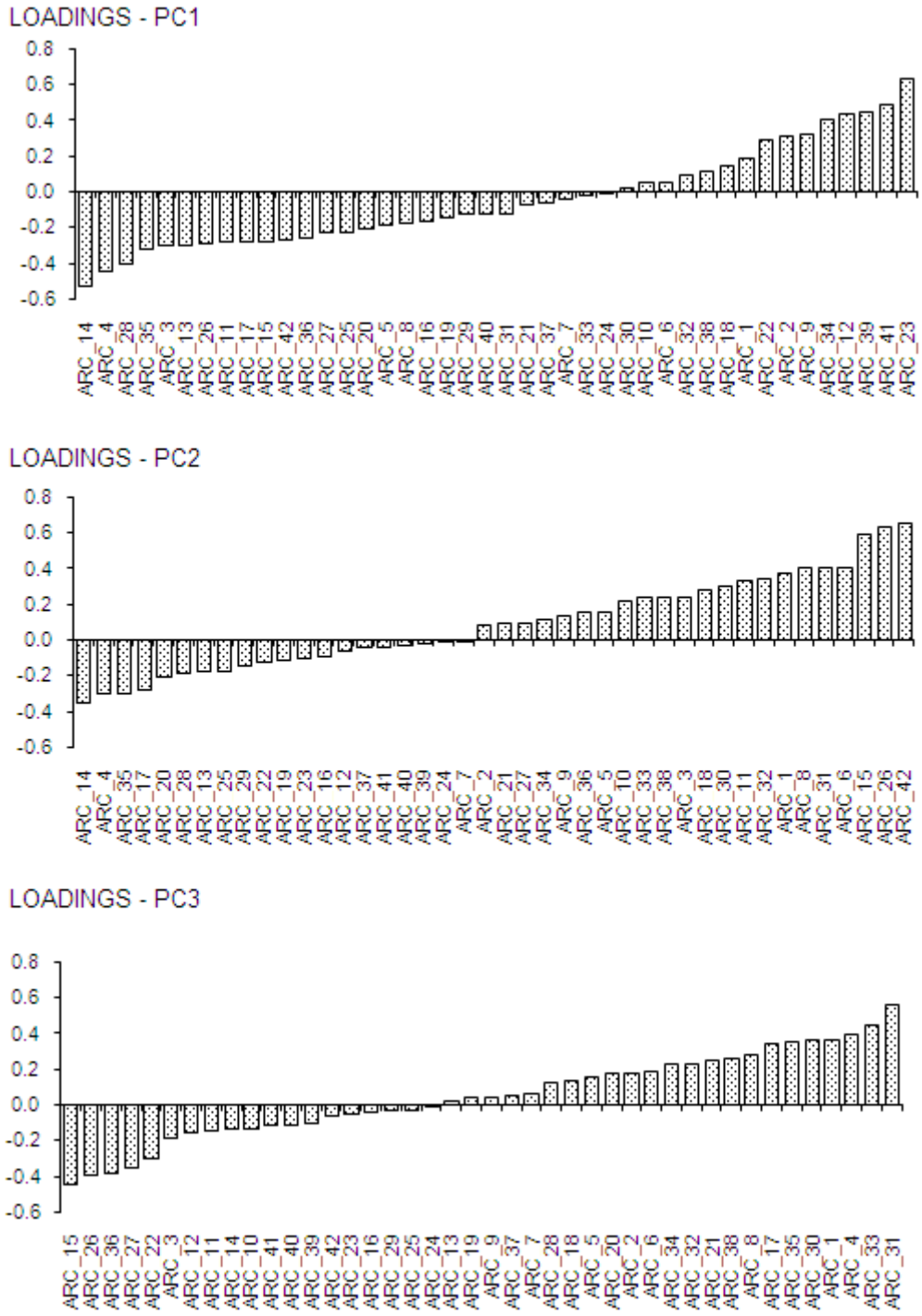
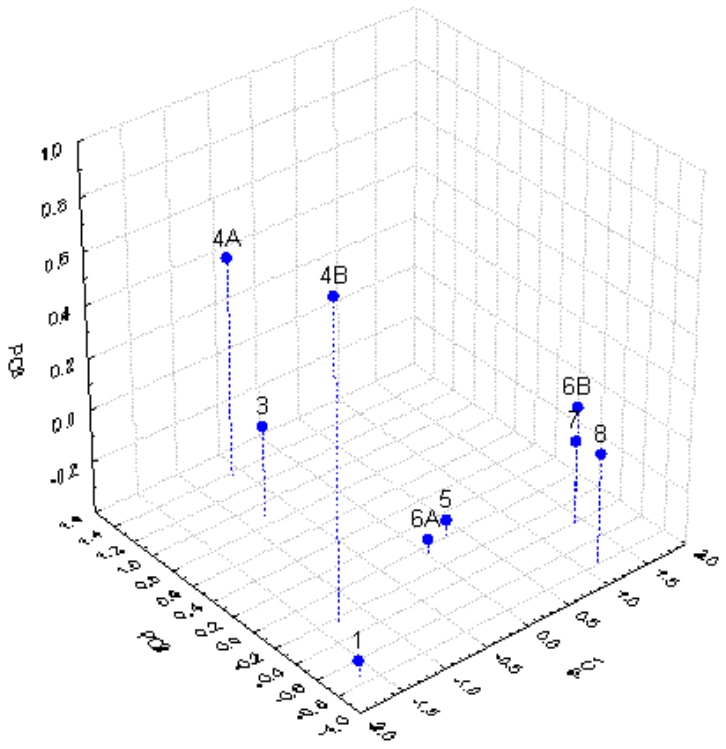


Figure 6-21 3D plot of land use results from the first three axes of PCA for reduced set of archaeal TRFs

	PC1	PC2	PC3
%Eigenvalue	27	21	13
ANOVA Px	0.000	0.000	0.167

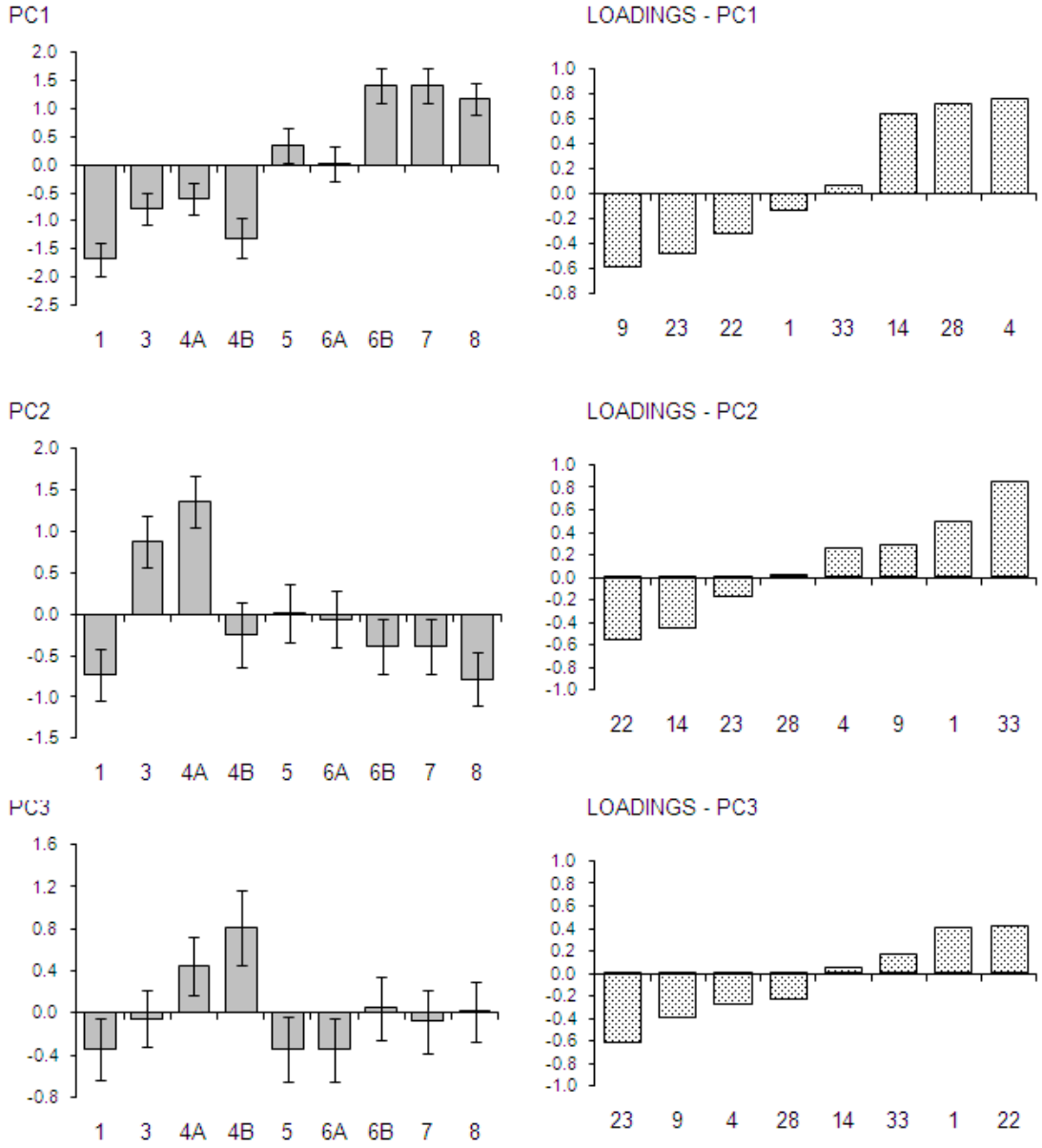


Key to land uses	
1	crops+weeds
3	fertile grass
4A	infertile acid grass
4B	infertile calcareous grass
5	lowland deciduous wooded
6A	upland deciduous wooded
6B	coniferous deciduous wooded
7	moorland grass mosaic
8	heath/bog

Figure 6-22 Summary results for PC1, PC2 and PC3 from PCA for reduced set of archaeal TRFs

(a) Land use means +/- 1 s.e

(b) Loadings for individual TRFs



1	Crops+weeds
3	Fertile grassland
4A	Infertile grassland (acid)
4B	Infertile grassland (calcareous)
5	Lowland wooded
6A	Upland wooded (deciduous)
6B	Upland wooded (coniferous)
7	Moorland grass mosaic
8	Heath / bog

6.2.3.3 Fungal TRFs

A total of 227 individual fungal ITS TRFs were characterized from the multiplex method. The average relative abundance for fungal ITS TRFs was < 6.5%, although 60 TRFs exceeded 10% relative abundance in individual samples. Given the large number of TRFs with low abundance, it would be inappropriate to statistically analyse individual TRFs. Diversity indices were used to characterise fungal community structure using ITS TRFs for univariate analyses.

Table 6-13 presents summary results for diversity indices of the fungal community by the nine land use classes. This illustrates that the average number of fungal TRFs ranged from 30 to 36 per land use. Analysis of variance showed that there were no significant effects of sampling month on the fungal diversity indices (Table 6-14). The patterns for three of these diversity indices are shown in Figure 6-23. This shows that there were no obvious land use patterns for the diversity indices.

Multivariate analyses by PCA utilized all the fungal TRF data. There were no significant effects of land use from analysis of variance of the PC axes. Therefore further analyses was carried out on a reduced set of fungal TRFs (19 in total) which were identified by including the most abundant TRFs which did not cross correlate with each other.

The 3D plot of this much reduced set of fungal TRFs (Figure 6-24) demonstrated discrimination of the land uses. Analysis of variance of the reduced set showed significant effects of land use on the first three principle components axes. The first axis (PC1) explained 23.13% of the variance associated with this discrimination while PC2 and PC3 explained a further 19.32% of the variation; a grand total of 42.45%. Summary results for the PC axes illustrate the effects of each axis on fungal TRFs by land use (Figure 6-25). The results show separation along PC1 of agricultural land uses from semi-natural land uses. PC2 produced separation of crops+weeds and infertile calcareous grassland from fertile and infertile grasslands, and lowland and upland deciduous wooded from the remaining semi-natural land uses. PC3 separated crops+weeds from all other land uses. The loading values for the individual TRFs (Figure 6-25) illustrate that the majority of the 19 fungal TRFs contributed to land use discrimination. This demonstrates that fungal community structure could be characterized by a much reduced number of ITS TRFs. However further information and analyses would be required to identify the truly distinctive characteristics of fungal community structure for individual land uses.

Table 6-13 Summary statistics for fungal TRFs diversity indices

	Diversity index	fungal richness		fungal Shannon H'		fungal Shannon E		fungal McIntosh E	
	Land Use	Means	s.e	Means	s.e	Means	s.e	Means	s.e
1	crops and weeds	33.25	2.75	2.88	0.10	0.83	0.01	0.84	0.01
3	fertile grassland	33.54	2.18	2.94	0.07	0.84	0.01	0.86	0.01
4A	infertile grassland (A)	32.62	1.82	2.86	0.10	0.82	0.02	0.84	0.02
4B	infertile grassland (B)	30.88	2.74	2.74	0.09	0.81	0.02	0.81	0.03
5	lowland wooded	29.90	3.69	2.81	0.16	0.85	0.03	0.87	0.03
6A	upland wooded (A)	26.64	2.22	2.63	0.15	0.81	0.04	0.81	0.05
6B	upland wooded (B)	36.18	2.37	3.07	0.09	0.86	0.01	0.89	0.01
7	moorland grass/mosaic	30.55	1.28	2.84	0.09	0.83	0.02	0.84	0.02
8	heath/bog	33.25	2.98	2.89	0.14	0.83	0.02	0.85	0.03

Table 6-14 Results from ANOVA to test for the significance of land use effects on fungal TRFs diversity indices

Fungal ITS	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
fungal richness	659.50	8	82.437	6419.5	92	69.777	1.181	0.319
fungal shannon H'	1.32	8	0.165	13.0	92	0.141	1.171	0.325
fungal shannon E	0.03	8	0.003	0.5	92	0.005	0.589	0.784
fungal McIntosh E	0.05	8	0.01	0.65	92	0.01	0.855	0.557

Figure 6-23 Mean diversity results by land use for fungal TRFs diversity indices

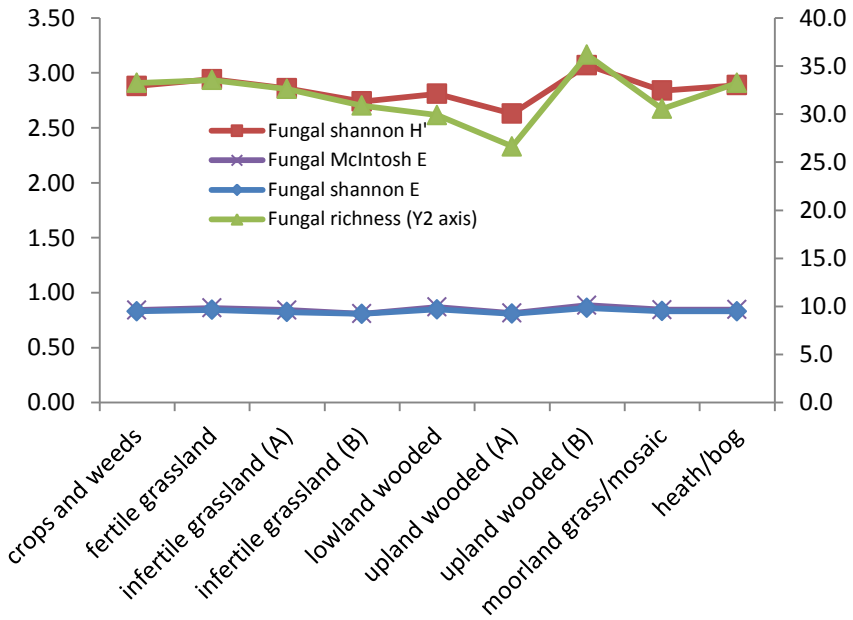
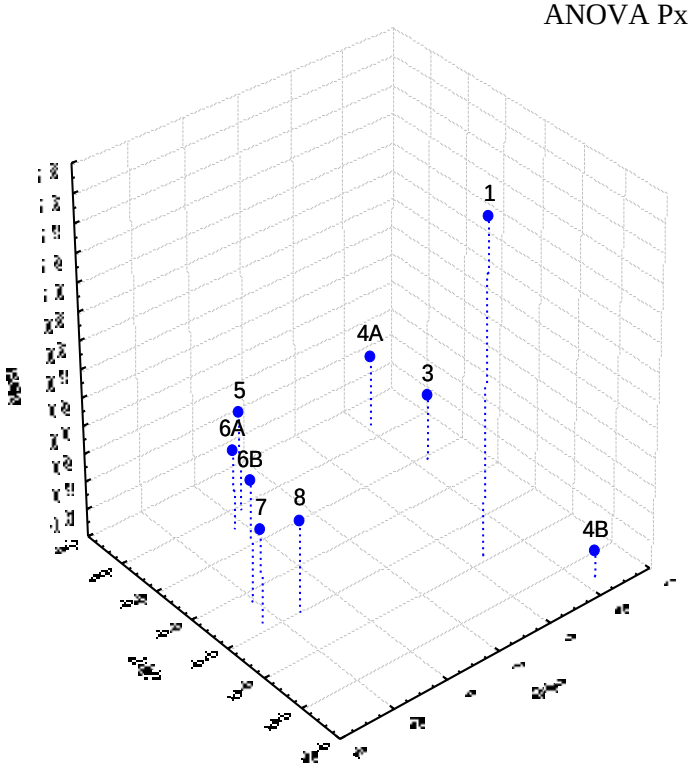


Figure 6-24 3D plot of land use results from the first three axes of PCA for reduced set of fungal TRFs

	PC1	PC2	PC3
%Eigenvalue	23.13	11.28	8.04
ANOVA Px	0.000	0.000	0.001

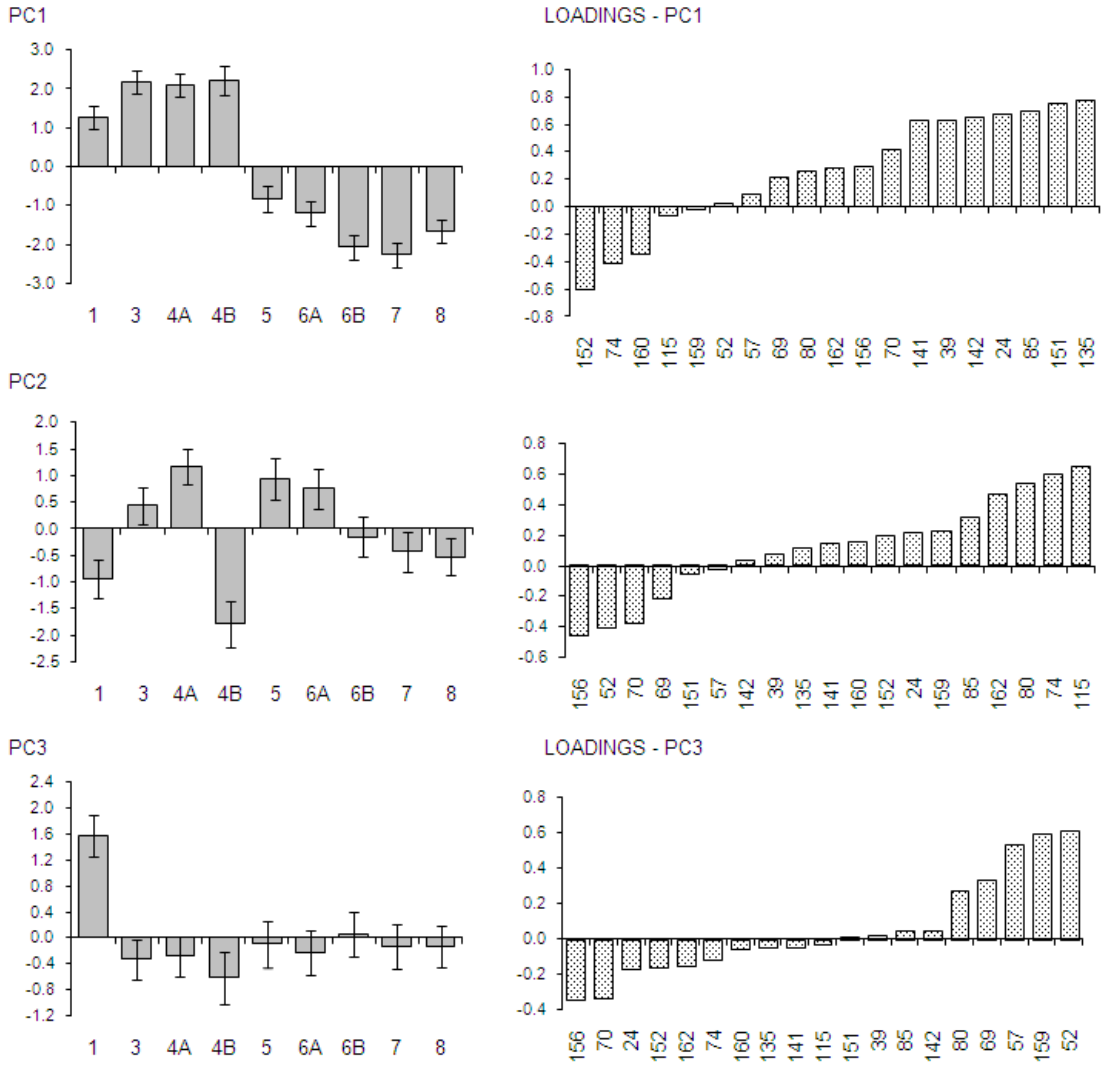


Key to land uses	
1	crops+weeds
3	fertile grass
4A	infertile acid grass
4B	infertile calcareous grass
5	lowland deciduous wooded
6A	upland deciduous wooded
6B	coniferous deciduous wooded
7	moorland grass mosaic
8	heath/bog

Figure 6-25 Summary results for PC1, PC2 and PC3 from PCA for reduced set of fungal TRFs

(a) Land use means +/- 1 s.e

(b) Loadings for individual TRFs



1	Crops+weeds
3	Fertile grassland
4A	Infertile grassland (acid)
4B	Infertile grassland (calcareous)
5	Lowland wooded
6A	Upland wooded (deciduous)
6B	Upland wooded (coniferous)
7	Moorland grass mosaic
8	Heath / bog

6.2.4 PLFA

The PLFA method was applied with a few modifications from the sensitivity trial to take into account the range in soil organic matter experienced (Appendix A). A total of 47 fatty acids were identified from the method. For statistical analyses, these data were analysed to produce diversity indices and categorized to produce abundance and relative abundance data for microbial groups. Abundance and relative abundance data were transformed (LN n+1 and sqrt+0.5, respectively) prior to statistical analysis. Table 6-15 and Table 6-16 present summary results for PLFA measures. Analysis of variance showed that there were no significant effects of sampling month on any of these PLFA measures. Univariate analysis of variance indicates that there were significant effects of land use on all but one of these PLFA measures (Table 6-17). The land use patterns for a select number of these measures are illustrated in Figure 6-26 and Figure 6-27, transformed data where relevant.

Figure 6-26 illustrates that biomass (log_{10} nmol g^{-1}+1) for the total microbial community and individual groups (bacterial, fungal and actinomycete) was lowest in crops+weeds and greatest in heath/bog and moorland grass mosaic, irrespective of biomass differences between the measures (total≅bacterial>fungal≅actinomycetes). There was little difference in biomass amongst the other land uses. The Shannon H diversity index demonstrated a contrasting pattern with lowest diversity in heath/bog and moorland grass mosaic, with a decline from fertile grasslands through infertile grasslands and woodlands to these land uses. Figure 6-27 illustrates that the relative abundance of fungal PLFAs and fungal/bacterial ratios of PLFAs follow similar land use patterns. These fungal measures were greater in the semi-natural land uses relative to the most intensive agricultural land uses (crops+weeds and fertile grasslands). There was an increasing gradient from fertile grasslands to infertile calcareous grasslands and a similar gradient from upland wooded to moorland grass mosaic. Actinomycete PLFAs displayed greatest biomass in moorland grass mosaic and heath/bog habitats. These results and the statistics in Table 6-15 and Table 6-16 suggest that it would be possible to define typical ranges for soil microbial community structure based on PLFAs to discriminate between crops+weeds and grasslands and between woodlands, moorland grass mosaic and heath/bog. Figure 6-28 illustrates that total PLFA (nmol g^{-1}), including 95% confidence intervals, in crops+weeds was distinctively lower to that in grassland land uses and distinctively higher in moorland grass mosaic and heath/bog than in woodland land uses while fungal/bacterial ratios (means +/- 1 s.e) were distinctive between grassland land uses and between crops+weeds and fertile grassland.

Multivariate analyses by PCA utilized all the PLFA data. The 3D plot of the full PLFA results (Figure 6-29) illustrates that the multivariate discrimination amongst the nine land uses is distinct. Analysis of variance showed significant effects of land use on the first three principle components axes. The first axis (PC1) explained 24% of the variance associated with this discrimination while PC2 and PC3 subsequently explained 21% of the variation; a grand total of 41%. Summary results for the PC axes illustrate the effects of each axis on PLFAs by land use (Figure 6-30). The results show separation along PC1 of agricultural land uses (crops+weeds and grasslands)

from the semi-natural land uses (woodlands, moorland-grass mosaic and heath bog). PC2 separated crops+weeds, moorland grass mosaic and heath/bog from grasslands and woodlands. PC3 produced further separation of woodlands from moorland grass mosaic and heath/bog and separation of crops+weeds from grasslands. The loading values for the individual PLFAs illustrate that the land use patterns in PC1, PC2 and PC3 were produced by a combination of all fatty acids.

Further multivariate analyses by PCA utilized a reduced set of the 20 most dominant PLFAs. The 3D plot of this reduced set of PLFA (Figure 6-31) illustrates that the multivariate discrimination amongst the nine land uses is again distinct. Analysis of variance showed significant effects of land use on the first three principle components axes. The first axis (PC1) explained 83.7% of the variance associated with this discrimination while PC2 and PC3 subsequently explained 9.28% of the variation; a grand total of 92.98%. Summary results for the PC axes illustrate the effects of each axis on PLFAs by land use (Figure 6-32). The results show separation along PC1 of crops+weeds from grasslands and these groups from moorland-grass mosaic and heath bog. PC2 separated crops+weeds from grasslands and grasslands from the semi-natural land uses. PC3 produced further separation of heath/bog from grasslands and lowland and upland deciduous woods. The loading values for the individual PLFAs illustrate that the land use patterns in PC1, PC2 and PC3 were produced by a combination of all fatty acids.

Figure 6-26 Means for PLFA microbial community structure measures by land use; abundance (log_{10} nmol g-1+1) and Shannon H' index. See Table 6-17 for key.

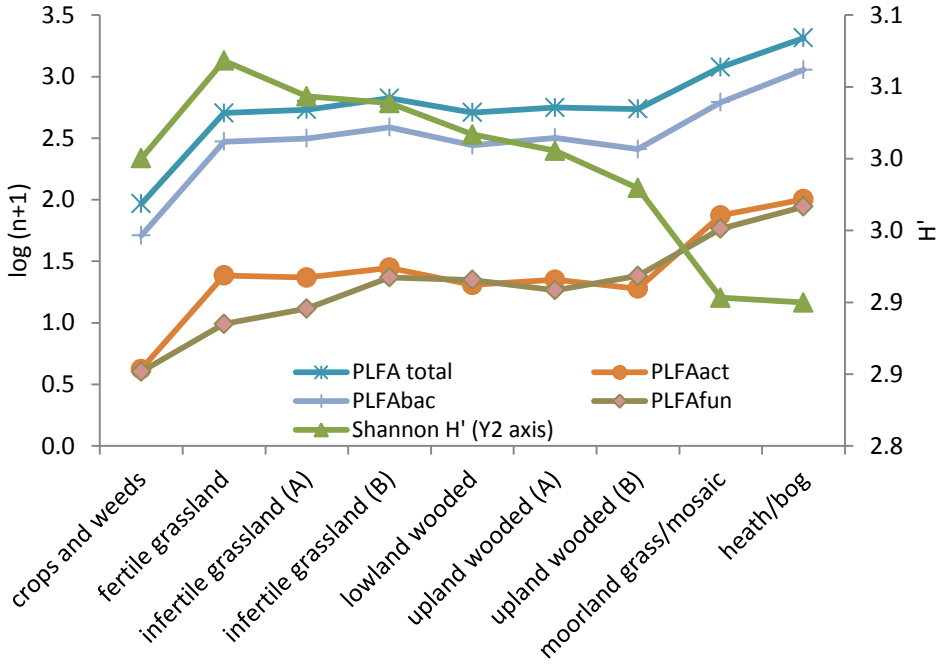


Figure 6-27 Means for PLFA microbial community structure measures by land use; relative abundance (%) and ratios of microbial groups. See Table 6-17 for key.

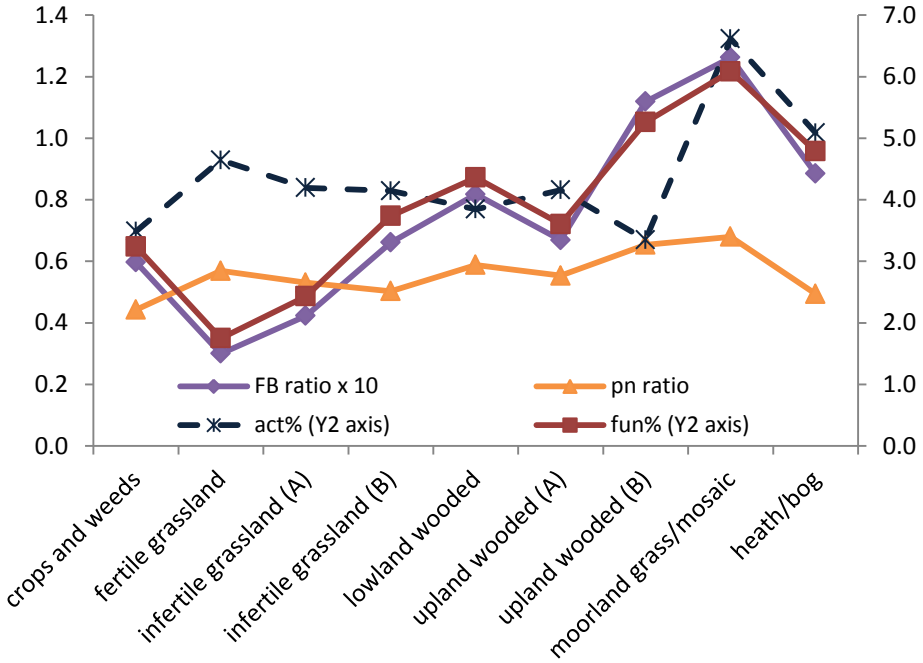


Figure 6-28 Box plot of total PLFA and fungal/bacterial ratios (nmol g^{-1}) by land use

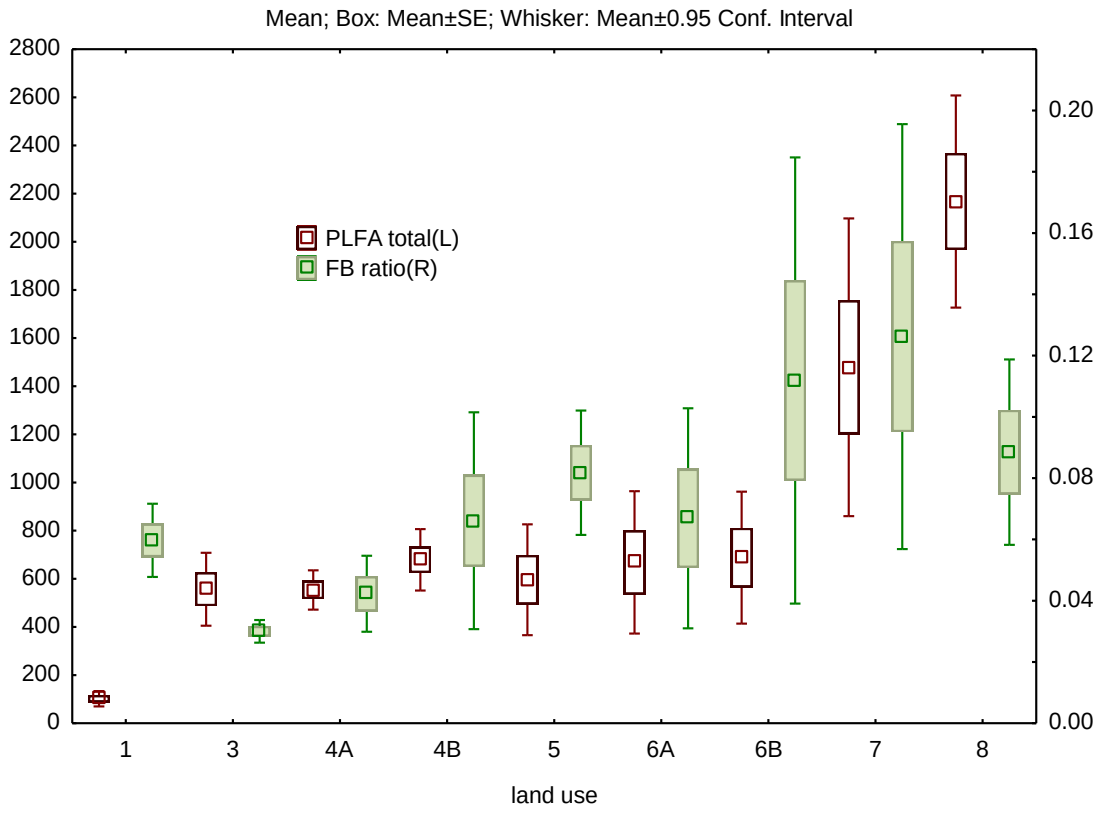


Table 6-15 Summary statistics for abundance PLFA measures

PLFA (nmol g^{-1})		Total PLFA		Bacteria		Fungi		Actinomycetes		Gram positive		Gram negative	
code	Land Use	Means	s.e	Means	s.e	Means	s.e	Means	s.e	Means	s.e	Means	s.e
1	crops and weeds	101.87	14.36	55.38	7.36	3.53	0.76	3.39	0.39	15.96	1.84	38.00	5.36
3	fertile grassland	556.83	69.55	325.74	41.30	9.94	1.56	25.53	3.35	116.13	17.10	200.89	23.75
4A	infertile grassland (A)	553.87	37.53	323.32	23.00	13.33	1.84	23.18	1.99	109.81	8.56	205.80	14.15
4B	infertile grassland (B)	679.03	53.94	392.42	24.95	27.55	8.04	28.09	3.17	128.55	8.65	255.90	15.89
5	lowland wooded	595.94	101.76	322.26	54.63	26.57	5.46	22.73	4.26	117.71	23.37	196.88	32.87
6A	upland wooded (A)	668.30	132.68	382.44	79.47	24.31	7.63	28.22	8.84	132.19	32.04	240.11	46.93
6B	upland wooded (B)	687.88	123.00	341.76	63.04	42.34	12.89	22.34	4.32	135.58	30.77	191.23	31.68
7	moorland grass/mosaic	1478.67	277.57	786.88	151.25	82.06	26.11	96.72	19.33	293.52	55.37	468.98	93.05
8	heath/bog	2167.39	200.23	1197.83	112.88	107.53	20.05	109.09	12.98	368.03	27.21	796.64	90.75

Table 6-16 Summary statistics for relative abundance and ratio based PLFA measures

PLFA (%) and ratios		FB ratio		pn ratio		Bacteria		Fungi		Actinomycetes		Gram positive		Gram negative	
code	Land Use	Mean	s.e	Mean	s.e	Mean	s.e	Mean	s.e	Mean	s.e	Mean	s.e	Mean	s.e
1	crops and weeds	0.06	0.01	0.44	0.02	54.79	0.60	3.24	0.26	3.49	0.16	16.31	0.68	37.14	0.60
3	fertile grassland	0.03	0.00	0.57	0.03	58.42	0.38	1.75	0.10	4.65	0.22	20.48	0.56	36.41	0.78
4A	infertile grassland (A)	0.04	0.01	0.53	0.01	58.31	0.77	2.43	0.30	4.19	0.19	19.68	0.26	37.24	0.73
4B	infertile grassland (B)	0.07	0.01	0.50	0.02	58.25	1.11	3.74	0.73	4.14	0.33	19.07	0.52	38.04	0.91
5	lowland wooded	0.08	0.01	0.59	0.06	54.24	1.31	4.36	0.43	3.85	0.27	19.04	0.84	33.85	1.83
6A	upland wooded (A)	0.07	0.02	0.55	0.06	56.90	1.36	3.60	0.72	4.16	0.57	19.06	1.04	36.36	1.81
6B	upland wooded (B)	0.11	0.03	0.65	0.09	48.22	2.92	5.26	1.26	3.35	0.25	17.91	2.24	28.37	2.00
7	moorland grass/mosaic	0.13	0.03	0.68	0.08	52.22	1.73	6.08	1.29	6.62	0.87	19.86	1.25	30.68	1.67
8	heath/bog	0.09	0.01	0.49	0.04	55.28	0.75	4.79	0.68	5.09	0.48	17.54	0.97	36.15	0.96

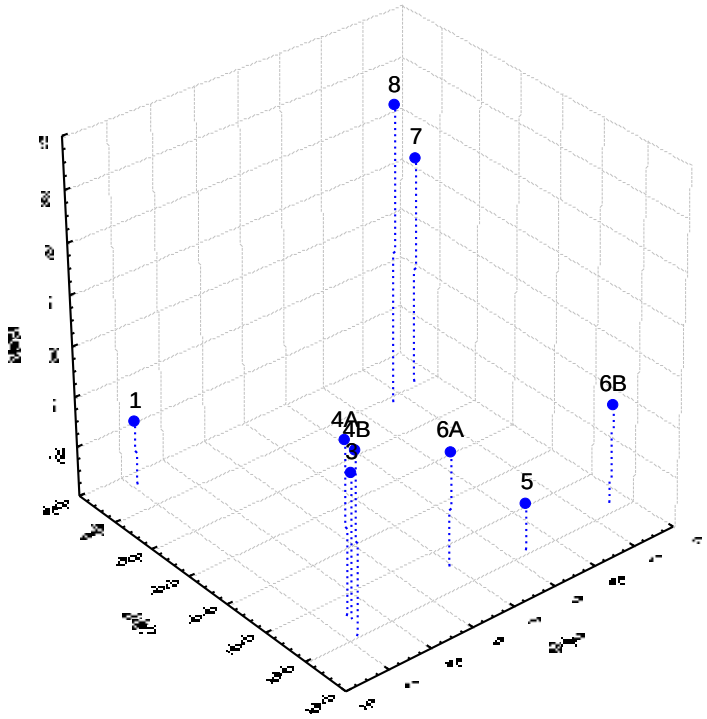
Table 6-17 Results from ANOVA to test for the significance of land use effects on PLFA measures of microbial community structure

PLFA measurre	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
Shannon H'	0.3	8	0.04	0.83	92	0.01	4.54	0.000
FB ratio	0.1	8	0.01	0.31	92	0.00	3.40	0.002
pn ratio	0.5	8	0.06	2.71	92	0.03	2.20	0.035
bac%	1011.8	8	126.48	1928.37	92	20.96	6.03	0.000
fun%	178.6	8	22.33	547.71	92	5.95	3.75	0.001
act%	87.3	8	10.91	185.97	92	2.02	5.40	0.000
Gpos%	162.7	8	20.34	1165.23	92	12.67	1.61	0.134
Gneg%	957.1	8	119.64	1804.26	92	19.61	6.10	0.000
PLFA total	12.4	8	1.55	5.04	92	0.05	28.37	0.000
PLFAbac	12.3	8	1.54	5.69	92	0.06	24.92	0.000
PLFAfun	15.0	8	1.88	10.15	92	0.11	17.01	0.000
PLFAact	14.4	8	1.79	5.22	92	0.06	31.62	0.000
Gpos	12.9	8	1.61	6.93	92	0.08	21.40	0.000
Gneg	11.8	8	1.47	5.47	92	0.06	24.70	0.000

Shannon H'	Shannon Weiner diversity index
PLFA total	total PLFA microbial biomass
PLFAact	PLFA biomass of actinomycetes
PLFAbac	PLFA biomass of bacteria
PLFAfun	PLFA biomass of fungi
Gneg	PLFA biomass of gram negative bacteria
Gpos	PLFA biomass of gram positive bacteria
FB ratio	fungal/bacteria ratio from biomass
pn ratio	gram positive/gram negative ratio from biomass
act%	% actinomycetes of total biomass
bac%	% bacteria of total biomass
fun%	% fungi of total biomass
Gneg%	% gram negative bacteriaof total biomass
Gpos%	% gram positive bacteria of total biomass

Figure 6-29 3D plot of land use results from the first three axes of PCA of full set of 47 PLFAs (ln nmol g-1+1).

	PC1	PC2	PC3
%Eigenvalue	24	11	10
ANOVA Px	0.000	0.000	0.000



Key to land uses	
1	crops+weeds
3	fertile grass
4A	infertile acid grass
4B	infertile calcareous grass
5	lowland deciduous wooded
6A	upland deciduous wooded
6B	coniferous deciduous wooded
7	moorland grass mosaic
8	heath/bog

Figure 6-30 Summary results for PC1, PC2 and PC3 from PCA for full set of 47 PLFAs (ln nmol g^{-1} +1)

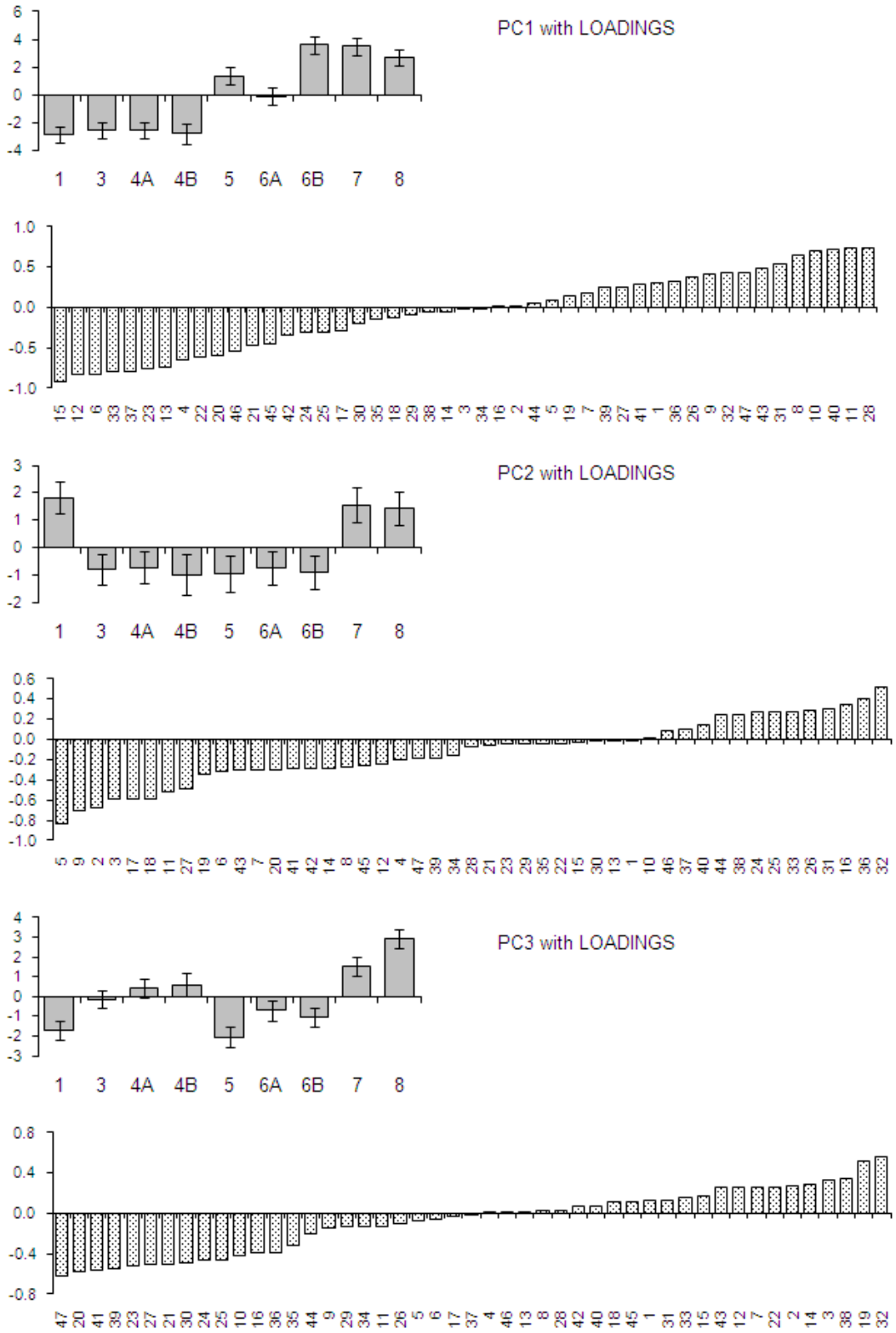
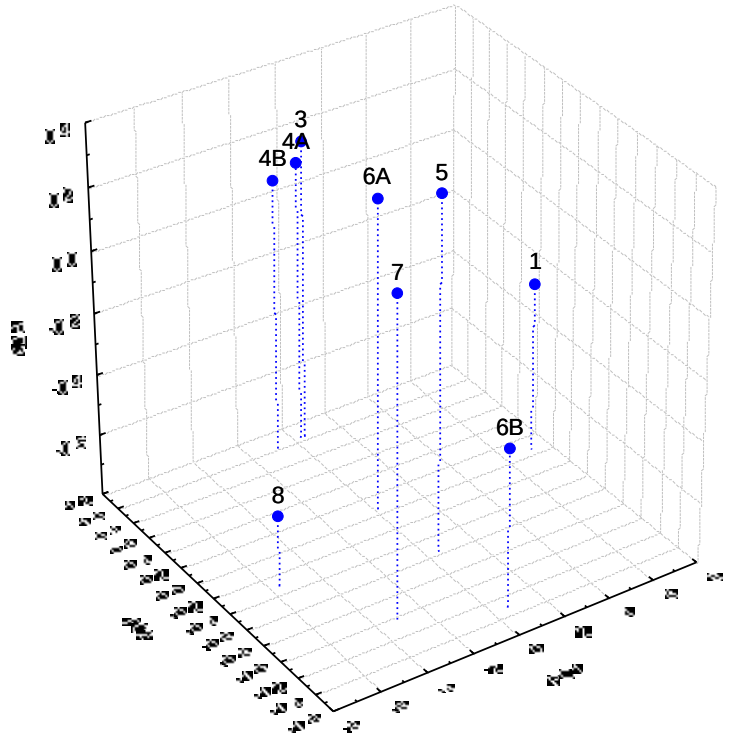


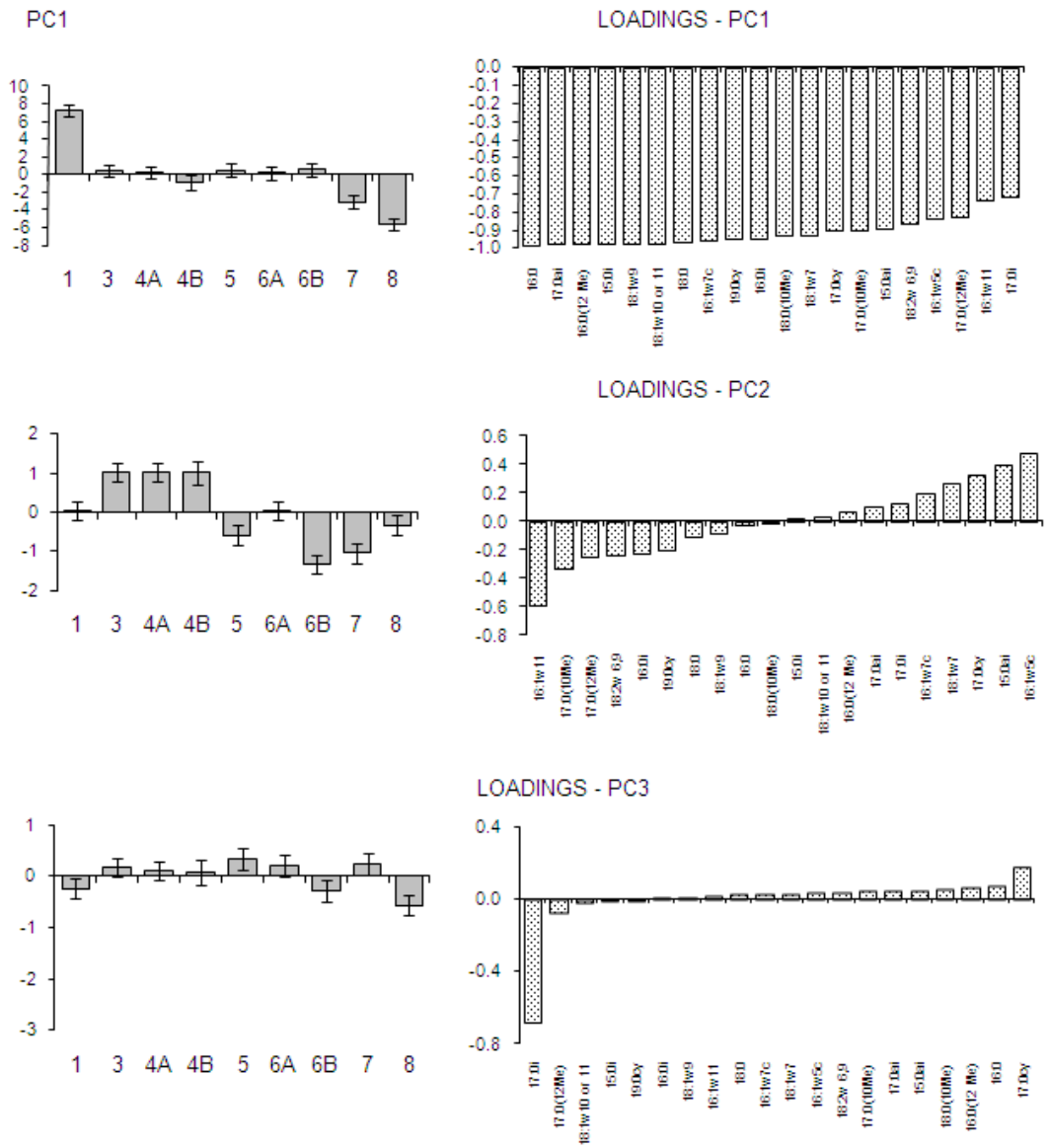
Figure 6-31 3D plot of land use results from the first three axes of PCA of 20 dominant PLFAs (nmol g^{-1}).

	PC1	PC2	PC3
%Eigenvalue	83.73	6.68	2.60
ANOVA Px	0.000	0.000	0.033



Key to land uses	
1	crops+weeds
3	fertile grass
4A	infertile acid grass
4B	infertile calcareous grass
5	lowland deciduous wooded
6A	upland deciduous wooded
6B	coniferous deciduous wooded
7	moorland grass mosaic
8	heath/bog

Figure 6-32 Summary results for PC1, PC2 and PC3 from PCA for 20 dominant PLFAs (nmol g^{-1})



1	Crops+weeds
3	Fertile grassland
4A	Infertile grassland (acid)
4B	Infertile grassland (calcareous)
5	Lowland wooded
6A	Upland wooded (deciduous)
6B	Upland wooded (coniferous)
7	Moorland grass mosaic
8	Heath / bog

6.2.5 Nematodes

Nematodes were extracted and identified with the same method used for the sensitivity trial, with no modifications (Appendix A). Taxonomic resolution was limited to feeding groups (bacterial, fungal, omnivores, carnivores and plant feeders). The results for these groups were then used to derive diversity indices and ratios. Table 6-18 and Table 6-19 present summary results for the abundance and relative abundance of nematode feeding groups by the nine land use classes. These data demonstrate that nematode communities were dominated by bacterial and plant feeders in all land uses. All abundance and relative abundance data required transformation (log10 n+1 and sqrt n+0.5 respectively) prior to statistical analysis.

Analysis of variance (Figure 6-33) showed significant effects of sampling month on three nematode measures (fungal feeders/fungal feeders+bacterial feeders, abundance of fungal feeders and relative abundance feeders) with these measures highest in May and lowest in June and/or July. Univariate analysis of variance indicates that there were significant effects of land use on nematode feeding groups but not ratios (Table 6-20). The abundance of total nematodes and all groups except fungal feeders were influenced by land use while the relative abundance of carnivores and omnivores were also influenced by land use. The influence of land use on the abundance of these groups is illustrated in Figure 6-34. This demonstrates similar patterns of abundance for total nematodes and individual feeding groups with highest numbers found in fertile and infertile acid grasslands and lowest numbers in upland coniferous wooded and heath/bog. Overlapping and relatively large standard errors and confidence intervals for the nematode measures across the land uses (Table 6-18, Table 6-19 and Figure 6-35) suggest that, using these data, it would only be possible to establish distinctive ranges for nematode measures for certain land use combinations. For example, to contrast between crops+weeds and grasslands but not within grasslands, or between moorland grass mosaic and heath/bog or upland deciduous wooded but not between woodland land uses.

Multivariate analyses by PCA utilized the individual feeding groups and ratio data. The 3D plot of the nematode results (Figure 6-36) illustrates that there is some multivariate discrimination amongst the nine land uses. Analysis of variance showed significant effects of land use only on the first principle components axes (PC1) which explained 32% of the variance associated with this discrimination. Summary results for the PC axes illustrate the effects of each axis on nematodes by land use (Figure 6-37). The result for PC1 demonstrate similar discrimination to that of the invidual feeding groups (Figure 6-34) with fertile and infertile acid grassland separated from crops+weeds and infertile calcareous grassland, and heath/bog and upland coniferous wooded separated from moorland grass mosaic and the other woods. PC1 produced distinct discrimination between heath/bog and upland coniferous wooded from the agricultural land use which was not obvious from the univariate analysis. High variability masked any discrimination in PC2 and PC3.

The loading values (Figure 6-37) for the individual measures illustrate that the land use patterns in PC1 were produced by a combination of several nematode measures. Loadings for PC2 and PC3 reflect the influence of fewer measures which may account for the high levels of variability.

Table 6-18 Summary statistics for nematode abundance (n $100g^{-1}$)

Nematode		carnivore		omnivore		bacterial feeder		fungal feeder		plant feeders		total	
code	Land Use	Means	s.e	Means	s.e	Means	s.e	Means	s.e	Means	s.e	Means	s.e
1	crops and weeds	2.08	1.50	4.75	2.76	56.58	17.69	2.58	1.29	61.08	19.30	127.08	30.91
3	fertile grassland	7.77	4.05	28.62	14.46	90.38	17.28	1.62	0.54	75.15	23.20	203.77	43.76
4A	infertile grassland (A)	5.77	2.83	21.00	5.78	92.23	19.46	1.08	0.59	123.54	31.10	243.77	46.32
4B	infertile grassland (B)	0.25	0.25	8.50	2.97	77.50	21.00	2.00	1.31	77.75	45.47	165.88	64.75
5	lowland wooded	1.10	0.64	4.70	1.67	57.00	17.35	2.30	0.92	41.50	14.65	106.60	29.27
6A	upland wooded (A)	0.64	0.47	5.64	2.15	51.18	16.52	2.09	0.65	32.00	6.55	91.45	22.22
6B	upland wooded (B)	0.00		1.64	1.18	33.73	8.31	0.91	0.67	13.09	3.84	49.36	11.83
7	moorland grass/mosaic	1.45	0.78	6.45	1.96	41.45	8.29	1.55	0.68	44.73	9.68	95.27	15.17
8	heath/bog	0.00		0.67	0.51	26.08	6.95	1.25	1.25	16.58	9.33	44.75	12.53

Table 6-19 Summary statistics for relative abundance of nematodes (%)

Nematode		Carnivore%		Omnivore%		bacterial feeder%		fungal feeder%		plant feeders%		Fungal/ bacterial+fungal	
code	Land Use	Means	s.e	Means	s.e	Means	s.e	Means	s.e	Means	s.e	Means	s.e
1	crops and weeds	1.54	0.90	2.81	1.79	41.04	8.40	1.54	0.83	52.74	8.25	0.05	0.03
3	fertile grassland	2.88	1.04	15.26	5.42	47.59	4.68	0.80	0.28	33.47	4.59	0.01	0.00
4A	infertile grassland (A)	2.48	1.42	9.16	1.96	41.29	6.04	0.42	0.25	46.58	5.77	0.01	0.00
4B	infertile grassland (B)	0.13	0.13	6.10	1.66	56.70	6.03	0.57	0.41	36.55	5.50	0.01	0.01
5	lowland wooded	0.84	0.52	8.51	4.80	49.94	7.39	1.43	0.55	39.36	4.90	0.03	0.01
6A	upland wooded (A)	0.97	0.85	8.44	3.41	46.41	5.82	4.68	2.29	39.41	5.86	0.09	0.05
6B	upland wooded (B)	0.00		3.12	1.77	56.59	9.95	1.02	0.69	30.18	8.23	0.02	0.01
7	moorland grass/mosaic	3.14	1.76	7.35	1.79	42.71	5.28	2.18	0.97	45.07	5.69	0.06	0.03
8	heath/bog	0.00		2.27	2.07	57.25	9.18	1.40	1.40	29.81	8.45	0.02	0.02

Table 6-20 Results from ANOVA to test for the significance of land use effects on nematodes

Nematode measure	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
carnivores	3.78	8	0.47	12.77	92	0.14	3.41	**0.002**
omnivores	12.71	8	1.59	21.04	92	0.23	6.95	**0.000**
bacterial feeders	5.31	8	0.66	25.17	92	0.27	2.43	**0.020**
fungal feeders	0.84	8	0.11	12.55	92	0.14	0.77	0.627
plant feeders	11.13	8	1.39	24.90	92	0.27	5.14	**0.000**
total nematodes	7.90	8	0.99	18.31	92	0.20	4.97	**0.000**
carnivores%	0.19	8	0.02	0.93	92	0.01	2.30	**0.027**
omnivores%	0.80	8	0.10	2.77	92	0.03	3.33	**0.002**
fungal feeders%	0.13	8	0.02	1.01	92	0.01	1.48	0.176
plant feeders%	0.66	8	0.08	4.24	92	0.05	1.80	0.088
bacterial/fungal	0.93	8	0.12	7.22	87	0.08	1.40	0.210
fungal/b+f	0.07	8	0.01	0.55	92	0.01	1.40	0.207

Figure 6-33 Mean nematode measures by sampling month

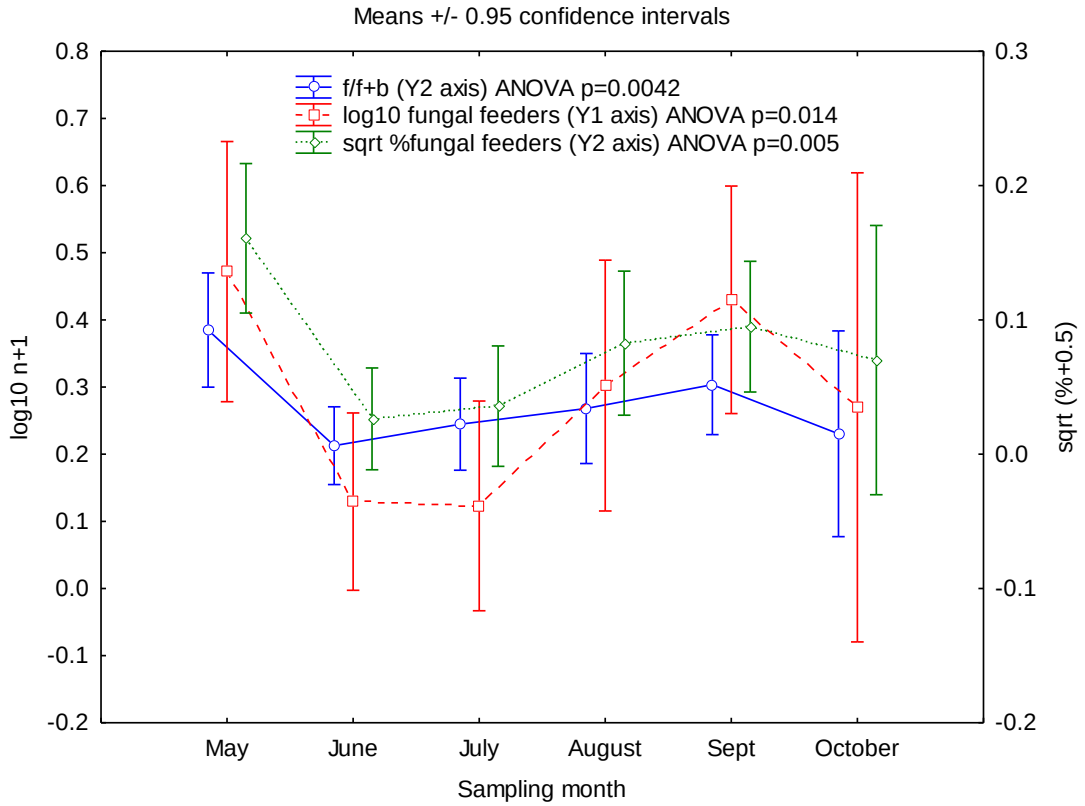


Figure 6-34 Mean nematode abundance measures (log_{10} n/100g +1) by land use

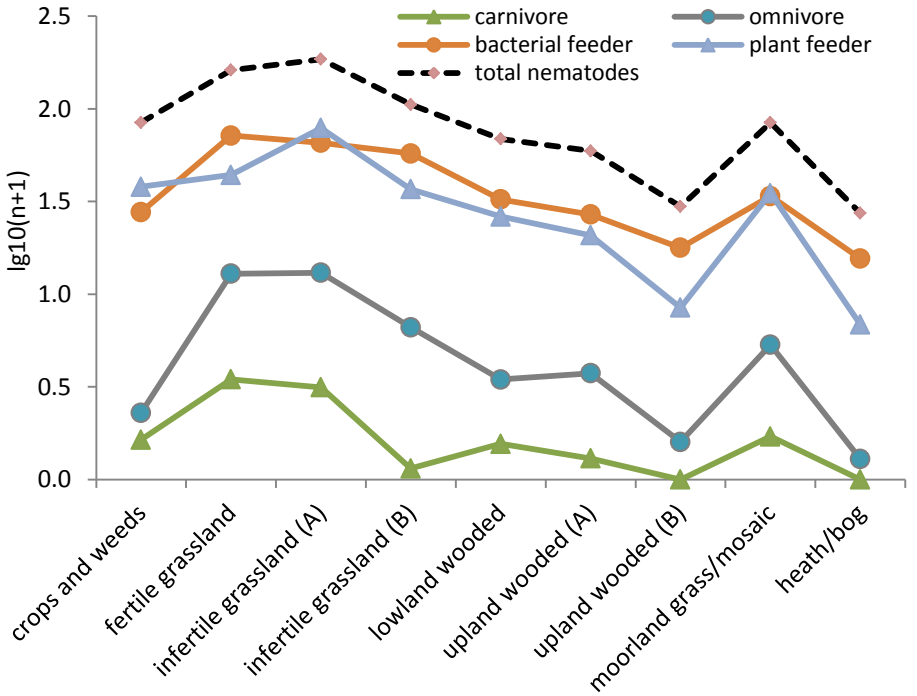


Figure 6-35 Box-plot of abundance of nematode feeding groups (log_{10} n+1) by land use

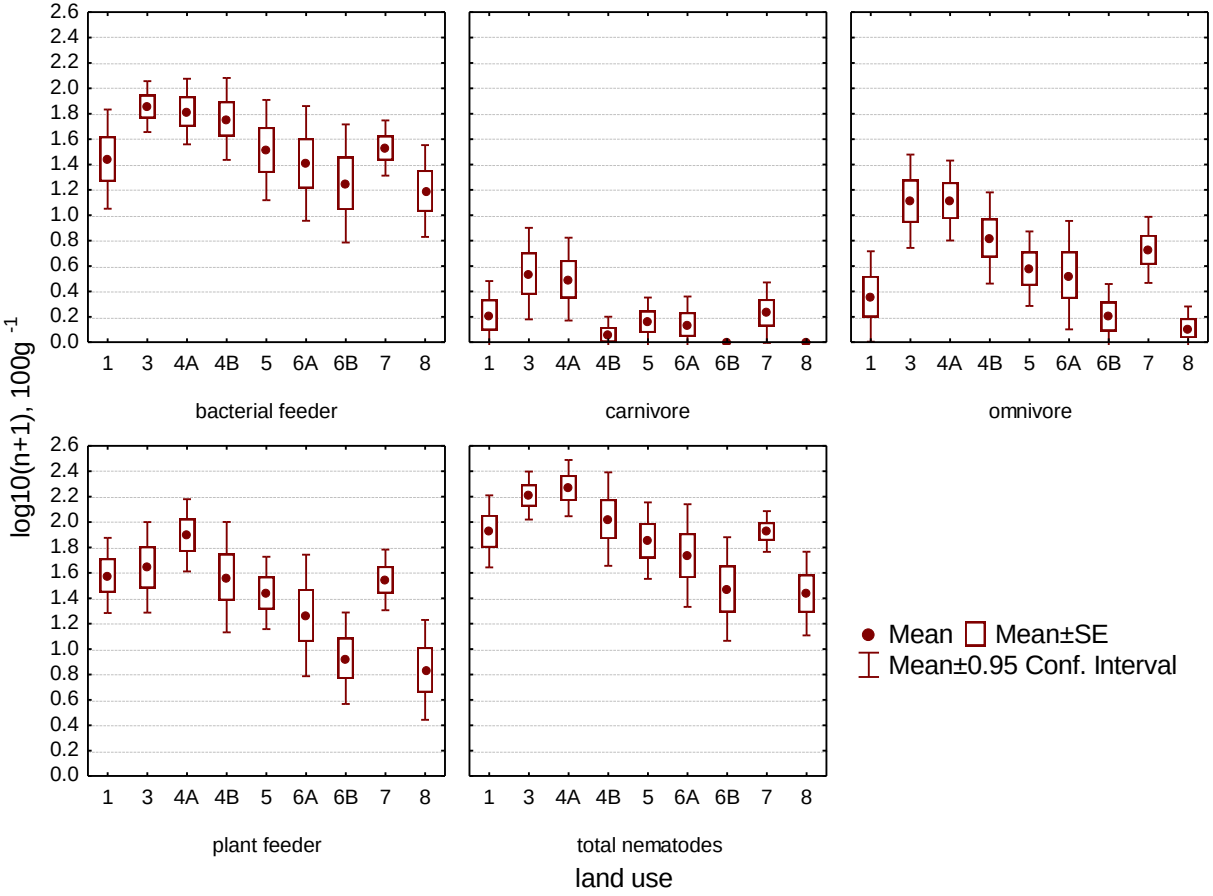
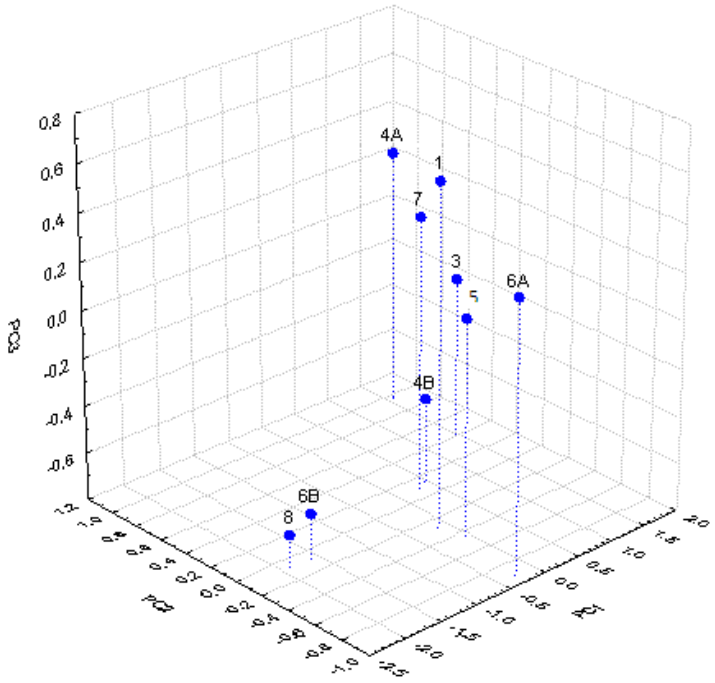


Figure 6-36 3D plot of land use results from the first three axes of PCA of the nematodes measures

	PC1	PC2	PC3
%Eigenvalue	32	22	20
ANOVA Px	0.000	0.177	0.345

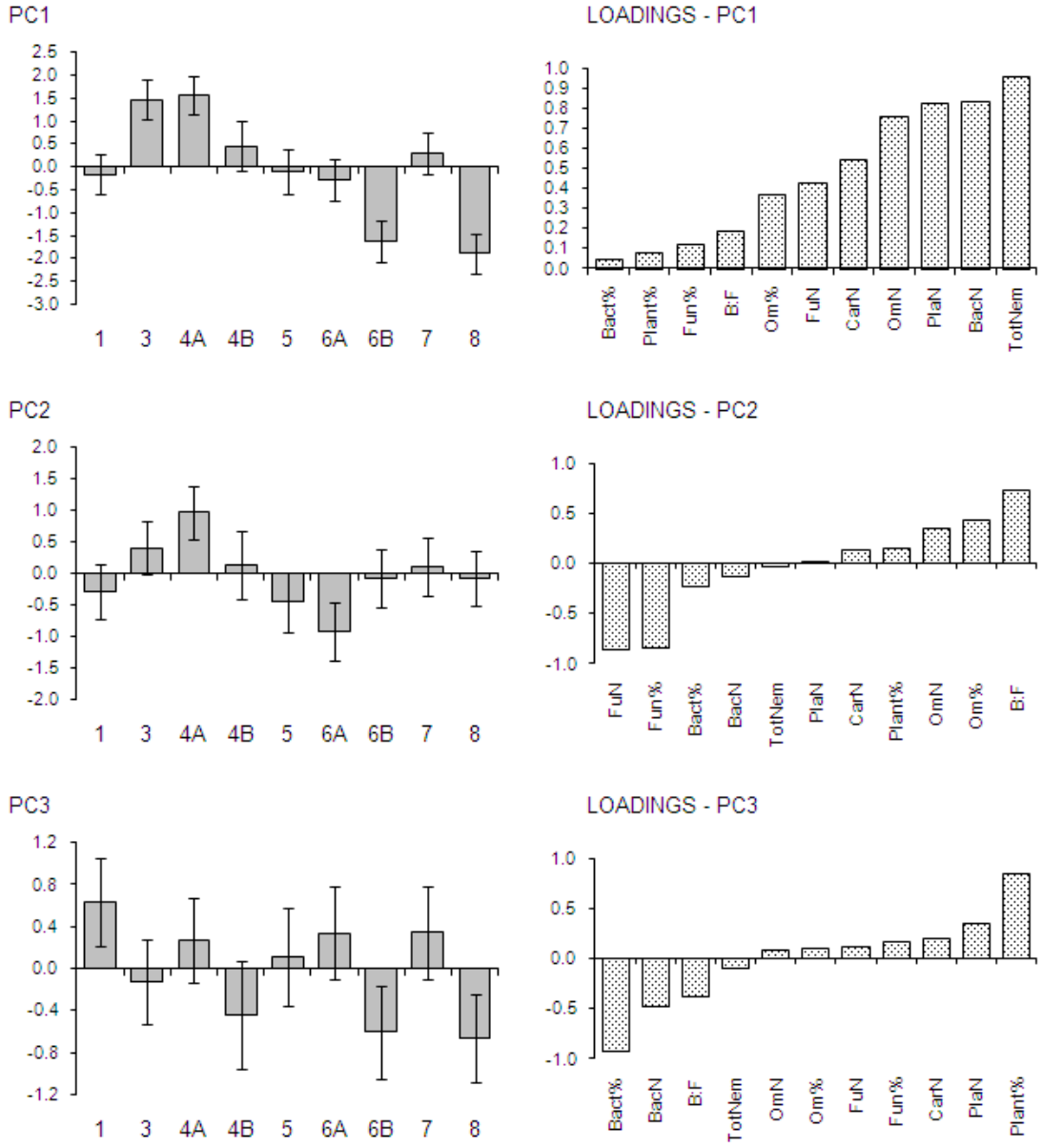


Key to land uses	
1	crops+weeds
3	fertile grass
4A	infertile acid grass
4B	infertile calcareous grass
5	lowland deciduous wooded
6A	upland deciduous wooded
6B	coniferous deciduous wooded
7	moorland grass mosaic
8	heath/bog

Figure 6-37 Summary results for PC1, PC2 and PC3 from PCA of the nematode measures

(a) Land use means +/- 1 s.e

(b) Loadings for nematode measures



1	Crops+weeds
3	Fertile grassland
4A	Infertile grassland (acid)
4B	Infertile grassland (calcareous)
5	Lowland wooded
6A	Upland wooded (deciduous)
6B	Upland wooded (coniferous)
7	Moorland grass mosaic
8	Heath / bog

6.2.6 Microarthropods

Invertebrates were extracted and identified with the same method used for the sensitivity trial, with no modifications (Appendix A). Abundance data were obtained for a range of taxonomic groups from higher-level invertebrate groups (e.g. mites, collembola, hemiptera, chilopoda, etc), microarthopods (collembola+mites) and individual mite and collembola groups. The results for these groups were used to derive relative abundance, diversity indices and ratios. Table 6-21 presents summary results for invertebrate abundance and a range of diversity indices for invertebrates for the nine land use classes. Table 6-22 and Table 6-23 present summary results for the abundance and relative abundance of mites and collembola, respectively, for the nine land use classes. These data demonstrate that invertebrate communities were dominated by mites (56.85 to 86.99%) and collembola (8.73 to 38.93%). Abundance and relative abundance data required transformation ($\log_{10}$ n+1 and sqrt n+0.5 respectively) prior to statistical analysis.

Analysis of variance showed significant effects of sampling month on four invertebrate groups (Figure 6-38); abundance of neelidae collembola, abundance of poduroidae collembola, invertebrate group richness and the relative abundance of microarthropods (collembola+mites). Invertebrate richness, neelidae and poduroidae demonstrated similar seasonal patterns with a dip in values during the summer and early autumn months. The relative abundance of microarthropods was lower in late autumn (October) relative to the earlier months. Univariate analysis of variance indicates that there were significant effects of land use on mites (Table 6-24), collembola (Table 6-25) and invertebrates (Table 6-26).

The abundance and relative abundance of the three mite groups were all influenced by land use. Figure 6-39 illustrates that there were contrasting patterns in abundance and relative abundance measures for mites amongst land uses. Mesostigmatid mites increased in abundance from crops+weeds through grassland to lowland wooded, with reverse in this pattern from lowland wooded through upland wooded, moorland grass mosaic to heath/bog where mesostigmatids were least abundant across all nine land uses. In contrast, the relative abundance of mesostigmatid mites was highest in crops+weeds with a gradual reduction in % across the subsequent land uses from grassland through woodland to moorland grass mosaic and heath/bog. The abundance and relative abundance of oribatid mites followed contrasting patterns with lowest values for both in crops+weeds and increasing values in both for fertile, infertile grasslands to semi-natural land uses. The transition from crops, grassland to semi-natural land uses was reflected in increasing numbers of mites and in the relative abundance of mites, with respect to the total number of invertebrates. There was a distinct change in mite community structure from agricultural to semi-natural land uses. In agricultural land uses, mesostigmatids and oribatids displayed similar abundance with a predominance of mesostigmatids in the most intensive agricultural land uses (crops+weeds and fertile grassland). Progression to semi-natural land uses shows a shift to an obvious dominance of oribatids.

Analysis of variance for collembola measures (Table 6-25) demonstrated far fewer significant effects of land use, compared to mites. Only four measures were influenced by land use; the abundance of neelidae and poduroidae and the relative abundance of neelidae and collembola. These significant results are illustrated in Figure 6-40. The proportion of collembola in the invertebrate community was higher in crops+weeds, fertile and infertile grasslands than the other land uses. The abundance of poduroidae was lower in crops+weeds and heath/bog than other land uses. The abundance and relative abundance of neelidae was lower in grassland, moorland grass mosaic and heath/bog than in crops+weeds and wooded land uses.

Analysis of variance for invertebrate measures (Table 6-26) demonstrated a range of significant effects of land use on different measures of community structure. The significant results for indices and ratios are illustrated in Figure 6-41. This illustrates a shift in invertebrate community structure from agricultural to semi-natural land uses with reduced diversity and evenness in the later, and lower and higher proportion of collembola and mites respectively. In contrast invertebrate richness, the number of higher level taxonomic groups, was higher in infertile calcareous grasslands, lowland wooded and upland deciduous compared to other land uses. The land use pattern was a little different for the abundance of invertebrates and microarthropods (Figure 6-42). Lowest abundances were recorded in crops+weeds which increased through fertile and infertile grasslands, with highest abundance in infertile calcareous grasslands (paralleling the highest invertebrate richness values). Abundances in woodlands, moorland grass mosaic and heath/bog were similar to abundances in infertile grasslands.

The overlapping and relatively large standard errors and confidence intervals for the collembola measures across the land uses (Table 6-23 and Figure 6-44) suggest that, using these data, it would be difficult to establish distinctive ranges for collembola measures within individual land uses. In contrast, it would be possible to define typical and distinctive ranges for invertebrate community and mites measures across land uses, in particular for crops+weeds and grassland land uses (Table 6-21, Figure 6-42, Figure 6-43 and Figure 6-44).

Two multivariate analyses by PCA were carried out on the invertebrate data. The first PCA utilised data for invertebrates along with mite and collembola data while the second PCA used data for collembola and mites only. The 3D plot of the invertebrate results (Figure 6-45) illustrates that there was some multivariate discrimination amongst the nine land uses. Analysis of variance showed significant effects of land use on the first and third principle components axes (PC1 and PC3) which explained 48% of the variance associated with discrimination. Summary results for the PC axes illustrate the effects of each axis on invertebrate measures by land use (Figure 6-46). The results for PC1 demonstrate similar patterns of discrimination to several of the individual measures with a gradient from crops+weeds through fertile grasslands to infertile acid grasslands to infertile calcareous grasslands. PC1 further extended this gradient into lowland wooded which in turn was separated from upland wooded,

moorland grass mosaic and heath/bog. High variation in PC2 contributed to a lack of significance. PC3 further separated the most intensive land uses (crops+weeds and fertile grassland) from the other land uses and heath/bog from all but lowland wooded land uses. The loading values (Figure 6-46) for the individual measures illustrates that the land use patterns in PC1 were produced by a combination of several invertebrate measures from invertebrate community measures to measures for mite and collembola. Loadings for PC2 and PC3 reflect the influence of fewer measures primarily from mites and collembola. In particular, the relative abundance of microarthropods (ACMIC%) contributed significantly to the discrimination in PC2. This is a group which demonstrated significant effects of sampling month which would contribute to the high variability in this axis.

The second multivariate analyses by PCA utilised abundance data for collembola and mites only. The 3D plot of these results (Figure 6-47) illustrates that this analysis produced a slightly different discrimination pattern amongst the nine land uses than that from the invertebrate measures combined, with upland wooded land uses more closely related than the previous analysis. Analysis of variance showed significant effects of land use on the first three principle components axes. PC1 explained 33% of the variance associated with discrimination while PC2 and PC3 accounted for a further 32%; a grand total of 65% variation explained. Summary results for the PC axes illustrate the effects of each axis on invertebrate measures by land use (Figure 6-48). The results for PC1 demonstrate similar patterns of discrimination to that from the invertebrate measures with a gradient from crops+weeds through fertile grasslands, infertile grasslands to lowland wooded. This analysis also demonstrated a clear reverse gradient from lowland wooded through upland wooded to moorland grass mosaic and finally to heath/bog. There was less variation in PC2 compare to the invertebrate analyses which highlighted a further gradient from crops+weeds to infertile calcareous grassland and a further gradient from upland wooded to heath/bog. PC3 separated agricultural land uses from the semi-natural land uses of upland wooded and heath/bog. The loading values (Figure 6-48) for the individual measures illustrates that the land use patterns in all three PC axes reflect a combination of the seven collembola and mite abundance measures. These results indicate that discrimination amongst land uses could be defined by a restricted set of invertebrate characteristics.

Table 6-21 Summary statistics for invertebrate measures

	Invertebrate measures	Total invertebrates (INVTOT m^{-2})		% mites of invertebrates (%ACTOT)		% collembola of invertebrates (%COLLTOT)		Higher level invertebrate group taxa richness (INV_S)		Invertebrate Shannon H' (INV_Sh H)		Invertebrate Shannon E (INV_Sh E)	
code	Land Use	Means	s.e	Means	s.e	Means	s.e	Means	s.e	Means	s.e	Means	s.e
1	crops and weeds	10922.5	2255.1	56.85	9.13	38.93	9.27	4.75	0.68	0.99	0.14	0.71	0.05
3	fertile grassland	35275.0	8048.1	70.59	5.74	25.81	6.06	5.67	0.77	1.18	0.12	0.76	0.05
4A	infertile grassland	39152.3	7661.6	72.58	4.19	23.67	4.24	6.15	0.41	1.35	0.06	0.76	0.03
4B	infertile grassland	77010.0	12066.1	83.20	2.48	11.29	2.47	8.50	0.65	1.19	0.12	0.56	0.05
5	lowland wooded	116994.0	17822.3	75.72	5.06	16.96	5.29	9.30	0.70	1.14	0.11	0.52	0.05
6A	upland wooded (A)	60520.0	16593.7	70.80	5.92	19.28	5.83	8.00	1.03	1.23	0.12	0.64	0.07
6B	upland wooded (B)	76296.0	13806.3	86.99	3.51	8.73	2.52	6.40	0.85	0.92	0.13	0.52	0.06
7	moorland	45760.9	9247.8	79.19	4.60	15.51	4.49	6.45	0.49	1.03	0.10	0.57	0.05
8	heath/bog	45482.7	10595.9	81.89	5.29	15.58	5.40	4.09	0.51	0.57	0.10	0.43	0.06

Table 6-22 Summary statistics for mites

Mite measures		Mesostigmatids (ACME m^{-2})		Oribatids (ACOR m^{-2})		Prostigmatids (ACPR m^{-2})		Total mites (ACTOT m^{-2})		%Mesostigmatids (%ACME)		%Oribatids (%ACOR)		%Prostigmatids (%ACPR)	
code	Land Use	Means	s.e	Means	s.e	Means	s.e	Means	s.e	Means	s.e	Means	s.e	Means	s.e
1	crops and weeds	2380.0	792.4	2805.0	1268.8	807.5	389.3	5992.5	1651.6	59.55	11.30	30.73	10.55	9.71	4.80
3	fertile grassland	7990.0	1721.9	9647.5	4031.6	10370.0	4779.8	28050.0	7508.6	45.72	9.38	30.56	8.94	23.64	7.69
4A	infertile grassland (A)	9533.1	1151.1	17379.2	5827.3	2000.8	567.2	28952.3	6407.3	42.47	5.15	49.00	5.87	8.14	2.82
4B	infertile grassland (B)	15045.0	3180.6	45581.3	7783.0	2422.5	459.7	63240.0	9207.3	25.37	5.18	70.05	5.76	4.29	0.99
5	lowland wooded	13515.0	1573.3	78540.0	18241.1	969.0	335.3	93024.0	18317.8	20.86	4.50	78.02	4.55	1.12	0.31
6A	upland wooded (A)	8443.3	2868.8	36890.0	11729.4	1586.7	682.4	47033.3	14500.1	21.92	6.11	72.71	7.49	4.77	2.36
6B	upland wooded (B)	9792.0	2898.8	53805.0	10091.3	2652.0	720.4	66249.0	11884.3	16.38	4.46	79.51	4.99	4.11	0.90
7	moorland grass/mosaic	4821.8	1604.5	28745.5	7413.4	2410.9	1260.7	36024.6	7984.0	14.57	4.75	77.00	6.23	8.32	5.30
8	heath/bog	695.5	365.7	36117.3	9177.6	741.8	353.1	37554.6	9351.3	1.91	0.82	96.14	1.41	1.95	0.81

Table 6-23 Summary statistics for collembola

Collembola measures		Entomybroidae (COEN m^{-})		Poduroidae (COPU m^{-2})		Sminthuridae (COSM m^{-2})		Total collembola (COLLTOT m^{-2})		Entomybroidae (%COEN)		Poduroidae (%COPU)		Sminthuridae (%COSM)	
code	Land Use	Means	s.e	Means	s.e	Means	s.e	Means	s.e	Means	s.e	Means	s.e	Means	s.e
1	crops and weeds	2975.0	1360.7	935.0	517.0	170.0	95.9	4377.5	1509.9	58.91	10.90	27.20	11.54	3.13	2.27
3	fertile grassland	3612.5	1288.8	2677.5	1060.0	255.0	117.4	6587.5	1812.3	43.98	11.10	47.49	10.70	8.32	4.66
4A	infertile grassland (A)	4354.6	1029.1	4629.2	1171.4	78.5	53.1	9062.3	2015.5	48.58	8.46	50.94	8.47	0.48	0.33
4B	infertile grassland (B)	6502.5	2336.6	1785.0	481.9	573.8	573.8	8861.3	2742.9	68.45	7.93	29.30	8.68	2.25	2.25
5	lowland wooded	10710.0	4871.0	4794.0	974.2	102.0	68.0	16116.0	4915.4	48.16	9.77	44.96	8.61	0.67	0.49
6A	upland wooded (A)	6856.7	3089.5	3400.0	1762.6	453.3	287.6	11050.0	4568.7	61.46	11.12	32.97	10.74	2.48	1.95
6B	upland wooded (B)	3060.0	1497.5	2754.0	1263.5	204.0	155.8	6222.0	2572.3	56.93	9.32	32.97	10.48	2.48	1.88
7	moorland grass/mosaic	4775.5	1836.6	2967.3	1042.5	46.4	46.4	7789.1	2779.9	48.67	8.81	50.92	8.95	0.41	0.41
8	heath/bog	6444.6	2186.9	834.5	596.6	0.0		7279.1	2439.2	76.96	12.12	23.04	12.12	0.00	

Table 6-24 Results from ANOVA to test for the significance of land use effects on mite measures

mites	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
Mesostigmatids (n)	50.30	8	6.29	95.48	87	1.10	5.73	**0.000**
oribatids (n)	73.64	8	9.20	76.80	87	0.88	10.43	**0.000**
Prostigmatids (n)	37.30	8	4.66	195.94	87	2.25	2.07	**0.047**
Total mites (n)	14.63	8	1.83	17.48	87	0.20	9.10	**0.000**
Mesostigmatids (%	296.34	8	37.04	354.10	87	4.07	9.10	**0.000**
Oribatids (%)	342.07	8	42.76	339.14	87	3.90	10.97	**0.000**
Prostigmatids (%)	61.28	8	7.66	256.17	87	2.94	2.60	**0.013**
Total mites (%invertebrates)	34.31	8	4.29	128.95	87	1.48	2.89	**0.007**

Table 6-25 Results from ANOVA to test for the significance of land use effects on collembola measures

collembola	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	P
Entomybroidae (n)	15.85	8	1.98	170.70	87	1.96	1.01	0.435
Neelidae (n)	32.68	8	4.08	86.44	87	0.99	4.11	**0.000**
Poduroidae (n)	62.40	8	7.80	141.14	87	1.62	4.81	**0.000**
Sminthuridae (n)	8.81	8	1.10	115.00	87	1.32	0.83	0.576
Total collembola	13.24	8	1.66	86.89	87	1.00	1.66	0.120
Entomybroidae (%)	50.68	8	6.34	653.24	81	8.06	0.79	0.617
Neelidae (%)	41.35	8	5.17	134.43	81	1.66	3.11	**0.004**
Poduroidae (%)	120.33	8	15.04	683.73	81	8.44	1.78	0.093
Sminthuridae (%)	14.82	8	1.85	108.82	81	1.34	1.38	0.218
Total collembola (%invertebrates)	68.62	8	8.58	349.50	87	4.02	2.14	**0.041**

Table 6-26 Results from ANOVA to test for the significance of land use effects on invertebrate measures

invertebrates	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
Invertebrates (n)	9.87	8	1.23	14.83	87	0.17	7.23	**0.000**
Microarthropods(n)	9.69	8	1.21	15.63	87	0.18	6.74	**0.000**
Collembola/mites	24.43	8	3.05	74.86	87	0.86	3.55	**0.001**
Mites/microarthropods	0.77	8	0.10	3.20	87	0.04	2.61	**0.013**
Mites/invertebrates	0.71	8	0.09	2.98	87	0.03	2.60	**0.014**
collembola/microarthropods	0.73	8	0.09	3.03	87	0.03	2.61	**0.013**
% microarthropods	1.12	8	0.14	8.69	87	0.10	1.41	0.205
Invertebrate richness	240.88	8	30.11	428.75	87	4.93	6.11	**0.000**
Invertebrate Shannon E	1.22	8	0.15	2.33	85	0.03	5.58	**0.000**

Figure 6-38 Mean abundance, relative abundance and richness of invertebrate groups by sampling month

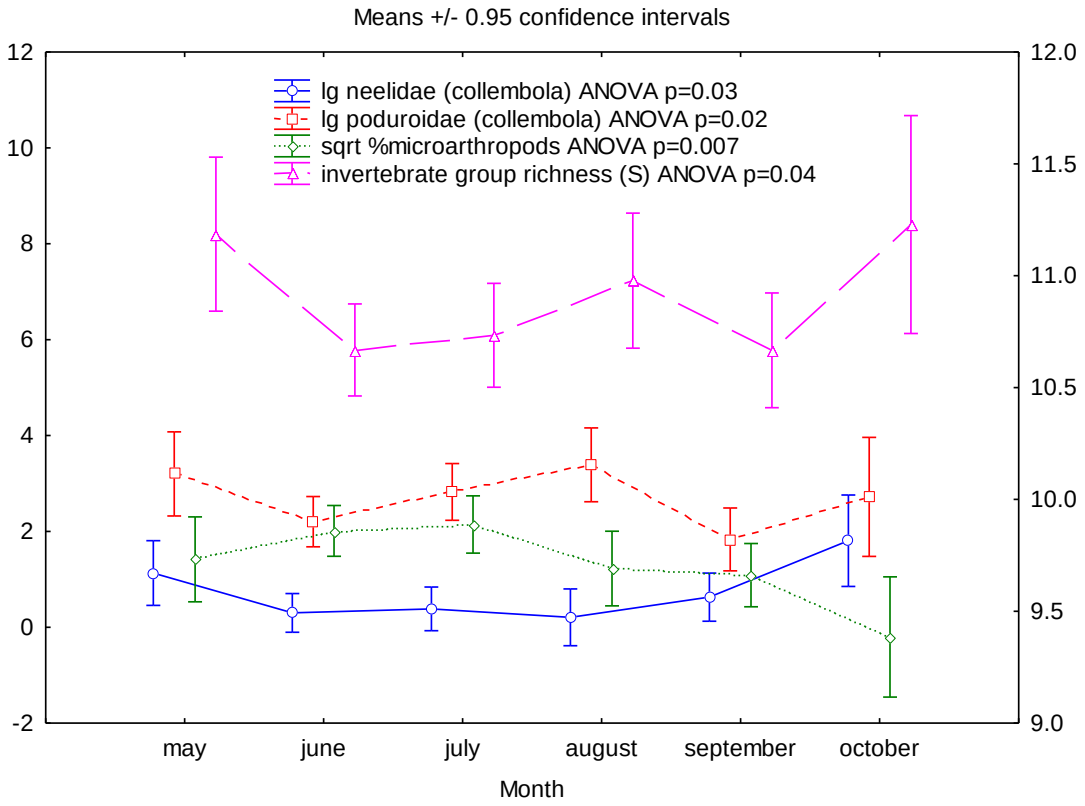


Figure 6-39 Mean abundance (log_{10} n+1 m^{-2}) and relative abundance (sqrt%+0.5) of mites by land use

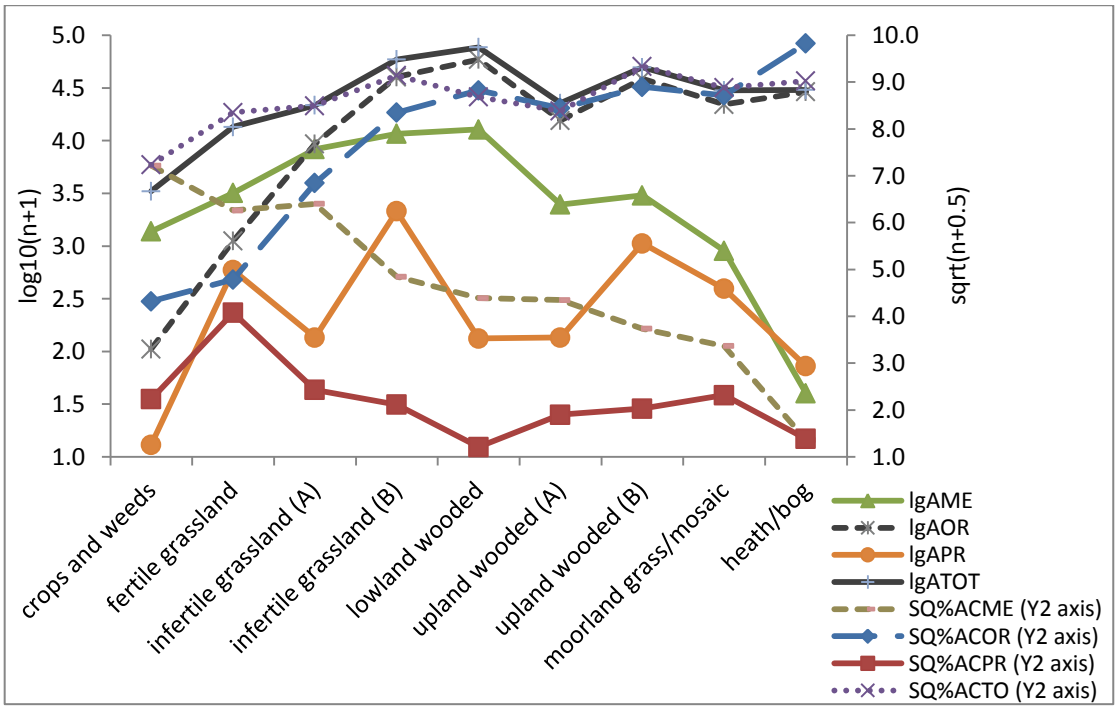


Figure 6-40 Mean abundance (m^{-2}) and relative abundance (%) of collembola by land use

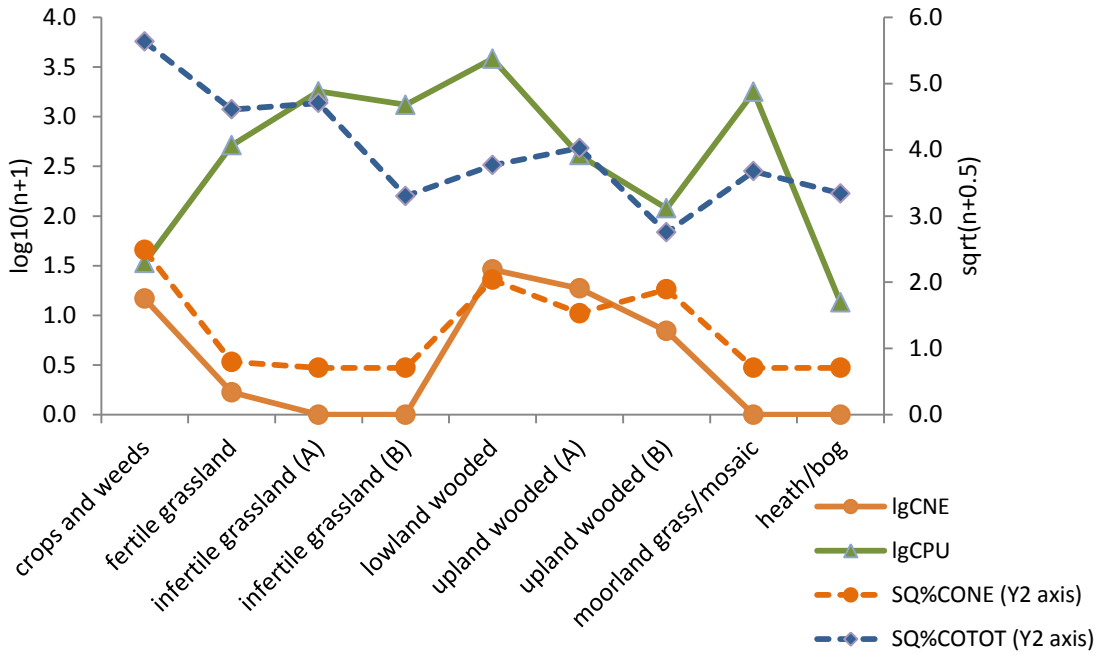


Figure 6-41 Mean diversity and community structure measures for invertebrates by land use

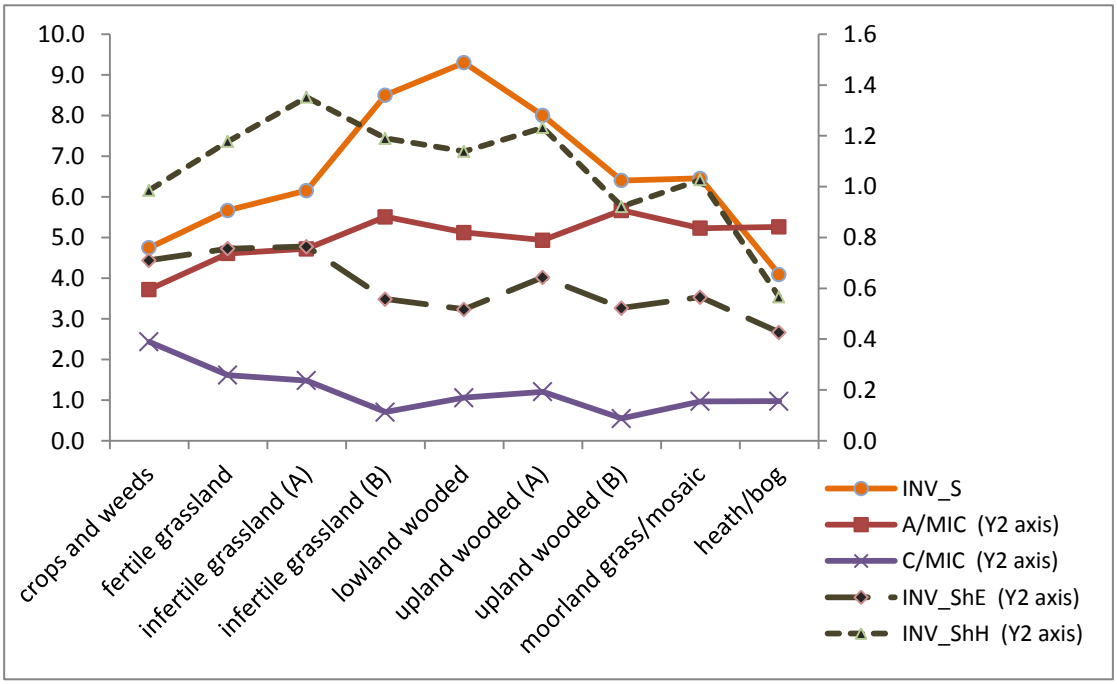


Figure 6-42 Mean abundance of invertebrates and microarthropods (collembola+mites) by land use (log_{10} n+1 m^{-2}).

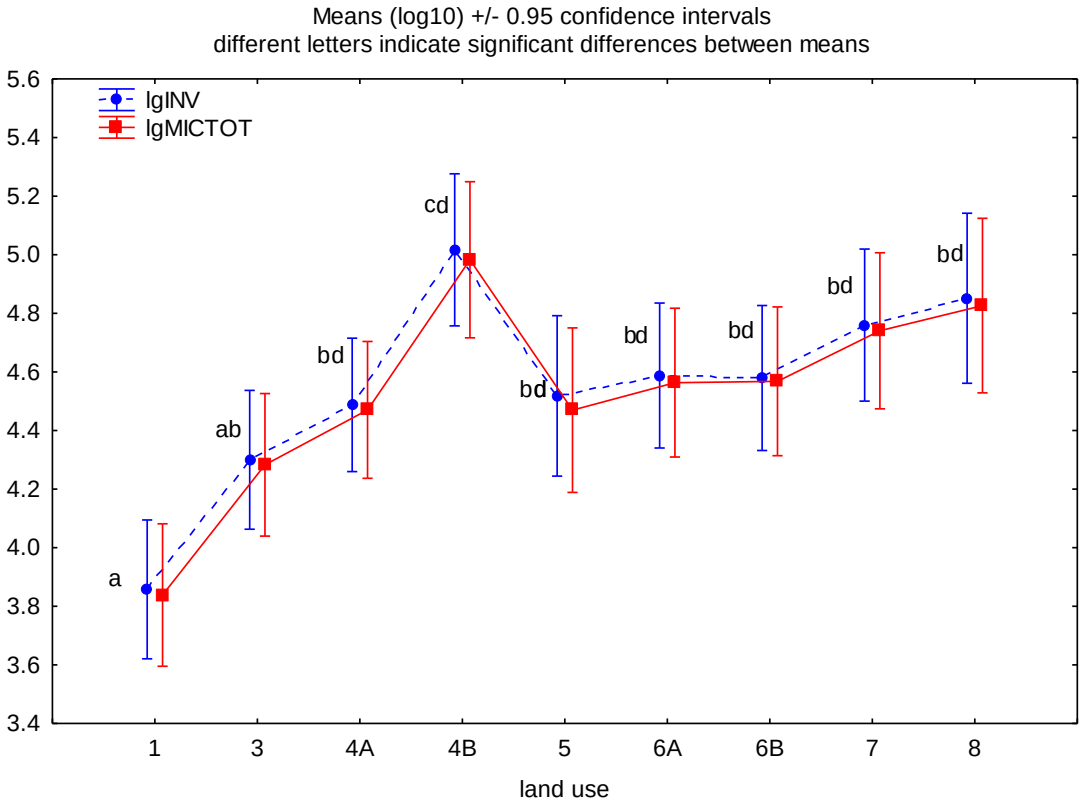


Figure 6-43 Box-plot of invertebrate and microarthropod taxa richness by land use

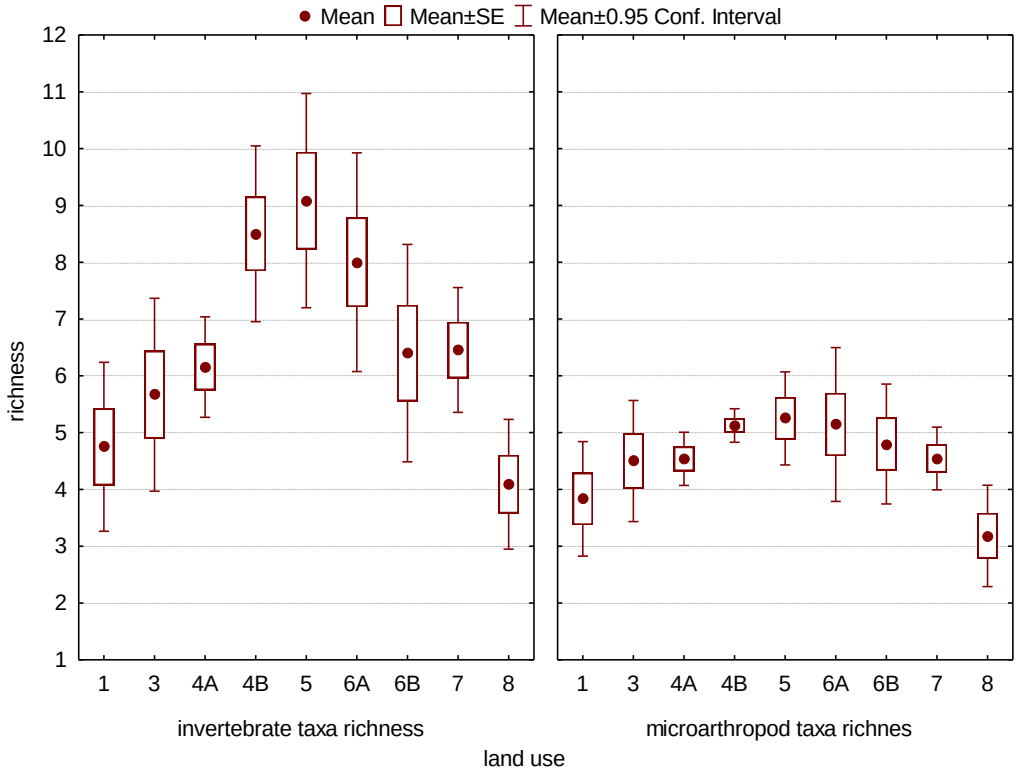


Figure 6-44 Box-plot of abundances (log10 n+1) of mites, collembola and invertebrates by land use

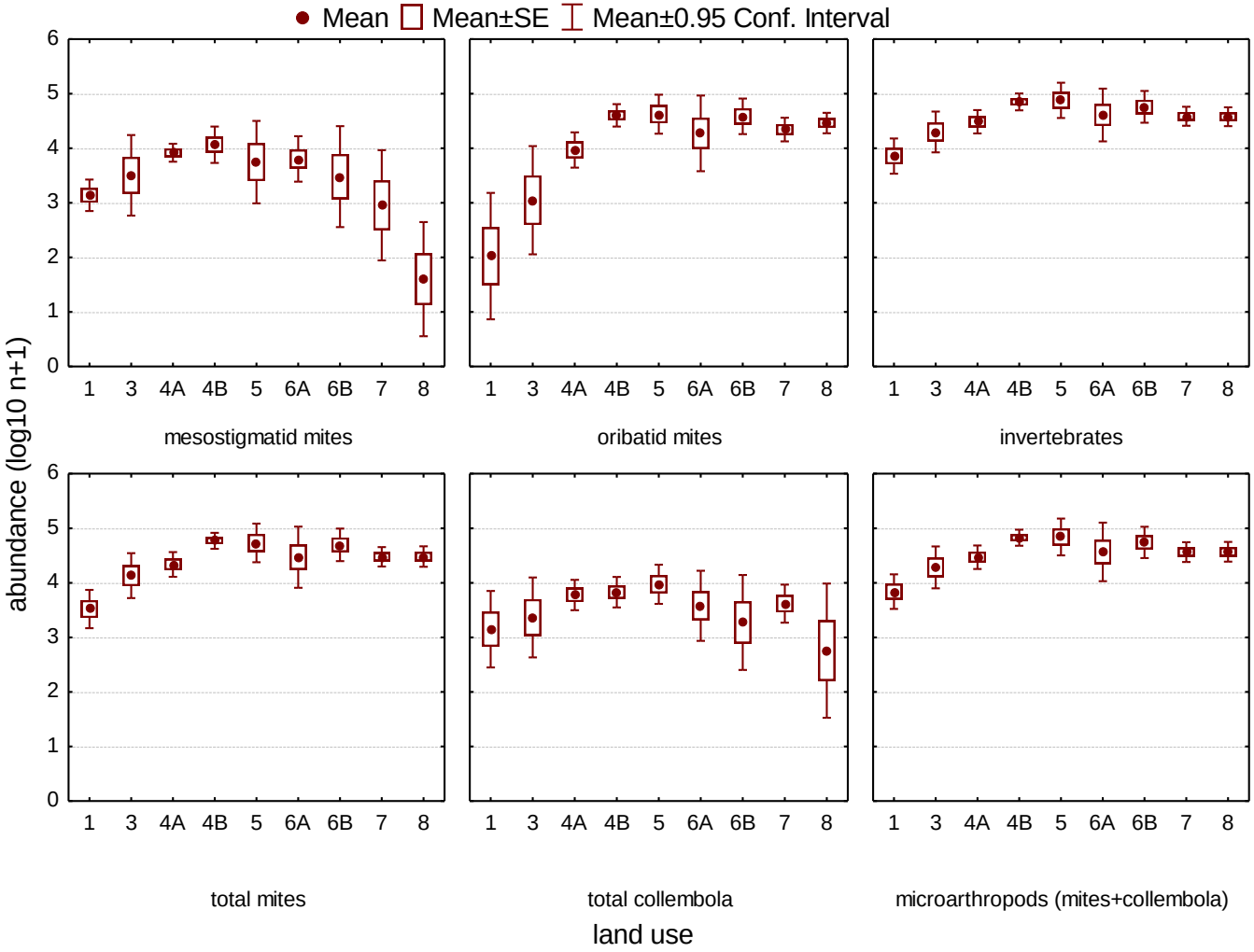
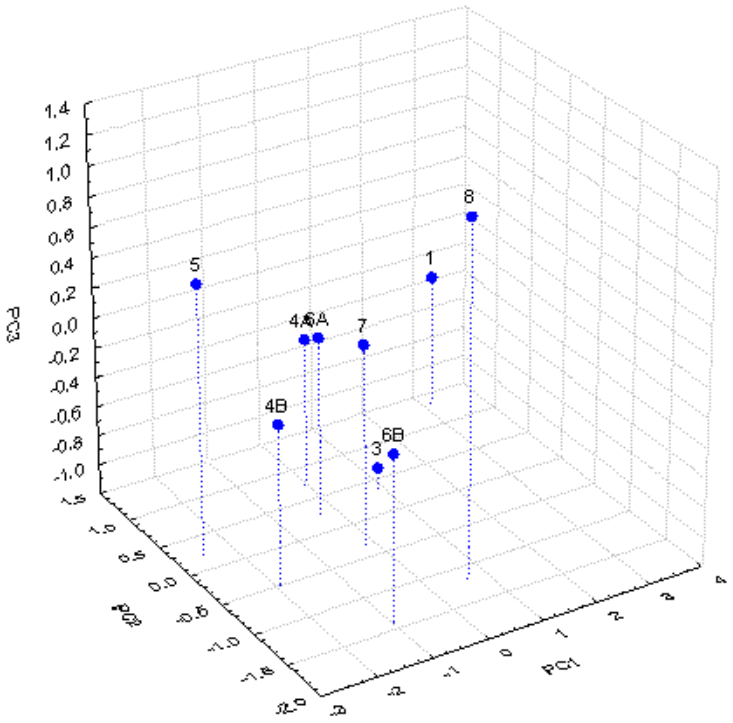


Figure 6-45 3D plot of results from the first three axes of PCA of invertebrate measures across nine land uses

	PC1	PC2	PC3
%Eigenvalue	41	34	7
ANOVA Px	0.000	0.09	0.000

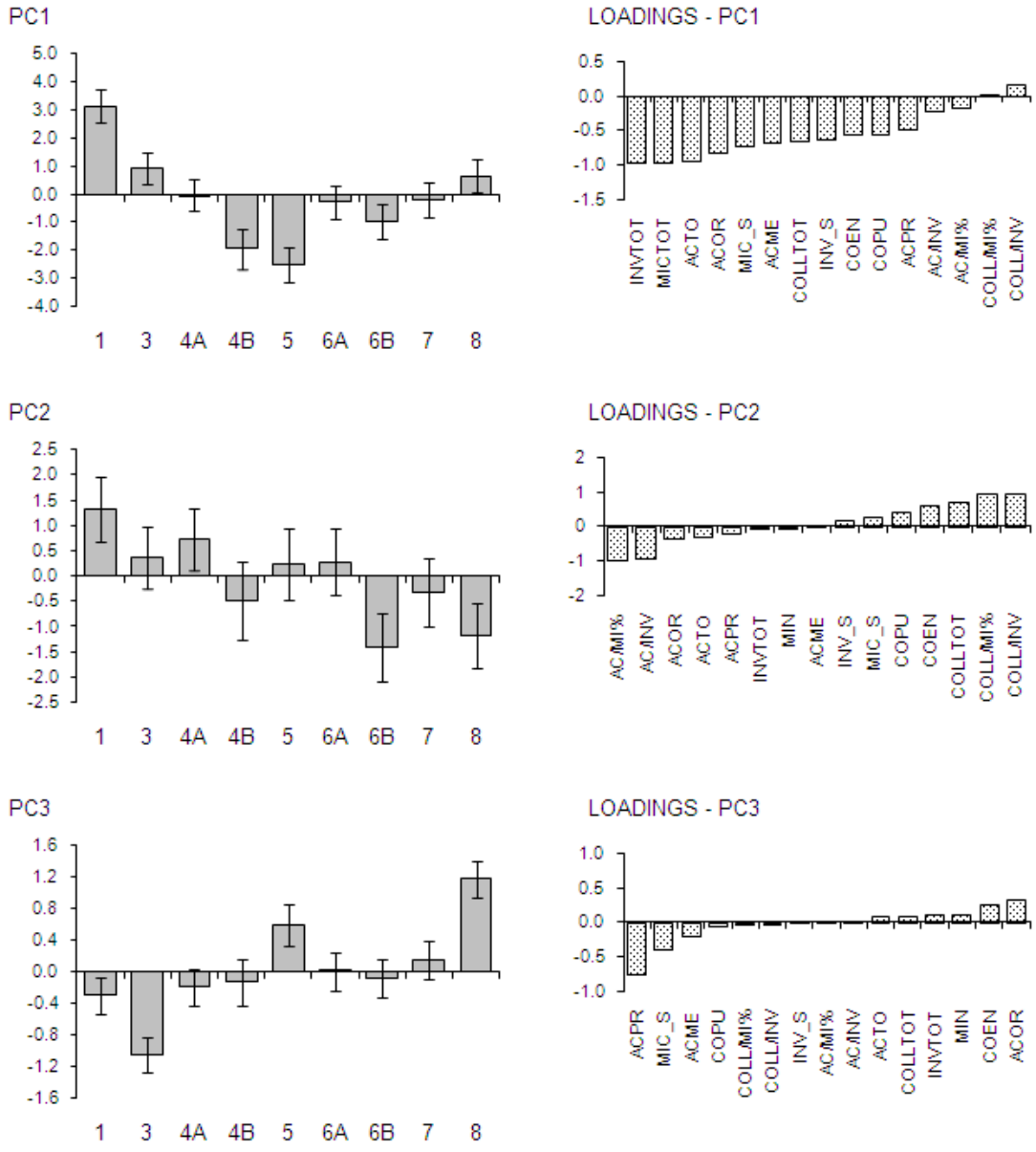


Key to land uses	
1	crops+weeds
3	fertile grass
4A	infertile acid grass
4B	infertile calcareous grass
5	lowland deciduous wooded
6A	upland deciduous wooded
6B	coniferous deciduous wooded
7	moorland grass mosaic
8	heath/bog

Figure 6-46 Summary results for PC1, PC2 and PC3 from PCA of the invertebrate measures

(a) Land use means +/- 1 s.e

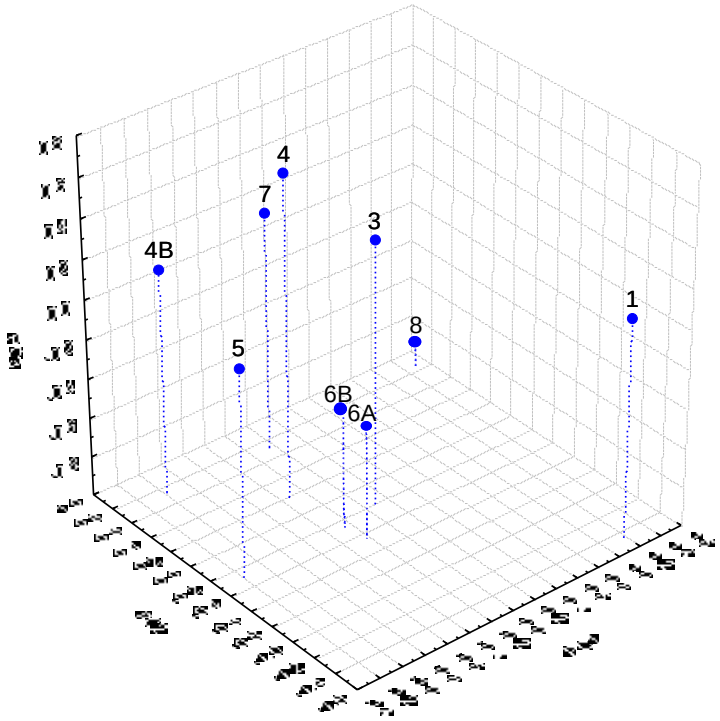
(b) Loadings for individual measures



1	Crops+weeds
3	Fertile grassland
4A	Infertile grassland (acid)
4B	Infertile grassland (calcareous)
5	Lowland wooded
6A	Upland wooded (deciduous)
6B	Upland wooded (coniferous)
7	Moorland grass mosaic
8	Heath / bog

Figure 6-47 3D plot of results from the first three axes of PCA of mite and collembola measures across nine land uses.

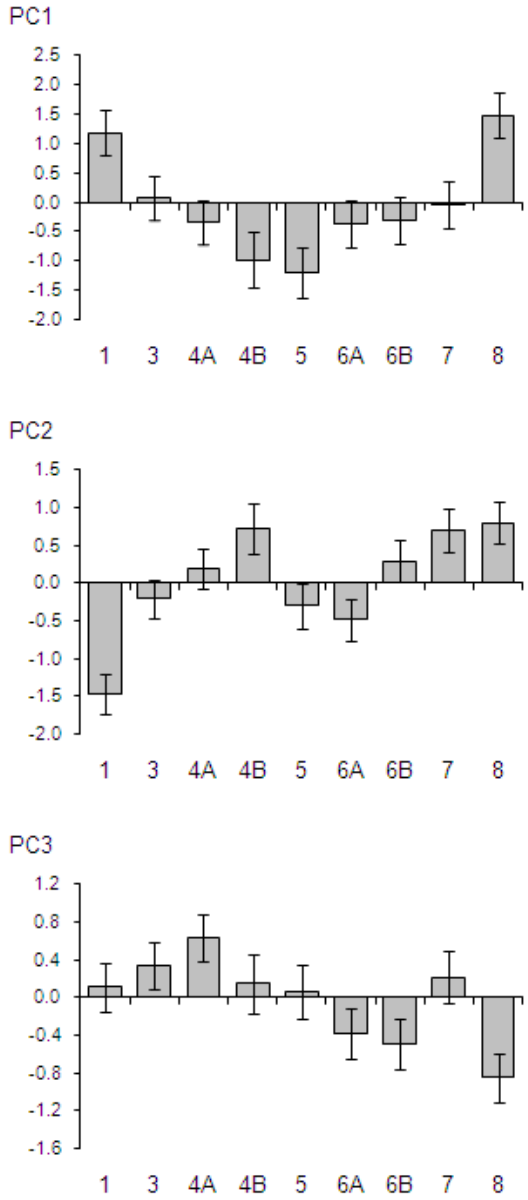
	PC1	PC2	PC3
%Eigenvalue	33	19	13
ANOVA Px	0.000	0.000	0.005



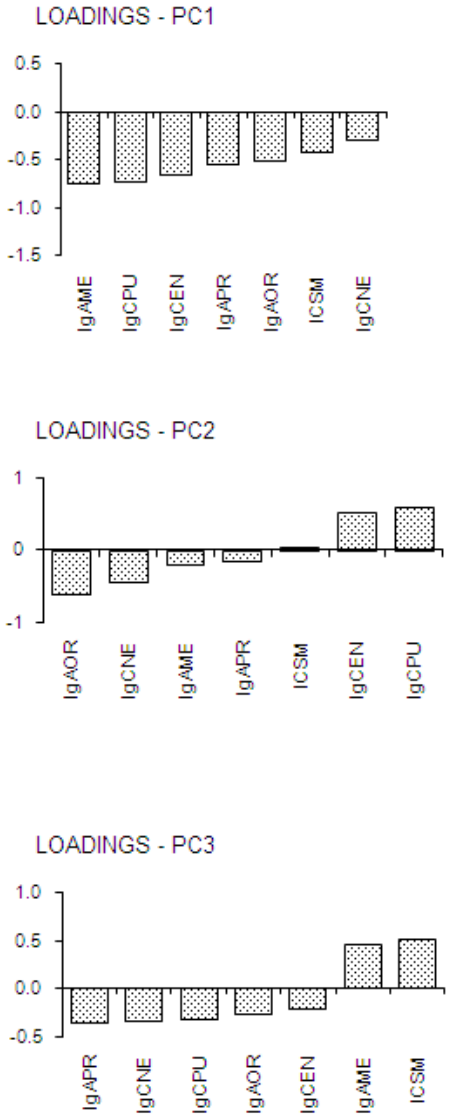
Key to land uses	
1	crops+weeds
3	fertile grass
4A	infertile acid grass
4B	infertile calcareous grass
5	lowland deciduous wooded
6A	upland deciduous wooded
6B	coniferous deciduous wooded
7	moorland grass mosaic
8	heath/bog

Figure 6-48 Summary results for PC1, PC2 and PC3 from PCA of mite and collembola measures

(a) Land use means +/- 1 s.e



(b) Loadings for individual measures



1	Crops+weeds
3	Fertile grassland
4A	Infertile grassland (acid)
4B	Infertile grassland (calcareous)
5	Lowland wooded
6A	Upland wooded (deciduous)
6B	Upland wooded (coniferous)
7	Moorland grass mosaic
8	Heath / bog

6.2.7 Combining the indicators

Individual methods or single measures can only provide a narrow perspective on the biological status of soil, or the sensitivity of soil biological properties and processes to specific pressures. The use of multiple soil chemical and physical indicators has been used widely to define soil quality for a range of purposes while the use of multiple measures have been explored elsewhere to profile the biological status of soils (e.g. Rutgers *et al.,* 2008). We examined two complementary approaches in combining phenotypic, genotypic and functional measures to characterize the biological status of soils. The first involved combining various measures from multiple indicators into a single display and the second involved an integrated statistical analysis of data from all of the indicators.

6.2.7.1 Multiple indicators – univariate data

Radar plots are widely used to combine different forms of data into a single diagram and can be used to visualize a baseline or typical range for different circumstances e.g. land use or management practices. The radar plot can consist of different measures from the same indicator method or different measures from different methods. These are demonstrated in radar plots of means from functional measures (respiration and enzyme responses) in Figure 6-49 and phenotypic measures (PLFA, invertebrates and nematodes) by individual land uses in Figure 6-50. The individual radar plots outline what could be defined as baseline values which could be used to monitor for statistically significant changes in status over time or to place a site within the context of a typical baseline for a particular land use. The differences between the plots illustrate that specific increases and/or decreases in measures would be expected (or required) when moving from one land use to another. Such changes could also be characterized for different management practices or pressures if sufficient information was available. The radar plots demonstrated here could be used to establish baselines for the specific land uses sampled during this project (see Table 6-1). Further data would be required to develop plots which would be application to broad land use types, or to other specific land uses. These data could be generated from further extensive surveys and from individual land use studies. The main constraint must be the generation of data using consistent and comparable methods.

The radar plot can be used to characterize and visualize the expected transition in soil biological measures from one land use to another. This is demonstrated in Figure 6-51 for agricultural land uses and lowland woodland, and in Figure 6-52 for semi-natural habitats moving from woodland through moorland grass/mosaic to heath/bog. For Figure 6-51, a transition from arable to grassland would involve a decrease of the fungal/bacterial ratio along with an increase in other measures while a transition from arable to woodland would require an increase in fungal/bacterial ratio as well as an increase in other measures (oribatids, invertebrate richness and glucose induced respiration). In Figure 6-52, a transition from upland coniferous woodland to deciduous woodland could be followed by an increase in invertebrate richness, total nematodes and fungal/bacterial ratio while a transition from moorland/grass mosaic to heath/bog could be followed by an increase in enzyme and respiration activities and

decrease in invertebrate richness. These results also suggest that a transition or restoration of soil biological properties/processes from one land use to another may not necessarily be a straightword increase or decrease over time since the transition may reflect the soil biological status of successional or intermediate land uses. For example, a transition from crops+weeds to lowland wooded may have an intermediate stage where fungal/bacterial ratios and glucose respiration are lower or greater, respectively, that the expected levels in lowland wooded (Figure 6-51).

A caveat with the radar approach is that it does not obviously account for variation or ranges in individual measures. For example a mean value may be statistically significant from the baseline but this may still be within a typical or acceptable range of variation for a land use. Further work is required to identify which statistics would be most useful for display in radar plots i.e. monitoring for status and change will need to define the levels at which change or differences from a preferred state become unacceptable for different purposes. These would then need to be considered alongside the radar plots, or integrated into the plots as lower and upper limits.

Ultimately, there are numerous ways to present and illustrate data on multiple measures and it would be sensible to work with end-users to identify the most appropriate approaches for specific purposes. In parallel, the results from this study illustrate that there are various measures from individual methods that could be incorporated into a multiple measure assessment of the biological status of soils. The selection of the measures can be based on statistical significance but should also include some consideration of the purpose of the assessment. An assessment of restoration success may be best served by a mix of measures that produce constrasting and interpretable differences between land uses while monitoring status or change within a land use may be best served by a mix of measures that best characterizes that land use. The measures used in this instance where selected to illustrate these issues rather as definitive multiple measures for the land uses presented.

Figure 6-49. Radar plots for each land use presenting mean values for univariate measures from the multi-enzyme assay and the multiple substrate induced respiration. MSIR data scaled 2.5 fold.

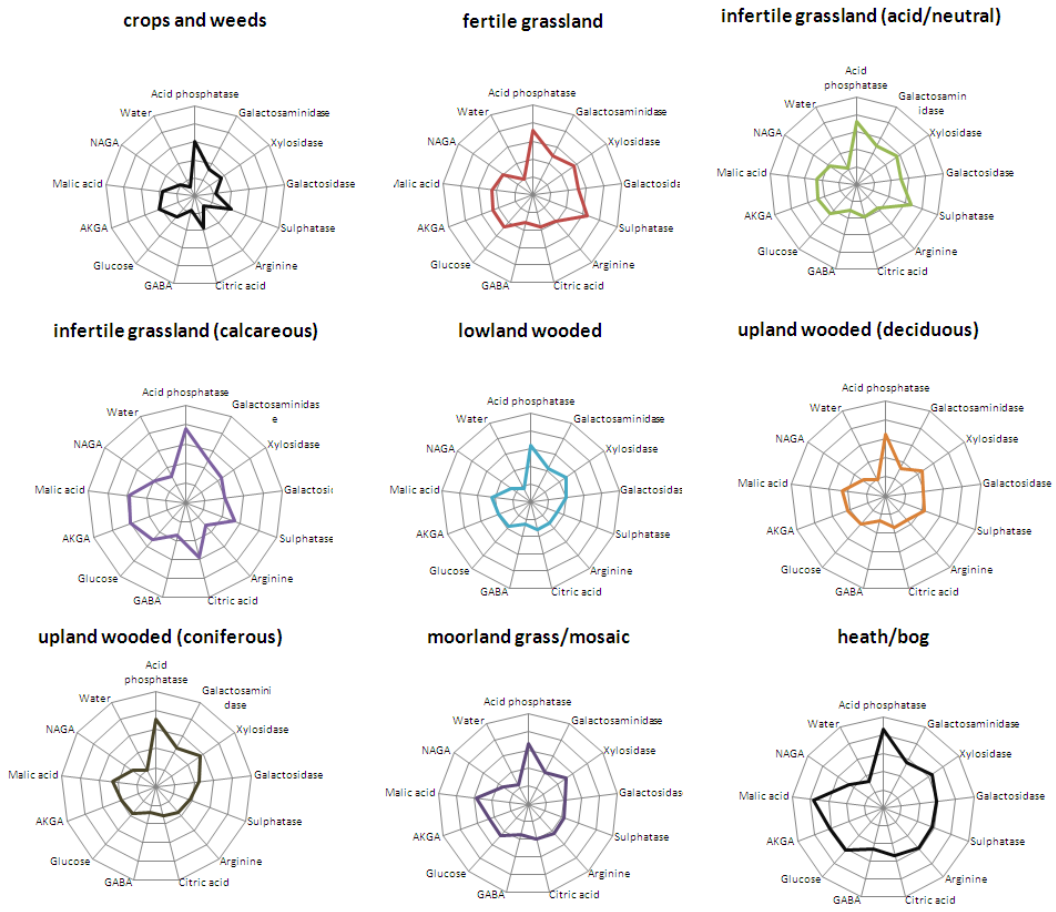


Figure 6-50. Radar plots for each land use presenting mean values for univariate measures from PLFA, invertebrate (dry extraction) and nematode analyses. F/B ratio scaled 60 fold and invertebrate and nematode measures by 2 fold (except invertebrate richness).

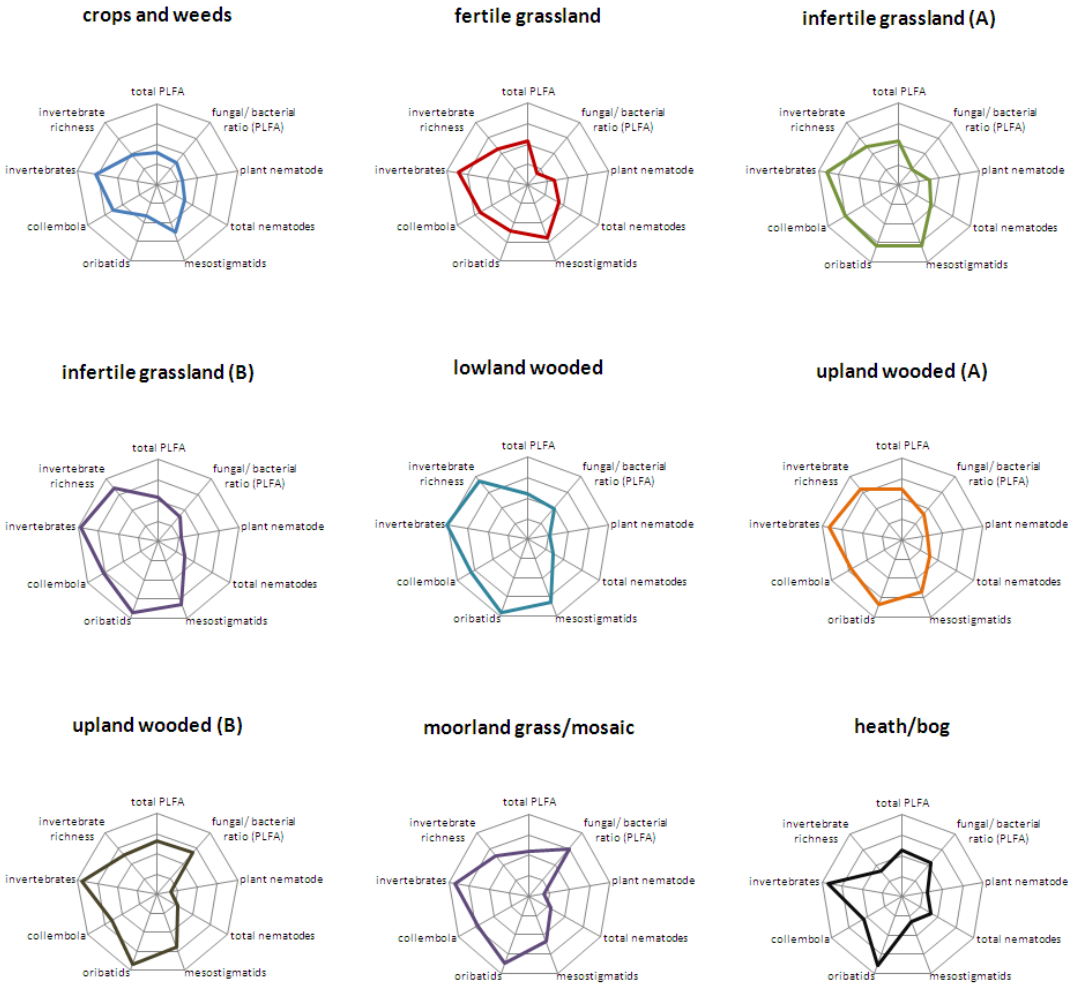


Figure 6-51. Radar plot constrasting mean values of univariate measures from PLFA, invertebrate (dry extraction) and nematode (wet extraction) methods for arable, grassland and woodland land uses.

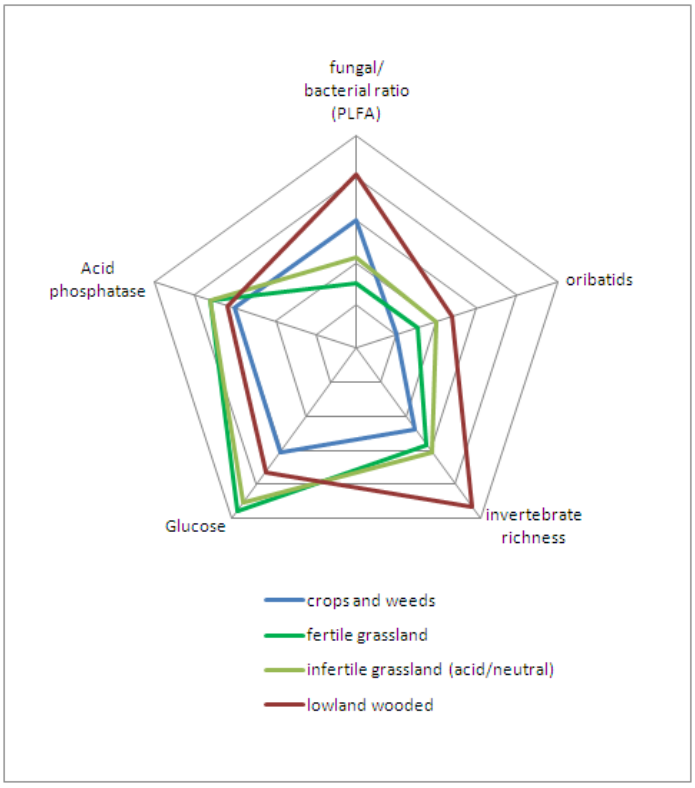
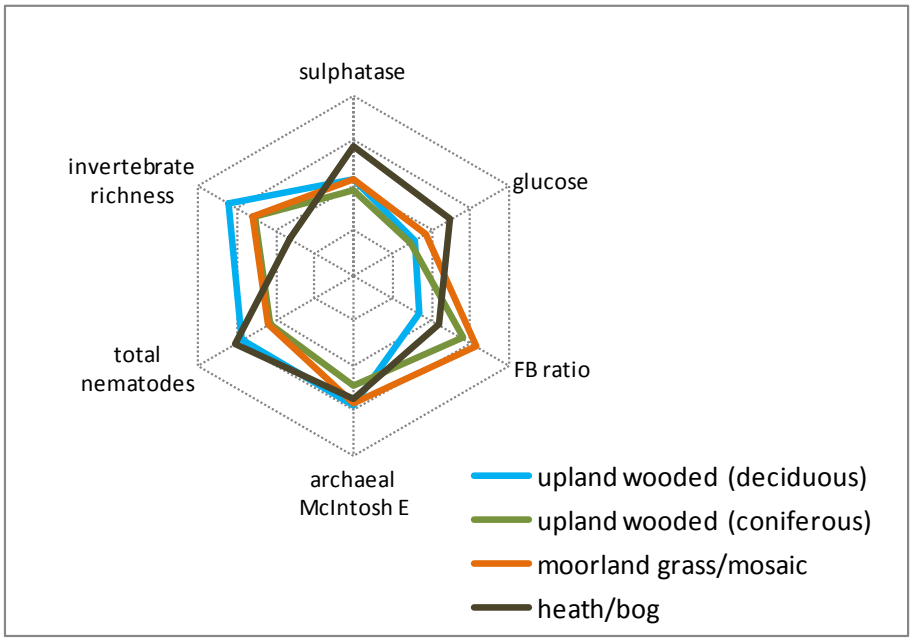


Figure 6-52. Radar plot contrasting mean values of univariate measures from the enzyme assay, MSIR, PLFA, invertebrate (dry extraction) and nematode (wet extraction) methods for upland wooded, moorland/grass mosaic and heath/bog land uses.



6.2.7.2 Multiple indicators – multivariate data

The 3D plots from the multivariate analyses of the individual measures are brought together in Figure 6-53. These figures illustrate that the multivariate analyses produced distinct land use discrimination patterns amongst the indicator methods. There were consistent discriminations across all the indicators (except nematodes) with clear separation of crops+weeds from semi-natural land uses (wooded, moorland grass mosaic and heath/bog). In addition certain indicators demonstrated separation of agricultural land uses (crops+weeds and grass) from all semi-natural land uses, and further clear separation between individual land uses (PLFA, fungal DNA and archaeal DNA). These separation patterns follow an interpretable transition from intensive to extensive land uses. The process methods (enzymes and MSIR) also demonstrated separation patterns although the transition within wooded or grassland uses was as clearly interpretable as those for PLFA, fungal DNA and archaeal DNA. The land use discrimination for invertebrates, nematodes and bacterial DNA were less distinct and not so easily interpretable.

Summary results from the multivariate analyses of the individual methods are presented in Table 6-27. The use of multiple measures from each of the methods resulted in a high percentage of the variance being explained, by the eigenvalues, for the first three PC axes (45.6 to 96%). There is no clear cut-off in the interpretation of variances, but the values obtained are relatively high and, in combination with the significant ANOVA results, add confidence that the land use discriminations are robust.

Where the ANOVAs were significant for all three PC axes, the percentages demonstrated a hierarchy following MSIR>enzymes>fungalDNA>archaeal DNA>PLFA. With MSIR and enzymes, the high percentages were associated with PC1 with clear discrimination between crops+weeds and heath/bog. Percentages for PLFA, archaeal and fungal DNA are more evenly spread across the first three PC axes which reflect the more distinct patterns of discrimination across all three axes.

Thus, the multivariate analyses of the individual methods demonstrated that each method produced a slightly different perspective on the biological status of soils across the nine land uses. These differences can be viewed in an alternate approach by plotting the mean values for the PC axes from the individual methods in radar plots. This approach is shown in Figure 6-54 with the indicators arranged in a clockwise arrangement to illustrate the three biological traits; genotypic (bacterial, fungal and archaeal), phenotypic (PLFAs, invertebrates and nematodes) and functional (MSIR and enzymes). These plots represent the configuration of soil biological properties and processes within each land use and amongst the nine land uses. Thus if two plots were identical in radar form, the composition of their genotypic, phenotypic and functional traits would essentially be the same, when taken in the context of the entire data set. Divergence from such similarity could be used to signify movement away from a typical state.

The radar plots reveal distinct configurations of the indicators within land uses (e.g. agriculture, grass, wooded) and a trajectory of change between the land uses which is reflected in changes in the relative contribution of the indicators to the radar structure. The distinct trajectory in radar morphology from crops to bogs suggests a form of succession in the integrated genotypic, phenotypic and functional structure of the associated soil biological communities in a remarkably consistent manner. This trajectory is easier to interpret in terms of land use succession compared to trajectories for the individual soil biological indicators, whether genotypic, phenotypic or functional, as illustrated in Figure 6-53.

As discussed previously, radar plots have been used to establish reference conditions for multiple soil biodiversity characteristics and to communicate the usefulness of soil biological measures in assessing soil quality (e.g. Breure *et al*., 2004). Our results using multivariate analyses advance this approach by providing the first experimental evidence that soil communities are organised with respect to a combination of their genotypic, phenotypic and functional traits according to the land use or habitat type across geographical scales, concomitant with differences in plant diversity and productivity, nutrient availability, physiological conditions and/or intensity of management (Bunce *et al*., 1999).

These data serve to illustrate that a sufficiently robust sampling strategy across geographical regions can overcome issues of seasonality and heterogeneity that influence the structure, function and dynamics of soil biodiversity at a local scales. Patterns in soil biology are clearly not solely driven by soil properties. Land use has a significant, if not dominant, role when considering a broad range of habitat types and when considering extensive geographical scales. It is only a historical lack of extensive and coherent spatial multivariate data that has left these patterns undiscovered until now.

Figure 6-53 3D plots of land use results from the first three axes of PCA of the individual and combined biological methods. Graphs show the first three principal components (PC1-3) derived from multivariate profiles within each habitat, determined according to a range of genotypic (a-c), phenotypic (d-f), functional (g-h) and a combination of all (i) properties. DNA = terminal restriction fragment length polymorphism (TRFLP) profile of community DNA amplified using group-specific primers; PLFA = phospholipid fatty acid profile; Multiple SIR = multiple substrate-induced respiration profile. Values in parentheses on axes denote percent variation account for by each respective principal component. Asterisks denote significance level for one-way ANOVA: No asterisk $P > 0.05$; ** $P < 0.01$; ***$P < 0.005$

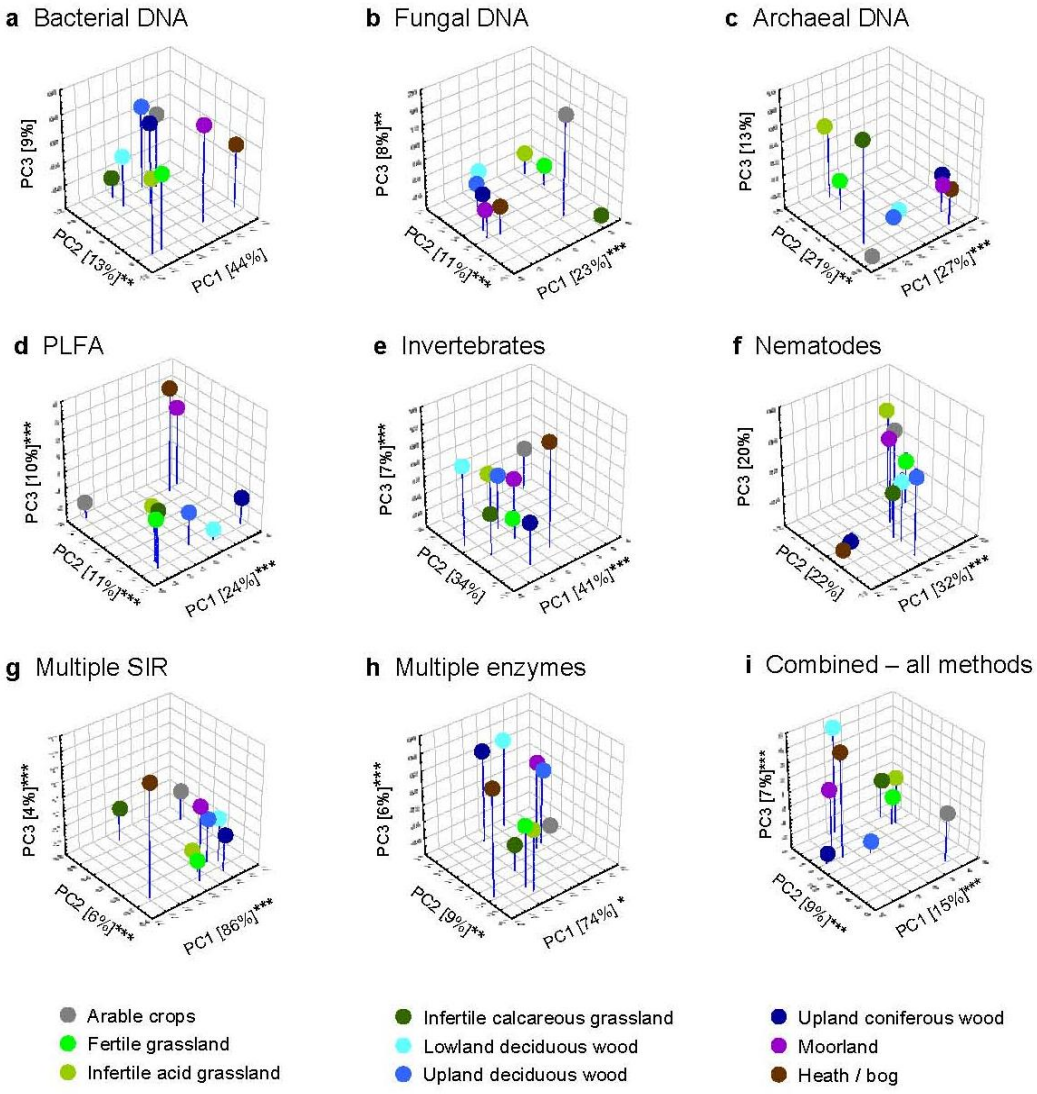
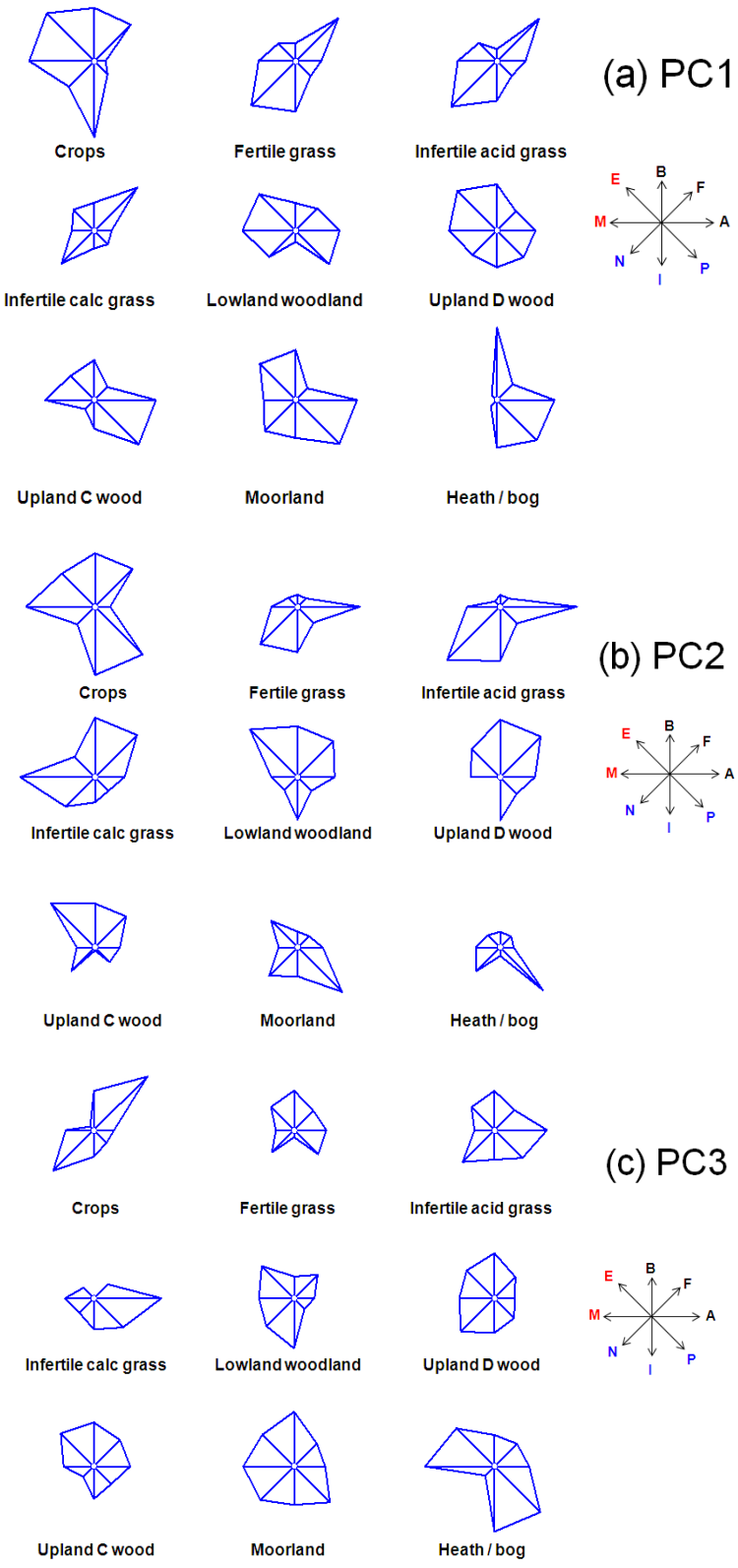


Table 6-27 Summary results from principal components analyses of individual and combined methods for the soil biological indicators across the nine land uses. Variance explained by principal components (PCs) 1, 2 and 3 and significance of ANOVA GLM analyses of land uses.

	Land Use	PC1	PC2	PC3	Sum (%)
Bacterial DNA	Eigenvalue %	44.2	12.8	8.84	65.8
	P	0.09	<0.01	0.61	
Fungal DNA	Eigenvalue %	23.1	11.3	8.0	63.5
	P	<0.001	<0.001	<0.05	
Archaeal DNA	Eigenvalue %	27.2	20.6	12.8	60.6
		<0.001	<0.001	<0.001	
PLFA	Eigenvalue %	23.7	11.5	10.4	45.6
	P	<0.001	<0.001	<0.001	
Invertebrates	Eigenvalue %	41.3	34.4	6.5	82.2
	P	<0.001	0.09	<0.001	
Nematodes	Eigenvalue %	31.8	21.8	19.7	73.3
	P	<0.001	0.18	0.34	
MSIR	Eigenvalue %	85.8	6.47	3.69	96.0
	P	<0.001	<0.001	<0.001	
Enzymes	Eigenvalue %	74.0	9.32	5.56	88.8
	P	<0.05	<0.01	<0.001	
Combined	Eigenvalue %	14.5	8.96	7.35	30.8
	P	<0.001	<0.001	<0.001	

Figure 6-54 Radar plots for the nine land uses displaying PC1, PC2 and PC3 from the PCA analysis of each soil biological method. The scaling of each axis is consistent for each method between each plot. B = Bacterial TRFs; F = fungal TRFs; A = archaeal TRFs; P = PLFAs; I = invertebrates; N = nematodes; M = MSIR; E = multiple enzymes. The methods are ordered in a clockwise manner, as shown on the wheel keys, according to genotypic (black), phenotypic (blue) and functional (red).



6.2.7.3 Integrated multivariate analysis

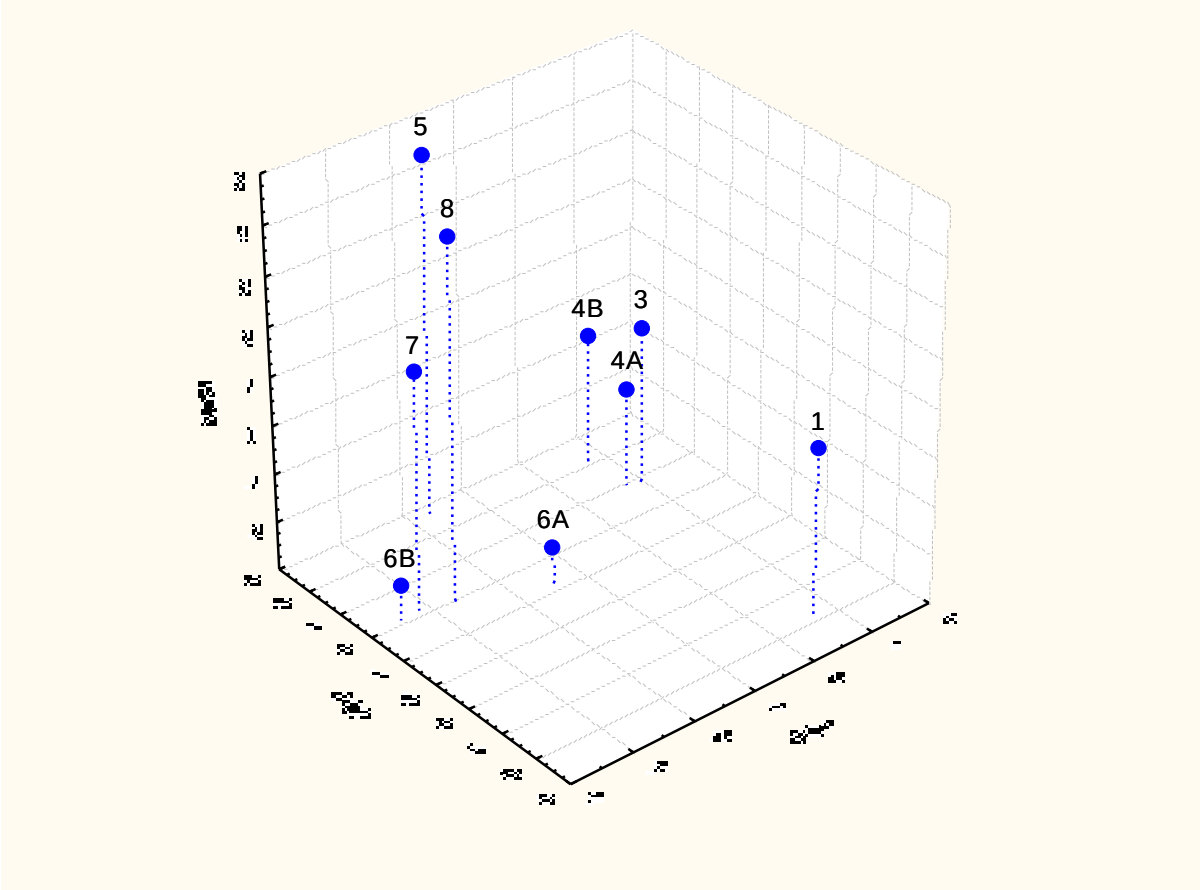
To investigate the relative significance of the different genotypic, phenotypic and functional traits on land use discrimination, the data for individual measures that were used for the PCA analyses for the individual methods were combined into a single PCA analysis. The data included a selection of respiration rates, enzyme activities, abundance, relative abundance and ratio data. Data transformations for the individual analyses, carried out to address normalization, were retained (logarithmic, sqrt and asin).

The 3D plot of the combined analysis (Figure 6-55) illustrates multivariate discrimination across the nine land uses. Analysis of variance showed significant effects of land use on all three principle components axes (PC1, PC2 and PC3) which explained 31% of the variance in total. Summary results for the PC axes illustrate the effects of each axis on combined measures by land use (Figure 6-56). The results for PC1 demonstrate clear separation of agricultural land uses from semi-natural land uses, and the separation of deciduous woods from other semi-natural habitats. PC2 demonstrates separation of grasslands from crops+weeds and separation of lowland deciduous woodland from upland deciduous woodland. PC3 demonstrates separation of upland wooded (deciduous and coniferous) from the other semi-natural habitats. The resultant 3D plot produces a pattern of succession from intensive arable through grasslands, lowland wooded to upland wooded and ultimately to moorland grass mosaic and heath/bog. These statistically significant results indicate, for the first time, that an integrated perspective of the entire soil community reflects an ecological succession that has, until now, only been demonstrated for a few soil biodiversity characteristics. The factor loadings for the PC axes (Table 6-28) indicate that the patterns are generated by the combined influence of genotypic, phenotypic and functional methods and not from the dominance of specific or a few measures.

In Table 6-27 the results from ANOVA analyses of the first three PC axes from the combined measures PCA are compared with the ANOVA results for the first three PC axes from the PCA of the individual methods. These results show that the combined measures produced land use discrimination of equivalent statistical significance to that from the individual methods. The variance explained by the eigenvalues in the combined measures (30.8%) was lower than for the individual methods (45.6 to 96%) but still suggests that the measures are fairly effective at discriminating land uses. It also suggests that constrasting land use discrimination patterns amongst the individual measures could have reduced the variance explained in the integrated analyses which combined the measures. If this is the case then the variance explained by the integrated analysis could be improved through sub-selection of the measures across the methods.

Figure 6-55 3D plot of land use results from the first three axes of PCA of the combined measures

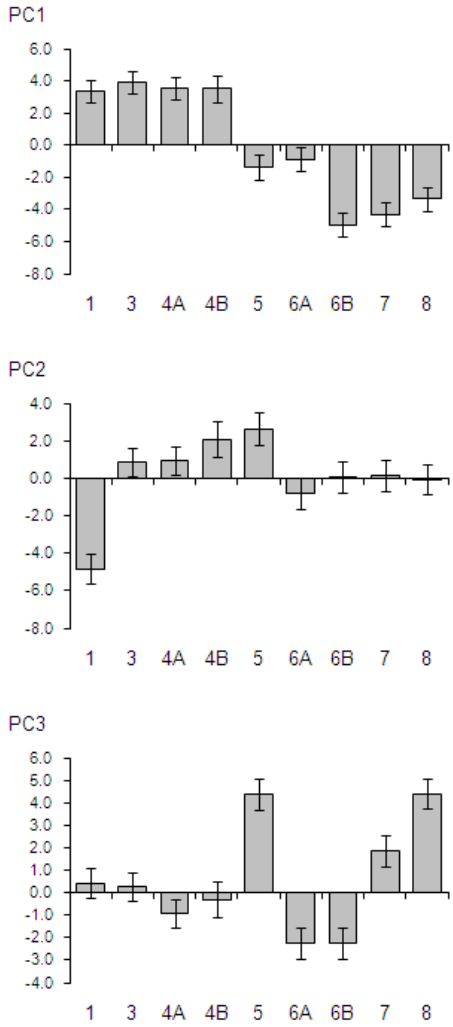
	PC1	PC2	PC3
%Eigenvalue	15	9	7
ANOVA Px	0.000	0.000	0.000



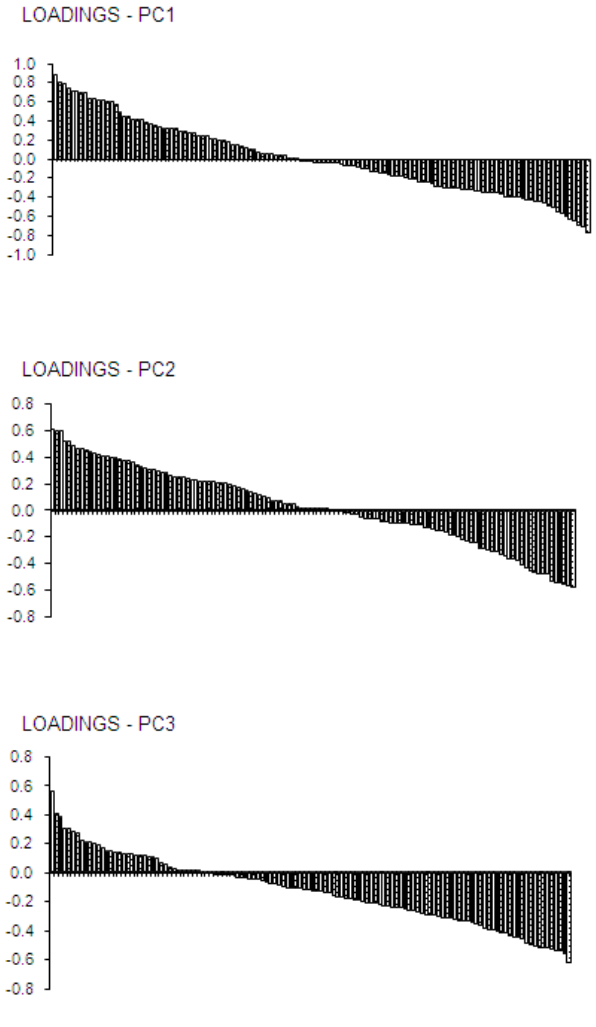
Key to land uses	
1	crops+weeds
3	fertile grass
4A	infertile acid grass
4B	infertile calcareous grass
5	lowland deciduous wooded
6A	upland deciduous wooded
6B	coniferous deciduous wooded
7	moorland grass mosaic
8	heath/bog

Figure 6-56 Summary results for PC1, PC2 and PC3 from PCA of the combined measures

(a) Land use means +/- 1 s.e



(b) Loadings for individual measures



1	Crops+weeds
3	Fertile grassland
4A	Infertile grassland (acid)
4B	Infertile grassland (calcareous)
5	Lowland wooded
6A	Upland wooded (deciduous)
6B	Upland wooded (coniferous)
7	Moorland grass mosaic
8	Heath / bog

Table 6-28 Loadings of the individual measures from the PCA of combined measures sorted from lowest to highest values.

Method	Measure	PC1	Method	Measure	PC2	Method	Measure	PC3
TRFLP	arcsqrt14	-0.78	MSIR	lnH2O	-0.58	MSIR	lnMAL	-0.63
PLFA	sqrtP10	-0.7	MSIR	lnCIT	-0.57	MSIR	lnGLC	-0.57
PLFA	sqrtP_40	-0.69	MSIR	lnNAG	-0.56	MSIR	lnARG	-0.54
PLFA	sqrtP_28	-0.64	MSIR	lnGLC	-0.55	MSIR	lnGAB	-0.54
PLFA	sqrtP11	-0.62	MSIR	lnGAB	-0.54	MSIR	lnNAG	-0.53
PLFA	sqrtP_31	-0.6	MSIR	lnKGA	-0.54	PLFA	sqrtP_41	-0.53
PLFA	sqrtP_8	-0.57	PLFA	sqrtP24	-0.48	MSIR	lnH2O	-0.53
inverts	lg_ACOR	-0.55	PLFA	sqrtP25	-0.48	enzymes	lnS4	-0.52
TRFLP	arcsqrt28	-0.5	MSIR	lnMAL	-0.47	MSIR	lnCIT	-0.51
TRFLP	arcsqrt4	-0.5	enzymes	lnS4	-0.46	MSIR	lnKGA	-0.5
TRFLP	sqrtITS152	-0.46	TRFLP	16S33	-0.46	inverts	ACME	-0.49
inverts	lg_ACTO	-0.45	MSIR	lnARG	-0.43	TRFLP	sqrtITS115	-0.46
PLFA	sqrtP_47	-0.44	TRFLP	16S97	-0.4	PLFA	sqrtP_47	-0.45
inverts	asin_A/INV	-0.43	TRFLP	16S10	-0.37	inverts	A/C_S	-0.45
PLFA	sqrtP_32	-0.43	enzymes	lnS5	-0.37	enzymes	lnS7	-0.44
inverts	asin_A/MI	-0.41	PLFA	sqrtP13	-0.36	enzymes	lnS5	-0.41
inverts	lg_N	-0.39	enzymes	lnS2	-0.35	PLFA	sqrtP20	-0.41
inverts	lg_MI_N	-0.39	enzymes	lnS3	-0.34	enzymes	lnS3	-0.4
PLFA	sqrtP_43	-0.39	enzymes	lnS7	-0.31	inverts	sqrt_COPU	-0.4
TRFLP	16S97	-0.39	PLFA	sqrtP16	-0.31	PLFA	sqrtP23	-0.39
enzymes	lnS4	-0.37	PLFA	sqrtP23	-0.3	PLFA	sqrtP_39	-0.38
enzymes	lnS7	-0.35	enzymes	lnS8	-0.29	TRFLP	16S94	-0.37
enzymes	lnS8	-0.35	enzymes	lnS6	-0.28	inverts	INV_S	-0.35
MSIR	lnARG	-0.35	enzymes	lnS1	-0.24	enzymes	lnS6	-0.34
MSIR	lnGLC	-0.34	NEMs	sqrt_b:F	-0.24	PLFA	sqrtP27	-0.33
TRFLP	sqrtITS74	-0.34	PLFA	sqrtP_46	-0.23	PLFA	sqrtP_5	-0.33
PLFA	sqrtP26	-0.33	PLFA	sqrtP_36	-0.22	inverts	lg_COLL	-0.33
PLFA	sqrtP_9	-0.33	PLFA	sqrtP21	-0.19	PLFA	sqrtP21	-0.33
TRFLP	16S10	-0.32	TRFLP	arcsqrt9	-0.19	enzymes	lnS2	-0.32
enzymes	lnS6	-0.31	NEMs	Bact%	-0.18	enzymes	lnS8	-0.31
MSIR	lnGAB	-0.31	PLFA	sqrtP22	-0.17	TRFLP	16S58	-0.31
MSIR	lnKGA	-0.3	PLFA	sqrtP_33	-0.15	inverts	lg_N	-0.3
MSIR	lnNAG	-0.3	PLFA	sqrtP15	-0.15	inverts	lg_MI_N	-0.3
PLFA	sqrtP_36	-0.3	TRFLP	arcsqrt23	-0.14	TRFLP	16S86	-0.29
PLFA	sqrtP_41	-0.3	inverts	asin_C/INV	-0.13	TRFLP	16S100	-0.29
MSIR	lnMAL	-0.29	NEMs	lg10_bact	-0.12	PLFA	sqrtP_9	-0.28
enzymes	lnS5	-0.26	TRFLP	arcsqrt22	-0.11	inverts	LG_ACPR	-0.28
enzymes	lnS1	-0.25	PLFA	sqrtP10	-0.1	TRFLP	16S135	-0.27
enzymes	lnS3	-0.25	NEMs	lg10_total	-0.1	TRFLP	16S118	-0.26
MSIR	lnCIT	-0.24	PLFA	sqrtP_44	-0.1	inverts	lg_ACTO	-0.25
PLFA	sqrtP1	-0.21	PLFA	sqrtP_29	-0.1	enzymes	lnS1	-0.25
PLFA	sqrtP_7	-0.21	PLFA	sqrtP_32	-0.09	TRFLP	16S8	-0.24
enzymes	lnS2	-0.19	TRFLP	sqrtITS151	-0.09	TRFLP	16S140	-0.24
MSIR	lnH2O	-0.18	PLFA	sqrtP_37	-0.09	PLFA	sqrtP_30	-0.23
PLFA	sqrtP_39	-0.18	PLFA	sqrtP12	-0.09	PLFA	sqrtP17	-0.23
NEMs	lg10_total	-0.17	NEMs	sqrt_Omni	-0.08	TRFLP	16S66	-0.23
NEMs	sqrt_b:F	-0.17	PLFA	sqrtP_4	-0.07	NEMs	sqrt_Omni	-0.21
PLFA	sqrtP27	-0.15	PLFA	sqrtP_31	-0.06	PLFA	sqrtP_42	-0.21
TRFLP	16S33	-0.15	NEMs	lg10_omni	-0.06	PLFA	sqrtP_6	-0.21
NEMs	lg10_bact	-0.13	PLFA	sqrtP_30	-0.06	TRFLP	16S56	-0.2
NEMs	lg10_plant	-0.13	NEMs	lg10_carniv	-0.05	PLFA	sqrtP10	-0.19
NEMs	lg10_omni	-0.1	PLFA	sqrtP_6	-0.03	inverts	lg_COEN	-0.19
TRFLP	16S85	-0.1	PLFA	sqrtP1	-0.02	PLFA	sqrtP_45	-0.18
PLFA	sqrtP16	-0.09	PLFA	sqrtP_35	0	TRFLP	arcsqrt9	-0.18
PLFA	sqrtP19	-0.07	PLFA	sqrtP_42	0	PLFA	sqrtP_35	-0.17
PLFA	sqrtP_44	-0.07	PLFA	sqrtP_7	0.01	inverts	lg_C/MI%	-0.17
inverts	INV_S	-0.06	NEMs	lg10_plant	0.01	PLFA	sqrtP18	-0.16
PLFA	sqrtP2	-0.05	PLFA	sqrtP_34	0.01	PLFA	sqrtP16	-0.14
inverts	A/C_S	-0.04	TRFLP	arcsqrt1	0.01	TRFLP	16S19	-0.14
inverts	sqrt_COPU	-0.04	inverts	lg_C/MI%	0.01	PLFA	sqrtP_37	-0.14
NEMs	lg10_fungal	-0.04	PLFA	sqrtP20	0.01	PLFA	sqrtP11	-0.13
NEMs	Plant%	-0.04	TRFLP	sqrtITS74	0.02	NEMs	Bact%	-0.13

Table 6-28 cont. Loadings of the individual measures from the PCA of combined measures

Method	Measure	PC1	Method	Measure	PC2	Method	Measure	PC3
PLFA	sqrtP_5	-0.04	PLFA	sqrtP26	0.02	inverts	lg_ACOR	-0.12
TRFLP	16S34	-0.04	PLFA	sqrtP_38	0.02	TRFLP	arcsqrt33	-0.12
NEMs	lg10_carni	-0.03	TRFLP	sqrtITS142	0.02	inverts	asin_C/IN	-0.11
inverts	LG_ACPR	-0.01	PLFA	sqrtP_47	0.03	TRFLP	sqrtITS85	-0.11
NEMs	Bact%	-0.01	TRFLP	sqrtITS159	0.05	PLFA	sqrtP_4	-0.11
inverts	ACME	0.01	TRFLP	16S34	0.06	NEMs	lg10_omni	-0.1
PLFA	sqrtP_34	0.01	TRFLP	sqrtITS24	0.06	TRFLP	sqrtITS24	-0.1
TRFLP	sqrtITS159	0.01	PLFA	sqrtP_39	0.07	TRFLP	sqrtITS74	-0.09
NEMs	sqrt_Fung	0.04	TRFLP	16S85	0.07	TRFLP	arcsqrt23	-0.08
PLFA	sqrtP14	0.04	TRFLP	sqrtITS135	0.08	TRFLP	16S28	-0.08
TRFLP	sqrtITS115	0.04	TRFLP	arcsqrt28	0.09	PLFA	sqrtP_46	-0.07
inverts	lg_COEN	0.05	NEMs	Plant%	0.11	TRFLP	sqrtITS152	-0.06
inverts	lg_COLL	0.06	TRFLP	sqrtITS115	0.12	PLFA	sqrtP2	-0.05
NEMs	sqrt_Omni	0.06	inverts	asin_A/MI	0.13	PLFA	sqrtP_34	-0.05
PLFA	sqrtP_3	0.07	inverts	asin_A/IN	0.14	TRFLP	sqrtITS151	-0.05
PLFA	sqrtP_29	0.1	TRFLP	arcsqrt4	0.15	TRFLP	arcsqrt4	-0.04
PLFA	sqrtP_38	0.1	PLFA	sqrtP_45	0.16	PLFA	sqrtP_3	-0.04
PLFA	sqrtP_35	0.12	TRFLP	arcsqrt14	0.18	PLFA	sqrtP12	-0.04
TRFLP	16S94	0.13	PLFA	sqrtP_41	0.18	PLFA	sqrtP_44	-0.02
PLFA	sqrtP18	0.14	PLFA	sqrtP_40	0.19	TRFLP	sqrtITS135	-0.02
TRFLP	16S86	0.14	PLFA	sqrtP_8	0.21	TRFLP	sqrtITS142	-0.02
TRFLP	16S90	0.18	PLFA	sqrtP19	0.21	TRFLP	16S90	-0.01
PLFA	sqrtP_30	0.19	TRFLP	arcsqrt33	0.21	PLFA	sqrtP24	-0.01
TRFLP	16S118	0.2	TRFLP	sqrtITS152	0.22	PLFA	sqrtP25	-0.01
TRFLP	16S8	0.21	NEMs	lg10_fung	0.22	PLFA	sqrtP_7	0
TRFLP	arcsqrt1	0.22	TRFLP	sqrtITS85	0.22	NEMs	sqrt_b:F	0
TRFLP	16S135	0.24	PLFA	sqrtP2	0.23	PLFA	sqrtP14	0
TRFLP	16S58	0.24	inverts	LG_ACPR	0.23	PLFA	sqrtP13	0
TRFLP	arcsqrt22	0.25	NEMs	sqrt_Fung	0.23	PLFA	sqrtP15	0
TRFLP	16S100	0.27	PLFA	sqrtP17	0.24	TRFLP	arcsqrt14	0.01
TRFLP	arcsqrt33	0.28	PLFA	sqrtP_43	0.25	PLFA	sqrtP_36	0.01
PLFA	sqrtP24	0.29	TRFLP	16S90	0.25	PLFA	sqrtP26	0.02
PLFA	sqrtP25	0.29	TRFLP	16S100	0.26	PLFA	sqrtP_29	0.02
PLFA	sqrtP17	0.31	PLFA	sqrtP_28	0.27	PLFA	sqrtP_43	0.04
PLFA	sqrtP_42	0.32	PLFA	sqrtP27	0.29	TRFLP	sqrtITS159	0.05
TRFLP	arcsqrt23	0.32	TRFLP	16S66	0.29	NEMs	lg10_bact	0.07
TRFLP	16S66	0.32	TRFLP	16S86	0.3	PLFA	sqrtP_8	0.09
TRFLP	16S19	0.33	inverts	lg_COEN	0.31	PLFA	sqrtP_40	0.1
TRFLP	16S140	0.34	PLFA	sqrtP18	0.31	TRFLP	16S85	0.11
TRFLP	16S56	0.37	PLFA	sqrtP_3	0.33	TRFLP	arcsqrt1	0.11
TRFLP	16S28	0.38	TRFLP	16S19	0.33	PLFA	sqrtP_31	0.12
inverts	asin_C/IN	0.41	PLFA	sqrtP14	0.35	inverts	asin_A/IN	0.12
inverts	lg_C/MI%	0.42	TRFLP	16S28	0.37	NEMs	lg10_fung	0.12
PLFA	sqrtP_45	0.42	PLFA	sqrtP_5	0.38	PLFA	sqrtP_28	0.13
PLFA	sqrtP21	0.44	TRFLP	16S8	0.38	inverts	asin_A/MI	0.13
TRFLP	arcsqrt9	0.44	TRFLP	16S94	0.39	TRFLP	arcsqrt22	0.13
PLFA	sqrtP_46	0.48	inverts	sqrt_COP	0.4	TRFLP	16S34	0.14
TRFLP	sqrtITS24	0.57	TRFLP	16S118	0.4	PLFA	sqrtP_33	0.14
PLFA	sqrtP20	0.6	inverts	A/C_S	0.41	NEMs	sqrt_Fung	0.15
TRFLP	sqrtITS85	0.61	inverts	ACME	0.41	TRFLP	16S33	0.16
PLFA	sqrtP22	0.62	TRFLP	16S140	0.42	NEMs	lg10_total	0.19
PLFA	sqrtP_4	0.62	inverts	lg_COLL	0.43	PLFA	sqrtP22	0.2
TRFLP	sqrtITS135	0.63	inverts	INV_S	0.45	PLFA	sqrtP1	0.21
TRFLP	sqrtITS142	0.64	PLFA	sqrtP_9	0.46	PLFA	sqrtP19	0.21
PLFA	sqrtP13	0.69	TRFLP	16S135	0.46	NEMs	lg10_carni	0.22
TRFLP	sqrtITS151	0.69	PLFA	sqrtP11	0.47	NEMs	lg10_plant	0.27
PLFA	sqrtP23	0.71	TRFLP	16S58	0.5	NEMs	Plant%	0.28
PLFA	sqrtP_37	0.72	TRFLP	16S56	0.52	TRFLP	arcsqrt28	0.3
PLFA	sqrtP_33	0.74	inverts	lg_ACOR	0.53	TRFLP	16S97	0.3
PLFA	sqrtP12	0.79	inverts	lg_ACTO	0.61	PLFA	sqrtP_38	0.38
PLFA	sqrtP_6	0.8	inverts	lg_N	0.61	TRFLP	16S10	0.4
PLFA	sqrtP15	0.88	inverts	lg_MI_N	0.61	PLFA	sqrtP_32	0.56

6.3 Outcomes from the discrimination trial

The objective was to test whether the indicators would produce characteristic results for the nine land uses sampled across mainland UK, irrespective of geographical location or other potential determinants e.g. soil chemical or physical properties, altitude, etc. The strength of the design is that the sampling was constrained to sites where the defined land use had been consistent over a period of 30 years or more. This reduced the discrimination analysis to what would be typical of a particular land use without the interference of significant management (e.g. vegetation) or land use changes.

Consistency in land use results from the indicators would imply that it would be possible to establish a baseline or status for a soil biological indicator that could then be used to monitor against. The benefit of distinctive indicator values for individual land uses is manifest when examining the significance of change in an indicator or when managing within a particular land use to achieve a particular goal e.g. restoration, biodiversity targets. The scale and trajectory of change from one land use to another can be used to assess the success of any intervention or as a signal of degradation towards an unacceptable state.

6.3.1 Specific indicator methods

Multi-enzyme assay. Sulphatase, acid phosphatase, xylosidase, galactosidase and galactosaminidase produced significant land use discriminations, particularly within the agricultural land uses. Similar to the results from the sensitivity trial, high spatio-temporal variability affected the discrimination power of the individual enzymes, in particular celliobiohydrolase, glucosaminidase and glucosidase. Variability in all enzymes was higher in the semi-natural land uses compared to the agricultural land uses. The multivariate PCA using all eight enzymes produced a clear significant pattern of discrimination across semi-natural land uses as well as agricultural land uses. Overall these results suggest that a small set of enzymes could be used to characterize the status of soil carbon, sulphur and phosphorus dynamics for individual land uses, particularly in agricultural land uses. The application of the enzymes in a multi-enzyme assay would be more efficient and better suited to quality control than individual enzyme tests. Further work would be required to determine the acceptable levels of sulphatase, acid phosphatase, galactosidase or galactosaminidase with respect to the soil functions (biomass productivity, habitat maintenance or environmental regulation).

Multiple substrate induced respiration. The seven carbon substrates and water only all produced significant land use discriminations with similar respiration patterns across agricultural and semi-natural land uses. The month of sampling influenced substrate respiration rates but this did not effect land use discrimination. The multivariate PCA using all substrate respirations rates produced a significant pattern of discrimination across the land use with more distinct discrimination between grassland and woodland/moorland than demonstrated by the univariate results.

Overall these results suggest that a limited set of carbon substrates could be used to characterize the status of carbon dynamics within individual land uses and to compare between arable, fertile and infertile grassland land uses and between heath/bog and other semi-natural land uses. The application of the carbon substrates in a multiple substrate assay would be more efficient and better suited to quality control than individual respiration tests. Further work would be required to determine the acceptable ranges of respiration for individual substrates and from multivariate analyses, particularly with respect to the soil functions (biomass productivity, habitat maintenance or environmental regulation).

Bacterial TRFs. There were similar trends in the individual diversity measures across the land uses, i.e. highest diversity in grasslands and lowest in heath bog. However, none of the individual diversity measures or the multivariate PCAs (all derived from 148 TRFs) produced consistent significant land use discrimination patterns. The lack of land use discrimination may reflect that other factors, such as soil pH or organic matter, are known play an important role in dictating the structure of the soil bacterial community. However it is also possible that the sampling strategy was not intensive enough to effectively capture the variability in the soil community at the individual sampling locations.

Archaeal TRFs. Land use discrimination was demonstrated by the archaeal community through significant effects on two diversity measures (Shannon E and richness of TRFs) with highest diversity in heath/bog. A more distinct discrimination between agricultural and semi-natural land uses was observed from the multivariate analyses. Lowland wooded land uses were distinct from upland land uses while there was a transition from fertile grasslands to infertile grasslands. These results suggest that characteristic measures of the archaeal community could be developed for individual land uses.

Fungal TRFs. None of the individual diversity measures produced significant land use discrimination. The multivariate PCA produced distinct discrimination patterns with clear separation between crops+weeds, individual grasslands and semi-natural land uses and between upland and lowland semi-natural land uses. These results suggest that there are characteristic fungal communities for individual land uses that could be used to develop baseline/target community structures. However further work is required to define the boundaries to typical land use community structures using a more comprehensive statistical analysis of fungal TRFs. Further work could also explore whether alternate individual (diversity) measures could be used to define fungal community structure within and across land uses.

PLFAs. The majority of individual PLFA measures demonstrated significant land use discrimination with consistent differences amongst land uses. The PLFA measures for heath/bog and moorland grass mosaic demonstrate that these land uses have different microbial community structures to those in crops+weeds, grasslands and the other semi-natural land uses, with highest PLFA abundance, fungal/bacterial ratios, lowest

Shannon H' indices and higher relative abundance of certain microbial groups (e.g. fungi and actinomycetes). Within agricultural land uses, the combination of total PLFA abundance and fungal/bacterial ratios discriminate between crops+weeds and grasslands and between fertile and infertile grasslands, and lowland wooded. The variability around means within these land use is sufficiently low to be able to define characteristic values for the individual land uses. This is also the case for heath/bog relative to upland wooded and moorland grass mosaic land uses. A more distinct discrimination of semi-natural land uses was demonstrated from multivariate analyses although this was produced a less distinct discrimination amongst grassland land uses compared to individual measures including total PLFA abundance and F/B ratios.

Nematodes. The total abundance of nematodes and abundances of four out of the five nematode feeding groups demonstrated significant land use discrimination, with highest abundance in fertile and infertile acid grasslands. Multivariate analysis demonstrated discrimination between grasslands and crops+weeds and semi-natural habitats. However, all nematode measures demonstrated high variability which reflected seasonal dynamics and site-level spatial heterogeneity. This variability limits the ability to identify target values/ranges for nematode measures. The sampling strategy was insufficient to effectively reduce the influence of spatial variability in the nematode community at the individual sampling locations. Further research could examine alternate sampling strategies and extraction methods to determine whether these would reduce spatio-temporal variability sufficiently. Nematode analyses was constrained to simple feeding groups and did not examine the range of nematode indices derived from allocation of taxonomic groups to feeding groups (due to a lack in available skills). DNA based methods for the identification of nematode species have now been developed and offer a solution to this constraint.

Microarthropods. Few collembola measures demonstrated land use discrimination while in contrast all mite measures demonstrated land use discrimination, which reflects that collembola demonstrated higher variability than mites. Two mite groups, oribatids and mesostigmatids, produced contrasting discrimination patterns which, in combination (with or without invertebrate taxa richness), effectively discriminate within and between agricultural and semi-natural land uses. These measures could be used to define characteristic ranges for individual land uses. Multivariate analysis using mite and collembola measures also demonstrated clear discrimination within the agricultural land uses and within the semi-natural land uses.

6.3.2 Multiple indicators

Individual univariate measures across all the indicator methods produced remarkably similar discrimination patterns across the nine land uses, with crops+weeds distinctive from grasslands and heath/bog and moorland grass mosaic distinctive from wooded land uses. In several instances there was a gradient from fertile grasslands to infertile grasslands often with crops+weeds at the lowest point of the gradient. The radar plots using the univariate measures demonstrated that using more than one indicator would provide greater scope in defining, assessing and interpreting changes to the biological

status of soils. For all indicator methods, it would be possible to define target/baseline values and ranges for individual land uses using one or more univariate measures. The data generated here could be used to examine which combination of measures produced the most robust baselines for different purposes. Compatible data, obtained using comparable methods, would be required to develop these statistics for a wider range of land uses or for management practices within land uses.

Multivariate analyses of the multiple measures for the individual methods tended to reinforce and enhance discrimination between agricultural and semi-natural land uses. All indicators demonstrated significant discrimination using multivariate analyses even where univariate measures were not significant e.g. for bacterial TRFs, fungal TRFs and collembola. There were similar patterns of land use discrimination from multiple enzymes, MSIR, PLFAs, archaeal TRFs, fungal TRFs and invertebrates. For these indicators, the effectiveness of this discrimination can be demonstrated by the variability accounted for in the first three PC axes which was, in decreasing order, MSIR (96%), multiple enzymes (88.8%), fungal TRFs (63.5%), archaeal TRFs (60.6%), invertebrates (62%) and PLFAs (45.6%). Radar plots to compare and contrast the results from the multivariate analyses further demonstrate that several indicators can be used to profile the different traits of soil biological properties and processes (genotypic, phenotypic and functional) within and between land uses.

Multivariate analyses for bacterial TRFs, nematodes and invertebrates did not produce such significant or clearly interpretable discrimination patterns as the other indicator methods. This reflected greater variation in these groups which in turn may be due to high spatial variation (bacterial TRFS and nematodes), temporal dynamics (nematodes and aspects of invertebrates e.g. collembola) and taxonomic resolution (nematodes and invertebrates). Modifications to the sampling intensity and/or soil volume collected and more detailed identification are required to determine whether these could improve the discrimination patterns and the statistics within and between land uses.

6.3.3 Integration of indicators

The combination of measures across the indicators into a single multivariate analysis produced the clearest interpretable land use discrimination with distinct separation between agricultural and semi-natural land uses and within agricultural and semi-natural land uses. The loadings from this analysis indicate that these patterns were produced by combination of measures from the genotypic, phenotypic and functional traits. The patterns produced by the integration of different measures demonstrate that there is potential to develop an approach to characterizing land uses (or habitats) according to the soil biological properties and processes that they possess. This would be equivalent to classifications developed for habitat vegetation or water quality which are now widely used in the management, protection and restoration of habitats and water bodies. These approaches use information on which properties change when moving from one land use (state) to another and how these changes can be related to different pressures. With sufficient information on the likely responses of

individual measures to pressures, as demonstrated in the sensitivity trial, it should be feasible to develop such an approach for soils. However a soil biological scheme would differ by incorporating functional and genotypic measures alongside the more traditional phenotypic measures. An integrative approach would be suited to a monitoring scheme aiming to interpret changes in soil biological properties and processes for soil quality across different land uses, and when considering wider consequences e.g. for the supply of ecosystem services.

7 Conclusion

7.1 Standard operating procedures, sampling and data analyses

Six laboratory standard operating procedures (SOPS) were applied in this study (multienzyme assay, MSIR, TRFLP, PLFA, dry extraction of invertebrates and wet extraction of nematodes). Each of these was developed from existing methodologies and adapted to improve efficiencies for UK soils, where possible. The benchmarking of MSIR by Microresp™ with MSIR by GC proved that this colorimetric approach is entirely comparable to the more widely used GC approach and more amenable to processing large sample numbers. All of the methods proved logistically suitable for the analyses of large numbers of samples and for use in a broad range of environmental conditions with suggestions for method optimization. The final SOP for each method is provided in the Appendix to this report and available for application elsewhere.

Potential future developments of these methods have been proposed as a consequence of the projects experiences. For the process (functional) methods, there is the potential to improve the efficiencies of both the multienzyme assay and MSIR. These may also reduce the relatively high variability compared to other methods. In addition, these methods used eight enzymes or eight carbon substrates, which were selected to reflect differences in soil nutrient and carbon dynamics. There are many other enzymes or C substrates that could be used. Further research should investigate the potential of these substrates to produce sensitivity to pressures and land use discrimination. For the genotypic method (TRFLP for bacteria, fungi and archaea), it is proposed that information on soil organic matter would be useful prior to DNA extraction to support the use of the most efficient extraction techniques. In addition, the ability to extract DNA efficiently from freeze-dried soils would add flexibility to the method. For the PLFA (phenotypic) method applied to soil microbial community structure, information of soil organic matter content in samples would also be useful to optimise extraction and GC analyses. For the extraction (phenotypic) methods applied to invertebrates (microarthropods and nematodes), it is difficult to introduce quality control in the extraction phases. Therefore it is important that comprehensive SOPs (optimal for the invertebrate groups of interest) are widely adopted to produce comparable results. The method used here for nematodes could clearly be improved to produce more consistent data e.g. by increasing the amount of soil extracted. In parallel, there is obvious potential to develop DNA based methods to identify and

quantify soil invertebrates which would negate issues associated with current extraction procedures.

An overarching issue for all the SOPs was the availability and use of reference materials, or standards, for quality control. Historically there has been limited use of reference materials in soil biological analyses as they can be difficult to define or maintain. However the use of common reference materials is essential for the monitoring of soils over time. Any biological method adopted will require a reference to ensure that data obtained for one sampling occasion will be entirely comparable with data collected on subsequent occasions, or when comparing data between different sampling regimes. With further development, the use of a quality control soil (freeze dried or air dried) could be applied to PLFAs, enzymes, MSIR and potentially TRFLP. The application of quality control to the invertebrate extraction methods is more problematic and requires further consideration. As identified above, an alternative is to develop molecular approaches for the identification and enumeration of invertebrates from soil samples, removing the requirement for QC on wet or dry extraction procedures.

This study adopted soil sampling procedures that were compatible with previous studies and Countryside Survey. In general, these procedures were sufficient to limit the influence of spatial and temporal variability on the soil biological properties and processes. The results indicate that sampling across large geographical scales can be carried out across several months to assess the status of soil biological properties and processes where there are sufficient sample numbers per land use or where the sampling strategy are defined to reduce variability i.e. selection of similar vegetation classes or land uses. In a few instances, namely enzymes, nematodes and bacterial TRFLP, alternative sampling methods at the sampling locations could be tested to determine if they would reduce variability and improve the sensitivity or discrimination of the measures from these methods.

Investigation of the effects of sampling month on the sensitivity and discrimination of the soil biological measures suggests that constraining sampling to a narrow temporal window will serve to reduce spatial and temporal variability and thus aid in the interpretation of monitoring results. Given that practicalities of extensive soil survey or monitoring are likely to require extended sampling over some months, the optimal sampling window for sampling biological measures in UK soils will fall between May and July. The sensitivity trial highlighted that seasonal (temporal) dynamics were significant in the majority of measures although patterns of seasonal dynamics differed across the measures both within and between methods. However, constraining sampling to a short sampling window would help to reduce the influence of temporal dynamics on responsiveness to pressures. The discrimination sampling was carried out from May until November, with the majority of soil samples collected during May, June and July. Although there were significant effects of sampling month on certain methods (e.g. enzymes, MSIR, nematodes), there was little influence of sampling month on land use discrimination across the indicators. This suggests that

May to July should provide an adequate sampling window.

Statistical analyses of the project data focused on standard parametric approaches including analyses of variance and principal components analyses. These required consideration of data normality and the application of data transformations to address normality. Non-parametric approaches could be used as alternatives to parametric approaches which would reduce the constraints imposed by data normality or linear relationships. Such approaches have been developed, and now widely applied, in aquatic, mainly marine, environments for environmental assessments and are gaining in use for terrestrial and genetic studies.

7.2 Surrogacy between indicators

At the start of the project it was proposed that there may be surrogacy amongst indicators, whereby one indicator could be selected to represent the status or responses of more than one indicator. The results obtained have identified that there is no clear surrogacy between the different indicator methods. Each method produced different responses to the pressures and different discrimination amongst the land uses.

Each of the methods produces a number of soil biological measures and therefore there is potential for surrogacy within a method. This could be explored if the objective is to use only one or a few measures from each method e.g. to establish baselines for individual land uses. However, the results illustrate that there was no straightforward surrogacy between measures from the same method. If a selection of specific measures was required then it should be determined by a combination of statistical significance and interpretation of difference in measures amongst land uses or in response to pressures, for defined purposes. However, limiting the number of measures used from an individual method is unlikely to reduce the financial costs substantially since the methods used in this study can all be used to produce multiple measures with little extra effort compared to single measures. Where finances are limiting, then a reduction in effort can only be achieved by constraining the number of methods used. The results suggest that, if this is required, then there would be merit in retaining methods to reflect a spectrum of genotypic, phenotypic and functional traits. Indeed the results from this study suggest that a range of measures, either in multiple or integrated approaches, could be more informative about the soil biological status and changes as a result of land use change or distinct pressures.

7.3 Sensitivity of indicators to pressures

This project investigated a limited number of constrasting pressures to establish whether variability (temporal and spatial) would be a major constraint in identifying the responsiveness of soil biological indicators to typical and widespread pressures within the UK environment. The results clearly demonstrated that temporal dynamics are significant in the responsiveness of most measures from all methods, whether genotypic, phenotypic and functional. Dynamics were most significant for nematodes and multienzymes and likely masked effects of the three pressures. In the remaining

methods, the influence of temporal dynamics was dependant upon the pressure and the measure and rarely masked the sensitivity of a soil biological measure to a pressure. A few instances, pressure-sensitive measures did not display significant temporal dynamics which suggests that these would be the most flexible in terms of sampling window and more straightforward to interpret from monitoring. These measures were from PLFA, TRFLP, MSIR and microarthropod methods. The following methods were associated with the most obvious sensitivity to individual pressures at the locations sampled; MSIR, PLFA, TRFLP fungi and TRFLP archaea (restoration at Sutton Courtenay), PLFA, microarthropods and TRFLP bacteria (sludge metals at Hartwood) and microarthropods and MSIR (N deposition at Pwllpeiran). Further field assessments would be required to determine whether these methods, and associated measures, would be as sensitive to the same pressures at other locations with different environmental conditions. Overall, the results indicate that there is no universal indicator (measure) or method that will provide sensitivity to a range of constrasting pressures. The results suggest that a suite of soil biological methods would be more informative approach to monitoring changes in soil biological status where multiple pressures are at play, or where the pressures influencing soil are unknown. From the sensitivity results, this suite would include: PLFAs, TRFLP (for fungi, bacteria and archaea), MSIR and microarthropods.

To support the interpretation of soil monitoring results, there will need to be more information on the sensitivity of soil biological indicators to different pressures. The sensitivity of multiple measures (from individual or multiple methods) to individual or multiple pressures could be explored further through the use of multivariate statistical approaches where there is supporting information on environmental conditions and pressure levels. In parallel, to support comparability and interpretation of results, the sampling and statistical approaches adopted in this project provides a template for a comprehensive assessment of the sensitivity of soil biological indicators to different pressures, including management practices and different forms of contamination.

7.4 Land use discrimination

This project investigated the power of individual, multiple and integrated soil biological measures to discriminate between constrasting land uses from intensive arable to native habitats. This was considered important since land use (or habitat) is the primary management unit. The capacity to identify characteristic soil biological measures for different land uses is the basis for defining a baseline from which to monitor status and change over time.

The results clearly demonstrated that all methods could be used to discriminate amongst land uses to a greater or lesser extent. Numerous univariate measures could be used to establish baselines or target values for soil biological status for genotypic, phenotypic and functional traits.

Table 7-1 summarises, for each method, the individual, ratio/indices and multivariate

measures which demonstrated signficant land use discrimination and sensitivity to pressures. Radar plots could be used to visualize these traits for different land uses, with further consideration of variation or ranges. The use of radar plots clearly demonstrated that there are distinct differences in the relative contribution of genotypic, phenotypic and functional traits to characteristics of soil biology under different land uses. These differences could be used to monitor and interpret status and changes in soil biological quality in much the same way that shifts in community structure have been used to develop approaches to good ecological status for habitats and water quality.

Overall, the discrimination results complement the results from the sensitivity trial in that they suggest that a suite of soil biological methods would be an informative approach to monitoring the biological status of soils, as opposed to relying on a single method or a single measure. From the discrimination results, this suite would include: PLFAs, TRFLP (for fungi and archaea), MSIR and multi-enzymes. These methods produced the most significant and interpretable land use discrimination patterns from statistical analyses of univariate, multiple and integrated measures. Furthermore, the results suggest that there would be a clear rationale for selecting methods which would provide information on the three characteristics of soil biology, namely genotypic, phenotypic and functional traits. The results suggest that the use of multiple measures from these methods could be used to define characteristic baselines of soil biological status for different land uses. The data obtained in this study could be used as baselines for the specific vegetation classes of the land uses studied. It is important to consider that this project tightly constrained the sampling of land use to sites which where vegetation composition had been consistent for over 30 years, as far as possible (i.e. no obvious land use or major management changes had occurred). Thus, further work is required to build up a comprehensive dataset for a broader range of land uses across UK and to investigate the influence of management or pressures on the variability within these land uses. These data could be generated in different ways, either through extensive survey or through targeted sampling of key land uses. This work should complement the determination of the sensitivity of soil biological measures to different pressures. The primary issue must be to ensure that any data collected are entirely compatible with existing and future data through the use of common SOPs, reliable reference materials and complementary statistical approaches.

Further work is also required in the interpretation of the results from monitoring soil biological status. More specifically there needs to be careful consideration and determination of action points. These action points can be taken as negative or positive indications of use or management. An action point can identify where data obtained from monitoring indicate an unacceptable level of change in soil biological status or unacceptable shift in soil biological characteristics, and thus a point at which action should be taken to address such changes. In contrast an action point could be used to follow a desired direction of change over time towards an ultimate target (e.g. restoration success). There are various options to consider in establishing these action points. A simple approach would be to set limits with no consideration of whether

these levels are optimal for soil quality in a particular land use. This approach could be investigated initially using the data obtained for the specific land uses in this project. The assumption is that if a soil displays soil biological characteristics within these typical values then soil functioning will be typical for this land use. Following approaches used elsewhere, such action points could be defined from current baselines as typical ranges for individual or multiple measures or typical envelopes from integrated multivariate analyses.

A more sophisticated approach to defining action points could consider the (upper and/or lower) levels of soil biological properties and processes needed to maintain soil functions (biomass productivity, habitat maintenance or environmental regulation). This approach would be more suited to protecting soil quality and restoring soil functions. In both instrances, quantitative research linking soil biological characteristics to soil functions is needed to progress the development of action points.

Table 7-1 Summary of measures which demonstrated significant land use discrimination or significant responses to pressures (italics) or both (italics and bold).

Method	Individual measures	Ratios / Indices	Multivariate measures
PLFA profiles	total biomass (microbial); fungi ; bacteria; ***gram negative bacteria;*** gram positive bacteria; actinomycetes; (all as % and nmol g-1)	***fungal/bacterial ratio; gram positive/gram negative ratio***	***PC axes***
TRFLP - ITS fungal			PC axes
TRFLP - Archeae	*richness*	*Mcintosh E, Shannon E and Shannon H*	PC axes
MSIR by MicroResp™	basal respiration (water); AKGA; Arginine; Citric acid; GABA; Glucose; ***AKGA***; Malic acid; NAGA		PC axes
Multi-enzyme fluorometric assay	acid phosphatase; galactosaminidase; xylosidase; galactosidase; sulphatase		PC axes
TRFLP - Bacteria	richness	*Shannon E*	PC axes
Nematode Baermann extraction procedure	carnivores; omnivores; bacterial feeders; plant feeders; total nematodes		PC axes
Microarthropods Tullgren dry extraction	Mesostigmatids (n); oribatids (n); Prostigmatids (n); Total mites (n); Mesostigmatids (%); Oribatids (%); Prostigmatids (%); %***mites;*** Invertebrates (n); Microarthropods(n); %microarthropods; Invertebrate richness; *%collembola; poduroidae%*	***Collembola/mites;*** Mites/microarthropods; Mites/invertebrates; collembola/ microarthropods; Invertebrate Shannon E	PC axes

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Appendices

Appendix A Standard Operating Procedures

A1. Multi-enzyme fluorometric assay

A2. Multiple substrate induced respiration using MicroResp™

A3. Multiplex TRFLP

A4. Phospholipid fattay acid analysis

A5. Dry extraction of soil invertebrates

A6. Wet extraction of soil nematodes

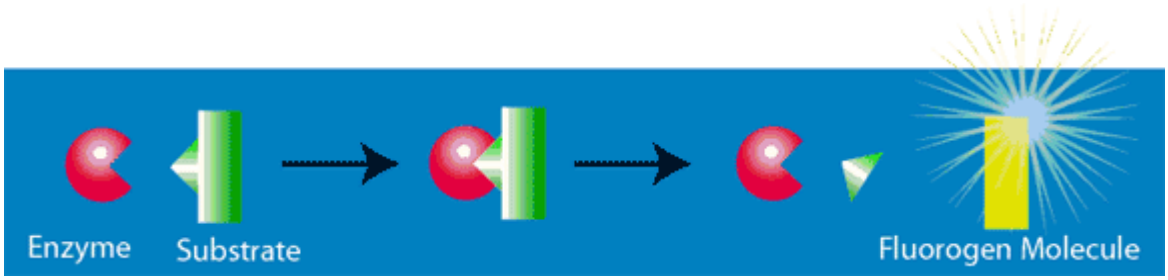
A7. MSIR by GC

A1 Multienzyme assay

Hydrolytic Enzyme Activities in Soil

Introduction

This method is based on that of Marx *et a.l* (2001). Esters of 4-methylumbelliferone (4-MUF) do not fluoresce unless cleaved to release the fluorophore. Hydrolysis of a 4-MUF containing substrate, such as 4-methylunbellifererone- β—D-cellobioside by the enzyme B-cellobiohydrolase, yields the fluorescent molecule 4-MUB that emits light at 460 nm when excited by wavelengths of 360 nm (Figure 1).



E + S → ES complex → E + P

Figure 1: Diagrammatic representation of enzyme kinetics

Buffer solutions are used to ensure that the enzymes operate in their optimal *p*H range. For many enzymes this is around *p*H 6.1. As such, 2-N-(morpholino) ethanesulfonic acid (MES buffer) has a pKa of 6.16 at 20°C (useful *p*H range of 5.5 to 6.7) and so is used to buffer enzymes within this *p*H range.

Each substrate is added in excess (i.e. greater than V_{max}) to ensure that all enzymes are assayed. Each soil sample is compared to a standard curve containing the same soil, such that each soil has a paired standard curve. This takes into account the degree of fluorescent quenching as a result of soil particles and organic matter.

When using micro-plate readers it is important to organise the plate set-up prior to the analysis. This should take into account the number of soil samples, enzymes, analytical replicates and standards. A suggested plate set-up is given below.

Health and Safety Considerations

- Read and sign all relevant risk assessment forms
- Wear suitable eye protection, lab coat and protective gloves when handling dangerous chemicals and solvents
- All solvents should be handled in an appropriate fume hood

Reagents

NB: Ensure the substrates and buffer are at room temperature before use

- *0.1 M MES buffer*: MW= 195.16: dissolve 19.5 g of 2-N-(morpholino) ethanesulfonic acid in one litre of deionised water.

- *Substrate solution (1mM):* dissolve each substrate (Table 1) in 300 µl of dimethyl sulfoxide (DMSO). When dissolved adjust to 10ml with MES-buffer. Split the substrate solution into 1ml aliquots and freeze until required. Prior to use, defrost the substrate and dilute x10 with MES-buffer to achieve a final concentration of 1mM. The substrate solutions can be stored at 4°C for up to one week.

Table 1: List of substrates and mass required for 10mM solution.

	Substrate	Enzyme	Molecular formula	Molecular weight	Mass (mg)
S1	4-methylunbellifererone-β—D-cellobioside	B-cellobiohydrolase	$C_{22}H_{28}O_{13}$	500.5	50.0
S2	4-methyl-lumbelliferone-N-acetyl- β-glucosaminide	N-acetyl-β-glucosaminidase	$C_{18}H_{21}NO_8$	379.4	37.9
S3	4-methylumbelliferone-β-D-glucoside	β-glucosidase	$C_{16}H_{18}O_8$	338.3	33.8
S4	4-methylumbelliferone-phosphate	Acid phosphatase	$C_{10}H_9O_6P$	256.2	25.6
S5	4-Methylumbelliferyl N-acetyl-β-D-galactosaminide	β-Galactosaminidase	$C_{18}H_{21}NO_8$	379.3	37.9
S6	4-Methylumbelliferyl β-D-xyloside	β-xylosidase	$C_{15}H_{16}O_7$	308.2	30.8
S7	4-Methylumbelliferyl β-D-galactopyranoside	β-galactosidase	$C_{16}H_{18}O_8$	338.3	33.8
S8	4-Methylumbelliferyl sulfate	sulfatase	$C_{10}H_7KO_6S$	294.3	29.4

Notes:

- Some substrates are difficult to dissolve. A sonic bath is useful for dissolving such substrates.
- Some substrates go out of solution when stored in a refrigerator. If this occurs re-dissolve in a sonic bath.

- *5 mM 4-MUB Standard:* MW=176.2: dissolve 0.0881 g of 4-MUB in 50 ml of methanol. Make up to 100 ml with MES buffer. Store at 4°C away from light

- *50 µM 4-MUB standard:* Dilute the 5mM 4-MUB standard x 100 to 50 µM using MES buffer (i.e. 1ml of 4-MUB in 100ml of buffer). Store at 4°C away from light for up to one week.

Soils preparation

i. Sieve soils through a 2.0 mm stainless steel sieve, removing roots and stones.
ii. Determine the soil moisture content by taking a sub-sample of 5 g soil and dry at 105^{o}C for 24 h.
iii. Incubate the fresh soil samples at 25^{o}C for 7-14 days

Standard soil preparation

I. Prepare a standard soil as above by sieving soil through a 2.0 mm stainless steel sieve, removing roots and stones as for the samples.
II. Mix thoroughly
III. Freeze-dry an adequate mass of the soil for long-term application
IV. Store at -20°C.
V. Analyse the standard soil daily.

Analysis of Soils

i. Disperse 0.5 g of soil/standard soil (prepared as above) in 50 ml of de-ionised water in suitable plastic container.
ii. Shake the sample for 30 minutes on a rotary shaker.
iii. Transfer the sample to a 100ml beaker
iv. Place a magnetic stirrer bar into each beaker and stir at a constant rate to obtain a homogenous soil suspension.
v. Withdraw 50 µl aliquots of the soil suspension while continuously stirring. Each sample will be analysed in triplicate. The best way to do this is to use a multi-channel pipette with three tips, thereby withdrawing all three replicates in one motion.
    a. **NB.** Ensure that the correct volume has been withdrawn. The pipette tip can easily become clogged with organic material from the soil.
vi. Dispense the 50 µl soil suspension to the microplate (refer to Figure 2 for the design of the plate).
vii. Prepare a **substrate control** by substituting the sample with 50 µl of sterile water.
viii. Add 50 µl of 0.1M MES buffer
ix. Add 100 µl of the 1 mM substrate solutions to the corresponding well (it is important to add the substrate last).
x. Mix the solution
xi. Cover the plate to prevent any contamination of the samples. A convenient and cheap way to do this is to place an old plate on top of the one being analysed.
xii. Incubate the samples for 3hr at 30°C.

Standard Curve

1. Add 50µl of soil suspension to each well that will contain the standard curve for that soil (Figure 2).
2. Add the appropriate amount of 50 µM 4-MUB stock standard and buffer to obtain final concentrations of 0, 10, 30 and 50 µM 4-MUB (0, 0.5, 1.5 and 2.5 nmole of 4-MUB per reaction). Refer to Table 2: Standard Curve for the volumes of standard and buffer required.

Table 2: Standard curve details

	4-MUB- (µM)	4-MUB per reaction (nmol)	Volume of 50uM stock standard (µl)	Volume of buffer (µl)
STD1	**0**	0	0	50
STD2	**10**	0.5	10	40
STD3	**30**	1.5	30	20
STD4	**50**	2.5	50	0

3. Add a further 100 µl of MES buffer

Figure 2: Plate set-up

Plate set-up

			Substrates								Standard Curves			
			S1	S2	S3	S4	S5	S6	S7	S8	STD1	STD2	STD3	STD4
			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Soil A	**Rep (i)**	**A**	A1(i)	A2(i)	A3(i)	A4(i)	A5(i)	A6(i)	A7(i)	A8(i)	A-1 (i)	A-2 (i)	A-3 (i)	A-4 (i)
	Rep (ii)	**B**	A1(ii)	A2(ii)	A3(ii)	A4(ii)	A5(ii)	A6(ii)	A7(ii)	A8(ii)	A-1 (ii)	A-2 (ii)	A-3 (ii)	A-4 (ii)
	Rep (iii)	**C**	A1(iii)	A2(iii)	A3(iii)	A4(iii)	A5(iii)	A6(iii)	A7(iii)	A8(iii)	A-1 (iii)	A-2 (iii)	A-3 (iii)	A-4 (iii)
Soil B	**Rep (i)**	**D**	B1(i)	B2(i)	B3(i)	B4(i)	B5(i)	B6(i)	B7(i)	B8(i)	B-1 (i)	B-2 (i)	B-3 (i)	B-4 (i)
	Rep (ii)	**E**	B1(ii)	B2(ii)	B3(ii)	B4(ii)	B5(ii)	B6(ii)	B7(ii)	B8(ii)	B-1 (ii)	B-2 (ii)	B-3 (ii)	B-4 (ii)
	Rep (iii)	**F**	B1(iii)	B2(iii)	B3(iii)	B4(iii)	B5(iii)	B6(iii)	B7(iii)	B8(iii)	B-1 (iii)	B-2 (iii)	B-3 (iii)	B-4 (iii)
Blank	**Rep (i)**	**G**	S1(i)	S2(i)	S3(i)	S4(i)	S5(i)	S6(i)	S7(i)	S8(i)	MUB 1(i)	MUB 2(i)	MUB 3(i)	MUB 4(i)
	Rep (i)	**H**	S1(ii)	S2(ii)	S3(ii)	S4(ii)	S5(ii)	S6(ii)	S7(ii)	S8(ii)	MUB 1(ii)	MUB 2(ii)	MUB 3(ii)	MUB 4(ii)

A1 (i) denotes Soil A, Substrate 1, Replicate 1 etc.

Columns 1 through to 8 contain separate substrates.

Each soil has its own standard curve (run in triplicate) to calculate the 4-MUB concentration of the samples.

In addition a third standard curve is prepared which contains 50µl of deionised water as a substitute for the soil extract. This standard curve is used to calculate the 4-MUB concentration of the substrate blanks (rows G-H).

For substrate blanks, replace the 50µl of soil with 50µl of sterile, de-ionised water

Plate readings

Read the fluorescence after 3 hrs of incubation with the plate reader set at 30°C.

Read each plate with an excitation wavelength of 360 nm and emission wavelength of 460 nm.

Calculation

1. Convert fluorescence into amount of MUB (µM) matched to each soil, using the soil-based MUB standard curve
2. Convert fluorescence into amount of MUB (µM) for the substrate blanks, using the control MUB standard curve
3. MUB concentration (µM) for each sample is then calculated by subtracting the substrate blank (2 above) from the sample reading (1 above)
4. Calculate release of MUF in nmol g^{-1} soil h^{-1}

   i.e. nmol g^{-1} soil h^{-1}= (4-MUB µM x (50ml/soil dry weight))/time (hrs)

Limit of Detection

The following limit of detection was calculated from the average standard deviation of 22 blanks x 3.

Substrate		LOD (uM)	LOD (pmol in 50µl reaction)
S1	4-MUB β—D-cellobioside	2.17	108
S2	4-MUB-N-acetyl- β-glucosaminide	2.97	149
S3	4-MUB- β-D-glucoside	4.23	212
S4	4- MUB -phosphate	1.89	95
S5	4- MUB N-acetyl-β-D-galactosaminide	2.07	103
S6	4- MUB β-D-xyloside	3.23	161
S7	4- MUB β-D-galactopyranoside	4.14	207
S8	4- MUB sulfate	2.71	135

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Ordering Information

Nunc plates (ref: 237108) plates are ordered from Fisher. Fisher ID no= MPA-560-060R, at £154.65 for a case of 180.

Chemicals	Supplier	ID no	Storage
Standard			
4-methylunbellifererone	Sigma	M1381-25G	
Substrates			
(S1) 4-methylunbellifererone- β—D-cellobioside	Sigma	M6018-100MG	2 to 8 °C
(S2) 4-methylumbelliferyl -N-acetyl- β-glucosaminide	Sigma	M2133-250MG	-20 °C
(S3) 4-methylumbelliferone- β-D-glucopyranoside	Sigma	M3633-250MG	-20 °C
(S4) 4-methylumbelliferone-phosphate	Sigma	M8883-250MG	-20 °C
(S5) 4-Methylumbelliferyl N-acetyl-β-D-galactosaminide	Sigma	M9659-100MG	-20 °C
(S6) 4-Methylumbelliferyl-β-D-xylopyranoside	Sigma	M7008-100MG	-20 °C
(S7) 4-Methylumbelliferyl β-D-galactopyranoside	Sigma	M1633-1G	-20 °C
(S8) 4-Methylumbelliferyl sulfate potassium salt	Sigma	69610-500MG	2 to 8 °C
Various			
MES buffer	Fisher	BPE 300-100	
Dimehyl sulfoxide	Fisher	BPE 231-1	

A2 Multiple substrate induced respiration using MicroResp™

This method was adapted from MicroResp™ Technical Manual version 1. Copyright The Macaulay Institute.

Created by: C Cameron, Macaulay Institute
Updated by R Creamer, Cranfield University
Version Date: 11/04/2007

Reference: Campbell, CD. *et al*., AEM, 2003, 69 (6), 3593 – 3599.

A. Preparation of Detection Plates

Ingredients	Amount of medium dissolved in de-ionised water	Final Concentration when combined
Purified Agar	15 g in 500 ml	1%
Indicator Solution: 7.4.1.1 Cresol Red 7.4.1.2 Potassium chloride (KCl) 7.4.1.3 Sodium bicarbonate ($NaHCO_3$)	18.75 mg 16.77 g 0.315 g } in 1000 ml	12.5 μg ml^{-1} 150mM 2.5mM

N.B. Concentration of purified agar and indicator solution (3%) so that the desired concentration of 1% is achieved when the two are combined.
The cresol red is yellow in solution and turns pink when the bicarbonate is added.

Method for preparing stock:

1. Prepare agar by dissolving the required amount in de-ionised water and autoclave.
2. Once autoclaved, aliquot into 10 batches of 50 ml, allow to cool and store at 4°C.
3. For the indicator dye solution, dissolve ingredients in 900ml dH_2O over a low heat before diluting to 1000ml in a volumetric flask.
4. Aliquot the indicator dye mixture into 10 batches of 100 ml and store at 4°C.

N.B. The cresol red is yellow in solution and turns pink when the bicarbonate is added.

Method for preparing detection plates

1. Remove one aliquot each of noble agar and indicator dye from storage – this will provide 8 plates.
2. Melt the noble agar in a water bath, microwave or by re-autoclaving. Use a measuring cylinder to check the volume of agar is 50 ml, make up with dH_2O.
3. Transfer the indicator dye solution into a 1L wide-necked beaker, place on a hotplate containing a magnetic stirrer and keep warm on a hot plate (65°C).
4. Warm the pipette tips in an oven before use as this will aid in a more uniform dispensing of agar into the microplates.

5. When agar has cooled slightly, add to the warmed dye indicator solution and allow the temperature to equilibrate before dispensing. Maintain at 65^{o}C with constant stirring.
6. Using a multi-channel pipette (8 x 1500μl) and warmed sterile tips dispense 150μl aliquots into each column, discarding the first and last dispenses, dispense half the plate at one time. If using automatic pipette – set to 8 x 150μl dispenses.
7. Store plates at room temperature, in the dark, in a dessicator cabinet containing soda lime and bowl of water. After 24h cover each detection plate with parafilm. 7 days incubation is recommended to ensure that any CO_2 absorbed from the preparation of the plates has been adsorbed by the soda lime prior to use.
8. Replace the soda lime when necessary and keep the atmosphere in the dessicator moist.

N.B. Do not autoclave the indicator solution. The agar is autoclaved to ensure it has properly melted and not to ensure sterility.

B. Calibration of MicroResp^{TM} using GC

N.B. Calibration of MicroResp^{TM} only needs to be completed once for each spectrophotometer used.

Materials

120-125 ml glass bottle with screw cap (Dimensions – Height ~ 100mm (+/- 5mm), overall diameter ~ 55mm (+/- 1mm))
Rubber stopper (bungs) with holes filled with clear multipurpose silicone sealant
Light-free box
Soda lime
Beaker of de-ionised water
Glucose solution (30 mg g^{-1} soil water)
MicroStrip plates (Fisher Cat# DIS-948-040Y, pk 50)
Cresol Red
Potassium chloride
Sodium bicarbonate
Purified agar
1000ppm Carbon Dioxide in Nitrogen (57L Lecture bottle, CK Gas Products or Scotty 14, Scott Specialty Gases)

Preparation of MicroStrip plates

1. Prepare indicator/agar solution as for MicroResp^{TM}.
2. Dispense 150 μl indicator/agar solution into each well of the MicroStrip plates using 8 x 1500 μl multi-pipette (dispense ½ plate at a time).
3. Store in dessicator with soda lime, covering the base, and a beaker of water for 24h uncovered, then cover with parafilm and store for further 6 days.

Sample Preparation

1. The soil moisture needs to be in the range of 40–60 % of the soil's WHC.

2. Use two replicates per soil sample. It is preferable to use a range of soils with different activities to achieve good range of responses.
3. Both basal (water) and substrate induced (glucose) measurements should be taken on separate samples.
4. A range of soil:headspace measurements will be taken, at 30 ml, 20 ml and 10 ml soil volume. (A weight can be determined by using the average weight of soil in the microplate deepwell cells (400 µl), instructions for measurement are given in section 4.1.
5. Weigh out the soil equivalent for the various volumes, replace the cap and screw on lightly to allow gaseous exchange.
6. Place the samples in a light-free box containing a beaker of soda lime and a beaker of deionised water – this stops the soil drying out and also absorbs CO_2 that is produced. Incubate the samples for 2 - 3 days at 25^{o}C.
7. Ensure the silicon in the rubber bungs are intact, if not, remove completely and refill the hole with silicon gel.

Analysis

This is best carried out with two persons, one to measure the MicroStrips and one to measure the GC samples.

1. Follow the instructions for setting up the GC.
   GC Conditions - Carrier gas: Helium
   Oven temperature: 60°C
   Pressure: 60 psi
2. Switch on Spectrophotometer plate reader and set-up to read at 570nm.
3. Remove required number of MicroStrip plates from the dessicator (you will be using 4 wells per soil jar).
   a. Carefully remove each strip (8 wells) from the holder and snap the strip in half (2 x 4 wells) and replace back in the holder.
   b. Place an evaporating dish (or similar) containing soda lime into a zip-lock polythene bag along with the MicroStrip plates for use.
4. Remove any seedlings that may have germinated in the soil jars during incubation.
5. Add either water or glucose solution (10% of the soil volume, for example to 30 ml soil volume add 3 ml solution to each soil jar, for 20 ml soil, 2ml and for 10 ml soil volume 1ml solution).
6. Inject three standards – 1000ppm Carbon Dioxide in Nitrogen - into the GC.
7. Remove 1 x 4 well strip, place in an empty strip-holder, read on the plate reader and immediately save the file.
8. Using forceps carefully place the 1 x 4 well strip into the soil jar on top of the soil surface, ensuring that the gel does not come in contact with the soil.
9. Place the syringe needle into a bung and insert the bung into the jar of the first sample pressing firmly to ensure a tight fit. Attach the syringe to the needle.
10. Flush the syringe several times before taking up 2 ml of gas from the headspace.
11. Before injection flush out the gas until there is 1ml left in the syringe then inject.

12. Clean out the syringe by pulling out the plunger and pushing air into the syringe through the needle – do this several times.
13. Repeat steps 7 -12 for the second sample and so on until you are finished.
14. After 6h incubation, inject three standards into the GC.
15. Insert the needle and syringe into the first soil jar, flush the syringe several times before removing 2 ml of gas from the headspace.
16. Flush the syringe to 1 ml and inject into the GC.
17. Once the CO_2 peak has come off and you are happy with the injection, carefully using forceps remove the 1 x 4 well strip (from the jar you have taken the GC sample from), clean off any soil particles by rinsing with water and dry with paper towel.
18. Place the 1 x 4 well strip into the plate holder, read on the plate reader at 570nm and save the file immediately.
19. While this is carried out the next sample is taken for the GC.
20. Repeat steps 15 – 19 until finished.

Calibration Curve

1. Calculate the $\%CO_2$ from the GC data using the formula:

$$\%CO_2 = \frac{\text{sample } CO_2 \text{ peak} \times \%CO_2 \text{ standard}}{\text{Standard } CO_2 \text{ peak}} \times \frac{\text{Standard injection peak}}{\text{Sample injection peak}}$$

2. Calculate a mean Absorbance (A_{570}) value for the detection 4-well strip.
3. Using GenStat 8, copy the 6h data for $\%CO_2$ and mean A_{570} values into the program and carry out a Regression Analysis using a Linear-by-linear (rectangular hyberbola) as the standard curve. Select $\%CO_2$ as the response variate and A_{570} as the explanatory variate.
4. The calculation for the conversion of A_{570} to $\%CO_2 = A + B / (1 + D*Ai)$, where Ai is the A_{570} value. The parameters A, B and D are given in the output file of the regression analysis.

C. Preparation of soil samples

1. Sieve soils through a 2.0 mm stainless steel sieve, removing roots and stones. A minimum of 100 g is required to allow for the following soil properties to be determined - moisture content, loss-on-ignition, pH and water holding capacity (WHC). The methods are detailed in Supplement 1. The acceptable range for the moisture content of the soil is 30 – 60% of it maximum WHC. To carry out MicroResp™ approximately 30 - 35 g fresh weight will be required.
2. Once the soil is in an acceptable condition, a wick (wetted paper towel) is placed in the bag containing the remaining soil and secured above the sample using elastic band.

Incubate the soil samples at 25^{o}C for 7-14 days in the soil conditioning unit (incubator), with a beaker of water and a beaker of self-indicating soda lime, prior to carrying out the MicroResp™ method.

N.B. Soils must not be too wet, as this restricts gaseous exchange, nor too dry, as this may adversely affect the microbial activity. Soils with ideal moisture content should fall easily through the filling device.

D. Preparation of carbon source stock solutions

The carbon sources are prepared as 30 mg carbon source per gram of water in the soil of each well. Need to know: i. weight (g) of soil in each well *
ii. weight (g) of water in soil per well
iii. weight (mg) of carbon source per well

**Place the filling device over a deep well plate (wells are blanked off with tape) and weigh whole assembly. Fill all 96 wells with soil as described (section G), re-weigh and divide the soil weight by the number of wells filled.*

Example: Carbon solutions were prepared according to soil [A] as follows:

- moisture content 24.36 g H_2O/100 g soil $\therefore$ 0.244 g H_2O/ g soil
- 0.32 g soil/well => 0.078 g H_2O/0.32 g soil
- C source @ 30 mg/ g H_2O => 2.34 mg Carbon source/0.078 g H_2O
- 2.34 mg C-source delivered in 25 μl aliquots $\Rightarrow$ <u>2.34 g/25 ml</u>

The carbon sources are stored at 4^{o}C for up to 2 weeks.
Carbon sources include (order and storage details in Supplement 2):
Cs1 Water (distilled H_2O)
Cs2 L-Arginine
Cs3 L-Malic Acid
Cs4 Gamma amino butyric acid
Cs5 n-acetyl glucosamine
Cs6 D(+) glucose
Cs7 Alpha ketogluterate
Cs8 Citric acid

E. C-source addition to deepwell plates

1. Remove prepared C-source stock from fridge.
2. Dispense 25μl of each desired carbon source (or water) into the appropriate wells of an empty deepwell plate following the template shown below.
3. Deepwell plates can be covered in parafilm and left overnight in the fridge.

Template

It is best to prepare a template of the deepwell plate demonstrating the positioning of the carbon source replicates and soils. Remember, the dye plate, which will be inverted on top of the deepwell, will read in reverse of deepwell. Therefore, deepwell plate is usually set-up in reverse of the desired display of the dye plate.

Within each plate 4 blocks have been identified with 3 repeat substrates per block allocated using a randomised pattern.

Example:
Deepwell filled as follows:

	1	2	3	4	5	6	7	8	9	10	11	12
A	cs2	cs2	cs2	cs7	cs7	cs7	cs1	cs1	cs1	cs3	cs3	cs3
B	cs4	cs4	cs4	cs5	cs5	cs5	cs6	cs6	cs6	cs1	cs1	cs1
C	cs3	cs3	cs3	cs2	cs2	cs2	cs8	cs8	cs8	cs8	cs8	cs8
D	cs1	cs1	cs1	cs3	cs3	cs3	cs2	cs2	cs2	cs7	cs7	cs7
E	cs7	cs7	cs7	cs1	cs1	cs1	cs5	cs5	cs5	cs6	cs6	cs6
F	cs8	cs8	cs8	cs6	cs6	cs6	cs3	cs3	cs3	cs4	cs4	cs4
G	cs5	cs5	cs5	cs4	cs4	cs4	cs7	cs7	cs7	cs2	cs2	cs2
H	cs6	cs6	cs6	cs8	cs8	cs8	cs4	cs4	cs4	cs5	cs5	cs5
	BLOCK 1			**BLOCK2**			**BLOCK3**			**BLOCK4**		

Dye plate reads as follows:

	12	11	10	9	8	7	6	5	4	3	2	1
A	cs3	cs3	cs3	cs1	cs1	cs1	cs7	cs7	cs7	cs2	cs2	cs2
B	cs1	cs1	cs1	cs6	cs6	cs6	cs5	cs5	cs5	cs4	cs4	cs4
C	cs8	cs8	cs8	cs8	cs8	cs8	cs2	cs2	cs2	cs3	cs3	cs3
D	cs7	cs7	cs7	cs2	cs2	cs2	cs3	cs3	cs3	cs1	cs1	cs1
E	cs6	cs6	cs6	cs5	cs5	cs5	cs1	cs1	cs1	cs7	cs7	cs7
F	cs4	cs4	cs4	cs3	cs3	cs3	cs6	cs6	cs6	cs8	cs8	cs8
G	cs2	cs2	cs2	cs7	cs7	cs7	cs4	cs4	cs4	cs5	cs5	cs5
H	cs5	cs5	cs5	cs4	cs4	cs4	cs8	cs8	cs8	cs6	cs6	cs6
	BLOCK4			**BLOCK3**			**BLOCK2**			**BLOCK 1**		

F. Checking Detection Plates

It is important to check each detection plate before use.

1. Check that the amount of agar in each of the wells is even and the colour is consistent.
2. Measure each plate on the spectrophotometer at A_{570}.
3. Calculate the % coefficient of variance (%CoV) for each of the plates. The %CoV of each plate must be $\leq$ 5%, discard any plates out with this range.

$$\frac{\text{Standard Deviation}}{\text{Average}} \times 100 = \%\text{CoV}$$

G. MicroResp™ Set-up

1. Remove deepwell plates from the fridge and allow to warm-up to room temperature.
2. Switch on the microplate reader and the computer.
3. Place the filling device directly over the deep-well plate and slide the Perspex sheet between them.
4. Sprinkle an excess of soil over the filling device and gently brush the soil into the wells until evenly filled, tapping the whole system once to gently compact the soil before using a ruler to level off the soil and a brush to remove excess soil. Do not force or press the soil into the filling plate, if it is at the right moisture content it should fall easily into the wells.
5. Remove the perspex sheet and place on top of the filling device. Using the fingerholds, gently but firmly tap the assembly on the bench so that the soil falls through to the wells.
6. Soil should fall into the deepwell plate but any soil particles that have stuck may need to be pushed lightly down into the deepwell plate using a clean wire, inoculating needle or rod.
7. Remove the filling device and apply the MicroResp™ seal to deepwell plate.
8. Read detection plates (0 hour) on a microplate reader at a wavelength of 570nm and save to file. Softmax® software was used for the absorbance readings.

9. Invert detection plate on top of deepwell plate so that A1 of the detection plates corresponds to A12 on the deepwell plate, pressing down firmly to seal correctly.
10. Secure the plates in the specially designed clamps, including two black mats for sealing.
11. Incubate the plates for 6 hours at 25°C.
12. After incubation, re-read the detection plates at 570nm and save to file. Care must be taken removing the clamps as the spring mechanism can cause soil to contaminate the detection plate.

H. MicroResp™ Calculations

1. Both the 0h (At0) and 6h (At6) data are normalised (Ai) using the formula: Ai = (Atx/At0) * Mean (At0), where x = 0h or 6h data
2. The normalised 6h data is converted to $\%CO_2$ using the formula and parameters from the calibration (section 2.5).
3. The CO_2 rate is calculated by converting the 6h % CO_2 to µg/g/h CO_2-C using gas constants and constants for headspace volume in the well (945 µl), fresh weight of soil per well (g), incubation time (h) and soil sample % dry weight.

$$CO_2 \text{ rate } (\mu g\ CO_2\text{-C/g/h}) = \left\{ \frac{(6h\ \%CO_2/100) \times vol \times (44/22.4) \times (12/44)}{soil\ fwt \times (soil\ dwt/100)} \right\} / \text{time}$$

Supplement 1

Moisture Content (MC)

1. Record weight of crucible
2. Record combined weight of crucible and ~5g fresh soil.
3. Place in oven at 105°C for 24hrs.
4. Place sample in dessicator to cool.
5. Record combined weight of crucible and oven-dried soil.

$$\%\ \text{dry weight of sample (\% Dwt)} = \frac{(w_3 - w_1)}{(w_2 - w_1)} \times 100$$

where: w_1 = weight of the crucible
w_2 = weight of the crucible plus fresh sample
w_3 = weight of the crucible plus oven-dry sample

% Moisture Content (%MC) = 100 - % Dwt

Loss on Ignition (LOI)

1. Use the over-dried sample from determination of moisture content.
2. Place in muffle furnace at 450°C for 2 hrs.
3. Cool in dessicator and re-weigh.
4. Ash determination = $\frac{(w_3 - w_1)}{(w_2 - w_1)} \times 100$

where: w_1 = weight of the crucible

w_2 = weight of the crucible plus oven-dry sample
w_3 = weight of the crucible plus oven-dry sample after ignition

pH

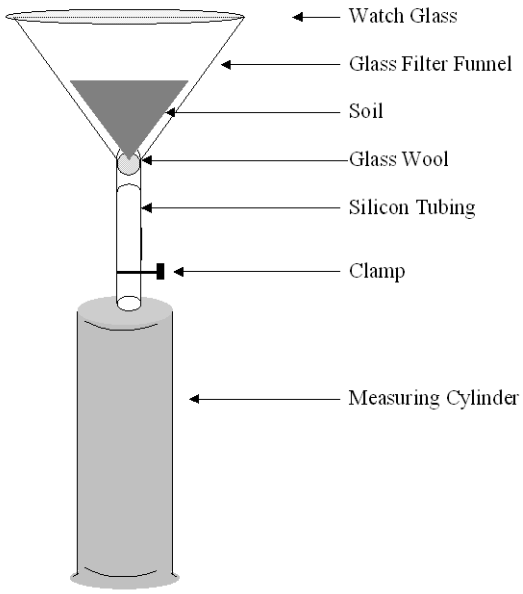
1. Weigh out 10 ml of fresh soil into soil jars.
2. Add 50 mls distilled water and mix well with glass rod.
3. Mix for 1 hr, using a horizontal shaker.
4. Leave to stand for 5 mins.
5. Read sample following instructions for pH meter.

Water Holding Capacity (WHC)

Method

1 Place a plug of glass wool in the funnel and moisten the glass wool with a little deionised water.
2 Close the clamp on the silicon tubing.
3 Weigh out 50g of fresh soil and transfer to the funnel.
4 Add 100mls deionised water to the soil, place the watch glass over the top of the funnel, to prevent evaporation, and leave for 30mins.
5 When the time has passed, open the clamp and let the water drain into the measuring cylinder for 30mins.
6 Measure the volume collected.

Apparatus



Calculation

1. Calculate the volume of water retained by the soil = 100mls – volume collected
2. Calculation of WHC :
   Need to know: %dwt and %mc of soil sample

Water retained * 2 = water retained /100g fresh soil
WHC = water retained/100g fresh soil + %moisture content of soil

3. Calculation of %WHC = %mc / WHC * 100

Example

Soil N^{o}.1: %dwt = 72.90; %mc = 27.10
Volume of water collected = 83.5mls
Volume of water retained = 100 – 83.5
= 16.5mls

Water retained /100g fresh soil = 16.5 * 2
= 33mls / 100g fresh soil
WHC = 33 + 27.1 = 60.1
(this value describes the amount of water the soil sample can hold)

%WHC = 27.1 / 60.1 * 100 = 45.09%
(this value describes the amount of water in the soil sample in relation to the amount of water it can hold)

4. Calculation for adjusting soil to target WHC

Loss/Gain of H_2O =
wt of fresh soil * (%dwt/100 * (target WHC/100 * WHC/100 +1) –1)

Example

Soil N^{o}.1: %dwt = 72.90; %mc = 27.10

Loss/Gain of H_2O = 50g * (72.9/100 * (40/100 * 82.44/100 +1) –1)
= 50 * (0.729 * (0.4 * 0.8244 +1) –1)
= 50 * (0.729 * 1.329) –1)
= 50 * -0.03
= -1.5g of H_2O

$\therefore$ 50g of Soil N^{o}.1 needs to loose 1.5g of H_2O to reach the target of 40%WHC.

5. The adjustment of soil moisture

You will need a large tray lined with benchcoate and balance with a large pan.
Wetting:

- Spread the soil thinly over the benchcoate and tray, take note of the weight.
- Using a water spray on a fine nozzle – spray once over the surface of the soil, then turn the soil to mix thoroughly, spread evenly again, and re-weigh.
- Continue to do this until you have added the required amount of soil
- THE AMOUNT OF WATER TO ADD IS ONLY AN ESTIMATE – do not worry if you cannot add all of it. You do not want the soil to become too sticky.
- Take a sub-sample of soil (5 g), dry at 105^{o}C for 24hrs to obtain the new moisture content.

Drying:

- Spread the soil thinly over benchcoate and take note of the weight.
- Leave the tray in a warm dry room preferably in the dark.
- Turn the soil and check the weight every 30 min.

- IF ANY SOIL PARTICLES HAVE DRIED OUT COMPLETELY REMOVE AND TAKE A NOTE OF THE WEIGHT – if less than 5g/ 100g fresh wt, you do not need to re-calculate.
- THE AMOUNT OF WATER TO BE LOSS IS ONLY AN ESTIMATE – you do not want the soil to become too dry.
- Once the soil has dried to the required weight (or as near to), sub-sample (5-10g) and dry at 105^{o}C for 24hrs to obtain the new moisture content.

Supplement 2

MicroResp™ Carbon Sources Supplier: Sigma

	Name	Other Details	Code	Wt	Storage	Risk	Safety
C2	L-Arginine		A5006	500g	RT	n/a	n/a
C3	L-Malic acid		M1000	100g	RT	36/37/38	26-36
C4	γAmino butyric acid		A2129	100g	RT	36/37/38	26-36
C5	n-Acetyl glucosamine		A8625	100g	Freezer	n/a	n/a
C6	D-(+)-Glucose	anhydrous	G8270	100g	RT	n/a	n/a
C7	α Ketoglutaric acid		K1750	100g	Fridge	37/38-41	26-39
C8	Citric Acid	anhydrous	C0759	500g	RT	41-37/38	26-36/37/39

Risk Phrases	Safety Phrases
37/38 - Irritating to respiratory system and skin	26 - In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
36/37/38 - Irritating to eyes, respiratory system and skin	36 - Wear suitable protective clothing
41 - Risk of serious damage to eyes	35/37/39 - Wear suitable protective clothing, gloves and eye/face protection

A3 Multiplex TRFLP

Method for Multiplex Terminal Restriction Fragment Length Polymorphism (M-TRFLP)

Created by: N Thomas, Macaulay Institute
Updated by L Robinson, Macaulay Institute
Version Date: 12/12/2007

Reference

1. Singh and Thomas, Nat Protoc. 2006; 1(5):2428-33.
2. Singh *et al*, Appl Environ Microbiol. 2006 Nov; 72(11):7278-85. Epub 2006 Aug 25.

Introduction and Scope

This method allows for the simultaneous analysis of the community composition of two or more microbial taxa[1]. The method can be applied to biological materials (in this case, soil, but could also be water or food) that are likely to contain microbial life.

Principle

DNA is extracted from biological samples using a proprietary kit. The DNA is

amplified by PCR with fluorescently labelled primers designed specifically for the microbial gene of interest. The resulting PCR product is firstly purified using a proprietary kit & then digested using an appropriate restriction enzyme. Finally, polymorphism information is obtained by processing the samples through an Applied Biosystems Genetic Analyzer & exporting fragment data for analysis.

The data are viewed as electropherogram traces (peaks) for each sample and dye used in the M-TRFLP. You can view a combined or split dye trace for each sample (i.e. multiplex or simplex views). Each peak represents a terminal restriction fragment (TRF). The height & area of each TRF are directly proportional to the number of copies of the target gene (subject to PCR bias) [1].

Reference material

No certified biological reference materials are available. However, quality controls should be derived from pure microbial cultures featuring the gene of interest, &/or well-characterised soils obtained from highly organic or mineral soil sites.

Health and Safety

The following Macaulay Institute COSHH assessments apply to this method:

SO730 Use of UltraClean Soil DNA Isolation kit (Mo Bio)
SO790 Polymerase chain reaction (PCR)
SO777 Hyperladder I
SO645 Ethidium bromide
SO671 Decontamination of ethidium bromide waste
SO675 Pouring and visualising agarose electrophoresis gels
SO775 Enzymatic restriction digest of DNA
SO777 Storage of hyperladders and restriction enzymes
SO763 Preparation and purification of DNA using ABI PRISM BigDye Terminator v3.1 cycle sequencing kit
CST PCR Clean-up Kit

MSDS data is available for proprietary kit components online at the manufacturers' websites or within the product packaging. As a basic requirement, personal protective equipment should consist of lab coat, nitryl gloves & safety spectacles. Access to fume/flow hoods should be considered for some parts of this protocol.

All solid waste should be autoclaved at least once at 120^{o}C for 20 minutes & then disposed via local authority approved routes. Liquid waste should be disposed via local authority approved routes.

Reagents/Kits

	Supplier	Catalogue Number
Power Soil DNA (4) 96 Well Format Kits	Mo Bio (Cambio in the UK)	UC-12955-4
SeaKem LE Agarose	Cambrex	50004
Tris Base	SIGMA	T8524

Boric Acid	Promega	H5003
EDTA	Promega	V4231
HyperLadder I	Bioline	BIO-33026
Loading Buffer (5x)	Bioline	BIO-33026
Ethidium Bromide (0.625mg)	Mo Bio	15006-10
10xNH_4 Buffer	Bioline	BIO-21060
dNTPs (20mM)	Bioline	BIO-39026
$MgCl_2$ (50mM)	Bioline	BIO-21060
BSA (20mg/ml)	Roche Applied Science	10711454001
Taq	Bioline	BIO-21060
UltraClean-htp™ 96 Well PCR Clean-up™ Kit	Mo Bio	12596-4
Enzyme Hha1	Promega	R6441
BSA (100x)	Promega	R6441 (comes with enzyme)
10xBuffer	Promega	R6441 (comes with enzyme)
dH_2O (autoclaved)	MilliQ filtered	-
12µl Hi-Di formamide	Applied Biosystems	4311320
0.3µl GeneScan™ 500 LIZ™ Size Standard	Applied Biosystems	4322682
100% Ethanol (Analytical Reagent Grade)	Fisher Scientific	E/0650DR/17
70% Ethanol (Analytical Reagent Grade)	Fisher Scientific + Milli Q Filtered water	E/0650DR/17
Sodium Acetate	VWR	102364Q
Glacial Acetic acid	VWR	100012K

Equipment

- Balance (to three decimal places).
- Centrifuge capable of handling two 96 Well blocks (13 cm x 8.5 cm x 6.0 cm) at 2500 x g.
- Mechanical Shaker for 96 Well Blocks and plate adapters (MO BIO Laboratories catalogue numbers: 11996 and 11999).
- Multichannel pipettes in the range 2µl - 1200µl & single channel pipettes in the range 2µl - 1000µl (with suitable tips).
- Programmable thermocycler (PCR) machine.
- Microwave oven.
- Electrophoresis kit, tray, combs and power supply.
- Ultraviolet transilluminator with safety cabinet and image capture system.
- Applied Biosystems 3130xl Genetic Analyzer (ABI part number 3130XL) plus compatible PC.
- Applied Biosystems GeneMapper® software plus compatible PC.
- Autoclave

1) Sample Preparation

Samples are received as fresh soil sealed in plastic zip-lock bags, delivered to the

laboratory. Samples are stored at 4^{o}C and processed within a 24-hour period (or as soon as practicable). It is important to consider volume of sample delivery when setting up a project. Too many samples arriving at once may be difficult to process. Sample processing occurs as follows:

- All soil information is recorded electronically on reception of samples. A unique sample identifier is given to each soil (e.g. analytical barcode).
- The soil is mixed in its sample bag & sampled into three 1.5ml labelled Eppendorf PCR tubes. One sample is reserved for DNA extraction, the other two are archived. Take a representative sample by mixing & collecting soil from different parts of the bag. The tube is filled as full as possible by tapping it on a hard surface to compress material & to remove air gaps.
- The sample is frozen at -80oC for long-term storage or -20^{o}C for short-term easy access storage (return to -80^{o}C as soon as possible). The original fresh bulk sample is stored at 4^{o}C until it is either archived or deemed to be no longer required.
- Make a plan of the sample layout in a 96-well format. The samples are randomised & include inter & intra-plate replicated controls (see point 2 below). Make sure to reserve at least eight empty wells for downstream application controls.
- Soils are defrosted at room temperature immediately prior to sampling for extraction. Remainders of samples are then returned to -80^{o}C storage.
- Following the Power Soil DNA 96 Well Format Extraction Kit protocol, samples are weighed (in the range 0.25g- 1.0g) into the initial 96-well bead plate. Samples may be stored in the plates at 4^{o}C overnight but must be extracted on the next day. Depending on soil type (i.e. ease of sampling), weighing out 96 soils should take two to three hours.

2) Isolate DNA

Use the UltraClean-htp™ 96 Well PCR Clean-up™ Kit following the "centrifugation-only" protocol.

Suggested positive controls to include in DNA extraction:

- Soils from highly organic, mineral & clay sites. Ideally these should be well characterised & standardised as controls for this application. Controls should be replicated within & between plates.

If your soils are highly organic, then it is advisable to perform an ethanol precipitation on them. Although some DNA will be lost, this is quite an effective procedure for removing some contaminants (e.g. humic acids) from the sample. It is advisable to precipitate an aliquot of the total extracted DNA as there is a small chance of the sample being lost during the removal of supernatant.

Ethanol Precipitation procedure for a 20ul aliquot of DNA:

1. Add 40ul 100% Ethanol + 2ul 3M NaAc to each tube or well of 20ul PCR product, vortex.

2. Incubate plate for at least 20 minutes at -20oC
3. Centrifuge for 45 minutes at 3000rpm (96 well plate) or 30 mins at 14100 x g (Eppendorf tubes).
4. Remove supernatant by gently inverting the plate onto blue towel & then centrifuging briefly, or use a pipette to remove supernatant from a tube.
5. Add 60ul 70% Ethanol to each sample & vortex.
6. Incubate plate for ten minutes at -20oC
7. Centrifuge for 30 minutes at 3000rpm (96 well plate) or 20 mins at 14100 x g (Eppendorf tubes).
8. Remove supernatant as before in point 4.
9. Add 8ul nuclease free water & mix to re-suspend.

Note: -20^{o}C incubation times are given as minimum only. The longer the incubation, the better the precipitation. DNA can be left in 70% ethanol at -20^{o}C indefinitely.
The sample may now be used for PCR.

3) Check Total DNA on Agarose
Make an agarose gel as follows:

<u>Make 5 x TBE Stock Solution:</u>

54g Tris Base
27.5g Boric Acid
up to 1 Litre
20mls 0.5M EDTA pH8.0
(Add the EDTA last)

} Dissolve in some Millipore dH_2O, and then make

- For working stock, dilute 5 x TBE stock solution to 1 x TBE with Milli Q water.

<u>Make 1% agarose:</u>

- Add 1g SeaKem LE Agarose to every 100mls 1 x TBE; melt & mix in a microwave oven. Make enough agarose/TBE to pour a 0.75cm thick gel big enough to fit the tray.
- Add 40ul ethidium bromide at 0.5ug/ul to the liquid gel before casting.
- Load gel with 4µl template + 4µl 2 x loading buffer per lane and 5µl Hyperladder 1 in a separate lane.
- Run gel in 1xTBE buffer at 100V for 30minutes.
- Using the UV transilluminator, check that all samples have worked. If some samples have failed, you may need to re-extract them using individual tubes from the Mo Bio PowerSoil™ DNA Isolation Kit, catalogue number 12888-50 (50 preps).

4) PCR gene of interest
PCR conditions may vary for each reaction, and optimising conditions for each gene can be an ongoing process. Multiplexing sets of fluorescently labelled compatible primers (i.e. primers that can use the same PCR conditions) allows considerable

savings in time & resources. However, if compatible primers are not available, it is possible to PCR amplify primer-pairs individually & pool the product post-PCR to allow savings during fragment analysis.

- Include PCR control samples – a positive simplex control for each marker plus a contrasting 'negative' (i.e. MilliQ water plus PCR mix).
- For TRFLP profiles of Bacteria, Fungi & Archaea, the genes for 16S, ITS, & 16S rRNA are amplified respectively.
- One of the primers in each pair should be fluorescently labelled (for example in 16S the 63F primer is labelled with green dye VIC).

Below is example set for a multiplex PCR of 16S, ITS and Archaeal genes:

	µl added per 1 x rxn	Supplier	Catalogue Number
10xNH_4 Buffer	5	Bioline	BIO-21060
dNTPs (20mM)	1	Bioline	BIO-39026
$MgCl_2$ (50mM)	2	Bioline	BIO-21060
BSA (20mg/ml)	1	Roche Applied Science	10711454001
63F (VIC) (20pmol/ul)	0.5	Applied Biosystems (usually)	-
1087R (20pmol/ul)	0.5	Applied Biosystems (usually)	-
ITS1(FAM) (20pmol/ul)	1	Applied Biosystems (usually)	-
ITS4 (20pmol/ul)	1	Applied Biosystems (usually)	-
Arch344F (20pmol/ul)	0.5	Applied Biosystems (usually)	-
Ar927(NED) (20pmol/ul)	0.5	Applied Biosystems (usually)	-
Taq (5u/µl)	0.5	Bioline	BIO-21060
Template	1	-	-
dH_2O	35.5	MilliQ	-
Total Volume	50	-	-

Conditions for the multiplex PCR:

Denature 95°C 5 mins
Denature 95°C 30 seconds
Annealing 55°C 30 seconds
Elongation 72°C 1 minute
} 30 cycles
Elongation 72°C 10 minutes
15°C hold

5) Visualise PCR Product

Run 1% agarose gel (as for point 3), and using the UV transilluminator to ensure that there is no contamination & that all samples have worked.

6) Clean up PCR product

Use Mo BioUltraClean-htp™ 96 Well PCR Clean-up™ Kit (Catalogue no. 12596-4 - 384 preps) as per manufacturer's protocol except for step 16 where the sample is eluted in 30ul.

If you only have a few samples, it is more cost effective to use the following kit:

- Invitrogen Charge Switch Kit (Catalogue no. CS1200010 - 960 preps) (Elute in 30ul in step 20)

7) Quantify DNA

Run 1% agarose gel (as above in part 3) except:

- Load gel with 1µl template + 4µl 2 x loading buffer per lane and 5µl hyperladder 1 in a separate lane.
- Estimate quantity of DNA in sample by comparing to the DNA marker (see manufacturer's instructions).

8) Digest Cleaned up PCR Product

	Supplier	Catalogue Number	µl added per 20ul reaction
Enzyme (Hha 1)	Promega	R6441	2
BSA (100x)	Promega	R6441 (comes with enzyme)	0.2
10xBuffer	Promega	R6441 (comes with enzyme)	2
Sample	-	-	up to 15.8µl (see below for explanation)
dH_2O (autoclaved)	MilliQ		Up to 20µl

- Initial DNA concentration will determine sample volume to add. If your initial DNA yield is quite low, then add the maximum amount of sample volume (15.8ul). Ideally ~500ng of DNA for a multiplex reaction. Use 200ng DNA for simplex reaction
- For 2 x 96 well plates, calculate a mix for 210 reactions

Restriction Enzyme Controls:

- Incubate a reaction that does **not** have enzyme but has everything else (undigested control).
- Incubate a reaction that has no template – but has everything else (negative

control).

Incubate digest on thermocycler:
37^{o}C 3hrs
95^{o}C 10mins
10^{o}C hold
It is not necessary to run a gel to check the digested samples.

9) Run TRFLP

This method uses the Applied Biosystems 3130xl Genetic Analyzer for fragment analysis:

- Up to 2µl of each digested sample are aliquoted into a 96-well 'skirted' plate (compatible with the 3130xl plate deck).
- The machine operator adds a mix of Formamide and internal size standard (LIZ) to each sample.
- If using FAM, PET, VIC and/or NED primer labels, then the LIZ-labelled standard is suitable.

The list of samples, size standards & their location in the 96 well plate must be entered into the Data Collection software. It is useful to set up an Excel template to list this information. Here is an example of a sample submission sheet template:

User			
Group			
RO			
Total number of samples		**Plate ID**	

Please fill in the sample ID and/or bar code and other details

					Internal use		
	Well position	ID	Size standard (ROX/TAMRA)		Filter set	Date sample run	result (Ok/ repeat)
			GS 500	GS 1000			
1	A1	Unique ID	LIZ-labelled				
2	B1		LIZ-labelled				
3	C1		LIZ-labelled				
4	D1		LIZ-labelled				
5	E1		LIZ-labelled				
6	F1		LIZ-labelled				

For internal use only

Date samples received/checked			
Full or partial plate		Plate No.	

Date results sent out	
Location of result files sent out	

Volume sample added to each well:
2ul
Dyes used on plate: VIC, FAM, NED

Aliquot the samples & then submit them with sample sheet to the machine operator. If necessary, store the plate at -20oC until the machine operator is ready.
Before sample is run, the following is added to each sample:

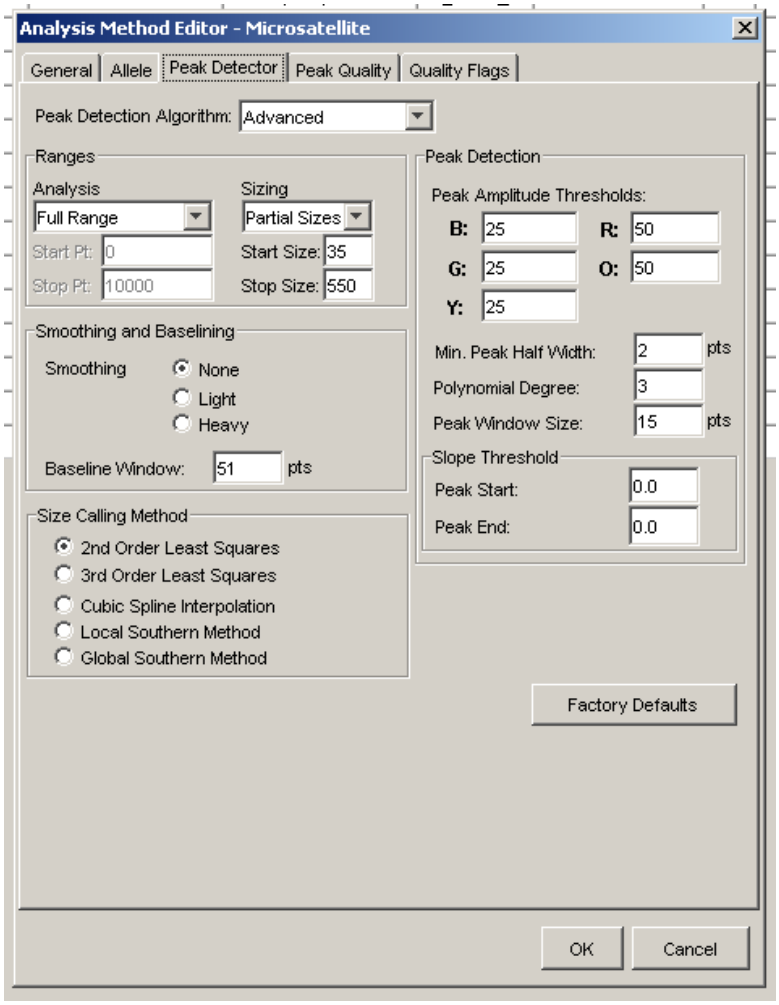
- 12µl Hi-Di formamide (ABI Part No 4311320)
- 0.3µl GeneScan™ 500 LIZ™ Size Standard (ABI Part No 4322682)
- Once the formamide/size standard mix has been added, the plates are processed as follows:
- The samples are then denatured on a heated block set to 95^{o}C for five minutes.
- The samples are cooled on ice for two minutes.
- The samples are then loaded onto the Genetic Analyzer.
- Samples are run on the Applied Biosystems 3130xl Genetic Analyzer under the following conditions:

Fragment Analysis

Description	POP 4	Unit
Oven Temp	60	^{o}C
Poly Fill Vol	7300	Steps
Current Stability	5	µAmps
Pre run volt	15	kVolts
Pre run time	180	sec
Inj volt	1.6	kVolts
Inj time	15	Sec
Volt steps	20	nk
Volt step interval	15	sec
Data delay time	750	Sec
Run volt	15	kVolts
Run time	2500	Sec

50cm capillary, Module: FragmentAnalysis50_POP4_1, Dye Set G5
Two 96 well plates (192 samples) will take approximately 12 hours to run through the machine.

8) Analyse data using GeneMapper® software. ?
Set up the Analysis Method Editor as shown below. The values shown are specific for Archeal 18S fragments.



For 16S rDNA and ITS fragments the size range is 35- 500. Peak amplitudes remain the same.

Once the data has been analysed by the GeneMapper® software, check the profiles to ensure that the standards return expected traces. It is necessary to re-submit any samples where the size standard has failed as they may be successful on a second attempt.
Once all data have been verified in the software, a table of size values can be exported ready for statistical analyses.

A4 Phospholipid fatty acid analysis

Method for the Estimation of Phospholipid Fatty Acids (PLFA) Content in Biological Materials by Gas Liquid Chromotography

Created by: C Cameron, Macaulay Institute
Version Date: 10/06/2010

Reference
Frostegård, Å., Tunlid, A. and Bååth, E. Applied Environmental Microbiology, 59, 3605 - 3617 (1993)

Introduction and Scope
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

Priniciple
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 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 (nonadecanoic acid).

Reference material
No certified reference materials are available.
Quality Control - Freeze-dried bulk sample of Countesswells soil is used for mineral soils, and Glensaugh Moor (NO652802) is used for organic soil.
A Fatty Acid Methyl Ester (FAME) standard (Supelco) is used for quality assurance of the GC run.

Health and Safety
The following COSHH assessment is associated with this method: SO577.
Solvent waste is collected in clean Winchester bottles the volume of each type recorded and disposed of through the Institute's Toxic Waste procedure.

Reagents
The grade of solvents should be HPLC.
The grade of reagents should be Sigma and Analar.

Chloroform	Potassium chloride	Nonadecanoic acid
Methanol	Citric acid	Sodium hydroxide
Acetone	Acetic acid	Butylated Hydroxy toluene
Iso-hexane	Toluene	Deionised water

Solutions
1. Citrate Buffer (0.15M)

Weigh accurately to two decimal places 14.41 g anhydrous Citric acid dissolved in 500 mls millipore 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 millipore water and make up to 100 ml.
7. C19:0 Internal Standard
   Weigh accurately 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 in cold room at 3-5^{o}C). The weight used must be recorded. 6 months 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%). 6 months expiry.

Equipment

Balance (5 decimal places)
Dri-block heater with sample concentrator and stainless steel needles
Vortex mixer
Water bath
Socorex dispenser
1000 µl Pipettor and tips
Mistral 3000 Centrifuge
Sample Rotator

Gas Chromatograph fitted with a flame ionisation detector and a split/splitless injector and a HP 7673 autosampler
Capillary gas liquid chromatography column: 50 m x 200 µm id x 0.33 µm film thickness, coated with 5 % phenyl methyl siloxane.

Gas Chromatograph Conditions for Agilent 6890

Carrier gas	Helium
Head Pressure	35 psi
Carrier Gas Source Pressure	50 psi
Split flow	6.4ml min^{-1}
Air flow	400 ml min^{-1}
Hydrogen flow	30 ml min^{-1}
Carrier gas flow rate	0.8 ml min^{-1}

Injector temperature	250°C
Detector temperature	300°C
Equilibrium time	1 min
Sample volume	2 µl
Purge time	1 min

Oven temperature:

120°C $\xrightarrow{20^{\circ}C/min}$ 160°C $\xrightarrow{5^{\circ}C/min}$ 270°C $\xrightarrow{20^{\circ}C/min}$ 290°C (47 min)

Sample 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°C before use. Pasteur pipettes and vial inserts are also muffled. Dispensers are flushed with methanol (water first if contained buffer) and left to dry before use. Taps and are soaked in decon overnight, thoroughly rinsed with water, and dried prior to MeOH soak overnight. Sample concentrator needles are soaked in clean MeOH.

All procedures are carried out in a fume cupboard.

Analytical Procedure

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. **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).**

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

For SQID Sensitivity Samples:

Pwllpeiran: 0.05g

Hartwood: 0.5g

Sutton Courtney: 1g

For SQID Discriminant samples (weights based on LOI from CS 2000)

<5% LOI: 1g

5-40% LOI: 0.5g

40-60% LOI: 0.1g

>60%LOI: 0.05g

2. Add 9.2 ml Bligh & Dyer to each sample.
3. Mix the sample on a vortex mixer, then leave for 2 hours to extract, vortex mixing every 30 mins. After the 2 hours, vortex the samples then centrifuge for 10 min at 1500 rpm, brake 9, 20°C, on the Mistral 3000.

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.

Note: The culture tubes containing the soil residue are left to dry in the fume cupboard then rinsed with water and the soil disposed of into the soil bucket.

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 mins 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 <u>top</u> aqueous layer.
    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.
11. Using a clean pasteur pipette, transfer all the lower organic phase to a clean scintillation vial.
12. Evaporate the sample to dryness under a stream of nitrogen on the dri-block heater set at 40^{o}C.
13. Once the sample is completely dry add 1 ml methanol and evaporate to dryness under stream of nitrogen.
14. Add another 1 ml methanol and again evaporate to dryness under the nitrogen.
    Note: At this stage the samples can be stored in the –20^{o}C freezer. The samples can be frozen after Step 12 if required.

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 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 $CHCl_3$, 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 <u>$CHCl_3$</u> and collect in a culture tube (at this stage <u>neutral lipids</u> are eluted).
7. Add 2 x 3 ml <u>Acetone</u> and collect in the collection vial (at this stage <u>glycolipids</u> are eluted).

8. Once the acetone has passed through the column, move the rack forward to clean culture tubes, and discard the previous collections into the appropriate waste bottle.
9. Add 4 ml and 2 x 3 mL Methanol and collect, the column is allowed to dry out once all the methanol has passed through the column (the phospholipids are eluted).
10. Evaporate the methanol eluate to dryness at 40^{o}C under stream of nitrogen on the dri-block heater.
11. Once the sample is completely dry add 200 µL Internal Standard and again evaporate to dryness under stream of nitrogen on the dri-block heater.
12. *Note: At this stage the samples can be stored in the –20^{o}C freezer.*

Mild Alkaline Methanolysis

1. Switch on the water bath, the incubation temperature is 37^{o}C.
2. Prepare the 0.2M KOH in methanol for the amount required that day.
3. Add 1 ml MeOH:Toluene (1:1 v/v) to each sample and vortex.
4. Add 1 ml 0.2M KOH to each sample and vortex.
5. Place the samples in a rack and incubate at 37^{o}C (+/- 3^{o}C) in the water bath for 15 min.
6. 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
7. Vortex and place on the sample rotator for 10 mins.
8. Centrifuge for 10 min at 1500 rpm on the Mistral 3000.
9. 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.
10. Add a further 2 ml Hexane:$CHCl_3$ (4:1 v/v) to the culture tube containing the lower aqueous layer.
11. Vortex and centrifuge as before.
12. 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.
13. Evaporate the sample to dryness under stream of nitrogen on the dri-block heater at 40^{o}C.
    Note: Once the sample has completely dried it can be stored in the –20^{o}C freezer until required for analysis.

Preparation of sample for GC

1. Add 3 x 150 µL iso-hexane to the sample and transfer to a GC vial (containing a glass insert) using a clean glass pasteur pipette.
2. Evaporate to dryness under nitrogen.
3. Add 250 µL iso-hexane (containing 0.001% butylated hydroxy toluene).
4. Place the cap on the vial and seal the cap using the 'crimper'.
5. Barcodes identifiers are placed on the vial.

Calculation of Concentration of C19 Internal Standard Used

e.g. 6.11mg C19 methyl ester in 250 ml methanol

- 6110 μg = 6.11 mg
- $\frac{6110}{250}$ = 24.44 μg ml^{-1}
- 24.44 μg ml^{-1} = 24.44 μg 1000μL^{-1}
- $\frac{24.44}{5}$ = 4.888 μg 200 μL^{-1}.

Calculation of Results

Identification of PLFA's is achieved using relative retention times (RRT's); the peaks having previously been identified by GC/MS.

The basic formula for the calculation of each phospholipid is:

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

The results are expressed as μg n-plfa g^{-1} sample.

Quality Control Limits

The PLFA results (ug g^{-1}) for the quality control soils are checked against the QC limits of 20 fatty acids (Table 1).

The quality control soil is considered a fail if more than 7 out of the 20 fatty acids fall outside the limits. The extraction for a batch of samples (40 or less) is considered as failed if more than one replicate of the QC soil has failed. If you have used both mineral and organic QC soils, you are allowed one fail for each soil type. Failure of the QC means that that batch of samples are extracted and analysed again.

Fatty Acid		Mineral			Organic		
Reported As	aka	Lower Limit ug g^{-1}	Target ug g^{-1}	Upper Limit ug g^{-1}	Lower Limit ug g^{-1}	Target ug g^{-1}	Upper Limit ug g^{-1}
C15:0i		1.650	2.656	3.662	5.687	10.970	16.253
C15:0ai		1.480	2.444	3.408	2.202	4.141	6.080
C16:1i		0.226	0.454	0.682	2.098	3.414	4.731
C16:0i		1.160	1.562	1.964	9.290	14.678	20.066
C16:1w7c		1.788	2.514	3.241	6.860	10.139	13.418
C16:1w5		1.256	1.746	2.236	2.132	2.920	3.708
C16:0		6.661	8.779	10.897	20.699	29.021	37.343
C17:0ai		0.866	1.071	1.276	3.279	4.602	5.926
C17:0brb	C16:0(12 Me)	0.790	0.973	1.156	2.309	3.206	4.103
C17:0cy		0.486	2.003	3.520	0.866	2.307	3.747
C17:0(12me)		0.572	0.747	0.923	5.185	7.260	9.334
C17:0(10me)		0.625	0.803	0.981	4.940	7.027	9.113
C18:3(5,10,12)	C18:3 w 6,8,13	0.000	0.657	1.360	2.907	4.645	6.383
C18:2(9,12)	C18:2w 6,9	0.730	1.235	1.741	6.309	8.065	9.822
C18:1w9		4.326	5.424	6.522	26.412	33.271	40.130
C18:1w7		6.897	8.592	10.287	14.493	17.598	20.702
C18:0		0.824	2.055	3.286	8.019	11.410	14.801
C18:0(10me)		1.704	2.062	2.421	7.291	10.463	13.636
C19:0cy		4.706	5.618	6.530	25.012	30.370	35.727
C20:0		0.103	0.648	1.193	3.308	5.394	7.480

Table 1. Quality Control Limits for Mineral (Countesswells) and Organic (Glensaugh) Soils.

A5 Dry extraction of soil fauna

Identification and enumeration of soil fauna through dry extraction using Tullgren funnels and taxonomic identification using microscopes.

Created by: C Wood, CEH
Date: 26/06/2006

1 Scope
This SOP specifies a method for the identification and enumeration of soil fauna to the lowest possible taxonomic level from soil invertebrate samples; first level based on Field Studies AIDGAP key, sorted into appropriate colour-coded vials. Tullgren funnel dry extraction is used to determine the abundance and composition of soil invertebrates in particular soil micro and meso-arthropods. This passive extraction method relies on the movement of soil organisms away from a heat source. The method is widely used and compares well with the extraction efficiencies of other methods (e.g. Edwards, 1991, Smith et al., 2008). The same method and equipment were used during Countryside Survey 1999 (Black et al., 2003) which would help in data compatibility between studies.

2 Normative references
None

3 Definitions
None

4 Principle
The soil sample is placed in the funnel apparatus for a defined period of time where it is exposed to heat (typically from a light source). The heating and drying of the soil causes the soil animals to move down the soil and ultimately to exit the soil sample and drop into a collecting vial containing preservative. The abundance and composition of the soil invertebrate community is then obtained through taxonomic identification of the organisms extracted by suitably trained people.

5 Test conditions
In this instance, the extraction follows the same procedure used for several years at CEH Lancaster and previously applied during Countryside Survey (Black et al., 2000). The extraction period is restricted to 5 days to support the extraction of large numbers of soil samples. 25W light bulbs are used for consistent temperature and light conditions. The funnel equipment used creates a temperature gradient of approximately 14°C in a litter or soil sample, stimulating downward movement of soil arthropods into a collecting vessel.

6 Reagents and materials
Replacement 25W clear light bulbs
50 ml clean extraction tubes (with close fitting lids) which will fit on the rubber end of the funnels

70% ethanol (typically made up from IMS)
Latex gloves, when handling soil samples
A4 sheets of clean paper to transfer samples into funnels
External sample identification labels for extraction tubes, or permanent marker to label tubes
Internal sample identification labels for extraction tubes
Storage boxes for samples post extraction

7 Apparatus

Tullgren funnels supplied by Burkard Scientific (http://www.burkardscientific.co.uk/). Image below showing a bank of 6 funnels. 10 banks of 12 funnels (120 funnels in total) were available for use by the project.

Stereo microscope with 10x to 20x objective lenses, ideally with base lighting
Fibre optics lights
Forceps, watch glasses and petri dishes for working through samples under the microscope

8 Procedure

<u>A Extraction</u>

1) Locate free positions in the Tullgren funnels and check funnel unit is complete and clean.
2) Check bulb is working and is on the correct setting for bulb wattage.
3) Put paper label with sample code into extraction tube and on sticky label on lid.
4) Fill extraction tube 3/4s full with 70% ethanol (use IMS).
5) Gently screw tube onto the rubber seal on the bottom of the funnel.
6) Place a wire unit on an A4 sheet of paper.
7) Remove core from the bag and remove caps from either end; take care not to lose soil from the core.
8) As gently as possible, push the soil out of the core.
9) Gently break core into smaller pieces.

10) Gently put the mesh minus paper onto the funnel and pour the loose soil on the paper into the mesh unit.
11) Holding the paper to prevent soil escaping, carry the mesh unit to the funnel base.
12) Turn light on and leave extractor on for 5 full days (or until Monday am if 5 days fall on a weekend).
13) After this time, remove tube from funnel, screw labelled lid tightly onto tube and store (N.B. For first sampling, replace tube and leave for a further two weeks to ascertain level of invertebrates extracted after the first week).
14) Put soil back into labelled plastic bag, seal and store in appropriate location.
15) Place a beaker under the funnel and clean the funnel using a spray bottle of deionised water.
16) Clean cores and lids; rinse soil off in sink and then wash in dishwasher.
17) When dry; store separately in cardboard boxes in the extraction lab.

B Identification

1) Note down the details of the sample to be processed on record sheet along with the present day's date (not that on which the sample was extracted)
2) Remove approximately 1-2 ml of alcohol and soil/organic matter from the sample tube using a plastic disposable pipette.
3) Place the liquid and soil into a suitable container, either a watch glass or small petri-dish
4) Initially scan the sample on low magnification and remove the identified fauna to the appropriately coloured, size and type of tube.

Species	Tube description	Lid colour
Oligochaeta	Small screw top bottle	yellow
Diptera adults and larvae	""	green
Coleoptera adults and larvae	""	red
Acari	""	purple
Araneae	""	purple
Pulmonata	""	orange
Isopoda	""	orange
Lepidoptera adults and larva	""	blue
Psocoptera	""	white
Copepods	""	black
Opiliones	""	brown
Pseudoscorpions	""	brown
Collembola – Entomobryoidea	0.5ml	Green
Collembola – Poduroidea	0.5ml	Purple
Collembola – Sminthuridae Neelidae	0.5ml	Orange
Hemiptera	0.5ml	Blue
Chilopoda or Diplopoda	0.5ml	Pink
Hymenoptera	0.5ml	Yellow
Symphyla	0.2ml	Clear

Nematoda	0.2ml	Blue
Pauropoda or Protura	0.2ml	Green
Thysanoptera	0.2ml	Pink
Diplura or Thysanura	0.2ml	Yellow

5) Label the specimen with sample ID and date of identification
6) Note group/species of organisms discovered in faunal ID book.
7) Each time an animal of the same species or group is added to the tube place a tally mark in the correct place in the faunal ID book
8) Once the sample has been completed, tally marks should be added up and a total for that group/species noted in the *Total* number column of the faunal ID book
9) After scanning the sample on a low magnification, it should be re-examined on a higher power to enable such organisms as small mites, pauropoda etc to be located.
10) Soil particles and organic debris should be moved around so that fauna hidden underneath can be located
11) When the initial sub-sample has been thoroughly searched and the soil fauna removed, identified and enumerated both the alcohol and soil/organic matter should be returned to an empty container containing a label giving full details of the sample and a note that the contents of the tube have been examined. If the label does not indicate that the tube contains checked material it may become confused with the tube bearing the same label from which it was withdrawn!
12) Next another sub-set of the sample should be removed and examined following steps 2-11
13) In addition another member of staff for the purpose of quality control should check every twentieth sample. Fauna will then be identified and enumerated by both members of staff to ensure that the identification and counting procedures employed be both individuals produces comparable results.
14) Record sheets to be copied and sent Claire Wood, CEH Lancaster.
15) Data from the record book, taxonomic name and number of individuals, to be entered onto MS Excel spreadsheets with allocated sample identification codes. Files to be sent Claire Wood, CEH Lancaster.

9 Calculation of results

Data within excel spreadsheets should be converted to abundance (numbers m^{-2}) using the dimensions of the sampling area or sampling core. In this instance, n m^{-2}= $1/(\pi r2)$ where r = 0.025m.

10 Test report

This shall contain the following information: the numbers of individuals counted for each soil invertebrate group and a total number of invertebrates, collembola and mites determined from the sums of the respective groups.

11. Quality Control See procedure above to include comparative identification of 5-15% of samples by another.

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Edwards, CA. (1991) The assessment of populations of soil-inhabiting invertebrates. Agric. Ecosyst. Environ. 34 (1-4) 145-176.

Smith, J., Pott, S. and Eggleton, P. (2008) Evaluating the efficiency of sampling methods in assessing soil macrofauna communities in arable systems. Eur. J. Soil Biol. 44(3); 271-276

A6 Wet extraction of soil nematodes

Identification and enumeration of soil nematodes through wet extraction using modified Baermann funnels and feeding-groups identification using microscopes.

Created by: P Chamberlain, CEH
Date:

1 Scope

This SOP specifies a method for the identification and enumeration of soil nematodes to feeding groups. Baermann funnel wet extraction is a passive extraction method which relies on the movement of soil nematodes in water away from a heat (and light) source. The method is widely used and compares well with the extraction efficiencies of other methods (e.g. Edwards, 1991). It is not as efficient as sieving methods but it is much less labour intensive and will support the processing on large numbers of samples in a short time-frame. The method and equipment used here is a modified Baermann method developed by Ruess (1995). The method also includes the addition of processing to allow storage of nematode samples for molecular DNA identification at a later date.

2 Normative references

None

3 Definitions

None

4 Principle

The soil sample is placed in the funnel apparatus for a defined period of time where it is exposed to heat (typically from a light source). The heating of the soil sample as its sits in water causes the soil nematodes to move down the soil and ultimately to exit the soil sample into the water where they settle into a vial at the bottom of the funnel. The abundance and composition of the soil nematode community is then obtained through identification of the organisms extracted by suitably trained people.

5 Test conditions

The extraction period is requires just over 1 day. 40W light bulbs are used for consistent temperature and light conditions. The funnel equipment used creates a temperature gradient from the soil to the water, stimulating downward movement of soil animals into a collecting vessel.

6 Reagents and materials

Nylon mesh squares (50/60 from Plastok, 50 microns)
Clean small tubes (e.g. Autoclaved Exetainers) plus storage rack
Log book to record dates/time of extraction
Beaker/jug
70% Ethanol
95% Ethanol

Glycerol
Distilled water
Pens/labels
Gloves
Forceps, slides and coverslips, watch glasses and petri dishes for working through samples under the microscope

7 Apparatus

Clean Baermann funnel unit with working bulbs (40W)
Baermann funnel unit. The equipment used here was designed and built at ITE Merlewood
Binocular and stereo microscope with base lighting
Fibre optics lights

8 Procedure

A Extraction

1. Check funnels are clean and in working order
2. Wearing gloves, gently screw suitable vials into the end of plastic tubes on the end of funnels
3. Place a mesh disc in each funnel
4. Fill funnel with water just past mesh
5. Squeeze plastic tube and funnel to expel air bubbles and top up water
6. Record funnel numbers and record in log book with date, extraction start and removal time
7. Mark tubes with sample ID, but aim to keep in order as labels are prone to fall/rub off
8. Wearing gloves, place 100 g soil sample on mesh, recording which sample number is on which funnel
9. Leave for 24 hours at room temperature (16oC). Extractor unit with lights off
10. After 24 hours, switch lights on for 1 hour
11. After 1 hour, (wearing gloves) remove samples using a jug or beaker beneath each funnel to catch the waste water. Discard water between samples.
12. Vortex to mix suspension and pipette half the sample (2 ml) into labelled micro-centifuge tubes for DNA assay leaving half in the Exetainer tube for preservation and microscope identification. 1 ml aliquots into 2 x 2ml micro-centifuge tubes.

For DNA extraction

13. Freeze samples for at least 24 hrs at -20oC
14. Next day, open caps and cover with Parafilm, pierce Parafilm and freeze dry for 48 hrs.
15. When freeze-drying is complete, remove Parafilm and close lids

For microscope identification

17. Preserve remaining 2 ml nematode samples by:

a) Place in a water bath at 60oC. Can leave out this stage

b) Pipette out most of the water, making sure to leave all the nematodes in the vial

c) Add 70% Ethanol and store for 5 days

d) Transfer to an ethanol mixture (70 parts 95% ethanol, 5 parts glycerol and 25 parts dist. water) and leave in a rack without lids to evaporate, leaving the nematodes in a strong glycerol mixture which can be rediluted prior to examination.

17. Sub sample if necessary for identification purposes

B Identification

1. Note down the details of the sample to be processed on record sheet along with the present day's date (not that on which the sample was extracted)
2. Note down the total volume of the sample and gently shake the sample
3. Remove 50μl of sample volume for identification
4. Place the liquid into a suitable container, either a slide, watch glass or small petri-dish
5. Initially scan the sample on low magnification to determine how many nematodes are in the sample
6. Proceed with identification of nematode feeding groups, plus unknowns, based on mouth-part characteristics.
7. Note group of organisms discovered in nematode ID book and tally count until the sample is entirely processed.
8. Once the sample has been completed, tally marks should be added up and a total for that group noted in the *Total* number column of the ID book
9. Record sheets to be copied and sent Claire Wood, CEH Lancaster.
10. Data from the record book, taxonomic name and number of individuals, to be entered onto MS Excel spreadsheets with allocated sample identification codes. Files to be sent Claire Wood, CEH Lancaster.

9 Calculation of results

Data within excel spreadsheets should be converted to abundance (numbers $100g^{-1}$) using the weight of the sample extracted.

10 Test report

This shall contain the following information: the numbers of individuals counted for each soil nematode feeding group with a total number of nematodes determined from the sums of the respective groups.

11. Quality Control

Bibliography

Ruess, L. (1995) Studies on the Nematode Fauna of an Acid Forest Soil: Spatial Distribution and Extraction. Nematologica, 41, 229-239.

Advice for preservation of samples given by Daniel Wright, PhD student working with Richard Bargett E-mail: d.g.wright@lancaster.ac.uk

Advice for preparing samples for molecular assay given by Bryan Griffiths, SCRI, Dundee. Bryan.Griffiths@scri.ac.uk

A7 MSIR by GC

CEH protocol for MSIR by GC (Modified from Degens & Harris, 1997)

Created by: P Chamberlain, CEH
Date:

Principle
Soils (4 g dry weight equivalent) were placed in 100 ml Wheaton bottles. Seven substrates (8 ml; L-arginine, L-malic acid, □-amino butyric acid, N-acetyl glucosamine, D(+) glucose, □-ketoglutarate and citric acid, in concentrations derived from Degens & Harris, 1997) and water were added to the soils. Bottles were crimp sealed and overpressurised with 10 ml lab air. Headspace samples (5 ml) were taken immediately and put into pre-evacuated exetainers (3 ml), and the all bottles placed on a shaker. Subsequent samples were taken at 3 and 6 hr. Carbon dioxide concentrations in headspace gases were analysed on a packed column GC against a standard of known concentration.

Soil Samples
9 soils, 3 reps, 8 C sources (inc. water)
This method will be carried out over 2 days, with one half of the samples used on the first day, and the other half on the second day.

Protocol
1. Make up appropriate solution concentrations (from Degens & Harris, 1997)
15 mM for amino acids, amines and amides; 60 mM for alcohols; 15 mM for aromatic chemicals; 75 mM for carbohydrate compounds; 190 mM for carboxylic acids; and 30 mM for the polymers.

Water	n/a
L-Arginine	15mM
L-Malic Acid	190Mm
Gamma amino butyric acid (GABA)	15mM
n-acetyl glucosamine (NAGA)	15mM
D(+) glucose	75mM
Alpha ketoglutarate (AKGA)	190mM
Citric acid	190mM

Concentrations for MSIR by GC

Compound	RMM	1M = x g l^{-1}	1mM = x g l^{-1}	1mM = x g in 300ml	Required conc (mM)	**Amt (g) required in 300ml**
L-Arginine	174.2	174.2	0.1742	0.05226	15	**0.78**
L-Malic Acid	134.1	134.1	0.1341	0.04023	190	**7.64**
GABA	103.1	103.1	0.1031	0.03093	15	**0.46**
NAGA	221.2	221.2	0.2212	0.06636	15	**1.00**
D(+) glucose	180.2	180.2	0.1802	0.05406	75	**4.05**

AKGA	146.1	146.1	0.1461	0.04383	190	**8.33**
Citric acid	192.1	192.1	0.1921	0.05763	190	**10.95**

2. Put 4 g equivalent dry weight soil in 100 ml Wheaton Bottles

Institute	Soil	Code	%MC	1 g dry wt = x g	**4 g dry wt = x g wet**
NSRI-	Arable	CA	5.47	1.06	**4.23**
NSRI-	Pasture	CP	36.74	1.58	**6.32**
NSRI-	Woodland	CW	14.21	1.17	**4.66**
CEH-	Arable	LA	20.92	1.26	**5.06**
CEH-	Pasture	LP	20.44	1.26	**5.03**
CEH-	Woodland	LW	44.90	1.82	**7.26**
Macaulay	Arable	MA	14.05	1.16	**4.65**
Macaulay	Pasture	MP	39.93	1.66	**6.66**
Macaulay	Woodland	MW	56.45	2.30	**9.19**

Note

Day 1		**Day 2**	
CEH-Lancaster	LW	CEH-Lancaster	LP
CEH-Lancaster	LA	Macaulay Institute	MA
Macaulay Institute	MP	Macaulay Institute	MW
NSRI-Cranfield	CW	NSRI-Cranfield	CA
		NSRI-Cranfield	CP
4 sites x 3 reps x 8 C	96	5 sites x 3 reps x 8 C sources	120
Total exetainers	**288**	**Total exetainers**	**360**
Total exetainers	**648**		

On the day

1. Evacuate Exetainers
2. Put soils in bottles

One tray at a time:

3. Add C sources (2 ml per 1 g dry wt soil)
4. Put on seal and crimp
5. Overpressurise with 10ml air
6. Extract sample every 30 sec
7. Put tray on shaker
8. Repeat steps 3-6 for each tray
9. Extract again after 3 and 6 hrs
10. Dispose of the soil samples in the appropriate way.

Appendix B Soil sampling for the sensitivity trial

Three soil samples are needed from each of treatment plots at each site (3 x 4 =12 per sampling time). Six sampling dates will be set at eight week intervals from July to the following May.

1. 8 x 4 cm white core for nematode extraction at CEH Lancaster.
2. 8 x 4 cm white core for microarthropod extraction at CEH Lancaster.
3. One soil sample of ~ 250 g taken with 2 cm auger (to be composed of 15 samples bulked, sieved and sub-sampled) for: MSIR and Multi-Enzymes for NSRI plus PLFAs and TRFLP for Macaulay

Sample Collection at each site
Collect samples on Monday or Tuesday of the designated sampling week. It is important that sampling is consistent by method and for date among the three sites.

Equipment
White pipes, knife, hammer, pliers, plate, trowel, appropriate 2cm diameter corer/screw auger, pre-labelled plastic bags, cold box and cool packs, 70% ethanol, cloth, brush, water and container (to clean corer/auger) .

Sampling
Collect all 3 samples (described above) from each of the 12 plots at each site.

1. In the field
1.1 Clear the surface of vegetation and fresh plant material. Cut vegetation to 1cm above soil surface if necessary to allow ease of access to sampling location.
1.2 Take the pipe and hold it upright on the soil surface while you cut round the bottom edge with the knife; cut vertically down into the soil through any roots. If the ground is very stony move the sampling point.
1.3 Push pipe firmly into the ground until it stands upright.
1.4 Place a plate (a piece of wood will do) on the top of the pipe and hammer into the soil until the plate is level with the soil surface.
1.5 If there is not enough depth of soil, move the sampling point and start again.
1.6 Make sure the pipe is full, if not hammer the pipe below the ground surface until completely full.
1.7 Use pliers to grip one edge and twist the pipe free from the soil, being careful not to lose soil from either end of the pipe (especially in dry/sandy soils). A trowel can be used to dig the pipe out or to stop soil falling from the bottom. Be careful not to lose soil from the bottom or top of the pipe (especially in sandy soils).
1.8 Carefully scrape/remove any lumps of soil from the exterior of the pipe. If needed, cut the bottom end of the core until it is level with the end of the plastic pipe.
1.9 Place into plastic bag and seal and store in cold box with cool packs.
1.10 Take 15 x 2 cm screw auger samples to a depth of 15 cm, according to pre-arranged pattern for each site, and bulk together for each plot.
1.11 Between plots, clean the auger/corer with a brush and water to remove soil,

then surface-sterilise by wiping the auger/corer with a cloth soaked in 70% ethanol.
1.12 Keep samples in a cold box with cool packs.

2. **On return to lab**
2.1 Send small white cores for extraction at CEH Lancaster. Send by courier in a cold box with ice packs to arrive within 48 hrs.
2.1.1 Store all soil samples at ~ 4^{o}C at all times.

3. Sieving, bulking and testing of the bagged soil sample
3.1 When sieving, remove one soil sample from cold storage at any one time.
3.2 Sieve the sample through 5 – 6 cm stainless steel sieve and remove vegetation, roots, stones and small animals.
3.3 Sieve through a 2 mm stainless steel sieve and remove any remaining roots, etc. Samples should be mixed thoroughly and kept cool where possible.
3.4 Between samples the sieve(s) should be cleaned then surface-sterilised by wiping with a cloth soaked in 70% ethanol.
3.5 Mix the samples thoroughly and 'cone and quarter' enough soil to carry out moisture content (MC), loss-on-ignition (LOI), pH and Water holding capacity (WHC) analyses on each soil sample (see appendix A2, supplement 1 for SOPs). Only MC will be required for subsequent soil sampling dates.
3.6 Mix the remaining samples thoroughly, and by coning and quartering', divide the sample so that there is approximately ¼ of the sample for Macaulay (minimum 25g) and ¾ for Cranfield (minimum 150g).
3.7 Adjust soil for Cranfield to 30-60 % WHC if necessary (using sterile deionised water)and re-sieve. (Do not adjust Macaulay samples) Moisture content should be adjusted to +/- 5% for all subsequent samples.
3.8 Send samples to the other labs to arrive no later than Tuesday a.m. the following week.

This would mean:
1) sampling mon/tue (day 1/2)
2) sieving wed (day 3),
3) moisture contents in and WHC completed wed/thurs – soil moisture adjusted.
4) Pick-up for delivery Thursday night, drop off Friday before 5pm.

Sampling	Date
1	Monday 10 July
2	Monday 11 Sept
3	Monday 13 Nov
4	Monday 15 Jan
5	Monday 19 Mar
6	Monday 14 May

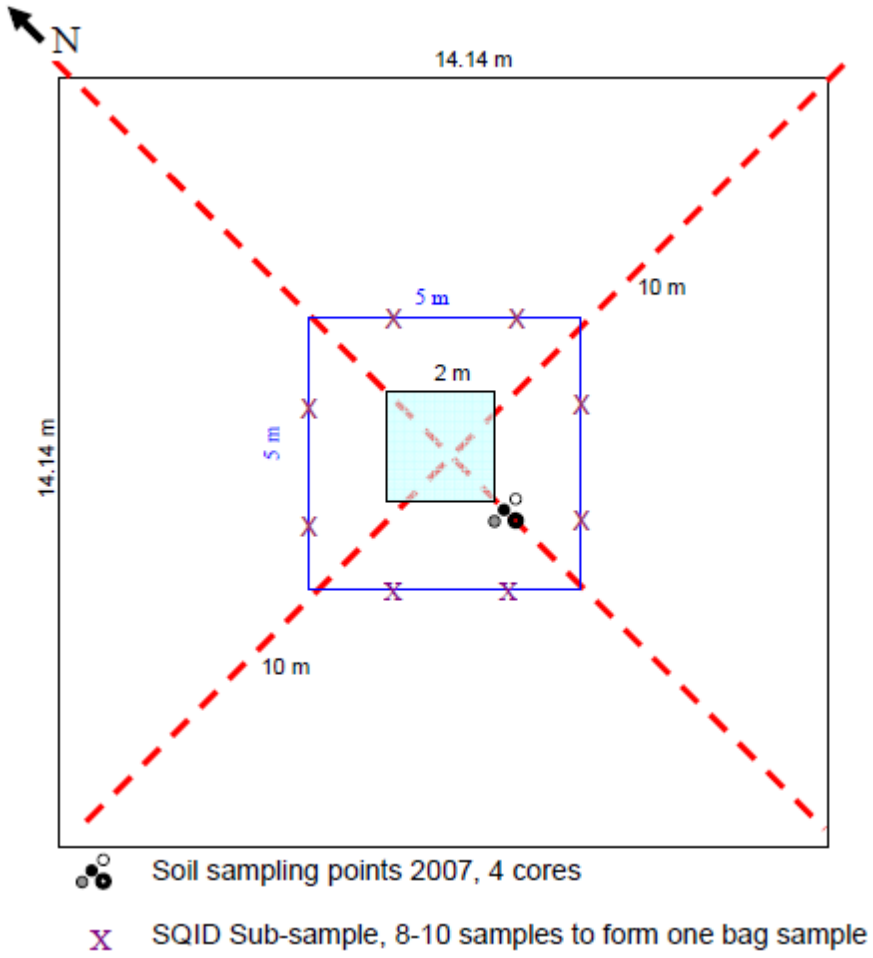
Appendix C Soil sampling for the discrimination trial

Sampling procedure for discrimination trial samples from Countryside Survey 2007. Task leader: Paul Chamberlain.
Adapted from CS Technical Report No. 3/07: Soils Manual v1.0 (Emmett et al., 2010)

The following is as handed to all field teams in 2007 survey

2.1. Equipment
Electric Cold Box, which should be kept cool by charging whenever the vehicle is being driven (connected to the vehicle lighter socket). An electrical mains charger is provided for use in accommodation. Additionally: 1 knife, 1 plastic plate, Hammer, 1 pair of pliers, Mallet, Regular trowel, Notebook & pen, Long thin trowel, Parcel tape, Spare cores, 1 pack for the appropriate square containing: · 5 X-plot packs with cores, end caps & labelled bags, Stamped & addressed mailbags for 5 X-plots.
SQID. 126 X plots are identified for bag sampling and small white core samples (see below)

2.2. Soil core samples The cores will be taken approximately 15 cm S of the south corner of the centre quadrat in each X-plot of every square. Sampling procedures for each core are detailed below. If there are problems taking any of the soil samples or a specific comment needs to be made regarding the sampling then a note must be placed in the envelope (e.g. “large tree roots - 1st soil core taken 1 m E of centre quadrat”). If there is unusual vegetation, cow pat, boulder etc move minimum distance to get more homogenous sensible location and record problem.
Taking the cores Take the appropriate labelled bag for this X-plot from the pack and find the 4 cores and 4 sample bags. For each core: · Ensure that correct core is used in the correct position (see below) ·
All cores except short white: move vegetation and loose litter to gain access to the soil surface ·
Short white core *(126 for SQID)*: move vegetation, leaving the litter layer intact. · Take the pipe and hold it upright with the bevelled end on the soil surface, while you cut round the bottom edge with the knife; cut vertically down into the soil through any roots. If the ground is very stony move the sampling point and record as above. · Push pipe firmly into the ground until it stands upright. · Hammer the pipe into the soil until the core is level with the soil surface. If there is not enough depth of soil, or the soil core is less than ¾ full when extracted from the ground, move the sampling point and start again. Record as above. · If pipe breaks or distorts significantly, use one of the spare pipes provided. · Use pliers to slowly twist and pull the pipe free from the ground, being careful not to lose soil from either end of the pipe (especially in dry/sandy soils). The trowel can be used to dig the pipe out or to stop soil falling from the bottom. · Carefully scrape/remove any lumps of soil from the exterior of the pipe · If needed, cut the bottom end of the core until it is level with the end of the plastic pipe · See below for storage requirements for different cores · Repeat for each centre quadrat in each X-plot (giving a total of five soil sampling locations in each square).



● **Core C: BLACK 15cm long x 5cm diameter** Locate the point 15cm south of the corner of the centre quadrat (USEFUL TIP: use the black core for distance as it is 15 cm long). Once collected place into plastic bag and seal

○ **Core F: SHORT WHITE 8cm long x 4cm diameter NOTE**: Remember to leave the litter layer intact for this core Core F is located 15cm to the east of the black core When the sample is obtained, push the caps over each end of the pipe. Carefully seal the sample in its bag and return to the plastic bag

● **Core N: LONG WHITE 15cm long x 4cm diameter** Core 4 is located 15 cm to the south of the black core (30cm from the south corner of the centre quadrat). When sample is obtained, push the caps over each end of the pipe. Carefully seal the sample in its bag and return to the plastic bag

● **Core P: LONG GREY 15cm long x 4 cm diameter** Core P is located 15 cm to the west of the black core It is vital that this core is the right way up, with the bevelled end placed on the soil surface. When sample is obtained, push the caps over each end of the pipe. Carefully seal the sample in its bag and return to the plastic bag.

2.3. Bag sample: SQID These samples are to be taken in 120 squares only. A labelled sealable bag and mail bag will be provided in the pack for X-plots where these samples are to be collected. One composite soil sample which fills the plastic bag up to the top of the top white panel. The sample consists of 8-10 soil sub-samples taken using the long thin trowel to a depth of 15 cm. The sub-samples will be taken along the boundary of the 5m quadrat, spaced evenly around the sides of the square. If

there are problems taking any of the samples or a specific comment needs to be made regarding the sampling then a note must be placed in the envelope. If there is unusual vegetation, cow pat, boulder etc move minimum distance to get more homogenous sensible location and record problem. Sampling procedures for each SQID sample are detailed below. · Move vegetation, as required, to gain access to the soil surface · Insert the long thin trowel into the soil four times up to the end of the trowel blade (15 cm) to form a square the width of the trowel · Lever the soil out of the ground and place into the labelled bag · If the ground is very stony move the sampling point and record as above. · If there is not enough depth of soil, move the sampling point and start again. Record as above. Repeat 8-10 times with at least 2 on each side of the quadrat, placing the soil into the same bag · The soil should now fill the bag to the top of the top white panel. If it does not, take more samples (as above) until the level is reached. · When the bag is full to the top of the top white panel, seal bag and enclose this bag in another bag and seal this

2.4. Soil sample storage and dispatch Take all 4 cores back to the vehicle and store:
Core C: BLACK Store this core in its sample bag. Once all black cores for a square have been collected, store together in a spare plastic bag. Store in a cardboard box in the vehicle; keep out of direct sunlight. Return to your regional base.
Core F: SHORT WHITE; Core P: GREY; Core N: WHITE Store these cores in their plastic bag in the coolbox immediately. When all 5 samples have been taken, place them in a spare larger plastic bag (short white, long white and grey cores separately) and put them in the appropriate mailbag, seal and post as soon as possible.
Soil Sample Bag Place this sample in the cool box immediately. As soon as possible place in mailbag labelled for CEH Lancaster, seal and post.
Posting Post samples as soon as possible. If samples cannot be posted by last post on Thursday, place them in the cool box over the weekend and post on Monday. Do not post any samples on a Friday. If the nearest post boxes will not take these packages please find a convenient Post Office. Check the OS map data for this square or Road Atlas for Post Offices.

Soil Processing Protocol for SQID-II bagged soil samples collected in CS2007

1. Overview
126 soil samples are being collected for SQID-II in CS2007, and are returned ASAP to CEH Lancaster, where they will be processed and appropriate sub-samples sent to the Macaulay Institute and Cranfield University.

2. Sample arrival and login
Log in the samples at the same time as other CS2007 samples which arrive, using the protocols in the CS2007 Lab Processing Document. Store all SQID samples at 4°C (in the cold room) for a maximum of **two working days** before processing (see below)

3. Processing of SQID-II samples
IMPORTANT All trays, sieves and paddles must be sprayed and wiped with IMS prior to use. Wear gloves at all times.

1. Take SQID samples from cold room and empty soil on to large foil tray

2. Gently crumble the soil and mix so it is homogenous
3. Quarter the soil until 100 g is selected
4. Put this 100 g in a small foil tray and move to one side. This sample is for nematode extractions – **see separate protocol**.

IF THE SAMPLE IS TOO WET TO SIEVE

5. Take a second representative sub-sample of 120 g (to account for extra water) for Macaulay, and leave the rest of the soil to dry **AT ROOM TEMPERATURE** until it is dry enough to sieve
6. Sieve the remaining soil using a 2 mm sieve
7. Take 250 g from the sieved soil to send to Cranfield

IF THE SAMPLE IS DRY ENOUGH TO SIEVE

5. Sieve the soil using a 2 mm sieve
6. Take 100 g soil for Macaulay
7. Dry the remaining soil **AT ROOM TEMPERATURE** for a few days
8. Take 250 g from the sieved soil to send to Cranfield

Soils must only be sent to Cranfield if they are sufficiently dry that they will not re-coagulate in the post.

WHEN SAMPLES ARE READY TO SEND TO OTHER SITES

9. To send to other sites, put soils in plastic bags and label with: SQID-II, destination, SQXN and date of arrival at Lancaster
10. **Every Tuesday**, pack the samples into appropriately sized boxes, with bubble wrap and freezer packs to keep the samples cold. Send the samples to Macaulay and Cranfield (first class) using the addresses below. DO NOT post samples on a Friday. (You will need to take the samples to post to reception at the main building for weighing by 3pm on the day of postage).

Send samples on a Tuesday, or if necessary another day – but DO NOT send samples on a Friday. Send in a box with freezer packs, and bubble wrap around the outside of the packs and soils. Email contacts at Cranfield and Macaulay to tell them how many samples to expect.
Paul Chamberlain 29/05/07