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ELUCIDATING THE RELATIVE IMPORTANCE OF THE BACTERIAL AND FUNGAL FEEDING CHANNELS WITHIN THE SOIL FOOD WEB UNDER DIFFERING LAND MANAGEMENTS

by

FELICITY VICTORIA CROTTY

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Elucidating the relative importance of the bacterial and fungal feeding channels within the soil food web under differing land managements.

Abstract

FELICITY VICTORIA CROTTY

The overall aim of this thesis was to elucidate the relative importance of the bacterial and fungal energy channels within the soil food web and to differentiate and appreciate the differences in niche of the soil fauna when affected by differing land management. Feeding niche of the soil fauna is ambiguous and has been previously determined by observation, inference or biochemical studies. One method that can determine feeding preferences *in situ* is the use of stable isotopes. Stable isotopes trace the passage of C and N through different trophic levels, both at natural abundance and by the addition of a pulse of enrichment.

The work described within this thesis describes the development of methods of culturing and labelling organisms for use in stable isotope studies. Bacteria, protozoa and fungi were cultured with stable isotopes enriched to 99 atom% and their growth and survival monitored. Utilising stable isotope enriched organisms means that empirical testing of the feeding interactions can occur and that differences between the bacterial and fungal energy channels can be explored. Two field sites were chosen to assess how management changes affect the food web, both sites were historically grassland with the same soil type, but one was converted to a willow woodland twenty years ago.

The results of these studies have shown, at natural abundance a grassland and woodland habitat with very different stable isotope signatures, reflecting plant and soil composition, as well as differences in trophic niche and C drivers. The introduction of enriched bacteria illustrated that bacterial feeding was more widespread than normally portrayed in food web diagrams. The introduction of enriched protozoa highlighted that protozoan feeding by soil fauna was more prevalent in the grassland habitat; reflecting differences in linkages between trophic levels within the two habitats. Methods were also developed to "grow" enriched fungal hyphae back into soil food webs in a comparable way to the investigation of the bacterial energy channel. Different species of saprotrophic fungi were found to fractionate to differing extents when grown on the same natural abundance media and the fungus *Absidia cylindrospora*'s growth was impacted when grown on a dually enriched medium.

We can now infer that the bacterial energy channel is not as divergent from the fungal energy channel as previously hypothesised. The majority of soil fauna were found to be omnivorous through empirical results, consuming bacteria and protozoa, even when they were considered to be fungal feeders by the literature. The different habitats within the study were found to have different C drivers, with roots and soil being the primary driver in the grassland whilst litter was in the woodland, consequently favouring different food webs. This work makes a first step in measuring the contribution of the different feeding channels and feeding interactions occurring within the different trophic levels in the two habitats and shows the effect that one change in management has had over the entire faunal assemblage.

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Finally, thank you to the 21,643 invertebrates that died in the name of science!!!

Collembola: Neelipleona

"Land then, is not merely soil; it is a fountain of energy flowing through a circuit of soils, plants and animals. Food chains are the living channels which conduct energy upward; death and decay return it to the soil." Aldo Leopold, 1949.

Oribatida: Phthiracaridae

Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee.

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Publications resulting from this thesis:

- **Crotty, F. V.,** Blackshaw, R. P., and Murray, P. J. (2011) Tracking the flow of bacterially derived ^{13}C and ^{15}N through soil faunal feeding channels. *Rapid Communications in Mass Spectrometry* **25:** 1503-1513 **(Chapter 5)**
- **Crotty, F. V.,** Adl, S. M., Blackshaw, R. P., and Murray, P. J. (2011) Ecological linkages between indigenous protozoa and the soil faunal food web. *Microbial Ecology* – **under review (Chapter 6)**
- **Crotty, F. V.,** Blackshaw, R. P., and Murray, P. J. (2011) Differential growth of the fungus *Absidia cylindrospora* on $^{13}C/^{15}N$ labelled media. *Rapid Communications in Mass Spectrometry* **25:** 1479-1484 **(Chapter 7)**

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Relevant scientific seminars and conferences attended at which the work listed below was presented (as an oral presentation unless otherwise stated):

- **Crotty, F. V.,** Clegg, C. D., Blackshaw, R. P., and Murray, P. J., (2010) Elucidation of the C and N flow through the soil invertebrate food web, a comparison of grassland and woodland soil. European Congress of Entomology: Soil Entomology Symposium, Budapest.
- **Crotty, F. V.,** Clegg, C. D., Blackshaw, R. P., and Murray, P. J., (2010) Discriminatory growth of $^{13}C/^{15}N$ enriched fungi over time. Stable Isotope Mass Spectrometry User Group (SIMSUG) Exeter (Poster presentation).
- **Crotty, F. V.,** Clegg, C. D., Blackshaw, R. P., and Murray, P. J., (2010) Tracking the flow of ^{13}C and ^{15}N through faunal feeding channels within the soil food web. Stable Isotope Mass Spectrometry User Group (SIMSUG) Exeter.
- **Crotty, F. V.,** Adl, S., Clegg, C. D., Blackshaw, R. P., and Murray, P. J., (2009) Tracking the translocation of ^{13}C and ^{15}N through microbial interactions within the soil. Soil Ecology Society, Vermont, USA. Abstract published in *Journal of Nematology* **41**: 321-322
- **Crotty, F. V.,** Clegg, C. D., Blackshaw, R. P., and Murray, P. J., (2009) The translocation of microbial C and N through the soil invertebrate food web. UK/Brazil Workshop on soil quality and processes, Brazil.
- **Crotty, F. V.,** Clegg, C. D., Blackshaw, R. P., and Murray, P. J., (2009) Unearthing links within the soil food web: following microbial carbon and nitrogen flow through the trophic levels. Royal Entomological Society Postgraduate Forum, York.
- **Crotty, F. V.,** Clegg, C. D., Blackshaw, R. P., and Murray, P. J. (2008) Tracking the bacterial faunal feeding channel within soil food webs. Royal Entomological Society Annual Conference, Plymouth.

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Chapter 1: Literature Review

The biodiversity of soil animal communities may exceed above-ground diversity by several orders of magnitude in many habitats (Anderson, 2009). Soil fauna are ubiquitous and can be found in any soil, anywhere in the world from Antarctic soils (Tiedje, 1995; Adams et al., 2006), to the tropics (Illig et al., 2005) and even in deserts (Santos et al., 1981; Polis, 1991). In terrestrial ecosystems, 90% of above-ground primary production enters the belowground system to form the base of the detrital food web (Gessner et al., 2010). For example, grasslands have a relatively stable and permanent plant cover providing a habitat for an abundant and diverse invertebrate fauna that contribute to effective soil functioning, including the maintenance of sustainable agricultural fertility (Kibblewhite et al., 2008).

The above-ground and below-ground systems are not separate, but linked by different biotic factors such as plants, which are the source of carbon (C) for the soil food web, and the soil biota within the web break down both the labile and recalcitrant plant compounds releasing the nutrients bound up within them, so that they can be exploited by the plant (Wardle, 1999) and the cycle continues. The soil fauna also utilise these detrital inputs and are of fundamental importance, due to their involvement in the biogeochemical cycling of nutrients (Wall et al., 2010). However, knowledge of the interactions occurring between the fauna occupying the same and different, trophic levels is limited.

The study of food webs linked to ecosystem function has been occurring since the late nineteenth century, at first focusing on aquatic systems (e.g. Forbes, 1887). Research in the 1950s focused on the importance of the organisms' spatial distribution within the ecosystem (Hutchinson, 1957), and the drivers of the interactions (Hairston et al., 1960) (Hairston, Smith and Slobodkin (HSS) hypothesis – linking resources and

predation) as two separate topics. It is only now that attempts are being made to link the spatial and functional disciplines (Massol et al., 2011) within the food web. However, in contrast to interactions in aquatic systems, the three-dimensional structure of the soil habitat adds to the complexity of trophic interactions in the rhizosphere (Bonkowski, 2004). The disparity between systems continues when considering that all death and defecation in aquatic systems is lost (to join the detrital system on the sediment bottom), whereas in the soil habitat it remains (where it was left) making it available to be utilised by other organisms (within the same system).

The dynamics of the C and N cycle directly affect the growth of the plant and organic matter pools within the soil itself (Bardgett et al., 2009). The soil biota mediate processes like nutrient cycling which they can affect directly through the comminution and incorporation of litter into soil (Ponge, 1991). Soil structure can be affected by changes in porosity and aggregate formation in soils through burrowing, casting, and faecal deposits (Davidson et al., 2006). Indirect effects include altering microbial function through grazing of the soil microbial biomass and through excretion of nutrient rich wastes (Petersen and Luxton,1982; Cole and Bardgett, 2002), thus improving plant production (Lavelle et al., 2006). The interactions between groups of organisms and physical and chemical processes shape the soil as a habitat, and influence the nature of the soil food web with consequences for the vegetation the habitat supports.

1.1 Soil food webs

Food webs are used to depict the complex array of feeding relationships between animals in the same community (Scheu, 2002) and vary from actual maps of all feeding interactions (measured through laboratory culturing and field surveys) (Henneman et al., 2001) (Figure 1.1) to a complex map of the known (from the literature) feeding

interactions that occur (Polis, 1991) (Figure 1.2) to model systems where the majority of interactions have been amalgamated (Hunt et al., 1987) (Figure 1.3).

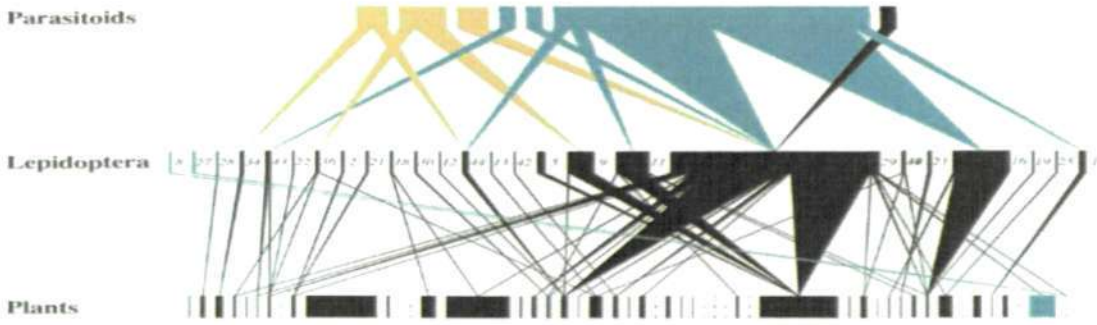


Figure 1.1: Quantitative food web from Hawaii (Henneman et al., 2001).

Bars represent species and width is its relative abundance. The width of connection between trophic levels represents the predator prey dynamics. Native species are black, accidental immigrants are yellow, and biocontrol species are blue.

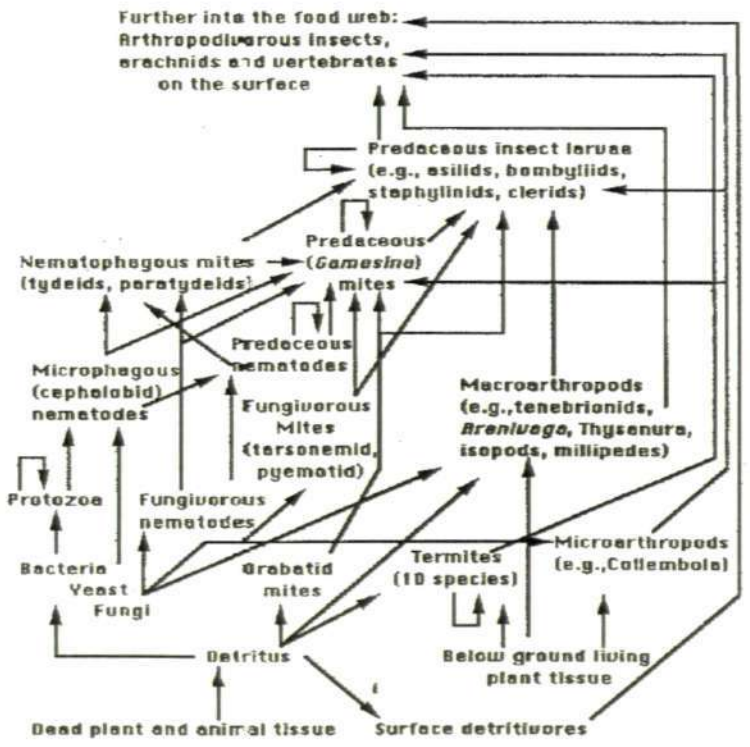


Figure 1.2: Trophic interactions within sandy soils in the Coachella Valley (Polis, 1991). An arrow returning to a taxon indicates cannibalism.

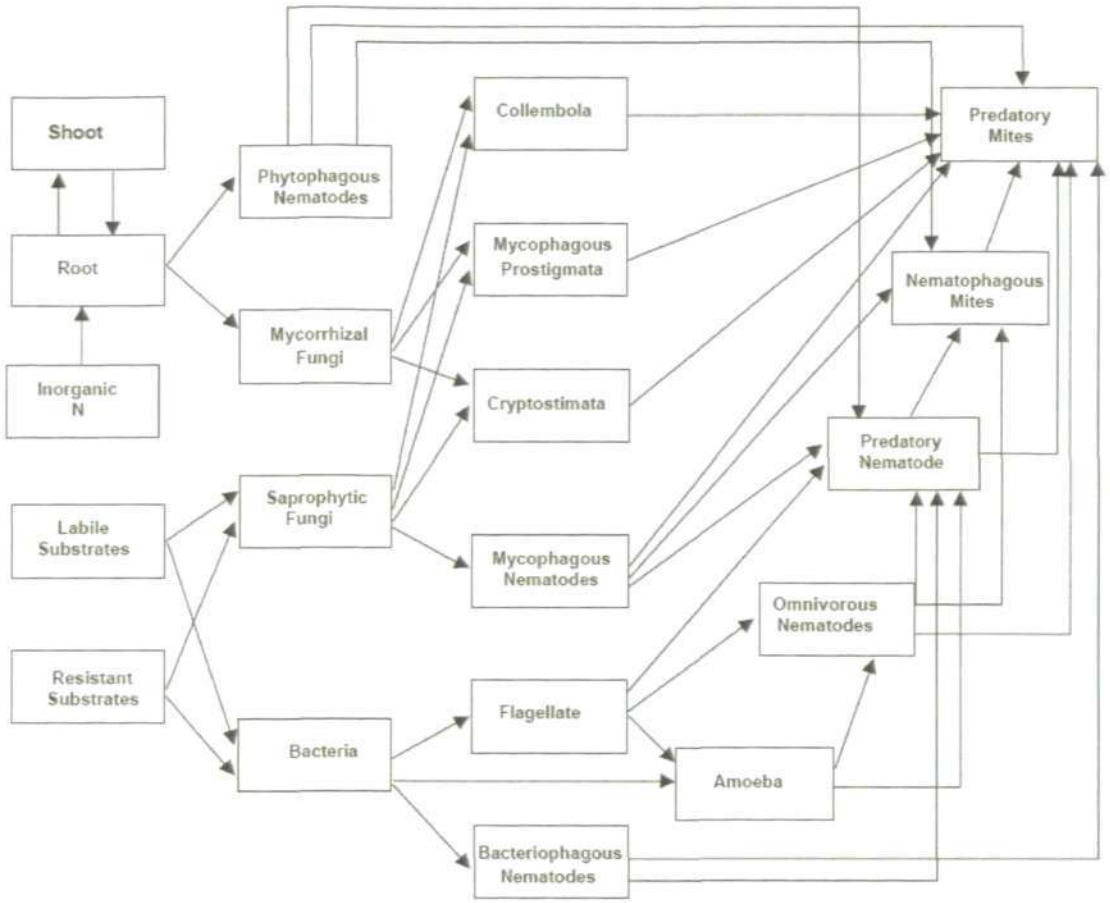


Figure 1.3: Representation of the detrital food web in shortgrass prairie (Hunt et al., 1987).

Webs that map all the interactions in addition to abundance and diversity have to date only been drawn for above-ground food webs (e.g. Figure 1.1) although some studies on belowground food webs have started to attempt this (Bezemer et al., 2010) (Figure 1.4). However, this still does not map all the interactions that occur or the individual species interactions. The complexity of soil ecosystems, including the huge diversity of microbial and faunal species, has made modelling decomposition processes difficult and inaccurate because of the number of assumptions made regarding all the interactions that are occurring. An alternative approach towards quantifying the soil microbes and fauna in decomposition and nutrient cycling is to define food webs, aggregating species on the basis of functional characteristics (Andren et al., 1990).

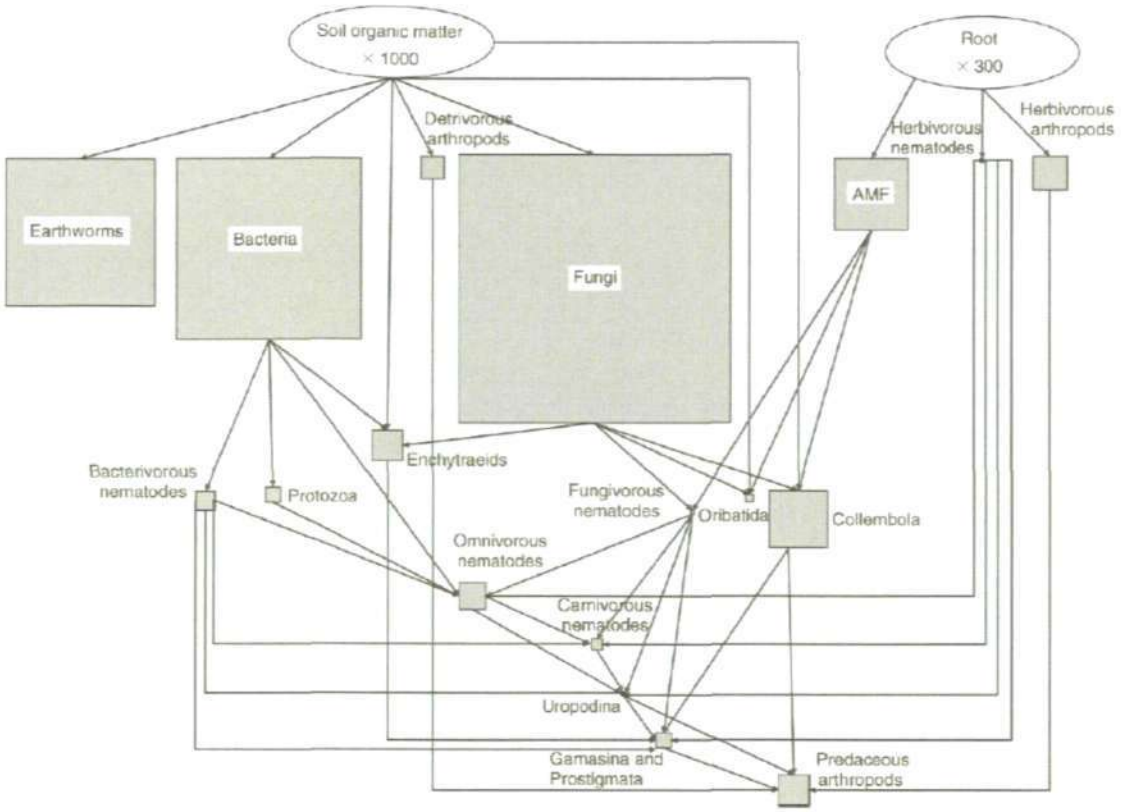


Figure 1.4: Schematic representation of the soil food web in natural grasslands (Bezemer et al., 2010).

Soil organisms were grouped into feeding guilds and the biomass of each group was calculated (g C per g soil). Boxes represent feeding groups, and their surface areas represent feeding group biomass. Arrows indicate feeding relations with the arrow head pointing toward the consumer. AMF stands for arbuscular mycorrhizal fungi.

The soil food web has been referred to as a "black box" (Bonkowski et al., 2009), a "poor man's tropical rainforest" (Giller, 1996) and an "enigma" (Anderson, 1975), due to the opaque nature of the environment, diversity of species and the limited insight into feeding specificity. It has been postulated that there are two main energy channels within the soil – bacterial and fungal (Hunt et al., 1987; Moore et al., 1988b) (visible in Figures 1.3 and 1.4). It has been hypothesised that the food web diverges

from these basal resources, and that there are minimal linkages between these channels, with different faunal assemblages branching from each (Hunt et al., 1987; De Ruiter et al., 1993) and compartmentalisation within each channel (De Ruiter et al., 1998). The bacterial route contains many "aquatic" organisms – bacteria, protozoa and nematodes, whereas the fungal route includes organisms that generally require high humidity but not free soil water or water films (Moore et al., 1988a).

A number of studies have discussed the dominance of the bacterial or fungal energy channel in relation to the other channel (e.g. Doblas-Miranda et al., 2008; Maharning et al., 2009; Strickland et al., 2010). It has been hypothesised that the differences in dominance of energy channel are due to management practices, for example, conventional tillage is thought to promote the bacterial energy channel by the redistribution of plant residues within the soil during ploughing, in comparison to no tillage systems which are thought to promote the fungal energy channel and the immobilization of plant nutrients (Hendrix et al., 1986). Soil systems may also differ in decomposition pathways dependent on plant type, with grasslands promoting the bacterial energy channel, whilst forests with more acidic soils generally showing a predominance for the fungal energy pathway (Ruess, 2003). It has been suggested that the bacterial energy channel will dominate under fertile or productive ecosystems, and the fungal energy channel will dominate in more infertile or unproductive ecosystems (Wardle et al., 2004).

The majority of soils are thermally buffered and at certain depths the soil atmosphere is saturated with water (Giller, 1996) affecting the distribution and community structure within a soil horizon. If, as already discussed, the bacterial channel is more "aquatic" than the fungal channel, the differences in management regime leading to the dominance of one channel over another, may only be affected by differences in soil moisture content. Or, where the fungal channel "dominates" there

may be differences in the available pore space within the soil (Nielsen et al., 2008). Understanding how management, plants and the soil itself, can affect the energy channels and thus decomposition and nutrient cycling is the reason this experimental work is necessary.

One of the most important features of the soil food web is that the fauna are immersed in the environment they live in, surrounded by their food, living space and excretion products. The habitable space is essentially the pore space. As large pores with large diameters are less abundant than pores with smaller diameters, large animals have access to fewer crevices than smaller animals (Kampichler, 1999) (Figure 1.5) leading to the abundance of a vast array of mesofauna of different diameters.

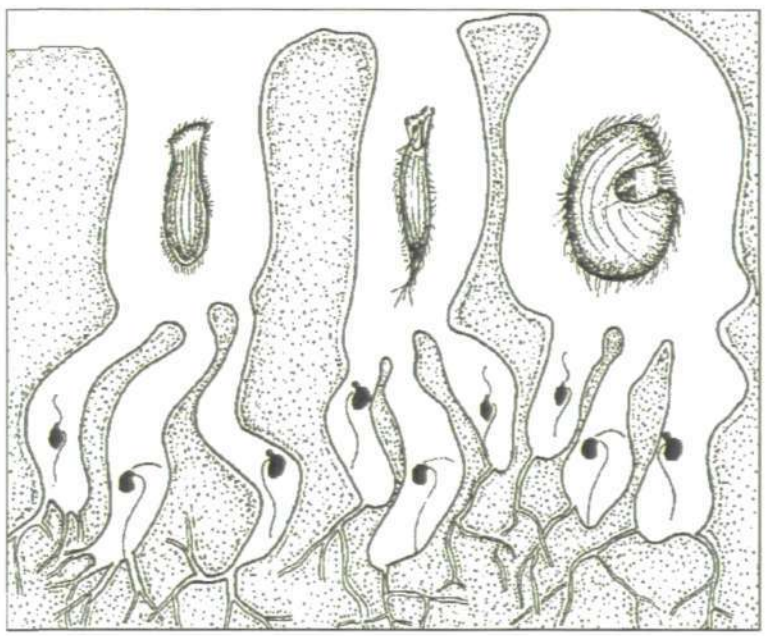


Figure 1.5: Schematic representation of protozoa in a fractal soil environment (Finlay et al., 2001).

The three larger protozoa are different species of ciliates. The smaller protozoa are flagellates, and their habitat space is bigger. The flagellates are more abundant than ciliates, but their habitat is qualitatively similar to that of ciliates, so there are still only three species. (Reproduced from Finlay et al., 2001)

Swift et al. (1979), was the first to discuss the size of soil fauna and use body width to classify the different invertebrates (Figure 1.6), and began to refer to the "microfauna", "mesofauna" and "macrofauna". The spatial dimension in soil spans at least ten orders of magnitude, considering that soils are three dimensional entities (Brussaard et al., 2006). The study of soil organic matter and nutrient availability is usually studied from the perspective of the particle whilst the study of soil organisms is usually studied from the perspective of the pore space (Elliott et al., 1988). Soil organisms exhibit a patchy distribution within the soil, even where topography and soil texture are relatively uniform, mainly due to their limited dispersal (Ettema et al., 2002) and small size. It is unknown how much space is needed for a soil organism to live comfortably within the ecosystem.

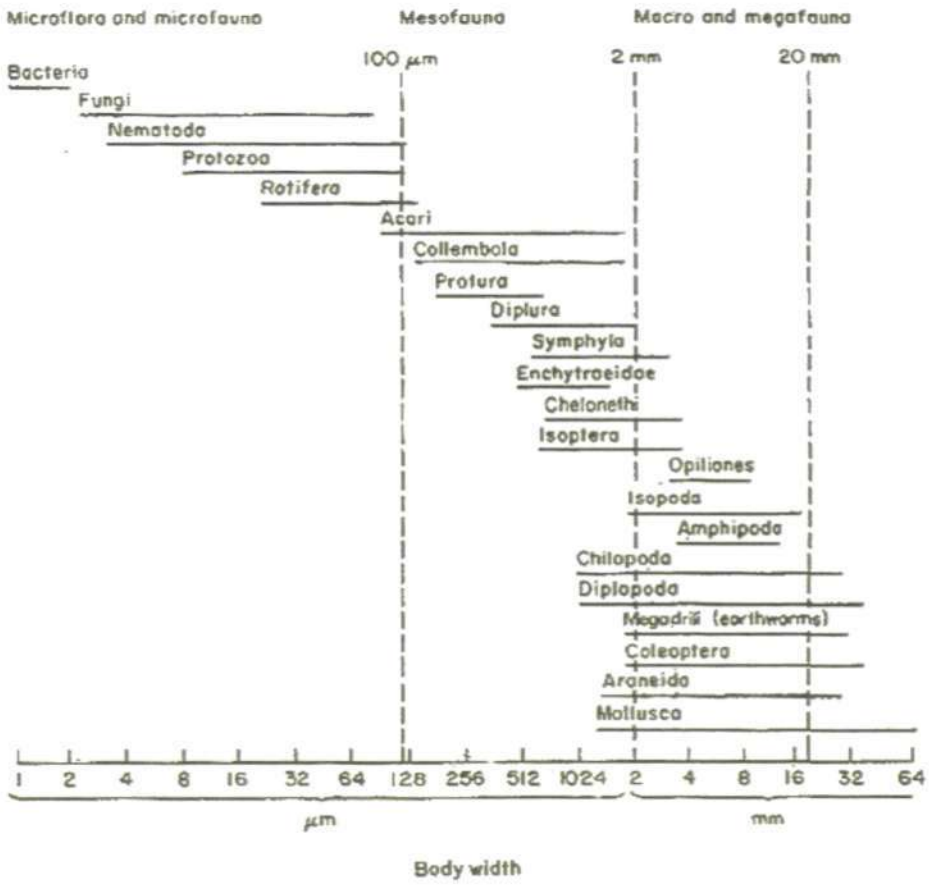


Figure 1.6: Size classification of organisms in decomposer food webs by body width (Swift et al., 1979).

1.1.1 Soil microflora and fauna

The composition of the community within a soil food web, involves interactions between a diverse array of microflora and fauna, mesofauna, and macrofauna as well as with plants and the soil itself. Bacteria have been estimated to coexist at densities of 20,000 – 40,000 species within 1 g of soil (Tiedje, 1995), consisting of many different functional groups e.g. autotrophs, heterotrophs, nitrifiers and denitrifers etc (Brussaard et al., 1997). Estimates of fungal species are thought to be in excess of 1.5 million different species worldwide (Hawksworth, 1991). The filamentous structure of fungal mycelium provides an extensive pathway for C and nutrient fluxes through the soil, often exceeding tens of metres per gram of soil (Leake et al., 2003). The basal consumer trophic level of the soil food web – the microflora (the bacteria and fungi) governs most of the mineralisation of nutrients within the soil (Wardle, 1999). Bacterial and fungal activities determine the rate of nutrient cycling, therefore detritivore-microbial interactions are pivotal when assessing faunal impacts on the nutrient dynamics of litter and soil (Seastedt et al., 1984).

The water films covering aggregates and filling soil pores are the centre of decomposition activity, where the microflora break down organic matter and are grazed by protozoa and nematodes (Bamforth, 1988) (Figure 1.5). The microfauna operate at the next stage within the soil food web. Protozoa are highly diverse and abundant group within the soil. For example, an intensive study of an upland grassland in Scotland recorded one third of known global protozoan diversity within a 1 hectare sample area over 1 year (Esteban et al., 2006). Protozoan grazing activity has been found to stimulate rates of C and N cycling in soil, promoting plant growth, as part of the microbial loop (Bonkowski, 2004). The microbial loop is considered to be the positive effect of protozoan grazing on rhizosphere bacteria (stimulated to multiply by root

exudates), this releases nutrients which can be used by the plant for growth (Coleman, 1994).

Nematodes can also be referred to as microfauna and are the most abundant metazoan, ubiquitous in soil environments (Ferris et al., 2001) although trophically they operate at a number of different trophic levels. Nematodes have been classified into eight different feeding guilds: 1) herbivore, 2) mycovore, 3) bacteriovore, 4) detritivore, 5) predator, 6) protistovore, 7) parasite, and 8) omnivore (Yeates et al., 1993). Nematodes can therefore be influential at all stages within the soil food web, operating at different levels within both energy channels. Nematodes are also prey for a number of different orders, e.g. Mesostigmatid mites (Koehler, 1999); but they are also predated by some unexpected ones, e.g. protozoa (Bjørnlund et al., 2008) and tardigrades (Sánchez-Moreno et al., 2008), leading to a more convoluted food web developing.

1.1.2 Soil mesofauna

The mesofauna predominantly consist of springtails (Collembola) and mites (Acari), which together can be found at densities of 50,000 to 300,000 individuals per m^2 in UK soils (Bardgett et al., 1998). The exact taxonomic position of Collembola is still under debate, previously they were considered to be insects (Hopkin, 1997), although their current status is as a sister group that evolved prior to Insecta (Timmermans et al., 2008). Current taxonomical hierarchy has separated the Collembola into four orders – Entomobryomorpha, Poduromorpha, Symphypleona and Neelipleona (Deharveng, 2004).

The majority of publications consider all the Collembola to be predominantly fungal feeders (e.g. Faber, 1991; Gange, 2000; Hedlund et al., 2000; Jorgensen et al., 2003; Scheu et al., 2004a; Jonas et al., 2007; Ladygina et al., 2008; Rotheray et al., 2009). However, Collembola are known to have other feeding preferences within the

soil food web e.g. herbivory (Endweber et al., 2009), omnivory (Rusek, 1998), or even predatory (Lee et al., 1996; Chernova et al., 2007). The conflict in the overall definition of feeding preferences for the Collembola has underestimated their importance within the soil food web, as they are usually portrayed in food web diagrams as fungal feeders only (Figure 1.3). The Collembola are also one of the main food sources for a large number of predators within the food web including Carabid and Staphylinid beetles (von Berg et al., 2010), ants (O'Grady et al., 2010) and spiders (Lawrence et al., 2000), as well as mites (Koehler, 1999).

Mites (Acari) are the major arthropod lineage found in soils (Behan-Pelletier et al., 1999) and unlike other arachnids, the mites have evolved far beyond saprophagy and predation (Krantz et al., 2009). Within the soil dwelling Acari, there are two main superorders, *Parasitiformes* and the *Acariformes*. The *Parasitiformes* include the order Mesostigmata, the majority of which are predatory (Koehler, 1999). Within the *Acariformes* there are two main orders – the *Trombidiformes* which includes the suborder Prostigmata, and the *Sarcoptiformes* which includes the suborder Oribatida (Krantz et al., 2009) and includes the cohort Astigmata (Norton, 1998). Within these three groups there are roughly 15,000, 10,000 and 5,000 described species respectively (Domes et al., 2007).

The suborder Prostigmata has a diverse array of feeding guilds including predators, phytophages, saprotrophs, omnivores and parasites (Krantz et al., 2009). Most Oribatida are thought to be predominantly free-living detritivores and fungivores (Maraun et al., 2003; Schneider et al., 2005a; Schneider et al., 2005b), which suffer little from predation in the field (Peschel et al., 2006). The Astigmata exploit a more diverse range of resources than the rest of the Oribatida. There is some doubt over their place in the phylogenetic tree, as they may be more closely related to the Prostigmata (Domes et al., 2007). The diversity of feeding preferences within the Acari has again

complicated current understanding of the soil food web, with different groups of mites being referred to as for example panphytophages, microphytophages, (Behan-Pelletier et al., 1983), necrophages, or zoophagous (Behan et al., 1978), herbofungivorous, fungivorous grazers or fungivorous browsers (Siepel et al., 1994).

1.1.3 Macrofauna

All other invertebrate groups within the soil are usually referred to as the macrofauna (body width > 2mm (Swift et al., 1979) Figure 1.5), and include the immature stages of many above-ground insects (e.g. Diptera and Coleoptera larvae). Although less abundant, their biomass is greater; some can have large effects on soil properties (Cole et al., 2006) and can be considered "ecosystem engineers" (Lavelle et al., 2006). The macrofauna food web operates on a larger scale to the mesofauna and the rest of the detrital food web reflecting the differences in scale and adds a nested (compartmentalised) structure (Pokarzhevskii et al., 2003), as a further complication when disentangling soil food webs.

Although the focus of research within this thesis investigates the detrital soil invertebrate food web, root herbivores also need to be considered. Root herbivores are ubiquitous in grassland in the UK and can cause significant damage to particular plant species (Murray et al., 2005), even small localised populations (Blackshaw, 1984). Below-ground herbivores can have a large impact on the soil food web in general, through the severing of roots and the consequent disruption to the fungal network (Johnson et al., 2005), changing root C exudation and the amount of dead roots present leading to changes in the microbial community (Dawson et al., 2004). Conversely, low amounts of herbivory have also been found to enhance the flux or leakage of N into the soil, increasing the N availability for neighbouring plants leading to higher yields (Murray et al., 1994; Murray et al., 1998; Bardgett et al., 1999). The soil fauna have

also been found to influence plant succession and diversity (De Deyn et al., 2003). Plant interactions with soil fauna have been found to affect the level of above-ground herbivory affecting the plant (Schutz et al., 2008; Wurst, 2010). Thereby both the herbivory and detrital invertebrate channels are intimately linked and thus will be considered as a whole within the soil food web.

1.1.4 Soil fauna other considerations

Soil ecologists cannot hope to become experts in all invertebrate groups inhabiting the soil, and it is unlikely that all species in a single ecosystem will ever be all identified and counted (Bengtsson, 1998). Unfortunately due to the level of diversity found in the soil food web, there is the potential for taxonomic bias of identifications (Andre et al., 2002), with larger or "easier" to identify taxa taking priority. When research focuses at the level of the soil ecosystem, two things are required: the cooperation of zoologists and the lumping of animals into functional groups (Coleman et al., 2003).

Studies investigating the effect of the belowground food web on ecological processes found a high level of functional redundancy at the species level within the web (Laakso et al., 1999) possibly due to the study being affected by "bottom-up" effects (Lenoir et al., 2007; Scherber et al., 2010). Relationships between soil organisms are complex and the cryptic nature of the soil environment makes disentangling feeding interactions between the soil fauna problematic. Consequently, the exact food source of each species or taxon within the soil is not known (Scheu et al., 2001) and because of this, the true trophic status of many soil animals remains uncertain or still at a conceptual stage (Schmidt et al., 2004).

1.2 Diet investigation techniques

Investigation of the feeding preferences of the soil fauna is historically based on three methods – direct observation, gut content analysis, and experimental/inference (Walter et al., 1991). As gut content analysis is also mainly observational, a more accurate description of the methods used to classify the feeding preferences of the soil fauna could be either observational, inference, or biochemical.

1.2.1 Observational

Direct observational studies of the soil faunal food web in the field are rare, mainly due to the impenetrability of the environment, small size and patchy distribution of the organisms involved. Installing an observational "window" within the soil itself to make observations at a three dimensional scale, without disturbing the environment is also extremely difficult. A published example is the study by Gunn and Cherrett (1993), who identified seven food resources for the soil fauna, these were 1) algae, 2) roots, 3) fungal hyphae and mycorrhizae, 4) dead vegetation, 5) carrion, 6) faeces, detritus and soil surfaces, and 7) live animals. Conspicuously absent from this list are bacteria: a major energy provider within the soil food web, but too small to visualise. The utilisation of carrion was corroborated by Braig et al. (2009), but the main focus of this study was not the soil food web in general.

Other forms of "direct" observation include maintaining, rearing and culturing soil fauna on a food source to ascertain their trophic status (Walter, 1987; Walter, 1988a; Walter, 1988b). The analysis of gut contents, which shows that an organism has consumed the food source at the time of sampling, has been performed on many different fauna including mites (Behan et al., 1978; Behan-Pelletier et al., 1983), earthworms (Judas, 1992; Bernier, 1998) and symphyla (Walter et al., 1989b). Also whole food webs can be assessed in this way (Ponge, 1991); this study included the

examination of faecal pellets, thus analysis of invertebrates that were not present in the soil cores at the time of the study could also be assessed.

Gut content analysis is problematic due to the level of digestion of the food particles, the level of fragmentation or if fluid feeding has occurred (Walter et al., 1991). Recent advances in molecular techniques has led to the adaptation of gut content analysis by observation, so prey items can be identified through sequencing gut content DNA (Renker et al., 2005; Read et al., 2006; Juen et al., 2007), therefore even if the prey item structure has been completely digested and it is just fluid remaining, it can still be recognised as being consumed. However, all these methods of observation only assess single points in time, rather than the whole feeding profile of an organism.

1.2.2 Inference

Inferring feeding preferences by testing whether an invertebrate prefers one food source over another is relatively common for studies involving the soil fauna (e.g. Walter et al., 1988; Maraun et al., 1998; Klironomos et al., 1999; Patt et al., 2003; Koukol et al., 2009). The use of mouthpart morphology has also been implemented to identify feeding preferences (Yeates et al., 1993; Rusek, 1998). Another method utilising the presence of specific digestive enzymes has been used to infer feeding guild in Collembola (Urbasek et al., 1994; Berg et al., 2004). Again all these methods show the organisms do exploit a particular resource, but they do not clarify the interconnectedness of the soil food web or whether the results would be consistent if offered a different choice.

1.2.3 Biochemical

The investigation of biochemical properties of the soil fauna provides a more long-term analysis of feeding preferences, although it is not as specific. Phospholipid fatty acids are components of cell membranes and show specific patterns making them effective markers of microorganisms (Frostegard et al., 1993), whilst in contrast neutral lipid fatty acids are predominantly storage lipids and therefore closely relate to the nutritional requirements of the soil fauna (Ruess et al., 2004). Neutral lipid fatty acid analysis can provide answers relating to bacterial, fungal or nematode feeding (Chamberlain et al., 2004; Ruess et al., 2005a; Haubert et al., 2006), but this can be ambiguous because of *de novo* synthesis of fatty acids (Ruess et al., 2005b) rather than dietary routing. Furthermore, only recent studies have started to track the feeding preferences through more than one trophic level using this method (Pollierer et al., 2010). Most experiments tracking fatty acids through the soil faunal food web do so in conjunction with stable isotope analysis (Ruess et al., 2010).

Using isotopes offers novel insights into trophic relationships, that are independent of previous knowledge derived from conventional techniques such as those stated above. Historically radio isotopes have been used to trace feeding interactions as they were occurring, starting with the work of de Hevesy in the 1930's. The use of radio isotopes has measure the consumption of fungi by the soil fauna (Coleman et al., 1970). The use of ^{14}C tracers have been particularly useful in measuring the movement of ^{14}C photosynthate into roots and exudates (McDougall, 1970), as well as the impact of aboveground herbivory on rhizosphere microbial growth and the soil food web (Coleman et al., 2002). However, to date all radioisotope tracer studies haven't fully differentiated between the two feeding channels within the soil, and due to increases in health and safety concerns these studies are now harder to implement. Stable isotopes occur naturally in the environment, they are safe, non-radioactive and do not decay –

making them useful natural tracers (Hood-Nowotny et al., 2007). Stable isotopes can be utilised to study diets because the organism's tissues bear a fixed isotopic enrichment (or depletion) in relation to their diet, and are a measure of the assimilated (not just ingested) diet, reflecting the diet over the long-term (Peterson et al., 1987). The application of stable isotope techniques is particularly important in soil zoology as the majority of processes are hard to study *in situ* (Tiunov, 2007).

1.3 Stable isotope ecology

Many elements including carbon (C), nitrogen (N), oxygen (O), hydrogen (H), and sulphur (S), are found naturally within the environment to occur in two or more different forms (isotopes), where they have an extra neutron within the nucleus of the atom. These isotopes can either be stable or radioactive. Of the stable N atoms on earth, 99.6337% are ^{14}N, the remaining 0.3663% are ^{15}N (Robinson, 2001). There are also two stable isotopes of C, ^{12}C which make up 98.8%, and ^{13}C which is only 1.11% (Ehleringer et al., 1986) and two radioactive isotopes of C, ^{11}C and ^{14}C. All organic matter in nature contains some of all the stable isotopes of an element, and this ratio is changeable depending on species, feeding preference or trophic level. Isotopic fractionation occurs during most biochemical reactions due to differences in mass of the isotopes causing reactions to occur at different rates (Peterson et al., 1987). Reactions discriminate against the heavier isotope, which in turn leads to it decreasing in concentration within biochemical compounds.

Using the differences in ^{15}N and ^{13}C between trophic levels, allows for a fast standardised evaluation of the trophic structure of the soil food web, without prior knowledge of predator-prey relationships (Maraun et al., 2011). This is particularly advantageous for analysing food web structure in cryptic communities like the soil. Stable isotopes, usually ^{13}C and ^{15}N have been used to assess the feeding preferences of

organisms as they provide a time-integrated measure of food resources (Pollierer et al., 2007).

There is little enrichment of the ^{13}C signal in the tissue of consumers (0.5 – 1‰), and C stable isotope ratios in animal tissue reflect those of their diet (DeNiro et al., 1978). Studies have found ecosystem specific patterns for ^{13}C enrichment (France et al., 1997), thus ^{13}C can be used for directly tracing food sources (Neilson et al., 1998). The ^{15}N enrichment is thought to increase at a fixed amount within animal tissue in comparison to diet, at a rate of +3.4‰ (± 1.1) per trophic level and appears to occur independently of habitat, form of N excreted and growth rate (DeNiro et al., 1981; Minagawa et al., 1984). The enrichment in ^{15}N is due to a preference in enzymatic reactions for ^{14}N leading to an accumulation of ^{15}N within the system (Macko et al., 1986). The ^{15}N isotopic content of an organism does not reveal which species it consumes but indicates the mean number of trophic transfers that occurred between the basal species and the organism investigated (Ponsard et al., 2000). Differences in ratios at natural abundance use δ (delta) or ‰ (per-mil) representing the per-thousand difference between the sample and a known standard (e.g. Pee Dee Belemnite (for C and O) or air (for N)). When using artificially enriched stable isotopes the atom percentage (atom%), the percentage of heavy isotope in a sample, is referred to, this can provide an overview of the movement of an element within the system.

1.3.1 Natural abundance

Stable isotopes at natural abundance have been used to define trophic levels within the soil food web (where the difference in $\delta^{15}N$ has been used to infer trophic position), concentrating on individual taxa e.g. Collembola (Chahartaghi et al., 2005; Hishi et al., 2007), Oribatida (Schneider et al., 2004; Erdmann et al., 2007), Elaterid larvae (Traugott et al., 2007; Traugott et al., 2008), ants (O'Grady et al., 2010), termites

(Tayasu et al., 1997) and earthworms (Schmidt et al., 1997) and for the whole food web (Ponsard et al., 2000; Scheu et al., 2000; Okuzaki et al., 2009). The use of stable isotopes at natural abundance to directly assess feeding preferences is more complicated within the soil than other ecosystems. This is due to the diversity of organisms (microbes and faunal species) and also because of variable discrimination/fractionation of the soil fauna and their diet. Collembolan isotopic discrimination is not constant for $\delta^{13}C$, but varies depending on type of food ingested (Ruess et al., 2005b) and does not consistently increase in $\delta^{15}N$ per trophic level (Scheu et al., 2004b).

These studies of stable isotopes at natural abundance can be affected by artefacts of the method when interpreting the data. Predators consuming internal tissues only may obtain different isotopic compositions (Denno et al., 2003). Predators consuming substances assimilated during the juvenile stage of an insect (when feeding on different substrates) could also affect the overall isotopic analysis (Eggers et al., 2000). Food availability and starvation have been found in some studies to affect the isotope levels: for example, during over-wintering (non-feeding stage), raspberry beetles (*Byturus tomentosus*) were found to become progressively enriched (Scrimgeour et al., 1995). Exuviae eating (consuming the cuticle after moulting) (Mira, 2000), coprophagy (organisms feeding on faeces/nitrogenous wastes) , or cannibalism (Halaj et al., 2005) are common occurrences amongst soil inhabiting organisms and could affect the interpretation of natural isotope concentrations within different trophic levels, as the organisms would become progressively more enriched relative to their "trophic level".

The digestive tract should be removed prior to isotopic analysis as this represents a large proportion of the whole body of some organisms (Ponsard et al., 2000) and could, depending on contents, affect the overall isotopic analysis if testing the bulk organism. For example *Bacillus* endospores passed through the gut of the earthworm *Lumbricus terrestris* without digestion (although gut passage commenced

germination of cells) (Fischer et al., 1997). Presence of "food" within the gut can not be used as proof of digestion and assimilation. For some soil fauna it is difficult to remove the gut contents during dissection e.g. Collembola. Collembola were found to contain around 13 nl of food when their guts were full and this gut volume equates to around 10^6 bacteria per individual (Tebbe et al., 2006), therefore it may be more reliable to wait until the invertebrate has moulted, and the gut epithelium is excreted (Berg et al., 2004), before isotopic analysis.

Different animal tissues have been found not to reflect the whole isotopic composition of the bulk diet (Gannes et al., 1997). For example, organisms with a high body fat content have been associated with low $\delta^{15}N$ values (Schmidt et al., 2004). Exoskeletons have a very high C:N ratio different to the internal organs (Denno et al., 2003), which could affect isotope results depending on the question being investigated. Mucus has been found to rapidly reflect dietary changes in $\delta^{13}C$ compared to tissue (Schmidt et al., 1999), and therefore could provide a method of testing changes in feeding preference.

There is little experimental proof that ^{15}N is really enriched by 3.4‰ per trophic level in soil animals (Robinson, 2001; Scheu, 2002), possibly due to the occurrence of omnivory (Ponsard et al., 2001), coprophagy or fractionation efficiencies of soil animals. In a laboratory study and review of published results McCutchan et al. (2003) found isotopic shifts for N which varied depending on food source e.g. invertebrate fed +1.4‰, compared to herbivory +2.2‰, whilst predatory was +3.3‰. Whereas a recent review found overall isotopic shifts of 0.75‰ (± 0.11) for $\delta^{13}C$ and 2.75‰ (± 0.10) for $\delta^{15}N$ (Caut et al., 2009). In a field study, soil primary decomposers were not enriched in ^{15}N by 3.4‰ compared to their food source, but were similar to the detritus (Illig et al., 2005). The activity of microorganisms may lead to a progressive shift in the isotopic composition of detritus (Vanderklift et al., 2003), leading to a gradual shift in

decomposers. Primary and secondary decomposers appear to form a continuum, from those feeding predominantly on detritus to those feeding predominantly on microorganisms on the detritus (Scheu et al., 2000; Scheu, 2002). The $\delta^{13}C$ and $\delta^{15}N$ values of organic substrates have been found to increase significantly with depth (Hishi et al., 2007). This did not appear to affect the soil fauna, as their delta values did not vary significantly with depth (Scheu et al., 2000).

Using stable isotopes at natural abundance is limited in defining the C and N transfers between the microbial soil component and the decomposers, particularly when the above caveats are considered. Many of the natural abundance studies for the soil fauna refer to a continuum of decomposers feeding on a range of litter/decomposition products/microorganisms, and this is reflected by the range of stable isotope values of the soil fauna. However, the majority of studies do not distinguish between the fungal or bacterial energy channels, although some studies are beginning to try to differentiate this (e.g. Paterson et al., 2008b) with the use of enriched tracers.

1.3.2 Enrichment of isotopes

Many studies have utilised enrichment of stable isotopes as tracers to elucidate the interactions occurring within soil food webs (Leake et al., 2006; Ostle et al., 2007; Pollierer et al., 2007; Elfstrand et al., 2008) and directly trace feeding in the natural environment. The addition of a substrate with a distinct isotopic signature can be traced as an "isotopic fingerprint" in newly synthesised faunal compounds (Elfstrand et al., 2008). Methods have been developed to enrich bacteria (Murray et al., 2009), earthworms (Dyckmans et al., 2005), slugs (Hakvoort et al., 2002), plant litter (Schmidt et al., 2001), and the growing plant (Paterson et al., 2008a), to trace the passage of ^{13}C and ^{15}N through soil ecosystems and act as an isotopic baseline (Schmidt et al., 2004). Although again, most of these studies focus on the origin of C compounds – whether

they have been obtained from the rhizosphere or decomposing plant material (e.g. Pollierer et al., 2007; Elfstrand et al., 2008), but are unable to ascertain whether an intermediary microbial step is involved (Ostle et al., 2007).

The introduction of a food source which is highly enriched with stable isotopes into the soil food web can elucidate feeding interactions as they are happening. Dissimilar to using natural abundance isotopes, the enriched pulse will be diluted as it travels through the soil food web, thus providing an estimate of consumption at the first trophic level above the introduced enriched food source and of predation at the next level within the food web. Utilising an enriched food source as an isotopic tracer helps to define trophic levels within the soil food web and also shows categorically that the food source has been consumed and assimilated.

1.4 Ecosystem characteristics

Ecosystem properties may have an influence on the soil faunal community which in turn could influence ecosystem processes. For example the soil biota are responsible for the decomposition of plant compounds mineralising the nutrients and making them available for uptake by the plants (Wardle, 1999), thus increasing net primary production. Abiotic factors such as soil type or climate, and biotic factors such as plant community or intensity of herbivory, may affect the soil fauna to the extent that their influence over ecosystem processes (e.g. nutrient cycling) may be changed.

Other methods that can be incorporated in food web analysis include allometry which has previously bee used to link food web topology with environmental variables (Mulder et al., 2006). Mulder et al. (2005), showed a relationship between the average body mass of soil fauna and numerical abundance and how this is affected by differences in the environment (including bacterial biomass). This method may be a

useful tool for comparing ecosystem characteristics, but does not elucidate the food web linkages within the individual habitats.

In a study by Proulx et al. (2010), plant diversity increased stability across trophic levels, at the community level. However, Scherber et al. (2010), showed that plant diversity can impact the soil biota and their functions, although this effect weakens with increasing trophic position. It is thought that the combination of taxonomic diversity and rapid C flux makes the soil ecosystem highly resilient to change (Fitter et al., 2005). Bezemer et al. (2010), found the whole soil food web to be influenced by plant species identity both locally, immediately above the web, and in relation to the surrounding plant community. This localised effect of plant species on the soil food web has the potential to continuously feed back influencing the food web, which influences nutrient cycling, which influences plant growth, influencing the food web and so on.

In a recent meta-analysis investigating the impacts of soil fauna on plant productivity and microbial biomass (Sackett et al., 2010), it was found that the soil fauna had significant positive effects on plant productivity both above- and below-ground, and negative effects on microbial biomass, across ecosystems. Predators significantly reduced the density of grazers, however this did not cascade further through the food web (Sackett et al., 2010). Sackett et al. (2010) concluded that the response of aboveground plant biomass changed in relation to the different plant and soil types, for example in N-limited ecosystems the effects of increases in soil faunal biomass were significant in relation to plant productivity, whilst the effects of the soil fauna were not significant where N was more available. In a study assessing the impact of the soil faunal community including macrofauna the differences in community composition markedly affected microbial and root biomass, and plant community

composition (Bradford et al., 2002). However plant productivity was unaffected and different ecosystems were not tested.

Testing whether there are differences between ecosystems is vital to enhance our understanding of the soil food web. For example, a study investigating the amount of microbial biomass C showed significant differences between different habitats (grassland had the highest amounts, then woodland, whilst arable had the least) (Abaye et al., 2006). Microbial grazers have been shown to promote plant growth, therefore ecosystems with lower amounts of microbial biomass will have comparatively less microbial grazers, and thus the comparative level of nutrients released may be less, reducing the potential level of plant growth. The effect of differences in management, nutrient availability and plant composition will be important considerations when investigating two different habitats within this thesis.

1.5 Aims

The overall aim of this thesis was to gain a better understanding of the bacterial and fungal energy channels within the soil invertebrate food web and to further develop the techniques to do this. Specifically, it was sought to reveal whether there are distinct "energy channels" and whether the feeding preferences of the soil fauna are independent of habitat or if they are affected by land management.

1.6 Objectives

To achieve the aims and to further improve the understanding of the central theme of this research – soil food webs, the following objectives are to be met:

1) Previous research (Murray et al., 2009) introduced enriched bacteria (*Pseudomonas* spp.) into soil cores with different N managements to elucidate the soil faunal food web. The survival of this bacterium when enriched to 99 atom% in ^{13}C and ^{15}N will be ascertained in preparation for future food web experiments where knowledge of the bacterial life span will be critical for developing standard incubation times, (Chapter 3).

2) Utilising stable isotopes at natural abundance, the "control" level of stable isotope abundance by the resident soil fauna will be discerned. The number of trophic levels in the two test habitats and whether the level of enrichment is at a similar level for the same taxa in each habitat will be assessed, (Chapter 4).

3) To quantify the difference in bacterial feeding between soil fauna inhabiting a grassland and a woodland soil, a highly isotopically enriched bacterium will be introduced to test for bacterial feeding *in situ,* (Chapter 5).

4) To quantify the importance of protozoa within the soil food web, as a food source for the rest of the soil fauna, an isotopic enrichment technique to test for protozoan feeding will be developed and employed, (Chapter 6).

5) Both bacterial and protozoan stable isotope enrichment trace part of the bacterial energy channel within the soil food web. A method will be developed to introduce highly isotopically enriched fungi into the soil food web and track the progress through the soil, (Chapter 7).

6) Investigating the temporal changes of the soil fauna (utilising the duration of the separate food web experiments), population change within a grassland and woodland soil over time will be assessed in relation to climate and community statistics, (Chapter 8).

Chapter 2: Materials and Methods

2.1 INTRODUCTION

The work described in this thesis attempts to elucidate the bacterial and fungal feeding pathways within the soil food web, and to distinguish the different feeding interactions and networks co-existing within the soil ecosystem. To do this a suite of methods were utilised and modified or built upon, throughout the proceeding chapters. The most common complement of methods are described below, including the two field sites focused on within the main experiments, where other field sites or methods were used these are described in the respective chapters.

2.2 ROUTINE PROTOCOLS

2.2.1 Soil core collection / field site location

For the majority of experiments within this thesis, intact soil cores (10 cm diameter, 10 cm deep; weighing on average 1.2 kg (± 0.02) (wet weight)) were taken from a permanent grassland site (Rowden Platform, N 50,46,54.55019 W 3,55,1.03173) and a willow woodland (N 50,46,15.69154 W 3,54,21.51714) both at Rothamsted Research North Wyke in the South West of England (Figure 2.1). Both sites were of the same soil type Hallsworth series (Harrod et al., 2008) (Figure 2.2), which is a clayey pelo-stagnogley soil in head from clay shale, located mainly under gentle low lying slopes. The main properties of the soil were organic C 3.7%, nitrogen 0.5% and phosphorus 0.1%, with a clay content of 38%, bulk density of 0.99 gcm^{-3} and a pH of 5.3 (Harrod et al., 2008) (see Table 2.1 for the full characteristics of soil, obtained during the study period).

Both sites were originally grazed agricultural grassland 20 years ago, after which changes in management were implemented. The grassland site had received no inorganic-N fertiliser for the last 20+ years (0N), although had been grazed regularly by cattle.

Figure 2.1: Field site location

Floral assemblage in the grassland was made up predominantly of grass species such as *Lolium perenne* (Linnaeus 1753), *Agrostis stolonifera* (Linnaeus 1753), and *Holcus lanatus* (Linnaeus 1753). The willow woodland was planted approximately 20 years ago and although coppiced regularly in the first 5 years after planting, it has not been coppiced or disturbed in the last ten years. The botanical composition of the cores taken consisted of under-canopy woodland forbs including *Pteridium spp* (Gleditsch 1760), *Hedera spp* (Linnaeus 1753), and *Anemone nemorosa* (Linnaeus 1753).

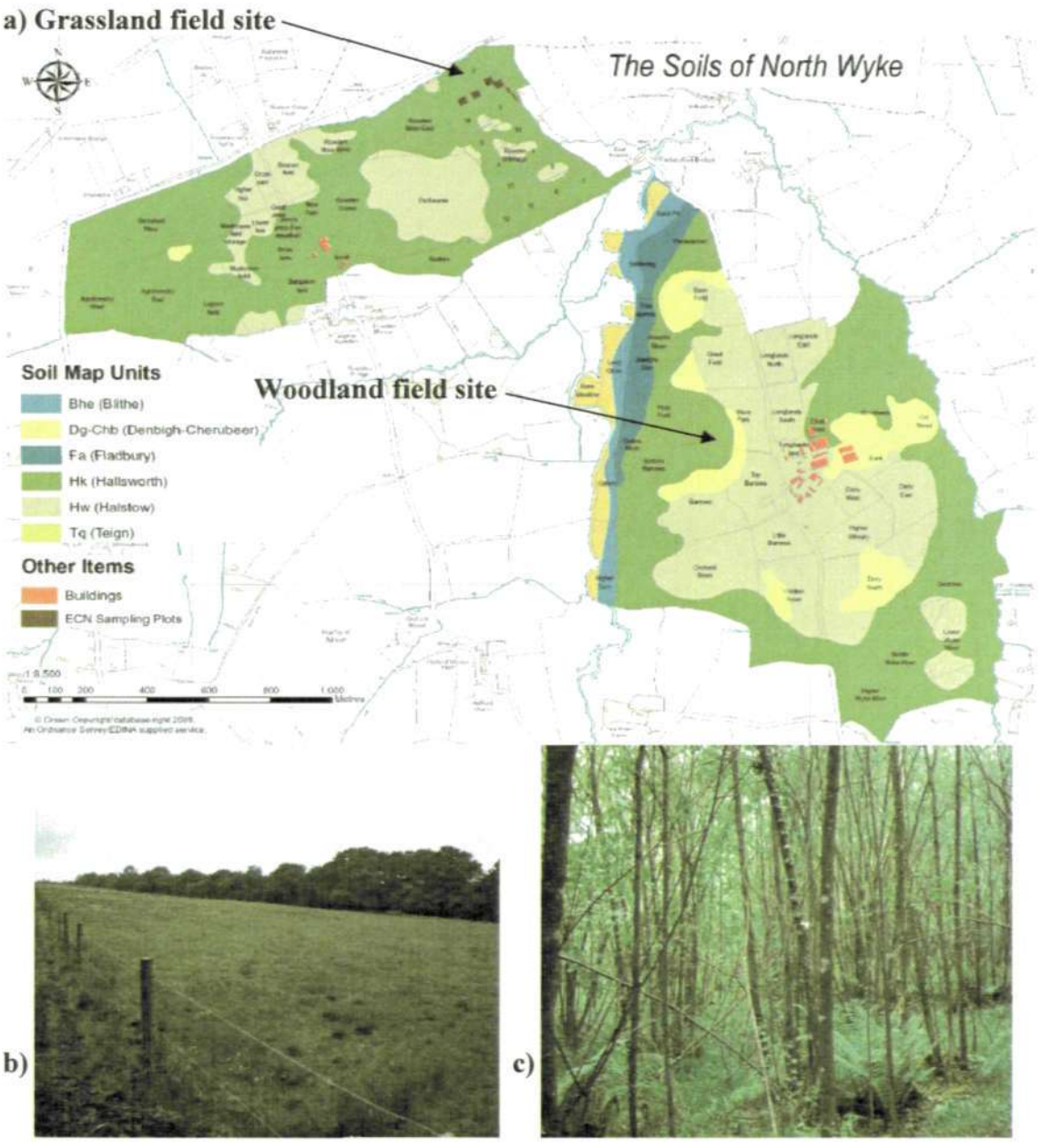


Figure 2.2: Field site location a) soil map, b) photograph of grassland field site, c) photograph of woodland field site.

The cores were removed by driving individual polypropylene sleeves (11.4 cm diameter, 10 cm deep) into the soil, to retain the entire faunal assemblage within the core and to leave the flora remaining intact on top of the core. Each core was stored within an individual Sun bag (Sigma-Aldrich, St Louis, USA), which allows gas

exchange through a 0.02 μm filter, but prevents movement of invertebrates between cores before extraction.

		Grassland	Woodland
Location: SW England	Latitude	50,46,54.55019	50,46,15.69154
	Longitude	3,55,1.03173	3,54,21.51714
	Hectares	0.68	1.1
Weather conditions			
Average annual rainfall (mm)		1085 (± 8.5)	
Average air temperature (°C)		9.8 (± 0.65)	
Average yearly total sunlight (hours)		1419 (± 38.78)	
Average soil temperature (°C)		10.9 (± 0.77)	
Maximum air temp (04/07-03/10) (°C)		23.95	
Minimum air temp (04/07-03/10) (°C)		-1.00	
Soil Analysis			
Organic Matter (%DM)		12.7 (± 0.70)	10.6 (± 1.07)
Total P (μg/g)		1084.7 (± 13.78)	840.5 (± 45.82)
Total C (%)		6.6 (± 0.22)	5.5 (±0.56)
Total N (%)		0.6 (± 0.01)	0.4 (± 0.02)
pH		5.7 (± 0.15)	5.0 (± 0.10)
Olsen's P (mg/kg)		23.9 (± 1.84)	12.0 (± 1.04)
^{13}C atom% soil		1.079 (± 0.0001)	1.080 (± 0.0001)
^{15}N atom% soil		0.368 (± 0.0001)	0.368 (± 0.0001)
Total PLFA (ng/g)		4.10	2.30
PLFA Analysis (proportion bacteria)		81.4 %	83.0 %
PLFA Analysis (proportion fungi)		18.6 %	17.0 %
Vegetation			
^{13}C atom% plant cover		1.078 (± 0.0001)	1.078 (± 0.0002)
^{15}N atom% plant cover		0.367 (± 0.0001)	0.367 (± 0.0002)
%C plant cover		39.9 (± 0.56)	32.6 (± 2.26)
%N plant cover		2.0 (± 0.07)	1.4 (± 0.07)
^{13}C atom% litter		1.078 (± 0.00005)	1.078 (± 0.00006)
^{15}N atom% litter		0.366 (± 0.00003)	0.367 (± 0.00002)
%C litter		42.9 (± 0.24)	50.8 (± 0.15)
%N litter		1.5 (± 0.02)	1.8 (± 0.03)

Table 2.1: Site characteristics for the two main sites, data represented as mean ± standard error n = 3-6. Weather averages between April-07 to March-10.

2.2.2 Culturing *Pseudomonas lurida* in ^{13}C-glucose and ^{15}N-ammonium chloride

The bacterium *Pseudomonas lurida* was the originally enriched organism used in experiments performed by Murray et al., (2009), a culture was maintained using King B agar (Sigma-Aldrich, St Louis, USA) (considered to be a *Pseudomonas* selective agar). The bacterium had both natural resistance to ampicillin and induced resistance to rifampicin at concentrations of 50 μg ml^{-1}. This combination of characteristics and the absence of indigenous bacteria identified with these characters enabled identification and recovery of this bacterium when added to experimental soils. For it to become enriched in stable isotopes, it was cultured in minimal media broth enriched to 99 atom% in both ^{13}C and ^{15}N [7 g Na_2HPO_4; 3 g KH_2PO_4; 0.12 g $MgSO_4$; 0.011 g $CaCl_2$; 0.5 g NaCl; 2.5 g $^{13}C_6$-glucose and 1 g $^{15}NH_4Cl$ (both SerCon, Crewe, UK); deionised water l^{-1}] and grown for two days at 27°C in the presence of the antibiotics ampicillin and rifampicin (both at 50 μg ml^{-1}). Following 48 hours growth the bacterial culture had utilised the ^{13}C-glucose and ^{15}N-ammonium chloride as the sole C and N source to become 99 atom% enriched.

After the 48 hours growth period, the culture was centrifuged at 6440 *g* for 20 min and washed twice in sterile ¼ strength Ringer's solution, before resuspension in sterile ¼ strength Ringer's solution and overnight incubation. This starved the bacterial cells to ensure all remaining substrate had been utilised. The inoculum would then consist solely of bacteria. After overnight incubation, the culture was subsequently washed three times by centrifugation and resuspension in sterile ¼ strength Ringer's solution, before finally suspending the cells in sterile $^1/_{20}$ strength Ringer's solution, ready for microcosm inoculation.

2.2.3 Soil respiration sampling

To assess the level of soil respiration, two hours before further sampling, each microcosm was sealed and incubated at room temperature for two hours. Subsequently, a 12 ml headspace volume sample was taken using a 25 ml gas syringe (Thames Restek Ltd, Saunderton, UK) and stored in prepared 12 ml butyl septum-capped vials (Exetainers®, Labco Limited, High Wycombe, UK). Preparation of the Exetainers involved heating the caps to 105°C for 12 hours immediately prior to use to retain a stable $\delta^{13}C$ signature (Midwood et al., 2006) and reduce known storage effects (Knohl et al., 2004).

Ahead of gas sampling, after capping, the Exetainers were evacuated to remove CO_2 from the airspace within them; and then flushed with helium followed by a second evacuation to be ready for sampling. After sampling of soil respiration each Exetainer was analysed using a trace gas analyser (ANCA TGII, PDZ-Europa, Crewe, UK) linked to a stable isotope analyser mass spectrometer (20/20, PDZ-Europa, Crewe, UK) with a Gilson model 221 auto-sampler (Middleton, USA).

2.2.4 Bacterial plate counts

Plate counts of *P. lurida* bacteria were made at intervals to assess the survival of the bacteria when added to mesocosms, using the method of Clegg et al. (1995). For each treatment and replicate, 10 g of soil (wet weight) were added to conical flasks containing 90 ml sterile 0.1% (weight per volume) sodium pyrophosphate. The suspensions were agitated using a mechanical shaker for 30 min before 10-fold serial dilutions were made in sterile ¼ strength Ringer's solution. Aliquots of the dilutions were spread-plated onto King B agar containing the antibiotics ampicillin and rifampicin (at 50 $\mu g\ ml^{-1}$) and replicated in triplicate. Agar plates were incubated at 27°C for three days before colonies were counted.

2.2.5 Stable isotope analysis

All samples were weighed (Mettler Toledo MX5 microbalance (precision to 1 μg)) and $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratios were measured, along with analytical quality control samples. All soil, herbage and fungal samples were ground and analysed on an elemental analyser (N1500, Carlo Erba, Milan, Italy) linked to an isotope ratio mass spectrometer (20/20, PDZ-Europa, Crewe, UK) with a Gilson model 221 auto-sampler (Middleton, USA). The precision range was 800 – 1800 μg C, and 40 – 80 μg N, with an analytical precision for atom% measurements of ±0.00006 for ^{13}C and ±0.00004 for ^{15}N. Gas samples were analysed for ^{13}C only, using a trace gas analyser (TGII, PDZ-Europa, Crewe, UK) linked to the same mass spectrometer.

The invertebrate stable isotope concentrations were determined using a Flash EA 1112 Series Elemental Analyser connected via a Conflo III interface to a Delta^{Plus} XP isotope ratio mass spectrometer (all Thermo Finnigan, Bremen, Germany). The precision range was 20 – 300 μg C and 15 – 150 μg N (low C run) and 400 – 4000 μg C and 30 – 900 μg N (normal C run), with an analytical precision of ±0.29 ‰ for $\delta^{13}C$ and ±0.0002 atom% for ^{15}N. The use of two different mass spectrometers during the experiments was due to the differences in precision of C and N needed for accurate measurements, and the weight of the different sample types analysed. Using the Delta^{Plus} XP isotope ratio mass spectrometer, allowed smaller biomasses of invertebrates to be analysed precisely, providing a greater number of replicates within experiments. The isotope abundance in the soil, invertebrate and soil respiration samples, are expressed as Atom% Excess (*APE*) ^{13}C and ^{15}N calculated using equation 1 and 2.

$$APE\ ^{13}C = \text{Atom\%}_{sample}\ ^{13}C - \text{Atom\%}_{natural}\ ^{13}C \qquad (1)$$

$$APE\ ^{15}N = \text{Atom\%}_{sample}\ ^{15}N - \text{Atom\%}_{natural}\ ^{15}N \qquad (2)$$

Where $Atom\%_{sample}{}^{13}C = (R_{sample}/R_{sample} + 1) \times 100$ and $Atom\%_{sample}{}^{15}N = (R_{sample}/R_{sample} + 1) \times 100$, R_{sample} is the $^{13}C/^{12}C$ ratio or $^{15}N/^{14}N$ ratio determined by the mass spectrometer (Leake et al., 2006) and where $Atom\%_{natural}{}^{13}C$ and $Atom\%_{natural}{}^{15}N$ are the corresponding natural abundance samples from uninoculated cores. The total quantity of enriched organism derived isotope was calculated using equations 3 and 4.

$$^{13}C_{sample}\ (\mu g) = C_{sample} \times APE^{13}C / Atom\%_{ENR\text{-}org}{}^{13}C \qquad (3)$$

$$^{15}N_{sample}\ (\mu g) = N_{sample} \times APE^{15}N / Atom\%_{ENR\text{-}org}{}^{15}N \qquad (4)$$

Where C_{sample} and N_{sample} are the total quantity of C or N in the biomass of that sample and $Atom\%_{ENR\text{-}org}{}^{13}C$ and $Atom\%_{ENR\text{-}org}{}^{15}N$ are the atom% values of the enriched organisms added.

2.2.6 Assessment of enrichment of organisms

Enrichment of organisms was tested by taking a sub-sample of the enriched organism solution, washing it via the centrifugation and resuspension method described above, before finally resuspending in deionised water. Due to the detection thresholds of the mass spectrometer, certain modifications to the samples needed to be made before analysis. Samples need to be ≤ 5 atom% - although they had a potential enrichment of up to 99 atom% in both ^{13}C and ^{15}N and therefore they needed to be diluted. In order for samples to be diluted to an atom% ratio within the thresholds of the machine, a reference flour with a known C content of 41.96% and 1.0822 atom% ^{13}C was used to dilute the sample. The equation by Hauck et al., (1976) (equation 5) was rearranged (equation 6) to determine the amount needed for dilution.

$$A_2 = ((T * A_0) + (D * A_1)) / (T + D) \quad (5)$$

$$D = ((A_0 - A_2) * T) / (A_2 - A_1) \quad (6)$$

Where A_2 is the desired atom% of the sample; T and A_0 are the weight and atom% of the compound to be diluted (the enriched organism), and D and A_1 are the weight and atom% (natural abundance) of the diluting compound (the flour), respectively. Each replicate was diluted with the appropriate amount of flour and analysed by mass spectrometry. Once the mass spectrometry results were obtained it was possible to rearrange equation 5 again and back-calculate the atom% of the enriched organism before dilution (equation 7).

$$A_0 = ((A_2 * (T + D)) - (D * A_1)) / T \quad (7)$$

Where A_0 is the original atom% of the sample prior to dilution; A_2 is the atom% mass spectrometry result of the total sample (the enriched organism and the flour combined), T and D are the weight of the compound to be diluted (the enriched organism) and the diluting compound (the flour), respectively, and A_1 is the atom% of the diluting compound.

2.2.7 Nematode extraction

Nematodes were extracted using a similar method to the direct extraction of mobile nematodes described by Whitehead and Hemming (1965) and adapted by Bardgett et al., (1997). For each core, a vertical slice approx 100 g (fresh weight) of soil was taken from the centre of the core to the edge, encompassed approximately 10% of the cores circumference. This was roughly broken up and placed on a thin layer of coarse porosity tissue paper (Mills et al., 2006) (KIMWIPES Lite, Kimberly-Clark,

West Malling, UK). This was supported by two different sized meshes in a tray, with 400 ml deionised water (Figure 2.3). All trays were incubated overnight to allow the nematodes to move into the solution. Samples were repeatedly settled and their volume reduced by suction until a concentrated solution of nematodes $\leq$ 1.5 ml was achieved. Once concentrated, 10 µl samples of the nematode solution were taken in triplicate and the numbers of active nematodes were counted, the remaining nematode solution was decanted into a centrifuge tube and was centrifuged at 360 *g* for two minutes. The supernatant was removed and the residual pellet was transferred to tin capsules (8 x 5 mm, pressed, standard weight, Elemental Microanalysis Limited, Okehampton, UK) and allowed to dry in a 65°C oven before mass spectrometry to determine their $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratios. Nematodes were not divided into functional groups prior to analysis.

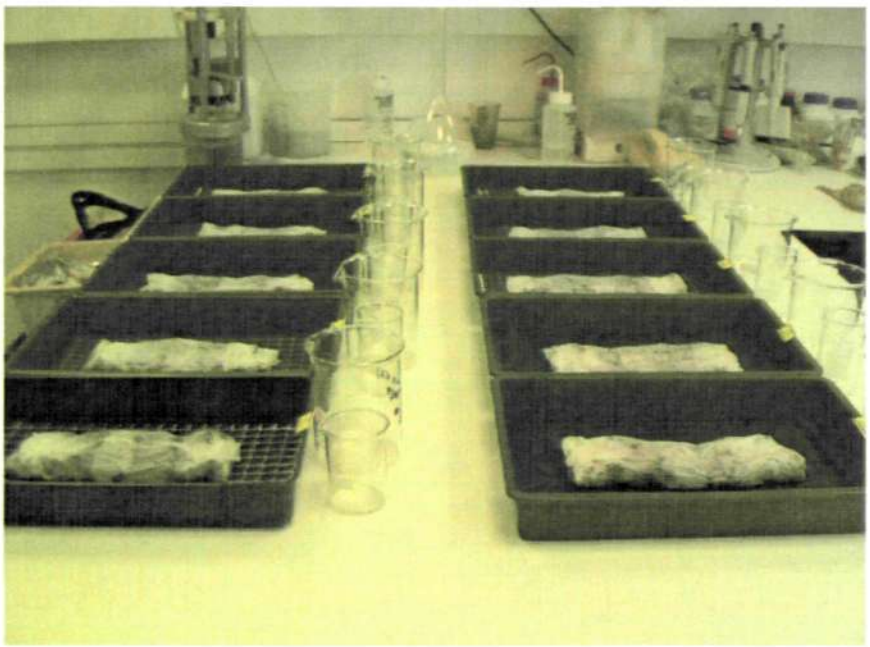


Figure 2.3: Nematode Extraction

2.2.8 Invertebrate extraction and separation

The remainder of the core was placed on a Tullgren funnel system (Burkard Manufacturing Co. Ltd, Rickmansworth, UK) (mesh 5 mm), where invertebrates migrated through each core via a temperature gradient over time and were collected (Figure 2.4).

Figure 2.4: Tullgren Funnel

The cores were held in the Tullgren funnels for a total of 5 – 21 days (depending on the experiment); invertebrate collections were a composite over the days of extraction. Invertebrates were collected in saturated salt solution to prevent chemical changes to isotopes. Studies investigating preservation methods/chemicals in soil fauna/insects found freezing or short term storage in salt solution had no effect on

isotopic composition (Fabian, 1998; Ponsard et al., 1999). Other studies looking at a range of organisms agree that freezing is the best method and organic chemicals (particularly formalin) should be avoided (Hobson et al., 1997; Feuchtmayr et al., 2003; Sweeting et al., 2004; Carabel et al., 2009).

Invertebrate groups were identified and separated under a microscope prior to drying and mass spectrometry. Invertebrates were transferred to tin capsules and dried at 65°C for 48 hours to be ready for mass spectrometry. These were the four main Collembola orders – Entomobryomorpha and Poduromorpha (*sensu* Deharveng (2004) which were both considered superfamilies within the order Arthropleona (Hopkin, 1997) until recently) and the orders Neelipleona and Symphypleona were also separated, but due to their limited abundance in the different habitats both were not represented in all samples. Other invertebrates were separated into taxonomic groups determined by abundance, this included the four main groups of soil dwelling Acari (Astigmata, Mesostigmata, Oribatida and Prostigmata), as well as families Damaeidae, Phthiracaridae, and Uropididae. All other invertebrates collected were separated to order, apart from Coleoptera where the majority were separated to family – Carabidae, Chrysomelidae, Cuculionidae, Elateridae, Ptilidae and Staphylinidae. Diptera were sorted to order apart from Tipulidae larvae which were analysed separately. All fauna were sampled with their gut contents intact (apart from earthworms, Tipulidae larvae and slugs), therefore the mass spectrometry results may be biased towards the prey item. (See Appendix A for example pictures of soil fauna).

Separating invertebrates to family (Coleoptera), superfamily (Collembola), lineage (Acari) or order (all other invertebrates), may weaken the results as different species may have different feeding preferences. This was because of invertebrate abundances, their small biomass and mass spectrometry constraints (≥ 20 μg C and ≥ 15 μg N were required for reliable results). This taxonomic resolution was chosen as a

compromise, so that feeding could be assessed across the whole soil food web, and the organisms within each core could be kept separate and used as individual replicates.

2.2.9 Statistical analysis

Throughout this thesis, all data was analysed using the statistical package GenStat (GenStat 13, VSN International Ltd., Hemel Hempstead, UK). Statistical treatment of the data for each experiment is described in full in the relevant chapters. All data presented as mean ± standard error, unless otherwise stated.

Chapter 3: Developing bacterial enrichment: monitoring bacterial survival in soil.

3.1 INTRODUCTION

Microbial communities play a major part in grassland ecosystems, regulating organic matter decomposition and plant nutrient availability. Soil organic C is made up of a complex mixture of plant and microbial residues. Agricultural grassland soils have a high turnover of shoot and root biomass, and thereby a large pool of labile organic matter at the soil surface (Bardgett et al., 1998). Soil organic matter is broken down and contributed to by microorganisms, through the metabolic transformation of plant derived material. This C turnover and mineralisation drives both the bacterial and fungal energy channel (Moore, 1988) within the soil decomposer food web. Insight into the structure of the food webs relies on understanding the interactions occurring between soil organisms and their environment, their survival, and their activity within the soil.

Stable isotope ecology can be used as a natural way to directly follow and trace details of element cycling *in situ* (Fry, 2006). Soil bacteria such as *Pseudomonas* spp. are readily extractable and culturable from the soil and are considered important for plant health (Clark et al., 2008). Previously a *Pseudomonas lurida* bacterium was isolated from the rhizosphere of an agricultural grassland (Murray et al., 2009), so that it could be used as a tool for soil food web investigation. However, its ability to grow in ^{13}C and ^{15}N enriched media and its survival when re-introduced back into different types of soil was initially unknown.

Due to the interdisciplinary nature of soil science many experiments performed within one area are not always considered by researchers in another area. This has led to different standards being accepted as the norm in one area but opposed in another. An example of this is the use of freeze-thaw cycles by soil zoologists to defaunate soils

(Stenberg et al., 1998; Kampichler et al., 1999; Schutz et al., 2008), to remove soil arthropods whilst theoretically, leaving the soil microbial community intact. Whereas soil microbiologists use a freeze-thaw cycle as part of a method for rapid extraction of microbial DNA (Tsai et al., 1991), through microbial cell rupture. These differences between research groups start at the moment of sample preparation, making the standardisation of experiments using soil microcosms more complicated.

Investigations of microbial activity within the soil are typically performed in laboratory microcosms using sieved soil incubated over varying time periods (Abaye et al., 2006; Gutierrez Lopez et al., 2008; Sheehan et al., 2008). Sieved soil is used mainly to increase the homogeneity of samples, but these experiments are often used to assess how the microbes affect soil fauna (Bradford et al., 2008) and their contribution to food web activity (e.g. Cole et al., 2004). The results of such investigations are often extrapolated to represent the microbial activities and interactions occurring within the field. The differences between the survival of the microbial community in intact undisturbed soil and the unnatural sieved equivalent are assumed to be negligible.

It is impossible to have exactly the same community composition of soil fauna in a sieved and repacked soil core with invertebrate additions. Only intact cores taken straight out of the field to be tested will have an indigenous community composition that matches nature. For example there may be up to 250 different species of mites and up to 150,000 individuals per m^{-2} occupying grassland soil (Behan-Pelletier, 2003), but of these at most 10% are thought to have been studied (Andre et al., 2002), to introduce even a fraction of these species and individuals into repacked soil cores for microcosm experiments is unfeasible. Therefore studies investigating the microbial component and its interactions to the soil food web whilst using sieved soil microcosms cannot be considered accurate in any way.

In this study the survival of the bacterium *P. lurida* was tested. It had been previously isolated from the rhizosphere of a regularly fertilised agricultural grassland, and was to be introduced into both intact and re-packed sieved soil microcosms, after being cultured with ^{13}C and ^{15}N to 99 atom%. This was so that survival of *P. lurida* under natural conditions could be assessed and comparison between treatments in relation to standard methods of microcosm preparation could be made. Measurements of basal respiration of the added bacterium also needed to be obtained so that the amount of enrichment left in the system over time could be assessed.

The bacterium was also introduced into sterile sand for a second comparison. Sterile sand was used rather than sterile soil due to the large impact the sterilisation process has on compounds within soil, creating entirely artificial conditions which would not be useful for comparison. In future experiments, *P. lurida* will be introduced into soil cores as a food source for the soil fauna and therefore knowledge of its survival is imperative for incubation times and assimilation within the food chain before extraction of invertebrates.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial isolation and growth

The bacterium used in the study was identified as *P. lurida*. It was isolated from the rhizosphere of a typical grassland soil that had received continuous N inputs over the last 20 years at a rate of 200 kg ha^{-1} $year^{-1}$ at Rothamsted Research, North Wyke (N 50, 46, 46.22523 W 3, 55, 0.96998). This soil is of the Hallsworth series (Harrod et al., 2008) and had undergone the same management treatments for the last 20 years.

3.2.2 Microcosm preparation

Using a metal corer, intact soil cores (2.7 cm diameter and 3 cm deep) were removed from two different fields; one had received no inorganic nitrogen fertilizer inputs (0N) for over 20 years, (described fully in Chapter 2.2.1), and one had received 200 kg N ha^{-1} $year^{-1}$ (200N) and was also the field the bacterium had originally been isolated from. Additionally, ten 10 cm diameter cores were removed for sieving. Soil was manually sieved using a 2 mm diameter sieve at the soil's current gravimetric water content. All soils were of the Hallsworth series, with the same soil properties as previously stated. These cores were taken to the laboratory and stored at +4°C for 7 days.

Intact cores and sieved soil moisture contents were kept at field conditions during storage and sieving before the gravimetric water content was determined and adjusted so that approximately 40% of the pore space was filled. The soil was not sterilised or defaunated (as in Stenberg et al., 1998; Kampichler et al., 1999; Schutz et al., 2008) prior to the experiment as the survival of the bacteria whilst in competition with the indigenous community was desired. Sand (Sigma Aldrich, St Louis, USA), was washed in a 0.1 molar hydrochloric acid solution before being rinsed in deionised water, this process was repeated three times to sterilise the sand.

Due to the differences in pore size between sieved, intact soil and sand, the aim during microcosm preparation was to have the same dry weight for all treatments. This meant they would differ in bulk density and in gravimetric water content, due to differences between the plots, but the bacterial solution added would be altered so that each sample would have 50% of its pore spaces water filled. Fifty intact soil cores for each treatment were weighed and measured and found to have approximately 16 g wet weight and a bulk density of 0.92 g cm^{-3} for the 0N soil and 15 g wet weight and a bulk density of 0.94 g cm^{-3} for the 200N soil. Fifty sieved soil microcosms were also

prepared for each treatment and had a wet weight of 16 g but a bulk density of 0.51 g cm^{-3} (0N and 200N) when they had been repacked, the 50 sand microcosms had a wet weight of 12 g and a bulk density of 1.49 g cm^{-3}. All soil was contained inside plastic microcosms, 3cm diameter and 4 cm deep, 50 microcosms were prepared for each treatment, so that there was five replicates of each treatment, destructively sampled at each time point (on days 0, 5, 12, 14, 21, 28, 35, 40, and 63).

3.2.3 Bacterial culture

The bacterium was cultured in minimal media broth enriched with stable isotopes, and prepared for inoculation as explained in Chapter 2.2.2. The enrichment was tested by taking a sub-sample of bacterial solution and, following the methods described in Chapter 2.2.6, analysed with a stable isotope analyser mass spectrometer.

Each microcosm was inoculated with 3×10^7 bacterial cells per gram of soil (dry weight) in suspension with $^1/_{20}$ strength Ringer's solution to fill the pore spaces to 50% pore-space capacity, this represented 110 μg ^{13}C g^{-1} soil per microcosm in all treatments. The bacterial suspension was introduced into the centre of each microcosm, using a Gilson pipette. A hole was made in the centre of each microcosm and the bacterial solution inoculated at this point, where the pipette was fully inserted into the hole and was slowly released as it was withdrawn from the soil, thus achieving a vertical distribution of the suspension. The weights of the microcosms after injection were recorded and were maintained throughout the experiment by the addition of sterile deionised water as needed, prior to measuring the soil $^{13}CO_2$ and destructive sampling. The microcosms were kept covered and incubated at 18°C prior to sampling.

3.2.4 Analysis of CO_2, bulk soil organic C/N and survival of bacteria

Two hours before destructive plate count sampling, each microcosm was placed inside a 500 ml Kilner jar. Each jar was sealed and incubated at room temperature for two hours, before a 12 ml sample of the headspace atmosphere was taken using a 25 ml gas syringe, according to the methods in Chapter 2.2.3. Destructive plate counts of the bacteria were made at intervals of five to seven days (see section 3.2.2 within this chapter for exact sampling days), using the method of Clegg et al. (1995), for each treatment and replicate thereof, as explained in Chapter 2.2.4.

The remaining soil from each sample was freeze-dried inside polyethylene grinding tubes (Fisher Scientific, Loughborough, UK) which were half filled with fresh soil before emersion in liquid N for approximately five seconds, to freeze the soil instantly in its current state before freeze-drying. After freeze-drying, the soil samples were ground and analysed on an elemental analyser linked to an isotope ratio mass spectrometer. The isotope abundance in the soil samples are expressed as Atom% Excess (*APE*) ^{13}C and ^{15}N calculated as equation 1 and 2 (Chapter 2.2.5).

3.2.5 Statistical analysis

Data are presented as mean ± standard error. A two sample paired Student's T-test was used to compare enrichment levels obtained. Data for bacterial survival were normalised using the transformation ($\log_{10}(x+1)$), prior to analysis. A generalised linear model was used in a regression analysis for bacterial survival data, analysis of variance (ANOVA) with day, soil type and method of preparation as the main factors was also used. A nonlinear regression analysis was performed to analyse soil $^{13}CO_2$ modelling an exponential (or asymptotic regression) with day and soil treatment as the main factors. ANOVA was used to assess the change in bulk soil *APE,* and % C and N content over time, with day and soil treatment as the main factors.

3.3 RESULTS

3.3.1 Bacterial enrichment

P. lurida was found to be enriched on average by 86.2 atom% (± 6.24 s.e.) for C and 93.0 atom% (± 7.02 atom%) for N. There was no significant difference between these results and the expected 99 atom% for C or N. The bacterium had a C and N content (37.8% C ± 1.00; 7.0% N ± 0.13; C:N ratio 5.4 ± 0.12), which was similar to the literature (e.g. Fagerbakke et al., 1996; Vrede et al., 2002).

3.3.2 Bacterial survival

The numbers of *P. lurida* declined significantly over time, the greatest decrease being in the first seven days after which it was more gradual – reaching a decline in cell numbers of > 99%. The overall decline for bacteria (all results considered together) in both N treatments, intact and sieved soil microcosm treatments and sand was significant ($P < 0.001$) (Figure 3.1). There were no significant differences between the decline in survival of bacteria within intact and sieved soil in either the 0N soil ($F_{1,71} = 3.34$; $P = 0.072$) or 200N soil ($F_{1,72} = 3.93$; $P = 0.051$). Although the interaction between the soil preparation and time of sampling in the 200N soil was significant ($F_{8,72} = 11.25$; $P < 0.001$), with a slower decline in the survival of the bacteria in intact soil microcosms over time. The difference between the survival of the bacterium in the two intact soil microcosm treatments was not significant, although the difference between the survival of the bacterium in the two sieved soil microcosm treatments was significant ($F_{1,72} = 10.60$; $P = 0.002$), with greater survival over time in the 200N soil.

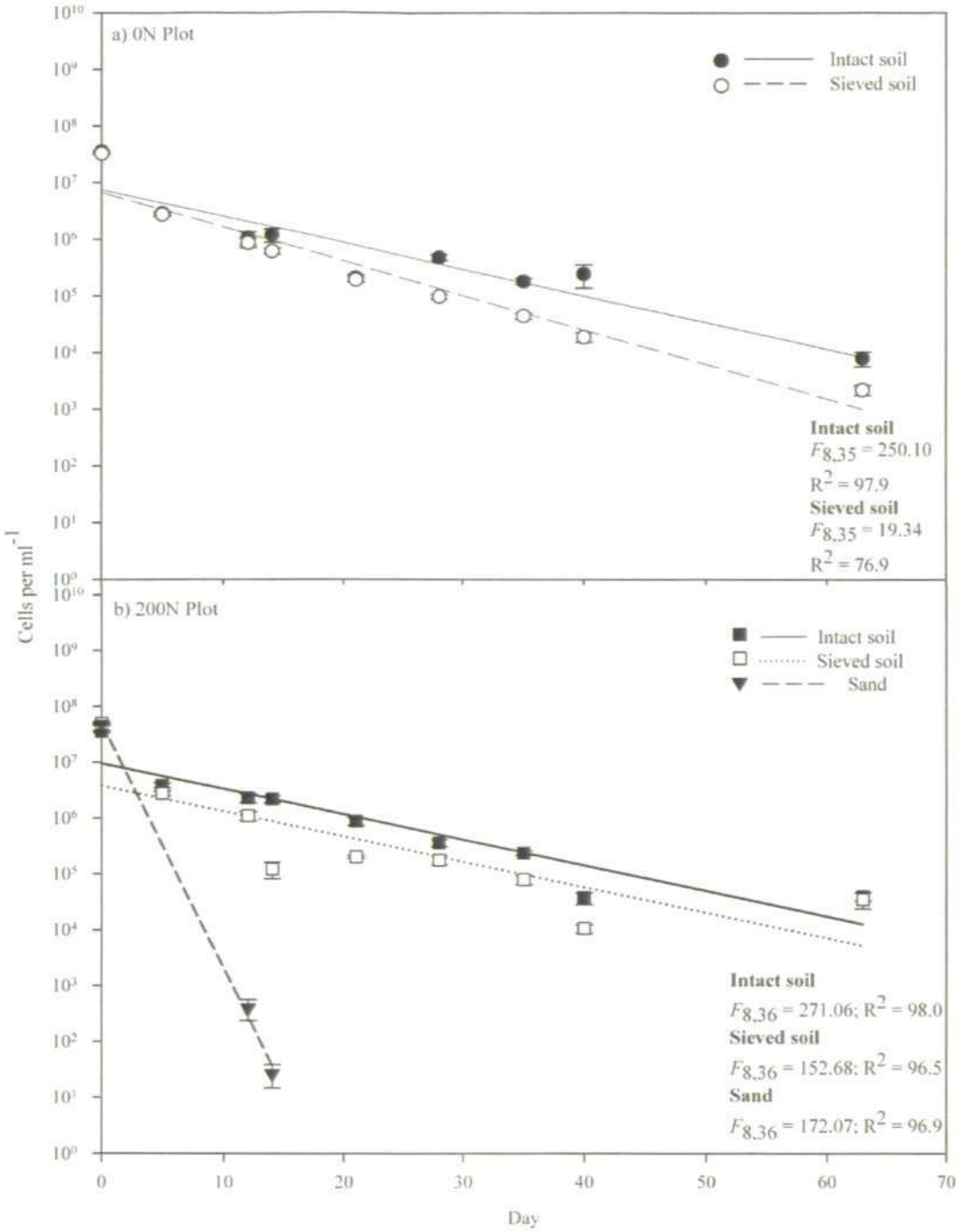


Figure 3.1: Decline in survival of bacteria in intact and sieved soil microcosms over time from two different N managed soils, with two different preparation treatments and sand. Data presented as mean ± standard error (n = 5).

The survival rate of the bacterium in sand was very low and it was significantly different to all the other treatments ($F_{1,71}$ = 209.36; $P < 0.001$). During the incubation time, there appears to be a similar rate of survival up to day 14 before there starts to be a

divergence between survival in intact soil microcosms and the repacked sieved ones, with higher survival rates from this point onwards for intact soil microcosms.

The *APE* ^{13}C signature of the headspace CO_2 is a consequence of the respiration from the added enriched bacteria and shows the level of $^{13}CO_2$ released over time. Measurements taken in the first week of the experiment were considerably higher than during the rest of the time. Overall, there was a significant exponential decline in *APE* $^{13}CO_2$ signal collected in the headspace atmosphere over time ($F_{1,197} = 169.27$; $P < 0.001$), for both intact and sieved microcosms in all soil types (0N, 200N and sand) (Figure 3.2). There was no difference in the *APE* $^{13}CO_2$ signal between the two soil treatments (0N and 200N), although there was a significant difference between the two preparation methods – intact or sieved soil ($F_{1,154} = 37.08$; $P < 0.001$) with much lower *APE* ^{13}C signals in the intact microcosms. The *APE* $^{13}CO_2$ signal from sand was significantly different to all other soil treatments ($F_{1,194} = 87.67$; $P < 0.001$) with a very low *APE* $^{13}CO_2$ signal found at all times in sand.

Comparison of the change in *APE* $^{13}CO_2$ signal with the number of surviving bacterial cells g^{-1} obtained from the plate counts, showed there was a significant relationship ($F_{1,197} = 112.30$; $P < 0.001$) for both intact and sieved microcosms in all soil types (0N, 200N and sand) (Figure 3.3). Here, increased survival resulted in increased *APE* $^{13}CO_2$ signal. The multivariate analysis of variance showed the interaction between *APE* $^{13}CO_2$ signal, survival of bacterial cells and the two soil treatments was not significant ($P = 0.779$). However, there was a significant difference between the two preparation methods – intact or sieved microcosms ($F_{1,154} = 60.25$; $P < 0.001$), with increased survival but reduced *APE* $^{13}CO_2$ signal in the intact microcosms.

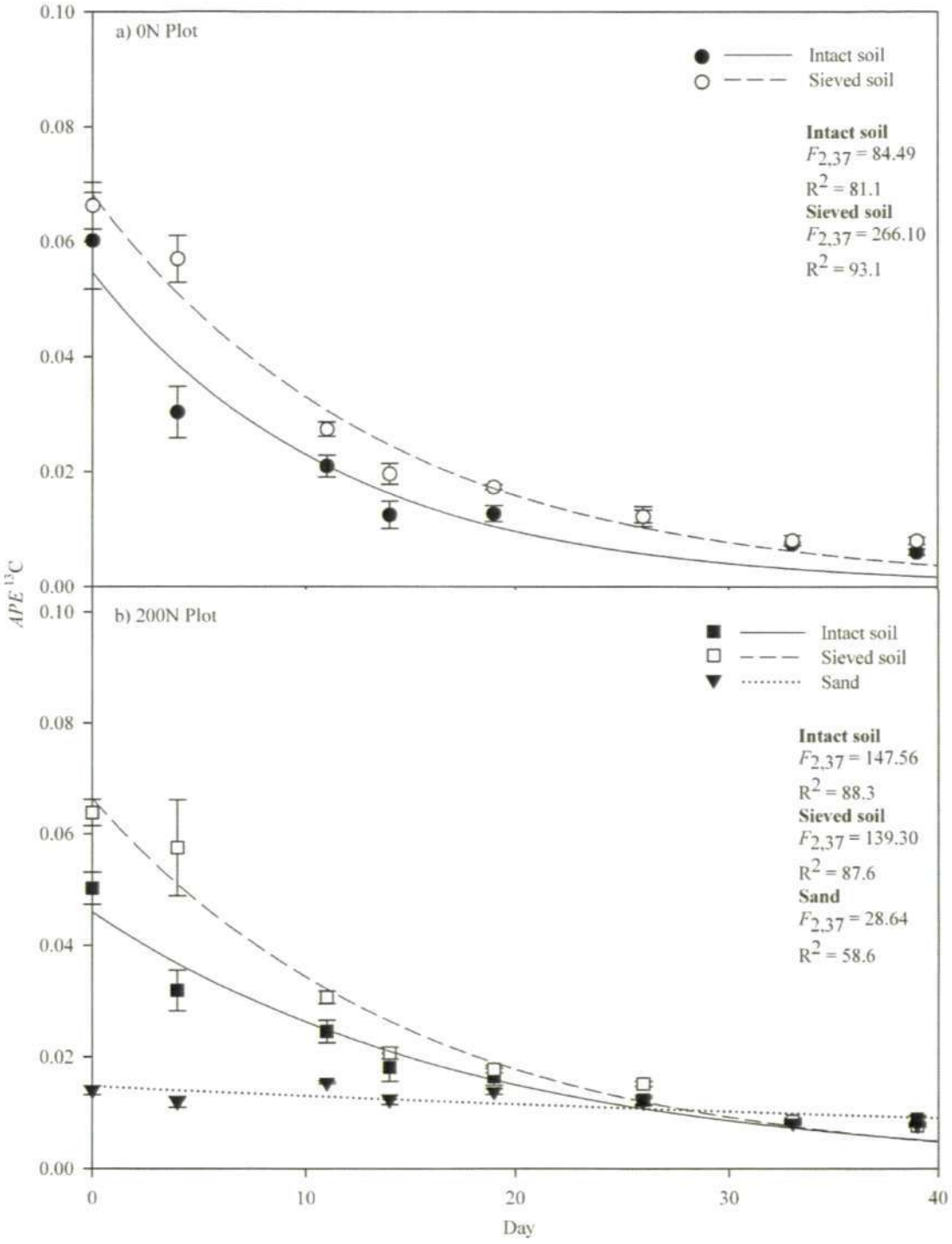


Figure 3.2: The decline in *APE* ^{13}C signal in the headspace atmosphere from the respiration of bacteria from two different N managed soils with two different preparation treatments and sand. Data presented as mean ± standard error (n= 5).

When the different soil types were combined, the multivariate analysis of variance showed the interaction between *APE* $^{13}CO_2$ signal, survival of bacterial cells and the two preparation methods was significant ($P < 0.001$). The multivariate analysis of variance showed the interaction between *APE* $^{13}CO_2$ signal, survival of bacterial cells and the soil compared to sand was also significant ($P < 0.001$).

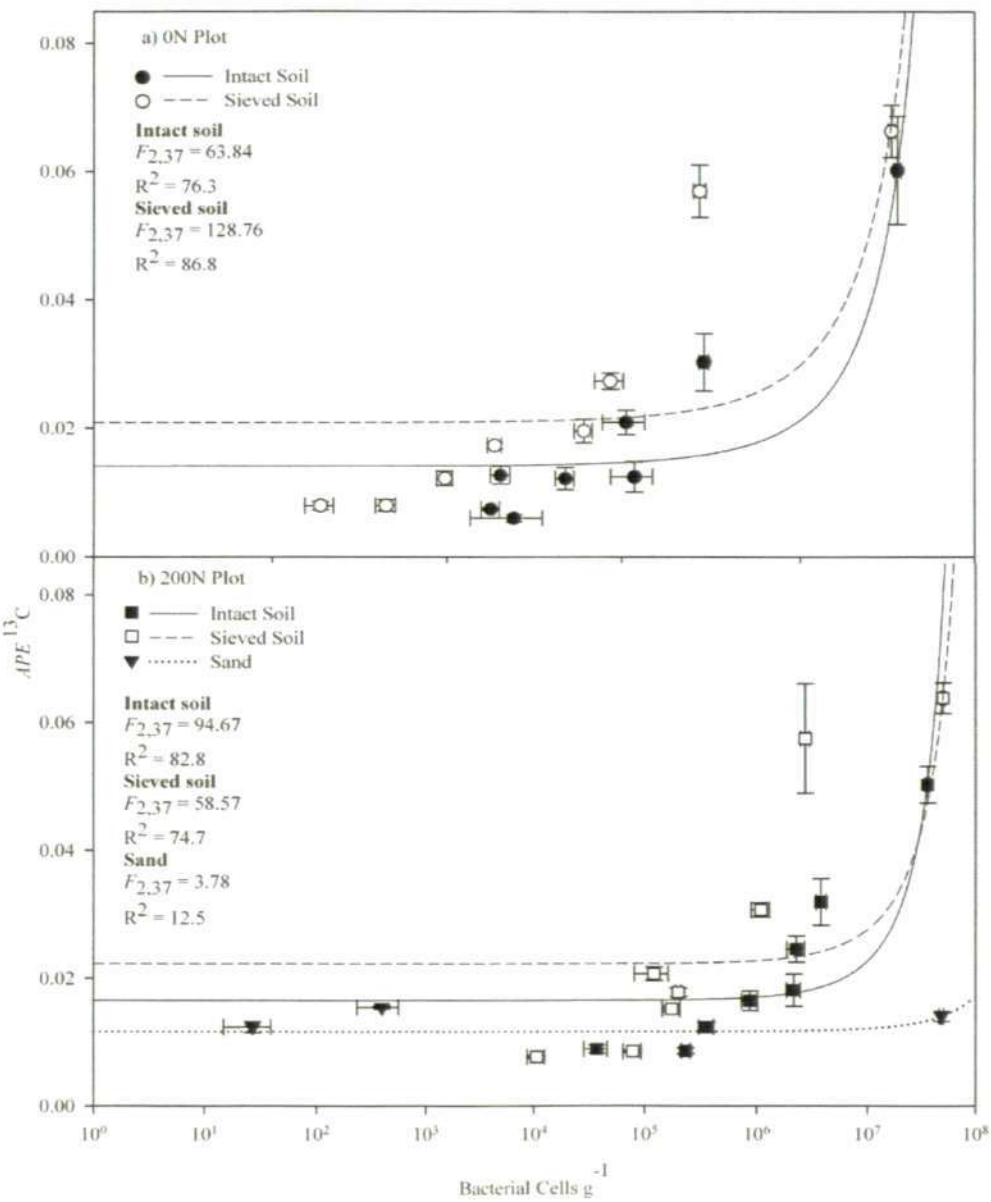


Figure 3.3: The relationship between *APE* $^{13}CO_2$ and bacterial cell survival from two different N managed soils with two different preparation treatments and sand. Data presented as mean ± standard error (n = 5).

Assessing the remaining bacterial signal through *APE* of the bulk soil within the microcosms, there were significant declines in *APE* ^{13}C for the bulk soil within the microcosms over time ($F_{1,158} = 10.62$; $P = 0.001$) (excluding sand from the analysis) (Figure 3.4a). Looking at the treatments separately, the differences between N treatments was not significant, however the difference between microcosm preparation methods was significant ($F_{1,156} = 5.53$; $P = 0.020$), with the sieved microcosms retaining a greater *APE* over time.

The difference in *APE* ^{15}N between all microcosms remained relatively static throughout the experimental period, and was not significant over time (Figure 3.4b). When analysing separately, the change in *APE* ^{15}N over time was not significant between N treatments, but it was significantly different between the two microcosm preparation methods ($F_{1,156} = 23.28$; $P < 0.001$), again with the sieved microcosms displaying greater *APE* levels over time. The *APE* level of the bulk soil within the microcosms was very low, and was lower for ^{13}C than ^{15}N, this was due to the loss through respiration of ^{13}C through $^{13}CO_2$ (Figure 3.2). The *APE* level was variable between microcosms in all treatments, although appeared to be more variable in the 0N treatment. In both N treatments the intact soil microcosms retained a lower *APE* signal for both ^{13}C and ^{15}N.

Due to the negligible level of C and N within the sand microcosms prior to introduction of the enriched bacteria, the *APE* results are not reliable, due to the mass spectrometry thresholds for minimum C and N biomass required in samples. Evaluating the *APE* level in "Bulk" sand in comparison to bulk soil overall (all treatments combined), there were significant differences to the soil for both ^{13}C and ^{15}N ($F_{1,196} = 190.66$; $P < 0.001$ and $F_{1,196} = 235.48$; $P < 0.001$ respectively) although time was not significant for either (Figure 3.4c).

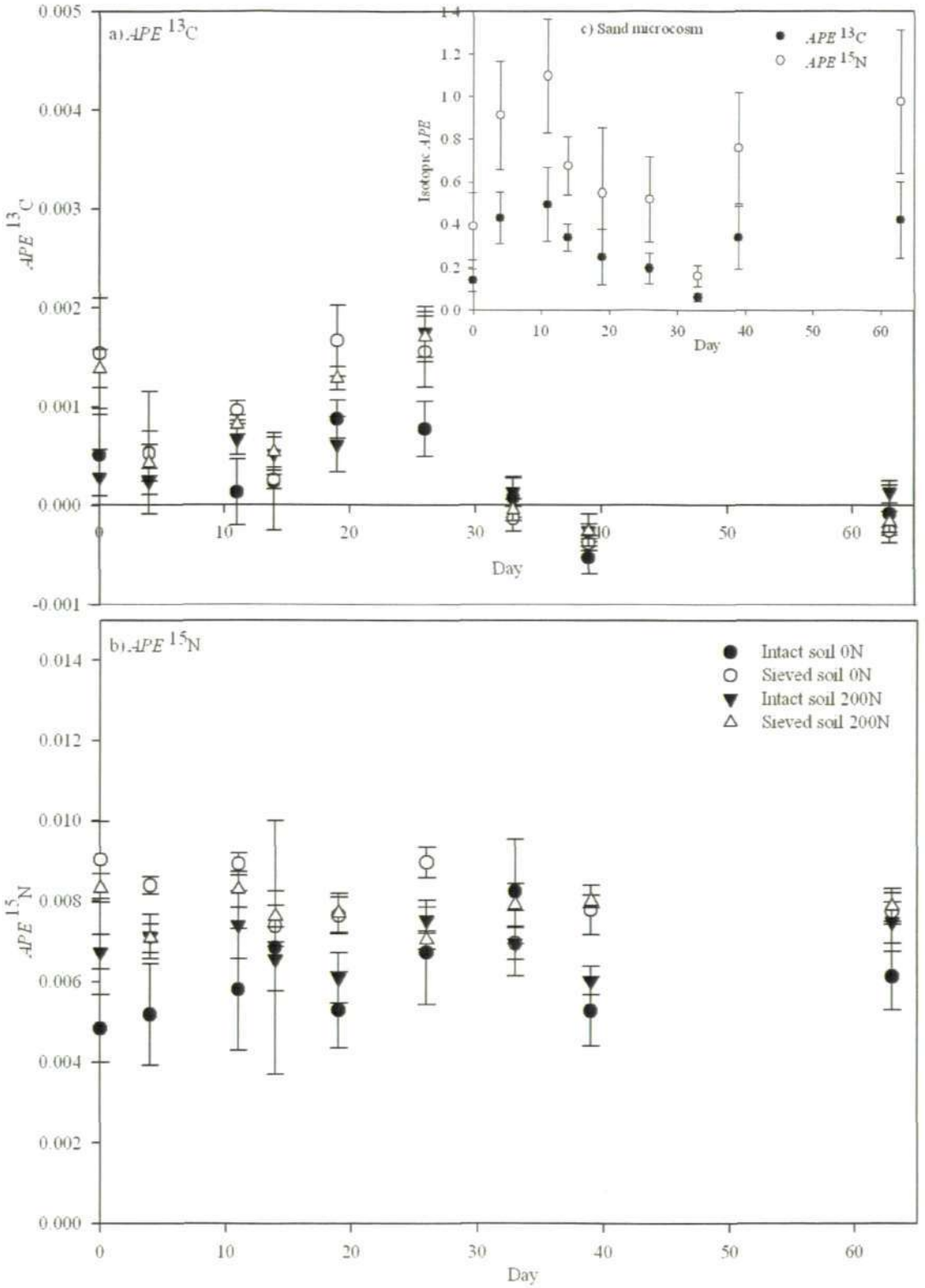


Figure 3.4: The relationship between bulk soil *APE* within the microcosms over time, a) *APE* ^{13}C, b) *APE* ^{15}N, and insert c) sand microcosm.

Data presented as mean ± standard error (n = 5).

Using the amount of ^{13}C and ^{15}N of bacterial origin remaining in the soil (in μg) is a more robust method of analysis, because it uses the percentage of C and N within the soil treatments. Overall (all treatments and preparation methods combined), there was a significant decrease in the amount of μg ^{13}C over time ($F_{1,156} = 10.76$; $P = 0.001$), although this difference was not significant between N treatments, or preparation methods, when considered separately. However, the pattern was different for the change in μg of ^{15}N over time, overall the amount of μg of ^{15}N across all treatments remained relatively static. Nevertheless, there were significant differences when N treatments were considered separately ($F_{1,156} = 5.21$; $P = 0.024$), with greater amounts of μg ^{15}N in the 200N treatment in comparison to the 0N soil treatment, although there were no significant differences between preparation methods.

Multivariate analysis of variance for the amount of ^{13}C and ^{15}N retained in the soil was significantly different between plots (N treatments) ($P = 0.025$ d.d.f$_{155}$), but was not significant for preparation method ($P = 0.696$ d.d.f$_{155}$). Combining the amount of ^{13}C and ^{15}N retained in the soil with the *APE* $^{13}CO_2$ headspace signature and the survival of bacterial cells within the soil for multivariate analysis of variance showed that overall there were no significant differences between the N treatments ($P = 0.078$ d.d.f$_{153}$) but there was a significant difference for preparation method ($P < 0.001$ d.d.f$_{153}$). These differences can be seen using Principal Component Analysis (Figure 3.5 and Table 3.1). The least variation was found for the *APE* $^{13}CO_2$ soil headspace signatures between the different soil treatments (Figure 3.5c) whilst the most variation was found for the differing amounts of ^{15}N of bacterial origin (Figure 3.5b). The sand treatment was always very different from the other treatments. There was also more variability within the intact soil microcosms (0N compared to 200N) than there was between the two sieved microcosms.

	PCA-axis 1	PCA-axis 2
0N Intact soil	87.36	9.84
0N Sieved soil	95.97	2.59
200N Intact soil	92.00	5.11
200N Sieved soil	92.84	4.46
Sand	93.36	6.40

Table 3.1: Principal Component Analysis – variation accounted for within each treatment.

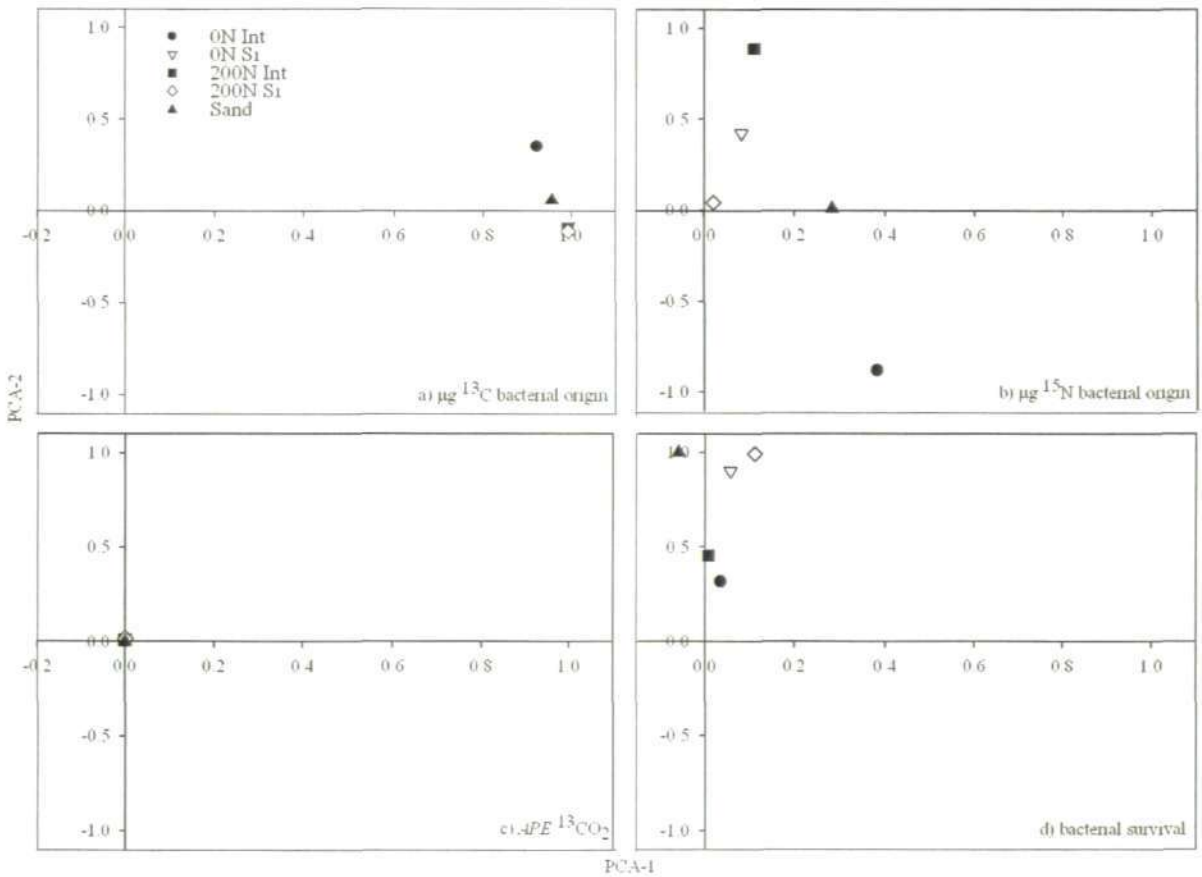


Figure 3.5: Principal Component Analysis showing the variation between treatments for the variables a) amount of µg ^{13}C of bacterial origin retained within microcosms; b) amount of µg ^{15}N of bacterial origin retained within microcosms; c) *APE* $^{13}CO_2$ respired by microcosms; d) bacterial survival within microcosms. Variation accounted for within each treatment displayed in Table 3.1.

3.4 DISCUSSION

The majority of bacterial cells added to all microcosm treatments did not survive the first seven days. This decrease in survival was faster in sieved soil compared to the intact soil microcosms for both plot treatments. The differences in survival in this study may be attributed to the greater porosity of the sieved soil, providing a less favourable habitat for the bacteria to survive in, with greater temperature, humidity and water content fluctuations. The methods used in this study did not dry the soil before sieving so the results could provide insight into how the introduction of enriched bacteria would survive during soil food web studies as in other microbial-invertebrate microcosm experiments (Sheehan et al., 2008; Tordoff et al., 2008), except no invertebrates were used at this stage of the study.

There was a significant decrease in the *APE* ^{13}C headspace atmosphere over time, for all treatments. The *APE* ^{13}C gas signatures are inextricably linked to enriched bacterial survival and there was a significant relationship for all soil treatments. Although there were differences between soil treatments, and these differences in survival continue throughout the time-span of the experiment. However, after 20 days there was little difference between the *APE* ^{13}C signature of the headspace CO_2 results, though the differences in survival remained.

After 30+ days there was less than 1% of the introduced bacteria living in each microcosm treatment, but the *APE* ^{13}C measured from the soil respiration was still 10% of the maximum ^{13}C recorded, suggesting the microbial community in general were utilising the mineralized introduced bacteria and thereby releasing an enriched $^{13}CO_2$ signal. This is possibly why the differences between microcosms reduced as, over time, the indigenous microflora started utilising the added ^{13}C and/or ^{15}N via the enriched bacteria, rather than the added bacteria themselves.

The change in isotopes of the bulk soil within the different microcosms shows how ^{15}N is mainly retained within the system over time, whereas ^{13}C is lost from the system. These results are similar to those found for easily degraded substances such as glucose C. For example, in a study by Baldock et al. (1989) 65% of the added substrate was mineralised after 34 days of incubation. These differences for ^{13}C and ^{15}N were not different between soil treatments (0N and 200N) but were significantly different between preparation methods for both isotopes.

Using the amount of ^{13}C and ^{15}N of bacterial origin (as opposed to just *APE* alone) shows that stable isotope ecology is more nuanced than at first glance. The significant change in the amount (μg) of ^{13}C remaining within the bulk soil over time, was not affected by soil treatments (0N and 200N) or preparation method. Conversely, the amount of ^{15}N retained within the bulk soil was affected by soil treatments (0N and 200N). In all probability, this difference in ^{15}N retention may be because within the 0N treatment, N is a more limiting factor (than within the 200N soil, which had received 200 kg ha^{-1} N per year), and was therefore utilised more by the indigenous microbial community.

Sieving is supposed to act as a "disturbance" within the soil system, releasing a C and nutrient pulse (Bradford et al., 2002), which should promote bacterial growth and survival, but this was not the case within this experiment. Historically the process of sieving soil was to homogenise soil treatments and reduce variability, these results are in agreement with this. Sieved microcosms appeared less variable between N treatments, but were very different compared to the intact soil microcosms within the same N treatment (Figure 3.5). The sieving process also removed (destroyed) many invertebrate taxa e.g. nematodes (Wardle et al., 2005) (one of the main consumers of bacteria), disrupting food chains, which should also be considered in studies that assess

bacterial survival and extrapolate to field conditions (e.g. Clayton et al., 2005; Kindler et al., 2006).

3.5 CONCLUSIONS

These results show that bacterial survival declines rapidly between 10-14 days after addition. The bacteria survived in a similar pattern in all soil microcosms, but not within the sand microcosm. Preparation method for the microcosms had more of an effect on survival, *APE* ^{13}C soil respiration, and amount of isotopes remaining within the soil, than plot (N treatment). Although, variation was less between sieved soil microcosms, survival was lower in sieved soil microcosms when compared to intact ones.

Consequently, experiments using sieved soil, because of its uniformity and ease in handling when creating microcosms to test different soil parameters on survival, have the disadvantage of not relating directly to undisturbed soil and should not be used to extrapolate to the natural environment. For further studies, this experiment has shown that relatively high numbers of bacteria remain detectable within soil microcosms up to 14 days after introduction. Hence, in future food chain experiments, tracing bacterial incorporation in the soil fauna, the incubation period needs to be less than 14 days to standardise treatments.

Chapter 4: Abundance and trophic structure of the soil food web under differing land managements

4.1 INTRODUCTION

One of the most noticeable features of a soil food web is that there is a large diversity of organisms which are densely packed in space, leading to a huge number of animals of a variety of species and trophic groups occurring in a small piece of soil (Scheu et al., 2000). However, little is known about the actual soil volume all the different species occupy, potentially it may be restricted to the rhizosphere (Petersen, 2002). The factors responsible for this high diversity of soil animals on small spatial scales are still not fully understood (Maraun et al., 2011). Especially the high α-diversity, which implies the existence of a large number of niches in a very small area (Maraun et al., 2007), possibly because below-ground animal taxa are generalists that inhabit wide niches.

The development of stable isotope ratios as a tool for the assessment of the feeding ecology of invertebrates has come far over the past two decades, with studies ranging from major taxonomic groupings (Neilson et al., 1998), to individual species within one order (Schneider et al., 2004). Studies of the whole soil food web have shown the food chains to be relatively short, with decomposers being clearly separated from predators (Ponsard et al., 2000), but contrary to this, individual species analysis has shown a continuum of stable isotope signatures (Chahartaghi et al., 2005). Most of the studies to date have investigated just one habitat – often forested, or habitats of differing humus or soil type, few have compared differences between habitats of the same soil type, using the same taxonomic parameters for separation.

To start to elucidate the different feeding channels within the soil food web, the current stable isotope signatures of the soil fauna within the food web need to be revealed to act as a reference point for future enrichment studies. To assess whether the soil fauna have similar stable isotope signatures independent of habitat, stable isotope analysis was performed on two habitats, a grassland and a woodland which were both of the same soil type and where 20 years ago the woodland was originally also grassland (see Chapter 2.2.1 for full description), to act as a control for future experiments. When investigating stable isotopes at natural abundance, results are described in delta (δ) or per mil (‰) whilst when dealing with enrichment of stable isotopes, atom% is used. In this study, the results were obtained in both formats and will be discussed in the natural abundance format within this chapter, but will be used as atom% as the control for enrichment experiments.

Overall the aims of this study were firstly to assess the optimum time for extraction of invertebrates on the Tullgren Funnel System; secondly to assess the abundance, biomass and diversity within the two habitats and identify differences between them. Finally, to begin to define the trophic structure of the soil food web using stable isotopes at natural abundance, for both habitats. This can evaluate whether the same invertebrate has different functions within the different habitats.

4.2 MATERIALS AND METHODS

4.2.1 Soil preparation and sampling

Intact soil cores (10 cm Ø, 10 cm deep) were taken from the grassland and woodland sites (Rothamsted Research (North Wyke)) in October 2009, see Chapter 2.2.1 for a full site description. Dead plant material was removed from the two field sites for future stable isotope analysis, and prepared as live plant samples (see below).

The cores were removed by driving individual polypropylene sleeves (11.4 cm diameter, 11 cm deep) into the soil, to retain the entire faunal assemblage within the core and to leave the flora remaining intact on top of the core. After removal of the cores, they were weighed and the water content was measured using a moisture meter (ThetaProbe Soil Moisture Sensor - ML2x from Delta-T Devices Ltd). Each core was stored within an individual Sun bag, in a controlled environment chamber (Chapter 2.2.1), to stabilise for seven days. Water was not added to the soil (as had occurred in previous experiments - see Murray et al., 2009) due to the starting level of wetness, greater than 40% mineral soil moisture (volumetric water content). Throughout the acclimation period the percentage mineral soil moisture content was reduced from 44% to 38%, and the mean weight of the cores was 1031 ± 15 g fresh weight.

Prior to invertebrate sampling of each core, the vegetation (grass/under-canopy forbs) was cut to ground level and dried and ground before analysis by mass spectrometry. The core was removed from the plastic sleeve and a vertical slice (approximately 150 g) of the core was removed and homogenized. Of this homogenized sample, 100 g was used for nematode extractions, and 50 g for dry weight and isotope analysis. For full descriptions of stable isotope analysis and nematode extraction methods see Chapter 2.2.5 and 2.2.7 respectively.

4.2.2 Meso- and Macrofauna Sampling

The remainder of the core was placed on a Tullgren funnel system (mesh 5 mm), where invertebrates migrate through each core via a temperature gradient over time and are collected. A preliminary experiment was performed to assess the optimum time for extraction over a five day period, as based on previous experiments (e.g. Murray et al., 2009) this was all that was needed. Invertebrates were collected daily in saturated salt solution. Invertebrates were identified and separated into three main categories at this

preliminary stage; Collembola, Acari or "Others" (which included all other invertebrates extracted).

The results from the first preliminary experiment were inconclusive for providing an optimum time for Tullgren funnel extraction therefore, another set of cores collected from both field sites using the methods described above, were placed on the Tullgren funnels for a total of 21 days. Collection vessels were changed daily (days 1-9) and every ~four days thereafter, to assess the abundance and assemblage collected over time. Invertebrate groups were identified and separated as described in full in Chapter 2.2.8, under a microscope prior to drying and mass spectrometry.

4.2.3 Stable isotope analysis

For stable isotope analysis appropriate amounts of invertebrate material, soils and foliage were dried and weighed in tin capsules before analysis, as explained in Chapter 2.2.5. The invertebrate samples were analysed for total N and total C contents and the ^{15}N:^{14}N and ^{13}C:^{12}C isotope ratios, along with analytical quality control samples. For stable isotopes at natural abundance, they are expressed using the δ notation with $\delta^{13}C$ (‰) and $\delta^{15}N$ (‰) calculated as equation 8.

$$\delta^{n}E\ (‰) = (R_{sample} - R_{standard}) / R_{standard} \times 1000 \qquad (8)$$

Where E is the element (C or N), n is the weight of the heavier (rarer) isotope and R is the ratio of the heavy to light isotopes (Tiunov, 2007). R_{sample} and $R_{standard}$ represent the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratios of the sample and standard, respectively, for ^{15}N atmospheric N_2 served as the primary standard and for ^{13}C it was Vienna Pee Dee Belminite (VPDB). The standards for C and N $R_{standard}$ are equal to 1.1237×10^{-2} and 3.6764×10^{-3} respectively.

Other studies comparing soil fauna from different habitats have normalised the results so that isotope signatures can be directly comparable. Methods that have been used in some of these studies include, either setting the stable isotope signatures of the main "habitat" (the soil) to zero and calibrating all other results accordingly (Erdmann et al., 2007); or to correct the delta signatures of animals from different habitats by the difference between the delta values of the soil (or litter) of those habitats to that of one them (Schneider et al., 2004; Chahartaghi et al., 2005; Illig et al., 2005). Both methods account for the differences between habitats, the former method described by Erdmann et al., (2007) is clearer for graphical representation and will therefore be used if necessary.

4.2.4 Statistical analysis

All population data was normalised by transformation ($\log_{10}(x + 1)$) prior to analysis. All data was analysed by a general regression analysis when assessing the differences in number of organisms extracted over time. Student's T-test was used to assess the overall differences for the invertebrates extracted between the two habitats. The Student's T-test (two-tailed) was also used to show differences between the C:N ratio of invertebrates and standard values from the literature. An analysis of variance (ANOVA), with habitat as the main factor, was applied to determine differences in organism numbers, and delta values within the different habitats. Results are expressed as mean ± standard error.

4.3 RESULTS

4.3.1 Tullgren funnel extraction efficiency – part 1

In the first preliminary experiment testing Tullgren funnel efficiency a total of 2,790 invertebrates were extracted from ten cores, of these 1,789 were from the grassland habitat and 1,001 from the woodland. These are relatively low numbers, equating to 45,500 (± 7,800) invertebrates per m^2 in the grassland compared to 25,500 (± 4,400) invertebrates per m^2 in the woodland and could be due to the time of extraction (five days only), or the soil conditions at the time of sampling. There were significant differences in the distribution of invertebrates extracted over time ($F_{29,120}$ = 5.03; $P < 0.001$) although this only accounts for 43.9% of the variation. It was decided from this preliminary experiment that five days extraction was not long enough to assess whether all the invertebrates had been extracted (Figure 4.1). There was a decline in numbers from days 3-4, however, the numbers extracted appeared to start increasing again around days 4-5, depending on habitat.

Analysis of variance of the results showed that time was not significant over the extraction period and neither was the relationship between habitat and time or the relationship between invertebrates extracted (Acari, Collembola, or other) and time. The difference between the numbers extracted in the two different habitats was also not significant. There were significant differences in the number of invertebrates extracted in the different groups (Acari, Collembola, or other) ($F_{2,120}$ = 45.62; $P < 0.001$); 61% of the invertebrates extracted were Acari, 31% were Collembola and 8% were other invertebrates. These differences in the number of invertebrates extracted was also significant when the interaction with habitat was also accounted for ($F_{2,120}$ = 11.50; $P <$ 0.001).

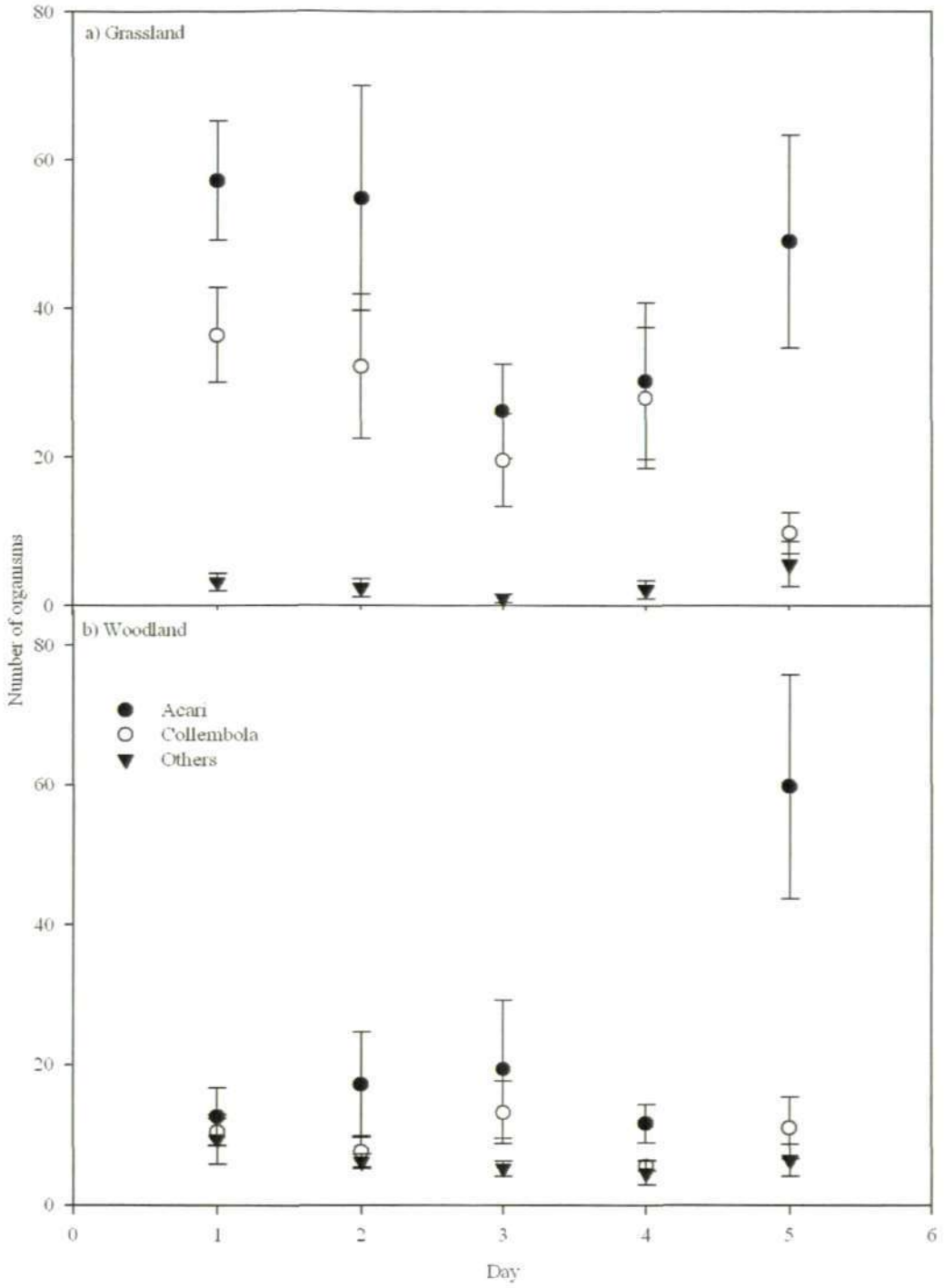


Figure 4.1: Number of invertebrates collected over a five day period from soil from two different habitats a) grassland, and b) woodland.

Data represent mean ± standard error, (n = 5).

Assessing the differences between habitats for the number of invertebrates extracted, showed there were no significant differences between habitats for the number of Acari extracted (T-test statistic = 1.71), although there was a significant difference between the number of Collembola extracted ($P = 0.004$) with a much greater number extracted in the grassland. There was also a significant difference between the number of "other" invertebrates extracted ($P = 0.022$), although here the number was greatest in the woodland. Overall, the interaction between habitat and invertebrate extracted over time was not significant.

4.3.2 Tullgren funnel extraction efficiency – part 2

In the second assessment of Tullgren funnel efficiency, a total of 5,564 invertebrates were extracted from twelve cores, of these 2,458 were from the grassland habitat and 3,106 from the woodland, this equates to 53,500 (± 8,300) invertebrates per m^2 in the grassland compared to 71,400 (± 10,300) invertebrates per m^2 in the woodland. There were significant differences in the distribution of invertebrates extracted over time ($F_{71,360} = 16.75$ $P < 0.001$) and this accounted for 72.2% of the variation. Over the 21 day period there were large differences in the number of invertebrates collected daily from the Tullgren funnel system over the time scale of the experiment (Figure 4.2).

In the grassland (Figure 4.2a) there was a decline in the number of Collembola and mites collected over the first five days. Subsequently, there was an increase up to 14 days, after which Collembola numbers declined, although mite numbers remained high; (all the rest of the invertebrates collected remained relatively static throughout). In the woodland habitat (Figure 4.2b) numbers of invertebrates extracted daily remained constant for the first five days before increasing for all groups (Collembola, mites and

others). These numbers continued to rise until day 14, after which there was a rapid decline in Collembola and other invertebrate numbers. Although the mite numbers extracted remained high, they were also decreasing.

There were significant differences in the number of invertebrates collected over the time of the collection period ($F_{11,360} = 43.62$; $P < 0.001$) and the relationship between habitat and time ($F_{11,360} = 7.02$; $P < 0.001$). Also, the relationship between invertebrates collected (Acari, Collembola, or other) and time ($F_{22,360} = 9.04$; $P < 0.001$) were significant. The difference between the numbers extracted in the two different habitats was also significant ($F_{1,360} = 23.58$; $P < 0.001$), with greater numbers collected in the woodland. There were significant differences in the number of invertebrates extracted in the different groups (Acari, Collembola, or other) ($F_{2,360} = 178.87$ $P < 0.001$).

The overall proportions of invertebrates extracted were 58% Acari, 37% Collembola and 5% other invertebrates. However, these differences in the number of invertebrates were not significant when the relationship with habitat was also accounted for. Assessing the differences between the number of invertebrates collected and habitat, showed there was no significant difference between habitats for the total number of Acari, Collembola or "others" extracted (T-test statistic = -0.06, 0.25, and -0.66 respectively). Overall, the interaction between habitat and invertebrate extracted over time was significant ($F_{22,360} = 2.35$; $P < 0.001$) with different numbers of invertebrates collected over time in each habitat.

For the individual orders of Collembola, the pattern of collection changes, depending on order (Figure 4.3). Of the Collembola orders only the Entomobryomorpha numbers were significantly different over time in the grassland and woodland ($F_{2,69} = 11.45$; $P < 0.001$ $R^2 = 22.7$ and $F_{2,69} = 21.95$; $P < 0.001$ $R^2 = 37.1$) (Figure 4.3a). With numbers collected in both habitats peaking around day nine, before decreasing.

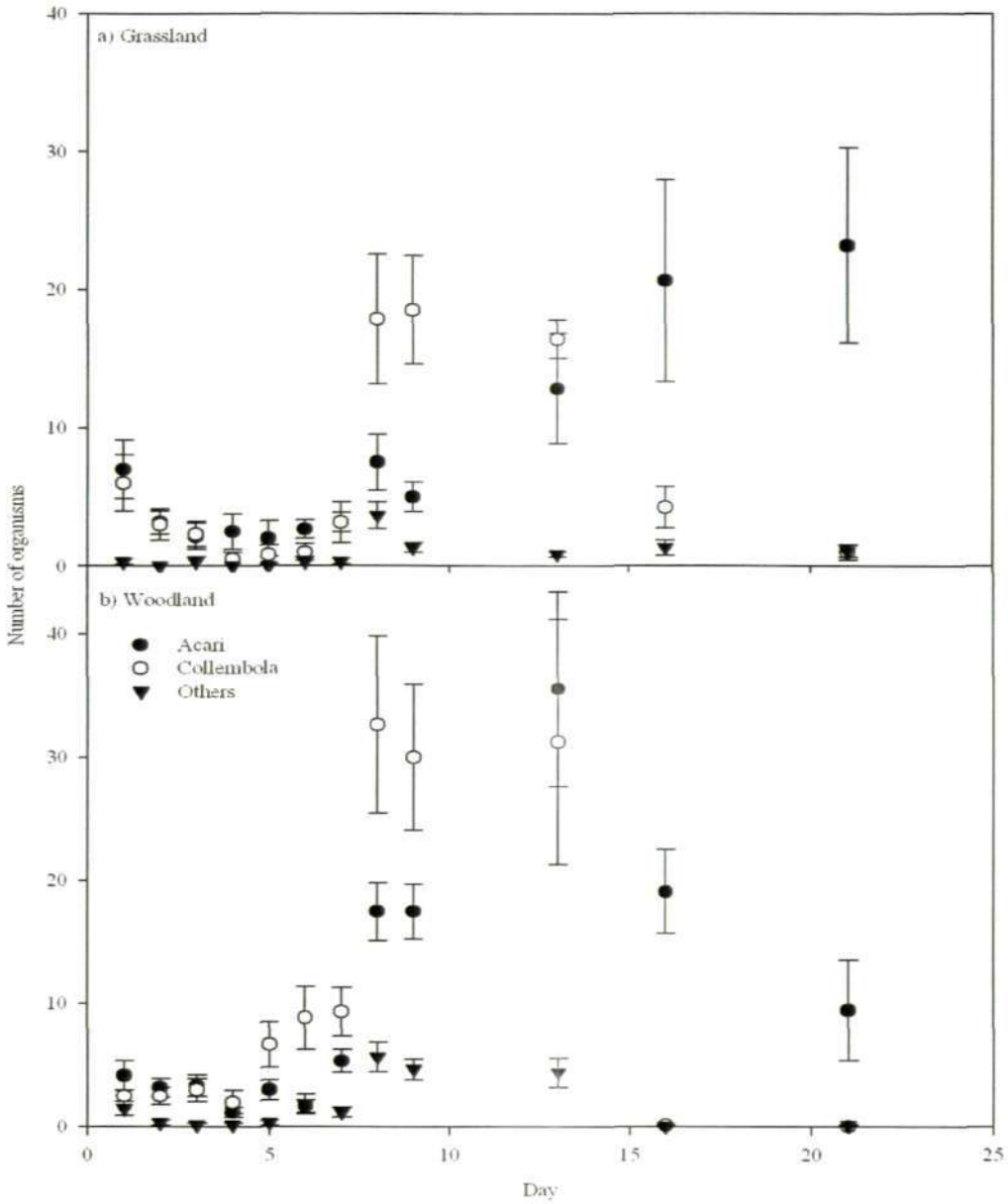


Figure 4.2: Number of invertebrates collected on average per sample per day, over 21 day period from soil from two different habitats a) grassland, and b) woodland. Data represent mean ± standard error, (n = 6), days where a composite number of days were collected have been divided by the number of days that collection was for (e.g. day 13 invertebrates were collected for four days (day 10-13) abundance has therefore been divided by four to show the number collected per day).

The other orders of Collembola were extracted in similar numbers daily and were not significantly different over time in the grassland. However, in the woodland all the orders of Collembola were collected in significantly variable amounts over time (Poduromorpha: $F_{2,69} = 12.08$; $P < 0.001$ $R^2 = 23.8$; Neelipleona: $F_{2,69} = 17.62$; $P < 0.001$ $R^2 = 31.9$; and Symphypleona: $F_{2,69} = 3.97$; $P = 0.023$ $R^2 = 7.7$) with increased numbers extracted between day nine and thirteen compared to the rest of the time (Figure 4.3b-d).

The Acari lineages had similar distributions between habitats, and all were collected in significantly variable numbers over time (Figure 4.4). With relatively high numbers extracted in the first few days of the experiment before a decline, then numbers increased between day nine and sixteen, before another rapid decline. The Mesostigmata numbers were significantly different over time in the grassland and woodland ($F_{2,69} = 28.07$; $P < 0.001$ $R^2 = 43.3$ and $F_{2,69} = 6.73$; $P = 0.002$ $R^2 = 13.9$) with increased numbers from day thirteen to day sixteen, although there were large differences in the variation accounted for (Figure 4.4a).

The Prostigmata numbers were significantly different over time in both habitats ($F_{2,69} = 24.99$; $P < 0.001$ $R^2 = 40.3$ and $F_{2,69} = 16.56$; $P < 0.001$ $R^2 = 30.5$) (Figure 4.4b). Prostigmata numbers decreased up to day five, before large increases in the number collected in the woodland up to day thirteen before sharp declines, whilst in the grassland there was a gradual increase from day thirteen onwards.

Oribatida numbers collected over time in both habitats were also significantly different ($F_{2,69} = 11.82$; $P < 0.001$ $R^2 = 23.4$ and $F_{2,69} = 35.42$; $P < 0.001$ $R^2 = 49.2$) (Figure 4.4c) with gradual increases from day eight onwards in both habitats before a decline appears to start on day twenty-one. The number of Astigmata collected over time was also significant for both habitats ($F_{2,69} = 7.47$; $P = 0.001$ $R^2 = 15.4$ and $F_{2,69} =$

3.38; $P = 0.040$ $R^2 = 6.3$), however only a small percentage of the variation was accounted for (Figure 4.4d) and numbers remained relatively low throughout.

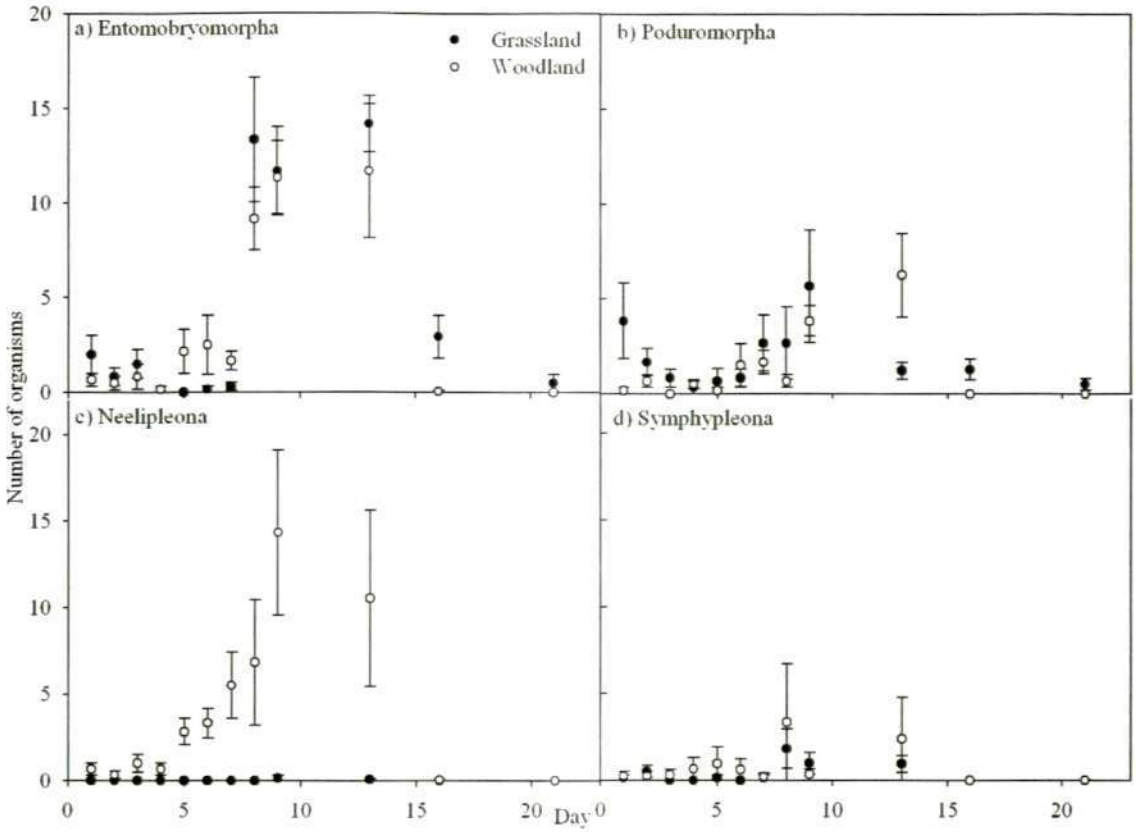


Figure 4.3: Number of Collembola collected on average per sample per day, over a 21 day period from soil from two different habitats, grassland (filled black circles), and woodland (unfilled circles), a-d represent the different collembolan orders, a) Entomobryomorpha, b) Poduromorpha, c) Neelipleona and d) Symphypleona. Mean ± s.e., (n = 6).

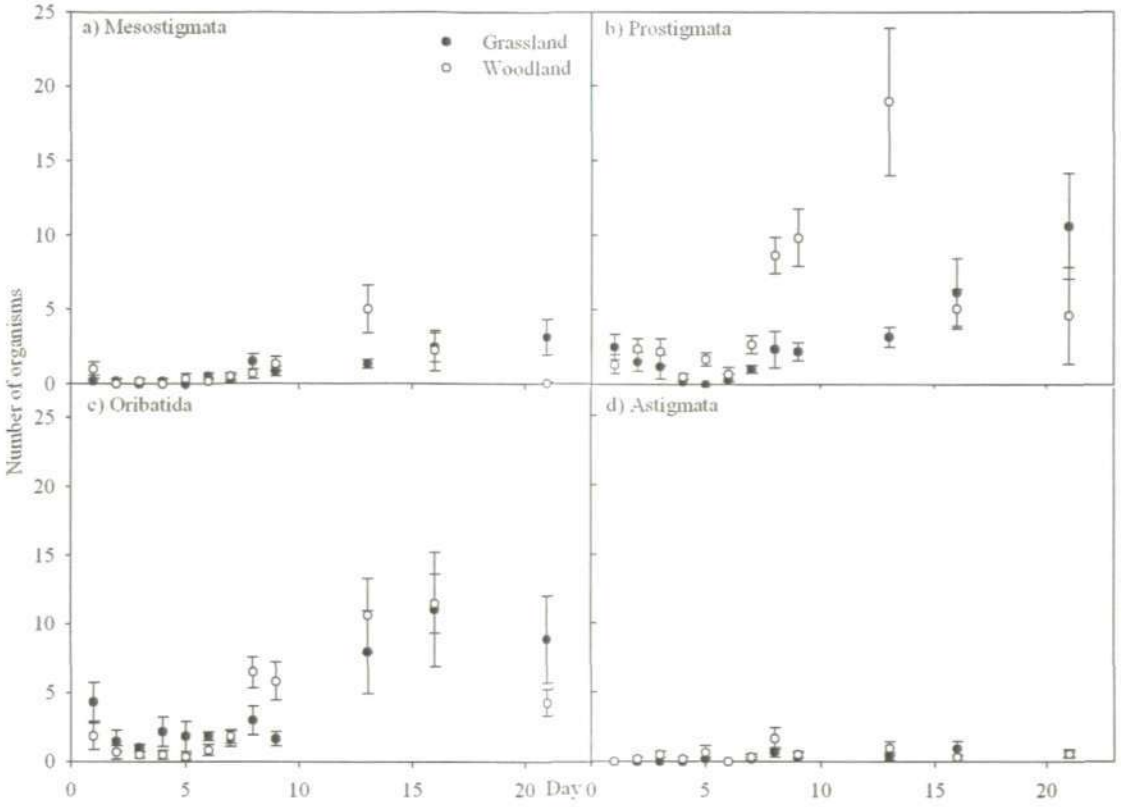


Figure 4.4: Number of Acari collected on average per sample per day, over a 21 day period from soil from two different habitats, grassland (filled black circles), and woodland (unfilled circles), a-d represent the different Acari lineages, a) Mesostigmata, b) Prostigmata, c) Oribatida and d) Astigmata. Data represent mean ± standard error, (n = 6).

4.3.3 Soil and vegetation characteristics

The soil within each habitat was assessed during both the 1981 and 2008 soil surveys (Harrod et al., 2008), and was considered to be of the Hallsworth series, on both occasions. There were significant differences between the C and N content of the soil in the grassland and woodland habitat (%C: $F_{1,10} = 36.81$; $P < 0.001$ and %N: $F_{1,10} = 82.21$; $P < 0.001$), with the grassland having a higher C and N content (6.6% ± 0.21 C and 0.7% ± 0.02 N, C:N ratio of 10.1 ± 0.15), when compared to the woodland (4.9% ±

0.21 C and 0.5% ± 0.01 N, C:N ratio of 10.3 ± 0.21). However, C:N ratios were not significantly different between habitats and neither were the differences in bulk densities. The $\delta^{13}C$ and $\delta^{15}N$ signatures though, were different between habitats ($F_{1,10}$ = 86.10; $P < 0.001$ and $F_{1,10} = 43.09$; $P < 0.001$ respectively), with the grassland having a lower $\delta^{13}C$ and $\delta^{15}N$ signature (-29.5 ‰ ± 0.09 $\delta^{13}C$ and 3.5 ‰ ± 0.17 $\delta^{15}N$) compared to the woodland (-28.1 ‰ ± 0.13 $\delta^{13}C$ and 5.2 ‰ ± 0.20 $\delta^{15}N$).

The C and N content of the vegetation in the two habitats were both significantly different (%C: $F_{1,8} = 9.82$; $P = 0.014$ and %N: $F_{1,8} = 35.50$; $P < 0.001$), although the C:N ratios were not. The grassland had on average a higher C and N content, although a lower C:N ratio (39.9% ± 0.56 C and 2.0% ± 0.07 N, C:N ratio 20.1 ± 0.68), when compared to the woodland (32.6% ± 2.26 C and 1.4% ± 0.07 N, C:N ratio 23.1 ± 1.79). The vegetation $\delta^{13}C$ and $\delta^{15}N$ signatures were not significantly different from each other between habitats, with the grassland being slightly lower in $\delta^{13}C$ and $\delta^{15}N$ (-30.4 ‰ ± 0.13 $\delta^{13}C$ and 1.3 ‰ ± 0.33 $\delta^{15}N$), compared to the woodland (-30.4 ‰ ± 0.20 $\delta^{13}C$ and 2.3 ‰ ± 0.54 $\delta^{15}N$).

Dead plant material was also collected from both habitats (grass/hay and willow senesced leaf litter) to assess whether there were any differences in comparison to live plant material/under-canopy forbs. There were significant differences between the two habitats for C and N content ($F_{1,10} = 778.82$; $P < 0.001$ and $F_{1,10} = 102.23$; $P < 0.001$ respectively), although the C:N ratios were not significantly different. Conversely to live plant material, the grassland had on average a lower C and N content, with a higher C:N ratio (42.9% ± 0.24 C and 1.5% ± 0.02 N, C:N ratio 29.2 ± 0.33), when compared to the woodland (50.8% ± 0.15 C and 1.8% ± 0.03 N, C:N ratio 28.7 ± 0.45). The $\delta^{13}C$ of the litter was not different between habitats, however the $\delta^{15}N$ signatures were significantly different between the two habitats ($F_{1,10} = 563.02$; $P < 0.001$). The

grassland was higher in $\delta^{13}C$ and lower in $\delta^{15}N$ (-30.0 ‰ ± 0.04 $\delta^{13}C$ and -0.4 ‰ ± 0.10 $\delta^{15}N$), compared to the woodland (-30.1 ‰ ± 0.06 $\delta^{13}C$ and 2.1 ‰ ± 0.05 $\delta^{15}N$).

Comparison of the differences between living and dead plant material, shows that significant changes have occurred during senescence. There were significant differences in C content and C:N ratio between the living and dead plant material (%C: $F_{1,18} = 98.11$; $P < 0.001$ and C:N ratio: $F_{1,18} = 63.25$; $P < 0.001$ respectively), with lower %C and C:N content in the living plant material. Furthermore, the $\delta^{13}C$ and $\delta^{15}N$ signatures were also significantly different between the living and dead plant material ($F_{1,18} = 10.36$; $P = 0.005$ and $F_{1,18} = 39.82$; $P < 0.001$ respectively), with lower delta values in the dead plant litter for both $\delta^{13}C$ and $\delta^{15}N$ in both habitats.

4.3.4 Community composition within the soil food web

There were significant differences between the population numbers and biomass for some of the macro- and mesofauna taxa (Table 4.1), although these were not consistent, with one habitat having a greater number present than the other, in all orders. These differences represent differences in community structures in these two habitats, indicating potential different functional food web interactions occurring.

Despite the fact two different habitats are under consideration there were few significant differences between the C and N content and C:N ratio for the soil invertebrates (Table 4.2). The only exceptions where there were significant differences between the two habitats were aphids for C content, Collembola Entomobryomorpha for N content and Collembola Poduromorpha and aphids for C:N ratio.

All the aforementioned taxa were significantly higher in the grassland (C content: aphids ($F_{1,2} = 427.128.88$; $P = 0.002$); for N content: Entomobryomorpha ($F_{1,4} = 8.42$; $P = 0.044$); and for C:N ratio: aphids ($F_{1,2} = 77.12$; $P = 0.013$)), apart from the

Poduromorpha C:N ratio ($F_{1,2} = 43.01$; $P = 0.022$) which was significantly higher in the woodland.

There were significant differences between all the Acari from both habitats to the expected C:N ratio of 8 ($t = -13.76$; df_{19}; $P < 0.001$) (as stated by Hunt et al., (1987)) with overall averages in both habitats being significantly lower (grassland 5.2 ± 0.19; woodland 5.3 ± 0.36) and all individual lineages being significantly lower (Table 4.2). The Collembola were not significantly different to the results presented by Hunt et al., (1987) (C:N ratio of 8), when grouped as one taxon and both habitats were combined ($t = -0.88$; df_{13}; $P = 0.397$). However, when the habitats were tested separately, Collembola inhabiting the grassland were significantly different ($t = -4.50$; df_5; $P = 0.006$), whilst Collembola inhabiting the woodland were not ($t = 0.11$; df_7; $P = 0.915$), with overall averages being significantly lower in the grassland (6.6 ± 0.31) in comparison to the woodland (8.1 ± 1.02) which were very similar to the C:N ratio reported by Hunt et al., (1987), although they were quite variable. The majority of individual superfamilies tested were not different to those reported by Hunt et al., (1987), apart from the Symphypleona which had lower C:N ratios in both habitats. Within this study the nematodes were not found to be significantly different from the ratio's reported by Hunt et al., (1987), however the percent C and N appear to be lower than expected , this may affect future results based on these figures.

The mean biomass of each of the different orders varied greatly from 0.3 to 3591 µg per individual for the grassland, and 0.6 to 7438 µg per individual for the woodland (Table 4.3). In the grassland the invertebrates with the smallest biomass were the nematodes, followed by the Prostigmata and the biggest were earthworms. In the woodland the orders with the smallest biomass were the Collembola: Neelipleona, followed by nematodes whilst the biggest were the Diplopoda: Julidae.

Table 4.1: Community composition, abundance and biomass (dry weight, mg[#]) of the macro- and mesofauna taxa from a grassland and woodland habitat. Data presented as mean ± standard error (n = 6), and *F*-values of a single factor ANOVA, * $P < 0.05$; ** $P < 0.001$ indicating significant differences between habitats ($df_{1,4}$). [#] data presented as g for Earthworms due to biomass per m^2 being an order of magnitude greater, even with gut content removed.

	Number per m^2			Biomass per m^2 (mg)		
	Grassland	**Woodland**	***F-values***	**Grassland**	**Woodland**	***F-values***
Acari: Astigmata	1082	1167 (± 21.2)	17.40*	3.2	3.3	0.00
Acari: Mesostigmata	3204 (± 341.5)	3883 (± 510.6)	1.04	37.7 (± 7.27)	77.5 (± 13.96)	7.55
Acari: Mesostigmata: Uropodidae	934 (± 479.7)	0	4.00	13.9 (± 7.00)	0	3.99
Acari: Oribatida	16913 (± 5254.4)	13475 (± 1118.7)	0.05	54.5 (± 18.45)	73.2 (± 8.92)	0.88
Acari: Oribatida: Damaeidae	0	170 (± 21.2)	4.00	0	21.6	4.00
Acari: Oribatida: Phthiracaridae	0	1231 (± 275.9)	1156.76**	0	66.8 (± 16.78)	326.03**
Acari: Prostigmata	10674 (± 3427.2)	16510 (± 693.8)	1.83	9.0 (± 4.04)	12.6 (± 2.37)	0.86
Aphids (Hemiptera: Aphidoidea)	700 (± 287.1)	42 (± 42.4)	6.86	34.4 (± 5.47)	10.5 (± 10.53)	4.12
Chilopoda: Geophilomorpha	0	85 (± 21.2)	370.52**	0	171.2 (± 75.56)	65.35**
Coleoptera Larvae	64 (± 36.8)	106 (± 21.2)	1.11	23.9 (± 11.93)	14.6 (± 2.91)	0.06
Coleoptera Larvae: Elateridae	85 (± 42.4)	0	4.00	6.9 (± 3.47)	0	4.00
Coleoptera Larvae: Staphylinidae	191 (± 127.3)	85 (± 84.9)	2.30	90.6 (± 7.94)	21.1 (± 21.05)	5.05
Coleoptera: Carabidae	0	64 (± 36.8)	3.93	0	6.9 (± 3.97)	3.80
Coleoptera: Ptilidae	0	127 (± 63.7)	4.00	0	5.8 (± 2.88)	4.00
Coleoptera: Staphylinidae	42 (± 42.4)	212 (± 76.5)	4.37	19.9 (± 19.88)	201.2 (± 82.97)	6.06
Collembola: Entomobryomorpha	13284 (± 2717.5)	9401 (± 720.6)	2.25	33.9 (± 2.7)	21.8 (± 6.37)	2.28
Collembola: Neelipleona	0	11226 (± 5684.6)	4.00	0	7.2 (± 4.45)	3.69

	Number per m^2			Biomass per m^2 (mg)		
	Grassland	**Woodland**	***F-values***	**Grassland**	**Woodland**	***F-values***
Collembola: Poduromorpha	2674 (± 1508.3)	3353 (± 1724.5)	0.00	4.9 (± 2.99)	17 (± 8.5)	0.39
Collembola: Symphypleona	1061 (± 21.2)	1358 (± 21.2)	94.05**	1.4	2.9	0.00
Diplopoda: Julidae	0	42 (± 21.2)	4.00	0	315.7 (± 234.03)	3.82
Diplopoda: Polydesmidae	0	276 (± 148.5)	114.96**	0	169.3 (± 53.22)	145.90**
Diptera	42 (± 42.4)	297 (± 112.3)	5.55	1.5 (± 1.51)	21.3 (± 6.91)	14.62*
Diptera Larvae	403 (± 76.5)	255 (± 160.2)	1.51	317.6 (± 190.67)	4.0 (± 1.3)	11.79*
Earthworm	42 (± 21.2)	64	1.00	1.2 g (± 0.49)	1.4 g (± 0.11)	0.72
Enchytraeid worms	64 (± 0)	42 (± 42.4)	2.49	12.5 (± 0)	2.8 (± 2.82)	6.14
Pseudoscorpion	0	42 (± 21.2)	4.00	0	6.5 (± 3.25)	4.00
Snail	0	21 (± 21.2)	1.00	0	3.6 (± 3.57)	1.00
Spider	21 (± 21.2)	170 (± 139.2)	0.80	8.9 (± 8.93)	16.9 (± 8.57)	0.47
Thrips	615 (± 21.2)	0	33555**	6.3 (± 0)	0	0.00
Woodlice	21 (± 21.2)	2207 (± 424.4)	20.06*	2.7 (± 2.72)	208.6 (± 51.67)	33.65*

Table 4.1 continued: Community composition, abundance and biomass of the macro- and mesofauna taxa from a grassland and woodland habitat.

Table 4.2: Analysis of C and N content of the macro- and mesofauna taxa from the grassland and woodland habitats.

Data presented as mean ± standard error (n = 3). Single factor ANOVA indicating differences between habitats was not significant for the majority of invertebrates apart from those labelled. Student's T-test was performed to assess whether the invertebrates had different C:N ratio's in comparison to Hunt et al., (1987) which has been used over the last 20 years for modelling soil fauna ecological interactions; where Acari and Collembola have a C:N ratio of 8, and Nematodes have a C:N ratio of 10; (df 1-5) habitats were combined for analysis.

	%C		%N		C:N ratio		Hunt C:N ratio
	Grassland	Woodland	Grassland	Woodland	Grassland	Woodland	T-test
Acari: Astigmata	21.5	16.9	3.5	3.4	6.1	5.0	-7.63; $P = 0.005$
Acari: Mesostigmata	39.5 (± 1.36)	42.5 (±3.48)	9.7 (± 0.73)	10.0 (± 0.54)	2.9 (± 1.47)	2.7 (± 1.36)	-33.97; $P < 0.001$
Acari: Mesostigmata: Uropodidae	43.8 (± 1.01)		8.6 (± 0.46)		5.1 (± 0.16)		-18.42; $P = 0.035$
Acari: Oribatida	40.4 (± 1.22)	37.6 (±1.09)	7.7 (± 0.12)	7.3 (± 0.40)	5.2 (± 0.14)	3.3 (± 1.64)	-20.77; $P < 0.001$
Acari: Oribatida: Phthiracaridae		21.4 (±0.20)		2.9 (± 0.04)		7.3 (± 0.02)	-19.20; $P = 0.033$
Acari: Prostigmata	25.6 (± 1.95)	30.0 (±4.19)	4.9 (± 0.54)	5.8 (± 1.07)	5.2 (± 0.24)	5.2 (± 0.22)	-15.22 $P < 0.001$
Aphids (Hemiptera: Aphidoidea)[a,c]	40.5 (± 0.48)	26.5	4.8 (± 0.19)	4.9	8.5 (± 0.25)	5.4	
Chilopoda: Geophilomorpha		30.9 (±1.32)		5.0 (± 0.88)		5.0 (± 2.49)	
Coleoptera Larvae		21.4	6.6	4.9		4.4	
Coleoptera Larvae: Elateridae	29.2		6.6		4.4		
Coleoptera Larvae: Staphylinidae	15.0 (± 0.38)	16.5	3.4 (± 1.11)	3.6	4.4 (± 2.22)	4.6	
Coleoptera: Carabidae		37.2		5.5		6.7	
Coleoptera: Ptilidae		37.4		4.8		7.8	
Coleoptera: Staphylinidae	28.5	31.2 (±1.22)	4.5	4.9 (± 0.28)	6.4	6.4 (± 0.45)	
Collembola: Entomobryomorpha[b]	45.0 (± 0.45)	36.1 (±3.34)	7.2 (± 0.42)	5.0 (± 0.65)	6.3 (± 0.32)	7.3 (± 0.30)	-2.68; $P = 0.075$

	%C		%N		C:N ratio		Hunt C:N ratio
	Grassland	**Woodland**	**Grassland**	**Woodland**	**Grassland**	**Woodland**	**T-test**
Collembola: Neelipleona		16.1 (±5.49)		2.1 (± 0.67)		7.7 (± 0.15)	-2.07; $P = 0.286$
Collembola: Poduromorpha[c]	49.3 (± 5.83)	48.7 (±1.53)	6.8 (± 1.36)	4.0 (± 0.36)	7.3 (± 0.60)	12.3 (± 0.71)	1.22; $P = 0.309$
Collembola: Symphypleona	16.7	15.2	2.8	3.1	5.9	4.9	-8.74; $P = 0.003$
Diplopoda: Julidae		24.3 (±4.44)		3.7 (± 0.60)		6.5 (± 0.13)	
Diplopoda: Polydesmidae		27.7 (±2.71)		4.4 (± 0.41)		6.3 (± 0.06)	
Diptera	27.5	23.2 (±1.36)	6.3	5.0 (± 0.25)	4.4	4.7 (± 0.12)	
Diptera Larvae	18.7 (± 3.08)	14.0 (±3.11)	3.8 (± 1.12)	3.7	5.8 (± 1.63)	5.4	
Earthworm	30.5 (± 7.36)	32.1 (±5.76)	6.8 (± 1.92)	7.0 (± 0.96)	4.6 (± 0.20)	4.6 (± 0.34)	
Enchytraeid worms	22.8	34.1	5.4	6.9	4.3	5.0	
Nematodes	11.2 (± 0.13)	9.6 (± 0.81)	1.2 (± 0.16)	1.1 (± 0.21)	9.7 (± 1.61)	8.8 (± 0.84)	-0.90; $P = 0.411$
Pseudoscorpion		23.5		5.2		4.5	
Snail		13.0		1.5		8.7	
Spider	34.8	38.2 (±6.40)	6.3	7.7 (± 2.80)	5.6	5.4 (± 1.11)	
Thrips	37.0		5.8		6.4		
Woodlice	15.1	16.5 (±1.64)	2.7	2.6 (± 0.11)	5.7	4.3 (± 2.17)	

[a] **for %C –Aphids $F_{1,2} = 427.128.88$; $P = 0.002$;**
[b] **for %N – Collembola Entomobryomorpha $F_{1,4} = 8.42$; $P = 0.044$;**
[c] **for C:N ratio – Collembola: Poduromorpha $F_{1,2} = 43.01$; $P = 0.022$; and Aphids $F_{1,2} = 77.12$; $P = 0.013$.**

Table 4.2 continued: Analysis of C and N content of the macro- and mesofauna taxa from the grassland and woodland habitats.

Table 4.3: Analysis of individual dry weights and number of individuals needed for mass spectrometry for the macro- and mesofauna taxa from the grassland and woodland habitats. For all fauna apart from Earthworms sample includes gut contents, Earthworms had gut contents removed prior to dry weight analysis.

Data presented as mean ± standard error (n = 3), for single factor ANOVA * $P < 0.05$; ** $P < 0.001$ indicating significant differences between habitats ($df_{1,4}$).

	Dry weight per individual (µg)		Number of individuals needed for 20µg C (rounded to whole organisms unless ≤ 0.5)	
	Grassland	Woodland	Grassland	Woodland
Acari: Astigmata	3	3 (± 0.1)	32	43 (± 0.77)
Acari: Mesostigmata	12 (± 2.6)	21 (± 3.9)	6 (± 2.03)	2 (± 0.25)
Acari: Mesostigmata: Uropodidae	15 (± 1.0)		4 (± 0.13)	
Acari: Oribatida*	3 (± 0.2)	5 (± 0.5)	17 (± 1.51)	11 (± 0.92)
Acari: Oribatida: Phthiracaridae		54 (± 2.1)		2 (± 0.08)
Acari: Prostigmata	0.8 (± 0.2)	0.8 (± 0.2)	109 (± 23.14)	94 (± 8.49)
Aphids (Hemiptera: Aphidoidea)	91 (± 50.7)	248	1 (± 0.34)	0.3
Chilopoda: Geophilomorpha		2586 (± 1277.7)		0.12 (± 0.10)
Coleoptera Larvae	422 (± 140.5)	137		1 (± 0.00)
Coleoptera Larvae: Elateridae	82		1 (± 0.00)	
Coleoptera Larvae: Staphylinidae	945 (± 353.3)	248	0.33 (± 0.22)	0.49
Coleoptera: Carabidae		108		0.50 (± 0.00)
Coleoptera: Ptilidae		45		2 (± 0.00)

	Dry weight per individual (µg)		Number of individuals needed for 20µg C (rounded to whole organisms unless ≤ 0.5)	
	Grassland	**Woodland**	**Grassland**	**Woodland**
Coleoptera: Staphylinidae	469	1582 (± 998.5)	0.15	0.14 (± 0.10)
Collembola: Entomobryomorpha	3 (± 0.6)	2 (± 0.6)	19 (± 5.29)	29 (± 7.29)
Collembola: Neelipleona		0.6 (± 0.2)		225 (± 2.05)
Collembola: Poduromorpha*	2 (± 0.2)	5 (± 0.7)	24 (± 0.09)	9 (± 0.84)
Collembola: Symphypleona**	1	2	88 (± 1.76)	61 (± 0.95)
Diplopoda: Julidae		7438 (± 4701.5)		0.02 (± 0.01)
Diplopoda: Polydesmidae		1200 (± 546.9)		0.22 (± 0.17)
Diptera	36	77 (± 11.8)	3	2 (± 0.22)
Diptera Larvae	854 (± 430.6)	24 (± 6.2)	1 (± 0.91)	8 (± 0.90)
Earthworm	3591 (± 367.0)	2770 (± 378.3)	0.02 (± 0.00)	0.02 (± 0.01)
Enchytraeid worms	197 (± 0.0)	67	0.45 (± 0.00)	0.88
Nematodes	0.3 (± 0.08)	0.7 (± 0.16)	7.6×10^5 ($\pm 2.3 \times 10^5$)	3.4×10^5 ($\pm 7.3 \times 10^4$)
Pseudoscorpion		153		1 (± 0.00)
Snail		168		1
Spider	421	245 (± 194.1)	0.14	1 (± 0.57)
Thrips	10 (± 0.4)		5.25 (± 0.18)	
Woodlice	128	94 (± 13.8)	1.03	2 (± 0.23)

Table 4.3 continued: Analysis of individual dry weights and number of individuals needed for mass spectrometry for the macro- and mesofauna taxa from the grassland and woodland habitats.

Most taxa weighed between 1 µg and 100 µg. To have the correct amount of C in each sample for mass spectrometry, on average there needed to be a minimum of 16 (± 6.7) individuals for 20 µg C in the grassland, and 19 (± 6.7) in the woodland. The C, N and C:N ratios of the invertebrates were not correlated with individual body dry weight in general or at the habitat level (%C: $F_{3,101} = 1.40$; $R^2 = 1.2$; %N: $F_{3,107} = 1.18$; $R^2 = 0.5$; and C:N ratio: $F_{3,105} = 0.68$; $R^2 = 0.0$).

4.3.5 $\delta^{13}C$ and $\delta^{15}N$ signatures of the soil fauna

There were isotopic differences between the soils within these two habitats which may effect the isotopic composition of the soil faunal community, hence isotopic results may need to be calibrated accordingly. However, at this stage it is not certain whether calibration is necessary, as the stable isotope signatures of fresh vegetation in each habitat were almost the same.

Prior to calibration of stable isotope signatures to the soil within each habitat, an analysis of variance was performed of the $\delta^{13}C$ and $\delta^{15}N$ (Table 4.4). A number of differences were found between the two habitats, although there was a noticeably large overlap between the two habitats (Figure 4.5). However, Oribatid mites were significantly different for both ^{13}C and ^{15}N variation between the two habitats ($F_{1,3} = 62.95$; $P = 0.004$; and $F_{1,4} = 90.71$; $P < 0.001$ respectively), having lower $\delta^{13}C$ values but higher $\delta^{15}N$ values in the grassland (Table 4.4).

Table 4.4: Average delta signatures for δ ^{13}C and δ ^{15}N for the macro- and mesofauna taxa from the grassland and woodland habitats.

Data presented as mean ± standard error (n = 3), and *F*-values of a single factor ANOVA * $P < 0.05$; ** $P < 0.001$ indicating significant differences between habitats ($df_{1,4}$ unless otherwise stated).

	Grassland		Woodland		*F-values*	
	δ ^{13}C	δ ^{15}N	δ ^{13}C	δ ^{15}N	δ ^{13}C	δ ^{15}N
Acari: Astigmata	-26.23	5.46	-26.73	4.16		
Acari: Mesostigmata	-26.61 (± 0.198)	9.73 (± 0.659)	-26.55 (± 0.047)	7.52 (± 0.543)	$0.15_{1,2}$	6.64
Acari: Mesostigmata: Uropodidae	-26.69 (± 0.204)	10.45 (± 0.204)				
Acari: Oribatida	-28.08 (± 0.085)	5.92 (± 0.370)	-27.08 (± 0.119)	2.16 (± 0.138)	$62.95^{*}_{1,3}$	90.71**
Acari: Oribatida: Phthiracaridae			-22.94 (± 0.029)	3.03 (± 0.022)		
Acari: Prostigmata	-27.63 (± 0.656)	6.72 (± 0.598)	-28.47 (± 0.268)	4.20 (± 0.345)	1.41	13.40*
Aphids (Hemiptera: Aphidoidea)	-30.66 (± 0.266)	2.56 (± 0.623)	-33.28	4.32	$48.46^{*}_{1,2}$	$3.97_{1,2}$
Chilopoda: Geophilomorpha			-27.51 (± 0.207)	7.54 (± 0.973)		
Coleoptera Larvae			-26.20	5.87		
Coleoptera Larvae: Elateridae	-27.21	4.70				
Coleoptera Larvae: Staphylinidae	-28.32 (± 0.293)	6.42 (± 0.221)	-26.17	4.28	$40.60_{1,2}$	$46.54^{*}_{1,2}$
Coleoptera: Carabidae			-28.09	4.66		
Coleoptera: Ptilidae			-28.15	2.99		
Coleoptera: Staphylinidae	-28.87	4.62	-27.61 (± 0.272)	5.93 (± 0.677)	$10.81_{1,2}$	$1.88_{1,2}$
Collembola: Entomobryomorpha	-29.08 (± 0.412)	5.15 (± 0.176)	-28.96 (± 0.135)	1.18 (± 0.900)	0.07	18.71*
Collembola: Neelipleona			-27.25 (± 0.306)	4.30 (± 1.665)		
Collembola: Poduromorpha	-28.15 (± 0.440)	6.66 (± 0.204)	-27.79 (± 0.045)	9.23 (± 0.687)	$0.98_{1,2}$	$19.26^{*}_{1,2}$
Collembola: Symphypleona		2.10	-27.64	-0.16		

	Grassland		Woodland		*F-values*	
	δ ^{13}C	**δ ^{15}N**	**δ ^{13}C**	**δ ^{15}N**	**δ ^{13}C**	**δ ^{15}N**
Diplopoda: Julidae	-28.56		-25.87 (± 1.252)	1.89 (± 0.278)		
Diplopoda: Polydesmidae			-25.61 (± 0.255)	4.25 (± 0.814)		
Diptera	-27.62	7.23	-28.96 (± 0.359)	10.44 (± 0.875)	$6.95_{1,2}$	$6.70_{1,2}$
Diptera Larvae	-27.27 (± 0.603)	5.40 (± 0.9777)	-32.66 (± 3.517)	4.9	2.28	$0.13_{1,2}$
Earthworm	-28.26 (± 0.188)	4.55 (± 1.474)	-26.24 (± 0.366)	3.89 (± 0.128)	$21.04^{*}_{1,3}$	$0.43_{1,3}$
Enchytraeid worms	-26.92	4.99	-27.14	3.15		
Nematodes	-26.75(± 1.018)	7.44 (± 0.331)	-27.65 (± 0.279)	3.29 (± 2.076)	0.72	4.09
Pseudoscorpion			-27.07	3.90		
Snail			-21.02	0.82		
Spider	-28.40	6.90	-26.83 (± 0.596)	8.78 (± 1.066)	$5.23_{1,2}$	$2.33_{1,2}$
Thrips	-29.50	3.75				
Woodlice	-29.10	3.43	-25.86 (± 0.847)	3.16 (± 0.033)	$0.06_{1,2}$	$33.37^{*}_{1,2}$

Table 4.4 continued: Average delta signatures for δ ^{13}C and δ ^{15}N for the macro- and mesofauna taxa from the grassland and woodland habitats.

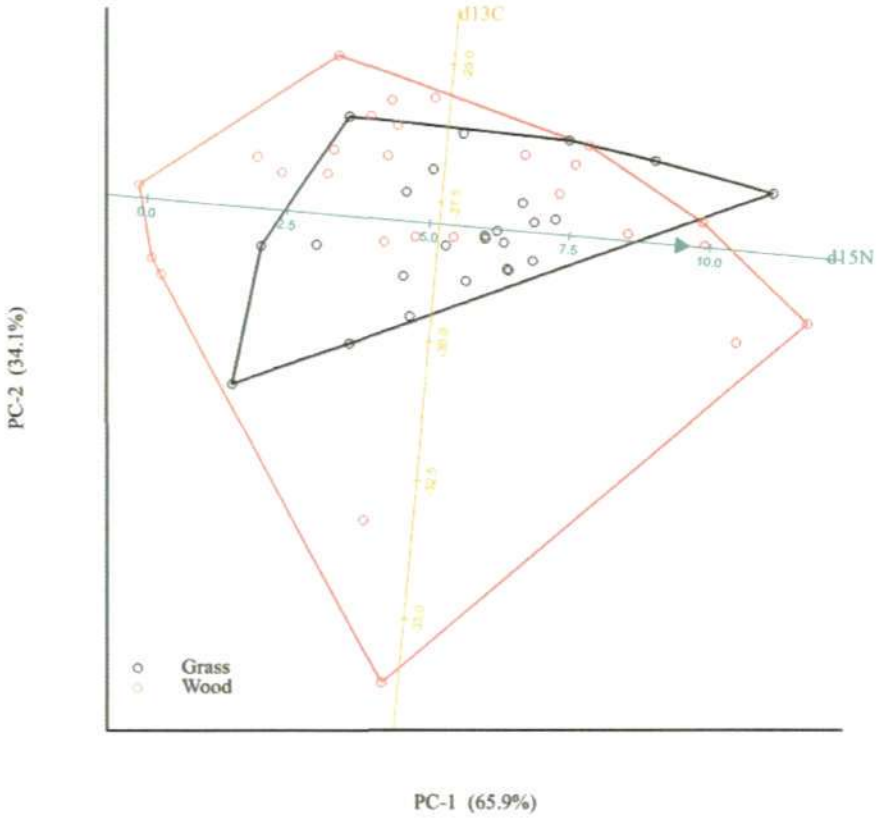


Figure 4.5: Principal component analysis (PCA) of the $\delta^{13}C$ and $\delta^{15}N$ stable isotope signatures of invertebrates within the grassland (black circle) and woodland (red circle) with convex hull.

Earthworms also had lower $\delta^{13}C$ values, whilst aphids had higher $\delta^{13}C$ values in the grassland compared to the woodland ($F_{1,3} = 21.04$; $P = 0.019$; and $F_{1,2} = 48.46$; $P = 0.020$ respectively). Whilst for $\delta^{15}N$ there were many invertebrates that were significantly different, these were Prostigmata ($F_{1,4} = 13.40$; $P = 0.022$); Staphylinidae larvae ($F_{1,2} = 46.54$; $P = 0.021$); Entomobryomorpha ($F_{1,4} = 18.71$; $P = 0.012$); and woodlice ($F_{1,2} = 33.37$; $P = 0.029$), all of which had $\delta^{15}N$ values that were higher in the grassland. Interestingly the $\delta^{15}N$ signatures of soil were lower in the grassland than the woodland. The Poduromorpha were the only group which had significantly higher $\delta^{15}N$ values ($F_{1,2} = 19.26$; $P = 0.048$) in the woodland (Table 4.4).

To distinguish whether these differences in $\delta^{13}C$ and $\delta^{15}N$ between habitats are due to differences in the interactions occurring between invertebrates the stable isotope signatures of the soil in each habitat were set to zero and all the other results were calibrated accordingly (*sensu* Erdmann et al., 2007), (Figure 4.6). The differences between the two habitats became more striking using this method, looking at all the delta values together within each habitat there were no significant differences prior to calibration for $\delta^{13}C$ and $\delta^{15}N$. However, after calibration both $\delta^{13}C$ and $\delta^{15}N$ were significantly higher in the grassland soil compared to the woodland soil habitat ($\delta^{13}C$ $F_{1,66} = 10.97$; $P = 0.002$ and $\delta^{15}N$ $F_{1,66} = 16.55$; $P < 0.001$), there is also less overlap between the PCA plots (Figure 4.7).

There are significant differences in delta signatures between fauna present in both habitats after calibration. This includes the majority of the mesofauna (Table 4.5). The Mesostigmata, Diptera and aphids all had significantly higher $\delta^{13}C$ values in the grassland, whilst the Oribatida, Prostigmata, Staphlinidae larvae, Entomobryomorpha, and woodlice all had significantly greater $\delta^{15}N$ values in the grassland (Table 4.5). Focusing on the differences in $\delta^{15}N$ signatures (Figure 4.8), there appears to be different numbers of trophic levels in the two different habitats.

In the grassland (Figure 4.8a), there are only three trophic levels (using the conservative estimate of a 3.4‰ increase in $\delta^{15}N$ per trophic level (DeNiro et al., 1981; Minagawa et al., 1984)), with the majority of invertebrates having a $\delta^{15}N$ value greater than soil. Whilst in the woodland (Figure 4.8b), there appears to be four trophic levels, here, the majority of invertebrates have a $\delta^{15}N$ value less than soil, yet greater than dead plant litter. There was no correlation between C:N ratio of soil fauna and $\delta^{15}N$ value and habitat ($F_{3,64} = 1.17$; $R^2 = 0.7$). There was a significant correlation when considering the soil fauna separately ($F_{3,64} = 7.99$; $P < 0.001$; $R^2 = 77.5$) (habitats combined).

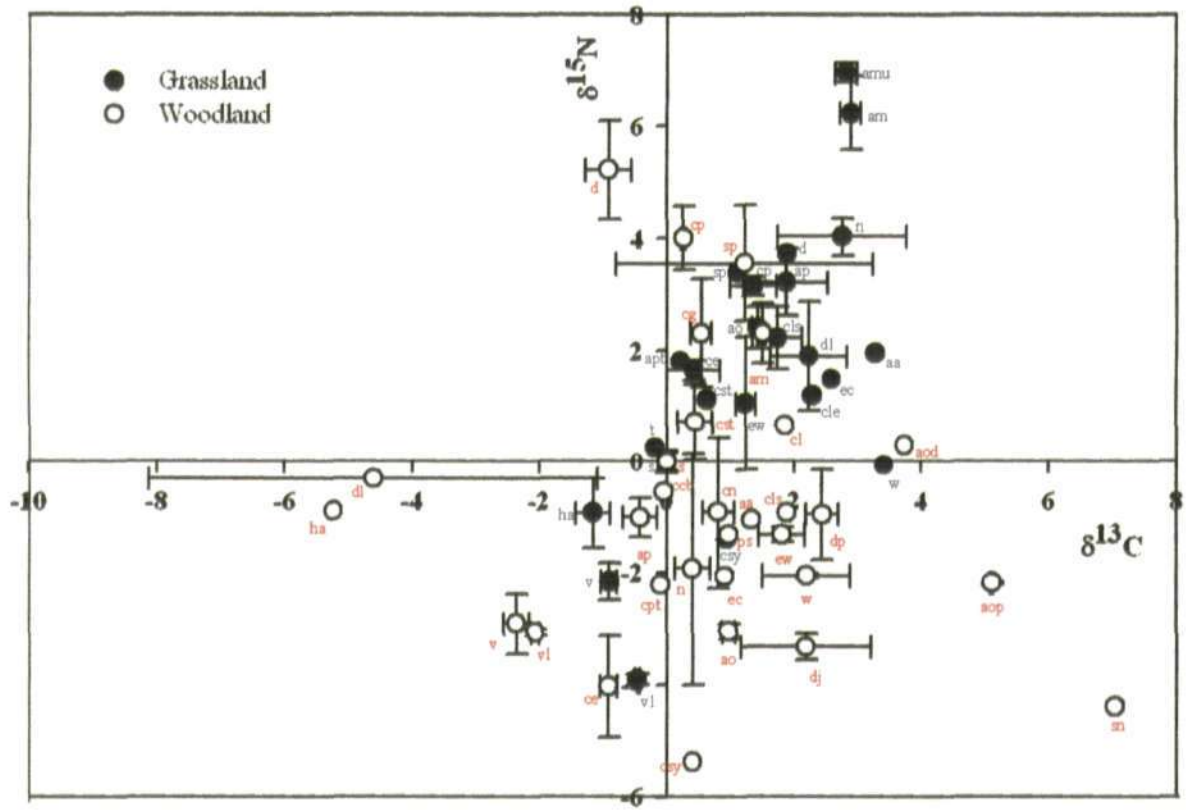


Figure 4.6: Isotopic composition of soil fauna within a grassland (black circles; blue labels) and a woodland (open circles; red labels) habitat with the soil stable isotope signature for each habitat set to zero and all the other results calibrated accordingly.

Data presented as mean ± standard error, n=3. s = soil for all other label codes see Table 4.5.

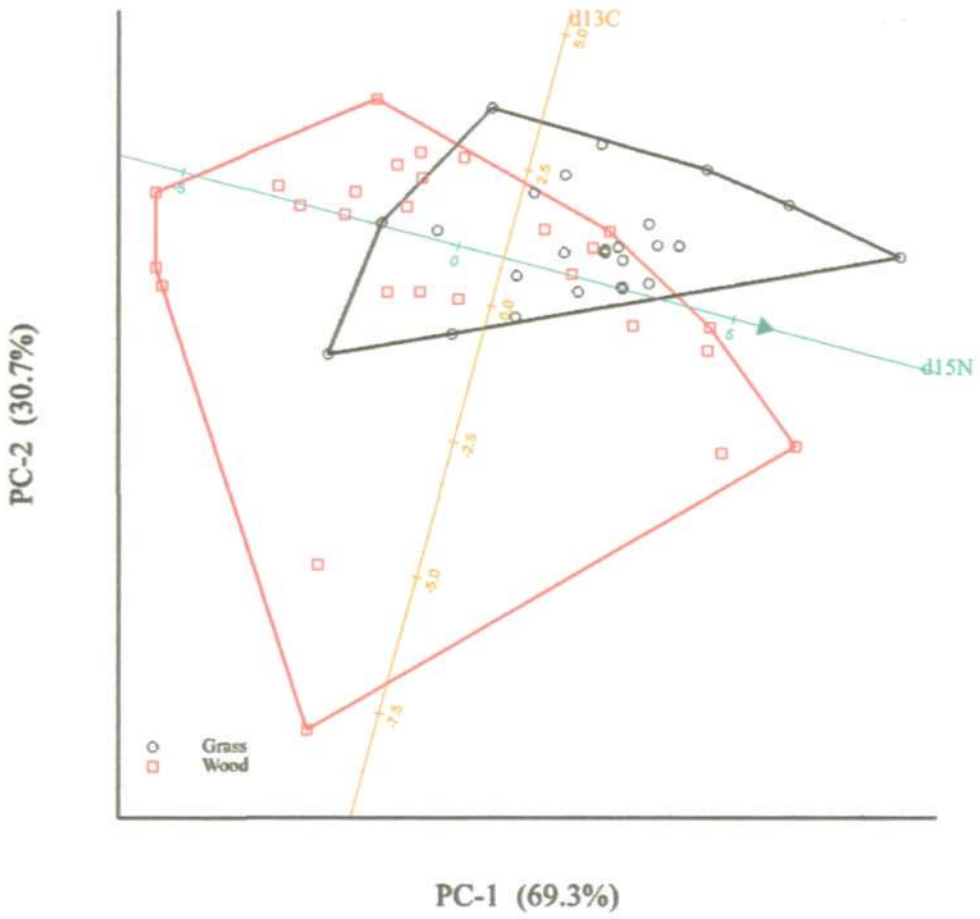


Figure 4.7: Principal component analysis (PCA) of the $\delta^{13}C$ and $\delta^{15}N$ stable isotope signatures of invertebrates, with the signature of soil in each habitat set to zero and all the other results calibrated accordingly, within the grassland (black circle) and woodland (red square). PCA accounting for 100% of variation in two axis.

A visual assessment of the differences between the two habitats for both $\delta^{13}C$ and $\delta^{15}N$ shows a continuum of isotope signatures for both elements (Figure 4.9). Bearhop et al., (2004) thought that stable isotope analysis can identify trophic niches within an ecosystem. Layman et al., (2007), developed methods to test for these community wide metric values, two of the methods were the $\delta^{15}N$ range and the $\delta^{13}C$ range. The $\delta^{15}N$ range of results (including plant litter, herbage and soil) for the two

habitats was very similar (10.86‰ for the grassland, compared to 10.60‰ in the woodland). However, when plant litter and herbage are excluded from the $\delta^{15}N$ range, there is almost one trophic level difference (8.35‰ for the grassland, compared to 10.60‰ in the woodland). The $\delta^{13}C$ range between the two habitats was very different, with signatures spanning 4.56‰ in the grassland, whilst in the woodland the range was 12.27‰ (the $\delta^{13}C$ range was unaffected by inclusion of plant litter, herbage and soil in the analysis).

Many studies using the isotopic composition of invertebrates at natural abundance categorise the invertebrates as herbivores (Schmidt et al., 2004) (T0), primary (T1) or secondary decomposers (Hishi et al., 2007) (T2), and micro (T3) or macro-predators (Koehler, 1999; Okuzaki et al., 2010) (T4). Using these literature classifications the invertebrate $\delta^{13}C$ and $\delta^{15}N$ results were grouped into "trophic levels" T0-4 (Brussaard et al., 1997; Hopkin, 1997; Halaj et al., 2005; Krantz et al., 2009) (Table 4.6) and the differences between these average trophic level $\delta^{13}C$ and $\delta^{15}N$ were assessed.

There was a significant difference between the grouped "trophic levels" for $\delta^{13}C$ ($F_{4,87} = 7.99$; $P < 0.001$) and $\delta^{15}N$ ($F_{4,87} = 9.52$; $P < 0.001$). When habitat was considered separately there were no significant differences in $\delta^{13}C$ or the relationship between habitat and trophic level for $\delta^{13}C$. However, when the differences in $\delta^{15}N$ were considered for the whole food web there were significant differences between habitat ($F_{1,82} = 6.33$; $P = 0.014$). Although the relationship between habitat and trophic level for $\delta^{15}N$ was not significant suggesting similar effects are occurring.

Table 4.5: Variation in stable isotope signatures of δ ^{13}C and δ ^{15}N for the macro- and mesofauna taxa from the grassland and woodland habitats. Where the stable isotope signature of soil for each habitat was set to zero and all the other results were calibrated accordingly.

***F*-values of a single factor ANOVA indicating significant differences between habitats (all $df_{1,2}$, apart from vegetation which was either $df_{1,8}$ for live plant material, or $df_{1,10}$ for dead plant material). Where there are no values the sample size was too small to perform the test, when the invertebrates were not present – np-g = not present in the grassland habitat; np-w = not present in the woodland habitat. Includes abbreviations used in Figure 4.6.**

		$\delta^{13}C$		$\delta^{15}N$	
	abbreviation	***F-value***	***P-value***	***F-value***	***P-value***
Acari: Astigmata	aa				
Acari: Mesostigmata	am	46.83	0.021	16.11	0.057
Acari: Mesostigmata: Uropodidae	amu	np-w		np-w	
Acari: Oribatida	ao	11.03	0.080	599.74	0.002
Acari: Oribatida: Damaeidae	aod	np-g		np-g	
Acari: Oribatida: Phthiracaridae	aop	np-g		np-g	
Acari: Prostigmata	ap	4.19	0.177	42.92	0.023
Aphids (Hemiptera: Aphidoidea)	ha	81.49	0.012	0.04	0.859
Chilopoda: Geophilomorpha	cg	np-g		np-g	
Coleoptera Larvae	cl				
Coleoptera Larvae: Elateridae	cle	np-w		np-w	
Coleoptera Larvae: Staphylinidae	cls	5.73	0.139	234.10	0.004
Coleoptera: Carabidae	ccb	np-g		np-g	
Coleoptera: Ptilidae	cpt	np-g		np-g	

		$\delta^{13}C$		$\delta^{15}N$	
	abbreviation	***F-value***	***P-value***	***F-value***	***P-value***
Coleoptera: Staphylinidae	cst	0.16	0.727	0.00	0.978
Collembola: Entomobryomorpha	ce	5.57	0.142	528.50	0.002
Collembola: Neelipleona	cn				
Collembola: Poduromorpha	cp	6.13	0.132	1.41	0.357
Collembola: Symphypleona	csy				
Diplopoda: Julidae	dj	np-g		np-g	
Diplopoda: Polydesmidae	dp	np-g		np-g	
Diptera	d	188.15	0.005	13.94	0.065
Diptera Larvae	dl	1.10	0.404	3.85	0.189
Earthworm	ew	14.08	0.064	2.38	0.263
Enchytraeid worms	ec				
Herbage	v	7.67	0.024	0.22	0.654
Litter	vl	504.81	<0.001	59.82	<0.001
Nematodes	n	4.35	0.172	8.77	0.098
Pseudoscorpion	ps	np-g		np-g	
Snail	sn				
Spider	sp	0.04	0.859	0.02	0.891
Thrips	t				
Woodlice	w	2.04	0.289	3639.84	<0.001

Table 4.5 continued: Variation in stable isotope signatures of $\delta^{13}C$ and $\delta^{15}N$ for the macro- and mesofauna taxa from the grassland and woodland habitats. Where the stable isotope signature of soil for each habitat was set to zero and all the other results were calibrated accordingly.

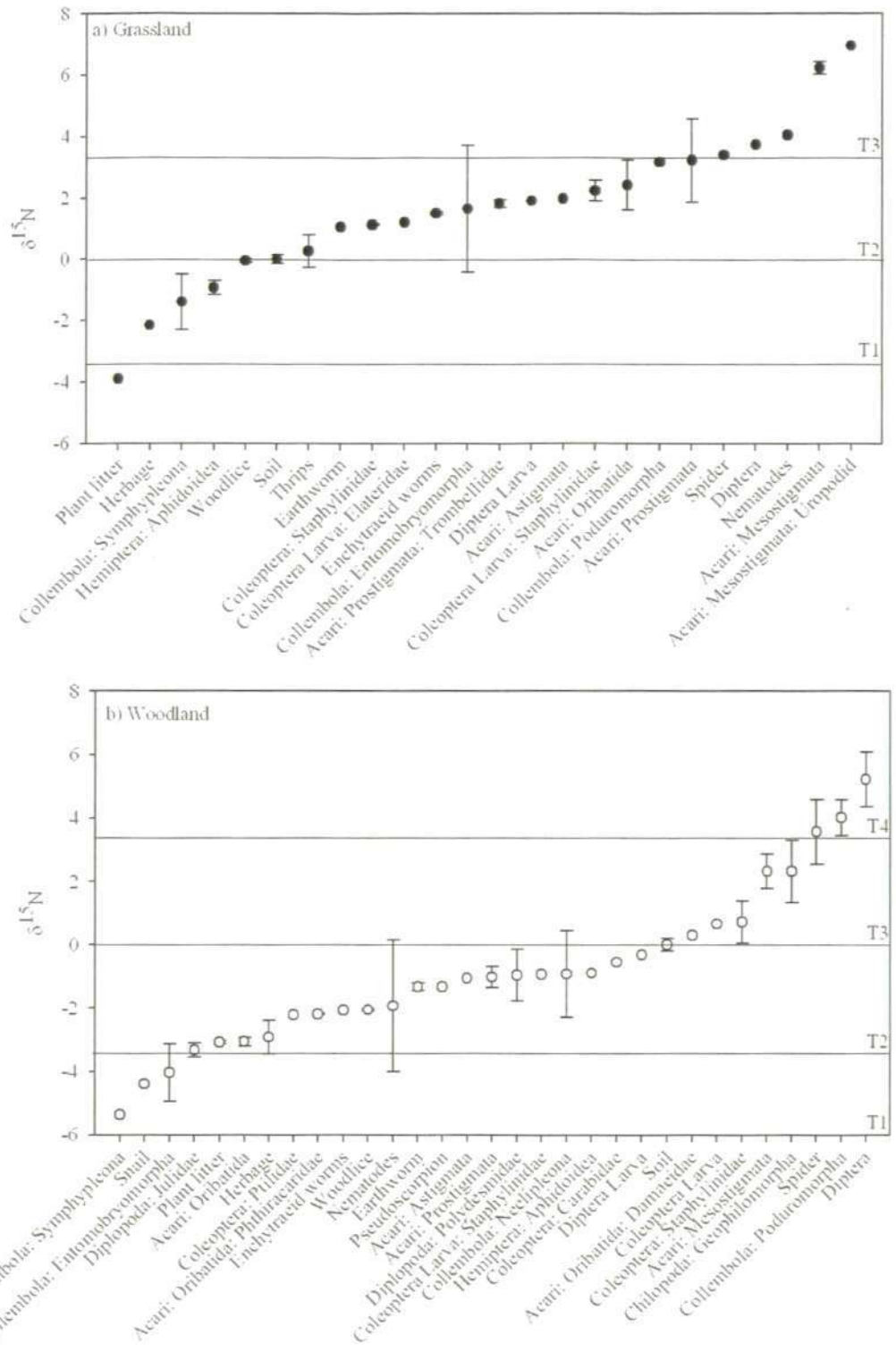


Figure 4.8: Variation in $\delta^{15}N$ signatures for the soil food web in a) grassland and b) woodland habitats; all results normalised with soil as baseline.

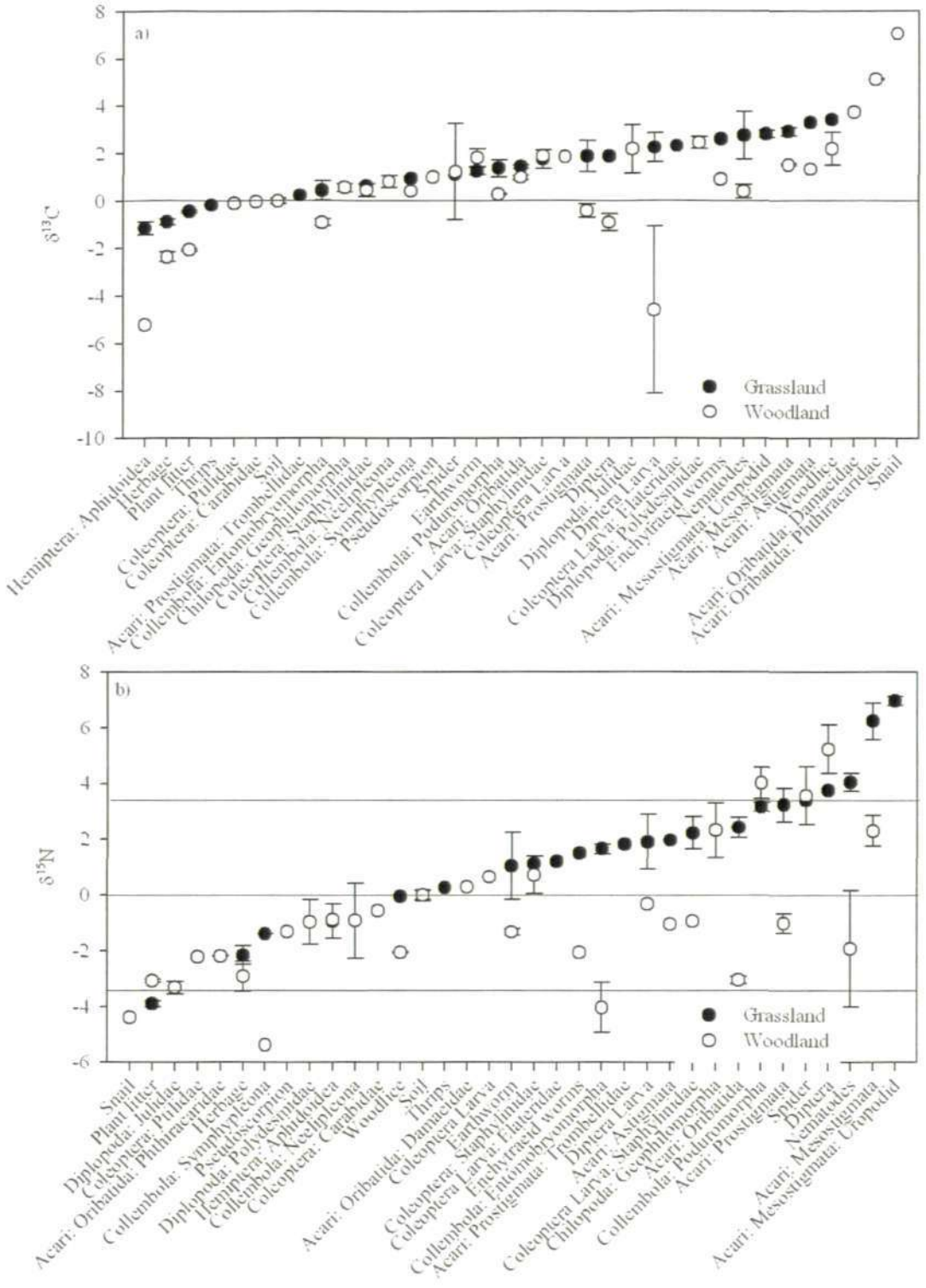


Figure 4.9: Variation in stable isotope signatures for the soil food web in a grassland and woodland habitat for a) $\delta^{13}C$ and b) $\delta^{15}N$; all results normalised with soil as baseline.

Assessing the differences between habitats for the grouped "trophic levels" separately, clear relationships can be seen between predators and prey in both $\delta^{13}C$ and $\delta^{15}N$ (Figure 4.10). Assessing the change in delta signatures for each habitat individually showed that there were significant differences for both habitats, for both $\delta^{13}C$ and $\delta^{15}N$ (in the grassland $\delta^{13}C$ $F_{4,35} = 5.74$ $P = 0.001$; $\delta^{15}N$ $F_{4,35} = 16.57$ $P < 0.001$; and in the woodland $\delta^{13}C$ $F_{4,47} = 4.56$ $P = 0.003$; $\delta^{15}N$ $F_{4,47} = 3.73$ $P = 0.010$).

The $\delta^{15}N$ signatures of the invertebrate groups within the grassland show clear delineation between trophic levels (Figure 4.10a). Herbivores had the lowest $\delta^{15}N$ signature and were significantly different to all other trophic levels. Primary decomposers were significantly different to all other trophic levels apart from macro-predators. Secondary decomposers were also significantly different to the other trophic levels apart from the macro-predators suggesting that the macro-predators are feeding on both the primary and secondary decomposers, forming part of the continuum of delta values. The micro-predators were significantly different to all other trophic levels, therefore all the other soil fauna are a potential food source, possibly the delta signature is large due to intraguild predation.

The differences in the $\delta^{13}C$ signatures varied between trophic levels (Figure 4.10b), again with the herbivores having the lowest delta signature which was significantly different to all other trophic levels apart from the macro-predators. Primary decomposers had the highest $\delta^{13}C$ signature and were significantly different to the rest of the soil fauna apart from the micro-predators, whilst the secondary decomposers formed a link between the micro- and macro-predators, being not significantly different to either.

Organism	Trophic level
Aphids (Hemiptera: Aphidoidea)	0
Coleoptera Larvae: Elateridae	0
Collembola: Symphypleona	0
Thrips	0
Acari: Astigmata	1
Acari: Oribatida - Damaeidae	1
Acari: Oribatida - Phthiracaridae	1
Collembola: Neelipleona	1
Diplopoda: Julidae	1
Diplopoda:Polydesmidae	1
Diptera Larvae	1
Earthworms	1
Enchytraeidae	1
Woodlice	1
Acari: Oribatida	2
Acari: Prostigmata	2
Coleoptera: Ptilidae	2
Collembola: Entomobryomorpha	2
Collembola: Poduromorpha	2
Diptera	2
Nematodes	2
Acari: Mesostigmata	3
Acari: Mesostigmata: Uropodidae	3
Coleoptera Larvae	3
Coleoptera Larvae: Staphylinidae	3
Chilopoda: Geophilamorpha	4
Coleoptera: Carabidae	4
Coleoptera: Staphylinidae	4
Pseudoscorpion	4
Spider	4

Table 4.6: Groupings of invertebrates used for trophic level analysis.

Trophic level 0 = herbivores, TL1 = primary decomposers, TL2 = secondary decomposers, TL3 = micro-predator, and TL4 = macro-predator. (Groupings ordered according to literature (Brussard et al., 1997; Hopkin, 1997; Halaj et al., 2005; Krantz et al., 2009))

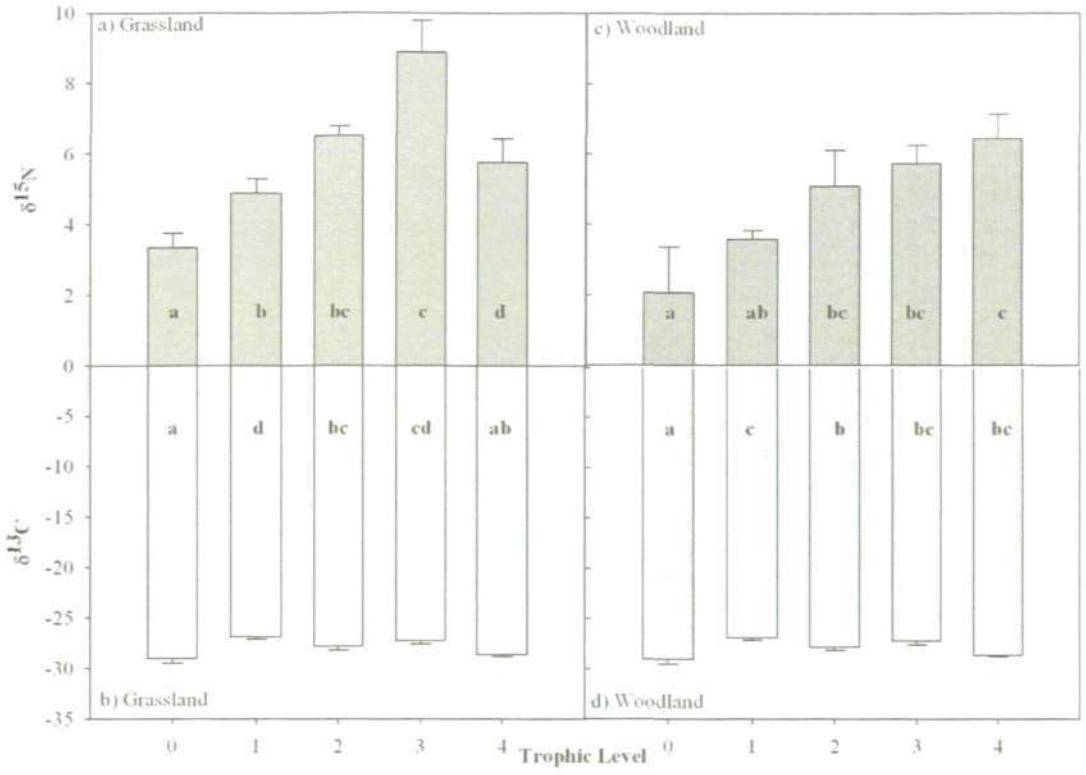


Figure 4.10: Isotopic composition of the grouped "trophic levels" for the different habitats, average $\delta^{13}C$ and $\delta^{15}N$ (± standard error, n = >4). a) Grassland $\delta^{15}N$; b) Grassland $\delta^{13}C$; c) Woodland $\delta^{15}N$; d) Woodland $\delta^{13}C$.

See Table 4.6 for Trophic level groupings TL0 herbivores, TL1 primary decomposers, TL2 secondary decomposers, TL3 micro-predators, and TL4 macro-predators. Difference in letters within each graph show significant differences between trophic levels – Fisher's protected least significant difference test.

Within the woodland habitat the $\delta^{15}N$ signatures show a step-wise enrichment up through the trophic levels, suggesting that the herbivores, primary and secondary decomposers form a continuum of decomposition, whilst being predated upon by micro- and macro-predators (Figure 4.10c). Here, herbivores were significantly different to all other trophic levels apart from the primary decomposers. Whilst the primary

decomposers were only significantly different to the macro-predators, again suggesting the macro-predators are feeding mainly on the "lower" primary decomposer trophic level as opposed to the secondary decomposer level. The secondary decomposers and micro-predators form a continuum of $\delta^{15}N$ enrichment.

Looking at the $\delta^{13}C$ signatures of the trophic levels within the woodland (Figure 4.10d) there is a clear split between the herbivores and the rest of the food web. The herbivores were significantly different to all the other trophic levels, primary decomposers are significantly different to the secondary decomposers but neither was significantly different to the predators.

4.4 DISCUSSION

4.4.1 Tullgren funnel extraction efficiency

Assessment of the optimum time for extraction of invertebrates on the Tullgren Funnel System, proved harder than expected due to the probable within core population changes during the time of extraction. The abundance, biomass and diversity within the two habitats varied depending on invertebrates identified. Principally there appeared to be greater diversity within the woodland habitat, but greater numbers of individuals within the grassland, although this varied with sampling.

Overall the results from the Tullgren funnel efficiency test advocate that cores should remain on the Tullgren funnel system for a minimum of five days. During this time a relatively low number of invertebrates will be extracted. From five to thirteen days the number of invertebrates extracted generally increased, before reducing rapidly after thirteen days (although there were exceptions e.g. grassland Prostigmata (Figure 4.4b)). It is feasible that the numbers continued to increase, due to the rapid

developmental time of the organisms involved, e.g. some species of Prostigmata can complete their life cycle in approximately one week (Behan-Pelletier, 2003). Most likely the reason for this continued increase in the number of soil fauna extracted is due to eggs laid within the soil cores hatching during the study period (Hopkin, 1997). It is also plausible, that as conditions changed on the Tullgren funnel system there was increased availability of secondary food sources for the mesofauna e.g. excystment of protozoa (Adl et al., 2005) or increased microbial numbers (Bonkowski et al., 2000), boosting the reproductive capacity of invertebrates.

For these reasons, future experiments should extract soil fauna on the Tullgren funnels for a minimum of five days and a maximum of twelve days, particularly where the addition of an enriched food source has been introduced into soil cores. In tracer studies, the results need to reflect the faunal assemblage at the time of addition of the tracer, and not newly hatched/fast developing fauna as these will affect overall isotopic compositions and mask feeding preferences.

4.4.2 Community composition and stable isotope signatures of the soil fauna and habitat

Generally, the results from these experiments have begun to show the complexity of the soil food web and the intricate nature of the study it entails. The trophic structure of the soil food web has started to be elucidated through the use of stable isotopes at natural abundance. At this stage, differences between the trophic structures of each habitat can be perceived, although more investigation is needed.

The significant differences in soil delta values between the two habitats may affect the signatures of the invertebrates within these habitats. Although the live vegetation did not have different isotopic compositions, which initially indicated that the calibration of the soil fauna's delta values was unnecessary. However, the dead plant

material collected from both habitats was significantly different. This was possibly due to differences that occur during decomposition or because different plants were sampled, i.e. within the woodland the dead plant litter sampled was from the willow leaves, whilst the live plant material was under-canopy forbs. Although, there are differences between the live and dead plant litter collected from the grassland, suggesting that changes in isotopic composition occurred during senescence. These differences in soil and vegetation highlight the effect of different management practices on the two systems over time since conversion, and the change decomposition has over time. It also indicates that calibration of the two habitats should be performed.

The differences between the abundance and biomass of invertebrates between the two habitats were significant. Twenty years ago, these two habitats were the same type and it was only a change in management that created the woodland, therefore differences in the community composition must have occurred since this point in time. There were a greater number of predators occurring within the woodland habitat, this may reflect the differences in plant diversity between the two habitats (Szanser et al., 2011). There were also a greater biomass of decomposer invertebrates within the woodland (e.g. Diplopoda: Polydesmidae and woodlice) possibly due to a greater amount of resources (Berg et al., 2001).

Studies investigating lake food webs found that the number of trophic levels within a food web was correlated with either the productivity or habitat heterogeneity (Persson et al., 1992). Furthermore, the trophic position of a species or group within a food web is not determined by the length of the food chain itself but by the available amount of resources to the consumer (Hairston et al., 1993). If theories like this are applicable to soil, then the results from this study suggest the woodland habitat is more heterogeneous with greater resources.

Interestingly, although there were significant differences between the two habitats for soil and vegetation C and N content and C:N ratios there were very few differences between the soil fauna. These similarities in C, N, and C:N between habitats implies that the constitutions of the fauna are relatively constant across space and feeding guild. Comparison of the C:N ratio results found through mass spectrometry, to the seminal paper published by Hunt et al., (1987), which stated that C:N ratios of both Collembola and Acari were eight, highlights differences that may affect some of the many models and papers which have used this data (e.g. De Ruiter et al., 1993; De Ruiter et al., 1998; Moore et al., 2005). The paper by Hunt et al., (1987), has since been cited nearly 300 times. The C:N ratios for all Acari were significantly different to those stated by Hunt et al., (1987), as were the Collembola in the grassland. Whilst most Collembola in the woodland, and Nematodes in both habitats, were found to be similar to the C:N ratios stated by Hunt et al., (1987). The significant differences found here could have a large bearing on the overall impact of soil fauna on the global C and N cycle. However, caution needs to be used when considering the percent C and N values of the Nematodes, as they were much lower than expected (around 10% C and 1% N), this could be due to the mineral gut contents, additional soil or salt crystals within the samples affecting the overall weight.

Preliminary analysis of the stable isotope composition of the soil fauna was performed before the habitats were calibrated as there was no difference in the vegetation signatures. Whilst the C and N contents of the soil fauna were similar between habitats, the stable isotope values for many groups of the soil fauna were not. Some of these differences may be due to different feeding preferences, different species assemblage or different life stage, but it is difficult to compare without calibrating one habitat to the other. The $\delta^{13}C$ signature of a habitat is considered to be "ecosystem specific" although there are only small differences between ecosystems with the same

plant type (Peterson et al., 1987). There are large differences between ecosystems with different plant types (C_3 or C_4) (Albers et al., 2006) or different environmental conditions (Ekblad et al., 2005).

Post calibration the isotope signatures became more obvious, these differences in delta values allude to different feeding preferences of the invertebrates occurring between the two habitats. Looking at the overall isotopic composition of the soil fauna (Figure 4.6) the two habitats are relatively separated by their $\delta^{15}N$ values. These results have a striking similarity to those described by Hobson (1999), who investigated the isotope signatures of songbirds in agricultural wetlands and boreal forests. Clearly this separation of delta values globally across habitats has important implications for stable isotope ecology studies where many samples are analysed (e.g. Kohzu et al., 1999).

Differences in delta values can imply feeding guild; mycotrophs/phycophages are thought to have the lowest $\delta^{15}N$ values – lower than plant litter, suggesting the organisms are feeding on algae or lichens (Schneider et al., 2004; Tiunov, 2007). Algae and lichens are unlikely to be present in grassland systems, and the results reflect this as there were no grassland invertebrates with $\delta^{15}N$ signatures lower than plant litter. Whilst in the woodland both Collembola: Symphypleona and snails had lower $\delta^{15}N$ signatures, suggesting they may consume algae and lichens.

The main food source of primary decomposers is dead plant litter and the $\delta^{15}N$ signatures are usually found to be very similar to this (Illig et al., 2005). Noticeably within the grassland habitat there appears to be very few "litter" feeders, with the majority of organisms forming a continuum with delta values greater than soil. Whereas in the woodland a different food web emerges with the majority of invertebrates clustering (and forming a continuum) from litter to soil. The secondary decomposers main food sources are thought to be humified plant materials or the microbial community associated with plant litter and detritus (Hyodo et al., 2010). Secondary

decomposers' delta signatures are usually enriched by 1-3‰ more than plant litter (Tiunov, 2007), in the grassland the majority of invertebrate "decomposers" tested had delta signatures greater than plant litter, whilst in the woodland these secondary decomposers were less common.

A study by Bonkowski et al., (2009), found the majority of soil invertebrates to be relying on C inputs from roots, breaking with the dogma that soil food webs are fuelled by plant litter inputs from above ground. The results here agree with this, particularly in the grassland, where the stable isotope signatures suggest that the majority of soil fauna appear to be utilising other sources than litter.

Potentially where the invertebrate orders are significantly different from one another (Table 4.5), they are utilising different food sources within each habitat or there are differences in fractionation between the individual species within each group (Tiunov, 2007). Usually within an ecosystem there is little variation between C isotopes and a $\geq$ 5‰ difference is used to distinguish between food sources (Staddon, 2004). Here, there is around 5‰ difference in $\delta^{13}C$ signatures in the grassland – suggesting all the invertebrates are utilising the same baseline food source (Figure 4.9a). However, there is a 12‰ difference in $\delta^{13}C$ signatures in the woodland, indicating that there is a more complex food web existing within this habitat. The woodland food web appears to be based on more than one primary resource (Pollierer et al., 2009), providing for niche diversification at the base of the food web (Layman et al., 2007). The differences in $\delta^{13}C$ signatures agree with the distribution of primary and secondary decomposers within the woodland, suggesting that within this food web there are soil-feeders and litter-feeders, as well as secondary decomposers. Predators are thought to have the highest $\delta^{15}N$ signatures within the food web, here assessment of "predators" would indicate that the Mesostigmatid mites in the grassland were predators, which agrees with Koehler (1999). Other known predators such as Chilopoda: Geophilomorpha or

pseudoscorpions had lower $\delta^{15}N$ signatures than some decomposers; looking at the $\delta^{15}N$ signatures alone therefore would not have classified them as predators. However, the lower $\delta^{15}N$ signatures could be due to their feeding preferences in that habitat, where all $\delta^{15}N$ signatures were much lower and their main food source was a litter feeder/primary decomposer. These results agree with other stable isotope assessments of the soil food web where predators range from about 2‰ greater than soil (Halaj et al., 2005) to around 10‰ greater than the litter layer (Ponsard et al., 2000). The continuum of predatory species also needs to be accounted for when discussing differences in stable isotope signatures between the different trophic levels.

To confirm whether literature classifications of invertebrates at particular trophic levels were accurate, these results were grouped to form "trophic levels" and proved to be acceptable means of defining this food web. This is considered to be classifying the organisms *a priori* (Eggers et al., 2000), which could lead to wrong conclusions being drawn regarding the true trophic structure of a community. However, because it was performed post-stable isotope analysis (rather than lumping the organisms together before mass spectrometry), it provided insight into the true functional role of the organisms (without masking individual results). These results highlight the possible differences in food source at each trophic level. The primary decomposers are likely to be utilising plant litter or soil whilst the secondary decomposers are more likely to be utilising the microorganisms growing on the plant litter and/or soil. These differences increase the amount of fractionation occurring resulting in different delta signatures between trophic levels.

The difficulty within the study of soil food webs is disentangling the different individual feeding preferences, from the general trophic levels. Here the trophic levels can be seen, but the number of linkages between different organisms within the food web is unknown. Polis (1991) depicted how a desert food web is much more

complicated than the food web literature suggests (e.g. Hunt et al., 1987), with a greater number of total actual linkages and a greater number of linkages between species. Estimating the number of trophic links within each food web (comparing the number of different taxa (Table 4.1) with hypothesised trophic links) the results agreed with Polis (1991), rather than Hunt et al., (1987), with over 70 trophic links in both the grassland and the woodland, and had more than 4 links per group in both habitats.

There is a gap in the current understanding of stable isotope ecology linking the relationship between individual species and trophic level variation with the connectivity of food webs (Vanderklift et al., 2003). There is still limited agreement about how much fractionation occurs per trophic level for $\delta^{15}N$ within the soil food web. Historically it was thought to be 3.4‰ (DeNiro et al., 1981; Minagawa et al., 1984), but recent studies suggest it is closer to 2‰ (McCutchan et al., 2003), particularly when analysing the food web in the field (Illig et al., 2005). It is likely this difference in delta signatures across trophic levels within the soil food web is due to the mixing of food within the environment, with all "waste" being utilised by other organisms increasing the potential for mixing the isotopic signatures. Furthermore, although it is known that the soil biota play integral roles in biogeochemical processes there is limited understanding in the global patterns of community structure (Fierer et al., 2009).

4.5 CONCLUSIONS

The results from this community assessment have shown differences between the two habitats, in invertebrate numbers, biomass and stable isotope signatures. These differences have occurred due to the changes in management practice that has happened over the past 20 years. A first step in understanding the subtle differences in the soil

food web within these two habitats has begun. Nevertheless, only a more empirical approach will be able to distinguish the different links between the soil fauna.

While the method of stable isotope analysis at natural abundance allows detection of trophic niches, it is of limited use for identifying precise food resources (Maraun et al., 2011), therefore there is the need to manipulate stable isotopes to start to track the trophic transfers *in situ*. Further experiments within this thesis will expand on this avenue of investigation and try to track the transfer of C and N through the different organisms within the soil food web, from a known starting point.

Chapter 5: Introduction of Enriched Bacteria into the Soil Food Web

(Part of this Chapter is reproduced from Crotty et al., (2011) Rapid Communications in Mass Spectrometry 25: 1503-1513)

5.1 INTRODUCTION

Soil animals are notoriously flexible in their diet, making it difficult to assign a certain species to just one trophic level (Scheu, 2002). In fact the majority of fauna within soil food webs have been shown to be mainly generalist feeders (Schneider et al., 2005a), due to the opacity of the environment and diversity of species, many different species have been lumped together within the same feeding guild, trophic level (Solow et al., 1998) or energy channel.

As previously discussed, the soil decomposer food web is thought to have two main energy channels (Moore et al., 1988a) beginning with fungi or bacteria. Testing the utilisation of these energy channels has often involved laboratory feeding preference tests or microcosm studies, but these only assess single points in time and space within one food web, rather than the whole feeding assemblage. Stable isotopes can provide a time-integrated measure of the trophic position of soil animals (Pollierer et al., 2009). Stable isotopes at natural abundance have been used to study feeding preferences of the soil fauna (Schmidt et al., 2004) (previously discussed in Chapter 4), but are limited in defining the C and N transfers between the microbial soil component and the decomposers. Some studies are beginning to try to differentiate this (e.g. Paterson et al., 2008b), but only look at one order of invertebrates within the soil food web, not the whole.

Methods were developed to enrich bacteria (Murray et al., 2009, and explained in Chapters 2 and 3), to trace the passage of ^{13}C and ^{15}N through the soil invertebrate

food web. In order to investigate the flow of C and N from the microbial step upwards, Murray et al. (2009) introduced an enriched bacterium and added this to grassland soil managed with two different nutrient regimes. In this thesis, the flow of C and N through the soil food web was tracked, through the addition of a highly ^{13}C and ^{15}N enriched native bacterium – *Pseudomonas lurida,* introduced to soil cores from two different habitat managements, a grassland and a woodland with the same basal soil type, both of which 20 years ago had been managed as grassland. This allowed the bacterially-derived C and N to be traced, facilitating some differentiation of the food chains and trophic levels occurring *in situ* in the test sites. Potentially, revealing whether the organisms function at the same trophic level independent of habitat type.

5.2 MATERIALS AND METHODS

5.2.1 Soil preparation

Intact soil cores (10 cm Ø, 10 cm deep) were taken from the permanent grassland and willow woodland sites (Rothamsted Research North Wyke), see Chapter 2.2.1 for full details. The cores were removed by driving individual polypropylene sleeves into the soil, to retain the entire faunal assemblage within the core and to leave the flora remaining intact on top of the core. Each core was stored within an individual Sun bag within a controlled environment chamber (see Chapter 2.2.1), to stabilise for seven days, prior to bacterial introduction and invertebrate extraction.

5.2.2 Bacterial culture and growth conditions

The naturally occurring bacterium *Pseudomonas lurida* originally isolated from the rhizosphere of a grassland soil at North Wyke was used in this study. The bacterial culture was prepared and introduced to the experimental soils using the method

developed by Murray et al., (2009) and according to Chapter 2.2.2. The enrichment was tested by sub-sampling the bacterial solution, and analysing with a stable isotope analyser mass spectrometer (as per Chapter 2.2.6). Prior to introduction to soils, the enriched bacterial culture was resuspended in sterile $^{1}/_{20}$ strength Ringer's solution for introduction to soil microcosms.

5.2.3 Inoculation

The cores were inoculated with the bacterial culture using a modified soil injector (Hatch et al., 2000) (Figure 5.1), consisting of 13 evenly spaced needles connected to a manifold and syringe. Depressing the syringe delivered the inoculum as the needles were withdrawn from the soil, delivering the inoculum in a regular distribution throughout the soil core matrix.

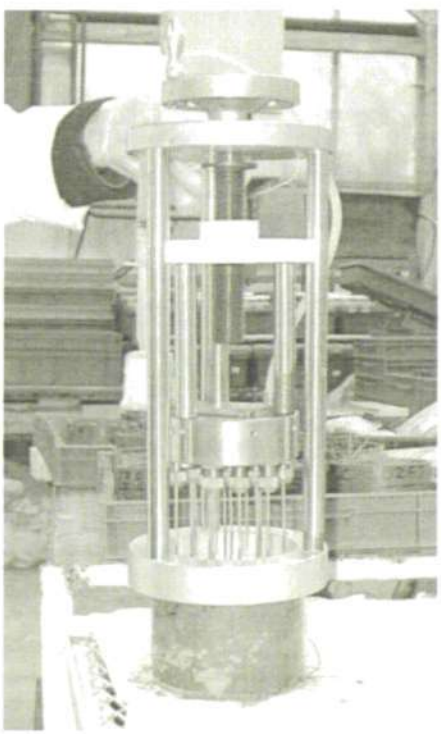


Figure 5.1: Photograph of soil injector used to inoculate soil cores with enriched bacterial solution.

Using this method 13.5 ml of bacterial suspension was delivered to each of the 15 cores from the two sites, inoculating approximately 3 x 10^7 cells g^{-1} soil.

5.2.4 Sampling

Post-inoculation, the cores were held under the same conditions as pre-inoculation. Five cores were destructively sampled immediately post-inoculation (D0) and five after a further six days (D6) and another five after eleven days (D11). Prior to each sampling the soil respiration was measured, by capping the cores and taking a sample of the resulting headspace atmosphere, according to the methods described in Chapter 2.2.3.

On each of the sampling days, the vegetation from each core was cut to ground level, dried at 105°C for 24 hours before being ground in a ball mill for 3 minutes prior to analysis by mass spectrometry. The core was removed from the plastic sleeve and a vertical slice (approximately 150 g) was removed and homogenised. The slice was taken from the centre of the core to the edge and encompassed approximately 15% of its circumference. Of this homogenised sample, 10 g was used for bacterial plate counts, 100 g for nematode extractions, and 40 g for dry weight and isotope analysis. On D6 and D11, nematodes were extracted using a similar method to the direct extraction of mobile nematodes described by Whitehead and Hemming (1965) and adapted by Bardgett et al., (1997), see Chapter 2.2.7 for full description of methods.

The remainder of the core was placed on a Tullgren funnel system and the invertebrates were extracted, as described in Chapter 2.2.8. Due to time constraints the cores were held on the Tullgren funnels for a total of 5 days only. Cores sampled on D6 and D11 were a composite from the 5 days of extraction (i.e. D6 equated to days 6–11; whilst D11 were days 11–15). Invertebrates were extracted and groups identified and separated under a microscope prior to drying and mass spectrometry (see Chapter

2.2.8). Samples for stable isotope measurement were transferred to previously weighed tin capsules (8 x 5 mm, Elemental Microanalysis Limited) and dried at 65°C. After 48 hours the samples were weighed and $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratios were measured. In order to determine the natural abundance of the stable isotopes for the different groups, samples of soil and invertebrates were extracted from each site, as above and analysed by mass spectrometry, from six cores (from each field site) that had not been inoculated with the labelled bacterium.

5.2.5 Bacterial Plate Counts

The abundance of the introduced bacterium in microcosms on each sampling day was determined using bacterial plate counts (Chapter 2.2.4). Bacterial colonies were counted after incubation at 27°C for three days.

5.2.6 ^{13}C and ^{15}N analysis

The invertebrate stable isotope concentrations were determined using an elemental analyser connected to an isotope ratio mass spectrometer (Chapter 2.2.5). The isotope abundance in the plant, soil, headspace gas and invertebrate samples are expressed as Atom% Excess (*APE*) ^{13}C or ^{15}N calculated using equations 1 and 2 (Chapter 2.2.5). The total amount of ^{13}C and ^{15}N of bacterial origin was calculated using equations 3 and 4 (Chapter 2.2.5). IsoError 1.41 (Phillips et al., 2001) source partitioning was performed on those fauna most enriched, this used a two source mixing model which included a discrimination factor to assess the overall contribution of bacteria to the diet.

5.2.7 Statistical analysis

Population data were normalised by the transformation log(x+1) prior to analysis. A general analysis of variance (ANOVA), with time and habitat as factors was used to assess differences between the two systems. Fisher's protected least significant difference test was used to assess the level of variance, when time as a factor was significantly different in the analysis of variance ($P < 0.05$). Fisher's unprotected least significant difference test was used when time as a factor was not significant at the 5% level, but had a P value less than the 10% level. The level of enrichment from bacterially-derived ^{13}C and ^{15}N was assessed using Student's T-test, to perceive whether the *APE* level was significantly greater than zero, and therefore greater than natural abundance. Multivariate analysis of variance (MANOVA) of the *APE* ^{13}C and ^{15}N, using habitat and day as the main factors was also used. A Lorenz curve analysis combined with a Gini coefficient and Chi squared analysis assessed the inequality between the variable amounts of ^{13}C and ^{15}N of bacterial origin obtained by the individual invertebrates within the grassland and woodland soils.

5.3 RESULTS

5.3.1 Bacterial survival and soil characteristics

There were no significant differences between the survival of the bacteria in the soil from the grassland and woodland habitats. The reduction in bacterial numbers (through plate counts) over time between D0 and D11 was not significant.

There were significant differences in $^{13}CO_2$ headspace atmosphere between the different habitats ($F_{1,24} = 27.43$ $P < 0.001$) and this was also significant over time ($F_{1,24} = 35.22$ $P < 0.001$), (Figure 5.2). For both habitat types there was a greater amount of ^{13}C atom% in the headspace atmosphere collected on D0, in comparison to D6 and D11

($P < 0.001$). The amount of $^{13}CO_2$ in the headspace atmosphere was significantly greater for the grassland than the control on D0 ($P < 0.001$), but not for D6 and D11, whereas the woodland $^{13}CO_2$ results are significantly greater than the control on all sampling days ($P < 0.001$).

Analysis of the soil characteristics showed that there was a significant difference between the grassland and woodland habitat for C and N content, with the grassland having a higher C and N content (7.0% ± 0.13 C and 0.7% ± 0.01 N, C:N ratio of 10) when compared to the woodland (5.0% ± 0.14 C and 0.5% ± 0.01 N, C:N ratio of 11), ($F_{1,28} = 51.98$ $P < 0.001$ and $F_{1,28} = 170.78$ $P < 0.001$ respectively), although C:N ratios appear similar for the two habitats, they were also significantly different ($F_{1,20} = 9.39$ $P = 0.006$).

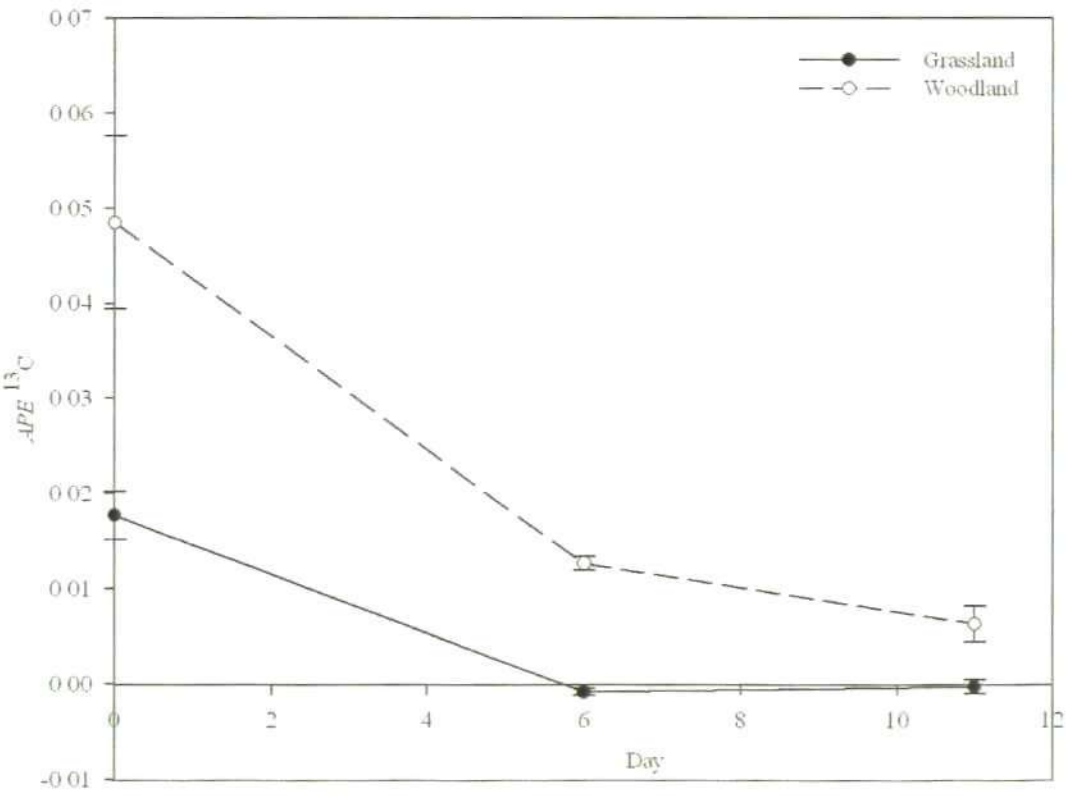


Figure 5.2: Change in $^{13}CO_2$ atom% excess enriched bacterial respiration in soil over time. Data presented as mean ± standard error (n = 5).

The variation in C and N content of the soil before addition of the labelled bacteria or immediately afterwards, or by the end of the experiment was not significantly different. Although there was a significant difference in both ^{13}C and ^{15}N between the control and the soil after enriched bacteria were added to both habitats ($F_{1,28} = 10.16$ $P < 0.001$ and $F_{1,28} = 6.09$ $P = 0.003$ respectively). This difference in the overall amount of ^{13}C in the soil decreased over time though (Table 5.1). The change in *APE* for ^{13}C was significant for both habitats ($F_{1,20} = 32.75$ $P < 0.001$) and time ($F_{2,20} = 5.56$ $P = 0.012$). The difference in amount of ^{13}C (µg) found in soil was significant for both habitat ($F_{1,20} = 21.66$ $P < 0.001$) and time ($F_{2,20} = 3.56$ $P = 0.048$), with greater quantities found in the woodland, although this reduced over time.

			Grassland	Woodland
a)	^{13}C (µg)	Day 6	907 (± 152.5)	1471 (± 39.4)
		Day 11	529 (± 286.6)	1256 (± 83.6)
Soil				
	^{15}N (µg)	Day 6	353 (± 62.4)	344 (± 40.1)
		Day 11	400 (± 231.6)	284 (± 42.6)
b)	^{13}C (µg)	Day 6	1.87 (± 0.437)	4.21 (± 1.801)
		Day 11	5.10 (± 2.941)	4.80 (± 0.732)
Plant				
	^{15}N (µg)	Day 6	1.79 (± 0.198)	1.09 (± 0.353)
		Day 11	2.10 (± 0.265)	1.02 (± 0.410)

Table 5.1: Total quantity of bacterially-derived isotopic enrichment of a) soil cores and b) plant material over time. Data presented as mean ± standard error (n = 5).

The differences in *APE* ^{15}N and amount of ^{15}N (µg) between the two habitat types and over time were not significant (Table 5.1) with relatively similar amounts in both habitats and variable fluctuations over time. The amount of ^{15}N within the soil

represents between 30 – 41% of the total ^{15}N added to the soil cores (as calculated through mass balancing) over time. This is relatively low, and could be due to leaching of the bacterium through the soil cores during inoculation.

Significant differences in C and N content of the vegetation were detected ($F_{1,28}$ = 15.07 $P < 0.001$ and $F_{1,28}$ = 26.93 $P < 0.001$ respectively), with the grassland having a higher C and N content (40.3% ± 0.17 C and 2.0% ± 0.09 N, C:N ratio of 20) when compared to the woodland (35.6% ± 0.99 C and 1.5% ± 0.04 N, C:N ratio of 24). It was also found that this difference changed significantly over the time of the experiment for both C content ($F_{3,28}$ = 3.40 P = 0.031) and N content ($F_{3,28}$ = 4.47 P = 0.011). There was a change in isotopic composition of the vegetation from both habitats over the time period of the experiment (Figure 5.3), with significant differences for *APE* ^{13}C ($F_{2,20}$ = 3.63 P = 0.045) but not *APE* ^{15}N ($F_{2,20}$ = 2.34). There was no difference in the *APE* ^{13}C and ^{15}N results when comparing the two habitats to each other.

Analysing the habitats individually, plant material from the grassland increased significantly in *APE* ^{13}C and ^{15}N over time ($F_{2,23}$ = 5.33 P = 0.013 and $F_{2,23}$ = 6.14 P = 0.007, respectively). The vegetation was most enriched at D11, and was significantly different in both *APE* ^{13}C and ^{15}N to D0, but only significantly different to D6 for *APE* ^{15}N (Figure 5.3a and b). Plant material from the woodland did not increase significantly in *APE* ^{13}C over time ($F_{2,23}$ =3.11 P = 0.064), nor *APE* ^{15}N, although the average D11 *APE* ^{13}C was significantly different to the other days (Figure 5.3c and d). The amount of bacterially-derived C found in the vegetation was not significantly different between the two habitats (Table 5.1), but there was a significant difference between the two habitats for the amount of bacterially-derived N found in the vegetation ($F_{1,20}$ = 5.04 P = 0.036), with greater amounts in the grassland.

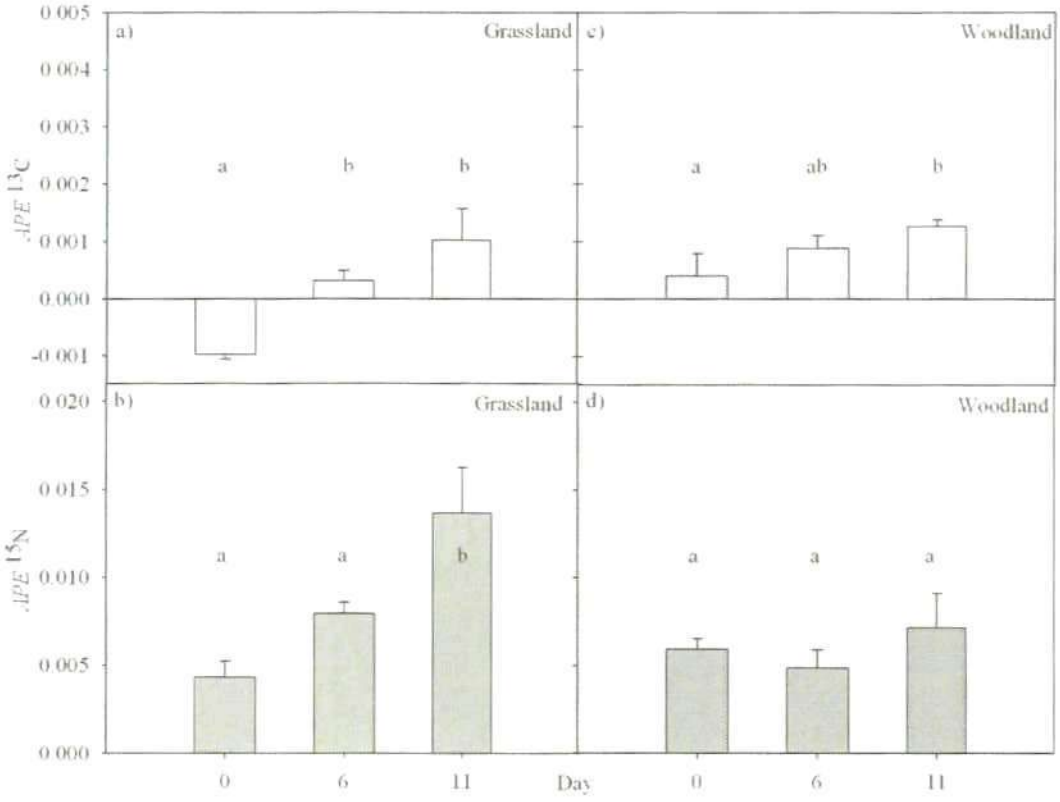


Figure 5.3: Changing isotopic composition of vegetation over time, average *APE* ^{13}C and ^{15}N (± standard error, n=5). a) Grassland *APE* ^{13}C; b) Grassland *APE* ^{15}N; c) Woodland *APE* ^{13}C; d) Woodland *APE* ^{15}N.

Difference in letters within each individual graph showing significant differences using Fisher's protected/unprotected least significant difference test.

5.3.2 Community Composition of the soil food web

A total of 4593 invertebrates were extracted via the Tullgren funnel system. The majority of invertebrates being extracted were either Collembola (60% of the woodland invertebrates extracted) or mites (46% of the grassland invertebrates extracted) (Table 5.2). The proportion of biomass however is mainly biased towards the macrofauna – earthworms, Tipulidae larvae and other dipterous larvae.

There were no significant differences between sampling days for the number of invertebrates extracted for all the different taxa. There were some differences between habitats, with the Poduromorpha, Enchytraeidae and dipterous larvae being extracted in significantly lower numbers in the grassland (Table 5.2). Whereas the Acari: Mesostigmata and Prostigmata were extracted in significantly lower numbers in the woodland (Table 5.2).

5.3.3 Bacterial C and N incorporation into the soil invertebrate food web

Assessing the *APE* level of the invertebrates, it was found that the results were similar for the two habitats, but not all invertebrates were enriched in both ^{13}C and/or ^{15}N, and not all to the same level (Table 5.3 and Figure 5.4 – grassland, Figure 5.5 – woodland). Multivariate analysis of variance for the *APE* ^{13}C and ^{15}N for all invertebrates, showed significant differences between the level of *APE* ^{13}C and ^{15}N between habitats $P = 0.047$. Therefore, the invertebrates in the two habitats appear to be incorporating bacterially-derived ^{13}C and ^{15}N at differing rates, and the relationship between this increasing *APE* ^{13}C and ^{15}N was not uniform between the two habitats.

Assessing the invertebrates in individual groups, the majority of invertebrates extracted showed no significant effect of sampling date or habitat types on the level of enrichment obtained for ^{13}C or ^{15}N (Table 5.3). Although, there were a few exceptions, suggesting that there are differences in the feeding preferences of these organisms in the two different habitats over time. The level of enrichment obtained by Poduromorpha was significantly different for *APE* ^{15}N between the two habitats ($F_{1,5} = 11.79$ $P = 0.019$). The enrichment obtained by oribatid mites was also significantly different between habitats for *APE* ^{15}N ($F_{1,17} = 5.26$ $P = 0.035$), and for *APE* ^{13}C ($F_{1,17} = 7.25$ $P = 0.015$).

Organisms	Grassland	Woodland	*F*-values
Acari: Astigmata	535 (± 361.0)	700 (± 474.6)	0.08
Acari: Mesostigmata	5055 (± 704.3)	1146 (± 306.6)	25.89**
Acari: Oribatida	4584 (± 4189.0)	4189 (± 672.2)	0.30
Acari: Oribatida - Damaeidae	0 0.0	102 (± 67.9)	2.25
Acari: Oribatida - Phthiracaridae	0 0.0	178 (± 124.8)	2.04
Acari: Prostigmata	5997 (± 1628.4)	1770 (± 609.6)	5.91*
Aphids (Hemiptera: Aphidoidea)	242 (± 151.2)	153 (± 82.3)	0.27
Chilopoda: Geophilomorpha	0 0.0	25 (± 17.0)	2.25
Coleoptera Larvae	904 (± 319.6)	866 (± 522.1)	0.00
Coleoptera Larvae: Elateridae	25 (± 25.5)	0 0.0	1.00
Coleoptera Larvae: Staphylinidae	13 (± 12.7)	0 0.0	1.00
Coleoptera: Carabidae	13 (± 12.7)	13 (± 12.7)	0.00
Coleoptera: Ptilidae	38 (± 27.2)	191 (± 69.1)	4.24
Coleoptera: Staphylinidae	89 (± 50.4)	51 (± 20.8)	0.49
Collembola: Entomobryomorpha	12822 (± 2526.1)	8594 (± 3665.4)	0.90
Collembola: Neelipleona	13 (± 12.7)	5449 (± 1050.4)	25.40**
Collembola: Poduromorpha	382 (± 183.0)	3056 (± 1256.7)	4.43*
Collembola: Symphypleona	3705 (± 894.0)	13 (± 12.7)	15.34**
Collembola: Tomoceridae	0 0.0	318 (± 255.5)	1.67
Diptera	76 (± 43.3)	344 (± 131.6)	3.73
Diptera Larvae	13 (± 12.7)	331 (± 95.3)	10.96*
Earthworms	115 (± 35.3)	76 (± 34.0)	0.61
Enchytraeidae	0 0.0	102 (± 45.7)	4.97*
Slugs	76 (± 28.2)	13 (± 12.7)	4.25
Aranea (Spiders)	0 0.0	13 (± 12.7)	1.00
Tipulidae Larvae (Leatherjacket)	13 (± 12.7)	0 0.0	1.00
Woodlice	0 0.0	13 (± 12.7)	1.00

Table 5.2: Population m^{-2} for all invertebrates extracted in the two habitats.

Data presented as mean ± standard error (n=10). *F*-values of a single factor ANOVA * $P < 0.05$; ** $P < 0.001$ indicating significant differences between habitats ($df_{1,18}$)

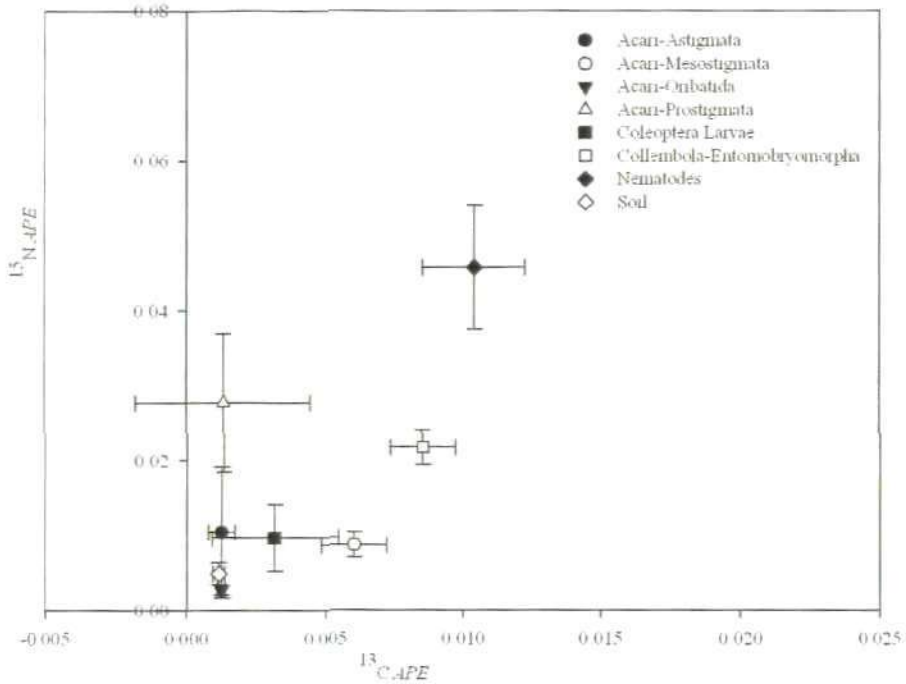


Figure 5.4: Isotopic composition of grassland invertebrates, average *APE* ^{13}C and ^{15}N (± standard errors, n = 2-10, sampling D6 and D11 pooled).

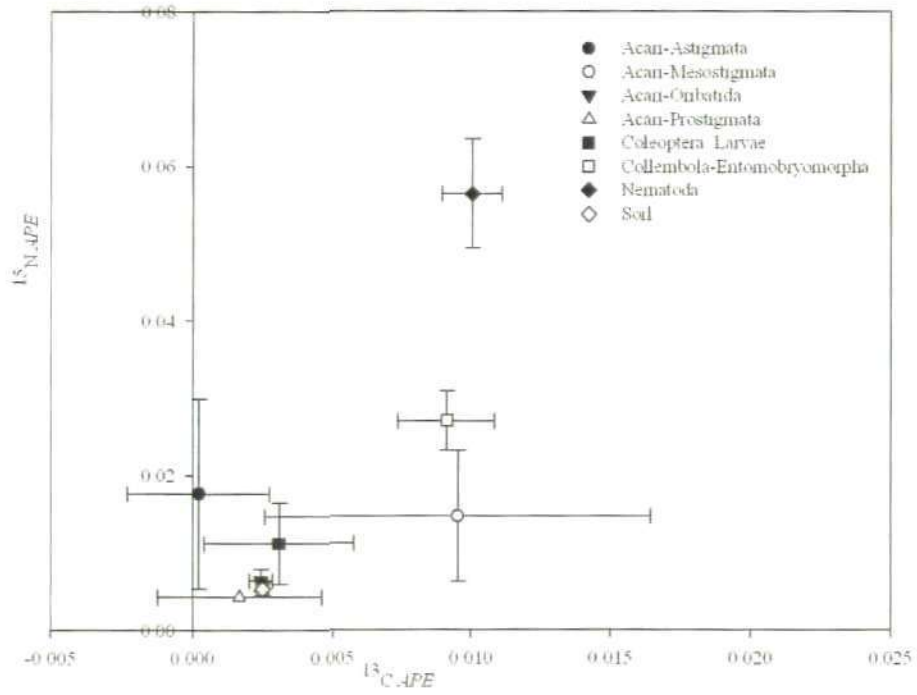


Figure 5.5: Isotopic composition of woodland invertebrates, average *APE* ^{13}C and ^{15}N (± standard errors, n = 2-10, sampling D6 and D11 pooled).

Organism	Grassland ^{13}C T stat	df	Grassland ^{15}N T stat	df	Woodland ^{13}C T stat	df	Woodland ^{15}N T stat	df
Acari: Astigmata	2.64	1	1.2	1	0.09	2	1.44	1
Acari: Mesostigmata	5.09**	9	5.19**	9	1.37	8	1.74	7
Acari: Oribatida	9.14**	8	4.14*	8	6.18**	9	4.53**	9
Acari: Oribatida - Damaeidae	np		np		-6.52	1	14.61*	1
Acari: Oribatida - Phthiracaridae	np		np		0.54	1	1.77	1
Acari: Prostigmata	1.95	7	3.01*	4	0.57	6		
Aphids (Hemiptera: Aphidoidea)	1.22	2	1.43	2	1.35	3	1.50	2
Chilopoda: Geophilomorpha	np		np		0.98	1	5.28	1
Coleoptera Larvae	1.40	4	2.18*	4	1.16	6	2.12	4
Coleoptera Larvae: Elateridae	-2.82	2	-0.05	2	np		np	
Coleoptera: Ptilidae	1.68	1	2.19	1	2.11*	5	4.03*	5
Coleoptera: Staphylinidae	-0.49	3	3.78*	3	1.42	3	2.33	3
Collembola: Entomobryomorpha	7.25**	9	9.43**	9	5.25**	9	7.02**	9
Collembola: Poduromorpha	-1.24	3			2.03*	8	5.51**	5
Collembola: Symphypleona	2.00	5	1.24	1	np		np	
Collembola: Tomoceridae	np		np		3.37	1	16.63*	1
Collembola: Neelipleona	np		np		1.65	5	1.44	1
Diptera	1.07	2	-0.52	2	2.82*	6	0.08	6
Earthworms	3.44*	8	2.07	8				
Enchytraeidae	-1.07	2			4.92*	3	2.21	3
Nematoda	5.61**	9	5.54**	9	9.26**	9	8.00**	9
Slugs	3.35*	7	5.32**	7	3.00	1	3.07	1
Tipulidae Larvae (Leatherjacket)	-25.22	2	-13.99	2	np		np	

Table 5.3: Students' t-test results * $P < 0.05$; ** $P < 0.001$ showing whether level of enrichment obtained from bacterially-derived ^{13}C and/or ^{15}N by invertebrates is significantly different from natural abundance levels, (testing whether *APE* results were greater than zero).

(df = degrees of freedom, np = not present in that habitat).

To test whether there were differences in oribatid mite feeding preferences between habitats or whether it was due to different species assemblages inhabiting the two sites, individual families of some Oribatid mites were analysed separately.

Damaeidae and Phthiracaridae mites were found in the woodland habitat only, as they are both relatively large they would have biased the overall Oribatid bulk sample. Both contained very little bacterially-derived C and N, with *APE* being only significantly different from zero for Damaeidae mites in ^{15}N ($P = 0.022$). Conversely, the bulked oribatid mite sample contained significant amounts of bacterially-derived C and N ($P < 0.001$ for both).

The analysis of variance showed that the effect of time was only significantly different for nematodes (for C $F_{1,16} = 8.24$ $P = 0.011$ and for N $F_{1,16} = 4.84$ $P = 0.043$), with those in the grassland habitat having significantly greater enrichment of ^{13}C and ^{15}N on D6, although D11 is also significantly greater than natural abundance ($P < 0.001$ for both C and N). In the woodland habitat this pattern is found for ^{13}C and ^{15}N but the enrichment between D6 and D11 is not significantly different, both days are significantly greater than natural abundance ($P < 0.001$ for both C and N).

The impact of bacterial consumption on the soil faunal food web was assessed, using the amount of ^{13}C and/or ^{15}N of bacterial origin incorporated by the soil fauna, to clarify which taxa consumed the most bacteria (equations 3 and 4). Multivariate analysis of variance showed that there was no significant difference between the amount of ^{13}C and ^{15}N of bacterial origin incorporated by the invertebrates in either habitat ($P = 0.051$). Although there were some differences when looking at individual orders (Figure 5.6, Tables 5.4 and 5.5), the amount of ^{13}C and ^{15}N of bacterial origin obtained by the Mesostigmata, Oribatida, Entomobryomorpha and Poduromorpha were significantly different between the two habitats (Tables 5.4 and 5.5 – *F*-values).

The Prostigmata had significantly different amounts of ^{15}N of bacterial origin only between the two habitats (Table 5.5 – *F*-values). The amount of ^{13}C and ^{15}N of bacterial origin incorporated by the invertebrates was significantly greater than zero for a similar range of invertebrates in both habitats (Figure 5.6 and Tables 5.4 and 5.5 – *T*-

statistic). Nematodes incorporated the greatest amount of ^{13}C and ^{15}N of bacterial origin in similar proportions in both habitats, whilst the Mesostigmata and Entomobryomorpha were the next to incorporate the most bacterial ^{13}C and ^{15}N, although they incorporated significantly more in the grassland (Tables 5.4 and 5.5 – *T*-statistic).

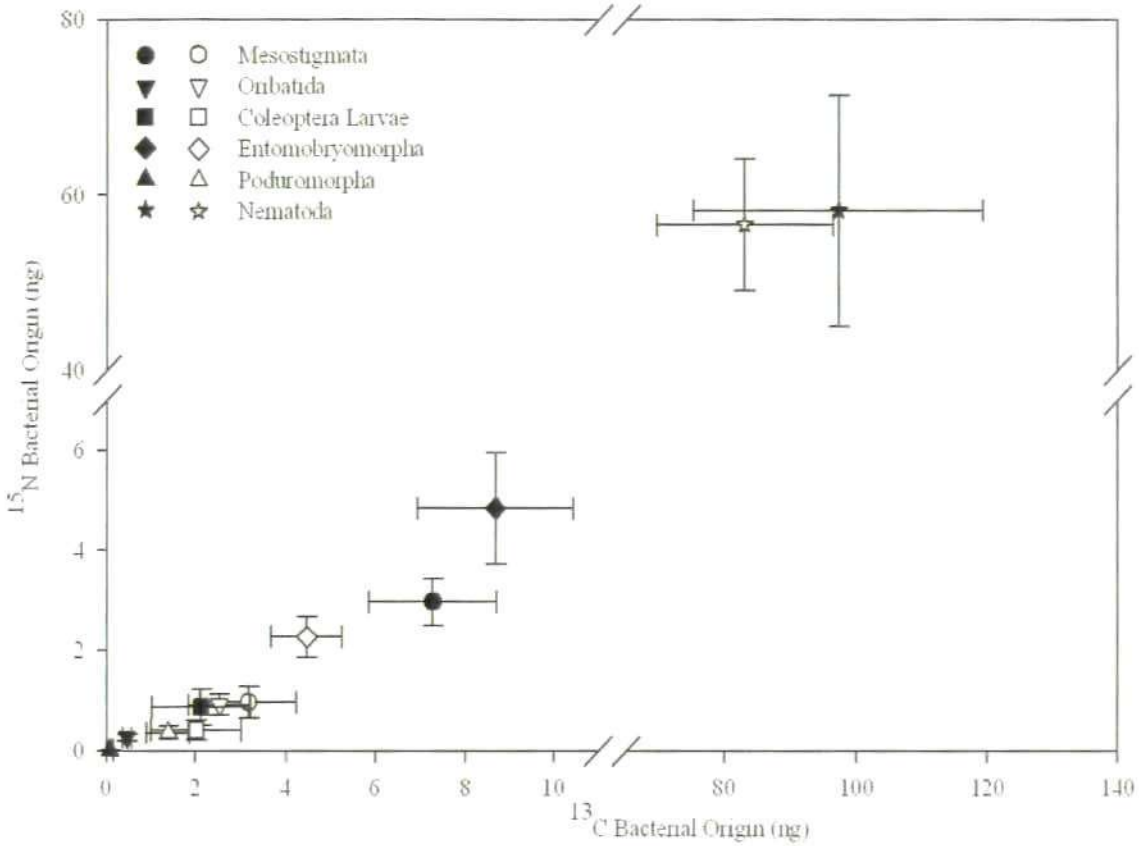


Figure 5.6: Amount of ^{13}C and ^{15}N of bacterial origin in grassland (closed symbols) and woodland (open symbols) invertebrates (ng). Data represented as mean ± standard error (n= 10).

	Grassland		Woodland		
	^{13}C (ng/m^2)	T stat	^{13}C (ng/m^2)	T stat	*F*-values
Acari: Astigmata	6 (± 4.0)	1.49	64 (± 55.3)	1.16	0.20
Acari: Mesostigmata	927 (± 181.2)	5.12**	403 (± 136.1)	2.96*	7.09*
Acari: Oribatida	60 (± 13.8)	4.38**	321 (± 86.4)	3.71*	11.54*
Acari: Prostigmata	77 (± 39.9)	1.92*	17 (± 8.0)	2.16*	1.80
Aphids (Hemiptera: Aphidoidea)	45 (± 35.9)	1.24	29 (± 19.6)	1.50	0.03
Coleoptera Larvae	270 (± 138.3)	1.96*	256 (± 127.0)	2.01*	0.00
Coleoptera: Ptilidae	16 (± 13.7)	1.18	226 (± 157.7)	1.44	2.98
Coleoptera: Staphylinidae	9 (± 9.4)		62 (± 40.9)	1.51	2.76
Collembola: Entomobryomorpha	1106 (± 222.7)	4.97**	568 (± 101.3)	5.61**	4.75*
Collembola: Poduromorpha	10 (± 10.3)	1.00	177 (± 61.0)	2.90*	10.06*
Diptera	137 (± 112.6)	1.22	315 (± 118.4)	2.66*	2.53
Nematoda	124000 (± 28000)	4.43**	106000 (± 17000)	6.20**	0.04

Table 5.4: Amount of ^{13}C of bacterial origin for selection of invertebrate groups per m^2 (ng).

Data presented as mean ± standard error (n=10). Student's t-tests where performed to assess whether the amount of ^{13}C of bacterial origin was significantly greater than zero (df_9) from core results, *F*-values of a single factor ANOVA indicating whether there are significant differences between habitats ($df_{1,18}$). (* $P < 0.05$; ** $P < 0.001$).

	Grassland		Woodland		
	^{15}N (ng/m^2)	T stat	^{15}N (ng/m^2)	T stat	*F*-values
Acari: Astigmata	7 (± 6.2)	1.15	27 (± 21.1)	1.49	0.02
Acari: Mesostigmata	378 (± 59.5)	6.36**	123 (± 40.9)	3.01*	13.83*
Acari: Oribatida	33 (± 8.6)	3.86*	117 (± 26.2)	4.44**	12.11*
Acari: Prostigmata	74 (± 30.9)	2.40*	2 (± 2.1)	1.00	6.02*
Aphids (Hemiptera: Aphidoidea)	176 (± 164.8)	1.07	47 (± 24.0)	1.95*	0.10
Coleoptera Larvae	110 (± 45.8)	2.41*	52 (± 25.5)	2.03*	1.38
Coleoptera: Ptilidae	5 (± 3.3)	1.49	63 (± 35.3)	1.79	4.05
Coleoptera: Staphylinidae	6 (± 5.8)	1.49	9 (± 5.4)	2.05*	0.44
Collembola: Entomobryomorpha	617 (± 140.8)	4.38**	289 (± 52.2)	5.54**	6.00*
Collembola: Poduromorpha	1 (± 1.4)	1.00	46 (± 16.8)	2.73*	7.84*
Diptera	3 (± 3.3)	1.00	13 (± 4.2)	3.05*	3.29
Nematoda	74000 (± 16800)	4.41**	72000 (± 9600)	7.52**	0.39

Table 5.5: Amount of ^{15}N of bacterial origin for selection of invertebrate groups per m^2 (ng).

Data presented as mean ± standard error (n=10). Student's t-tests where performed to assess whether the amount of ^{15}N of bacterial origin was significantly greater than zero (df_9) from core results, *F*-values of a single factor ANOVA indicating whether there are significant differences between habitats ($df_{1,18}$). (* $P < 0.05$; ** $P < 0.001$).

There appears to be a difference in the number of invertebrates within the two habitats and the amount of ^{13}C and ^{15}N of bacterial origin that was incorporated (Figures 5.7 and 5.8). In the grassland there appeared to be a greater variation in the amounts incorporated by the invertebrates, with many incorporating very little or zero and a small number incorporating larger amounts. Whilst in the woodland there were many invertebrates incorporating low levels of ^{13}C and ^{15}N of bacterial origin and less with high amounts.

Comparison of the frequency distribution (Figure 5.7) found that there were significant differences in the amount of ^{13}C obtained by invertebrates between the grassland and woodland soils ($P = 0.014$). This difference was significant for invertebrates obtaining between 0 – 10 ng ^{13}C of bacterial origin ($P = 0.006$). Although above 10 ng (between 10 – 200 ng ^{13}C) there were no significant differences in frequency distribution ($P = 0.380$).

Comparison of the frequency distribution for ^{15}N of bacterial origin obtained by invertebrates (Figure 5.8) showed there were also significant differences between habitats ($P = 0.023$). Again, this difference between habitats was significant for invertebrates obtaining between 0 – 10 ng ^{15}N of bacterial origin ($P = 0.007$), and above this amount (between 10 – 200 ng ^{15}N) there were no significant differences in frequency distribution ($P = 0.299$). There was little difference in the distribution of the Lorenz curve for ^{13}C (^{13}C Grassland Gini coefficient 0.8582, compared to ^{13}C Woodland Gini coefficient 0.8473) (Figure 5.7c insert); or for ^{15}N (^{15}N Grassland Gini coefficient 0.8725, compared to ^{15}N Woodland Gini coefficient 0.8871) (Figure 5.8c insert).

Using Isoerror's two source mixing model to assess the contribution of bacterial C and N involved running the model separately for C and then for N (Table 5.6) and using the natural abundance result for soil as one of the two sources and the enriched

bacteria as the other source. This is somewhat arbitrary as the actual food source for the soil fauna tested (Mesostigmata, Entomobryomorpha and nematodes) may be different or more than one source itself. The results using Isoerror to assess the contribution of bacterial C and N in comparison to the equation 3 and 4 (Chapter 2.2.5) suggests that these equations over estimate the results.

Fauna	Proportion C	C bacterial origin (ng/m^2)	Proportion N	N bacterial origin (ng/m^2)
Grassland				
Mesostigmata	0.0000406 (± 0.0000146)	7.33	0.0001096 (± 0.00002271)	67.75
Entomobryomorpha	0.0000449 (± 0.0000147)	5.71	0.0002283 (± 0.00003895)	93.39
Nematodes	0.0001416 (± 0.0000260)	168.94	0.0004742 (± 0.00010579)	46.86
Woodland				
Mesostigmata	0.0000679 (± 0.0000775)	4.89	0.0001565 (± 0.00007973)	38.75
Entomobryomorpha	0.0000327 (± 0.0000208)	2.26	0.0002550 (± 0.00005227)	70.76
Nematodes	0.0001281 (± 0.0000180)	151.84	0.0005716 (± 0.0001057)	18.07

Table 5.6: Isoerror results for proportion of bacterial C/N in diet and amount this equates to within the organism per m^2 (ng).

Many studies use the isotopic composition of invertebrates at natural abundance to categorise the invertebrates into different trophic levels (see Chapter 4), e.g. as herbivores (T0), primary (T1) or secondary decomposers (T2) and micro- (T3) and macro- (T4) predators. Using these aforementioned classifications the invertebrate *APE* ^{13}C and ^{15}N results were grouped (Brussaard et al., 1997; Hopkin, 1997; Halaj et al., 2005; Krantz et al., 2009) into "trophic levels" T0-4 (Table 5.7) and the differences between their *APE* ^{13}C and ^{15}N were assessed.

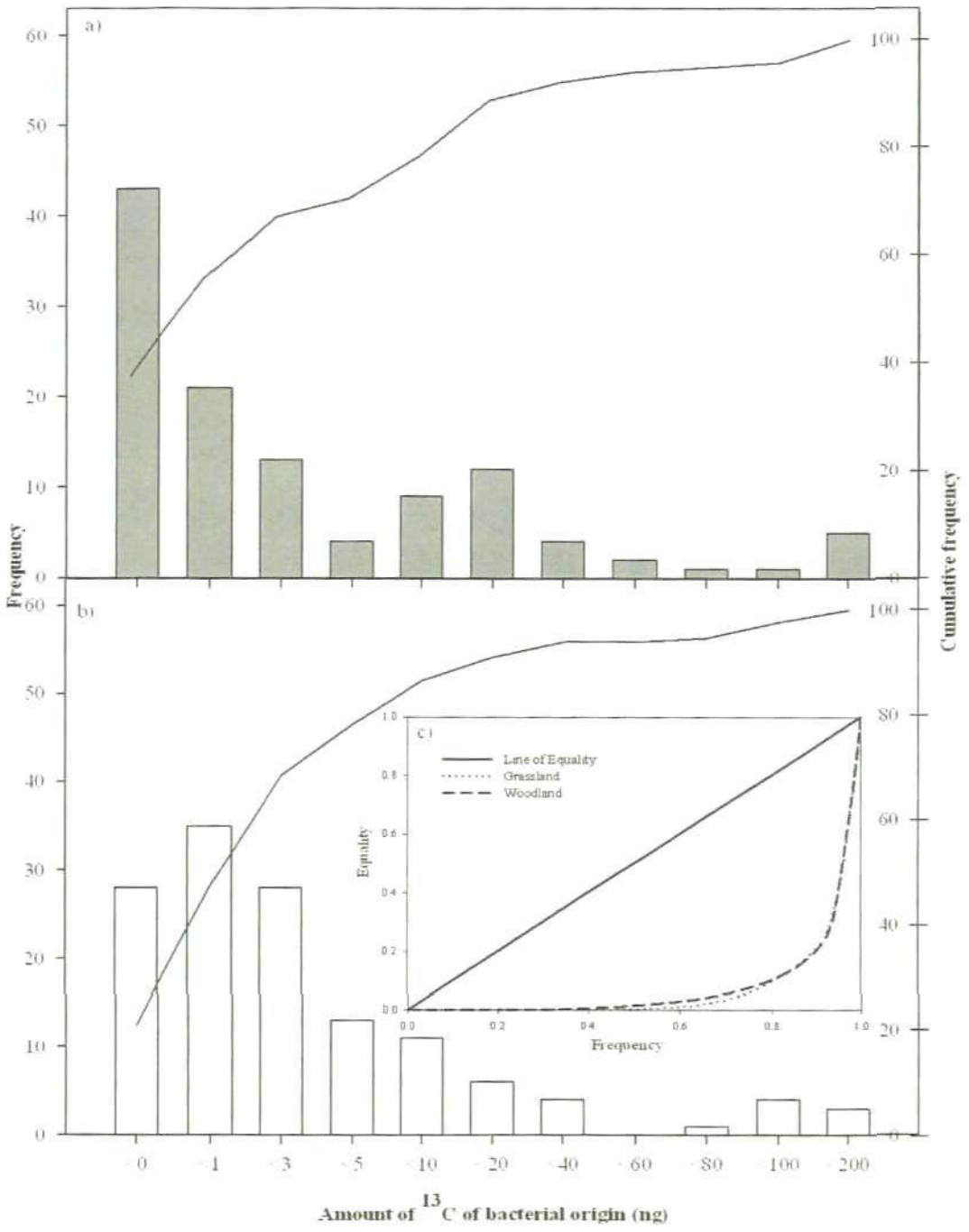


Figure 5.7: Histogram showing frequency and cumulative frequency of the amount of ^{13}C of bacterial origin obtained by invertebrates in a) grassland and b) woodland habitats – looking at the variation in bacterial interaction with the soil fauna as a whole. Insert c) Lorenz curve, grassland (dotted line) and woodland (dashed line) highlighting the moderate differences between habitats.

(Bars represent the frequency of individual results, line the cumulative frequency).

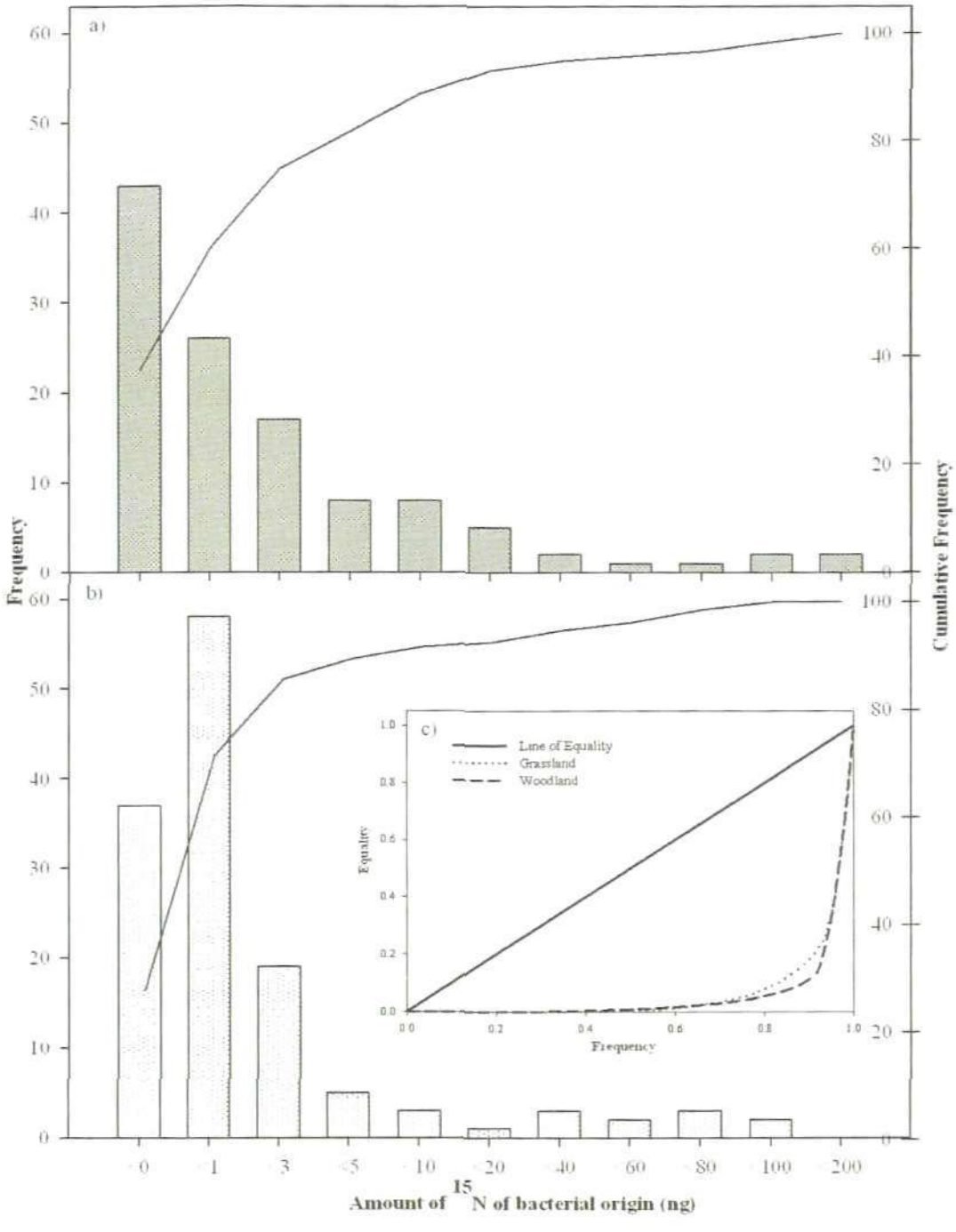


Figure 5.8: Histogram showing frequency and cumulative frequency of the amount of ^{15}N of bacterial origin obtained by invertebrates in a) grassland and b) woodland habitats – looking at the variation in bacterial interaction with the soil fauna as a whole. Insert c) Lorenz curve, grassland (dotted line) and woodland (dashed line) highlighting the moderate differences between habitats.

(Bars represent the frequency of individual results, line the cumulative frequency).

In theory the group which is considered to be "secondary decomposers" (T2) are supposed to base the majority of their diet on microbes (Illig et al., 2005) and therefore should have the highest level of enrichment, in comparison to primary decomposers (T1) (that feed mainly on litter material that is yet to be colonised by microorganisms). There was a significant difference between the grouped "trophic levels" for *APE* ^{13}C ($F_{4,220} = 3.54$ $P = 0.008$) and ^{15}N ($F_{4,184} = 8.34$ $P < 0.001$). There were no significant differences between the habitats for *APE* ^{15}N between these trophic groupings, but there was a significant difference for ^{13}C ($F_{1,220} = 7.77$ $P = 0.006$).

Due to these differences between habitats the grouped "trophic levels" were re-analysed separately for habitat (Figure 5.9), under these conditions, *APE* ^{15}N was significant for both habitats (in the grassland $F_{4,89} = 4.91$ $P < 0.001$ and in the woodland $F_{4,95} = 5.03$ $P < 0.001$) but *APE* ^{13}C was only significantly different for the grouped "trophic levels" for the grassland ($F_{4,103} = 4.77$ $P < 0.001$). The difference in *APE* ^{13}C from the grassland varied between trophic levels (Figure 5.9a), trophic levels T2 and T3 had the highest isotope enrichment and were significantly different from trophic levels T0 and T1 but not T4.

The *APE* ^{15}N from the grassland also varied in enrichment between the trophic levels (Figure 5.9b), and again trophic level T2 showed the highest isotopic enrichment and was significantly different from all other trophic levels apart from trophic level T0. In the woodland the differences between *APE* ^{13}C for the trophic levels were not significant, but again trophic levels T2 and T3 showed the highest enrichment of bacterial origin (Figure 5.9c). The *APE* ^{15}N for the different trophic levels was significant from the woodland, again trophic level T2 showed the highest isotope enrichment and was significantly different from trophic levels T1 and T4 (Figure 5.9d), but not from trophic levels T0 and T3 which had an intermediary level of enrichment.

Organism	Trophic level
Aphids (Hemiptera: Aphidoidea)	0
Coleoptera Larvae: Elateridae	0
Collembola: Symphypleona	0
Slugs	0
Acari: Astigmata	1
Acari: Oribatida - Damaeidae	1
Acari: Oribatida - Phthiracaridae	1
Collembola: Neelipleona	1
Diptera Larvae	1
Earthworms	1
Enchytraeidae	1
Tipulidae Larvae	1
Woodlice	1
Acari: Oribatida	2
Acari: Prostigmata	2
Coleoptera: Ptilidae	2
Collembola: Entomobryomorpha	2
Collembola: Poduromorpha	2
Collembola: Tomoceridae	2
Diptera	2
Nematoda	2
Acari: Mesostigmata	3
Coleoptera Larvae	3
Coleoptera Larvae: Staphylinidae	3
Chilopoda: Geophilamorpha	4
Coleoptera: Carabidae	4
Coleoptera: Staphylinidae	4
Spider	4

Table 5.7: Groupings of invertebrates used for trophic level analysis.

Trophic level 0 are herbivores, 1 are primary decomposers, 2 are secondary decomposers, 3 are micro-predator, and 4 are macro-predator. (Groupings ordered according to literature (Brussard et al., 1997; Hopkin, 1997; Halaj et al., 2005; Krantz et al., 2009)).

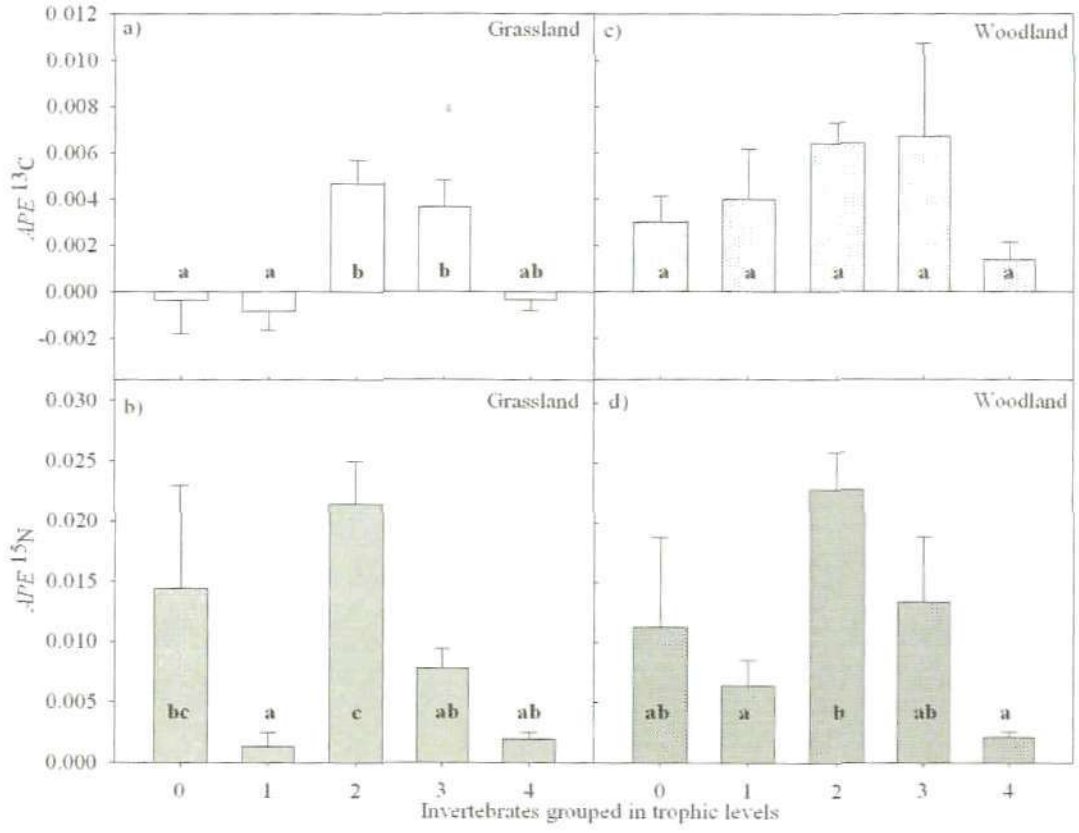


Figure 5.9: Isotopic composition of the grouped "trophic levels" for the different habitats, average *APE* ^{13}C and *APE* ^{15}N (± standard error, n = >4). a) Grassland *APE* ^{13}C; b) Grassland *APE* ^{15}N; c) Woodland *APE* ^{13}C; d) Woodland *APE* ^{15}N. See Table 5.7 for Trophic level groupings TL0 herbivores, TL1 primary decomposers, TL2 secondary decomposers, TL3 micro-predators, and TL4 macro-predators. Difference in letters within each graph showing significant differences between trophic levels – Fisher's protected least significant difference test.

5.4 DISCUSSION

Isotope labelling methods are vital for the understanding of soil food webs, providing a method to differentiate between the major trophic levels thought to occur in soil. This starts to distinguish between the bacterial and fungal based energy channels,

and provides a method to evaluate the importance of bacteria as a basal resource. Here, the flow of ^{13}C and ^{15}N of bacterial origin was tracked through the soil faunal feeding channels. During the timescale of this experiment the soil remained enriched in ^{13}C and ^{15}N due to the addition of the enriched labelled bacterium *Pseudomonas lurida*. This does not signify that all the bacteria added remained viable during this time span, but they were utilisable, either as a direct food source or after mineralisation. There was a reduction in the ^{13}C signal within the soil over time, this could be attributed to the amount lost through bacterial respiration, and also possibly due to assimilation and immobilisation of the ^{13}C in the body tissues of organisms.

The *APE* ^{13}C signature of the headspace CO_2 is a consequence of respiration from the added enriched bacteria. The $^{13}CO_2$ respiration results are different between the two habitats, with a rapid decline in the amount of $^{13}CO_2$ respired in the grassland, compared to a slower decline in the woodland. This decline is due to the initial large addition of living enriched organisms, and natural die-off rates (Kindler et al., 2006). This difference in activity of the microorganisms between the habitats may be because the bacteria in the grassland were multiplying faster, diluting the signal captured from headspace samples. *Pseudomonas* species in the soil obtain the majority of the C sources from roots exudates (Dawson et al., 2004). It is possible that within these two habitats the main C source driving them is different, in the grassland it may be root derived, whereas in the woodland it may be litter derived, and it is this that is reflected in the different ^{13}C gas signals. It would have been advantageous to measure the isotopic composition of roots to clarify this, but unfortunately only above-ground plant material isotopic compositions were obtained.

The vegetation changed in isotopic composition significantly over time. This increase implies that the plants were able to take up mineralised N from the added bacteria – characteristic of the microbial loop (Coleman, 1994). Although these changes

were not consistent between habitat, this may be due to differences in mineralisation rates of the bacteria within the soil, the availability of mineralised N of bacterial origin, or the different plant species present in the two habitats as well as the dominance of bacteria over fungi within the soils.

The invertebrates monitored during this experiment were obtained from intact soil cores, taken directly from the field to assess the movement of bacterially-derived C and N through the invertebrate food web in situ. This approach meant that there were slight variations between the population sizes within individual cores, due to the spatial heterogeneity of soil itself, which is largely affected by the scale of sampling (Benefer et al., 2010). In other studies it has been hypothesised that the heterogeneous distribution of the tracer may also influence the variability in isotopic ratios of soil organisms (Elfstrand et al., 2008). Using a modified soil injector reduces this affect, as it introduces the bacteria throughout the soil cores relatively evenly (Hatch et al., 2000).

The level of enrichment obtained by the invertebrates may be time dependent. However, for the majority of invertebrates there was no significant difference between the sampling days, which could be due to the total experimental time being too short to incorporate a high level of isotope label in all bacterially feeding invertebrates (Dyckmans et al., 2005). Generally the relative strengths of the ^{13}C and ^{15}N signals are not linearly related as shown by the multivariate analysis of variance results. There is a greater increase in ^{15}N enrichment in comparison to ^{13}C, in both habitats, although to a greater extent in the grassland. This trend is reversed when looking at the amount of ^{13}C and ^{15}N of bacterial origin incorporated by the fauna, with consistently greater amounts of ^{13}C in comparison to ^{15}N in all invertebrates tested apart from the aphids.

Only three of the invertebrate groupings were significantly enriched in both ^{13}C and ^{15}N atom% in both the grassland and woodland habitats. These were the Entomobryomorpha, Oribatida, and nematodes, implying that these organisms are

consuming the most bacteria within both systems. Bacterial feeding nematodes are found in high numbers within the soil (Ferris et al., 2004), but there are also other feeding specialisms, including plant and fungal feeding. In this study, the nematode samples were not divided into functional groups therefore these results are only an impression of nematode feeding preferences. The high level of enrichment obtained by the nematodes does, though, suggest that there are a large proportion of bacterial feeders within both habitats. Nematodes were the only group to be significantly affected by time during this experiment with greater enrichment earlier in the experiment. This is probably due to the fast turnover rates of the nematodes in comparison to other organisms within this experiment.

Collembola are generally considered fungal feeders, when individual species feeding preferences are considered (Chahartaghi et al., 2005), and in food web diagrams (Hunt et al., 1987; De Ruiter et al., 1993), but the results found here, show that this is not exclusively the case. The superfamily Entomobryomorpha was shown to consume significant amounts of bacterially-derived C and N in both habitats. It is unlikely that bacteria where within the gut contents of the Collembola at the time of sampling as gut transit time is on average one hour (Tebbe et al., 2006). Tomoceridae (a family within the Entomobryomorpha), only show enrichment in ^{15}N in the woodland habitat (not both C and N). The superfamily Poduromorpha were found to have a significant enrichment in *APE* ^{13}C and ^{15}N in the woodland habitat only, suggesting that they have different feeding preferences within the different habitats, possibly acting as primary decomposers in the grassland but as secondary decomposers in the woodland. The Neelipleona and Symphypleona do not show a significant enrichment in C or N in either habitat, indicating that they do not act as secondary decomposers but are more likely to be primary decomposers or possibly even herbivores.

Oribatid mites, like Collembola, are often portrayed in food webs solely as fungal feeders (Hunt et al., 1987), and even when individual species feeding preferences are considered (Schneider et al., 2004). However, the results show that food choice within the soil is more nuanced than this. Oribatida (excluding the families Damaeidae, and Phthiracaridae) were shown to have consumed a significant amount of bacterially-derived C and N in both habitats, whilst the Damaeidae, and Phthiracaridae did not. This implies that Damaeidae mites are more likely to be herbivorous or a primary decomposer. The Phthiracaridae mites *APE* for both ^{13}C and ^{15}N were not significantly different from zero indicating that these mites are primary decomposers consuming plant litter prior to microbial degradation.

The Astigmata did not obtain a significant level of enrichment in either habitat. The level of enrichment obtained by the Mesostigmata was only significant for *APE* ^{13}C and ^{15}N in the grassland habitat. The Mesostigmata are predatory (Koehler, 1999) and therefore were unlikely to gain large amounts of bacterially-derived C and N directly. However, in the grassland habitat they showed a significant enrichment in both *APE* ^{13}C and ^{15}N, either from consumption of the bacteria directly or indirectly through the consumption of microbivorous prey. In the woodland system due to the large variety of predators and prey, the Mesostigmata's feeding preferences were apparently different, possibly consuming the Neelipleona, or the Prostigmata more, which had consumed less enriched bacteria.

The amount of ^{13}C and ^{15}N of bacterial origin obtained by the invertebrate orders (in addition to the analysis of the *APE* results) suggests that bacterivory is more important than indicated by the *APE* results alone. Significant amounts of both ^{13}C and ^{15}N of bacterial origin were incorporated by invertebrates considered "secondary decomposers" (Oribatida, Entomobryomorpha, Poduromorpha, and Diptera) as well as by predators (Mesostigmata, Prostigmata, and Coleoptera larvae), and those considered

microbivores (Nematoda). Generally the invertebrates in the grassland obtained a greater amount of ^{13}C and/or ^{15}N of bacterial origin than those in the woodland (Figure 5.6), although the Poduromorpha and Oribatida are important exceptions. This indicates differences in feeding interactions that are occurring between the two habitats, and that the grassland is potentially more dependent on the bacterial channel.

However, when the Isoerror mixing model was used, incorporation of C and N of bacterial origin appears to be much lower than originally described using the equations without a discrimination factor. It is difficult to assess here, which is more accurate without further experimentation. Previous studies have found Collembola isotopic discrimination to change depending on food source (Ruess et al., 2005b), and not consistently increasing per trophic level (Scheu et al., 2004b). McCutchan et al. (2003) and Caut et al. (2009) found different isotopic shifts in laboratory feeding experiments. Whilst Illig et al. (2005) found primary decomposers not enriched compared to food source (i.e. not discriminating when incorporating food of a different isotopic composition). Only when the isotopic shifts of the soil fauna have been discerned can a discrimination factor be used with any certainty. Here, and within the rest of the thesis the equations from Chapter 2.2.5 will continue to be used within food web experiments, to prevent incorporation of a discrimination factor that is incorrect, when the evidence is currently mixed as to whether it is necessary.

When the organisms were grouped into hypothesised trophic levels (Table 5.7) the function of the organisms and the transfer of C and N through the food web became the focus of the results, rather than looking at the diversity of feeding preferences. First C transfers between the trophic levels appear to have different patterns in the grassland and in the woodland habitats – with only the change in ^{13}C *APE* in the grassland being significant. In the grassland, the secondary decomposers (T2) show a significant level of enrichment, as do the micropredators (T3), compared to the other trophic levels. Similar

results were found in the woodland, but without significant differences. For N however, the pattern was the same between the two habitats – the herbivores (T0) obtained large amounts of ^{15}N. This was most likely through mineralisation and the microbial loop effect (Bonkowski, 2004), whereby ^{15}N from the bacteria are mineralised and utilised by the plants within the cores before the herbivores consume the plant.

Primary decomposers (T1) had significantly less N of bacterial origin, because they consumed fresh dead plant matter which had not been colonised by enriched bacteria during the experiment. The plant litter had also already died prior to the beginning of the experiment therefore did not obtain any N through the microbial loop effect. The secondary decomposers showed the same pattern for N as for C, with the highest level of enrichment of all the trophic levels. Further attention should be given to the food choices found here in future work, as fungi are usually considered to be the main food source of secondary decomposers (Scheu et al., 2000), but may not be under field conditions. These results agree with other investigations where "microorganism" feeders showed differences in enrichment level in comparison to "litter" feeders (Caner et al., 2004). The micro- and macro-predators (T3 and T4) showed a reduction in ^{15}N, due in part to a dilution in bacterial signal with body mass, trophic transfers and the timescale of the experiment. It is likely that the micro- and macro-predators fed on a range of organisms within the soil from herbivores to secondary decomposers, where prey species consumed enriched bacteria prior to being consumed, and it is this signal that is visible in the predators' stable isotope results (Pollierer et al., 2010; von Berg et al., 2010).

5.5 CONCLUSIONS

Isotopic enrichment of a bacterium to track the trophic transfer between organisms within the soil food web is a novel way to elucidate interactions, which otherwise can not be proven. Almost all the invertebrates tested showed some level of bacterial enrichment, implying that bacterial feeding is a common mechanism within the soil. This is conceivable when considering the numbers of bacterial cells available and the limited movement of the organisms. It is difficult to comprehend why bacteria are not considered to be the main food source for more invertebrates. Potentially this misrepresentation of feeding preferences is due to historical methods of testing – including gut content analysis (Berg et al., 2004) and observation (Gunn et al., 1993), as these only record food that has not been digested or what was consumed at the time of observation.

The level of taxonomy within this experiment may have masked the full affect of bacterial feeding preferences amongst the different organisms within the soil food web, as individual species were not tested, therefore there is the potential mixing of feeding guilds. Other studies, looking at the affects of insecticides (Fountain et al., 2007) have shown the importance of identification to the appropriate taxonomic level for biodiversity estimates. Nevertheless, grouping the organism like this enabled the rapid extraction preparation and identification of fauna and enabled the determination of some of the interactions occurring within the soil food web. The amount of bacterially-derived ^{13}C and ^{15}N moving through the different groups of organisms and trophic levels, was also quantified.

The varying level of enrichment obtained by the same taxonomic group in the two different habitats, shows that there is the possibility that the same organisms may have different feeding preferences within different habitats and may even function at

different trophic levels dependent on habitat type, competition and opportunity. Isotopic enrichment of food sources allows the feeding preferences to be tested *in situ* and focus on the different energy channels within the food web to start to assess the intermediary steps from the base of the web, between primary and secondary decomposers and their micropredators. From this first step, the contribution of bacteria and fungi to the decomposer energy channels can begin to be assessed.

Chapter 6: Introduction of enriched protozoa into the soil food web

6.1 INTRODUCTION

Soil protozoa contribute to decomposition, mineralisation and nutrient cycling, and have a direct effect mainly due to the large proportion (~60%) of ingested nutrients excreted back into the soil environment (Bardgett, 2005). They are commonly found at abundances of around 10^6-10^8 cells g^{-1} soil but vary both spatially and temporally (Adl et al., 2005) and are hugely diverse communities (Esteban et al., 2006). Along with nematodes they are one of the most abundant groups of bacterivores within the soil. Although the predators of nematodes are known, for example Acari (Walter, 1988b), Symphyla (Walter et al., 1989b) and Collembola (Read et al., 2006), it is not known which invertebrates are predating on the protozoa.

It has been postulated that there are two main energy channels within the soil – bacterial and fungal (Hunt et al., 1987; Moore et al., 1988b). In the majority of published food webs protozoa are shown as consumers of bacteria and being consumed by nematodes only (De Ruiter et al., 1993). However, Adl & Gupta (2006) suggest this is not necessarily correct and Adl (2003) suggested, from the literature, that insect larvae, earthworms and nematodes were probably the main consumers of protozoa, but this remains untested. It is more probable that there are nested compartments within the soil food web (Pokarzhevskii et al., 2003), and that feeding preferences reflect opportunity as well as predilection.

Stable isotopes can be used to trace the feeding interactions occurring between the soil fauna, with minimal disturbance to the environment. Many studies have utilised enrichment with ^{13}C and ^{15}N stable isotopes as tracers to elucidate the interactions occurring within soil food webs (Leake et al., 2006; Ostle et al., 2007; Pollierer et al.,

2007; Elfstrand et al., 2008). Methods have been developed to enrich bacteria (Murray et al., 2009), invertebrates (Hakvoort et al., 2002; Dyckmans et al., 2005), and plant litter (Schmidt et al., 2001), to trace the passage of ^{13}C and ^{15}N through soil ecosystems. This work has been built upon throughout this thesis, with the development of an "isotopic baseline" (Schmidt et al., 2004), to trace the feeding interactions occurring from a fixed time point. To date, there have been no studies investigating the enrichment with stable isotopes of indigenous protozoa to trace linkages within the soil food web.

There are many studies which have shown soil fauna to consume one organism over another (Lee et al., 1996), or to have particular preferences (Schneider et al., 2005a). Some have shown different utilisation of litter by the fauna in different locations with similar habitats. An example of this is described in a study by Caner *et al.* (2004) where the soil fauna fed preferentially upon microorganisms colonising the litter in a beech stand at one location whilst feeding more on the litter itself at another location. Looking at soil food webs from a historical perspective, the need to model interactions and the necessary simplification has led to large quantities of literature on feeding preferences being ignored. An example is from reviews within the special issue of Agriculture, Ecosystems and the Environment in 1988, both Bamforth (1988) and Edwards and Fletcher (1988) discussed investigations where protozoa are a regular food source for other organisms, particularly earthworms, but this has not been tested empirically, using stable isotope ecology or other methods.

The aims of this chapter were firstly to enrich live indigenous protozoa with ^{13}C and ^{15}N stable isotopes to a detectable level above natural abundance. The second objective was to perceive if they would become integrated into the soil food web, through the measurement of stable isotope signatures of the resident soil fauna and thus the level of consumption of protozoa. This will provide a better understanding of the

trophic links of the soil dwelling protozoa in two systems. Finally, this stable isotope methodology was used to test the hypothesis that the same orders of invertebrates function in the same way independent of habitat type.

6.2 MATERIALS AND METHODS

6.2.1 Site descriptions

Intact soil cores (10 cm Ø, 10 cm deep) were taken from permanent grassland and willow woodland sites (Rothamsted Research North Wyke). Both sites were of the same soil type – Hallsworth series (Harrod et al., 2008) for a full description see Chapter 2.2.1. Each core was stored within an individual Sun bag, in a controlled environment chamber at 18°C for 10 days (as described in Chapter 2.2.1), prior to protozoa introduction and invertebrate extraction.

6.2.2 Protozoa enrichment and inoculation

Ten days prior to the start of soil food web experimentation, protozoa were cultured from both field sites separately, using 1.5 g of soil from each site, added to a conical flask with 70 ml deionised water, and 15 ml lettuce leaf solution (Sonneborn, 1970) [1 large lettuce leaf boiled in 150 ml water for 10 minutes, allowed to cool to room temperature before addition to cultures] and incubated at 18°C for three days. After three days, the protozoa had multiplied to around 4×10^6 ml^{-1} flagellates and 2.5×10^5 ml^{-1} ciliates, this was verified using a haemocytometer (Adl et al., 2005; Adl et al., 2007). The suspension within each flask was divided amongst four flasks and 100 ml sterile yeast-tryptone broth (2 g yeast malt broth (Sigma-Aldrich, St Louis, USA), 3 g tryptone soya broth (Oxoid, Cambridge, UK) per litre deionised water) was added to each flask. After a further three days, the protozoa had multiplied to around 4×10^7 ml^{-1}

of approximately equal proportions of flagellates and ciliates for each flask, this process was repeated to obtain 20 flasks with protozoa, from both field sites, which were incubated under the same conditions.

In order to ensure a high enrichment of the stable isotopes in the protozoa, a combination of two different isotope enrichment techniques was used; consumption of an enriched bacterial food source, and pinocytosis during incubation. To obtain dense cultures of enriched protozoa the forty flasks were centrifuged, so the contents of each flask was split into centrifuge tubes and spun at 360 *g* for 5 minutes. The supernatant was discarded and all sedimented protozoa were combined to normalise protozoa numbers between flasks. The protozoa were then divided between 20 flasks. Each flask contained around 8 x 10^7 ml^{-1} protozoa of approximately equal proportions of flagellates and ciliates. An additional 20 ml yeast-tryptone broth was added to each flask before overnight incubation at 18°C.

After overnight incubation the concentration process was repeated by centrifugation at 360 *g* for 5 minutes, with protozoa being combined to obtain an even protozoan assemblage when divided into 20 flasks with approx 25 ml protozoan solution per flask and 1.6 x 10^8 ml^{-1} protozoa of approximately equal proportions of flagellates and ciliates. To each flask 1 ml of enriched bacterial culture, prepared according to Murray *et al.* (2009) (and Chapter 2.2.2) was added. The bacterial inoculum had been grown with labelled glucose and ammonium chloride as the sole C and N sources, 2.5 g $^{13}C_6$ – glucose ($C_6H_{12}O_6$); 1 g ^{15}N – NH_4Cl (both 99 atom%, SerCon); to become 99 atom% enriched. To enrich the protozoa by pinocytosis, a 0.1 molar solution of $^{13}C_2$ – sodium acetate ($C_2H_3NaO_2$) (99 atom%, SerCon) (only enriched in ^{13}C no ^{15}N) was added to each flask, and all flasks were incubated for 24 hours.

Prior to inoculation of the soil cores, each flask of enriched protozoa was washed by centrifugation at 180 *g* for 5 minutes, the supernatant was removed and the

pellet resuspended in 20 ml lettuce leaf solution before a second centrifugation and resuspension in 20 ml lettuce leaf solution. Due to the motile nature of the ciliates and flagellates the supernatant was recovered and centrifuged again at 360 *g* for 10 minutes, these protozoan pellets were combined and 1.5 ml added to each of the flasks, with each flask obtaining around 4 x 10^7 ml^{-1} protozoa of approximately equal proportions of flagellates and ciliates. The enrichment of the protozoan cell concentration was verified by taking a sub-sample of solution and analysing with a stable isotope analyser mass spectrometer (as described in Chapter 2.2.6).

The soil cores were inoculated with the combined protozoa culture using a modified soil injector (Hatch et al., 2000), designed to deliver the inoculum in regular distribution throughout the soil core matrix. Using this method a 25 ml protozoan suspension was delivered to each of 10 cores from each of the two sites, introducing approximately 1.8 x 10^6 labelled protozoa g^{-1} soil. To check for remaining labelled bacteria in the medium, a 10 µl sub-sample was taken in triplicate, from the enriched protozoa solution at the time of soil core inoculation and was used for bacterial plate counts, as described in Chapter 2.2.4.

6.2.3 Experimental procedures

Immediately after inoculation, the soil respiration was measured, by capping the cores and taking a sample of the resulting headspace atmosphere, according to the methods described in Chapter 2.2.3. The cores were incubated under the same conditions as above for 72 hours before soil respiration was measured again prior to invertebrate sampling.

After 72 hours incubation, the vegetation was cut to ground level, dried and ground before analysis by mass spectrometry. Each core was removed from the plastic sleeve and a vertical slice (approximately 150 g) of the core was removed and

homogenized. Of this homogenized sample, 100 g was used for nematode extractions, and 50 g for dry weight and isotope analysis. Nematodes were extracted using a similar method to the direct extraction of mobile nematodes described by Whitehead and Hemming (1965) and adapted by Bardgett et al. (1997), according to the methods described in Chapter 2.2.7.

The remainder of the core was placed on a Tullgren funnel system and the invertebrates were collected. The cores were held in the Tullgren funnels for a total of 10 days; therefore, invertebrate collections were a composite from the 10 days of extraction. Invertebrates were collected in saturated salt solution to prevent chemical changes to isotopes as described in Chapter 2.2.8. Invertebrate groups were identified and separated under a microscope prior to drying and analysis by mass spectrometry. Separation of invertebrates is described in full in Chapter 2.2.8.

6.2.4 Stable isotope analysis

Samples for stable isotope analysis were transferred to tin capsules and dried at 65°C for 48 hours. Stable isotope concentrations were determined using an elemental analyser linked to an isotope ratio mass spectrometer (Chapter 2.2.5). The isotope abundance in the plant, soil, headspace gas and invertebrate samples are expressed as Atom% Excess (*APE*) ^{13}C or ^{15}N calculated using equations 1 and 2 (Chapter 2.2.5). The total amount of ^{13}C and ^{15}N of protozoan origin was calculated using equations 3 and 4 (Chapter 2.2.5). In order to determine the uptake of isotope by the soil animals it was necessary to determine the natural abundance of the stable isotopes for the different groups. Therefore, samples of soil and invertebrates were extracted as above from cores from each site, that had not been inoculated with the labelled protozoa and the fauna analysed by mass spectrometry.

6.2.5 Statistical analysis

The results were analysed using a general analysis of variance (ANOVA), with habitat as the main factor. The level of enrichment from protozoan derived ^{13}C and ^{15}N was assessed using the Student's T-test, to determine whether the *APE* level was significantly greater than zero, and therefore greater than natural abundance. Multivariate analysis was also used and included Principal component analysis (PCA) to assess the differences between population and biomass within the two habitats. Using PCA with convex hull incorporates the intraspecific variation due to the plasticity and genotypic differentiation into the analysis, whilst being able to compare the variation between two different habitats. Multivariate analysis of variance (MANOVA) of the *APE* ^{13}C and ^{15}N, with habitat as the main factor was also used. A Lorenz curve analysis combined with a Gini coefficient and Chi squared analysis assessed the inequality between the variable amounts of ^{13}C and/or ^{15}N of protozoan origin obtained by the individual invertebrates within the grassland and woodland habitats.

6.3 RESULTS

6.3.1 Protozoa enrichment

The mixed protozoa culture was made up of 1.8×10^6 protists g^{-1} soil and the mean enrichment (± s.e.) was found to be 82.8 atom% (± 2.97) for ^{13}C and 9.7 atom% (± 1.03) for ^{15}N. This level of enrichment is significantly greater than natural abundance ($P < 0.001$ and $P = 0.006$ respectively) and should therefore be detectable if protozoa are part of the soil food web. There was a negligible number of enriched bacteria within the solution inoculating the soil cores (5 orders of magnitude less than the protozoa).

6.3.2 *APE* ^{13}C and ^{15}N value of soil respiration, bulk soil and vegetation

The *APE* ^{13}C signature of the headspace CO_2 is a consequence of respiration from the added enriched protozoa, and shows the level of ^{13}C dissipation over time (Figure 6.1). Significant differences were found between the $^{13}CO_2$ respired in the different habitats with greater ^{13}C signal in the woodland, this was also significant over time, ($F_{1,27} = 27.72$; $P < 0.001$ and $F_{1,27} = 105.29$; $P < 0.001$). Looking at the amount of $^{13}CO_2$-C, there was no difference between habitats; there was a greater amount of $^{13}CO_2$-C respired at time 0, in comparison to after 72 h ($F_{1,28} = 96.14$; $P < 0.001$).

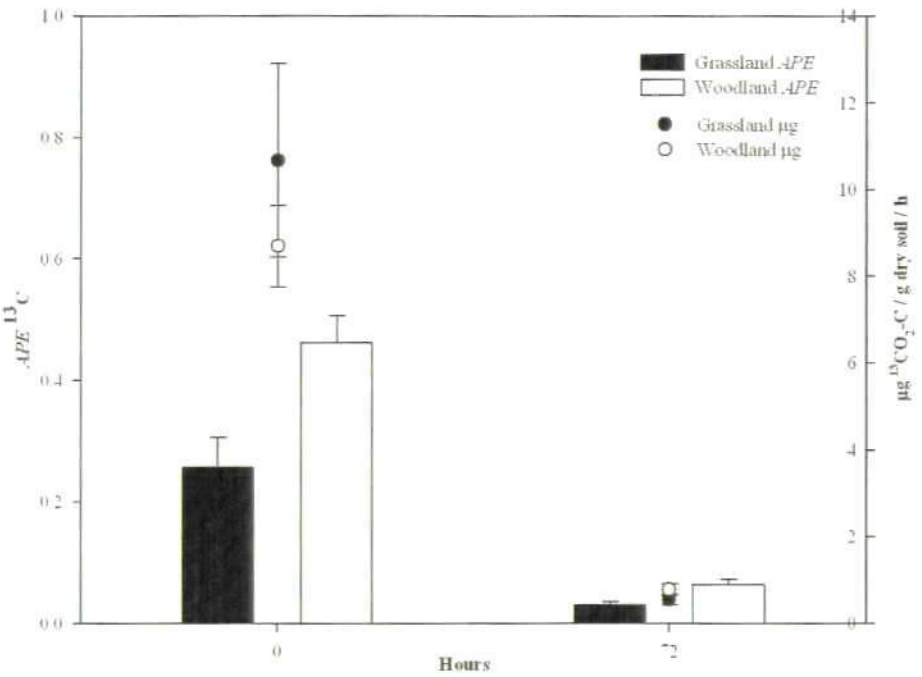


Figure 6.1: Differences in *APE* ^{13}C signatures of the headspace CO_2 due to the added enriched protozoa within the soil over time (Bar – left axis), with differences in µg $^{13}CO_2$-C / g dry soil over time (Dot –right axis).

Data are presented as mean ± standard error, (n = 5).

The *APE* ^{13}C respired was significantly greater than natural abundance for both the grassland and woodland at time 0 ($P = 0.003$ and $P < 0.001$ respectively). Although

there was a significant decline in *APE* ^{13}C after 72 h, the *APE* ^{13}C signature was also greater than natural abundance in both habitats at this time ($P < 0.001$).

The C and N content of the soil in the grassland and woodland habitat were significantly different ($F_{1,8} = 48.03$; $P < 0.001$ and $F_{1,8} = 214.08$; $P < 0.001$ respectively). The grassland had a higher C and N content (8.2% ± 0.20 C and 0.8% ± 0.01 N, C:N ratio of 10.6), when compared to the woodland (5.8% ± 0.28 C and 0.5% ± 0.01 N, C:N ratio of 11.2), although the C:N ratios were not significantly different between habitats. There were no significant differences between the *APE* ^{13}C or *APE* ^{15}N or the amount of ^{13}C (µg) or ^{15}N (µg) of protozoan origin of the soil between the two habitats. Due to the large dilution from organic C within the soils from both habitats, the *APE* ^{13}C was not significantly different from the natural abundance signature in soils. The *APE* ^{15}N was significantly greater than natural abundance in both the grassland and woodland soils ($P = 0.009$ and $P < 0.001$ respectively) (Figure 6.2). The amount of ^{15}N within the soil represents between 80 – 91% of the total ^{15}N added to the soil cores (as calculated through mass balancing). This result shows only a small proportion of the ^{15}N that was added has been lost from the system, particularly when the amount of ^{15}N contained within the vegetation is included (taking the total to 88 – 97% of the total amount added).

There was a significant difference in the C and N content and C:N ratio of the vegetation in the two habitats (%C was $F_{1,8} = 13.81$; $P = 0.006$, %N was $F_{1,8} = 26.57$; $P < 0.001$, and C:N was $F_{1,8} = 8.44$; $P = 0.020$). The grassland had a higher C and N content (41.3% ± 0.78 C and 3.2% ± 0.22 N, C:N ratio of 13.3) when compared to the woodland (31.7% ± 2.46 C and 1.8% ± 0.17 N, C:N ratio of 18.4). The vegetation *APE* ^{13}C and ^{15}N signatures at the grassland site were significantly greater than natural abundance ($P = 0.013$ and $P = 0.005$ respectively), as were those at the woodland site ($P = 0.001$ and $P = 0.039$ respectively). There were no significant differences between the

APE ^{13}C or *APE* ^{15}N or the amount of ^{13}C (µg) of protozoan origin in the vegetation between the two habitats. There was a significant difference between the amount of ^{15}N (µg) of protozoan origin between the two habitats ($F_{1,8} = 7.14$; $P = 0.028$) (Figure 6.2) though, this difference probably reflects the difference in N content.

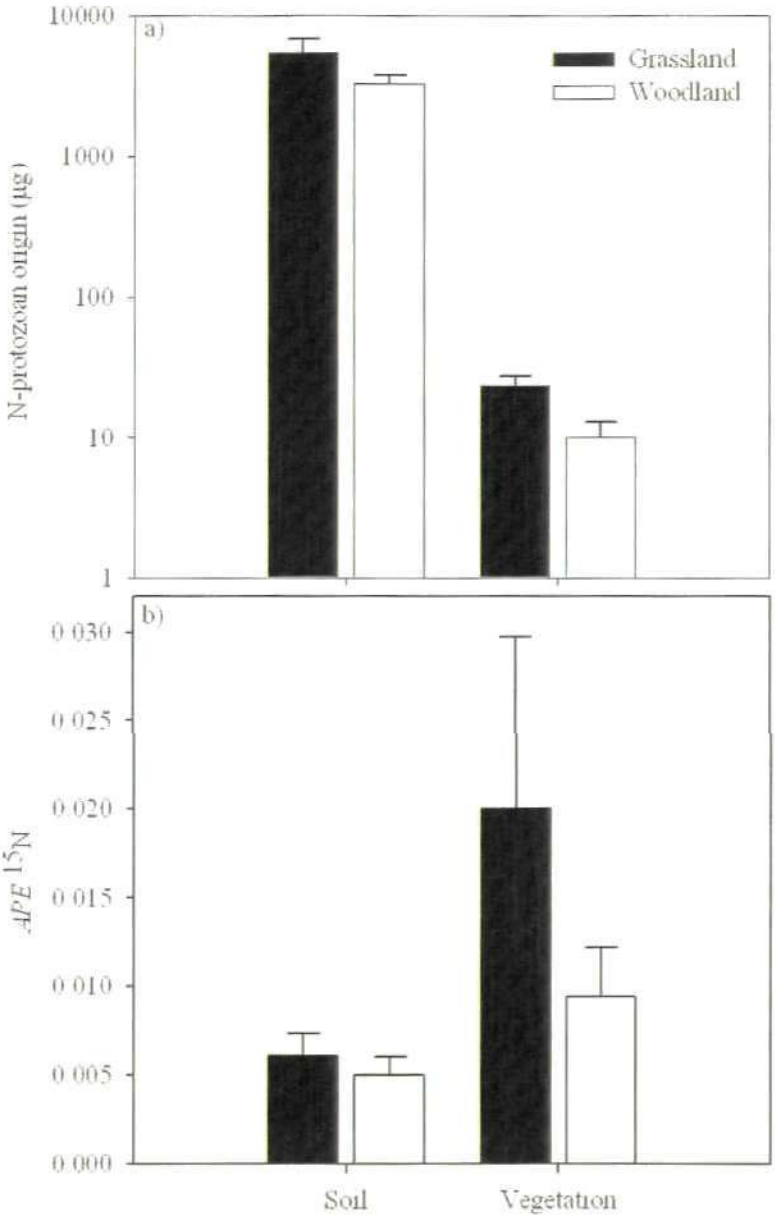


Figure 6.2: Differences in N of protozoan origin and *APE* ^{15}N values in soil and vegetation. Data are presented as mean ± standard error, (n = 5).

6.3.3 Community Composition of the soil food web

A total of 7,435 invertebrates were extracted using the Tullgren funnel system, of these 4,045 were from the grassland habitat and 3,390 from the woodland, this equates to over 100,000 invertebrates (± 20,000) per m^2 (Table 6.1). There were significant differences between the population numbers and biomass for some of the macro- and mesofauna taxa (Table 6.1), although these were not consistent. These differences represent potentially different community structures in these two habitats, indicating different functional food web interactions occurring, which may in turn affect the incorporation of protozoan ^{13}C and/or ^{15}N.

Principal component analysis of the Tullgren funnel results showed that there were seven groups which had a large effect on the variation between the two habitats (Figure 6.3). The invertebrates that had the greatest effect in the grassland were the Entomobryomorpha, Astigmata, aphids and the Prostigmata. Whilst in the woodland the invertebrates which had the greatest affect were the collembolan superfamilies Poduromorpha, Neelipleona and Symphypleona (Figure 6.3).

The differences in biomass between the two habitats showed there were only five groups which had an influence on the variation and there was also less variation between the two habitats (Figure 6.4). Although again, there were a small number of orders that had a large effect on the variation, and included Enchytraeidae and slugs for the grassland, and Tipulidae larvae, earthworms and Diplopoda: Polydesmidae for the woodland.

Table 6.1: Community composition, abundance and biomass of macro- and mesofauna taxa in the grassland and woodland habitats.

Data presented as mean ± standard error (n = 5), and F-values of a single factor ANOVA, * $P < 0.05$; ** $P < 0.001$ indicating significant differences between habitats ($df_{1,8}$).

	Number of organisms per m^2			Biomass per m^2 (mg)		
Organism	Grassland	Woodland	*F*-values	Grassland	Woodland	*F*-values
Acari: Astigmata	4342 ± 1882.3	764 ± 368.5	2.48	16.8 ± 6.18	1.5 ± 0.74	6.00*
Acari: Mesostigmata	4813 ± 567.1	5195 ± 992.9	0.11	56.7 ± 8.55	58.3 ± 12.25	0.01
Acari: Mesostigmata: Uropodidae	204 ± 118.1	0	2.98	4.0 ± 2.29	0.0	2.98
Acari: Oribatida	9867 ± 1519.9	9753 ± 2002.9	0.00	66.6 ± 19.71	36.3 ± 6.80	2.11
Acari: Oribatida: Humerobatidae	0	89 ± 89.1	1.00	0.0	10.7 ± 10.67	1.00
Acari: Oribatida: Phthiracaridae	0	649 ± 240.2	7.31*	0.0	34.3 ± 13.26	6.69*
Acari: Prostigmata	3298 ± 631.3	904 ± 365.9	10.76*	4.2 ± 0.85	1.4 ± 0.55	7.67*
Aphids (Hemiptera: Aphidoidea)	2712 ± 1119.3	0	5.87*	50.0 ± 18.21	0.0	7.53*
Chilopoda: Geophilomorpha	0	64 ± 34.9	3.33	0.0	146.7 ± 77.09	3.62
Coleoptera Larvae	102 ± 101.9	38 ± 25.5	0.37	3.1 ± 3.15	24.8 ± 16.50	1.65
Coleoptera Larvae: Carabidae	89 ± 89.1	0	1.00	4.0 ± 4.04	0.0	1.00
Coleoptera Larvae: Chrysomelidae	140 ± 42.2	38 ± 38.2	3.20	41.6 ± 12.07	15.8 ± 15.84	1.67
Coleoptera Larvae: Curculionidae	191 ± 117.4	0	2.65	7.7 ± 5.11	0.0	2.27
Coleoptera Larvae: Elateridae	13 ± 12.7	0	1.00	1.7 ± 1.67	0.0	1.00
Coleoptera Larvae: Staphylinidae	191 ± 66.8	25 ± 25.5	5.37*	16.5 ± 4.76	6.2 ± 6.24	1.72
Coleoptera: Carabidae	13 ± 12.7	13 ± 12.7	0.00	9.6 ± 9.64	48.1 ± 48.08	0.61
Coleoptera: Curculionidae	0	13 ± 12.7	1.00	0.0	0.1 ± 0.13	1.00

	Number of organisms per m^2			Biomass per m^2 (mg)		
Organism	Grassland	Woodland	*F*-values	Grassland	Woodland	*F*-values
Coleoptera: Ptilidae	13 ± 12.7	13 ± 12.7	0.00	0.6 ± 0.60	0.7 ± 0.71	0.02
Coleoptera: Staphylinidae	38 ± 15.6	51 ± 12.7	0.40	7.2 ± 5.08	40.6 ± 28.31	1.35
Collembola: Entomobryomorpha	18029 ± 1871.4	5628 ± 1163.5	31.67**	42.5 ± 6.83	13.3 ± 5.48	11.09*
Collembola: Neelipleona	153 ± 59.0	3794 ± 1076.3	11.41*	0.2 ± 0.09	1.4 ± 0.40	8.56*
Collembola: Poduromorpha	1986 ± 343.6	10555 ± 2925.3	8.46*	2.9 ± 0.49	52.8 ± 16.17	9.52*
Collembola: Symphypleona	4838 ± 971.3	9625 ± 2387.0	3.45	1.7 ± 0.35	3.7 ± 0.93	3.97
Diplopoda: Polydesmidae	0	127 ± 56.9	5.00	0.0	151.6 ± 51.22	8.76*
Diptera	140 ± 50.9	38 ± 38.2	2.56	44.8 ± 20.91	1.4 ± 1.43	4.27
Diptera Larvae	242 ± 54.8	178 ± 31.2	1.02	295.3 ± 151.88	159.5 ± 46.46	0.73
Earthworms (Lumbricina)	64 ± 20.1	38 ± 25.5	0.62	467.7 ± 175.88	1117.0 ± 775.82	0.67
Enchytraeidae	25 ± 15.6	280 ± 99.8	6.35*	11.8 ± 7.21	93.1 ± 81.37	0.99
Harvestmen (Opiliones)	0	25 ± 25.5	1.00	0.0	10.1 ± 10.11	1.00
Hemiptera	76 ± 31.2	25 ± 25.5	1.60	5.0 ± 2.39	0.2 ± 0.20	4.03
Hymenoptera: Apocrita: Parasitica	115 ± 50.9	13 ± 12.7	3.76	4.5 ± 3.84	0.6 ± 0.64	0.98
Slugs (Gastropoda: Limax)	25 ± 15.6	0	2.67	342.1 ± 317.30	0.0	1.16
Snails (Gastropoda: Helicoidea)	0	25 ± 15.6	2.67	0.0	8.7 ± 5.39	2.60
Spiders (Aranae)	51 ± 50.9	0	1.00	1.0 ± 1.02	0.0	1.00
Symphyla	13 ± 12.7	25 ± 15.6	0.40	0.3 ± 0.27	9.7 ± 9.18	1.06
Thysanoptera	89 ± 47.6	13 ± 12.7	2.40	0.4 ± 0.20	0.0 ± 0.03	3.97
Tipulidae Larvae	13 ± 12.7	51 ± 37.1	0.95	32.4 ± 32.38	573.1 ± 371.22	2.11
Woodlice (Oniscidea)	0	191 ± 72.6	6.92*	0.0	37.3 ± 14.30	6.82*

Table 6.1 continued: Community composition, abundance and biomass of macro- and mesofauna taxa in the grassland and woodland habitats.

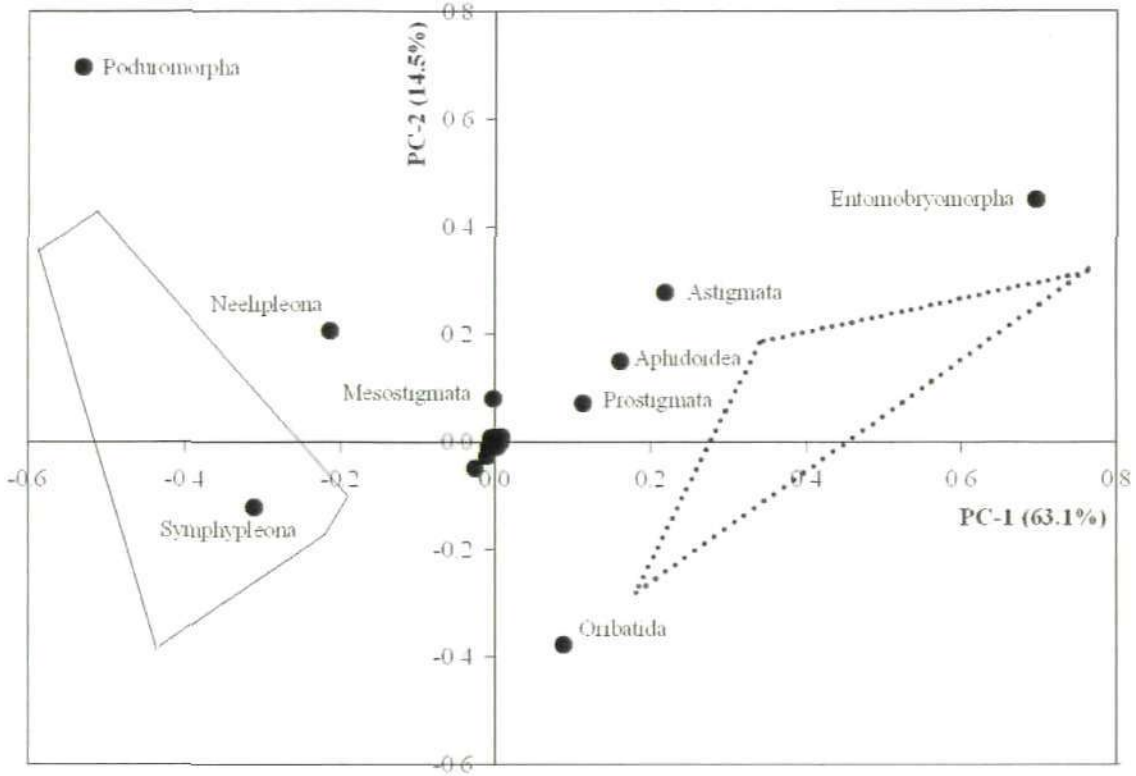


Figure 6.3: Principal component analysis (PCA) of the number of organisms per m^2 in the grassland and woodland. PCA accounting for 77.6% of the variation, habitats combined.

Reproduction of convex hull from GenStat – black dotted line represents grassland and solid black line represents the woodland. The graph highlights which invertebrates are effecting the variation between the two habitats, with those invertebrates on the left of the graph having the biggest effect on the woodland, whilst those on the right side have the biggest effect on the grassland.

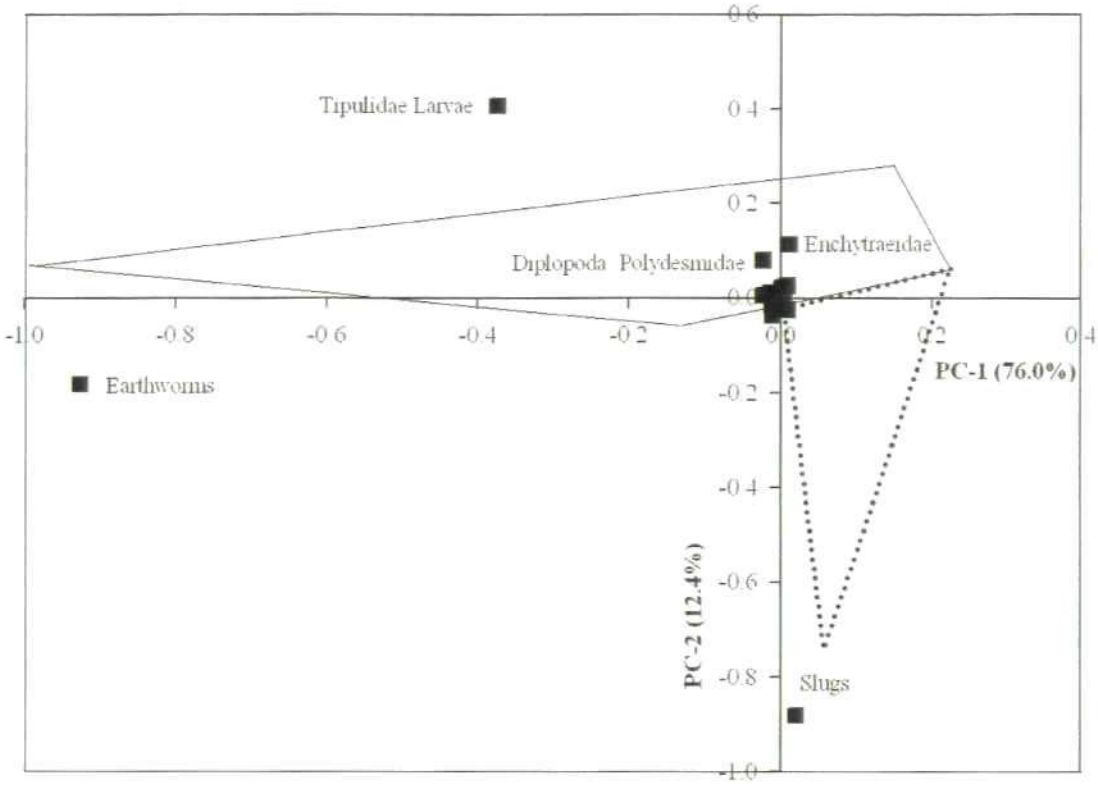


Figure 6.4: Principal component analysis (PCA) of the biomass of organisms per m^2 in the grassland and woodland. PCA accounting for 88.4% variation, habitats combined.

Reproduction of convex hull from GenStat – black dotted line represents grassland and solid black line represents the woodland. The graph highlights which invertebrates have the greatest effect on variation between the two habitats, with those invertebrates on the left of the graph having the greatest effect on the woodland, whilst those on the right side are effecting the grassland the most.

6.3.4 Protozoan C and N incorporation into the soil invertebrate food web

Multivariate analysis of variance for *APE* ^{13}C and ^{15}N for the total invertebrate population showed that there was no significant difference between the amount of *APE* ^{13}C and ^{15}N between habitats ($P = 0.531$). Therefore although there was a different assemblage of invertebrates within the two habitats those that were present were incorporating protozoan derived ^{13}C and ^{15}N at similar rates. There was some variation between the two habitats in the amount of enrichment obtained from protozoan consumption. The level of enrichment obtained by Staphylinidae larvae for *APE* ^{13}C was significantly different between the two habitats ($F_{1,3} = 150.57$; $P = 0.001$). Nematodes were also found to be significantly different for *APE* ^{15}N ($F_{1,9} = 9.04$; $P = 0.015$), and earthworms were found to be significantly different for *APE* ^{13}C and ^{15}N between habitats ($F_{1,20} = 6.44$; $P = 0.020$ and $F_{1,20} = 5.48$; $P = 0.030$ respectively).

The level of enrichment obtained varied between taxa and habitat (Figures 6.5 and 6.6, and Table 6.2). Orders that were the most enriched in ^{13}C and ^{15}N of protozoan origin in both habitats were nematodes, the Entomobryomorpha, and Mesostigmata, all of which were significantly greater than natural abundance for ^{13}C and/or ^{15}N (Table 6.2). In the grassland other invertebrates that were also significantly enriched were Coleoptera larvae (all combined) and earthworms (Figure 6.5), whilst in the woodland soil other invertebrates that were significantly enriched were the Polydesmidae, Poduromorpha, and Oribatida (Figure 6.6).

The Mesostigmata are also seen in both habitats to be highly enriched with ^{13}C and ^{15}N of protozoan origin. This enrichment was possibly due to consumption of prey organisms which had previously consumed protozoa. To test whether this was a valid hypothesis the correlation between enrichment level of predators and prey were assessed. Comparing the enrichment level of the Entomobryomorpha with the Mesostigmata, there was a significant linear relationship for both *APE* ^{13}C and ^{15}N

across habitats ($F_{1,8}$ = 6.45; P = 0.035 and $F_{1,8}$ = 9.46; P = 0.015 respectively). However, the percentage variance accounted for was low, and there was not a significant relationship in either *APE* ^{13}C or ^{15}N when accounting for the different habitats. According to the literature, the Mesostigmata are the main mesofauna predators, preying on Collembola and Nematodes (Walter et al., 1989a; Koehler, 1999). Testing nematophagy, there was no relationship between the enrichment levels of the Mesostigmata and Nematodes for both *APE* ^{13}C and ^{15}N across habitats or within habitats.

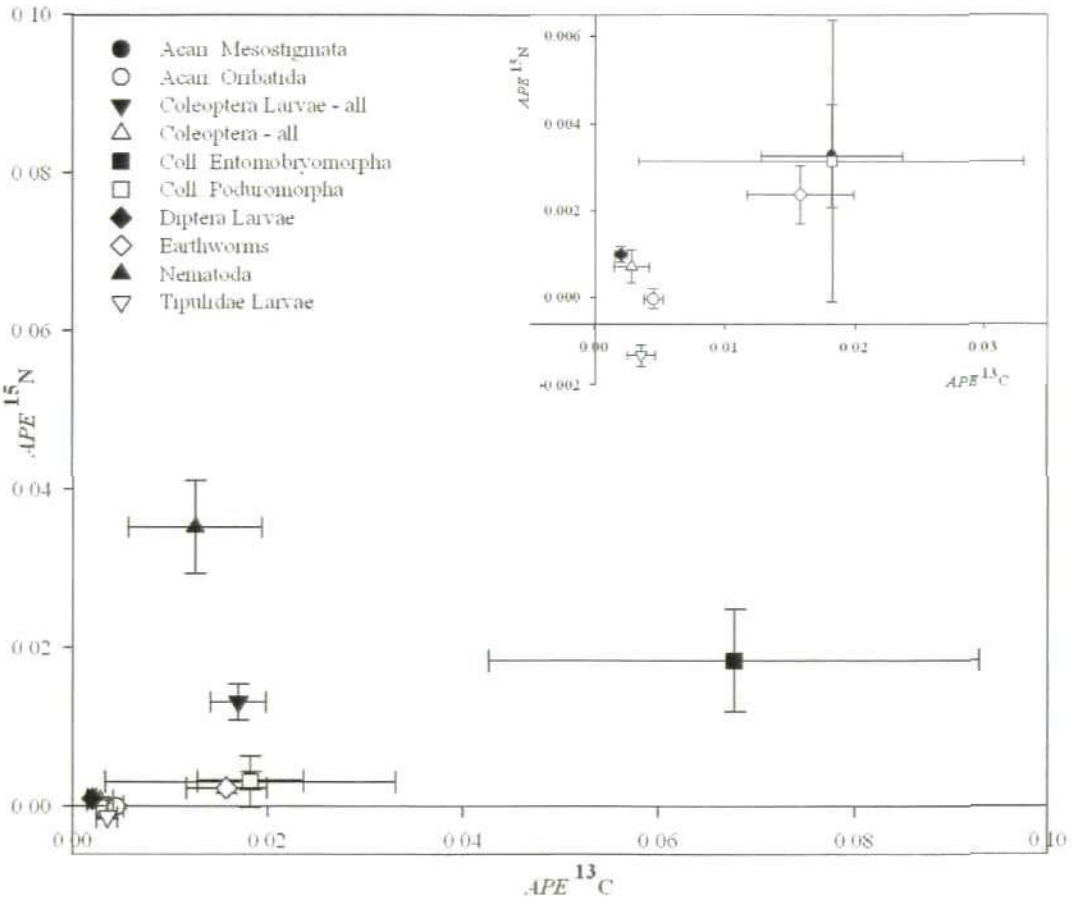


Figure 6.5: Isotopic composition of grassland invertebrates, *APE* ^{13}C and ^{15}N of invertebrate orders found in both habitats. Insert: magnified section near origin. Data represented as mean ± standard error (n= 2-10).

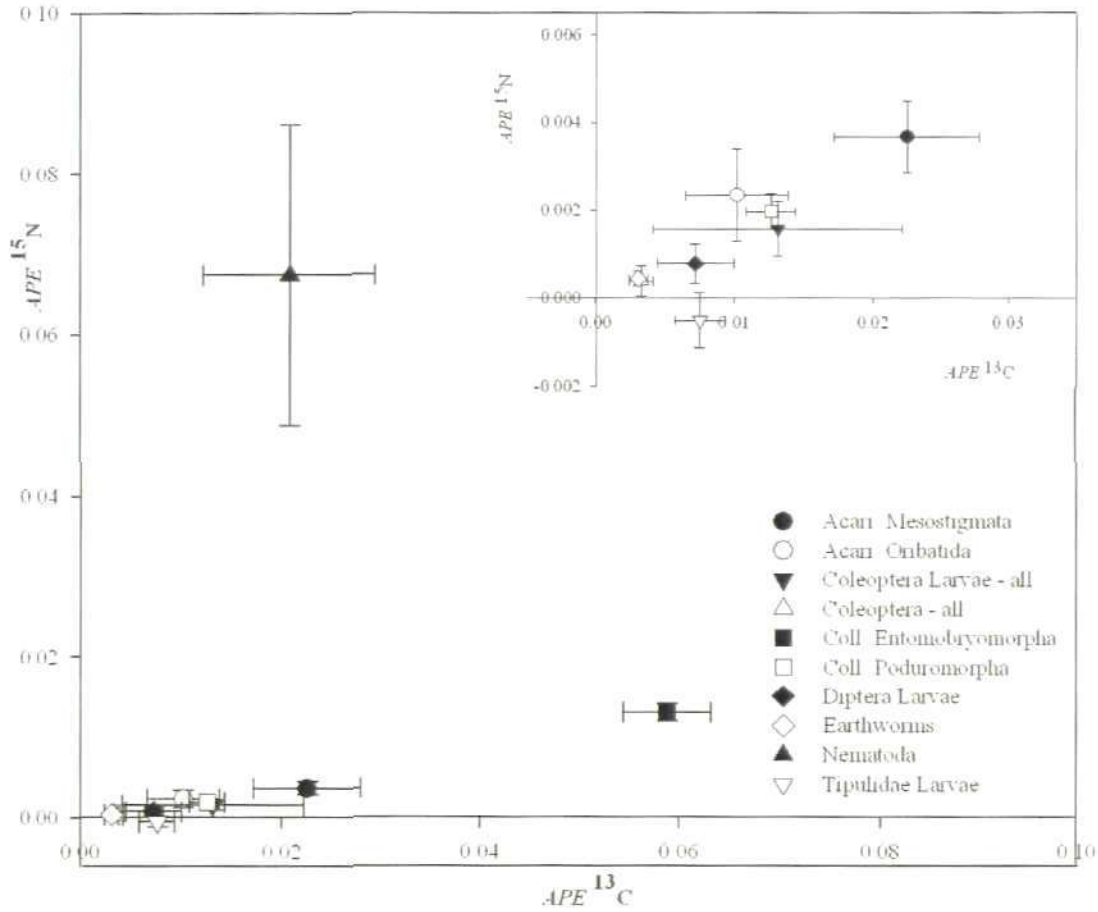


Figure 6.6: Isotopic composition of woodland invertebrates, *APE* ^{13}C and ^{15}N of invertebrate orders found in both habitats. Insert: magnified section near origin. Data represented as mean ± standard error (n= 3-10).

	Grassland			Woodland		
Organism	*APE* ^{13}C	*APE* ^{15}N	df	*APE* ^{13}C	*APE* ^{15}N	df
Acari: Mesostigmata	3.35*	2.74*	8	4.24*	4.51**	7
Acari: Oribatida	6.16**	-0.15	5	2.79*	2.23*	5
Acari: Oribatida: Phthiracaridae	np	np		3.48*	1.99	2
Aphids (Hemiptera: Aphidoidea)	1.52	1.23	11	np	np	
Chilopoda: Geophilomorpha	np	np		2.31*	3.89*	6
Coleoptera Larvae: all	4.21**	3.22*	12	1.46	2.52	2
Coleoptera Larvae: Chrysomelidae	7.29*	8.88*	3			
Coleoptera Larvae: Staphylinidae	5.27*	1.79	3			
Coleoptera: all	2.14	1.91	3	3.18*	1.10	5
Coleoptera: Staphylinidae	12.29*	0.53	1	7.06*	0.55	3
Collembola: Entomobryomorpha	2.7*	2.85*	9	13.51**	12.4**	3
Collembola: Poduromorpha	1.23	0.97	1	7.16**	4.87*	5
Diplopoda: Polydesmidae	np	np		2.73*	1.57	7
Diptera	0.02	-2.26	3			
Diptera Larvae	4.51*	4.99**	6	2.62*	1.73	4
Earthworms (Lumbricina)	3.84*	3.54*	12	4.63**	2.68*	8
Enchytraeidae				2.24*	3.62*	4
Nematoda	1.81	4.95*	5	1.72	8.76**	4
Slugs (Gastropoda: Limax)	34.29**	18.41**	4	np	np	
Tipulidae Larvae	3.33	-5.30	1	4.2*	-0.80	6

Table 6.2: Student's T-test results highlighting where the level of enrichment obtained from protozoan derived *APE* ^{13}C and/or *APE* ^{15}N by invertebrates was significantly different from natural abundance levels.

(* P < 0.05; ** P < 0.001, df = degrees of freedom, np = not present in that habitat). Where there are no values the sample size was too small to perform test.

To assess the impact of protozoan consumption on the soil fauna food web, the amount of ^{13}C and ^{15}N of protozoan origin incorporated by the soil fauna was calculated, to clarify which taxa consumed the most protozoa. Multivariate analysis of variance showed that there was no significant difference between the amount of ^{13}C and ^{15}N of protozoan origin in invertebrates between the two habitats ($P = 0.095$), although looking at the fauna individually there are differences visible (Figure 6.7).

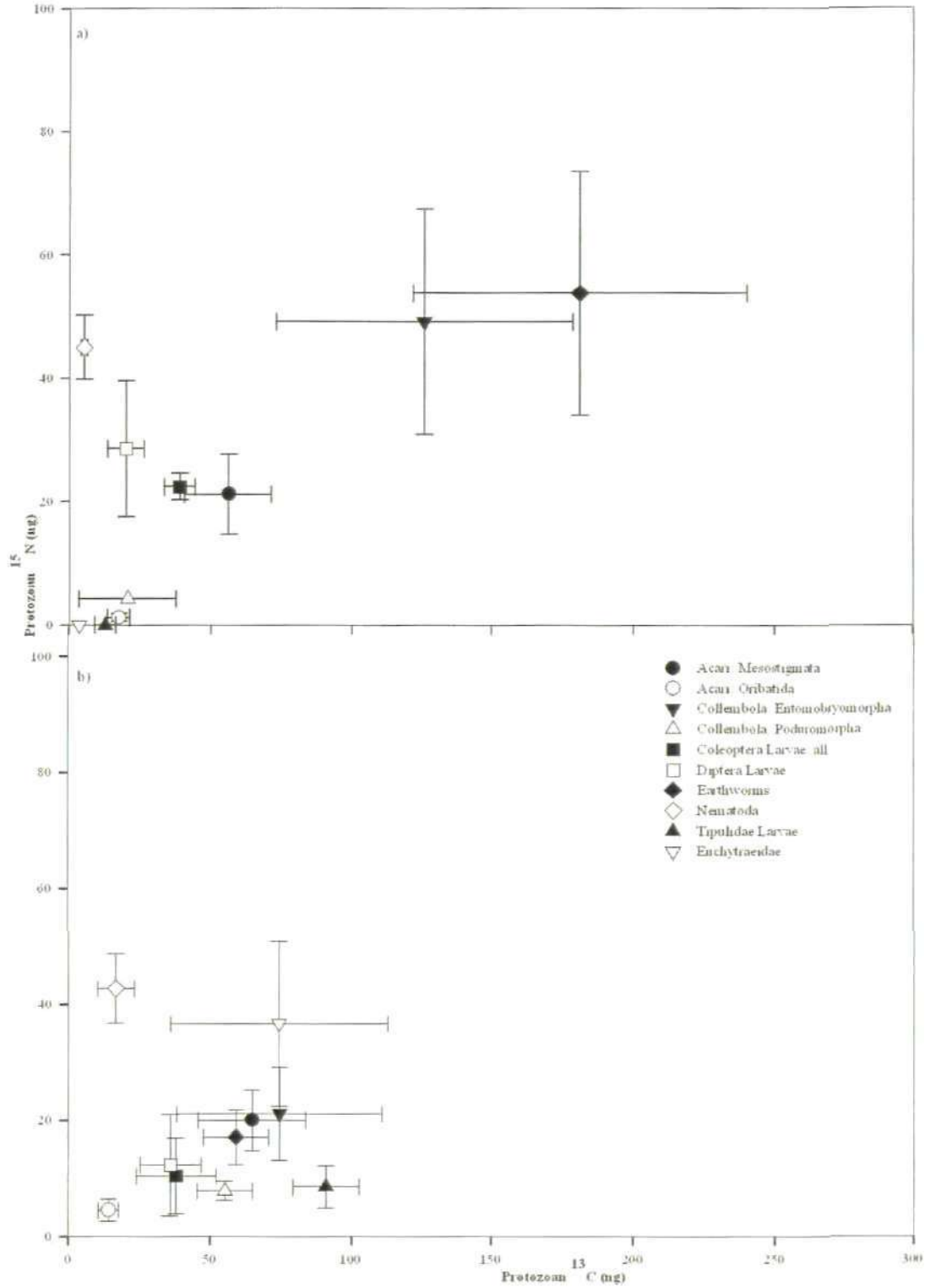


Figure 6.7: Amount of ^{13}C and ^{15}N of protozoan origin in invertebrates in a) a grassland habitat and b) in a woodland habitat.

Data represented as mean ± standard error (n= 2-10).

In the grassland those invertebrate orders which contribute the greatest proportion of biomass are nematodes (27%), earthworms (22%), and Diptera larvae (14%) (Table 6.1). However, the invertebrates that have incorporated the greatest proportion of ^{13}C of protozoan origin in the grassland were earthworms (30%), Entomobryomorpha (21%), and Mesostigmata (9%), whilst the nematodes and Diptera larvae accumulated less than 5% (Figure 6.7a). This trend is similar in the woodland, with the greatest proportion of biomass being in the earthworms (36%), Tipulidae larvae (18%), and nematodes (14%) (Table 6.1 and Figure 6.4). Whilst the invertebrates that have incorporated the greatest proportion of ^{13}C of protozoan origin in the woodland are Tipulidae larvae (11%), Enchytraeidae (9%), Entomobryomorpha (9%), and Mesostigmata (8%), whilst nematodes and earthworms accumulated less than 10% (Figure 6.7b). In the grassland soil (Figure 6.7a), the invertebrates have generally obtained a greater amount of ^{13}C and ^{15}N of protozoan origin than those in the woodland soil (Figure 6.7b).

There appeared to be some invertebrate groups with very high incorporation of ^{13}C and ^{15}N of protozoan origin in the grassland soil, whilst other groups had very low incorporation. Whereas, in the woodland soil there is a more uniform spread of incorporation across all groups, with more organisms with an intermediate level of enrichment (Figures 6.8 – 6.10). Comparison of the frequency distribution (Figure 6.8) found that there were significant differences in the amount of ^{13}C obtained by invertebrates between the grassland and woodland ($P = 0.013$). This difference was significant for invertebrates obtaining between 0 – 40 ng ^{13}C of protozoan origin ($P = 0.034$), although above this amount (between 40 – 800 ng ^{13}C) there was no significant differences in frequency distribution ($P = 0.149$). Comparison of the frequency distribution for ^{15}N of protozoan origin obtained by invertebrates (Figure 6.9) showed there were no significant differences between habitats.

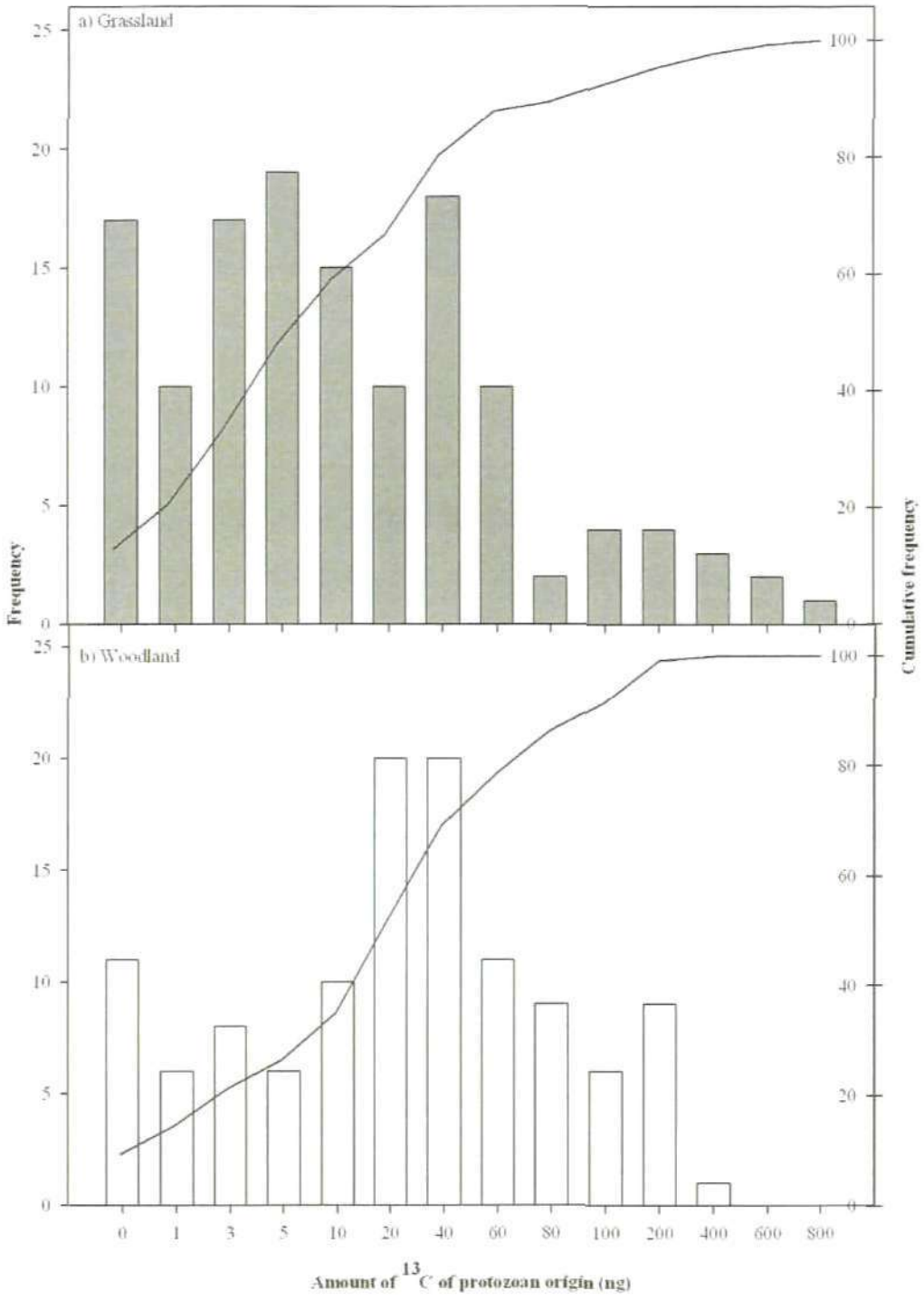


Figure 6.8: Histogram showing the frequency and cumulative frequency of the amount of ^{13}C of protozoan origin obtained by invertebrates in a) grassland soil and b) woodland soil – a community wide assessment of protozoan interaction with the soil fauna as a whole.

(Bars represent the frequency of individual results, line the cumulative frequency).

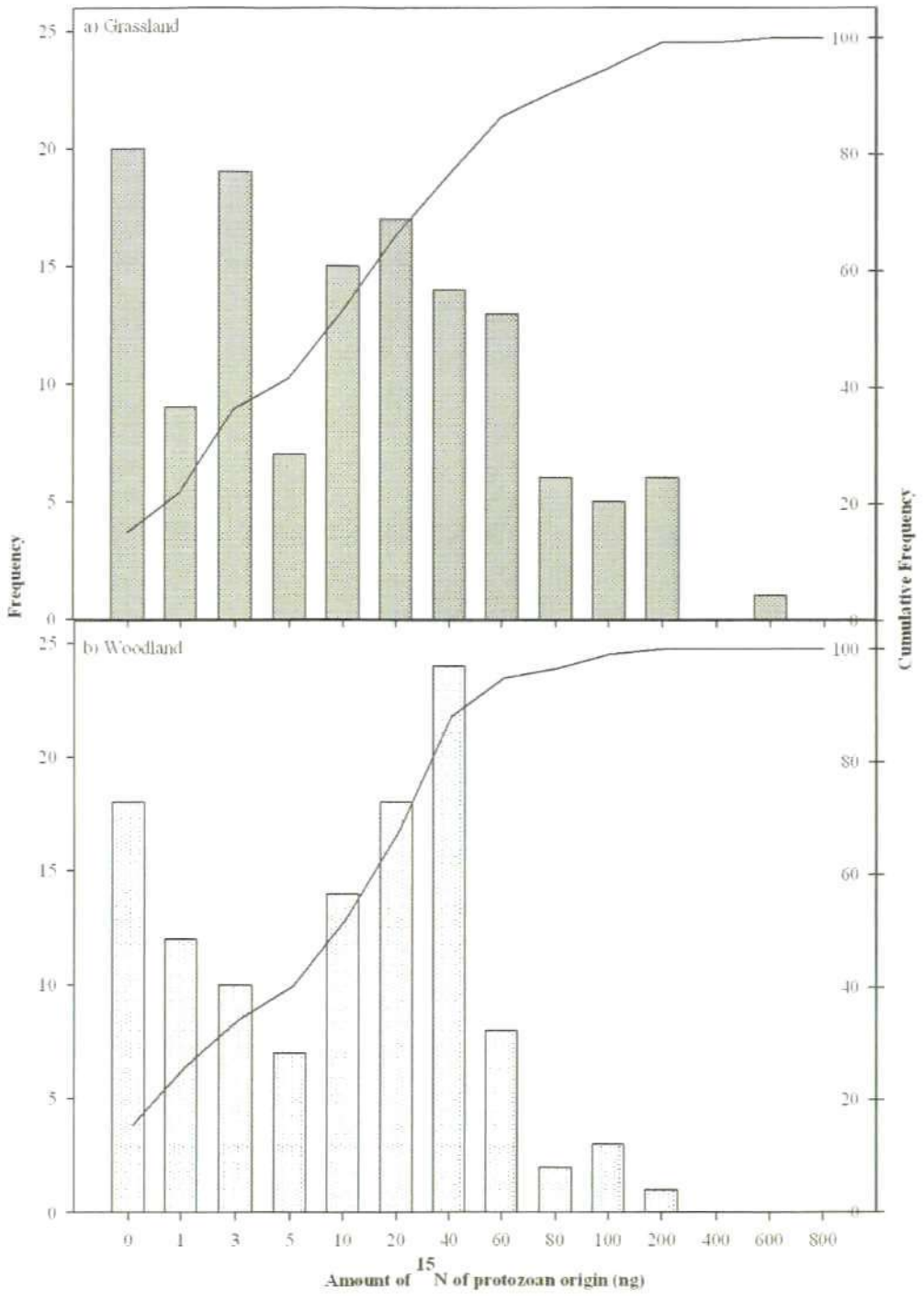


Figure 6.9: Histogram showing the frequency and cumulative frequency of the amount of ^{15}N of protozoan origin obtained by invertebrates in a) grassland and b) woodland soil – a community wide assessment of protozoan interaction with the soil fauna as a whole.

(Bars represent the frequency of individual results, line the cumulative frequency).

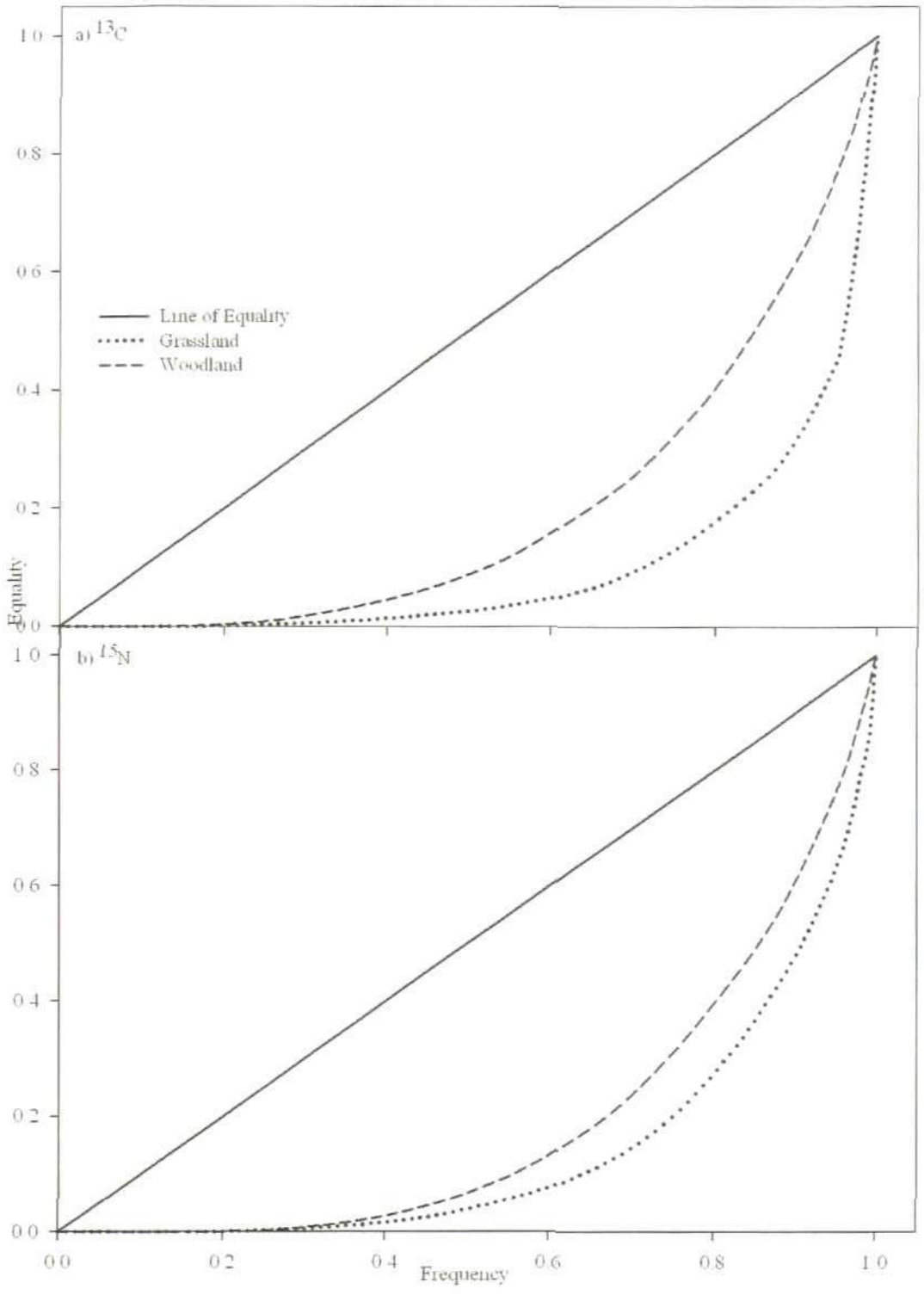


Figure 6.10: Lorenz curve showing difference in a) ^{13}C and b) ^{15}N of protozoan origin frequency distribution in the grassland (dotted line) and woodland (dashed line).

Assessment of the differences in distribution using a Lorenz curve, showed that there was greater inequality in the grassland habitat for the amount of ^{13}C (Grassland Gini coefficient 0.7949, Coefficient of asymmetry 0.9509, compared to Woodland Gini coefficient 0.5924, Coefficient of asymmetry 0.8892). This showed more variation in the amount of ^{13}C of protozoan origin obtained by the invertebrates in the grassland compared to the woodland (Figure 6.10a). Whilst this pattern was also visible for ^{15}N (Grassland Gini coefficient 0.7129, Coefficient of asymmetry 0.8902, compared to Woodland Gini coefficient 0.6149, Coefficient of asymmetry 0.8272), the variation was not as different between the two habitats (Figure 6.10b). This variation is another indication that different interactions are occurring between the two habitats.

In the two previous chapters the invertebrates were grouped into "trophic levels" and their isotopic compositions were assessed, in both chapters the isotopic signatures were as expected for the respective trophic levels. Here, the invertebrates were again grouped into trophic levels after being fed enriched protozoa (Table 6.3) as herbivores (T0), primary (T1) or secondary decomposers (T2) and micro- (T3) and macro- (T4) predators. Analysis of the differences between their *APE* ^{13}C and ^{15}N found there to be significant differences between the grouped "trophic levels" for *APE* ^{15}N ($F_{4,186} = 5.96$; $P < 0.001$) and T0 and T2 were found to be significantly different to T1, T3, and T4 (Fisher's least significant difference test). For *APE* ^{13}C the grouped trophic levels were not found to be significantly different ($F_{4,186} = 1.46$; $P = 0.217$), although T0 was different to T4 (Fisher's least significant difference test).

When each habitat was assessed individually, the trophic levels were even less distinct (Figure 6.11). In the grassland *APE* ^{13}C was not different between the trophic levels ($F_{4,98} = 2.37$; $P = 0.058$), although the highest enrichment was the secondary decomposers, nor was *APE* ^{15}N ($F_{4,98} = 1.93$; $P = 0.111$).

Organism	Trophic level
Coleoptera Larva: Curculionidae	0
Coleoptera Larva: Elateridae	0
Collembola: Symphypleona	0
Hemiptera	0
Aphids	0
Slug	0
Snail	0
Acari: Astigmata	1
Acari: Oribatida: Phthiracaridae	1
Collembola: Neelipleona	1
Diplopoda: Polydesmidae	1
Diptera Larva	1
Earthworm	1
Enchytraeid worm	1
Tipulidae Larva	1
Woodlice	1
Acari: Oribatida	2
Acari: Prostigmata	2
Coleoptera: Ptilidae	2
Collembola: Entomobryomorpha	2
Collembola: Poduromorpha	2
Diptera	2
Nematodes	2
Acari: Mesostigmata	3
Coleoptera Larva	3
Coleoptera Larva: Carabidae	3
Coleoptera Larva: Chrysomelidae	3
Coleoptera Larva: Staphylinidae	3
Chilopoda: Geophilomorpha	4
Coleoptera: Carabidae	4
Coleoptera: Staphylinidae	4
Opiliones	4
Spider	4

Table 6.3: Groupings of invertebrates used for trophic level analysis.

Trophic level 0 are herbivores, 1 are primary decomposers, 2 are secondary decomposers, 3 are micro-predator, and 4 are macro-predator. (Groupings ordered according to literature (Brussard et al., 1997; Hopkin, 1997; Halaj et al., 2005; Krantz et al., 2009)).

In the woodland, there were no significant differences found between trophic levels for *APE* ^{13}C ($F_{4,88}$ = 1.75; P = 0.147). However, there were significant differences found for the *APE* ^{15}N signatures between the different trophic levels in the woodland ($F_{4,88}$ = 4.92; P = 0.001), with the herbivores and secondary decomposers being significantly different to the other trophic levels. These differences in *APE* ^{15}N signatures for the different trophic levels are visible in the grassland habitat (Figure 6.11b) but were not significant.

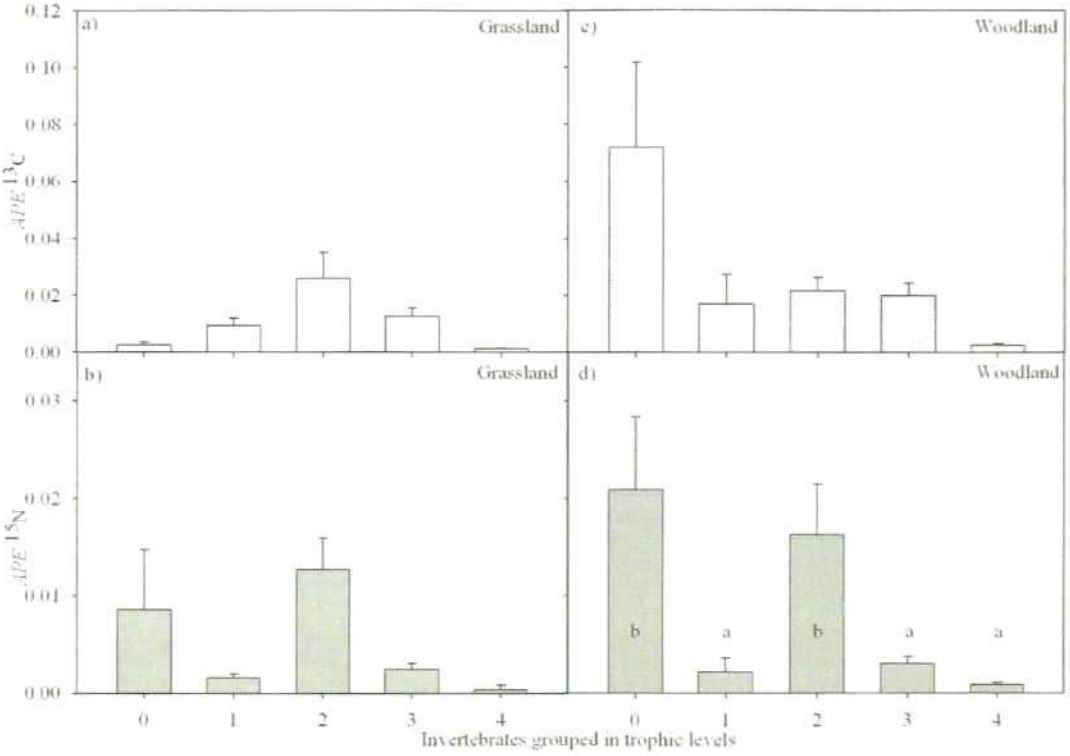


Figure 6.11: Isotopic composition of the grouped "trophic levels" for the different habitats, average *APE* ^{13}C and *APE* ^{15}N (± standard error, n = >4). a) Grassland *APE* ^{13}C; b) Grassland *APE* ^{15}N; c) Woodland *APE* ^{13}C; d) Woodland *APE* ^{15}N.

See Table 6.3 for Trophic level groupings TL0 herbivores, TL1 primary decomposers, TL2 secondary decomposers, TL3 micro-predators, and TL4 macro-predators. Difference in letters within each graph showing significant differences between trophic levels – Fisher's protected least significant difference test.

6.4 DISCUSSION

The main objective of this study was to quantify the ecological linkages between protozoa and other organisms within the soil food web. This was studied by enriching protozoa as a pulse label highly enriched in both ^{13}C and ^{15}N stable isotopes and introduced into the soil food web so its role could be analysed. Within this thesis, methods have been developed to enrich protozoa using stable isotopes to a significantly greater level than natural abundance in both ^{13}C and ^{15}N for the first time. At the time of inoculation the majority of the solution introduced to the soil cores was enriched protozoa, with only a negligible number of enriched bacteria. Therefore any enrichment found in the food chain can be assumed to be from protozoan consumption only, or from utilisation of protoxoan products (through mineralisation or the microbial loop effect). The flux of protozoan C and N through the soil invertebrate food web was also tested for the first time empirically, and to assess what role the protozoa hold in this decomposer based system.

The transient nature of ^{13}C as it dissipates through protozoan respiration and predator respiration after consumption, limits the potential incorporation into body tissues. The loss of ^{13}C through respiration necessitated the incubation period to be relatively short for this experiment. Although, as the protozoa were less enriched in ^{15}N than ^{13}C due to the methods used for culture (^{13}C enrichment through pinocytosis and consumption of enriched bacteria, whilst ^{15}N enrichment was by consumption alone) both isotopes may have been at relatively similar levels at the time of invertebrate extraction. The enrichment obtained by the soil flora and fauna during this 72 h time period indicates a high level of utilization. The respiration signal of ^{13}C by the protozoa decreased significantly over time (Figure 6.1), and this loss of ^{13}C to the atmosphere probably contributed to the non-significant bulk soil results. Bonkowski (2004) showed

that protozoa play a key role in the mineralisation of bacterial biomass by bacterivory and nutrient transfer to plant roots through the microbial loop. Here, protozoan N was transferred to the plant, made available through excretion by the protozoan predators, or through microbial turnover, demonstrating empirically the microbial loop.

Comparison of the enrichment obtained by the vegetation through uptake of mineralised C and N, to the bulk soil (which received the direct addition of the enriched protozoa) appears to show a discrepancy. As there was a large level of enrichment in the vegetation compared to the soil, when converting this enrichment into amount of N of protozoan origin (Figure 6.2), there is a significantly greater amount in the soil in comparison to the vegetation ($P > 0.001$). Using the amount of ^{13}C and ^{15}N of protozoan origin obtained is, in consequence, a more robust method to elucidate the feeding preferences of the organisms within the food web. Originally, these two habitats were managed grazed grassland systems, on the same soil type. The biodiversity and composition of the soil biota may be assumed to have been the same up to the point where the treatments diverged and therefore the differences seen in this study are due to this management change.

Although the habitats appear generally similar in food web structure, the grassland and woodland habitats differed in abundance and diversity of soil invertebrates. These differences in total number of organisms obtained from each habitat were not significant and neither were the differences in biomass. Some of the macro- and mesofauna when analysed separately did show differences in numbers and biomass between the habitats (Table 6.1), with the Prostigmata, Entomobryomorpha and aphids being found in significantly higher numbers and biomass in the grassland (Figure 6.3). Whilst in the woodland there were significantly higher numbers and biomass of Oribatida: Phthiracaridae, the Neelipleona, Poduromorpha and woodlice (Figure 6.3).

These differences in abundance of invertebrates are potentially due to different sources of C driving the food webs within the two habitats.

Here for the first time the role of protozoa within the soil fauna food web, can be seen and the ecological linkages between protozoa and the rest of the web can be determined, through the varying levels of enrichment obtained. Overall, the *APE* ^{13}C and ^{15}N signatures of the soil fauna showed a traceable flux of ^{13}C and ^{15}N of protozoan origin being incorporated into the soil food web. The ^{13}C and ^{15}N signatures of most of the invertebrates had increased through consumption of protozoa, or consumption of C and/or N of protozoan origin.

There was some variation in the amount of enrichment obtained between the two habitats, suggesting that there are differences in the feeding preferences in these habitats, or it could be due to the different population sizes within the habitats. Around 40% of the invertebrates from both habitats were significantly enriched in ^{13}C and ^{15}N (Table 6.2). There was a significant enrichment in the predators, but there was also enrichment in some of the organisms that are considered to be decomposers. The overall results of enrichment of the soil fauna through protozoan consumption shows the significant role protozoa have in the transfer of nutrients from the bacterial biomass to higher order consumers, through the soil food web. Arguably, the contribution of soil protozoa in general, is even greater than this study suggests, since amoeboid species were not included in this experiment.

The varying trophic position of the organisms showing the highest enrichment of C and N of protozoan origin highlights some of the interactions occurring within the soil food web. Nematodes are consistently seen as the main predators of protozoa and unsurprisingly showed a high isotopic enrichment, although they were only significantly enriched in ^{15}N in both habitats. The Entomobryomorpha are usually considered to be fungal feeders in the literature (e.g. Jorgensen et al., 2003; Scheu et al., 2004a;

Chahartaghi et al., 2005) but here they were one of the most enriched groups of organisms – indicating that protozoa are one of their food sources. It is unlikely that protozoa were mineralised by fungi, before the fungi were consumed by the soil fauna due to the short time span of the experiment (Paterson et al., 2008b). The Mesostigmata are one of the most voracious predators within the soil food web, and were highly enriched. It is possible that this enrichment was via consumption of protozoa by prey organisms at a lower trophic level. However, correlations between enrichment of the Mesostigmata with prey organisms were not significant. This implies that there are many feeding interactions between predators and prey within the soil food web, there are no "simple trophic linkages" from one prey taxon to one predator taxon, but this is a true web of interactions.

Calculating the amount of ^{13}C and ^{15}N of protozoan origin assimilated by the soil fauna is a more robust method than looking at *APE* alone, to elucidate the feeding preferences of the organisms within the food web. Fauna within the grassland soil obtained a greater amount of ^{13}C and ^{15}N of protozoan origin than those in the woodland soil. This difference in incorporation of ^{13}C and ^{15}N of protozoan origin could be an indication that different feeding interactions occurred in the two habitats, with the grassland soil being more dependent on the bacterial channel, including the protozoa. However, it was the same organisms within the soils from the two habitats that had the highest enrichment – the Entomobryomorpha, earthworms and Mesostigmata.

When the organisms were grouped into hypothesised trophic levels (as was previously performed in Chapters 4 and 5) the results did not clarify where protozoa were within the soil food web (Figure 6.11). This is most likely because the protozoa are an intermediary step between the basal food sources and the secondary decomposers, and protistovory is not as common as bacterivory. Interestingly the "trophic levels" showing the highest enrichment where the herbivores (another example

of the microbial loop affect), and secondary decomposers (usually considered to be fungal feeders). These results are a good example of why organisms should not be classified *a priori* (Eggers et al., 2000), into functional groups before stable isotope analysis. Here, if this had occurred the results would appear that protistovory is very uncommon, as there were very few significant differences between trophic levels. Also, that food web diagrams that depict protozoa's only predators as nematodes would have appeared to be correct. Whereas when you consider the varying levels of enrichment obtained across habitats, it is clear that protozoa have a pivotal role within the soil food web.

Using stable isotopes to differentiate trophic levels at natural abundance always falters when trying to define an isotopic baseline for the soil food web (Schmidt et al., 2004). Both bacteria and protozoa cannot be extracted *in situ* in large enough quantities to obtain accurate stable isotope measurements; they would have to be cultured first thereby acquiring the stable isotope signature of the media they grow on. The majority of food webs analysed to date show differences between the different invertebrates within the soil in comparison to litter or soil (Scheu et al., 2000; Illig et al., 2005), but not to the microorganisms that potentially form the main food source within the soil food web. Nearly half the soil invertebrates analysed from both habitats were significantly enriched in ^{13}C and ^{15}N from the introduction of enriched protozoa. Using an enriched organism has therefore shown feeding interactions occurring *in situ* within the soil and provided a measure of the proportion of feeding on indigenous protozoa that occurs by the meso and macrofauna.

6.5 CONCLUSIONS

The results of this experiment are able to show that soil protozoa can be cultured in enriched media and obtain an enrichment level significantly greater than natural abundance. The results show empirically that there are linkages between protozoa and many different taxa within the soil food web. The data suggest that indigenous flagellates and ciliates form a large food source for many different organisms within the soil food web, at many different "trophic levels". Furthermore, the incorporation of protozoan C and N can differ significantly between the two habitats, though there are also many similarities, particularly with decomposers and predators alike, obtaining high enrichment levels. In this present study, a first step has been taken to elucidate the links between fauna within the soil food web; only with more research and greater development of knowledge on the contribution of these different basal organisms to the nutrition of soil invertebrate species can our understanding of soil C and N turnover be increased.

Chapter 7: Part A – Developing fungal methods to track the fungal feeding channel within soil food webs

7a.1 INTRODUCTION

Within the terrestrial detrital system dead roots and plant litter deposited in the soil are subsequently decomposed by soil microorganisms and the soil fauna. This detritus is utilised to varying degrees by the different species and functional groups of fungi within the soil. Saprotrophic fungi commonly utilise freshly fallen litter mineralising carbon, whilst the mycorrhizal fungi specialise on more decomposed litter and humus, mobilising N and P and delivering it to their host plants (van der Heijden et al., 2008) in exchange for C (Elfstrand et al., 2008).

Fungi are considered to be one of the main decomposer energy channels within the soil food web, with little crossover between it and the other channels (Moore et al., 1988b). However, the energy channels within the soil are probably more inter-linked than first suggested; by many complex interactions including feedback mechanisms between the channels. For example, root herbivory has been found to increase subsequent mycorrhizal colonisation of the host plant (Currie et al., 2006). Although, these linkages are often not considered in soil food web experimentation.

There is very little evidence of adaptive evolution within the soil, with no one single decomposer animal species relying on one fungal species (Maraun et al., 2003). In some studies there appears to be some preferences within the soil, for non-mycorrhizal fungi amongst Collembola (Gange, 2000) and dark pigmented "dematiacea" fungi amongst oribatid mite species (Schneider et al., 2005a). Although other studies have found there to be limited occurrence of selective feeding (Koukol et al., 2009). Within the soil everything is intermingled – habitat, food sources, and

decomposition processes, this makes it difficult to determine the true interactions occurring.

For the majority of fungal species, most of the N present is found in the cytoplasmic constituents. Hyphal wall N contents generally range between 0.5-3% whilst that of the cytoplasm might be as high as 10% (Andren et al., 1990). Organisms feeding on hyphae will cause some cell content leakage creating a different environment or sphere of influence (Coleman, 1994) within the soil directly around this area, supporting the presence of different organisms. The use of stable isotope signatures is one method that can be used to directly asses the movement of C and N through the soil system, and to ascribe feeding preferences within soil food webs (Scheunemann et al., 2010) (also described in Chapters 5 and 6).

It is possible to use stable isotopes to differentiate the different functional groups of soil fungi (due to the different substrate utilisation and isotopic fractionation). For example, wood decomposing fungi and ectomycorrhizal fungi at the same site were completely separated on the basis of their $\delta^{13}C$ versus $\delta^{15}N$ plots (Kohzu et al., 1999). Moreover, the same species of mycorrhizal fungi utilising different C3 and C4 host plants could be separated by comparing isotopic values (Nakano et al., 1999). These studies suggest that stable isotopes could be used to study the input of fungi within the fungal feeding channel of the soil faunal food web.

To obtain a complete idea of the C and N cycle, the interactions occurring between the bacterial and fungal energy channels need to be defined. Interactions within the rhizosphere between root structure and microorganisms may favour different channels, for example it is thought highly branched root systems favour protozoa-bacteria interactions compared to large root cortices which favour mycorrhizae (Bonkowski, 2004).

Decomposer fungi have been found to translocate N and C simultaneously and bidirectionally, transporting significant quantities of C belowground (Frey et al., 2003), and N between fungi and host plant species (Wallander et al., 2006). Thus, there is the possibility of utilising these natural translocation mechanisms, to introduce a pulse of enrichment of ^{13}C and ^{15}N into soil food webs, to assess the feeding interactions that might be occurring.

Previously (in Chapter 3 and 5), enriched bacteria were injected into soil cores as a traceable pulse of enrichment within the soil. Again in Chapter 6, enriched protozoa were added to the soil as a traceable pulse to track the feeding preferences of the soil fauna. This method was appropriate for the introduction of bacteria and protozoa to soil cores as both inhabit the soil as part of a fluid matrix within the water-filled pore spaces and adhere to soil particles. However, fungi are filamentous and exist as part of the air-filled pore spaces within the soil, therefore this injection method would not work.

Consequently, a different approach is required to introduce fungi into the soil in a viable form, at a known level of stable isotope enrichment and this chapter concerns the development of such a method. Firstly, fungal species were isolated from the agricultural grassland, and growth conditions were manipulated to select a species that would be suitable for food web analysis. Secondly, these species were identified to aid in the assessment of suitable species. Lastly, selections of these species were tested to determine whether they would grow between different media, as a possible future introduction method.

7a.2 MATERIALS AND METHODS

7a.2.1 Fungal isolation from soil

Intact soil cores (10 cm diameter, 10 cm deep) were taken from three differently managed sites at the Rowden Research Platform at North Wyke within the South-West of England. One had received no inorganic nitrogen fertilizer input (0N) for over 20 years, although was annually grazed by cattle (described in full in Chapter 2.2.1). Another field had received 200 kg N ha^{-1} $year^{-1}$ (200N) (described in Chapter 3.2.1 N 50,46, 46.22523 W 3,55, 0.96998), the third site was from outside the field margin, under the trees at the northern boundary of the 0N field, it had received no inorganic fertiliser inputs or been grazed by cattle (0NPK), (N 50,46, 55.43246 W 3,55, 6.03291). Once collected from the field the soil cores were stored at 4°C for three days prior to use.

Each core was homogenised and 10 g wet weight was added to flasks with 90 ml sterile 0.1% w/v sodium pyrophosphate. The suspensions were agitated for 30 minutes using a mechanical shaker, before 10-fold serial dilutions were made, up to 10^{-5}, in sterile ¼ Ringer's solution. Aliquots of 100 µl of these dilutions were spread-plated on to yeast agar and repeated on Czapek Dox agar to isolate different fungal species, and incubated at 18°C for five days.

After five days the mixtures of fungal colonies were reinoculated individually onto separate plates to assess their individual growth characteristics. For each inoculation a triangular segment of agar was cut from the leading edge of the fungal growth and placed perpendicularly on to a new agar plate, of the same agar type. These isolates were incubated at 18°C for four days. As some of these isolates had slower growth rates than others, after four days they were reinoculated onto the other agar medium (yeast or Czapek Dox) to assess whether this affected growth. Additionally, any plates with more than one fungal species were separated, by inoculating each

separately onto a different plate. These new inoculations were incubated at 18°C for a further four days.

7a.2.2 Growth characteristics of fungal isolates

A visual assessment of the growth characteristics of all fungal cultures was performed over an incubation period of one week. For future assessment of reintroduction capacity, it was recorded whether the fungi were relatively fast growing, produced excess spores or hyphae, and their pigmentation. The isolates were also inoculated onto minimal media agar to assess whether they could grow on this limited nutrient medium – where the amount of C and N can be specifically controlled.

7a.2.3 Identification of fungal species

All the fungal isolates were cultured on either yeast agar or Czapek Dox agar, and allowed to grow for two weeks prior to DNA extraction so that the fungal isolates would sporulate. The fungal isolates were to be identified to species level by amplification of ribosomal RNA-encoding genes using a similar method to Thornton et al., (2004). This was through the sequencing of the internal transcribed spacer regions (ITS) (Faggian et al., 1999). These regions are highly divergent and can be used for differentiation between and within species (O'Donnell, 1992).

Each fungal isolate was identified to species level by amplification of ribosomal RNA-encoding genes using a modification of the method described by Sreenivasaprasad et al. (1996). The extended primers ITS-1 ext (5′-GTAACAAGGTTTCCGTAGGTG-3′) and ITS-4 ext (5′-TCTTTTCCTCCGCTTATTGATATGC-3′) were used to amplify both ITS regions. DNA was extracted using an "Extract N Amp Plant PCR Kit" (Sigma-Aldrich, St Louis,

USA) as follows. Approximately 5 mm^2 fungal mycelium was cut from the growing edge of the Petri dish and added to 100 µl extraction solution in a 2 ml Eppendorf tube without agar transfer. This was boiled for 10 minutes at 105°C and 100 µl of buffer solution was added, to make a fungal DNA solution for each isolate. Eppendorf tubes (0.2 ml) were prechilled to –20°C, before adding 10 µl "PCR mix" solution, along with 1 µl ITS1 primer, 1 µl ITS4 primer, 4 µl nuclease free water, as well as 4 µl fungal DNA solution to each tube. This was mixed thoroughly and the final reaction PCR amplification was performed in a thermal cycler (Hybaid Omn-E) using the following cycling conditions: 3 min at 94°C; 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 min; and a final extension of 10 min at 72°C.

Reaction products were fractionated using a 0.8% (w/v) agarose gel (with TBE buffer). DNA fragments were purified from gels using a commercial kit "Geneclean" (Promega, Wisconsin, USA) according to the manufacturer's instructions, through a three stage process of binding the DNA, washing and then elution. The entire ITS1–5.8S rRNA–ITS4 region was sequenced in both strands. Dye-labelled terminator cycle sequencing was performed on a Long Read IR 4200 DNA sequencer (Li-Cor Biosciences, UK, Ltd.) using the nested primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). Double-stranded DNA sequences were aligned and the gapped Blast program 2.0 (Altschul et al., 1990; Altschul et al., 1997) was used to compare nucleic acid sequences with those contained within databases held at the NCBI. Species identity was predicted based on > 95% sequence identity (E-value = 0.0) of the ITS1–5.8S–ITS4 region of the fungal isolate to species recorded in GenBank. The ITS sequences of representative isolates of the fungal isolates were submitted to GenBank and accession numbers obtained (see Appendix B).

7a.2.4 Growth between nutrient sources

To demonstrate that hyphae will grow over a gap between nutrient sources, empty Petri dishes were set-up with a series of agar squares in a cruciform formation within the dish (Figure 7a.1). Minimal media agar squares (approximately 1 cm^2) were cut and placed inside at varying distances from each other (not greater than 7 mm). The fungal isolate to be inoculated was cut from the leading-edge of a growing colony and transferred onto the central square within the Petri dish. Therefore the hyphae could grow in any direction to bridge the gap between nutrient sources. Care was taken to minimise spore transfer (to avoid germination of spores on the other nutrient sources without hyphal linkages). The Petri plates were incubated inside sealed bags at 18°C for five days, with a moistened tissue paper to maintain humidity and reduce dehydration of the agar.

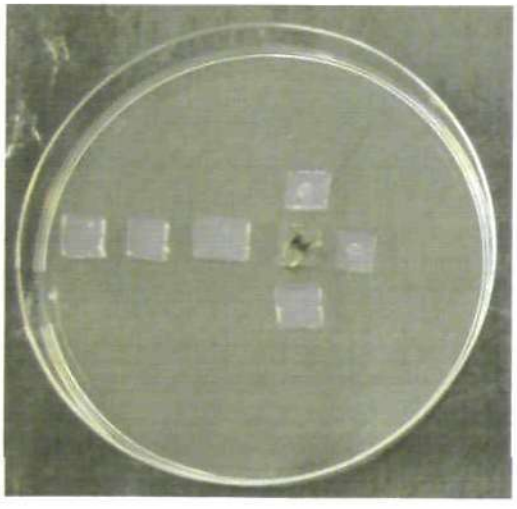


Figure 7a.1: Microcosm set-up to show growth between nutrient sources – agar squares in a cruciform distribution with the fungi inoculated on the central point.

7a.2.5 Reintroduction of fungi into soil – stage 1 sieved soil

To establish whether a fungal isolate would grow back into soil from its own (artificial) nutrient source, soil microcosms were set-up. Each microcosm was set up using non-sterile soil (0N), sieved using a 2mm diameter metal sieve, at its current gravimetric water content. The soil was added to a high lipped Pyrex dish (6.6 cm diameter), this was relatively small in diameter but had an increased depth (3.4 cm) compared to Petri dishes, allowing hyphae to grow without constraint (Figure 7a.2). The Pyrex dishes were covered with a Petri dish lid to reduce desiccation and all microcosms were kept on a tray inside a plastic bag. Due to the differences between the microcosms, all the isolates were tested and an assessment of differences in growth of the fungal isolates was recorded.

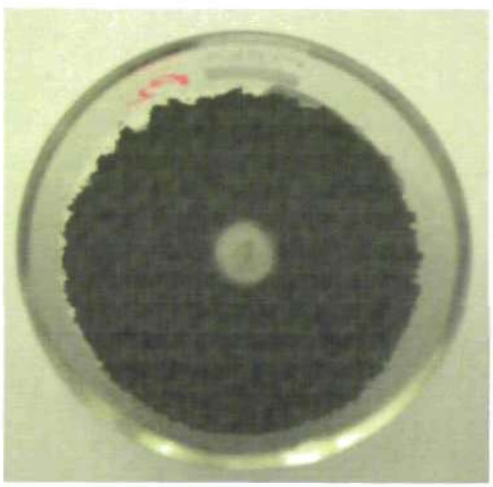


Figure 7a.2: Microcosm construction – reintroduction of fungi into sieved soil, the isolate was placed in the centre of the microcosm within an agar-filled tin capsule, surrounded by sieved soil and grew out from this point.

Aluminium tin capsules (10 mm by 10 mm, pressed, standard weight; Elemental Microanalysis Limited) were sterilised (autoclaved on liquid sterilisation cycle 121°C

for 15 minutes), prior to being filled with either minimal media agar or yeast agar. Each isolate was introduced individually onto the surface of the agar, as per previous inoculations (e.g. see Chapter 7a.2.1). These cuttings were all less than the diameter of the tin capsule so that the fungal isolate would be contained on top of the growth medium. This ensured that only the nutrients in the caps were used by the fungi until hyphal growth had overcome the gap between the inoculant and the soil. The inoculated agar was placed in the centre of each microcosm, so that the top of the capsule was flush with the level of the soil. These microcosms were incubated within a temperature controlled room at 18°C, and the growth of each fungal isolate was monitored over a three week period.

7a.2.6 Reintroduction of fungi into soil – stage 2: intact soil

Studies have found differences in organic matter content of soil with depth (Kramer et al., 2008) and that fungal hyphae may preferentially colonise organic patches (Hodge et al., 2001). Here, this experiment builds on the previous experiment using sieved soil and tests whether fungal isolates would readily bridge the gap between agar and intact soil cores, as it is intact soil cores that will be used in food web experiments. This experiment will also test whether depth or the organic matter content has an affect on the growth characteristics of the fungal isolates.

Non-sterile soil cores (10 cm by 10 cm) from the 0N field site were obtained and cut horizontally into four sections (with 2 cm depth per section), with the uppermost grass layer removed. Each separate layer was placed inside an individual tin tray (10 cm by 13 cm) within a plastic bag to maintain moisture content and increase humidity during incubation, it also prevents cross contamination of spores. Each fungal isolate was inoculated separately on each of the four soil layers, as before by inoculating the

minimal media tin capsule and situating it in the centre of each core section. All were incubated at 18°C over a period of two weeks (Figure 7a.3).

The fungal isolates chosen for this experiment were a selection that had shown good growth characteristics in earlier experiments. Growth of the fungal isolates was monitored over varying time intervals (2, 6 and 14 days). A control was also set up in triplicate, with soil cores sectioned and inoculated with a tin capsule containing minimal media agar, but without a fungal isolate, as a comparison. A further control, with soil cores sectioned but with no agar or fungi inoculated, was set up to assess the natural soil microbial community's ability for hyphal growth at the incubation temperature (18°C).

Figure 7a.3: Microcosm construction – reintroduction of fungi into intact soil, soil cores cut into four sections and each section inoculated with the same fungal isolate, to assess whether the isolate would bridge the gap between the agar and the intact soils at all levels of organic matter content.

7a.3 RESULTS

7a.3.1 Isolation of fungal isolates

Fifty fungi were isolated. At this stage species identifications were not performed as it was preferential to determine the growth characteristics desired of the

fungi. The majority were probably saprophytic soil fungi as these are the easiest to isolate via the methods used. Isolates that appeared identical (mycelium colour and type) and were from the same soil treatments were discarded. This left 38 "different" isolates for further examination.

7a.3.2 Growth characteristics of fungal isolates

The fungal isolates had many similarities and differences in growth characteristics (Table 7a.1). The isolates with the most positive attributes were used in further experimentation to assess the potential for these fungal isolates to be grown back into the soil.

Positive attributes included lack of visible spores (to reduce contamination when inoculating), and an abundance of hyphae or a fast growth rate (to increase the rapidity of food web inclusion). From monitoring the growth of all isolates on minimal media agar it was clear that they were able to grow on minimal media agar but this growth appeared to be at a slower rate than on the yeast or Czapek Dox agar and with less pigmentation.

7a.3.3 Identification of fungal species

ITS sequencing was used to identify all 38 fungal isolates, and all apart from four were identified to species (Table 7a.2). Nine isolates were unidentified after following the methods, due to a lack of DNA extracted, probably due to a deficiency in sporulating mycelium in that culture at that time. For these isolates the methods were repeated, identifying five of them, and leaving four which have remained unidentified. Overall the majority of fungal isolates were Ascomycetes (71%), and the rest were

Zygomycetes (29%), 18 different species of fungi where identified. Of those identified 30 isolates sequences were submitted to GenBank (see Appendix B).

Iso	Site	Media isolated on	Speed of growth	Spores	Abundant hyphae	Pigmentation
1	0NPK	Yeast	Slow	No	No	Light
2	0NPK	Yeast	Fast	Yes	Yes	Light (+ dark spores)
3	0N	Yeast	Fast	Yes	Yes	Light (+ dark spores)
4	0NPK	Czapek Dox	Slow	No	No	Light
5	0N	Yeast	Fast	No	Yes	Light
6	0NPK	Yeast	Fast	No	Yes	Dark
7	0N	Yeast	Fast	No	Yes	Dark
8	0NPK	Yeast	Fast	No	Yes	Light
9	0N	Yeast	Fast	Yes	Yes	Light (+ dark spores)
10	0NPK	Yeast	Fast	No	Half	Light
11	0NPK	Czapek Dox	Medium	No	No	Light
12	0NPK	Yeast	Fast	Yes	Yes	Light (+ dark spores)
13	0N	Yeast	Fast	No	Yes	Dark
14	200N	Yeast	Very fast	Yes	Half	Light (+ dark spores)
15	0N	Yeast	Fast	No	Yes	Light
16	200N	Czapek Dox	Fast	No	Half	Light
17	0N	Czapek Dox	Medium	No	No	Light
18	0NPK	Czapek Dox	Fast	No	Yes	Light
19	0N	Czapek Dox	Slow	No	No	Light
20	0N	Czapek Dox	Medium	No	No	Light
21	0N	Czapek Dox	Medium	Yes	No	Light (+ dark spores)
22	0NPK	Yeast	Medium	No	Yes	Dark
23	0NPK	Yeast	Medium	No	Yes	Light
24	0NPK	Yeast	Fast	No	Yes	Dark
25	0N	Yeast	Fast	Yes	Yes	Light (+ dark spores)
26	0NPK	Yeast	Fast	No	No	Light
27	0N	Yeast	V. slow	Some	No	Dark
28	0N	Yeast	Fast	No	No	Light
29	0NPK	Yeast	Fast	No	Yes	Light
30	0N	Czapek Dox	Slow	Some	No	Dark
31	0NPK	Czapek Dox	Medium	No	No	Light
32	0NPK	Czapek Dox	Medium	No	Half	Light
33	0NPK	Czapek Dox	Medium	No	No	Light
34	0NPK	Czapek Dox	Medium	Some	Half	Light
35	200N	Czapek Dox	Slow	Yes	No	Light
36	0N	Czapek Dox	Fast	No	Yes	Light
37	0N	Czapek Dox	Fast	No	Yes	Light
38	0N	Czapek Dox	Slow	Yes	No	Dark

Table 7a.1: Growth characteristics of fungal isolates (Iso) on agar media.

Iso	ITS Seq	Species Identified	Family	Phylum
1	18..557	*Penicillium ochrochloron*	Trichocomaceae	Ascomycete
2	11..622	*Mucor hiemalis*	Mucoraceae	Zygomycete
3	18..622	*Mucor hiemalis*	Mucoraceae	Zygomycete
4	CCCGCGTCACGGCTGCCGGGG		No Result on BLAST sequence too short	
5	25..85	*Absidia cylindrospora*	Mucoraceae	Zygomycete
6	26..83	*Absidia cylindrospora*	Mucoraceae	Zygomycete
7	29..98	*Absidia cylindrospora*	Mucoraceae	Zygomycete
8	18..526	*Gibberella zeae*	Nectriaceae	Ascomycete
9	12..623	*Mucor hiemalis*	Mucoraceae	Zygomycete
10	20..593	*Hypocrea virens*	Hypocreaceae	Ascomycete
11	21..578	*Trichoderma hamatum*	Hypocreaceae	Ascomycete
12	13..617	*Umbelopsis isabellina*	Umbelopsidaceae	Zygomycete
13	CCAGCTT		No Result on BLAST sequence too short	
14	87..610	*Umbelopsis isabellina*	Umbelopsidaceae	Zygomycete
15	27..631	*Fusarium sporotrichioides*	Nectriaceae	Ascomycete
16	14..518	*Gibberella zeae*	Nectriaceae	Ascomycete
17	24..521	*Microdochium phragmitis*	Hyponectriaceae	Ascomycete
18	20..514	*Fusarium cerealis*	Nectriaceae	Ascomycete
19	25..539	*Bionectria ochroleuca*	Bionectriaceae	Ascomycete
20	13..528	*Gibberella moniliformis*	Nectriaceae	Ascomycete
21	18..559	*Penicillium biourgeianum*	Trichocomaceae	Ascomycete
22	ATAAGCTTTTCAATAAGCGGAGGAAAACTAA		No Result on BLAST sequence too short	
23	25..522	*Fusarium cerealis*	Nectriaceae	Ascomycete
24	442..535	*Absidia cylindrospora*	Mucoraceae	Zygomycete
25	21..626	*Mucor hiemalis*	Mucoraceae	Zygomycete
26	26..589	*Trichoderma longipile*	Hypocreaceae	Ascomycete
27	16..562	*Penicillium simplicissimum*	Trichocomaceae	Ascomycete
28	25..585	*Hypocrea viridescens*	Hypocreaceae	Ascomycete
29	25..523	*Fusarium cerealis*	Nectriaceae	Ascomycete
30	14..529	*Cladosporium cladosporioides*	Mycospaerellaceae	Ascomycete
31	GCGGAGGAAAAAA		No Result on BLAST sequence too short	
32	16..590	*Trichoderma longipile*	Hypocreaceae	Ascomycete
33	22..584	*Hypocrea viridescens*	Hypocreaceae	Ascomycete
34	15..520	*Fusarium oxysporum*	Nectriaceae	Ascomycete
35	27..524	*Fusarium cerealis*	Nectriaceae	Ascomycete
36	15..523	*Fusarium cerealis*	Nectriaceae	Ascomycete
37	12..523	*Fusarium sporotrichioides*	Nectriaceae	Ascomycete
38	14..530	*Cladosporium cladosporioides*	Mycospaerellaceae	Ascomycete

Table 7a.2: Fungal isolates identified to species, thirty of which had sequences submitted to GenBank (Appendix B).

7a.3.4 Growth between nutrient sources by fungal isolates

All the fungal isolates were tested to see if they had the ability to grow hyphae between two separate nutrient sources at different distances. The hyphae of all fungal isolates bridged the gap between the separate agar squares, at varying degrees of speed and proficiency (Figure 7a.4).

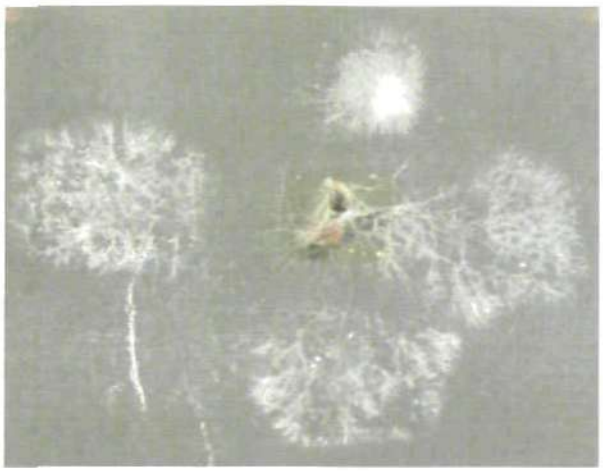


Figure 7a.4: Example of growth between nutrient sources – hyphal links are visible between the inoculation point in the centre and the other nutrient sources.

7a.3.5 Reintroduction of fungi into sieved soil

All fungal isolates grew from the agar plug back into the soil – with different proficiencies and hyphal masses (Table 7a.3 and Figure 7a.5). This ability to bridge the gap into the soil and speed of growth were assessed on an arbitrary scale of 1-5 (1 being a slow rate and 5 being a fast rate of growth) over time (6 – 14 days). This was a measure of the isolates ability in relation to the other isolates and the modal average of

these measurements was recorded for growth over time (Table 7a.3). For many of the isolates interactions with invertebrates, mainly nematodes and mites were also visible.

Fungal Gap Jumpers	Modal index of growth	Soil invertebrates present?
3: *M. hiemalis*	3	Yes – N
5: *A. cylindrospora*	5	Yes – C / M / N
6: *A. cylindrospora*	5	Yes – M / N
7: *A. cylindrospora*	4	Yes – M
8: *G. zeae*	3	Yes – M / N
9: *M. hiemalis*	3.5	Yes – N
10: *H. virens*	4	Yes – M / N
11: *T. hamatum*	4	Yes – C / M / N
12: *U. isabellina*	3	No
13: Unknown	4	Yes – M / N
14: *U. isabellina*	4	Yes – N
15: *F. sporotrichioides*	2	Yes – N
16: *G. zeae*	2	Yes – N
17: *M. phragmitis*	3	Yes – N
18: *F. cerealis*	3	Yes – C / N
19: *B. ochroleuca*	2	No
20: *Gibberella moniliformis*	2	Yes – N
21: *P. biourgeianum*	5	No
23: *F. cerealis*	3	Yes – M
24: *A. cylindrospora*	3	Yes – M / N (1000s)
25: *M. hiemalis*	4.5	No
26: *T. longipile*	4	Yes – M / N
27: *P. simplicissimum*	3.5	No
28: *H. viridescens*	3	Yes – N
29: *F. cerealis*	3	No
30: *C. cladosporioides*	2	No
32: *T. longipile*	3	Yes – M / N
33: *H. viridescens*	3	Yes – M
35: *F. cerealis*	2.5	Yes – C
36: *F. cerealis*	2.5	Yes – N (1000s)
37: *F. sporotrichioides*	2	No

Table 7a.3: Growth and invertebrate interactions over time for fungal isolates growing back into sieved soil. Key: C = Collembola; M = mites; N = Nematodes.

The soil was unsterilised and thus some invertebrate interactions did occur, although as the soil was sieved (2 mm diameter) at the soils gravimetric water content,

it was unlikely many of the mesofauna would be present and no macrofauna. The invertebrates present may not be equally distributed between microcosms, therefore this was not considered a defining characteristic.

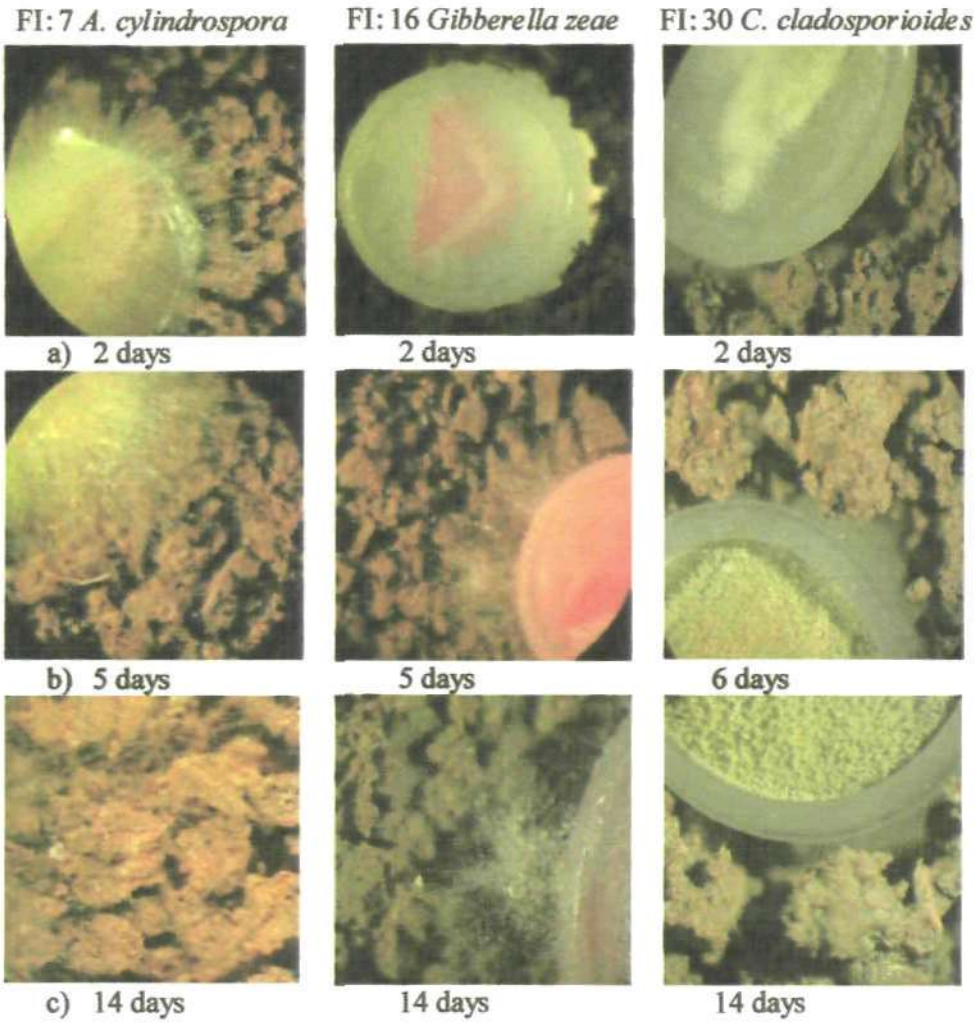


Figure 7a.5: Times series of fungal growth for three different isolates.

FI: 7 *Absidia cylindrospora* shows very proficient growth, and was isolated from 0kg N field; FI: 16 *Gibberella zeae* shows mediocre growth proficiency and was isolated from 200kg N field; FI: 30 *Cladosporium cladosporioides* shows a poor ability to grow back into the soil and was isolated from 0kg N field.

7a.3.6 Reintroduction of fungi into intact soil

Only 12 of the fungal isolates that had shown good growth proficiency were chosen to use during this experiment (Table 7a.4). All fungal isolates tested were seen to be growing from the agar plug back onto the intact soil – at varying aptitudes (Table 7a.4 and Figure 7a.6). The proficiency of growing across the gap between the agar and the intact soil (the hyphal mass and speed of growth) were assessed on an arbitrary scale of 1-5 (again 1 being a low rate and 5 being a high rate of growth) over time (2 – 14 days, Table 7a.4). On many of the isolates interactions with invertebrates were also visible – mainly Collembola and mites (Figure 7a.6 – FI1b, FI3b and FI3c).

Fungal Isolate	Top Layer growth	3-5 cm growth	5-7cm growth	7-9cm growth	Invertebrates present
5: *A. cylindrospora*	5.0	5.0	4.5	4.5	C/M/W/N
7: *A. cylindrospora*	5.0	5.0	5.0	4.5	M
9: *M. hiemalis*	5.0	5.0	5.0	4.5	C/W
10: *H. virens*	3.5	4.0	3.5	3.5	M
11: *T. hamatum*	4.0	4.0	4.0	4.0	C/W/N
12: *U. isabellina*	5.0	4.5	4.0	5.0	N
13: Unknown	4.0	4.2	3.8	4.0	N
14: *U. isabellina*	4.5	4.0	4.0	3.5	C/M/N
15: *F. sporotrichioides*	4.0	4.5	4.0	3.0	C
16: *G. zeae*	3.5	4.0	3.5	4.0	N
21: *P. biourgeianum*	4.0	4.0	3.5	3.5	N
24: *A. cylindrospora*	5.0	4.8	4.7	4.5	C

Table 7a.4: Modal index of growth and invertebrate interactions over time for fungal isolates reintroduced into intact soil.

Key: C = Collembola; M = mites; W = Enchytraeid worms; N = Nematodes.

It was difficult to assess how much of the hyphal network that developed was from the fungal plug at the centre of each microcosm; hence two different controls were also incubated. In the first control with the agar plug, a fungal hyphal network was visible on all soil sections and replicates there of, after 72 hours. There was also some

fungal growth bridging the gap, from the soil back onto the agar in many of the microcosms. There was also some fungal and bacterial growth visible on the agar plug in some microcosms, possibly transported on to the agar by invertebrates.

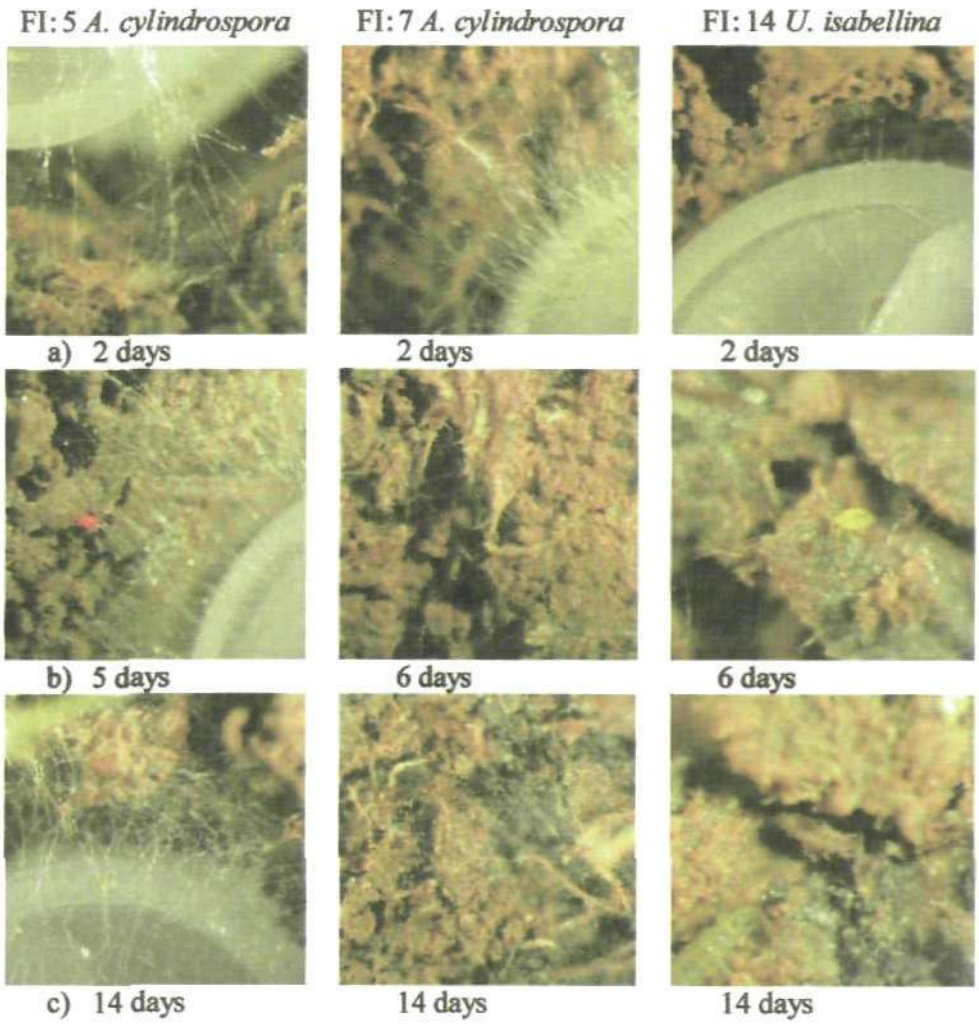


Figure 7a.6: Time series of fungal growth for three isolates (top section).

FI 5 - *Absidia cylindrospora* had good hyphal growth and many invertebrates were seen, including Mesostigmatid mite (photo b); FI 7 - *Absidia cylindrospora* had good hyphal growth from central point radiating outwards with time; FI 14 - *U. isabellina* had reasonable growth back into soil, but many invertebrates were seen, including Collembola - Sminthurid (photo b) and Entomobryomorpha (photo c).

A visual inspection of the second control samples found that there was also a hyphal network developing after 5 days incubation on all soil sections. These examples of fungal growth without inoculate, raise questions about the earlier results from visual assessment – showing the fungal inoculate bridging the gap between the agar plug back into soil. Visually it is impossible to differentiate between the test fungal isolate and indigenous fungal growth, therefore further experimentation was needed.

7a.4 DISCUSSION

Overall the main aim of this experimental series was to isolate a fast growing fungal species from the test soils which showed suitable characteristics for further experimentation. A large number of fungal isolates were obtained but many of these isolates were of the same species and some did not show suitable characteristics. For example, excess spore formation could lead to contamination of test soils with spores of an unknown enrichment level. It is impossible to distinguish between dead and viable spores visually (Johnson et al., 1991). Therefore, adding unknown quantities of spores could reduce consistency during experimental design and set-up, as there may be selective feeding by the fauna on living or dead spores, which would add another variable to the experiment.

A highly desirable characteristic is excess hyphal growth from a single point, which could maximise the speed of hyphal distribution through the soil matrix and consequently be available to the soil food web over a shorter time period. Another desirable characteristic is dark pigmentation of the hyphae. Some feeding preference studies have shown both Collembola and oribatid mites to prefer dark pigmented fungi ("Dematiacea") (Maraun et al., 2003; Schneider et al., 2005a), over hyaline forms suggesting some feeding specialisation (Maraun et al., 1998).

Assessment of these characteristics showed many of the extracted fungi to be unsuitable, but some did show potential for further experimentation (Table 7a.5). This table excludes all fungal isolates with more than two unsuitable characteristics, and highlights those with the most appropriate characteristics; these fungal isolates are the ones which should be chosen for use in further experiments. Interactions with invertebrates were not considered to be a defining characteristic, because of the artificial microcosm design (sieving and sectioning of soil cores). Therefore the number of invertebrates within the cores were not standardised in each microcosm for the different fungal species tested. Although, the invertebrates that were visible were on and around the fungal plugs within the microcosms, this potentially indicates some preference for the isolate and might make it easier to track the fungal feeding channel within the soil food web in the future, if invertebrates prefer to consume the introduced fungus.

Isolate	Site isolated	Excess hyphae	Pigmentation	Invertebrate interactions
5: *A. cylindrospora*	0N	Yes	Light	Yes – N / M / C / W
6: *A. cylindrospora**	0NPK	Yes	Dark	Yes – N / M / C
7: *A. cylindrospora**	0N	Yes	Dark	Yes – M
8: *G. zeae*	0NPK	Yes	Light	Yes – N / M
13: Unknown*	0N	Yes	Dark	Yes – N / M
15: *F. sporotrichioides*	0N	Yes	Light	Yes – N / C
16: *G. zeae*	200N	Half	Light	Yes – N
18: *F. cerealis*	0NPK	Yes	Light	Yes – N / C
24: *A. cylindrospora**	0NPK	Yes	Dark	Yes – N / M / C
29: *F. cerealis*	0NPK	Yes	Light	
36: *F. cerealis*	0N	Yes	Light	Yes – N
37: *F. sporotrichioides*	0N	Yes	Light	

Table 7a.5: Highlighting fungal isolates with most desirable characteristics synthesis of the information from Tables 7a.1 – 7a.4.

*** = most suitable characteristics, e.g. abundant hyphal growth or dark pigmentation. Key: C = Collembola; M = mites; W = Enchytraid worms; N = nematodes.**

Growing the fungi from an agar plug back into sieved or intact test soils showed that in all cases the fungus would readily grow back into soils when the experimental conditions were favourable. The area around the tin capsule where the hyphae bridged the gap between the agar and the soil was obviously colonised by the fungal isolate, unfortunately it was difficult to quantify whether the rest of the visible hyphal network was solely due to the growth of the fungal inoculant, or whether it was indigenous fungi. It should be noted, that these experiments did not quantify the amount of fungal hyphae (or C and N) that was translocated from a single growth point from the fungal isolate across the microcosm.

Further experimentation is needed to test whether the fungal isolates are growing back from the agar plug into the soil; one method of testing this is using stable isotope enrichment to track the fungal growth through the soil. The ability of the fungal isolates to grow using ^{13}C-glucose and ^{15}N-ammonium chloride as their sole C and N sources to 99 atom% enrichment has not been tested. Testing whether the fungi will utilise 99 atom% nutrient source is an essential next stage before determining whether this enrichment can be translocated through the growing hyphae into the soil.

Chapter 7: Part B – Fungal isotope enrichment

(Part of this Chapter is reproduced from Crotty et al., (2011) Rapid Communications in Mass Spectrometry 25: 1479-1484)

7b.1 INTRODUCTION

Filamentous fungi are unique amongst the soil biota in their ability to translocate nutrients, they establish extensive hyphal networks utilising spatially separated resources (Frey et al., 2003), and form one of the foundations of below-ground food webs. Decomposer fungi, have the ability to simultaneously translocate C and N bidirectionally (Fontaine et al., 2011), from source through hyphae to sink, and from sink through hyphae to growing tips. It is therefore potentially possible to utilise these natural translocation mechanisms and introduce a pulse of enrichment of ^{13}C and ^{15}N into soil microbial food webs, to assess the feeding interactions that might be occurring. Stable isotope analysis can be utilised to assess where an organism comes from, as well as its feeding preferences (Hyodo et al., 2010). It is possible to use stable isotope techniques to differentiate between functional groups of soil fungi, because of the utilisation of different substrates and isotopic fractionation.

Studies have differentiated between different functional groups of fungi, on the basis of their $\delta^{13}C$ and $\delta^{15}N$ ratios (Hogberg et al., 1999; Mayor et al., 2009). It has also been shown that there is significant fractionation of ^{13}C and ^{15}N in fungal hyphae, with isotopic levels differing between species (Griffith, 2004; Scheu et al., 2004b) and within the same species (Henn et al., 2002; Ruess et al., 2005b) including isotopic differences between different parts of the same organism (Taylor et al., 1997).

To date, there have been no studies investigating the movement of enriched soil saprophytic fungi as tracers, to attempt to identify the interactions occurring within the

soil food web, although this was suggested by Hobbie (2006) for extraradical ectomycorrhiza. In laboratory studies stable isotope signatures of fungi generally resemble that of the medium on which they are grown (Scheu et al., 2004b). The objectives of this part of the chapter, are to a) assess whether a saprotrophic fungus could grow on a medium which was enriched to 99 atom% with ^{13}C-glucose and ^{15}N-ammonium chloride, b) to determine the level of enrichment obtained and c) to examine whether there was any difference in the growth rate of this fungus on the enriched media compared to a natural abundance control.

It is also important to know the natural abundance values of the fungal isolates for comparison against the maximum level of enrichment for both ^{13}C and ^{15}N, as they appear to be very diverse depending on species, functional guild, nutrient uptake and habitat (Taylor et al., 1997; Hogberg et al., 1999; Kohzu et al., 1999; Nakano et al., 1999; Staddon et al., 1999; Griffith, 2004; Ruess et al., 2005b; and Keel et al., 2006). Using the results from the experiments from Part 1 (Table 7a.5) only a few isolates had the desired characteristics for further experimentation, and of these, (that were identified to species level) all were *Absidia cylindrospora*. In all growth tests, this species had the preferred growth characteristics as it was fast growing with dense colonies, and it had hyphae of a light brown colour. Additionally, the genus *Absidia* is ubiquitously distributed in soil (Hoffmann et al., 2007). For these reasons *A. cylindrospora* was chosen for further experimentation.

7b.2 MATERIALS AND METHODS

7b.2.1 Natural abundance measurements of fungal isolates

All fungal isolates were grown on agar to ascertain their natural abundance signatures. The agar gel was composed of 7 g Na_2HPO_4; 3 g KH_2PO_4; 0.12 g $MgSO_4$;

0.011 g $CaCl_2$; 0.5 g NaCl; 1 litre distilled water, with 1.2% w/v technical agar (number 3), which were combined and sterilised (autoclaved on the liquid sterilisation cycle 121°C for 15 minutes), before addition of filter sterilised 2.5 g $C_6H_{12}O_6$ and 1 g NH_4Cl; (at natural abundance). These inoculations were performed by cutting a thin section of agar and fungi from the growing edge of a previous culture and placing it perpendicularly in the centre of a Petri dish before incubating in darkness at 18°C for seven days. After seven days a complete mycelial mat had formed across the surface of the minimal media agar for each of the fungal isolates. To determine the natural abundance signature of the fungi, the mycelial mat was removed from the surface of the agar, and deposited inside tin capsules (8 mm by 5 mm), in preparation for analysis.

The tin capsules were weighed (Mettler Toledo MX5 microbalance), before addition of the fungal sample, and then filled with mycelium before oven drying at 80°C for 48 hours, after which they were re-weighed and the dry weight calculated. The stable isotope concentration was determined using an elemental analyser linked to an isotope ratio mass spectrometer (as described in Chapter 2.2.5). In addition to the fungal samples, samples of agar, pure glucose and pure ammonium chloride were also analysed.

7b.2.2 Enrichment measurements of fungal isolates

Prior to this experiment, it was not known whether the fungi would grow on media enriched with glucose and ammonium chloride to 99 atom%, or if its growth characteristics would appear different to those on natural abundance media. Enriched agar was prepared as previously described, exchanging the $C_6H_{12}O_6$ and NH_4Cl with the same amount $^{13}C_6$ – glucose and ^{15}N-ammonium chloride [both 99 atom%, SerCon]. Petri dishes were inoculated with *A. cylindrospora* in triplicate and incubated as before. After 10 days a complete mycelial mat had formed, this took around three days longer

than when grown on natural abundance media although the density of hyphal growth appeared similar.

To determine the enrichment level of *A. cylindrospora*, the top layer of mycelium was harvested (leaving a layer of hyphae adhering to the agar substrate) and sub-samples deposited inside three tin capsules per agar plate prior to analysis. Due to the detection thresholds of the mass spectrometer, the samples needed to be at a concentration of ≤ 5 atom%. Potentially the fungal isolates could be 99 atom% enriched with ^{13}C and ^{15}N, and therefore would need to be diluted to a suitable level. Although ^{13}C-glucose and ^{15}N-ammonium chloride were added as the sole nutrient sources it maybe possible for the fungus to breakdown some of the agar gel itself and utilise this as an energy source. Studies have shown that saprotrophic fungi can obtain up to 45% of their C from the agar gel (Hobbie et al., 2004), if this was the case here it would have had the effect of diluting the overall enrichment. Samples were diluted to have an atom% ratio within the thresholds of the machine, using the methods described in Chapter 2.2.5, and equations 5 and 6.

For the cultures of *A. cylindrospora*, different dilutions were considered to cover possible isotopic enrichments – three replicates estimated to have an enrichment of 99 atom%, three with 60 atom% and three with 40 atom%, these were chosen to range between the maximum possible enrichment and the minimum if utilisation of the agar gel had occurred. Each replicate was diluted with the appropriate amount of flour and analysed by mass spectrometry.

7b.2.3 Fungal growth rate experiment

To test whether there was a difference in growth rate on the enriched medium, three experimental treatments were set up together with a control. Treatment 1 (T1): 99 atom% $^{13}C_6$-enriched glucose, ammonium chloride at natural abundance; Treatment 2

(T2): glucose at natural abundance, 99 atom% ^{15}N-enriched ammonium chloride; Treatment 3 (T3): 99 atom% $^{13}C_6$-enriched glucose and ^{15}N-enriched ammonium chloride; all in a minimal medium agar. The control was as described previously for natural abundance. To standardise the experiment it was necessary to ensure that the amounts of glucose or ammonium chloride were the same given the different mass of the enriched molecules (99 atom% isotopically enriched glucose molecular weight 186.11 compared to 180.16 and 99 atom% enriched ammonium chloride molecular weight 54.49 compared to 53.49), therefore the amount added to the agar was adjusted accordingly.

Cultures of the fungus were established on small (5 cm diameter) Petri dishes containing agar with the three treatments or control, prior to the growth monitoring experiment, to avoid any lag in growth due to changes in the substrates. A 1 cm^2 piece of inoculum was then taken from these cultures and placed in the centre of each of the 9 cm diameter Petri dishes of the same treatments. This reduced the potential influence of the initial inoculums on the final isotopic pattern of the cultures, as suggested by Hobbie et al. (2004). There were four replicates for each treatment and the plates were incubated at 18°C in darkness. The growth of the fungal inoculum was assessed, by daily uni-directional measurement of fungal hyphae from the initial inoculums. Measurements were undertaken using a ruler under magnification (x4).

7b.2.4 Statistical analysis

All data were analysed using either a weighted two sample t-test (unpaired) or parallel curve analysis. To estimate the nutrient source composition of *A. cylindrospora*, source partitioning was performed using IsoError (Phillips et al., 2001) version 1.41, *which calculates the proportion of various food sources in a mixture, by their isotopic* contribution. This is based on a simple mixing model: $\delta_T = \alpha\delta_A + (1 - \alpha)\delta_B$, where δ_T is

the total isotopic signature (of the enriched fungus); δ_A and δ_B are the isotopic signatures of resources *A* and *B* (the ^{13}C-glucose/^{15}N-ammonium chloride and the inert agar base respectively); and α is the proportion of resource *A* in the diet. Hence, $\alpha = (\delta_T - \delta_B)/(\delta_A - \delta_B)$ (Tiunov, 2007). All analysis was performed in triplicate unless otherwise stated and results are shown as mean ± standard error.

7b.3 RESULTS

7b.3.1 Natural abundance measurements of fungal isolates

A selection of the isolates isotopic compositions were assessed, after being grown on the same minimal media for the same length of time, obvious differences were visible in the discrimination/fractionation between species (Figure 7b.1).

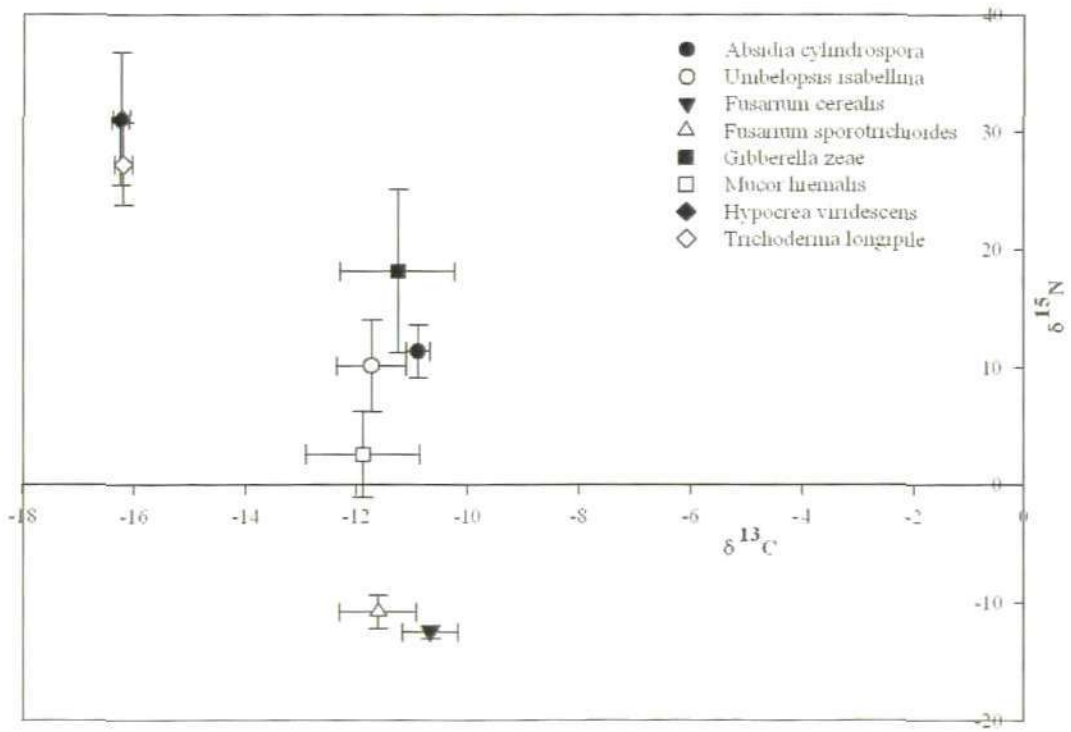


Figure 7b.1: Variation in δ^{13}C and δ^{15}N stable isotope signatures of the fungal isolates grown on the same minimal media agar over the same time period.

Multivariate analysis of variance for the $\delta^{13}C$ and $\delta^{15}N$ for the fungal isolates, showed significant differences between the $\delta^{13}C$ and $\delta^{15}N$ signatures between isolates $P < 0.001$ (Wilk's lambda = 0.09613; $d.d.f_{7,60}$). These differences in delta signatures were also significant between the individual fungal species when analysed separately for $\delta^{13}C$ and $\delta^{15}N$ ($F_{7,31} = 12.51$; $P < 0.001$ and $F_{7,31} = 8.03$; $P < 0.001$ respectively).

The mean isotopic signatures for *Absidia cylindrospora* at natural abundance were -10.9‰ (± 0.22) for ^{13}C (1.099 atom%) and 11.4‰ (± 2.25) for ^{15}N (0.371 atom%), with an average C content of 32.1% (± 0.39), N content of 4.5% (± 0.14) and C:N ratio of 8.0 (± 0.75). This reflected the isotopic signatures of the growth media – glucose ^{13}C 1.094 (± 0.00002) atom%, ammonium chloride ^{15}N 0.366 (± 0.0001) atom%, and the agar ^{13}C 1.092 (± 0.0001) atom% and ^{15}N 0.365 (± 0.0002) atom%.

7b.3.2 Enrichment measurements of the fungus

Absidia cylindrospora grew on the enriched agar from the inoculation point, and appeared to have a slower growth rate than it did when growing on a natural abundance medium, but showed the same growth characteristics. Each replicate was diluted with flour, and different dilutions were performed depending on the assumed starting enrichment level (between 40 – 99 atom% enrichment). Using equation 7 (Chapter 2), it was possible to back-calculate the atom% of the fungal isolate before dilution.

From equation 7, it was evident that *A. cylindrospora* did not solely use the added C and N for growth but had obtained some C and N from the agar gel itself. *A. cylindrospora* obtained an average enrichment of 64.2 (± 4.37) atom% for ^{13}C and 60.0 (±1.6) atom% for ^{15}N. Using the IsoError (Phillips et al., 2001) source mixing model it was deduced that the fungi obtained 36.1% (±5.12) of its C and 40.1% (±1.90) of its N from the agar.

7b.3.3 Fungal growth rate experiment

There was some differences in the growth rate between the treatments, although the fungus covered the whole plate by the end of the experiment in all cases (Figure 7b.2 and Figure 7b.3).

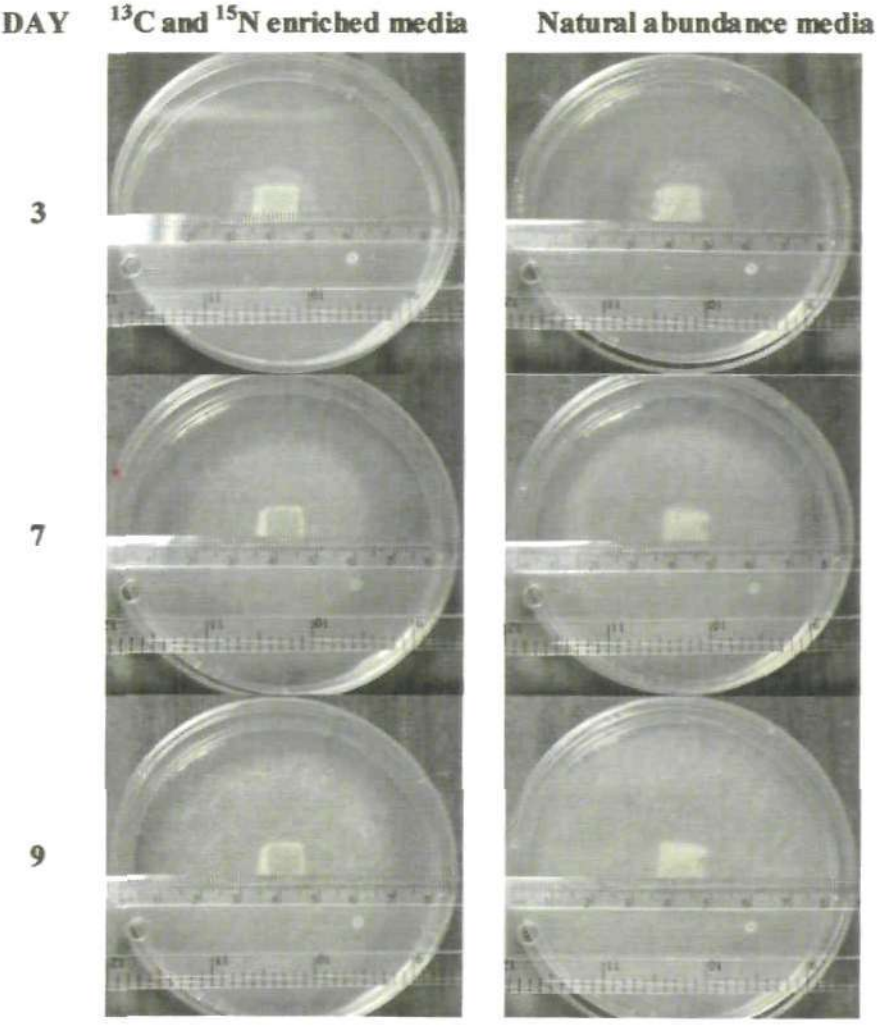


Figure 7b.2: Differences in growth rate of *A. cylindrospora* when grown on ^{13}C and ^{15}N enriched nutrient source (to 99 atom%) (left column) in comparison to natural abundance nutrient source (right column).

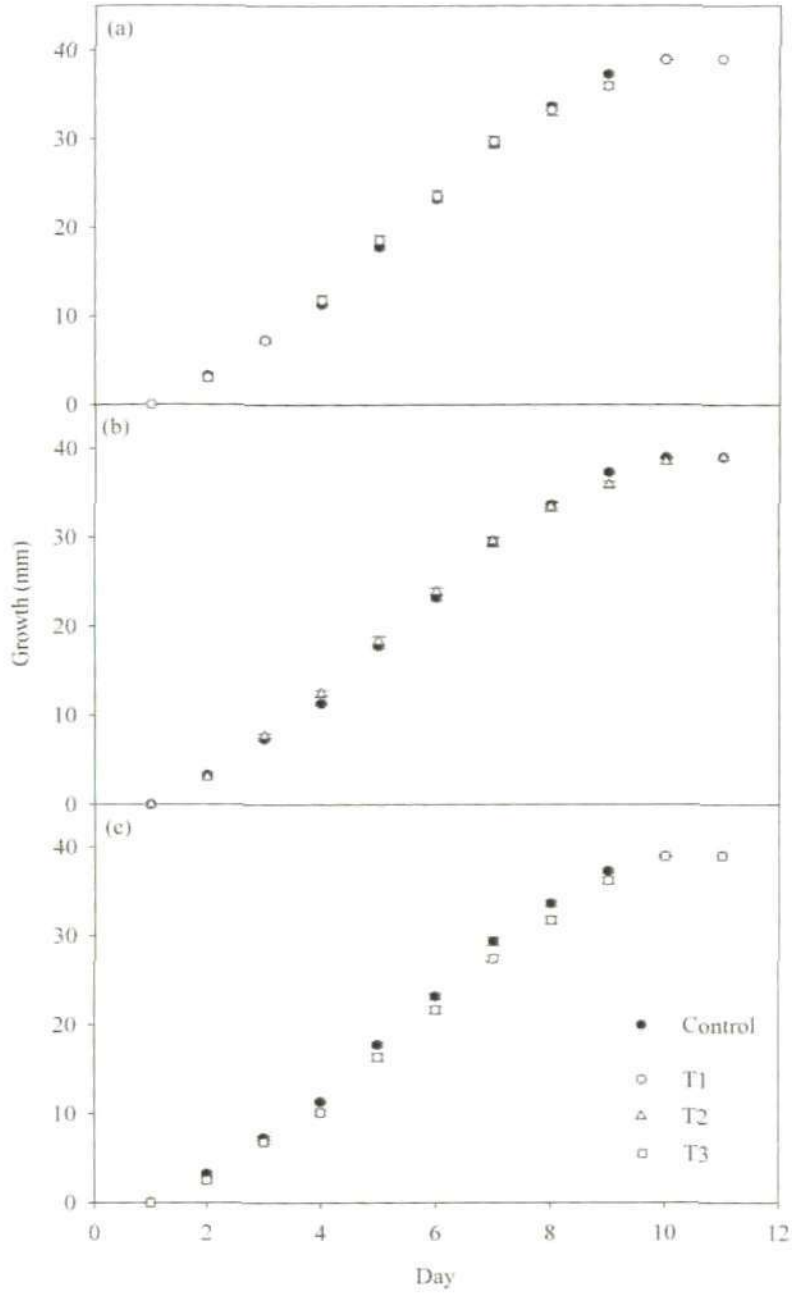


Figure 7b.3: Differences in growth of *A. cylindrospora* over time when utilising a) a nutrient source enriched in ^{13}C (99 atom%; ^{15}N at natural abundance) compared with a natural abundance nutrient source. b) a nutrient source enriched in ^{15}N (99 atom% ^{13}C at natural abundance) compared with a natural abundance nutrient source. c) a nutrient source enriched in both ^{13}C and ^{15}N (99 atom%) compared with a natural abundance nutrient source.

Data are represented as mean ± standard error (n = 4).

The accumulated analysis of variance showed there was no significant difference in growth rate between the control and T1 (Figure 7b.3a), but there was a significant difference between the control and T2, ($F_{2,80}$ = 3.76 P = 0.027), (Figure 7b.3b). Although, this difference appears to show that the growth rate is moderately increased when grown on ^{15}N-enriched media in comparison to the control. There was a significant difference between the growth rate of the fungus on the control and T3, ($F_{2,80}$ = 10.65 P < 0.001) (Figure 7b.3c), with the fungus on a natural abundance medium having a faster growth rate.

For the growth rates of *A. cylindrospora* on the singly-enriched media treatments, T1 and T2, there was no significant difference (Figure 7b.4a). However, when comparing the growth rate of the fungus on the single labelled media, ^{13}C or ^{15}N enriched (T1 or T2) in comparison to the dual-labelled medium, ^{13}C and ^{15}N enriched (T3), there was a significant difference ($F_{2,80}$ = 8.53 P < 0.001 and $F_{2,80}$ = 16.92 P < 0.001 respectively). The fungus grew faster when only one isotope was labelled than when both C and N were labelled (Figures 7b.4b and 7b.4c). The level of enrichment obtained by the fungal isolate for each of the different treatments varied depending on treatment (Table 7b.1), but this was not significantly different when comparing ^{13}C for T1 and T3.

The enrichment levels of the fungus were significantly different when comparing the enrichment of ^{15}N for T2 compared to the level of ^{15}N enrichment for T3 (P = 0.019). There was also a greater level of fractionation by the fungal isolate when grown on singly enriched media compared to natural abundance. When *A. cylindrospora* was grown on ^{13}C enriched medium (T1) it's ^{15}N atom% increased by 0.309, and when grown on ^{15}N enriched medium (T2) it's ^{13}C atom% increased by 0.463, in comparison to a 0.005 atom% (^{13}C and ^{15}N) fractionation at natural abundance (Table 7b.1).

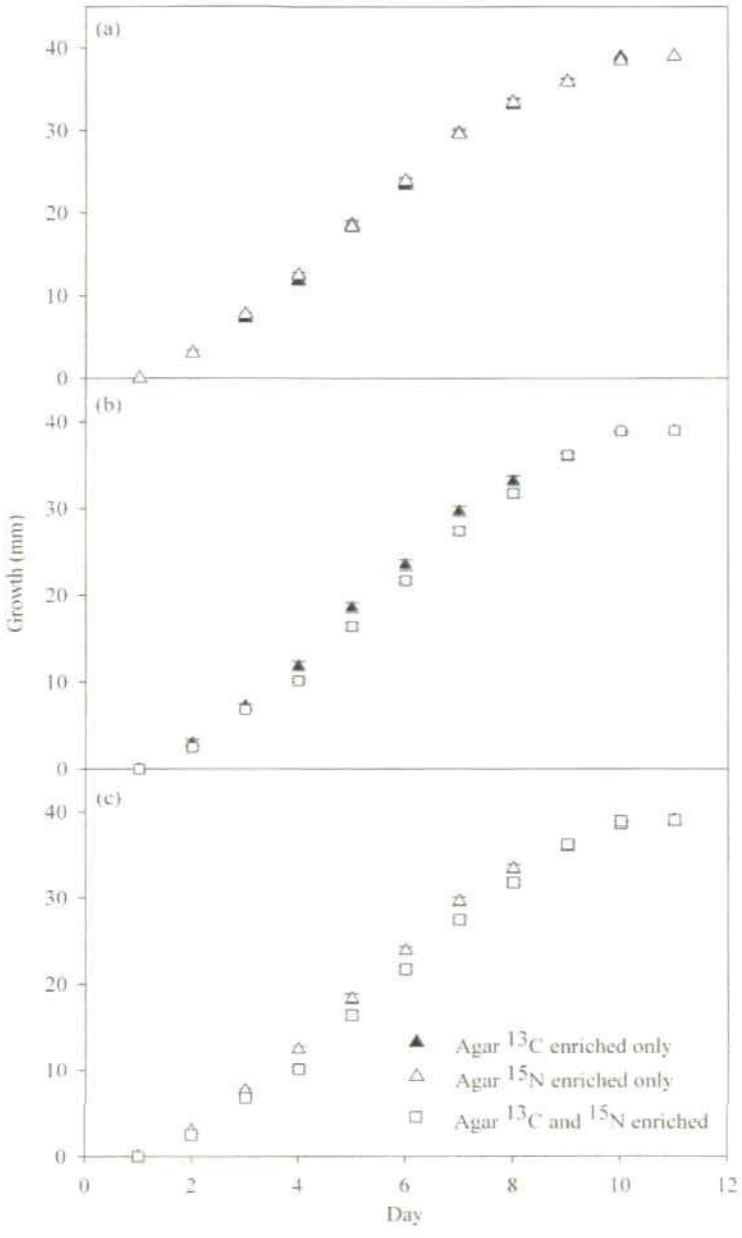


Figure 7b.4: Differences in growth of *A. cylindrospora* over time when utilising a) a nutrient source enriched in ^{13}C (99 atom%; ^{15}N at natural abundance) compared with nutrient source enriched in ^{15}N (99 atom%; ^{13}C at natural abundance). b) a nutrient source enriched in ^{13}C (99 atom%; ^{15}N at natural abundance) compared with a nutrient source enriched in ^{13}C and ^{15}N (both to 99 atom%). c) a nutrient source enriched in ^{15}N (99 atom%; ^{13}C at natural abundance) compared with a nutrient source enriched in ^{13}C and ^{15}N (both to 99 atom%).

Data are represented as mean ± standard error (n = 4).

	^{13}C enrichment	^{15}N enrichment
T1: ^{13}C enriched / ^{15}N at natural abundance	91.97 (± 2.700)	0.675 (± 0.0120)
T2: ^{15}N enriched / ^{13}C at natural abundance	1.557 (± 0.3640)	50.48 (± 2.704)
T3: Enriched in BOTH ^{13}C and ^{15}N	82.66 (± 2.867)	68.65 (± 3.925)
Control: ^{13}C and ^{15}N at natural abundance	1.099 (± 0.0003)	0.371 (± 0.0003)

Table 7b.1: Average enrichment obtained by *A. cylindrospora* growing on the three different treatments, where agar was labelled in ^{13}C and/or ^{15}N (± s.e.).

The proportion of enriched C and N in comparison to the agar gel within the mixture was calculated to determine whether the diet composition of *A. cylindrospora* was different for each of the treatments (Table 7b.2). The IsoError model shows the fungi obtained less C from the agar gel when only ^{13}C glucose was added (T1) compared to when ^{13}C and ^{15}N were added (T3). However, the fungus obtained more N from the agar gel when only ^{15}N was added (T2) compared to when both ^{13}C and ^{15}N were added (T3).

	% proportion C from agar	% proportion N from agar
T1: ^{13}C enriched / ^{15}N at natural abundance	8.0 (± 2.29)	Fractionation
T2: ^{15}N enriched / ^{13}C at natural abundance	Fractionation	49.7 (± 2.73)
T3: Enriched in BOTH ^{13}C and ^{15}N	17.5 (± 3.02)	31.4 (± 3.96)

Table 7b.2: Comparison of the proportion of C and N obtained from agar gel by *A. cylindrospora*, growing on agar which was labelled in ^{13}C and/or ^{15}N.

Fractionation occurs during metabolic reactions when heavier isotopes are discriminated against, the resulting reaction products are isotopically heavier or lighter than their precursor materials. Data presented as mean ± standard error.

7b.4 DISCUSSION

The three main aims of this study were firstly, to assess whether a saprotrophic zygomycete fungus could grow on a medium, which was enriched to 99 atom% for ^{13}C-glucose and ^{15}N-ammonium chloride, secondly to determine the level of enrichment obtained and thirdly to measure whether there was any change in the growth rate of this fungus when growing on the enriched media. Growing a selection of fungi on natural abundance minimal media prior to the enrichment study to obtain a "control" for the levels of enrichment obtained provided some unexpected results.

Looking at the differences in fractionation/discrimination of the fungi at natural abundance a number of related isolates clustered together e.g. the two Fusarium species, Hypocrea and Trichoderma species (teleomorph-anamorph connection) (Webster et al., 2007). These large differences could affect the isotopic composition of the soil fauna when considering natural abundance studies, for example in Chapter 4 the Poduromorpha are showing a relatively high level of enrichment implying they are feeding at a higher trophic level, but they may actually be consuming fungi that fractionate to a higher level than others e.g. Hypocrea and Trichoderma species. These results are similar to those described by Semenina et al., (2010), although here they found temperature and age of colonies to affect the level of fractionation.

Attempting to grow *A. cylindrospora* on an enriched nutrient source showed that it would grow on an agar medium enriched in both ^{13}C and ^{15}N gaining a level of enrichment that was significantly different from natural abundance. Although, this level of enrichment was not as high as expected, if the added C and N sources were the only food source. The level of enrichment obtained showed that the fungi were utilising a proportion of the C and N present in the agar gel, and this was in line with previous

studies which found saprotrophic fungi obtaining up to 45% of their C from the agar gel (Hobbie et al., 2004).

The results show that the growth rate of the fungal isolate was only affected when grown on the dual enriched medium, with the fungi able to compensate and maintain the same growth rate (in comparison to natural abundance) when on a singly enriched medium. This may be explained by the different proportions of agar gel utilised on the singly enriched medium in comparison to the dually enriched medium. Fungi have been shown to selectively incorporate lighter isotopes, for example, Rossmann et al., (1991) found that there was a non-random distribution of C isotopes in natural glucose, with a relative enrichment in position four (C-4) and a depletion in position 6 (C-6) of the C atoms within glucose. It has been shown (Hobbie et al., 2004) that fungi generally incorporate C-6 preferentially over C-4, this potentially explains some of these results.

The ability to selectively discriminate against the easily utilisable C and N sources supplied within the agar ($^{13}C_6$-glucose and/or ^{15}N-ammounium chloride) and actively utilise the agar gel, will vary between species, and will be affected by differences in environmental conditions. Actively utilising the agar gel over the supplied substrates for both C and N must incur a cost in the fitness of the fungal isolate in comparison to those grown on singly enriched (^{13}C or ^{15}N) and/or natural abundance media; this is visible in the differences in growth rate.

This apparent greater fractionation of stable isotopes for both ^{15}N (when grown on a ^{13}C enriched medium) and ^{13}C (when grown on a ^{15}N enriched medium) in comparison to fractionation on a natural abundance medium, is possibly due to the effect of metabolising the other heavy isotope. Studies have previously found that saprotrophic fungi are able to fractionate isotopes to varying levels for both ^{13}C and ^{15}N isotopes (Zeller et al., 2007; Semenina et al., 2010) and can have varying enrichment

levels within different areas of the fungi itself due to variation in internal cycling (Handley et al., 1996; Scandellari et al., 2009). This has implications for other studies where fungi are thought to be a preferred food source for microarthropods, which could appear to be feeding on different trophic levels (Chahartaghi et al., 2005), but may be selectively feeding on different fungi (Jorgensen et al., 2003) or different parts of the same fungus.

Chapter 7: Part C – Hyphal translocation of ^{13}C and ^{15}N through the soil

7c.1 INTRODUCTION

Fungi are filamentous and exist within the air-filled pore space within the soil. Most of the N present in the majority of fungal species is found within the cytoplasmic constituents of the hyphae. Using the soil injector like in Chapters 5 and 6 is inappropriate as the fungal mycelium would need to be broken up and if the hyphal network is disrupted cell contents will leak and a pulse of N will enter the soil system.

Previous studies using mesh to separate enriched leaf litter from the soil to assess the translocation of ^{13}C and ^{15}N by fungi have been conducted over 6 – 12 week periods and used sieved soil measuring the incorporation in Earthworms (Butenschoen et al., 2007). This timescale is too long to track the "fungal feeding channel" throughout the whole soil food web as there is the potential for isotopes to become mixed within the channels – due to fungal cell death and utilisation by bacteria prior to incorporation by soil fauna.

For a fungal isolate to be introduced into soil cores, to form a traceable part of the soil food web, alternative methods to the soil injector and long-term experiments had to be developed. Utilising the knowledge gained in Parts 1 and 2 above, all fungal isolates appeared to grow from an artificial nutrient source back into sieved soil (Part 1), although the amount of hyphae growing from the introduced fungal isolate back into the soil was unknown. *Absidia cylindrospora* obtained enrichment significantly greater than natural abundance when grown on ^{13}C and ^{15}N enriched media, although the growth rate was affected (Part 2). Here the broad aim of the experiment was to exploit this difference in enriched mycelium in comparison to natural abundance and test empirically the amount of enrichment translocated through the fungal hyphae back into

the soil. In this study our objectives were a) to determine whether *A. cylindrospora* could translocate ^{13}C and ^{15}N through the soil, b) to quantify the distance and magnitude of this translocation, and c) assess whether this is a viable option for food web introduction if extrapolated to a greater scale

When introducing a living fungus, enriched with ^{13}C and ^{15}N into a soil food web, the fungus will be competing with the natural microbial fauna residing in the soil at that time. The introduced fungi will need to infiltrate the entire area being tested in an even spatial distribution, so that consumption of the hyphae would be equally possible by all invertebrates located within the soil, therefore tracing their contribution to the food web. Overall the results here, will allow insight into whether this method of experimentation is suitable to track the fungal-faunal feeding channel within soil food webs.

7c.2 MATERIALS AND METHODS

7c.2.1 Soil collection and preparation of soil microcosms

Intact soil cores (10 cm Ø by 10 cm depth) were taken from a permanent grassland field (0N) (Chapter 2.2.1 for full details). Three cores had the top layer of grass removed before sieving – the soil was passed though a 2 mm diameter sieve at its current gravimetric water content, to remove stones and root debris. The sieved soil from the three cores was then pooled and homogenised.

7c.2.2 Defaunation of soil

The soil had to be non-sterile, as sterilisation changes the soil environment to such a large extent the growth of the fungus would have been affected and it would be impossible to extrapolate its ability to translocate ^{13}C and ^{15}N in natural conditions.

Using non-sterile soil keeps the microbial component of the soil intact, and allows potential interactions to occur between *A. cylindrospora* and the "normal" microbial community. To reduce variability, it was decided to defaunate the soil. The sieved soil was frozen at -20°C for 76 hours, allowed to thaw at room temperature before being refrozen at -20°C for a further 24 hours, before thawing at room temperature (Bradford et al., 2008). This freezing routine is thought to kill soil meso- and macrofauna such as Collembola but only have a minor affect on the soil microbial community and nutrient status within the soil (Stenberg et al., 1998; Kampichler et al., 1999; Schutz et al., 2008). The C and nutrient pulse associated with soil disturbance (inherent to microcosm construction) (Bradford et al., 2002) was not adjusted for, as the removal of roots and plant material from the sieved soil will have counteracted some of this pulse.

7c.2.3 Microcosm construction

Microcosms were constructed by adding 25 g defaunated sieved soil to sterile empty Petri dishes. Each Petri dish was 9 cm in diameter and held a maximum of 1cm depth of soil. Microcosms were to be destructively sampled at each time point, hence 36 microcosms were created, 18 to be inoculated with *A. cylindrospora* grown on ^{13}C and ^{15}N enriched media, and 18 to be inoculated with *A. cylindrospora* grown on natural abundance media. The microcosms were then grouped into sets of three and placed inside aluminium trays (Figure 7c.1) inside a clear plastic bag with a piece of wet tissue paper to maintain humidity. This reduced the potential for the fungal inoculate to spread between microcosms and to maintain the moisture content of the sieved soil.

Figure 7c.1: Microcosm set-up – sieved soil added to empty sterile Petri dishes and inoculated with *A. cylindrospora* enriched with ^{13}C and ^{15}N at the central point of the dish.

7c.2.4 Fungal culture and inoculation

Tin capsules (10 x 10 mm) were sterilised (autoclaved on liquid sterilisation cycle 121°C for 15 minutes), and minimal media agar (described in Part 2 above) both at natural abundance and enriched with ^{13}C-glucose and ^{15}N-ammonium chloride, was pipette aseptically into each capsule. A small sliver (≥ 5 mm diameter) of *A. cylindrospora* was cut from the growing edge of an enriched minimal media agar plate and inoculated individually onto the surface of the enriched agar contained within the capsules. Therefore the fungal isolate would only use the enriched agar as a nutrient source until there was growth within the sieved soil matrix. This method was repeated for *A. cylindrospora* grown on natural abundance minimal media and then inoculated onto natural abundance minimal media tin capsules to act as a control.

A small indentation was made at the central point of the microcosm and all soil particles from this area of the microcosm were removed. The fungal inoculated tin capsule was positioned there, in the centre of the Petri dish, with the lip of the capsule

level with the soil surface. Each Petri dish was incubated without its lid, to allow optimal growth of the hyphae. Once all the microcosms had been inoculated, they were incubated in darkness at 18°C until destructive sampling was performed.

7c.2.5 Sampling procedure

Over a period of 11 days, each set of three microcosms from both treatments was sampled, every 2-3 days. To sample each microcosm individually, a metal grid was placed over the Petri dish (Figure 7c.2), so that there would be uniformity in sampling. Bulk soil samples were taken using a metal corer (5 mm internal diameter) (to fit within each grid square – Figure 7c.2). At each time point a series of soil samples was taken, from one of four previously defined zones (Figure 7c.3). Each zone covered was a 1 cm^2 from the inoculation point (which was 1 cm^2), therefore all samples obtained from zone 1 are between 0-1cm^2 from the inoculation point, zone 2 between 1-2 cm^2 from the inoculation point and so on.

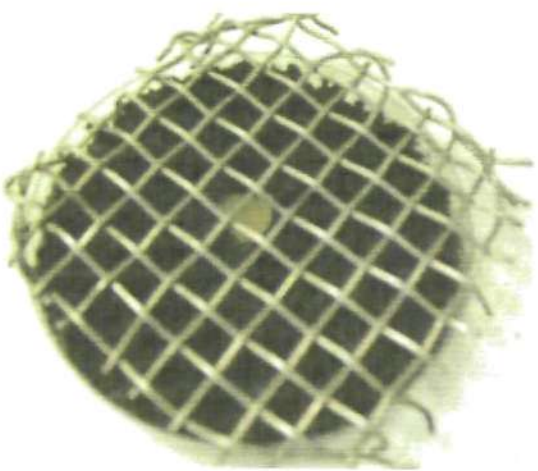


Figure 7c.2: Metal grid used for microcosm sampling to assess translocation of enrichment

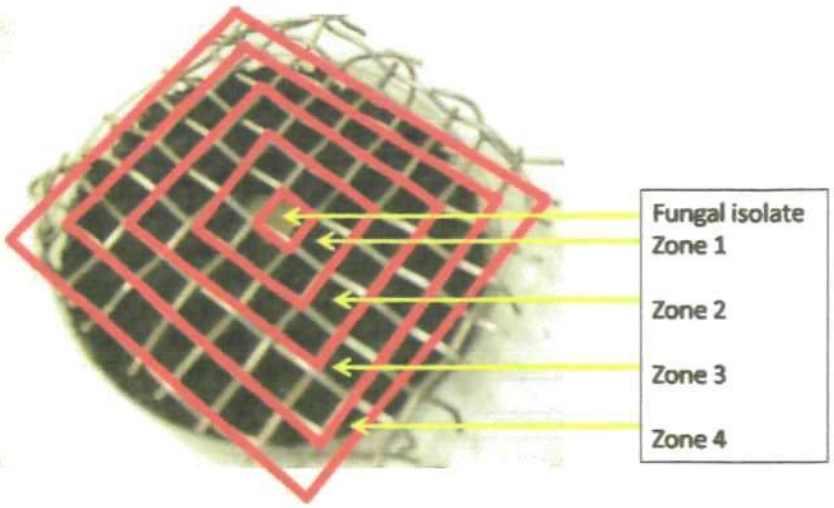


Figure 7c.3: Sampling locations within microcosm, all four zones from the inoculation point are highlighted.

At each time of sampling, six samples were taken at random from individual grid squares within each zone using the metal corer and soil from each zone was stored individually in sterile Eppendorf tubes. Bulk soil samples were a composite of soil and hyphae. After soil samples were taken from each zone, the metal corer was dipped in acetone and flamed, as was the metal grid between microcosms. Once all the soil samples had been taken, each Eppendorf was opened and dried in an oven at 80°C for 48 hours.

After 48 hours of oven drying, each soil sample was ground using a modified ball mill, following the methods of Murray and Hatch (1994), for 2 – 4 minutes. After all the soil samples had been finely ground, 10-12 mg of soil was weighed out from each Eppendorf into tin capsules (8 x 5 mm) for analysis by mass spectrometry (as per Chapter 2.2.5), with two replicates for each sample, and three replicates for each treatment.

7c.2.6 Statistical analysis

To analyse the change in enrichment of the fungi and soil samples over time with distance, polynomial quadratic regression analysis was used. Assessing the optimum time for fungal growth, with maximum potential ^{15}N and ^{13}C enrichment, the quadratic equation ($y = ax^2 + bx + c$) was differentiated and rearranged ($x = -b / 2a$) to obtain an equation for when x was not increasing (and therefore the maximum enrichment level of hyphae within the soil had been reached).

7c.3 RESULTS

Analysis of the mass spectrometry results shows that the fungal isolate is able to translocate ^{13}C and ^{15}N variable distances over time, with differing efficiencies depending on atom (C or N), distance and time. Greater quantities of ^{15}N were detected over a longer time period, and longer distance in comparison to ^{13}C, (Figure 7c.4). The ^{15}N signatures were detected at levels an order of magnitude greater than ^{13}C over the whole time period.

There was also a greater lag phase in the ^{13}C results with a detection of enrichment only noticeable in the 0-1 cm zone from the inoculation point four days after inoculation. Whereas with ^{15}N a small amount of enrichment was detectable after only one day's inoculation in zone one (Figure 7c.4). The control measurements showed very little variation over distance and time. At the 0-1 cm zone the ^{15}N detected at maximum enrichment is 274% greater than natural abundance. The ^{15}N detected decreases to only 18% greater than natural abundance when sampling at the 1-2 cm radial zone (Figure 7c.4). Whilst the difference in detectable enrichment in ^{13}C at the 0-1 cm zone from the inoculation point is only 8% greater than natural abundance, and this decreases to 0.6% greater than natural abundance when sampling 1-2 cm away from the inoculation point (Figure 7c.4).

Using a ^{13}C and ^{15}N biplot (Figure 7c.5) the differences in translocated enrichment can be visualised. From the 0-1cm zone all time points (except day 1) were significantly different from natural abundance ($P \leq 0.001$), whilst for the 1-2 cm zone, only days 6 and 11 were significantly different from natural abundance ($P \leq 0.001$ for both ^{13}C and ^{15}N). In the 2-3 cm zone, only day 11 was significantly different ($P \leq 0.001$), whilst in the 3-4 cm zone, only day 8 was significant ($P \leq 0.001$).

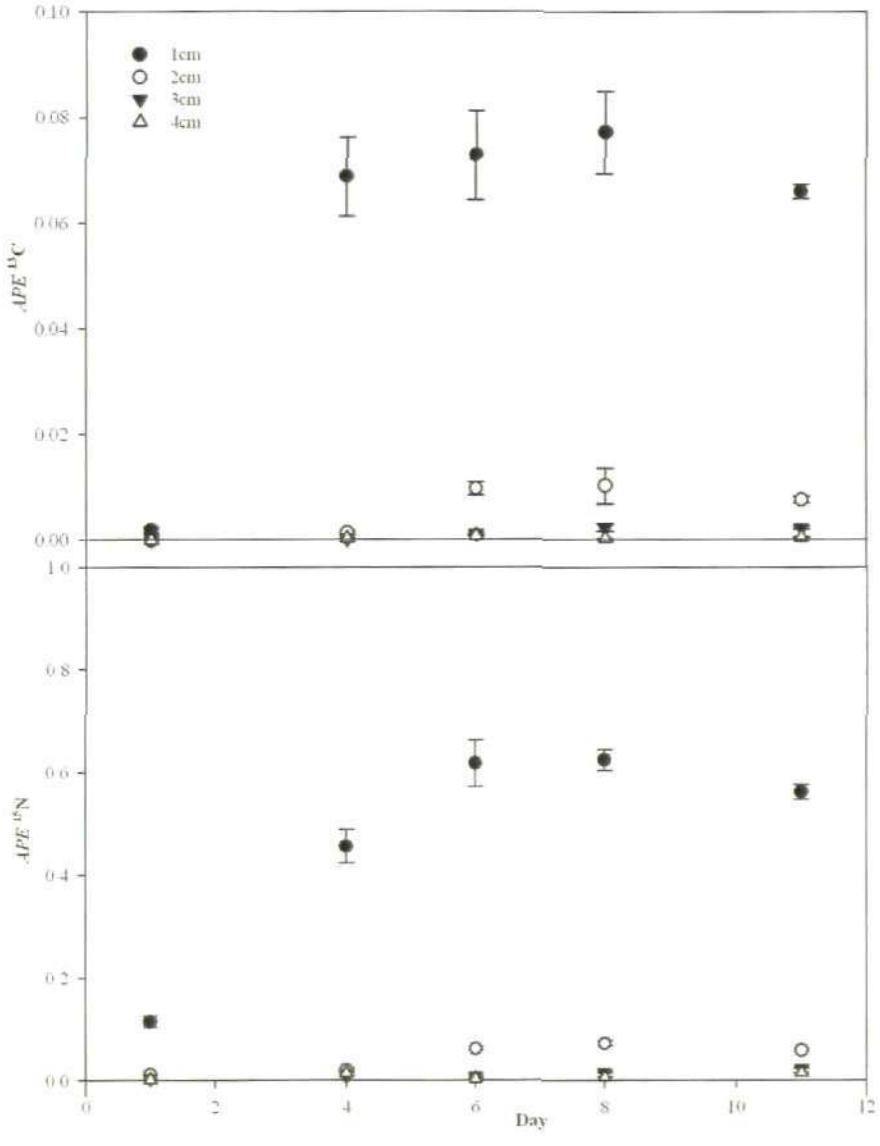


Figure 7c.4: Average change in *APE* ^{13}C and ^{15}N over time at varying distances from the fungal inoculation point.

There was a significant difference in the ^{13}C and ^{15}N signatures measured over time and distance from the fungal inoculation point ($P \leq 0.001$). The optimum time for maximum enrichment (± s.e.) was found for those squares that fitted the quadratic equation (Table 7c.1). Some results where unreliable as not enough enrichment was detected to fit a quadratic equation and these were not included in Table 7c.1.

The results from the quadratic equation were used to back calculate the maximum enrichment predicted at each distance at the optimum time point after inoculation (Table 7c.2). When comparing these to the control results obtained during this experiment and the optimum day results, it is clear that only samples within 0-2 cm radius of the fungal inoculation point will show a high enough level of enrichment to detect above background levels over time.

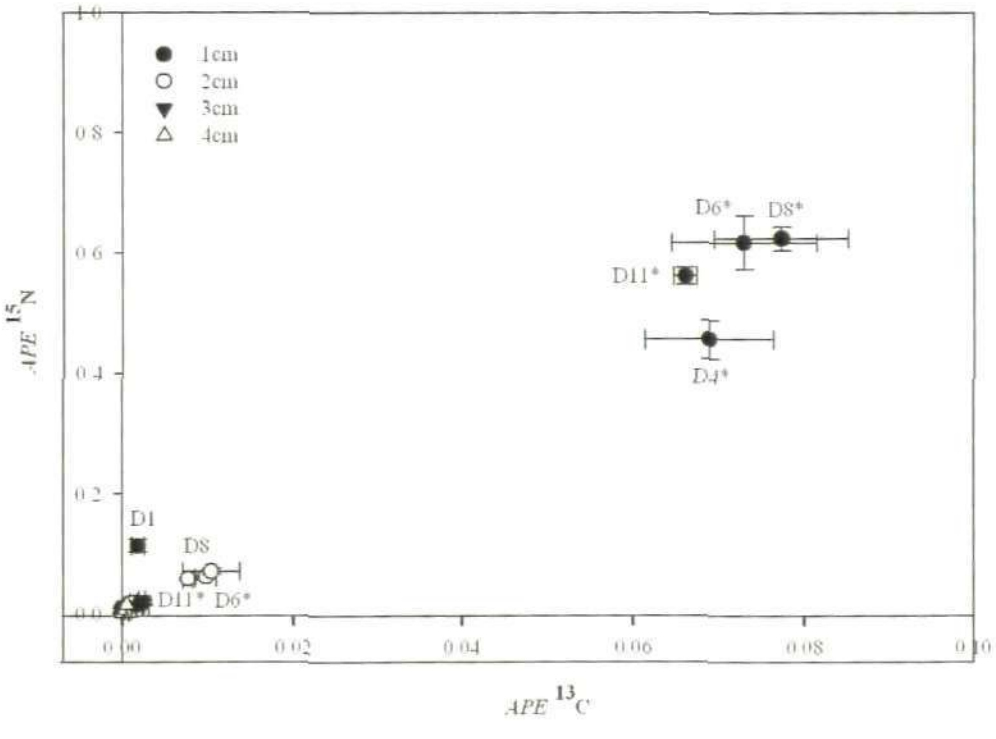


Figure 7c.5: Biplot showing change in *APE* ^{13}C and ^{15}N at varying distances from the fungal inoculation point. *Time points labelled were significantly different from natural abundance for both ^{13}C and ^{15}N at $P < 0.001$.

	^{15}N	^{13}C
0-1 cm radius	8.136 (±0.229)	7.688 (±0.496)
1-2 cm radius	9.692 (±3.51)	8.873 (±2.74)
2-3 cm radius	X	X
3-4 cm radius	X	X

Table 7c.1: Optimum time (days) for incubation to reach the maximum level of enrichment at each distance tested from inoculation point (± standard error, n = 3). X = results did not fit equation

	Maximum *APE* ^{15}N	Maximum *APE* ^{13}C
0-1 cm radius	0.641 (± 0.001)	0.084 (± 0.0004)
1-2 cm radius	0.065 (± 0.01)	0.007 (± 0.002)
2-3 cm radius	0.004 (± 0.002)	0.004 (± 0.002)
3-4 cm radius	0.004 (± 0.0002)	0.001 (± 0.0003)

Table 7c.2: Maximum enrichment possible at optimum time in relation to distance from inoculation point (± standard error n = 3).

7c.4 DISCUSSION

This experiment shows that *A. cylindrospora* can translocate limited amounts of both ^{13}C and ^{15}N through defaunated sieved soil. The level of enrichment follows a gradient dependent on distance from the inoculation point, when no additional enriched substrate was added. The results for the optimum time for maximum enrichment show, there will not be an even spatial distribution of enrichment regardless of incubation time. The furthest distance from the fungal inoculation point where enrichment was detectable was a 1-2 cm zone. This experiment has shown that the maximum level of

enrichment for both ^{13}C and ^{15}N occurs over a similar time period for the first 0-2 cm zone. Therefore an incubation of nine days will provide the maximum level of enrichment for both the 0-1 cm zone and 1-2 cm zone combined.

The difference in detectable enrichment between the 1-2 cm zone and 0-1 cm zone from the inoculation point is considerable particularly when looking at ^{13}C and ^{15}N combined (Figure 7c.5). The ^{15}N signal was detectable for a greater distance over a longer time than the ^{13}C signal. The difference between the control sample and the *APE* ^{13}C was minimal and would not be detectable as a pulse of enrichment if introduced into soil cores for feeding preference experiments and would not be distinguishable within the food chain.

Overall using this method the pulse of ^{13}C and ^{15}N enriched fungi, if grown back into the soil to be detectable in food web interactions, would only be appropriate if all the soil within the core tested was within a 0-1 cm radial distance from the inoculation point. Therefore within a 10 cm core with a surface area of 78.54 cm^2, the number of fungal inoculation points needs to be 12, when including the distance within the inoculation point as well as the radius around it. Interestingly, this is very similar to the soil injector – which has 13 needles evenly distributed to cover a surface area of 78.54cm^2. However, the soil injector introduces the pulse of enriched organisms both vertically and horizontally, throughout the core. Due to the design of this experiment, all soil microcosms had a depth of less than 1 cm; therefore the vertical translocation distance of the fungal hyphae in this experiment was not tested.

For this method to work effectively as a viable mode of introduction for food web investigations, the depth of translocation of the ^{13}C and ^{15}N by the hyphae also needs to be defined. Analysing the current results, it is unlikely that the enriched hyphae will be detectable at depths greater than 2 cm within a soil core, unless an additional enrichment source was provided. Methods need to be developed to provide enriched

nutrients so the growing mycelium retains a high level of enrichment whilst growing down throughout a soil core, but this could be problematic, due to losses of ^{13}C through respiration of the growing fungus over time. Moreover, considering the results obtained from Part 2, if the *A. cylindrospora* preferentially utilises non-enriched nutrient sources, it is likely that the fungus will utilise indigenous nutrient sources within the soil over the enriched agar. This would lead to a loss of detectable enrichment in the hyphal mycelium as the fungus grows through the soil.

There is also the possibility that the results showing a high level of enrichment in the 0-1 cm zone around the inoculation point and a low level of enrichment at the 3-4 cm zone are an artefact of the sampling methods used. As the 0-1 cm zone will contain more fungal mycelium in relation to soil, in comparison to the 3-4 cm zone which will probably have a greater soil to hyphae ratio, as the hyphal biomass had to grow through zone 0-1 cm to reach zone 3-4 cm. Therefore the enrichment levels may not be showing the translocation of ^{13}C and ^{15}N by the fungal hyphae but reflect the amount of hyphal biomass over time instead.

There is the need for more experiments to determine the amount of hyphae within each of the samples to assess whether it is just fungal biomass that has been measured or the dissipation of enriched isotope signal with growth. The vertical translocation of ^{13}C and ^{15}N by the fungal hyphae also needs to be tested. Possibly other methods of introducing a pulse of enrichment to track the fungal pathway will be necessary. For example Paterson et al. (2008b) differentiated between the bacterial and fungal pathway through the addition of enriched plant material which was utilised by different microbial communities within the soil dependent on whether it was the labile or more recalcitrant fraction of plant material. This method is based on proportions rather than absolute separation between the two pathways so would need to be adapted to be implemented for the current purpose.

Other studies have tested the C origin of spores using the isotopic differences between C_3 and C_4 plants and soil (e.g. Nakano et al., 1999). There is the potential to manipulate the isotopic signature of fungal spores and introduce these to soil cores to act as part of the fungal feeding channel within the soil faunal food web. These methods would need to be developed to ascertain whether spore feeding invertebrates or hyphal feeding invertebrates were being tested, as there may be different assemblages of organisms within these similar feeding guilds. Whether the isotopic signal dissipates as the spores germinate, would also need to be ascertained.

A simpler method of introducing an enriched fungus into the soil ecosystem would be to use a mycorrhizal fungus, as this could be enriched via symbiosis with a plant (e.g. using similar methods developed by Johnson et al., 2001). Do mycorrhizal fungi act as a main food source for the soil invertebrate food web? Studies have found arbuscular mycorrhiza acting like saprotrophic fungi (Hodge et al., 2001), whilst others have shown Collembola consuming mycorrhizal fungi in field experiments (Johnson et al., 2005) and Oribatid mites consuming mycorrhizae in feeding preference tests within the laboratory (Schneider et al., 2005b). These studies, however, do not consider the whole decomposer food web at the same time. The use of a mycorrhizal rather than saprotrophic fungus could potentially lead to ambiguous results which may not be appropriate to extrapolate to the fungal feeding energy channel within the soil food web. Also, labelling the plant to enrich the fungus, would need to be adapted to prevent leakage through root exudates which would be utilised by bacteria and again create ambiguity in the results.

7.5 CHAPTER CONCLUSIONS

A number of fungal isolates were obtained from the field sites; those that showed the most promising characteristics (abundant hyphal growth, dark mycelium, with minimal spore development) when identified to species were all *A. cylindrospora*. The results from testing whether the isolates would grow back into soil from an artificial inoculum showed that all would to varying degrees of proficiency, but it was impossible to assess visibly whether it was the introduced fungal isolate creating a hyphal network or whether it was an indigenous species. It was decided to use stable isotopes to assess the fungal isolate's ability to grow back into the soil from an artificial inoculum. To do this, it had to be tested whether the fungal isolates would grow on minimal media agar, utilise ^{13}C and ^{15}N enriched media and become enriched.

In Part 2 of this chapter, the results revealed that the fungal isolate *A. cylindrospora* can grow on enriched media and obtain an enrichment level that is significantly greater than natural abundance. However, *A. cylindrospora*'s growth rate was affected when grown on a dual labelled medium in comparison to a singly enriched medium, due to discriminatory utilisation of nutrient sources. On all enriched mediums the fungus was able to obtain a proportion of its C and N from the agar gel, and this varied with enrichment treatment (single: ^{13}C or ^{15}N or dual: ^{13}C and ^{15}N).

This discriminatory utilisation of the nutrient source by *A. cylindrospora* has implications for other studies, although as an example of a soil saprotrophic fungus it still has the potential to be used in tracer studies (e.g. Murray et al., 2009). However, where an enriched "nutrient" source is added to the soil, to monitor soil processes by fungi e.g. soil N cycling (Boyle et al., 2008), there is a need for caution, as there is the potential for the saprotrophic fungi within the soil to actively discriminate against the enriched nutrients utilising a source previously considered immobile or inert.

Furthermore, deviation from expected results caused by this discrimination is likely to be magnified in fungal feeders and higher trophic levels within the soil food web, particularly when an enriched nutrient source is hypothesised to be utilised by fungi, prior to consumption by soil invertebrates (Caner et al., 2004).

In Part 3 of this chapter the experiments demonstrate that *A. cylindrospora* can be introduced into soils in an isotopically enriched state, and this enrichment can be traced through the soil over time. The level of enrichment, the distance and magnitude of translocation was quantified. This is one of the first studies to do this, with most other studies interested in the destination of C and N transfer (e.g. Frey et al., 2003), rather than the translocation process itself. Whether this method is suitable for future experiments to track the fungal-faunal feeding channel within the soil food web needs to be investigated further.

Growing enriched hyphae back into the soil, has the advantage of only introducing a viable intact hyphal matrix within the soil, providing only one source of enrichment to the food web *via* direct consumption of living hyphae. Unfortunately, the actively growing fungi will respire $^{13}CO_2$, dissipating ^{13}C enrichment whilst also diluting with C obtained from the soil (at natural abundance). Although this may occur with the bacteria and protozoa previously added to the soil cores to track the feeding interactions of the soil fauna, at the time of injection all the bacteria and protozoa are highly labelled and are being introduced into the whole core at the same time. The ^{13}C and ^{15}N enriched hyphae are only significantly detectable over a very short distance (0-2 cm) from the inoculation point. Thus for this method to be used to track the fungal feeding channel, multiple inoculation points will be needed, and a method will need to be developed to replenish this level of enrichment to increase the movement of ^{13}C and ^{15}N horizontally and vertically. Therefore tracking the fungal feeding channel within the soil food web, will prove more difficult to do conclusively than the bacterial channel.

Chapter 8: Temporal changes affecting the community composition within a soil food web.

8.1 INTRODUCTION

Belowground interactions are essential for the overall C and N fluxes in the soil system. Arthropods have been found to speed up decomposition rates, whilst concurrently enhancing the nutrient concentrations available to the microbial component within the soil (Seastedt et al., 1984). The soil fauna have also been found to directly contribute 10% of C mineralisation and counteract N immobilisation by bacteria (Schroter et al., 2003). Soil faunal communities are among the most species rich components of terrestrial ecosystems; a hectare of productive soil may have a biomass of invertebrates and microbes weighing up to 10,000 kg (Pimentel, 2006).

There have been many studies and theories developed within population ecology, where all individual species have a unique niche (Hutchinson, 1957), or where populations are limited by their resources leading to strong top-down regulation (Hairston et al., 1960). However, these theories have limited applicability to the soil food web. Within the soil food web there is a highly diverse community exhibiting a low degree of food resource compartmentalisation, which has been referred to as "the enigma of soil animal species" (Anderson, 1975). The stability of this community over time is unknown and the effect of abiotic factors or competition between the taxa, may affect their overall abundance.

It has become clear that differences in management and abiotic conditions may have significant effects on the functioning of the soil ecosystem (Didden et al., 1998) and possibly the populations of invertebrates within the system. Spatial patterning of soil biota can occur both vertically, through the soil profile and horizontally; this spatial *heterogeneity of soil resources promotes microhabitat diversity and enables the*

separation of any potentially competing soil organisms (Ettema et al., 2002). Through regular random sampling the heterogeneous nature of a habitat can be assessed, and attempts to understand changes over time can be made.

The overall aim of this study was to assess differences between soil fauna populations over time and to see if the community composition and changes (if any) were affected by habitat type. Soil fauna were sampled on four occasions during a two-year period at two different field sites, a grassland and a woodland habitat, and measurements of the abundance and biomass were obtained.

8.2 MATERIALS AND METHODS

8.2.1 Site description and sampling strategy

Intact soil cores were collected from permanent grassland and willow woodland, (Rothamsted Research (North Wyke)). Both sites were of the same soil type (Hallsworth series). For a full site description and for sampling methods see Chapter 2.2.1. Soil cores were collected at varying intervals, from spring 2008 until spring 2010. Firstly in April 2008 and then again a month later, the third sampling was 17 months later in October 2009, before the final sampling was performed 6 months later in April 2010. The mean annual air temperature during the study was 9.5°C (± 0.74), and mean annual soil temperature was 10.8°C (± 0.85). The mean annual precipitation was 1032 mm (± 75.3) and mean monthly precipitation of 83 mm (± 6.7). The plot size was 0.68 ha in the grassland and 1.1 ha in the woodland, cores were taken using a "simple random sampling" protocol to obtain a random heterogeneous distribution of soil fauna within each habitat (Tan, 2005).

8.2.2 Precipitation and temperature

Air temperature (°C) was recorded automatically at hourly intervals using a temperature and relative humidity probe (CS215) placed 2 m above the ground connected to a datalogger, throughout the period of study. Soil temperature (°C) was recorded daily using a thermistor (107) embedded 10 cm deep within the soil and connected to a datalogger. Precipitation (mm) was also measured daily by a rain gauge (ARG100) connected to a datalogger. All equipment was MET office approved and calibrated by Campbell Scientific.

8.2.3 Community composition assessment

Measurements of the abundance and biomass of soil fauna were taken from the contents of soil cores. These soil cores were placed on a Tullgren funnel system and the invertebrates were collected, in saturated salt solution for the reasons previously described (Chapter 2.2.8). Invertebrate groups were identified to Order under a microscope, to assess the change in community composition over time. Densities of invertebrates were normalised to provide an estimated number per m^2. The biomass of each order was obtained from measurements taken of the organisms bulked dry weight prior to mass spectrometry (Results of mass spectrometry discussed in Chapter 4, 5 and 6).

8.2.4 Statistical analysis

Climatic data was analysed using a Spearman's rank correlation coefficient, as rainfall was not a continuous variable. Invertebrate numbers extracted over time were also correlated with climatic variables using Spearman's rank correlation coefficient, as the four sampling events meant the invertebrate data were not continuous variables.

Invertebrate composition was analysed based on order abundances log-transformed for each habitat. Community composition for each habitat was assessed using a variety of *diversity indices and rank/abundance plots. As invertebrates were only identified to* Order level for this analysis, it was somewhat arbitrary, but may provide an idea of composition differences between habitat and time.

To compare the effects of habitat and time on the abundance and biomass of invertebrate groups, log-transformed data was analysed using ANOVA. Relationships between the compositions of Orders in the different habitats were examined using principal component analysis, to see if the composition of Order per habitat in time was influenced by the differences in habitat. Abundance/biomass curves (ABC) were also created to assess the "disturbance" of populations within each habitat. The W-statistic associated with ABC curves measured the distance between the curves. A W-statistic of +1 would occur where there was complete biomass dominance (higher biomass than abundance curves) and an even abundance distribution across all species, and a W-statistic of -1 for the reverse case (Clarke, 1993).

Testing differences in aggregation three different methods were used, the variance mean ratio (VMR) to assess differences in scale of sampling (core or habitat scale), Taylor's power law (index of aggregation) to assess changes in the populations over time, and the coefficient of variation (CV) to assess the stability of populations over time. The VMR (*sensu* Benefer et al., 2010) was used to assess the differences in the distribution of individual taxa at each scale (core or habitat scale) and as an index of dispersion. Secondly, the full version of Taylor's index of aggregation (Taylor, 1961) was used (*sensu* Murray et al., 1995). Individuals within a natural population are not independent of each other, and their variance within the population is related to the mean by a simple power law ($s^2 = am^b$) where a and b are characteristic of the population under investigation.

Thirdly, the CV is similar to the above aggregation indices but tests the stability of the two habitats over time, with the 'reliability' of population sizes within each habitat as a measure of stability at each time point (Eisenhauer et al., 2011). The CV was calculated by dividing the standard deviation by the mean for each habitat separately at each time point. This meant the variability at one time point acted as a measure of stability, the lower the CV (the variability) the higher the reliability (the stability) at each time point. Having obtained four CV's for each habitat, an analysis of variance was used to assess differences between habitats over time.

8.3 RESULTS

8.3.1 Precipitation and temperature

The total monthly precipitation varied a great deal over time, data presented from prior to soil fauna sampling (April-2007) to seven months after final faunal sampling (December-2010) (Figure 8.1a). The minimum monthly total precipitation was 32.8 mm in May 2010 and the maximum total precipitation was 245.8 in November 2009. There appear to be greater fluctuations in monthly rainfall occurring from late 2009 to 2010 in comparison to the other years.

The average monthly temperature recorded varied through "normal" seasonal fluctuations (Figure 8.1b), although these fluctuations seem to be getting larger over time. The minimum monthly average temperature was 0.8°C in December 2010 and the maximum monthly average was 15.8°C in July 2010, suggesting there were more fluctuations in temperature in 2010 in comparison to the other years.

Total monthly rainfall and average monthly temperature showed a significant correlation, (Spearman's rank correlation coefficient $P = 0.050$) although the T-test approximation was not significant (*T-statistic* = -1.30; $P = 0.201$; df_{43}). Soil temperature and rainfall also showed a significant correlation (Spearman's rank correlation

coefficient $P = 0.046$) although again, the T-test approximation was not (*T-statistic* = -1.35; $P = 0.183$; df_{43}). These results link the increasing fluctuations between temperature and rainfall. As a preliminary test the total number of invertebrates extracted over time was assessed to see if there was a correlation with the climatic data, using the same methods (Figure 8.2).

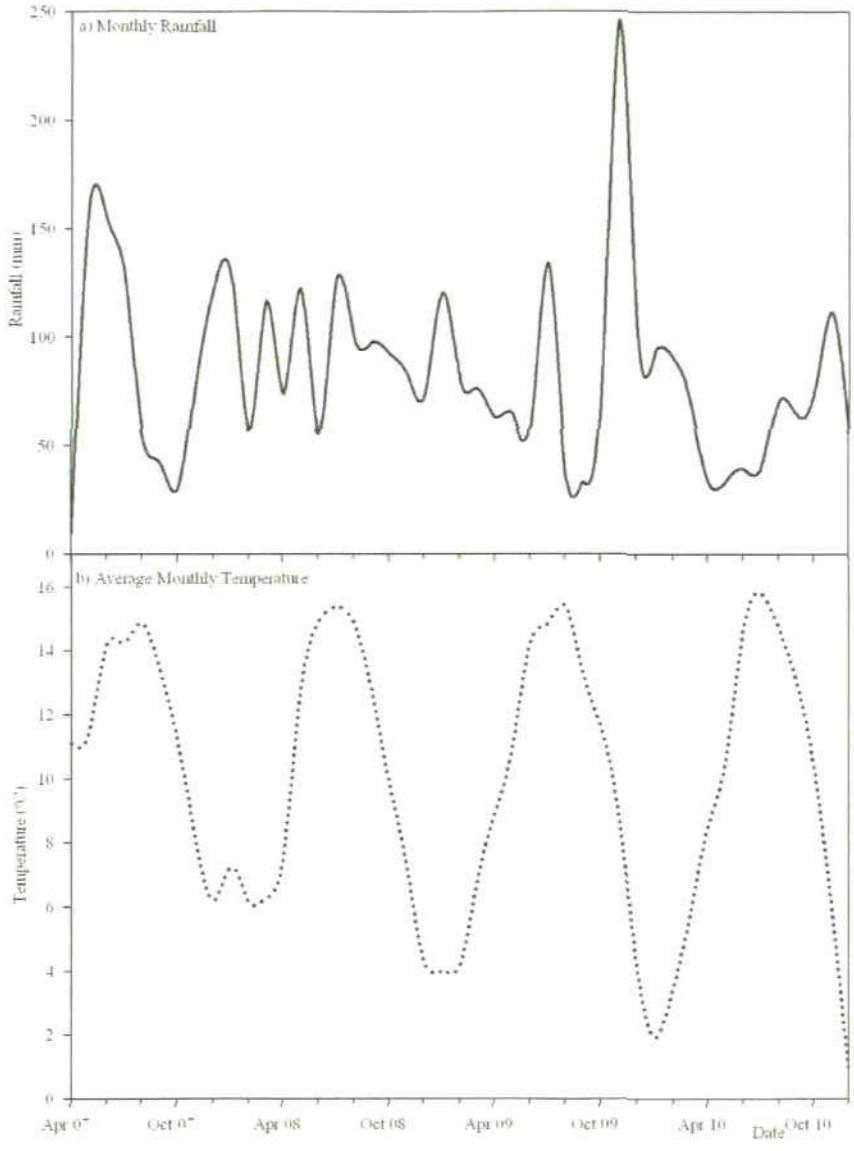


Figure 8.1: Climatic variation over time, a) total monthly rainfall and b) average monthly temperatures, from April 2007 until December 2010.

There was a correlation between the total number of grassland invertebrates collected over time with total monthly rainfall (Spearman's rank correlation coefficient $P = 0.021$) although there was no correlation for woodland invertebrate numbers (Spearman's rank correlation coefficient $P = 0.083$). There was no correlation for invertebrates collected from either habitat with air or soil temperature over time (Spearman's rank correlation coefficient grassland $P = 0.188$; woodland $P = 0.104$).

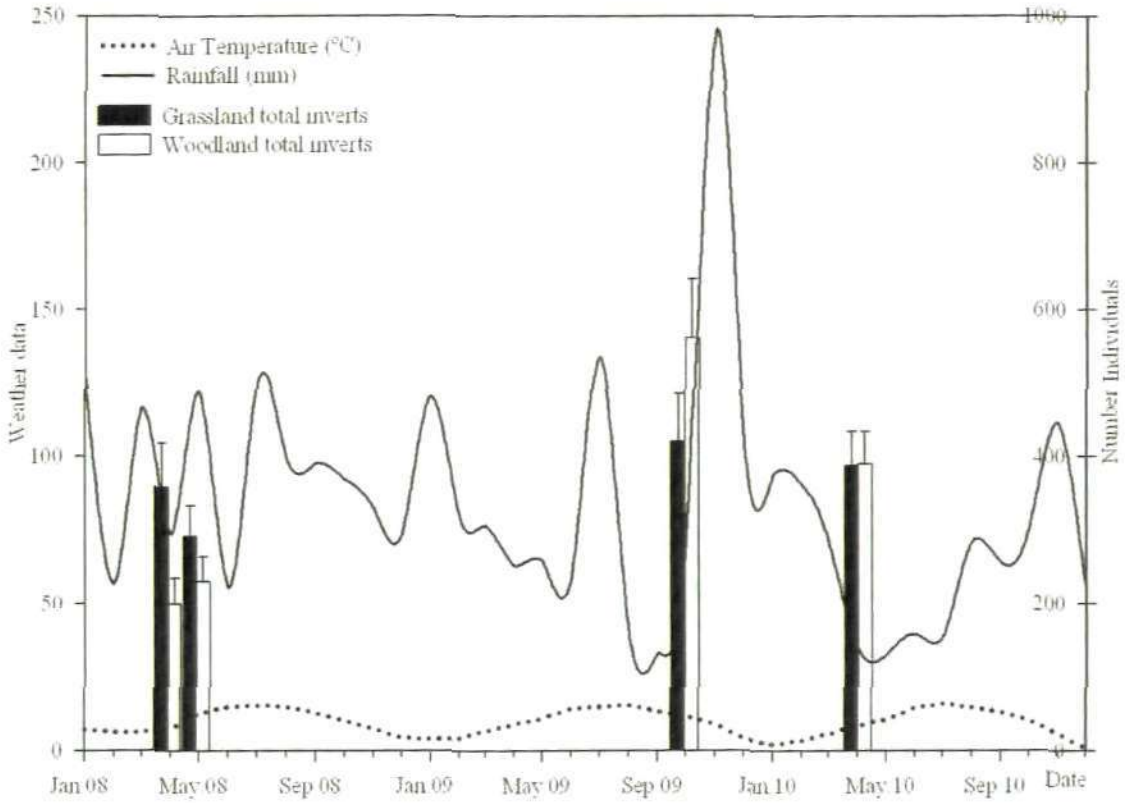


Figure 8.2: Total invertebrate abundance (mean ± standard error) per core in the grassland and woodland habitat, in relation to climatic variables.

8.3.2 Community composition assessment

During this temporal study 21,643 invertebrates were extracted via the Tullgren funnel system, there was no significant difference between the overall number of

invertebrates extracted between the two habitats ($df_{1,928}$ *T*-statistic = 0.27; $P = 0.788$); 11,095 invertebrates were extracted from the grassland and 10,548 from the woodland. Within each habitat the majority of invertebrates were either Acari (50.7% of invertebrates collected from the grassland and 42.6% of the ones collected from the woodland) or Collembola (43.5% of the grassland and 51.2% of woodland invertebrates collected) (Table 8.1). However, the majority of invertebrate biomass was biased towards the macrofauna, mainly earthworms (Table 8.1).

Although the invertebrates were only identified to order in this study of general community composition, the differences between habitats were calculated using a range of diversity indices described by Wheater et al., (2003); this was to determine if there were changes between habitats and/or time during the sampling period. These different indices were used as some are biased towards rare species (e.g. Shannon) whilst others are biased towards the more common ones (e.g. Simpson's) (Wheater et al., 2003). For the Shannon diversity index there was no significant difference between habitats or change in whole community composition over time. Similar results were found for the Simpson's Index (1-*D*) and Berger-Parker Index (1/*d*). Using a range of indices in this way makes the results more robust, and does indicate that at the taxonomic resolution tested there were no differences in the community composition.

Assessing the evenness of the community as an index, where the closer to zero the result the more dominated the community is by a single species (Wheater et al., 2003), showed that both habitats were very similar. The evenness index for the grassland was 0.31 ± 0.012, whilst the woodland was 0.34 ± 0.030, again there were no significant differences between habitats or time. A "rank/abundance" or Whittaker plot (Figure 8.3) shows the difference in community composition for the two habitats (all dates combined).

Order (including abbreviation)		Number of organisms per m^2		Biomass per m^2 (mg)	
		Grassland	Woodland	Grassland	Woodland
Acari	ac	23111 (± 2320)	18466 (± 2489)	274.9 (± 66.08)	490.9 (± 93.39)
Aphids	ha	1113 (± 472)	144 (± 49)	61.8 (± 22.75)	20.4 (± 7.11)
Chilopoda	ch	0 (± 0)	74 (± 24)	0.0 (± 0.00)	255.8 (± 89.91)
Coleoptera	c	86 (± 28)	308 (± 68)	41.0 (± 12.69)	158.5 (± 38.18)
Coleoptera Larvae	cl	542 (± 122)	444 (± 177)	396.1 (± 130.44)	73.4 (± 22.02)
Collembola	col	19817 (± 1662)	22191 (± 2746)	56.7 (± 6.93)	77.7 (± 10.22)
Diplopoda	dip	0 (± 0)	123 (± 40)	0.0 (± 0.00)	164.2 (± 56.36)
Diptera	d	107 (± 29)	193 (± 53)	50.0 (± 17.12)	34.1 (± 10.96)
Diptera Larvae	dl	324 (± 63)	435 (± 93)	431.4 (± 94.50)	171.0 (± 32.99)
Earthworms	ew	78 (± 18)	140 (± 32)	703.0 (± 184.22)	1025.3 (± 230.89)
Enchytraeidae	ec	29 (± 11)	103 (± 37)	8.9 (± 4.33)	24.8 (± 9.44)
Slugs	sl	42 (± 14)	4 (± 4)	381.3 (± 180.23)	105.3 (± 105.26)
Spiders	sp	94 (± 70)	82 (± 39)	2.9 (± 1.90)	6.4 (± 3.32)
Thrips	t	222 (± 75)	8 (± 6)	2.7 (± 0.90)	0.1 (± 0.04)
Woodlice	w	4 (± 4)	608 (± 181)	0.5 (± 0.53)	95.3 (± 26.36)

Table 8.1: Community composition, abundance and biomass of soil fauna from the grassland and woodland habitats, average over all sampling points. Data presented as mean ± standard error (n=31).

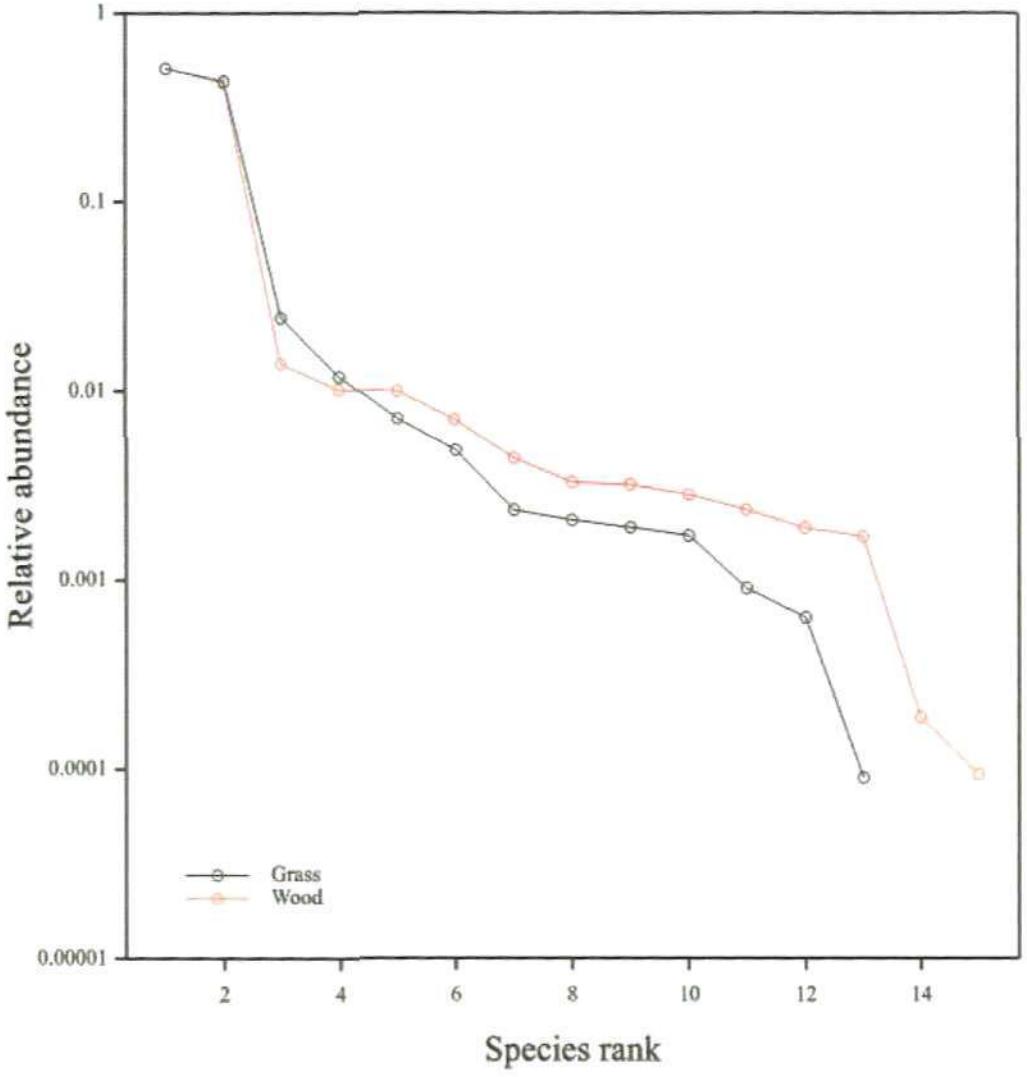


Figure 8.3: Rank/Abundance "Whittaker" Plot, showing community dominance within the grassland (black line and circles) compared to the woodland (red line and circles), for all sampling days combined.

A Whittaker plot provides an indication of dominance within a community, a steep plot (Figure 8.3) signifies that the assemblage is dominated by a few orders. When the slopes (Figure 8.3) become shallower (after the initial steep decline) this indicates that the rest of the community is relatively evenly proportioned.

A regression showed the number of invertebrates collected varied significantly over time ($F_{30,899} = 50.98$; $P < 0.001$), although the variation accounted for was at an intermediary level ($R^2 = 61.7$). The differences in rank/abundance over time (Figure 8.4 a-d) shows there are differences in the proportion of all invertebrates over time.

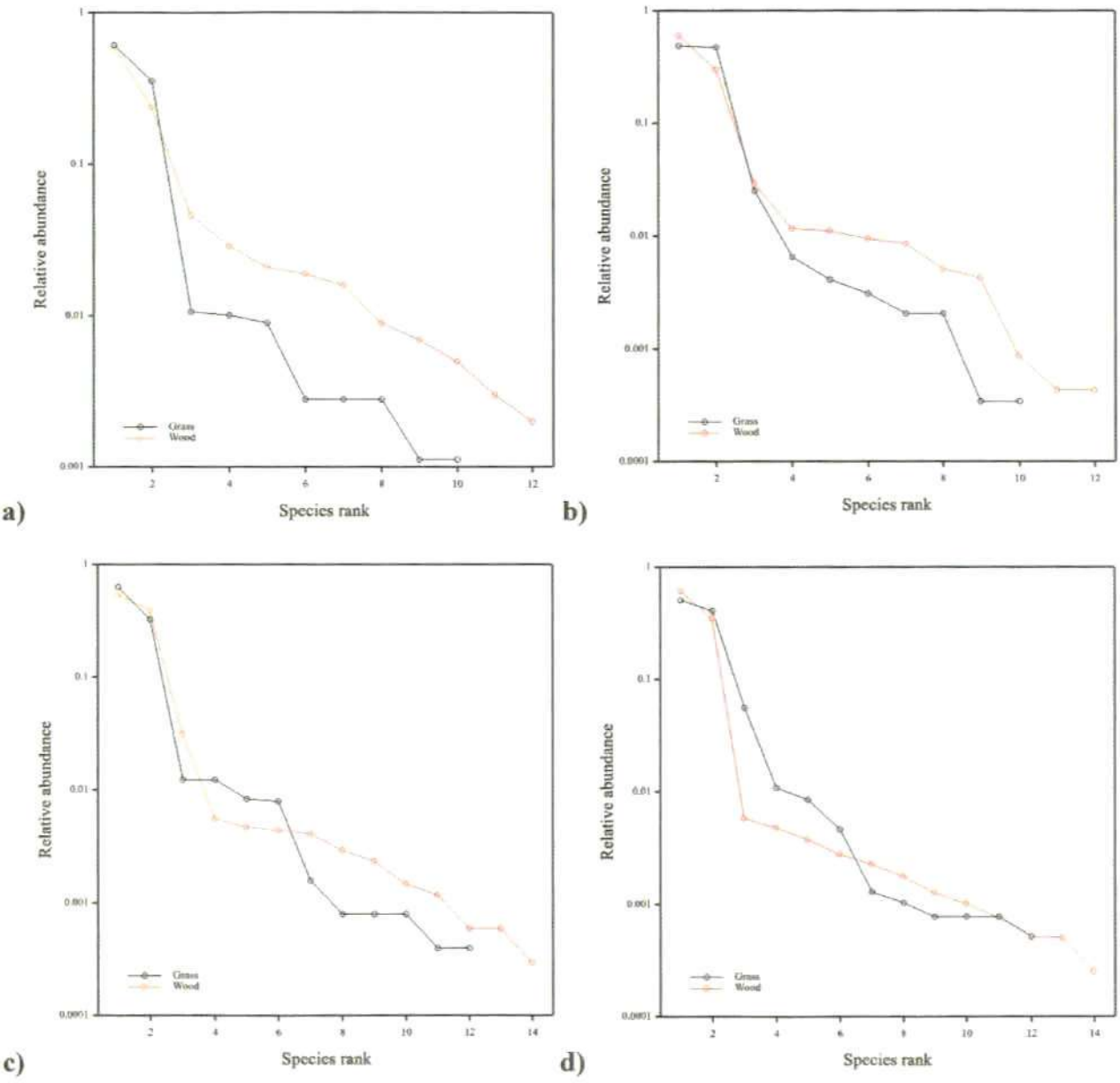


Figure 8.4: Rank/Abundance "Whittaker" Plot, showing community dominance within the grassland (black line and circles) compared to the woodland (red line and circles), for each sampling day separately a) day = 0; b) day = 18; c) day = 552; and d) day = 712.

Although Acari and Collembola remain dominant throughout the study period, the rest of the soil faunal numbers fluctuate. The slopes at the earlier sampling dates appear shallower (Figures 8.4a and 8.4b) in comparison to the ones at the later two sampling dates (Figures 8.4c and 8.4d) which appear to be steeper.

The number of individual invertebrates within each Order collected separately showed significant differences between the two habitats over time (Table 8.2). There were significantly greater numbers of Acari, aphids, slugs and thrips within the grassland habitat, whilst there were significantly greater numbers of Chilopoda, Coleoptera adults, Diplopoda, Diptera larvae and woodlice in the woodland (Table 8.2). The number of individual invertebrates collected during each sampling changed over time (Table 8.3). There were significant changes in the number of earthworms collected over time in both habitats (Table 8.2) and there was a significant decrease in the number of spiders collected over time in both habitats. There was also a significant change in the Acari numbers collected over time, with an increase in numbers in the woodland and a slight increase also perceivable in the grassland (Table 8.3).

A number of invertebrate Orders showed significant variation when the interaction between time and habitat were considered (Table 8.2), this is due to population changes over time which were different in each habitat. The Collembola had significant population changes over the time period (Table 8.2). The number of Collembola increased significantly in the woodland (Table 8.3), whilst in the grassland the numbers collected were relatively stable over time. Diptera larvae were found in significantly decreasing numbers in the woodland, although their numbers were more stable in the grassland. Enchytraeid worms were found in increasing numbers in both habitats. Whereas the thrips were seen in a more cyclical pattern in the grassland, and remained low in the woodland throughout. Generally very few woodlice were collected in the grassland, although they were found to become increasingly common in the woodland.

	Number of individuals per m^2						Biomass per m^2					
	Plot		Days		Plot x Days		Plot		Days		Plot x Days	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Acari	5.97	**0.018**	9.21	**< 0.001**	1.82	0.154	26.60	**< 0.001**	40.21	**< 0.001**	0.82	0.487
Aphids	4.35	**0.042**	1.55	0.212	3.91	**0.013**	2.26	0.139	0.36	0.783	3.83	**0.015**
Chilopoda	16.03	**< 0.001**	2.40	0.078	2.40	0.078	17.29	**< 0.001**	3.67	**0.018**	3.67	**0.018**
Coleoptera	14.12	**< 0.001**	2.54	0.066	1.93	0.135	13.98	**< 0.001**	2.66	0.057	1.40	0.254
Coleoptera Larvae	0.52	0.475	2.08	0.113	0.73	0.537	3.57	0.064	3.20	**0.030**	1.88	0.144
Collembola	0.01	0.941	8.61	**< 0.001**	3.64	**0.018**	7.88	**0.007**	6.82	**< 0.001**	1.66	0.186
Diplopoda	33.49	**< 0.001**	9.05	**< 0.001**	9.05	**< 0.001**	33.17	**< 0.001**	9.14	**< 0.001**	9.14	**< 0.001**
Diptera	1.35	0.250	2.36	0.081	9.18	**< 0.001**	0.10	0.755	1.80	0.158	16.39	**< 0.001**
Diptera Larvae	5.76	**0.020**	12.57	**< 0.001**	7.35	**< 0.001**	0.02	0.893	27.67	**< 0.001**	13.51	**< 0.001**
Earthworms	1.14	0.291	4.00	**0.012**	1.19	0.322	0.68	0.414	3.13	**0.033**	0.60	0.620
Enchytraeidae	2.54	0.117	11.90	**< 0.001**	8.17	**< 0.001**	2.52	0.118	12.35	**< 0.001**	7.97	**< 0.001**
Slugs	7.45	**0.009**	2.40	0.078	0.96	0.416	6.43	**0.014**	2.99	**0.039**	1.06	0.373
Spiders	0.79	0.378	4.03	**0.012**	0.97	0.413	1.03	0.315	2.54	0.066	0.83	0.482
Thrips	20.97	**< 0.001**	7.14	**< 0.001**	5.44	**0.002**	27.96	**< 0.001**	12.02	**< 0.001**	10.40	**< 0.001**
Woodlice	58.66	**< 0.001**	6.44	**< 0.001**	3.38	**0.025**	56.77	**< 0.001**	6.13	**0.001**	3.96	**0.013**

Table 8.2: ANOVA table of *F* values for the effect of day (0, 18, 552, 712) and plot (grassland or woodland) on the number and biomass per m^2 for the different groups of soil invertebrates. (Degrees of freedom – Plot $df_{1,54}$; Days $df_{3,54}$; and Plot x Days $df_{3,54}$).

	Number of organisms per m^2							
	Grassland				Woodland			
	Day 0	Day 18	Day 552	Day 712	Day 0	Day 18	Day 552	Day 712
Acari	217 (± 42.9)	137 (± 21.8)	264 (± 60.6)	158 (± 22.7)	121 (± 30.1)	70 (± 5.9)	304 (± 50.0)	137 (± 23.1)
Aphids	1 (± 0.5)	2 (± 1.2)	5 (± 4.6)	22 (± 10.3)	4 (± 1.6)	1 (± 0.6)	0.3 (± 0.33)	0.2 (± 0.13)
Chilopoda	0 (± 0.0)	0 (± 0.0)	0 (± 0.0)	0 (± 0.0)	1 (± 0.5)	0.2 (± 0.13)	0.7 (± 0.67)	0.5 (± 0.31)
Coleoptera	0.4 (± 0.25)	1 (± 0.6)	0.3 (± 0.21)	0.5 (± 0.31)	6 (± 2.2)	2 (± 0.7)	3 (± 0.9)	0.7 (± 0.30)
Coleoptera Larvae	1 (± 0.8)	7 (± 2.5)	4 (± 1.1)	3 (± 1.0)	4 (± 1.9)	7 (± 4.1)	2 (± 0.4)	0.9 (± 0.31)
Collembola	126 (± 17.5)	141 (± 24.2)	137 (± 17.2)	196 (± 27.0)	48 (± 1.8)	141 (± 34.1)	223 (± 43.2)	242 (± 36.7)
Diplopoda	0 (± 0.0)	0 (± 0.0)	0 (± 0.0)	0 (± 0.0)	0.6 (± 0.40)	0 (± 0.0)	3 (± 1.1)	1 (± 0.6)
Diptera	0 (± 0.0)	0.6 (± 0.34)	0.3 (± 0.21)	2 (± 0.5)	0.4 (± 0.2)	3 (± 1.0)	2 (± 0.76)	0.4 (± 0.31)
Diptera Larvae	3 (± 0.9)	0.1 (± 0.10)	3 (± 1.1)	4 (± 0.9)	9 (± 3.2)	3 (± 0.7)	3 (± 1.0)	2 (± 0.3)
Earthworms	1 (± 0.5)	0.9 (± 0.28)	0.3 (± 0.21)	0.3 (± 0.2)	3 (± 0.9)	1 (± 0.3)	0.8 (± 0.31)	0.3 (± 0.15)
Enchytraeidae	0 (± 0.0)	0 (± 0.0)	0.7 (± 0.21)	0.3 (± 0.2)	0 (± 0.0)	0 (± 0.0)	0.3 (± 0.33)	2 (± 0.7)
Slugs	0.4 (± 0.40)	0.6 (± 0.22)	0 (± 0.0)	0.2 (± 0.13)	0 (± 0.0)	0.1 (± 0.10)	0 (± 0.0)	0 (± 0.0)
Spiders	4 (± 3.3)	0 (± 0.0)	0.2 (± 0.17)	0.3 (± 0.30)	2 (± 1.6)	0.1 (± 0.10)	1 (± 0.8)	0.2 (± 0.13)
Thrips	4 (± 1.2)	0.1 (± 0.10)	5 (± 2.3)	0.4 (± 0.22)	0 (± 0.0)	0 (± 0.0)	0.2 (± 0.17)	0.1 (± 0.10)
Woodlice	0 (± 0.0)	0 (± 0.0)	0.2 (± 0.17)	0 (± 0.0)	1 (± 0.5)	2 (± 1.8)	18 (± 3.4)	2 (± 0.4)

Table 8.3: Community composition and abundance of soil fauna from the grassland and woodland habitats, average per core from each sampling point. Data presented as mean ± standard error (n=5-10).

To evaluate the relationship between population numbers over time for both habitats a Principal Component biplot was used (Figure 8.5). There was a clear difference in the number of invertebrates extracted between the woodland and grassland over time. The biplot is a graphical representation of the relationship between the individual populations within each core and the different soil faunal groups. From the biplot it is clear that there is a greater association between aphids, slugs, and thrips with the grassland. The invertebrates with a greater association with the woodland habitat are also visible, e.g. Diplopoda, woodlice, Coleoptera (adults and larvae), Chilopoda, Diptera (adults and larvae) and spiders.

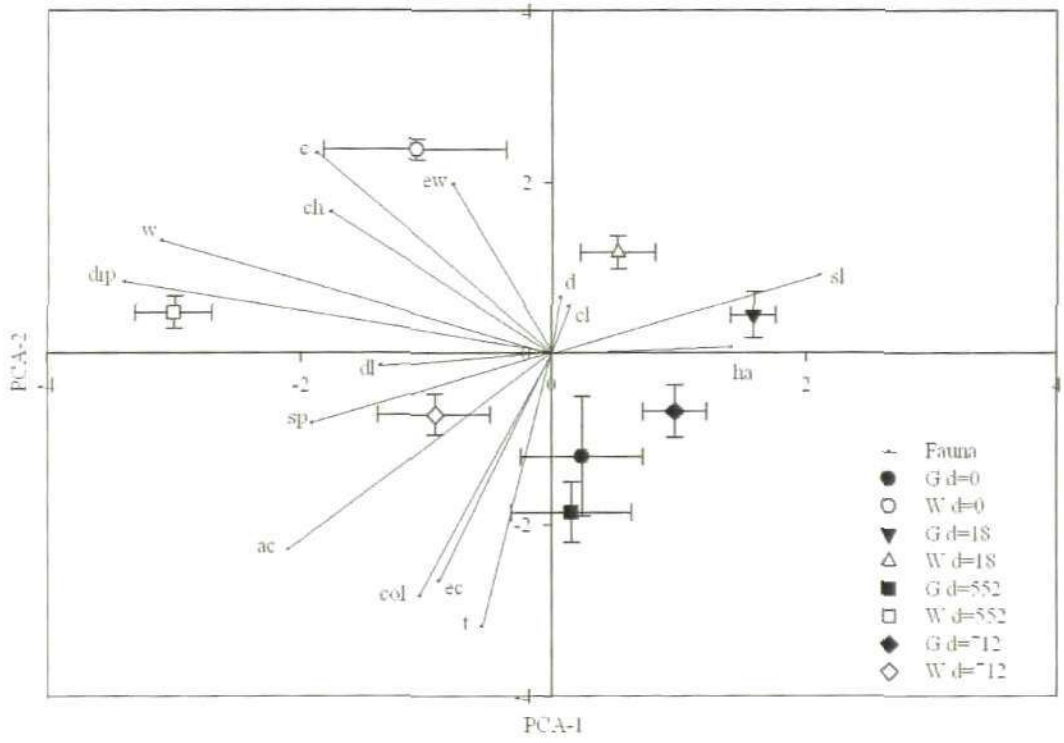


Figure 8.5: Principal component ordination diagram for the populations of soil fauna collected over time in the grassland (closed symbols) and woodland habitat (open symbols). The further the soil fauna are from the origin the greater the relative effect on overall population numbers. (For abbreviations see Table 8.1).

Acari and Collembola have a large effect on population numbers within the cores (Figure 8.5) and both appear to have a similar relationship with the woodland and the grassland. Looking at the number of fauna combined for each time point there are clusters around specific time points, particularly in the woodland. Generally, there were differences in the relationship between population numbers and particular soil fauna over time, however the points representing the core populations clustered together within each habitat, showing that the overall variation was low.

The abundance of soil fauna in relation to their biomass was significantly different between plots (MANOVA: Wilks' lambda 0.9788, $F_{1,809} = 8.78$ $P < 0.001$), between the different invertebrate orders (MANOVA: Wilks' lambda 0.2056, $F_{14,1618} = 69.66$ $P < 0.001$), and time (MANOVA: Wilks' lambda 0.9492, $F_{3,1618} = 7.12$ $P < 0.001$). Whilst the interaction between habitat type, invertebrate fauna and time was also significant (MANOVA: Wilks' lambda 0.7737, $F_{42,1618} = 2.64$ $P < 0.001$). Looking at a comparison of the abundance/biomass curves over time (Figures 8.6 and 8.7), all are dominated by a small number of taxa (the Acari and Collembola) with relatively low biomass, usually this sort of curve is characteristic of a disturbed community (Anticamara et al., 2010).

When the Acari and Collembola were excluded from the comparison, a different pattern is visible (Figure 8.8). Whereas previously with the Acari and Collembola the W-statistics were negative (grassland -0.233 and woodland -0.439 respectively), when the Acari and Collembola are excluded the W-statistics are positive (grassland 0.027 and woodland 0.193). Therefore when the Acari and Collembola are included there is complete dominance of abundance and the distribution of abundance is biased towards these two groups. Whereas, when the Acari and Collembola are excluded the biomass curve is dominant (to a greater extent in the woodland than the grassland) and there is a more even distribution of abundance across faunal groups.

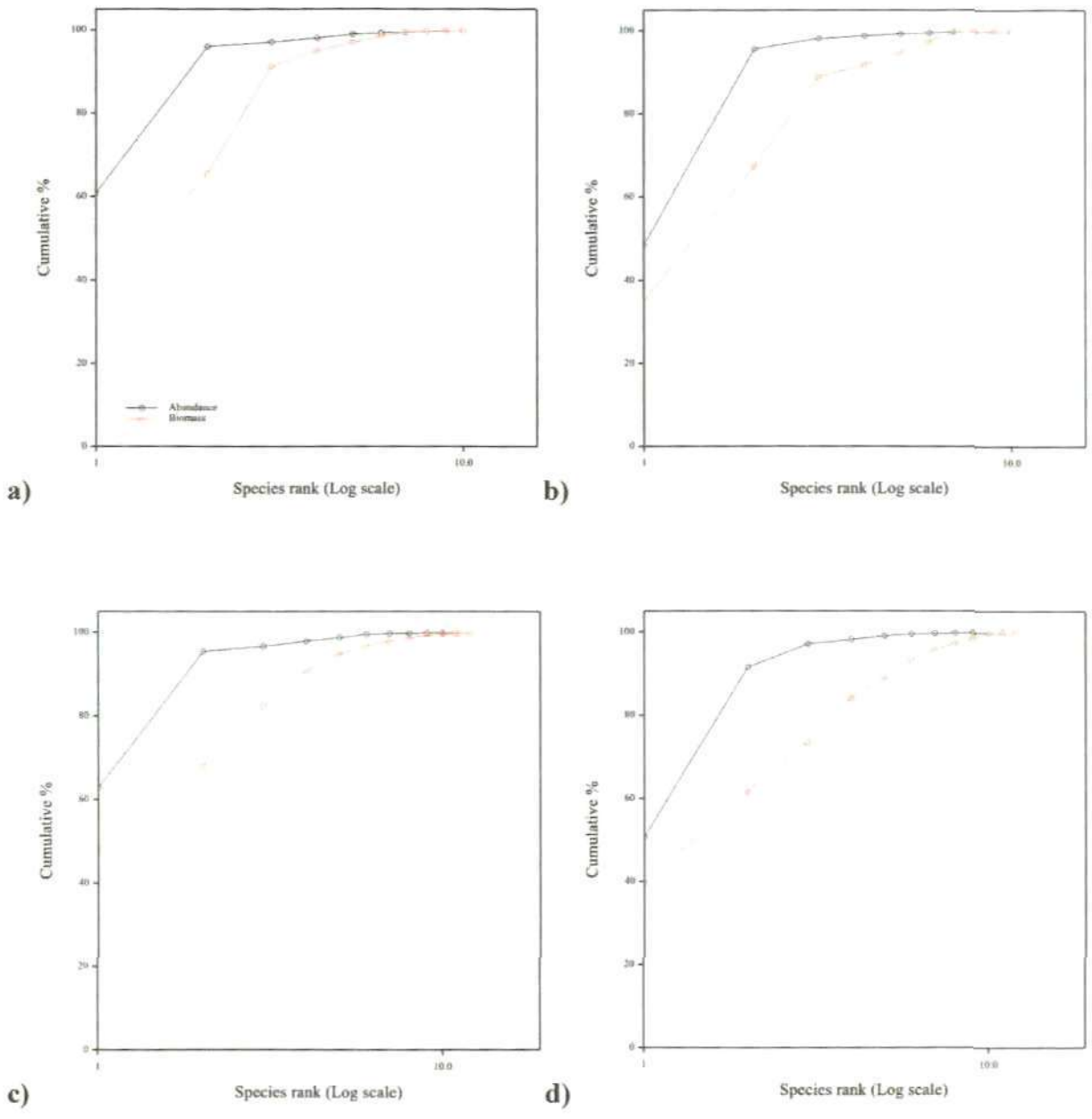


Figure 8.6: Abundance/biomass curve, showing cumulative proportion of abundant fauna (black line and circles) in comparison to biomass (red line and circles) for the grassland, for each sampling day separately a) day = 0; b) day = 18; c) day = 552; and d) day = 712.

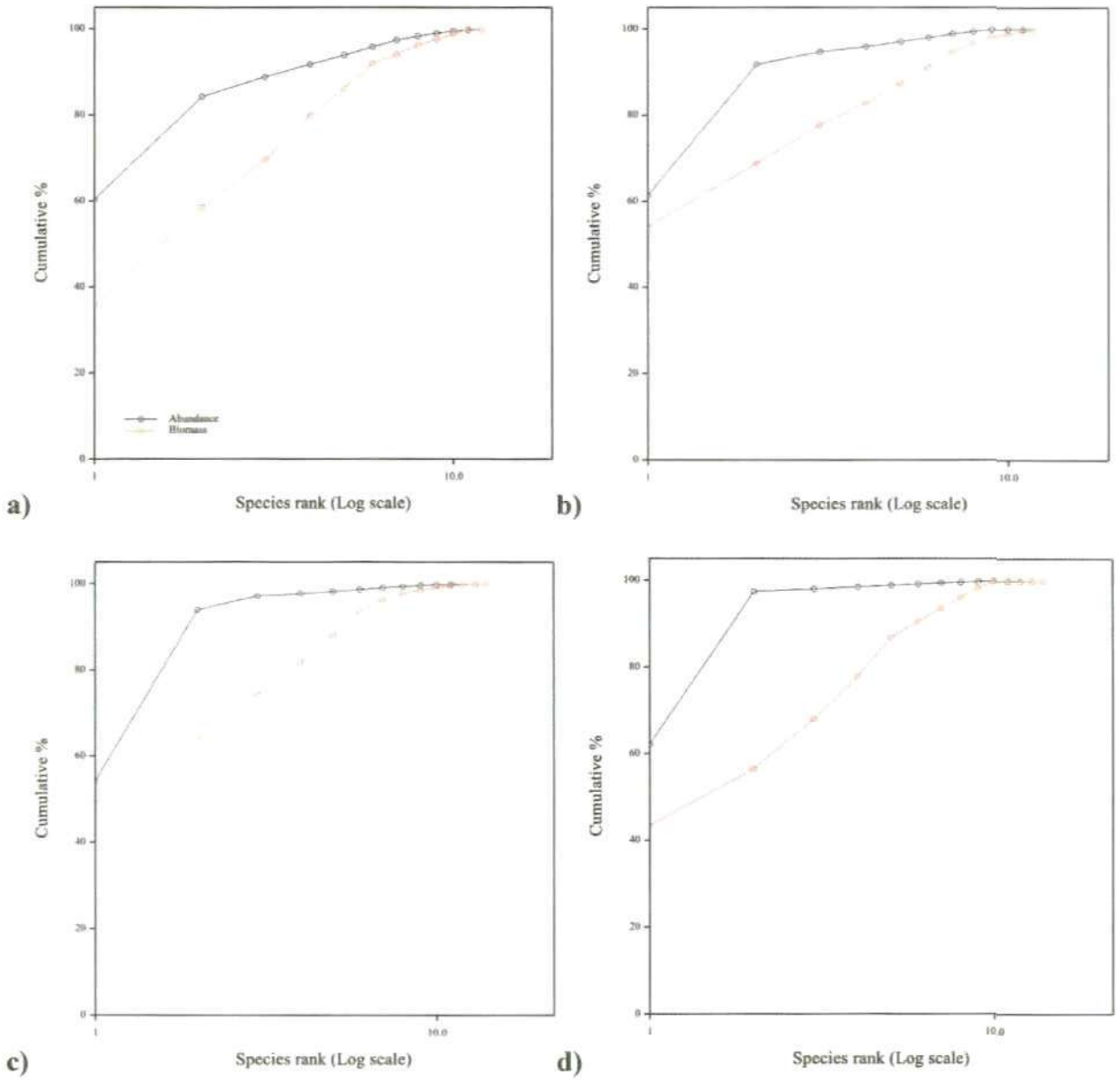


Figure 8.7: Abundance/biomass curve, showing cumulative proportion of abundant fauna (black line and circles) in comparison to biomass (red line and circles) for the woodland, for each sampling day separately a) day = 0; b) day = 18; c) day = 552; and d) day = 712.

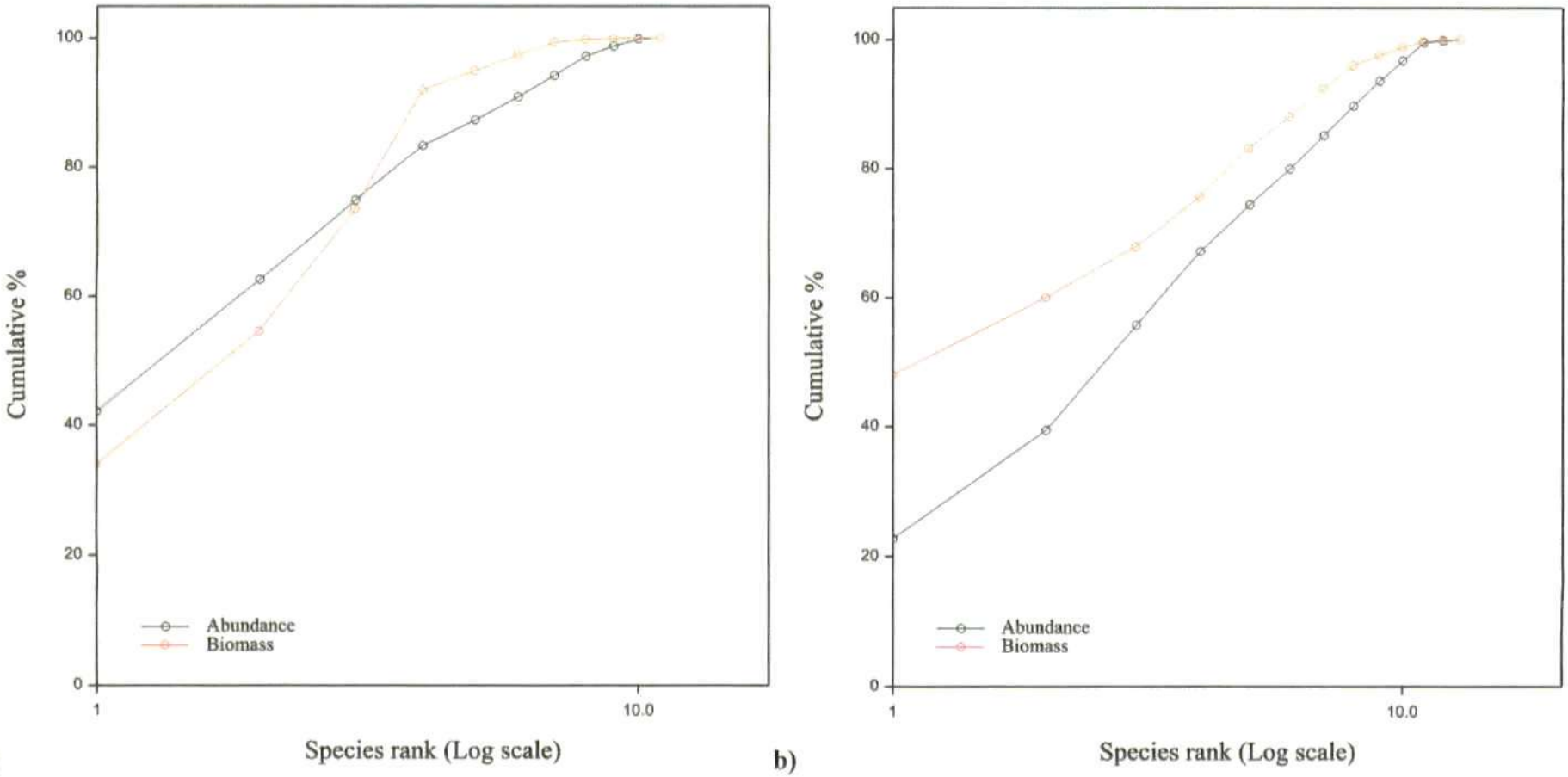


Figure 8.8: Abundance/biomass curve, showing cumulative proportion of abundant fauna (black line and circles) in comparison to biomass (red line and circles) (Collembola and Acari excluded from analysis) for a) the grassland and b) the woodland soils

Calculating the level of aggregation across the two habitats firstly by variance/mean ratio (VMR) (*sensu* Benefer et al., 2010) showed there were differences between the soil fauna. Generally the VMR increased from the habitat scale to the core scale (Table 8.4). Chilopoda and Diplopoda were aggregated at both the habitat and core scales (VMR >1), whilst the rest of the taxa appeared to be regularly distributed at the habitat scale (VMR = 0). At the core scales some of the taxa appeared to be randomly distributed (VMR = 1) e.g. Coleoptera (adults and larvae), Diptera (adults and larvae), and Earthworms (Table 8.4). However, the only taxa remaining regularly distributed at the core scale were Acari and Collembola, the rest appear to be aggregated e.g. Enchytraeidae, aphids, slugs, spiders, thrips and woodlice.

Taxa	Habitat scale	Core scale
Acari	0.0009	0.0194
Aphids	0.0965	1.6236
Chilopoda	3.3607	1.9784
Coleoptera	0.0413	1.1559
Coleoptera Larvae	0.0010	0.7925
Collembola	0.0002	0.0177
Diplopoda	3.5824	2.0021
Diptera	0.0090	1.2395
Diptera Larvae	0.0020	0.6318
Earthworms	0.0091	1.0888
Enchytraeidae	0.0474	1.8360
Slug	0.1906	1.8956
Spiders	0.0006	1.9860
Thrips	0.3272	1.9851
Woodlice	0.7356	1.8767

Table 8.4: The variance/mean ratio for the different invertebrate taxa collected at the habitat and core scale.

To assess the change in population numbers over time for aggregation within the two habitats, Taylor's index of aggregation was used (*sensu* Murray et al., 1995). For the index of aggregation a is a sampling factor whilst b gives a true measure of aggregation which is characteristic of that population. As previously described in the VMR, $b \approx 0$ the more regular the distribution, when $b = 1$ the distribution is random, and $b > 1$ shows increasing aggregation. Overall, the spatial distribution of the communities did change over time (Table 8.5) with the communities gradually becoming more aggregated.

Plot	Day	*a*	*b*	R^2	Student's T for log a	Student's T for log b	Aggregation
Grass	0	1.3435	-1.875	0.60	1.85	-1.13	Random
Wood		2.6948	-3.064	0.57			
Grass	18	0.6075	-0.509	0.18	-63.24	-76.64*	Regular
Wood		0.617	-0.485	0.10			
Grass	552	0.8058	-0.88	0.27	-2.50	-12.09φ	Aggregated
Wood		0.6038	-1.07	0.47			
Grass	712	0.6101	-0.772	0.22	-6.87	-12.04 φ	Aggregated
Wood		0.6917	-0.598	0.26			

* Significantly different from 1, $P < 0.05$
φ Significantly different from 1, $P < 0.1$

Table 8.5: Taylor's index of aggregation (Taylor, 1961) for the whole soil faunal community over time. Student's T-test was performed to assess aggregation; due to the small samples sizes (df_1), this represents a very conservative test, therefore distinct tendencies are also presented – where $P \leq 0.1$ ( ), and $P < 0.05$ (*).

There were only small differences in the "stability" of the habitats over time (Figure 8.9), as gauged using the coefficient of variation (CV) (Eisenhauer et al., 2011). The stability of population numbers between the two habitats were not significantly different. The change in population numbers over time remained stable and there were

no significant differences between CV's at the different time points. The average stability of population numbers was slightly lower, (the CV was slightly higher) in the grassland in comparison to the woodland. There was a larger variation in the average CV in the woodland, compared to the grassland, although the difference was not significant (Figure 8.9).

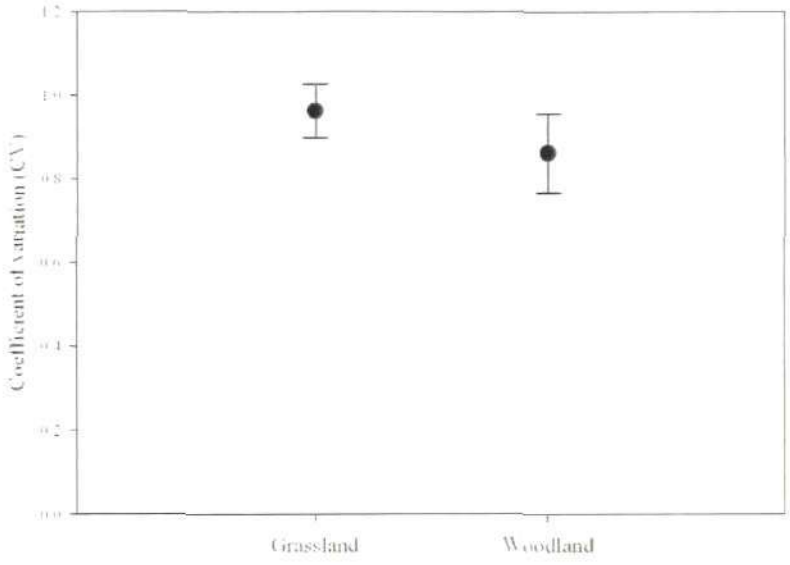


Figure 8.9: Stability of populations within two habitats, a grassland and a woodland.

8.4 DISCUSSION

Studies have found that soil communities specialise in decomposing the plant litter produced by the plant species growing above them (Ayres et al., 2009; Bezemer et al., 2010). Therefore radically changing the management processes of an agricultural grassland and converting it to willow woodland would have an impact on the soil community due to the effect of perturbation, and additionally they would need to adapt

to the new species of plants. It has been found that the underlying reasons for the inherent stability of the soil food web, is the high degree of spatial and temporal heterogeneity, together with the limited dispersal and colonisation capabilities of the soil organisms (Hedlund et al., 2004). Thus, soil communities have the ability to recover quickly from perturbations like ploughing, but with their limited dispersal and colonisation abilities the question remains how they adapt to habitat change.

Here, the results have shown that there was no difference in total population numbers of soil invertebrates over time. This implies that the optimum within the soil at these locations has been reached (as there was no effect of habitat on population numbers), possibly due to the limited ability of soil invertebrates to relocate in relation to biotic factors (Johnson et al., 2006). There were only weak correlations between weather variables, although during the experimental period a pattern of greater fluctuations in temperature and rainfall began to occur. The abundance of soil faunal populations collected over time did correlate with rainfall in the grassland, although not in the woodland. This correlation maybe due to vegetation cover being greater in the woodland, reducing the amount of precipitation reaching and remaining in the soil. Conversely other studies looking at C flux through the soil have found temperature to be the most important weather variable (Litton et al., 2008).

When diversity indices were used to assess differences in community composition it was found that there were no significant differences between habitats. However, as the taxonomic resolution tested was only to order level, these indices were unlikely to be very insightful. Both habitats had a low evenness index, with both the grassland and woodland being near to zero, suggesting that both communities are numerically dominated by a few orders i.e. Acari and Collembola, which was to be expected at this taxonomic resolution.

Although overall abundance of invertebrates remained relatively static throughout the study period, there were significant differences when looking at the individual Orders. For some of the individual Orders, population significantly increased over time (e.g. Acari in the woodland), or decreased (e.g. spiders collected in both habitats), or numbers fluctuated around a stable level over the study period (e.g. Collembola in the grassland). These differences suggest that there is more than one factor affecting the population at each sample time. There are many possible abiotic factors influencing abundances (e.g. weather or soil conditions) as well as differences in biotic factors (e.g. food abundance, or habitable space).

The Whittaker plots had a log-normal distribution, with the grassland plots having relatively steeper slopes, thus higher dominance of a few Orders (i.e. the Acari and Collembola), in comparison to the woodland which appeared to have a shallower slope on all sampling occasions, indicating more evenness (Magurran, 2004). The abundance/biomass curves (Figure 8.6 and 8.7) normally indicate the level of disturbance within a community, with relatively undisturbed sites dominated by a large number of large bodied organisms, whereas disturbed sites are dominated by a large number of small boded organisms. Theoretically as the woodland had been converted from grassland 25 years ago, it may have shown greater signs of disturbance in comparison to the grassland, whereas actually the reverse is visible (when the Collembola and Acari where excluded) (Figure 8.8). The abundance/biomass curves (Figures 8.6 and 8.7) do show some differences between habitats and time, but there are not enough differences to signify a "more disturbed" environment or time period. The results highlight the difficulty investigating the soil fauna using general community ecology theory, as these diverse communities are all dominated by small sized invertebrates, with a few macrofauna affecting the biomass curves, as could be seen when the two main groups of mesofauna were excluded (Figure 8.8).

Using aggregation indices to observe differences between the two habitats over time, it was clear that the Orders Acari and Collembola within both habitats were so abundant that they were considered to be "regularly" distributed throughout the soil habitats. However, consideration of the individual species within these Orders will produce an entirely different result. All other invertebrates' aggregation varied from random to aggregated depending on the taxa and habitats. Although the other invertebrates were not common throughout the habitats they had large affects as decomposers, prey and predators. There was little difference in stability between the two habitats and they remained similar over time. On a geographic scale, an increased stability of annual production may lead to an increased capacity for systems to support higher level predators (Paine, 1966). Here, the woodland had on average a slightly higher stability (lower CV), and more predatory taxa were found within this system.

High productivity within microbial communities has been found to sustain a greater number of trophic levels and increase population numbers (Kaunzinger et al., 1998). In a lake ecosystem, it was ecosystem size rather than productivity that determined food chain length (Post et al., 2000). The abundance and diversity of the soil fauna suggest that the soil as an ecosystem is highly productive; the habitable space is vast due to the heterogeneity, although it could be affected by perturbations.

It is possible that the nested structure within the soil food web, where small-scale communities are eaten as a whole by larger scale communities (Pokarzhevskii et al., 2003), has a greater impact on the community composition and abundance than the productivity or control mechanisms. The majority of organisms found within the soil were either Collembola or Acari, the results from previous chapters has shown that they should be considered to be omnivorous, as well as secondary decomposers. These two groups could rapidly graze a whole microbial community, leaving an empty niche space to be filled by another organism. Local diversity is directly related to the efficiency with

which predators prevent the monopolisation of the niche spaces by one species (Paine, 1966). These results suggest there were no dominant predator or decomposer invertebrates, allowing population numbers to increase and for organisms to diversify.

8.5 CONCLUSIONS

In soil ecosystems, the nature of resources, taxonomic constraints and scale of approach have made it difficult to integrate the study of soil communities into more general ecological theories at the community level (Giller, 1996). Within this study, the results have shown that there were some significant changes in abundance of invertebrates within the two habitats over time. However, these differences in abundance were not consistent between invertebrates or habitats, suggesting that there were many factors affecting the populations within the soil food web. It was suggested that climatic conditions may have affected the population, and for the grassland habitat there was a correlation between precipitation and invertebrate numbers, but this was not significant in the woodland.

Furthermore, the complete numerical dominance of Acari and Collembola fauna within both ecosystems masked the effects of other invertebrates on community composition, as could be seen when they were excluded from the analysis of the abundance/biomass curves. As these two groups hold such a pivotal role within the soil, exclusion from the analysis probably over estimates the importance of the other groups. To be able to distinguish population changes, aggregation and stability more clearly, future work needs to identify the soil fauna to a higher taxonomic resolution (family, genus or species) to fully elucidate which Acari and/or collembolan is dominant within the faunal assemblage.

Chapter 9: General Conclusions

9.1 INTRODUCTION

Since Anderson (1975) presented his description of the "Enigma of soil animal species" at the International Colloquium on Soil Zoology, research has focused on the feeding preferences of soil fauna. Over 35 years later we are still not much closer to revealing why soils are so rich in species (Nielsen et al., 2010) or defining separate niches for each species (*sensu* Hutchinson, 1957). In the introduction to this thesis, the key features of soil food webs were discussed including the premise that there are two "divergent" energy channels based on the bacterial or fungal decomposition pathways (De Ruiter et al., 1994). Soil fauna have been found to be part of either the bacterial or fungal energy channel, although results have found them to feed on parts of both channels in a number of studies, and this thesis. For example the same species of Collembola were shown to have consumed both bacteria and fungi in two separate fatty acid analysis studies (Haubert et al., 2006; Haubert et al., 2008), and here we have shown Collembola to consume bacteria and protozoa. However, the popular belief is that there is still very little crossover between the energy channels.

Thus the main aim of this thesis was to develop methods to test soil faunal feeding preferences to gain a better understanding of the bacterial and fungal feeding channels. It was also important to know whether these preferences were independent of habitat or if they were affected by land management. It was imperative to reveal the feeding preferences that were happening in the natural environment (not artificial microcosms); therefore in all food web experiments intact soil cores were used to test the *in situ* soil faunal population from a grassland and woodland habitat.

Organisms are considered one of the five major soil forming factors (along with minerals, organic matter, water and air (Coleman et al., 2003)), with life itself charactering a true soil (Coleman, 2008). Although the soil is teeming with life, the fraction of soil surface area inhabited is less than 0.000001% (Young et al., 2004). However, little is known regarding the searching and foraging behaviour of the fauna within the soil itself, and their response to the distribution and quality of food resources (Hassall et al., 2006). The spatial patterning of soil biota can occur both vertically through the soil profile and horizontally (Ettema et al., 2002). Consequently, although this thesis has started to elucidate the feeding interactions occurring with the soil food web, there is still more research needed.

Utilising stable isotope ecology to define feeding preferences within the soil food web allowed the feeding preferences to be shown through assimilation of enriched organisms. Using this method means the enriched food source signal will gradually be reduced as it spreads through the trophic networks within the soil (Lueders et al., 2006). Low levels of enrichment can therefore highlight how those organisms, which are part of the bacterial (or fungal) channel, form a network within the soil and should illustrate whether the channels are as divergent as hypothesised or whether they are interconnected throughout the web.

9.2 Thesis overview

The methodology implemented throughout this thesis was standardised for ease of comparisons between experiments (Chapter 2). All intact soil cores obtained from the two field sites were 10 cm Ø by 10 cm depth, this is average for soil fauna investigations with diameter of cores ranging from 5 cm (Sanchez-Moreno et al., 2009; Illig et al., 2010) to between 10 and 20 cm (Ngosong et al., 2009; Krab et al., 2010; Szanser et al., 2011). All soil core acquisition leads to some shearing of the soil pore

structure and boundary compression that may limit egress during extraction, this cannot be avoided. Taking 10 cm diameter cores was to partly reduce this effect, as cores with a height/diameter ratio < 2 have been suggested to have a minimal impact on compaction (Tan, 2005).

The taxonomic resolution the invertebrates were identified to was quite low (family (Coleoptera), superfamily (Collembola), lineage (Acari) or Order (all other invertebrates)) (Chapter 2). This level of resolution was chosen as a compromise so that stable isotope analysis could be performed quickly within the timescale, and the whole soil food web could be assessed with organisms within each core having a large enough biomass to be kept separate and used as individual replicates. An example of the time consuming nature of taxonomic identification to species was described by Wu et al., (2009) who calculated that it took six person-years to identify individuals from one soil animal group (mites) to species in a single experiment conducted at one grassland site (St John et al., 2006). Additionally, although the soil organisms were grouped taxonomically they were not separated into functional groups prior to stable isotope analysis. Only after the results were obtained for individual taxa were the isotopic composition of functional groups assessed. This reduced the likelihood that *a priori* classification masked the true trophic structure of the community (Eggers et al., 2000), and tested whether the *a posteriori* classifications were correct.

Culturing enriched bacteria and monitoring their survival when reintroduced into soil microcosms (Chapter 3) showed, that survival declined rapidly ten to fourteen days after addition in all microcosms. Hence, future food chain experiments, tracing bacterial incorporation in the soil fauna, the incubation period needs to be less than 14 days to allow for consumption of live bacteria. After 14 days the introduced bacteria are more likely to have died prior to consumption by the soil fauna, than dying due to consumption. Whether the microcosms were made up of intact soil cores or repacked

sieved soils had a greater effect, on survival, *APE* ^{13}C soil headspace atmosphere, and amount of isotopes remaining within the soil, than management (N treatment), although the sieved soil microcosms were more homogenous. Consequently, to track natural processes as they are happening microcosms with intact soil rather than sieved and repacked, should be used.

Stable isotope ecology was used to differentiate trophic levels amongst the soil fauna at natural abundance from two different habitats (Chapter 4). There were differences in soil faunal populations in terms of abundance and biomass as well as stable isotopes between the two habitats. Differences in isotopic composition revealed the woodland habitat to have one more trophic level in comparison to the grassland (using the theory that $\delta^{15}N$ increases 3.4‰ per trophic level (Minagawa et al., 1984)). Moreover, the $\delta^{13}C$ signatures varied between habitats, with a span of 5‰ in the grassland, compared to a 12‰ difference in the woodland. The $\delta^{13}C$ signatures are thought to be ecosystem specific (France et al., 1997) or rather "primary resource" specific (Pollierer et al., 2009). Food chain $\delta^{13}C$ signatures usually increase 0.4 – 1.3‰ per trophic level (Post, 2002) because of the low fractionation usually recorded. These natural abundance results suggest there is more than one primary resource being utilised in both habitats signified by the range in $\delta^{13}C$ signatures. Furthermore, considering the difference between the delta values for soil in both habitats with the soil fauna in comparison to plant litter, there appears to be a correlation between the soil and soil fauna in the grassland, whilst in the woodland the correlation is between plant litter and soil fauna. These two points in conjunction indicate that there are two different drivers in the two habitats, with the grassland being soil driven whereas the woodland is litter driven.

Further developing the methods of Murray et al., (2009) enriched bacteria were introduced back into the soil food web to test whether the bacterial "energy" channel

operates in both the grassland and woodland habitat in a similar way (Chapter 5). As there were potentially different C and N drivers within these habitats (described in Chapter 4); it was unlikely that the enriched bacteria would be consumed in similar amounts in the two habitats. Interestingly, all the invertebrates tested in both habitats showed some level of bacterial enrichment, implying that bacterial feeding is a common mechanism within the soil independent of C and/or N source. There were differences in enrichment level obtained by the same taxonomic group in the two different habitats, implying that the same organisms may have different feeding preferences within the different habitats and may even function at different trophic levels dependent on habitat type, competition and opportunity. Although the taxonomic resolution of the experiment may have affected this result, assessment of the enrichment levels if the same species occurred in both habitats would have tested this. These differences could also be due to the different C and N sources (soil verses plant litter) and the different assemblage of microorganisms that utilise these, leading to different adaptations amongst the soil fauna.

Protozoa were cultured in stable isotopically enriched media and used to track the feeding preferences of the soil fauna through the food web for the first time (Chapter 6). The flux of protozoan C and N through the soil invertebrate food web was tested empirically, and it was shown that protozoa hold a pivotal role in the web irrespective of habitat. Around 40% of the invertebrates from both habitats were significantly enriched in ^{13}C and/or ^{15}N; this enrichment was found in those fauna previously considered to be decomposers and also some considered to be mesofauna predators. The results within this thesis show that protozoa form an intermediary step between microbial biomass and higher order consumers within the soil food web.

To trace the fungal energy channel through the soil food web methods needed to be developed to introduce an enriched fungus back into the soil (Chapter 7). In the

natural environment fungi are filamentous and exist as part of the air-filled pore spaces within the soil. The methods previously developed introduce bacteria and protozoa into the soil food web as part of an aqueous solution and can therefore be injected into intact soil cores as viable organisms. Consequently, different methods were developed to introduce fungi into the soil in a viable form, with a known level of stable isotope enrichment. A fungal isolate identified to be *Absidia cylindrospora* (through ITS sequencing) was chosen as the most promising candidate for further experimentation to develop introduction methods (Chapter 7 – part 1). *A. cylindrospora* was chosen because of its speed of growth, large biomass of hyphae when growing on minimal media agar and dark pigmentation in comparison to other isolates.

A selection of fungal species were grown on minimal media agar (at natural abundance) and their stable isotope levels were tested. The results showed that not all fungal species discriminate/fractionate at the same rate whilst growing. These differences in stable isotope levels could influence the fungal isolate when grown on an enriched nutrient source. Although other studies have utilised enriched media to assess discrimination/fractionation of fungi (e.g. Scandellari et al., 2009) few have grown fungi on highly enriched media. This was necessary as a first step prior to introduction of the fungus to the soil as a tracer in the soil food web.

Experimentation revealed that *A. cylindrospora* could grow on enriched media and gain high levels of enrichment in stable isotopes (Chapter 7 – part 2). However, *A. cylindrospora*'s growth rate was affected when grown on a dual labelled medium in comparison to a singly enriched medium, due to discriminatory utilisation of nutrient sources. This result coupled to the differences in natural abundance levels of fungal isolates illustrates that investigation of the fungal channel will be more complicated than the bacterial channel, and has implications for food web theorists. The range of stable isotope signatures of the fungal isolates growing on the same medium could be

the reason that there appears to be a "continuum" of decomposers within the food web (Chahartaghi et al., 2005). Also where studies have added enriched substrate to the soil the microbial contribution to soil processes could be affected (e.g. Bull et al., 2000; He et al., 2009), here *A. cylindrospora* was found to actively discriminate against the enriched food source, this would skew results if not considered.

Enriched A. cylindrospora isolates were introduced into sieved soil microcosms to test the translocation of enrichment during fungal growth. (Chapter 7 – part 3). The level of enrichment, the distance and magnitude of translocation were quantified. This method had the advantage of only introducing a viable intact hyphal matrix within the soil, providing only one source of enrichment to the food web *via* direct consumption of living hyphae. This method showed promising preliminary results and needs to be developed further, so the level of enrichment of the fungus can be replenished whilst actively growing, as well as testing how far a traceable enrichment can move through the soil both vertically and horizontally.

The food web experiments within this thesis were performed over three years producing results which show the variability in abundances of invertebrates within the two habitats over time (Chapter 8). There were differences in abundance between populations over time, but these were not consistent between invertebrates or habitats, suggesting that there are many factors affecting the populations within the soil food web. These differences could just be part of natural variation within the populations over time.

9.3 Stable isotopes and soil food webs

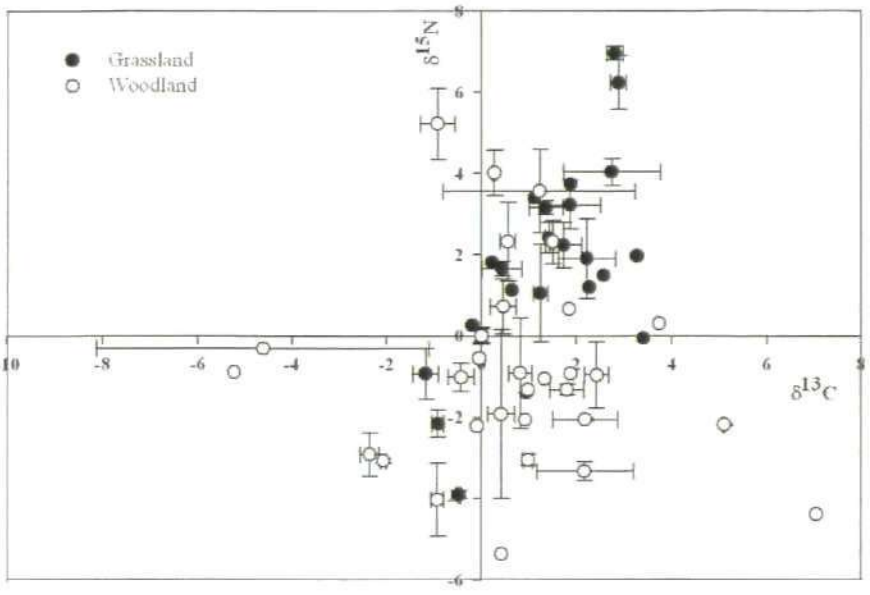
Stable isotopes provide a unique opportunity to assess trophic level within the soil food web in the natural environment and by tracking enrichment of food sources which provides proof of consumption and assimilation of the food source. Exploiting

natural abundance values can highlight ecosystem-wide differences between habitats. Where the average delta values for the soil fauna were presented together for the grassland and woodland habitats there was a clear separation for $\delta^{15}N$ (Figure 9.1a) with the woodland fauna generally more depleted in $\delta^{15}N$ signatures at natural abundance. These results agree with those found by Hobson (1999) (Figure 9.1b) for songbirds from agriculture and forest habitats, although here they attributed the isotopic differences to variation in plant and soil isotopic content, not invertebrates. The similarity of results across taxa and continent also concurs with a meta-analysis of Fierer et al., (2009) that found distinct global patterns in belowground communities across biomes.

When tracing the feeding preferences of the soil fauna through their isotopic composition, the results revealed that there are distinct differences in isotope levels for the mesofauna obtained from each of the separate isotope experiments (natural abundance, enriched bacterial injection, enriched protozoa injection) (Figures 9.2 and 9.3). The Collembola were more enriched in both the bacterial injection experiment (Chapter 5) and the protozoa injection experiment (Chapter 6) than the Acari, in both habitats. Here the results are portrayed using atom% therefore the natural abundances for all the mesofauna appear very similar (but using the delta values (Chapter 4) shows the fauna at different trophic positions).

The enrichment obtained in ^{13}C and ^{15}N by the Entomobryomorpha (Figure 9.2a) is greatest in the protozoa injection experiment and highlights a possible direct feeding interaction occurring. Whilst in comparison the Mesostigmata (Figure 9.3a) reveal a lower level of enrichment in the protozoa injection experiment, although it is much greater than their natural abundance levels highlighting a possible secondary feeding interaction; where their prey have consumed the enriched protozoa prior to consumption by the Mesostigmata.

a)



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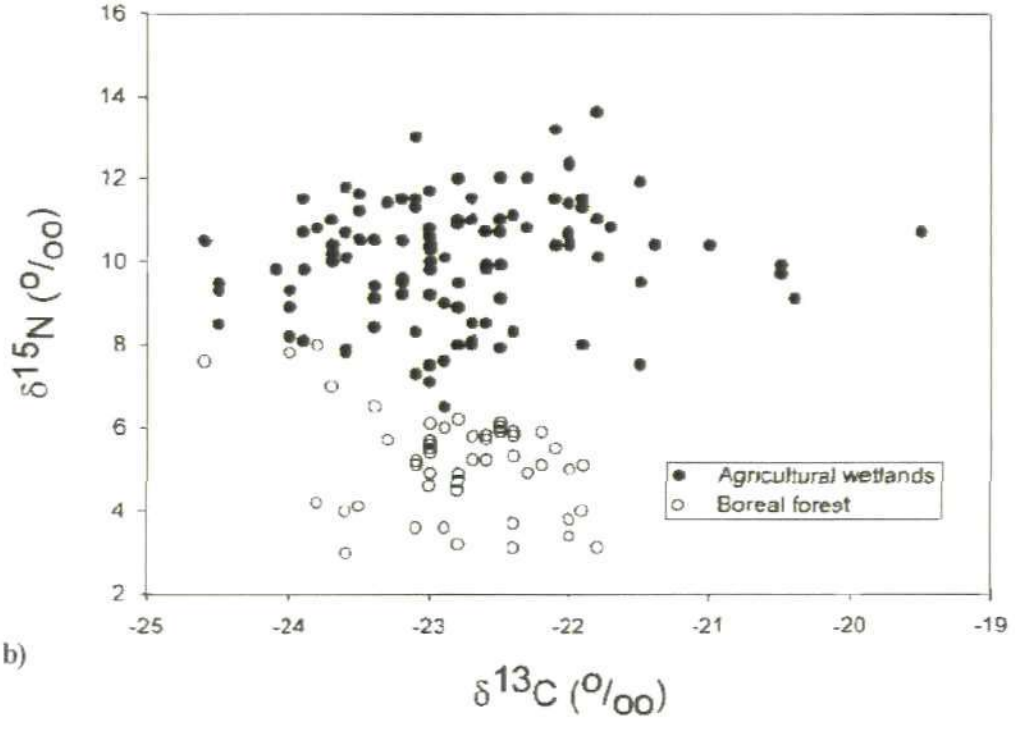


Figure 9.1: Isotopic composition of a) soil fauna (simplified version of Figure 4.6) within a grassland (closed circles) and a woodland (open circles), the scale has been normalised for soil; b) Results from Hobson (1999) of isotopic composition of feathers of songbirds from two landscape types – agriculture (closed circles) and forest (open circles).

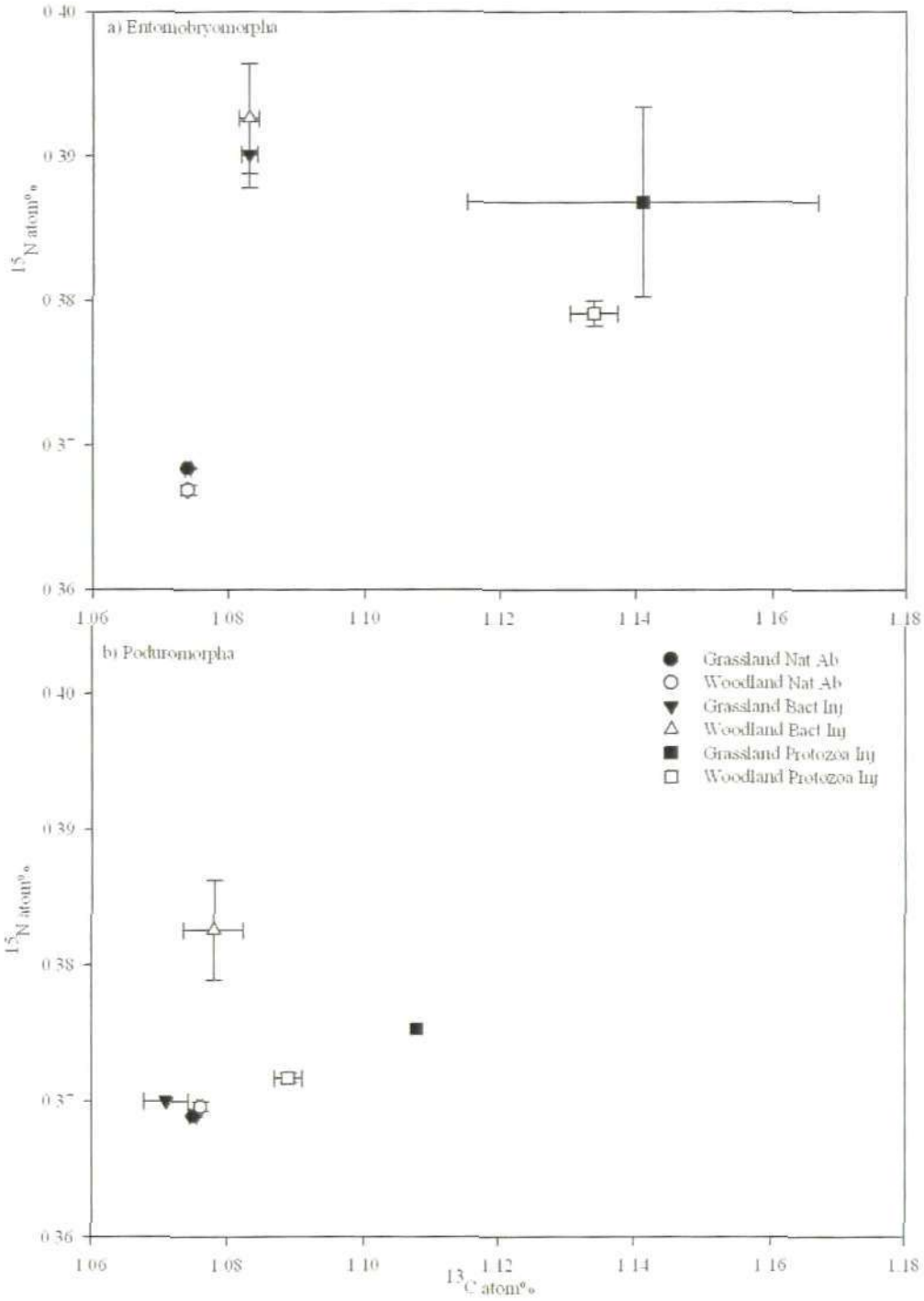


Figure 9.2: Isotopic composition of Collembola a) Entomobryomorpha and b) Poduromorpha from a grassland (closed symbols) and a woodland (open symbols) habitat. Results show average enrichment (± standard error) for natural abundance experiment (Chapter 4 – circles), enriched bacterial injection (Chapter 5 – triangles), and enriched protozoa injection (Chapter 6 – squares).

The Poduromorpha (Figure 9.2b) also have a lower level of enrichment compared to the Entomobryomorpha, for both the bacterial injection and protozoa injection experiments. This implies that the Poduromorpha have different feeding preferences to the Entomobryomorpha and that bacteria and protozoa are not their main food source.

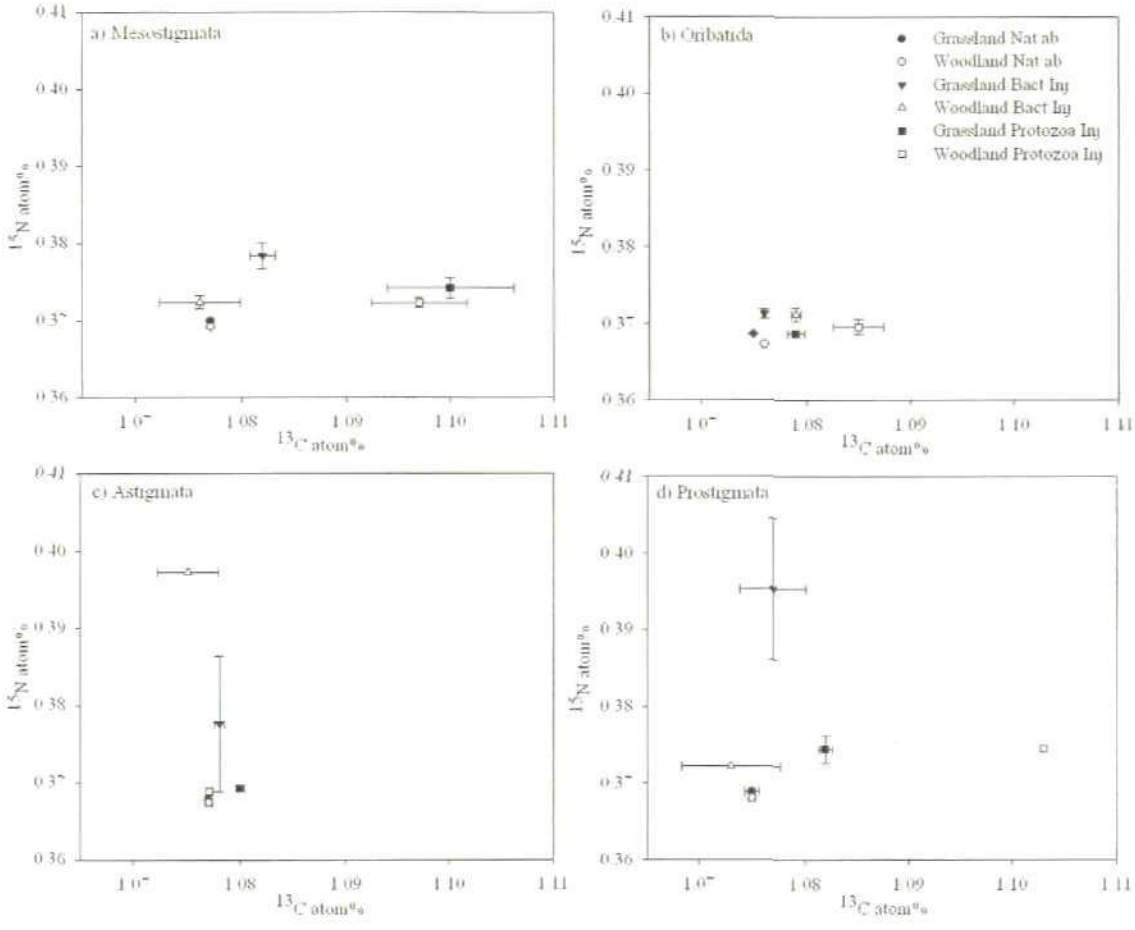


Figure 9.3: Isotopic composition of Acari a) Mesostigmata, b) Oribatida, c) Astigmata and d) Prostigmata from a grassland (closed symbols) and a woodland (open symbols) habitat. Results show average enrichment (± standard error) for natural abundance experiment (Chapter 4), enriched bacterial injection (Chapter 5), and enriched protozoa injection (Chapter 6).

The Oribatida (Figure 9.3b) are displaying lower levels of enrichment from the bacteria and protozoa injection experiments, indicating that their main food source is also not bacteria or protozoa. Oribatida are considered to be fungal feeders in the literature (e.g. Siepel et al., 1994; Maraun et al., 1998; Behan-Pelletier, 2003; Schneider et al., 2005a), therefore these low levels of enrichment agree that this may be their true feeding guild. The Astigmata (Figure 9.3c) and the Prostigmata (Figure 9.3d), display greater variation amongst results, both lineages are more enriched by bacterial consumption than protozoa. Both the Astigmata and Prostigmata are also more enriched in ^{15}N than ^{13}C indicating possible mineralisation of bacteria prior to consumption of an enriched nutrient source.

Comparison of the amount of ^{13}C and/or ^{15}N of bacterial or protozoan origin within the mesofauna (with the highest atom%) revealed greater variation in the amount obtained by the mesofauna in the two tracer experiments (Figure 9.4). Using the amount of ^{13}C and ^{15}N of bacterial/protozoan origin incorporates biomass of the consumer, therefore indicating which are the biggest consumers within the soil food web, i.e. if a group of consumers have a large biomass and a medium level of atom% enrichment the overall amount of ^{13}C and/or ^{15}N may be greater than that of a group of consumers with a small biomass and large atom% enrichment.

Within the four mesofauna groups assessed the amount of ^{13}C and ^{15}N of bacterial origin was very low (< 0.01 ng for both ^{13}C and ^{15}N) whereas the amount of ^{13}C and ^{15}N of protozoan origin varied between taxa. The Oribatida (Figure 9.4b) and Poduromorpha (Figure 9.4d) gained very little ^{13}C and ^{15}N of both bacterial and protozoan origin, indicating that neither protozoa nor bacteria are a preferred food source for these taxa. The Entomobryomorpha (Figure 9.4c) gained large amounts of ^{13}C and ^{15}N of protozoan origin in both habitats, although the greatest was in the

grassland habitat, indicating that protozoan feeding is a regular occurrence particularly in the grassland. The amount of ^{13}C and ^{15}N of protozoan origin gained by the Mesostigmata (Figure 9.4a) was at an intermediate level between the Poduromorpha/Oribatida and the Entomobryomorpha. As the Mesostigmata are known predators (Koehler, 1999) this implies that the Mesostigmata are feeding on a range of taxa, potentially including protozoa, although it is more likely they are consuming a range of organisms that have fed on protozoa with varying levels of partiality to them.

The enrichment of a bacterial tracer followed by a protozoan tracer provides a unique opportunity to show empirically the "microbial loop" in a natural system. The microbial loop has been described as the positive effect of microbial grazers (protozoa) on plant growth, through the release of nutrients during the consumption of rhizosphere bacteria (Coleman, 1994; Bonkowski, 2004) stimulated by root exudates. The change in atom% of the plant material after introduction of enriched organisms (Figure 9.5a), displayed little difference between the two tracer experiments, both are greater than natural abundance and are similarly enriched across habitats.

However, analysis of the amount of ^{13}C and ^{15}N of bacterial/protozoan origin incorporated by the plant material revealed clear differences between tracer experiments (Figure 9.5b). In the protozoan tracer experiment, plants acquired more ^{15}N than from the bacterial tracer experiment. As protozoa have a minimum generation time of 2-4 hours in soil (Coleman, 1994), this result implies that as well as protozoa consuming bacteria, releasing nutrients for plant uptake through death they are also providing nutrients for plant uptake through their own demise and this affect is larger. It has been suggested that it is increased N availability that stimulates plant growth rather than the "microbial loop" *per se* (Ekelund et al., 2009). These results do display a correlation with this hypothesis, as the grassland may have been more N limited due to its zero addition of N over the past 20 years.

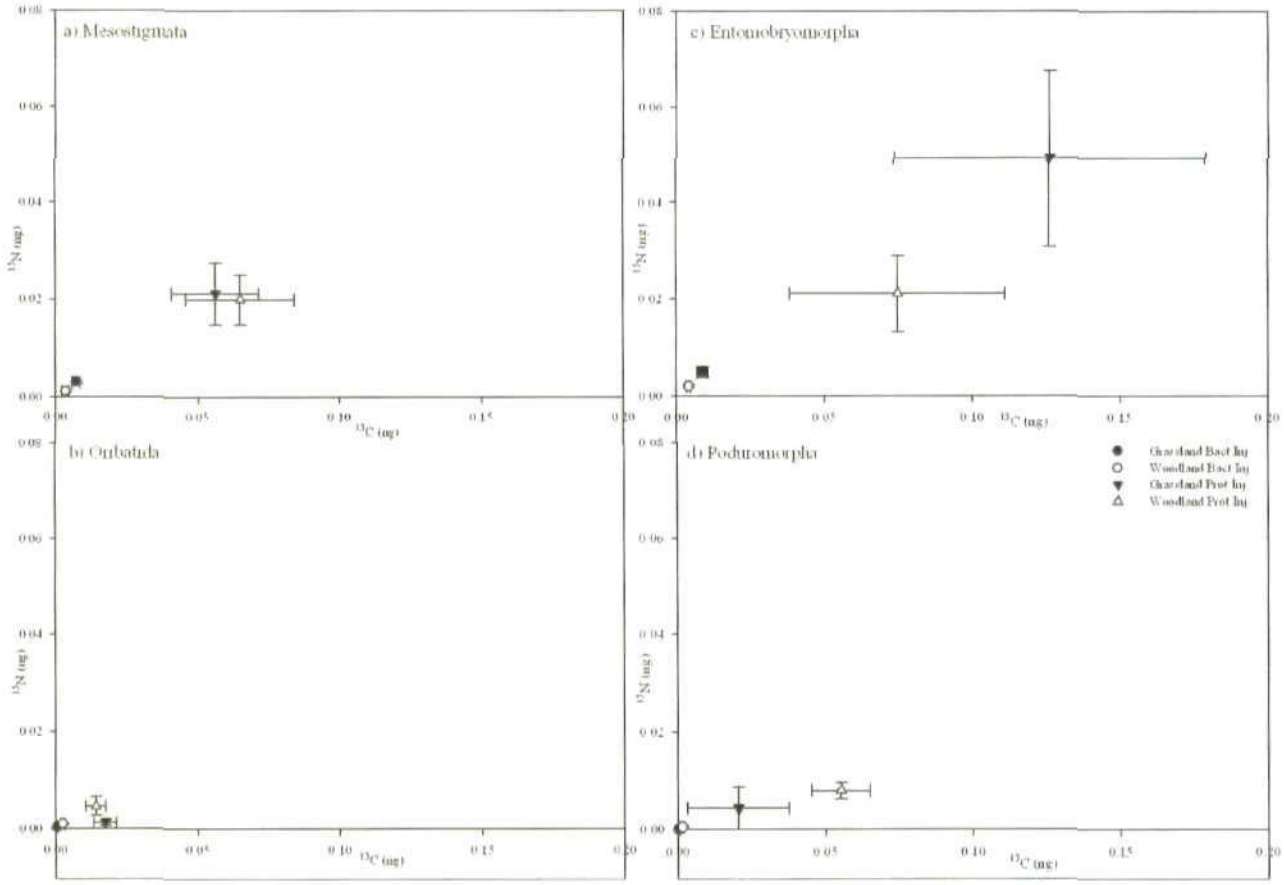
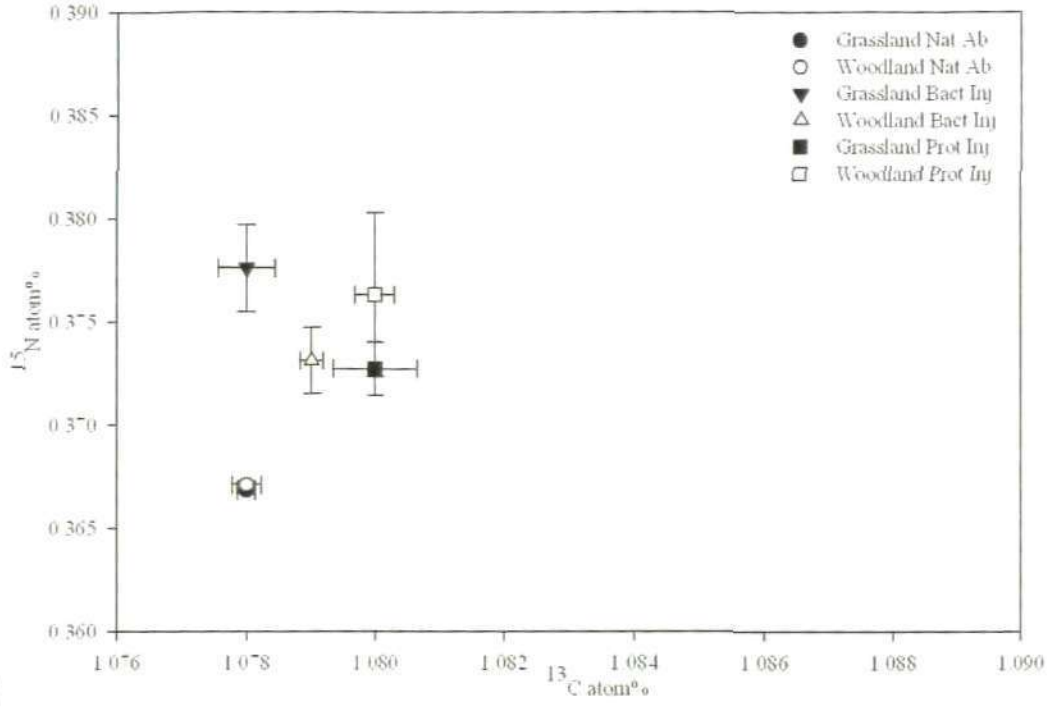


Figure 9.4: Amount of ^{13}C and ^{15}N of bacterial (circles) or protozoan (triangles) origin in the grassland (closed symbols) or woodland (open symbols) habitats for different mesofauna taxa, a) Mesostigmata, b) Oribatida, c) Entomobryomorpha and d) Poduromorpha.

a)



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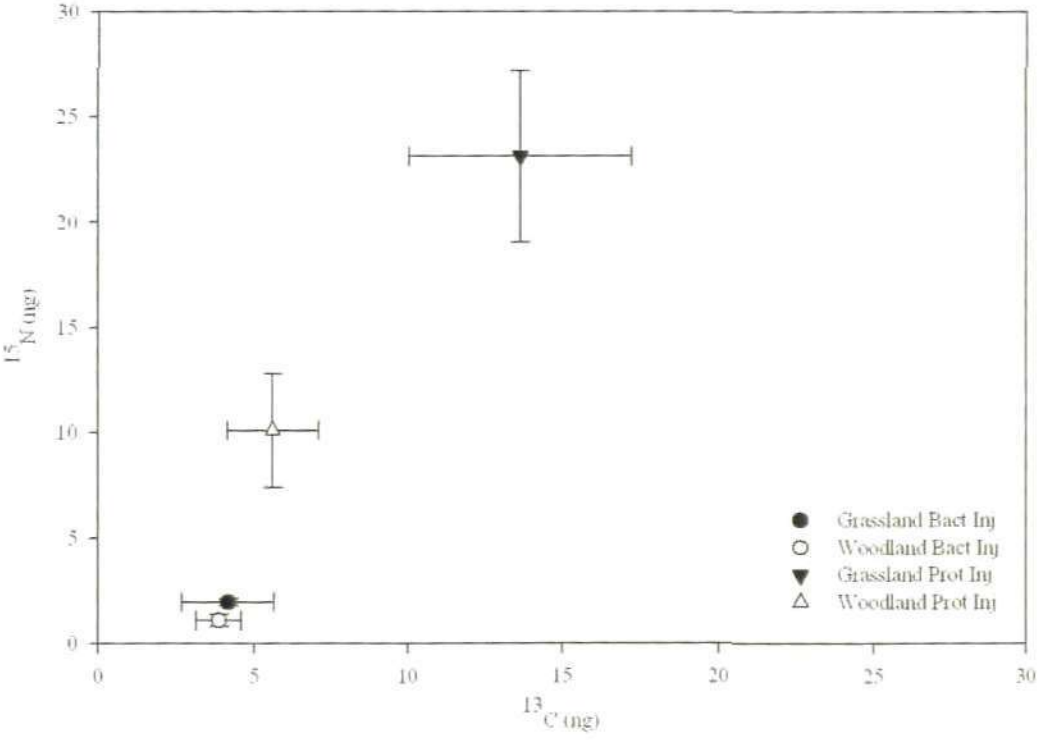

Figure 9.5: Differences in a) ^{13}C and ^{15}N atom% and b) amount (ng) ^{13}C and ^{15}N of bacterial or protozoan origin in vegetation from the grassland (closed symbols) and woodland (open symbols). Data are presented as mean ± standard error.

9.4 Further work

Generally this thesis provides a first step in the use of labelled organisms to trace the feeding preferences of soil fauna, but to fully elucidate the relative importance of the bacterial and fungal feeding channels within the soil food web more work is needed. Some studies have discussed the use of stable isotope variance as a measure to depict niche width (Bearhop et al., 2004). In Chapter 4 a number of measures have been utilised, but there is still more that can be applied (e.g. total area covered by a $\delta^{13}C$-$\delta^{15}N$ biplot (reflecting the total niche space occupied); mean distance to centroid (average degree of trophic diversity), mean nearest neighbour distance (overall density of species packing), and standard deviation of nearest neighbour distance (evenness of species packing) (Layman et al., 2007)). It would also be interesting to see the effect of an enriched food source on these trophic parameters.

Recently studies have examined the differences in stable isotope composition of above-ground vegetation compared to belowground vegetation (Werth et al., 2010), as well as differences in the "rhizosphere soil" (soil adhering to roots) in comparison to bulk soil (Paterson et al., 2008b). More samples would need to be taken, but it would be possible to look at these plant and soil parameters for the natural abundance analysis of the soil food web (Chapter 4), to assess whether there are isotopic differences between shoots and root material as well as "rhizosphere" soil compared to bulk soil, as this may correlate with the continuum of delta values in the two habitats. However, the break between invertebrate analysis and these parameters may mask relationships in isotopic composition.

Introducing living ^{13}C and ^{15}N enriched *Pseudomonas* bacteria and mixed cultures of protozoa has highlighted different entry-levels within the soil food web (Chapters 5 and 6). Future work on the bacterial energy channel, would be to culture an

enriched organism at the next level again, possibly bacterial feeding nematodes. Developing methods to dually label bacterial feeding nematodes could involve using the methods for labelling bacteria which could then be fed directly to the nematodes. The level of enrichment would need to be identified and manipulated so that it could work as a traceable enrichment source within the soil food web. Nematodes being part of the "aquatic" channel (Moore et al., 1988a), can be introduced into soil cores in the same way as both the bacteria and protozoa. Nematode predators would then be elucidated dependent on their isotopic composition after consumption. The results of a nematode enrichment experiment would be useful to quantify the differences in biomass of the introduced enriched organisms. As currently although the soil fauna are displaying greater enrichment from protozoa consumption than bacterial, it is possible that this is due to the greater biomass of a protozoan in comparison to a bacterium. Nematodes are again an order of magnitude greater in biomass than the protozoa in the previous experiment, so depending on results it would be possible to assess if this is a factor in the increasing enrichment levels.

Elucidating the importance of the fungal channel within the soil food web still needs to be developed fully. Within this thesis, progress has been made (Chapter 7), methods have been developed to enrich a fungal isolate and introduce it into soil cores, but this still needs to be tracked through the fauna within the soil food web. There were large differences in natural abundance measurements of different fungal isolates grown on the same media. Testing whether stable isotopes could be used to differentiate between saprotrophic fungal species would be a useful tool in microbial ecology, as current methods only look for differences between mycorrhizal and saprotrophic fungi (Kohzu et al., 1999).

Overall using the knowledge gained within this thesis to model interactions within the soil food web, as a method to test the affects of the change in management

regime, and whether this change affected nutrient cycling within the soil also needs to be investigated. Bezemer et al., (2010), focused on the differences in plant composition and found soil food webs to be different after only seven years since management changes were implemented. Bezemer et al., (2010), did not consider whether these differences in soil food webs reflected a stable/established community or whether it was a community in transition. The study sites investigated within this thesis had their main management change over 25 years ago, therefore they should reflect an established community. Experiments testing whether this is the case would further our understanding of community composition and food web interactions over time.

9.5 CONCLUSIONS

The overall aim of this thesis was to elucidate the relative importance of the bacterial and fungal energy channels within the soil food web and attempt to differentiate differences in feeding preferences of the soil fauna when affected by differing land managements.

The main results obtained through experimentation within this thesis that have increased our understanding of soil food webs were:

- Isotopic composition of grassland and woodland soil fauna were found to be different, possibly due to differences in C and N sources (root derived versus litter derived).
- The bacterial energy "channel" is not as divergent from the fungal energy channel as considered by the literature, as bacterivory was found to be widespread and independent of habitat.

- Protozoa hold a pivotal connection between bacteria and the soil fauna, and are an important food source for a number of different taxa within the soil.
- Omnivory is a key feature of most "trophic levels" within the soil food web.
- Different species of fungi can fractionate stable isotopes to a variable extent, therefore there is the potential for this to be utilised for identification purposes.
- Investigation of the fungal energy channel through enrichment of stable isotopes is achievable, although more development will be needed to trace the interactions occurring between the soil fauna and saprotrophic fungi.

Further investigation of this "ecological jigsaw puzzle" (Berg et al., 1998) is still needed. There is a need to unify studies, combining efforts of different disciplines (soil physics and chemistry) to focus on the soil food web as a whole. Only when this occurs can the interactions within the soil food web be fully untangled. The results obtained throughout this thesis provide key pieces of the puzzle to better understand the soil food web. They challenge the central dogma that the two main energy channels are as divergent as normally described, the two energy channels appear to be equally important, independent of habitat, with a true web of interactions being revealed.

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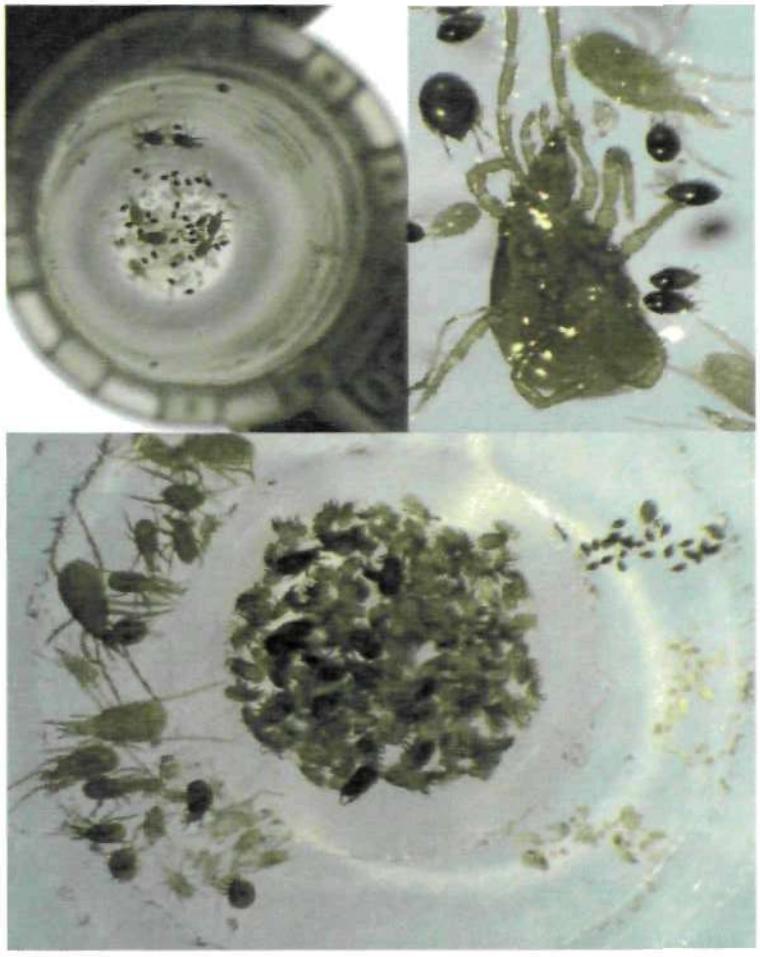
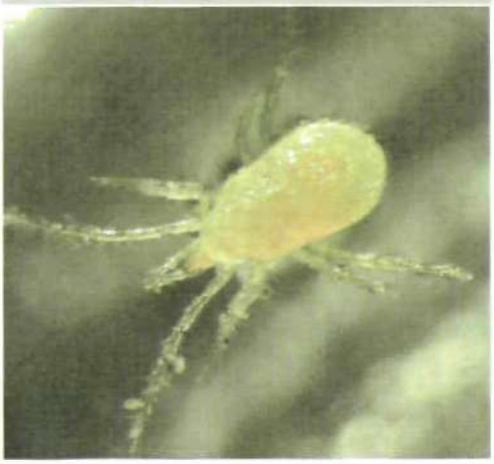
APPENDIX A: Examples of the different soil fauna extracted.

ACARI

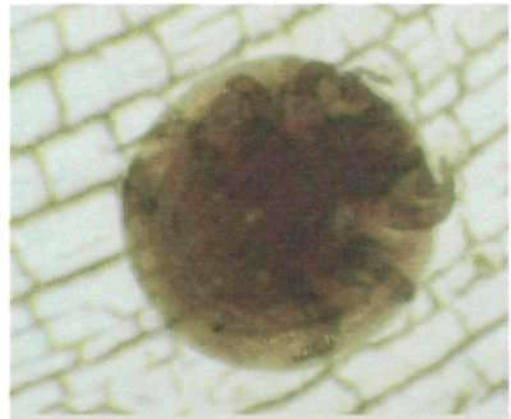


Acari: Astigmata

Acari Mesostigmata



Acari: Mesostigmata: Uropodidae

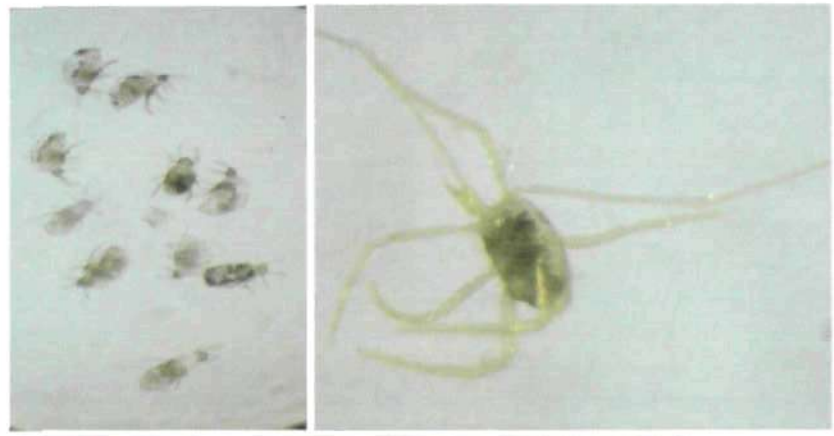


Acari: Oribatida

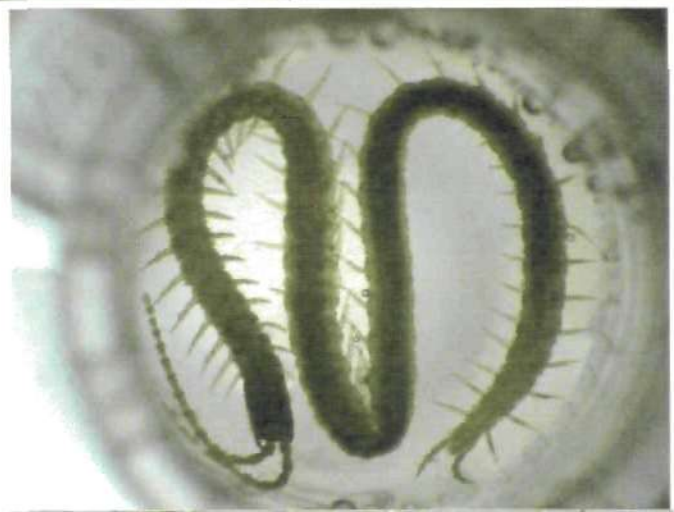
Acari: Oribatida: Damaeidae

Acari: Oribatida: Phthiracaridae

Acari Prostigmata

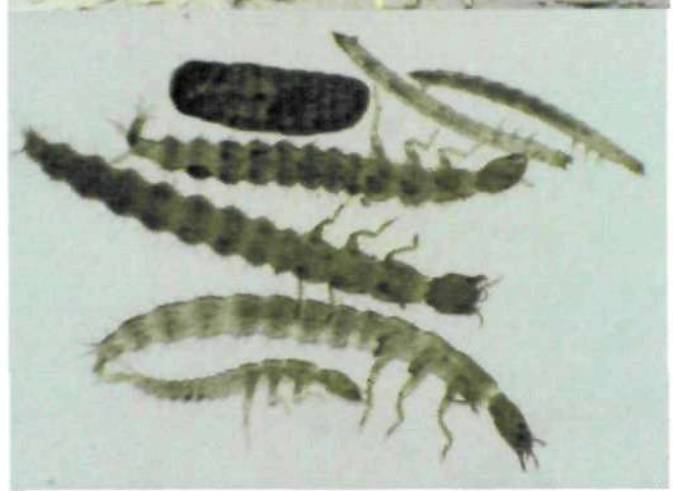


Chilopoda: Geophiomorpha



Aphids (Hemiptera: Aphidoidea)

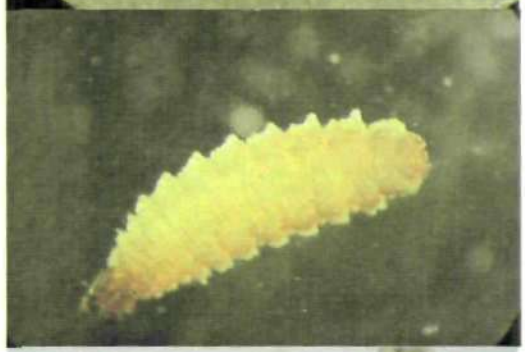
Coleoptera Larvae



Coleoptera Larvae:
Elateridae

Coleoptera:
Staphylinidae

Coleoptera Larvae:
Chryosomelidae



Coleoptera Larvae:
Curculonidae

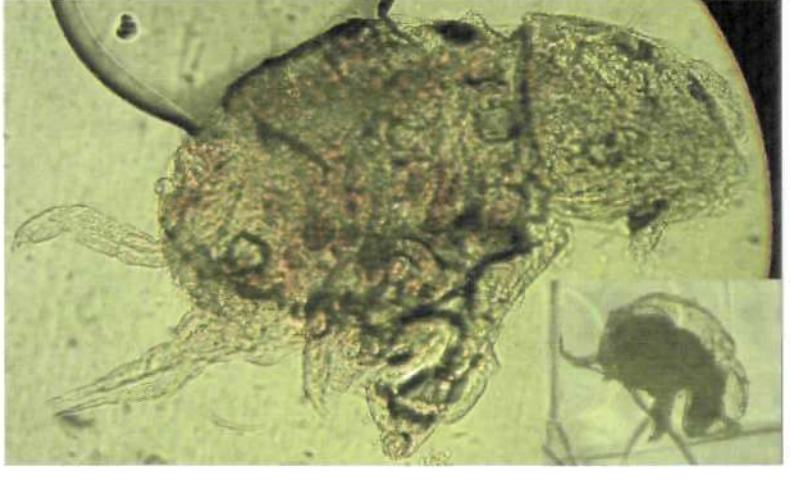
Coleoptera: Ptilidae

COLLEMBOLA

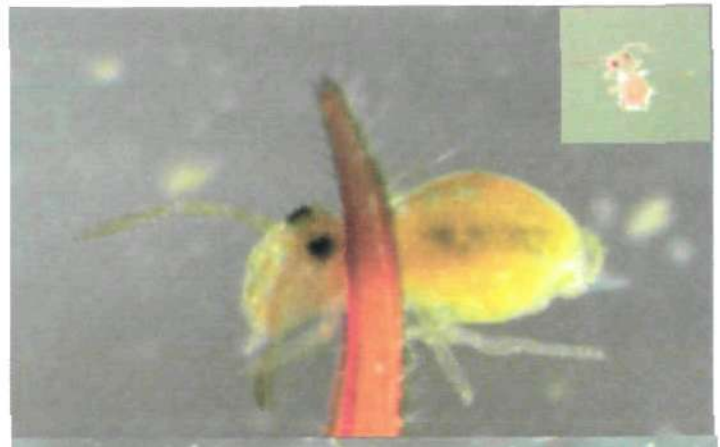
Collembola:
Entomobryomorpha

Collembola:
Poduromorpha

Collembola:
Neelipleona



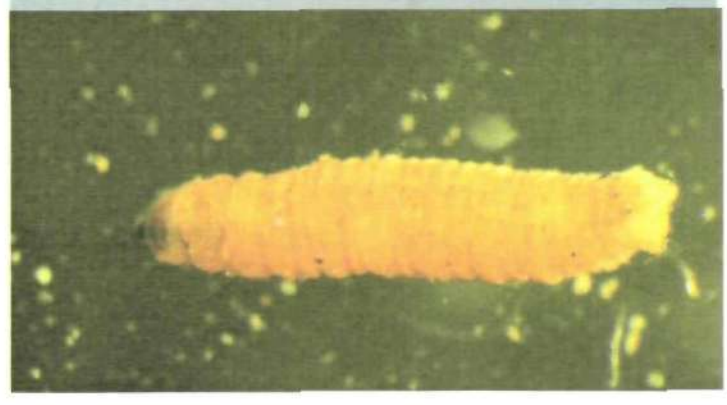
Collembola:
Symphypleona



Pseudoscorpion

Diptera Larvae

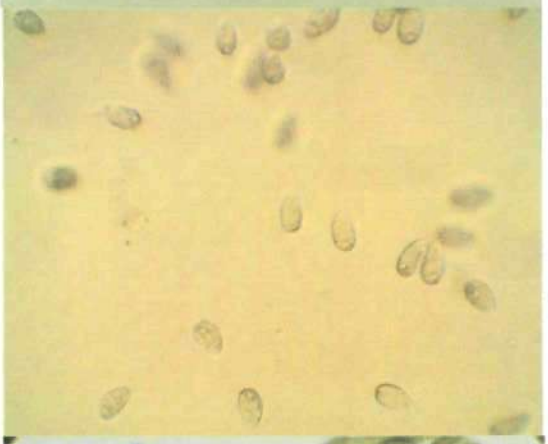
Tipulidae



Diplopoda

Nematode

Protozoa



Spider

Harvestmen

Fairy wasps (Chalcidoidea)

Diptera

Earthworm

Thrips

***APPENDIX B*: Fungal *ITS* Sequences and information sent to GenBank**

> Fungal isolate: 1 >GenBank accession number: JF303855
>Seq1 [organism=Penicillium ochrochloron strain PO1] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
CTGGGTCACCTCCCACCCGTGTTTATTTACCTTGTTGCTTCGGCGGGCCCGCC
TCACGGCCGCCGGGGGGCATCTGCCCCCGGGCCCGCGCCCGCCGAAGACAC
CATTGAACTCTGTCTGAAGATTGCAGTCTGAGCGATTAACTAAATCAGTTAA
AACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGA
AATGCGATACGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGA
ACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCAT
TGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCCCCGCCCCCCGGTCCCGGGG
GGCGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGG
GGCTTTGTCACCCGCTCCGTAGGCCCGGCCGGCGCCCGCCGGCGACCCCCA
ATCAATCTATCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTA
AGCATATCAATAAGCGGGGGAAA

> Fungal isolate: 2 >GenBank accession number: JF303856
>Seq2 [organism=Mucor hiemalis strain MH1] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
TAGATGGCCTTTGCTAGTTTTCTAGCGAATGGTTCATTCTTTTTTACTGTGAA
CTGTTTTAATTTTTCAGCGTCTGAGGAATGTCTTTTAGCCATAGGGATAGGC
TACTAGAATGTTAACCGAGCTGAAAGTCAGGCTTAGGCCTGGTATCCTATTA
ATTATTTACCAAAAGAATTCAGTATTATAATTGTAACATAAGCGTAAAAAA
CTTATAAAACAACTTTTAACAACGGATCTCTTGGTTCTCGCATCGATGAAGA
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AGTCTTTGAACGCAACTTGCGCTCAATGGTATTCCATTGAGCACGCCTGTTT
CAGTATCAAAAACACCCCACATTCATAATTTTGTTGTGAATGGAAATGAGA
GTTTCGGCTTTATTGCTGAATTCTTTAAAATTATTAGGCCTGAACTATTGTTC
TTTCTGCCTGAACATTTTTTTAATATAAAGGAATGCTCTAGTAAAAAGACTA
TCTCTGGGGCCTCCCAAATAAATCATTCTTAAATTTGATCTGAAATCAGGCG
GGATTACCCGCTGAACTTAAGCATATCAATAAGCGGGGGAA

> Fungal isolate: 3 >GenBank accession number: JF303857
>Seq3 [organism=Mucor hiemalis strain MH2] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
CCTTTGCAGTTTTCTAGCGAATGGTTCATTCTTTTTTACTGTGAACTGTTTTA
ATTTTTCAGCGTCTGAGGAATGTCTTTTAGCCATAGGGATAGGCTACTAGAA
TGTTAACCGAGCTGAAAGTCAGGCTTAGGCCTGGTATCCTATTAATTATTTA
CCAAAAGAATTCAGTATTATAATTGTAACATAAGCGTAAAAAACTTATAAA
ACAACTTTTAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGTAGCA
AAGTGCGATAACTAGTGTGAATTGCATATTCAGTGAATCATCGAGTCTTTGA
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AAACACCCCACATTCATAATTTTGTTGTGAATGGAAATGAGAGTTTCGGCTT
TATTGCTGAATTCTTTAAAATTATTAGGCCTGAACTATTGTTCTTTCTGCCTG
AACATTTTTTTAATATAAAGGAATGCTCTAGTAAAAAGACTATCTCTGGGGC
CTCCCAAATAAATCATTCTTAAATTTGATCTGAAATCAGGCGGGATTACCCG
CTGAACTTAAGCATATCAATAAGCGGGGGAAA

> Fungal isolate: 8 >GenBank accession number: JF303858
>Seq4 [organism=Gibberella zeae strain GZ1] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
CTCCAAACCCCTGTGACATACCTTATGTTGCCTCGGCGGATCAGCCCGCGCC
CCGTAAAAAGGGACGGCCCGCCGCAGGAACCCTAAACTCTGTTTTTAGTGG
AACTTCTGAGTATAAAAAACAAATAAATCAAAACTTTCAACAACGGATCTC
TTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAA
TTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAG
TATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCAGCT
TGGTGTTGGGAGCTGCAGTCCTGCTGCACTCCCCAAATACATTGGCGGTCAC
GTCGAGCTTCCATAGCGTAGTAATTTACACATCGTTACTGGTAATCGTCGCG
GCCACGCCGTTAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGA
ATACCCGCTGAACTTAAGCATATCAATAAGCGGGGAAAAAGAAAA

> Fungal isolate: 9 >GenBank accession number: JF303859
>Seq5 [organism=Mucor hiemalis strain MH3] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
TTAGATGGCCTTTGCTAGTTTTCTAGCGAATGGTTCATTCTTTTTTACTGTGA
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CTACTAGAATGTTAACCGAGCTGAAAGTCAGGCTTAGGCCTGGTATCCTATT
AATTATTTACCAAAAGAATTCAGTATTATAATTGTAACATAAGCGTAAAAA
ACTTATAAAACAACTTTTAACAACGGATCTCTTGGTTCTCGCATCGATGAAG
AACGTAGCAAAGTGCGATAACTAGTGTGAATTGCATATTCAGTGAATCATC
GAGTCTTTGAACGCAACTTGCGCTCAATGGTATTCCATTGAGCACGCCTGTT
TCAGTATCAAAAACACCCCACATTCATAATTTTGTTGTGAATGGAAATGAGA
GTTTCGGCTTTATTGCTGAATTCTTTAAAATTATTAGGCCTGAACTATTGTTC
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TCTCTGGGGCCTCCCAAATAAATCATTCTTAAATTTGATCTGAAATCAGGCG
GGATTACCCGCTGAACTTAAGCATATCAATAAGCGGGGAA

> Fungal isolate: 10 >GenBank accession number: JF303860
>Seq6 [organism=Hypocrea virens strain HV1] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
CCAACCCATGTGAACGTTACCAAACTGTTGCCTCGGCGGGATCTCTGCCCCG
GGCGCGTCGCAGCCCCGGACCAAGGCGCCCGCCGGAGGACCAACCAAAAC
TCTTATTGTATACCCCCTCGCGGGTTTTTTACTATCTGAGCCATCTCGGCGCC
CCTCGTGGGCGTTTCGAAAATGAATCAAAACTTTCAACAACGGATCTCTTGG
TTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC
AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATT
CTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGG

GGTCGGCGTTGGGGATCGGCCCTTTACGGGGCCGGCCCCGAAATACAGTGG
CGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACACTCGCATCGGGAG
CGCGGCGCGTCCACAGCCGTTAAACACCCCAAACTTCTGAAATGTTGACCTC
GGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGGA
AAAGAA

> Fungal isolate: 11 >GenBank accession number: JF303861
>Seq7 [organism=Trichoderma hamatum strain TH1] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
ACCATGTGACGTTACCAAACTGTTGCCTCGGCGGGGTCACGCCCCGGGTGC
GTAAAAGCCCCGGAACCAGGCGCCCGCCGGAGGAACCAACCAAACTCTTTC
TGTAGTCCCCTCGCGGACGTATTTCTTACAGCTCTGAGCAAAAATTCAAAAT
GAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAAC
GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGA
ATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCG
AGCGTCATTTCAACCCTCGAACCCCTCCGGGGGGTCGGCGTTGGGGATCGG
GACCCCTCACCGGGTGCCGGCCCTGAAATACAGTGGCGGTCTCGCCGCAGC
CTCTCCTGCGCAGTAGTTTGCACAACTCGCACCGGGAGCGCGGCGCGTCCA
CGTCCGTAAAACACCCAACTTCTGAAATGTTGACCTCGGATCAGGTAGGAA
TACCCGCTGAACTTAAGCATATCAATAAGCGGAGGGAAAAAGAAAA

> Fungal isolate: 12 >GenBank accession number: JF303862
>Seq8 [organism=Umbelopsis isabellina strain UI1] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
TATCTTGGTGCTTTACCGCCCACTATTATCTATTTACTGTGAACTGTATTATC
GCATGGCGCTTGAGAGATGCTTAAACACCATATGGATAGGTGTTTAAGATG
CTAATCGAGCCATGATCAAGCTTAGGCTTGGTATCCTATTATTATTTACCAA
AAGAATTCAGTATTAATATTGTAACATAGACCTAAAAAATCTATAAAACAA
CTTTTAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGTAGCAAAGT
GCGATAACTAGTGTGAATTGCATATTCAGTGAATCATCGAGTCTTTGAACGC
ATCTTGCACCTGTTGGTATTCCAACAGGTACGCCTGTTTCAGTATCAAAAAC
ATCCCTCTTTCAAATCTTTTTTTGAAAGGACTTGAGGGTATCTCGCTTAATAA
ACGAGAAACTCTTTAAACTACTAAGGCCTGGATTAGTTTACCTGCCTGAACT
TTTTTTTAATATAAAGGAAAGCTCTTGCGATTGAACTCCTGTTGAGGCCTCT
CAAACAATGCTTTTTTAAACTTGATCTGAAATCAGGTGGGATTACCCGCTGA
ACTTAAGCATATCAATAAGCGGAGGAAAAGAAA

> Fungal isolate: 14 >GenBank accession number: JF303863
>Seq9 [organism=Umbelopsis isabellina strain UI2] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
AGACACCATATGGATAGGTGTTTAAGATGCTAATCGAGCCATGATCAAGCT
TAGGCTTGGTATCCTATTATTATTTACCAAAAGAATTCAGTATTAATATTGT
AACATAGACCTAAAAAATCTATAAAACAACTTTTAACAACGGATCTCTTGG
TTCTCGCATCGATGAAGAACGTAGCAAAGTGCGATAACTAGTGTGAATTGC
ATATTCAGTGAATCATCGAGTCTTTGAACGCATCTTGCACCTGTTGGTATTC
CAACAGGTACGCCTGTTTCAGTATCAAAAACATCCCTCTTTCAAATCTTTTTT

TGAAAGGACTTGAGGGTATCTCGCTTAATAAACGAGAAACTCTTTAAACTA
CTAAGGCCTGGATTAGTTTACCTGCCTGAACTTTTTTTTAATATAAAGGAAA
GCTCTTGCGATTGAACTCTTGTTGAGGCCTCTCAAACAATGCTTTTTTAAACT
TGATCTGAAATCAGGTGGGATTACCCGCTGAACTTAAGCATATCAATAAGC
GGGGGAA

> Fungal isolate: 15 >GenBank accession number: JF303864
>Seq10 [organism=Fusarium sporotrichioides strain FS1] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
TGTGACATACCTTTATGTTGCCTCGGCGGATCAGCCCGCGCCCCGTAAAACG
GGACGGCCCGCCGCAGGAAACCCTAAACTCTGTTTTTAGTGGAACTTCTGA
GTATAAAAAACAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTG
GCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAAT
TCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGC
GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCAGCTTGGTGTTGG
GATCTGTGTGCAAACACAGTCCCCAAATTGATTGGCGGTCACGTCGAGCTTC
CATAGCGTAGTAATTTACACATCGTTACTGGTAATCGTCGCGGCCACGCCGT
TAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTG
AACTTAAGCATATCAATAAGCGGGGGAAAAAGAAACTATCTCTGGGGCCTC
CCAAATAAATCATTCTTAAATTTGATCTGAAATCAGGCGGGATTACCCGCTG
AACTTAAGCATATCAATAAGCGGAGGAAAAGAAATGCT

> Fungal isolate: 16 >GenBank accession number: JF303865
>Seq11 [organism=Gibberella zeae strain GZ2] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
ACTCCAAACCCCTGTGACATACCTTATGTTGCCTCGGCGGATCAGCCCGCGC
CCCGTAAAAAGGGACGGCCCGCCGCAGGAACCCTAAACTCTGTTTTTAGTG
GAACTTCTGAGTATAAAAAACAAATAAATCAAAACTTTCAACAACGGATCT
CTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGA
ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCA
GTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCAGC
TTGGTGTTGGGAGCTGCAGTCCTGCTGCACTCCCCAAATACATTGGCGGTCA
CGTCGAGCTTCCATAGCGTAGTAATTTACACATCGTTACTGGTAATCGTCGC
GGCCACGCCGTTAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGG
AATACCCGCTGAACTTAAGCATATCAATAAGCGGGGGAAAA

> Fungal isolate: 17 >GenBank accession number: JF303866
>Seq12 [organism=Microdochium phragmitis strain MP1] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
CCTGTGAACTTACCACTGTTTCCTCGGTGGAAGGTACCTGAAAGGGTGCTGG
AAGCCGGTGGACATTTAAACTCTTGTTAATTTTGTTAATTCTGAATCAAACT
AAGAAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATG
AAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAAT
CATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCC
TGTTCGAGCGTCATTTCAACCCTTAAGCCTAGCTTAGTGTTGGGAGACTGCC
TAATACGCAGCTCCTCAAAACCAGTGGCAGAGTTTTTACGTACTCTGAGCGC

AGTAATTCTATTCTCGCTTTTGAACACGTCTAGACGATAGCCAAAAACCGCT
TGCTTCGGCAGCACTTTTTTAATGGTTGACCTCGGATCAGGTAGGAATACCC
GCTGAACTTAAGCATATCAATAAGCGGGGGA

> Fungal isolate: 18 >GenBank accession number: JF303867
>Seq13 [organism=Fusarium cerealis strain FC1] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
CCTGTGACATACCTTATGTTGCCTCGGCGGATCAGCCCGCGCCCCGTAAAAA
GGGACGGCCCGCCGCAGGAACCCTAAACTCTGTTTTTAGTGGAACTTCTGA
GTATAAAAAACAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTG
GCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAAT
TCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGC
GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCAGCTTGGTGTTGG
GAGCTGCAGTCCTGCTGCACTCCCCAAATACATTGGCGGTCACGTCGAGCTT
CCATAGCGTAGTAATTTACATATCGTTACTGGTAATCGTCGCGGCCACGCCG
TTAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTG
AACTTAAGCATATCAATAAGCGGGGGAAAA

> Fungal isolate: 19 >GenBank accession number: JF303868
>Seq14 [organism=Bionectria ochroleuca strain BO1] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
ATGTGACATACCTACTGTTGCTTCGGCGGGATTGCCCCGGGCGCCTCGTGTG
CCCCGGATCAGGCGCCCGCCTAGGAAACTTAATTCTTGTTTTATTTTGGAAT
CTTCTGAGTAGTTTTTACAAATAAATAAAAACTTTCAACAACGGATCTCTTG
GTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTG
CAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
TCTGGCGGGCATGCCTGTCTGAGCGTCATTTCAACCCTCATGCCCCTAGGGC
GTGGTGTTGGGGATCGGCCAAAGCCCGCGAGGGACGGCCGGCCCCTAAATC
TAGTGGCGGACCCGTCGTGGCCTCCTCTGCGAAGTAGTGATATTCCGCATCG
GAGAGCGACGAGCCCCTGCCGTTAAACCCCCAACTTTCCAAGGTTGACCTC
AGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGGGAA

> Fungal isolate: 20 >GenBank accession number: JF303869
>Seq15 [organism=Gibberella moniliformis straim FM1] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
ACTCCAACCCCTGTGACATACCACTTGTTGCCTCGGCGGATCAGCCCGCTCC
CGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTATATG
TAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCTCT
TGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAAT
TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGT
ATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCTCGGG
TTTGGTGTTGGGGATCGGCGAGCCCTTGCGGCAAGCCGGCCCCGAAATCTA
GTGGCGGTCTCGCTGCAGCCTCCATTGCGTAGTAGTAAAACCCTCGCAACTG
GAACGCGGCGCGGCCAAGCCGTTAAACCCCCAACTTCTGAATGTTGACCTC
GGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGGGGAA
A

> Fungal isolate: 21 >GenBank accession number: JF303870
>Seq16 [organism=Penicillium biourgeianum strain PB1] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
TCTGGTCACCTCCCACCCGTGTTTATTTACCTTGTTGCTTCGGCGAGCCTGCC
TTTTGGCTGCCGGGGGACGTCAGTCCCCGGGTCCGTGCTCGCCGGAGACAC
CTTAGAACTCTGTCTGAAGATTGTAGTCTGAGATTAAATATAAATTATTTAA
AACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGA
AATGCGATACGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGA
ACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTCCGAGCGTCAT
TGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCCCCGTCCTCCTTCCGGGGGA
CGGGTCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCAAGCGTATGGGG
CTTTGTCACTCGCTTTGTAGGCCTGGCCGGCGCTTGCCGATCAACCAAACTT
TTTATCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATA
TCAATAAGCGGGGGAAAAAGAAAA

> Fungal isolate: 23 >GenBank accession number: JF303871
>Seq17 [organism=Fusarium cerealis strain FC1] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
CTGTGACATACCTTATGTTGCCTCGGCGGATCAGCCCGCGCCCCGTAAAAAG
GGACGGCCCGCCGCAGGAACCCTAAACTCTGTTTTTAGTGGAACTTCTGAGT
ATAAAAAACAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGC
ATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTC
AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGG
GCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCAGCTTGGTGTTGGGA
GCTGCAGTCCTGCTGCACTCCCCAAATACATTGGCGGTCACGTCGAGCTTCC
ATAGCGTAGTAATTTACATATCGTTACTGGTAATCGTCGCGGCCACGCCGTT
AAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGA
ACTTAAGCATATCAATAAGCGGAGGAAAAGAAA

> Fungal isolate: 25 >GenBank accession number: JF303872
>Seq18 [organism=Mucor hiemalis strain MH4] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
TGCTAGTTTCTAGCGAATGGTTCATTCTTTTTTACTGTGAACTGTTTTAATTT
TTCAGCGTCTGAGGAATGTCTTTTAGCCATAGGGATAGGCTACTAGAATGTT
AACCGAGCTGAAAGTCAGGCTTAGGCCTGGTATCCTATTAATTATTTACCAA
AAGAATTCAGTATTATAATTGTAACATAAGCGTAAAAAACTTATAAAACAA
CTTTTAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGTAGCAAAGT
GCGATAACTAGTGTGAATTGCATATTCAGTGAATCATCGAGTCTTTGAACGC
AACTTGCGCTCAATGGTATTCCATTGAGCACGCCTGTTTCAGTATCAAAAAC
ACCCCACATTCATAATTTTGTTGTGAATGGAATTGAGAGTTTCGGCTTTATT
GCTGAATTCTTTAAAATTATTAGGCCTGAACTATTGTTCTTTCTGCCTGAACA
TTTTTTTAATATAAAGGAATGCTCTAGTAAAAAGACTATCTCTGGGGCCTCC
CAAATAAATCATTCTTAAATTTGATCTGAAATCAGGCGGGATTACCCGCTGA
ACTTAAGCATATCAATAAGCGGAGGGAAAAGAA

> Fungal isolate: 26 >GenBank accession number: JF303873
>Seq19 [organism=Trichoderma longipile srain TL1] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
TGTGACGTTACCAAACTGTTGCCTCGGCGGGATCTCTGCCCCGGGTGCGTCG
CAGCCCCGGACCAAGGCGCCCGCCGGAGGACCAACCAAAACTCTTTATGTA
TACCCCCTCGCGGGTTTTTTACAATCTGAGCCATCTCGGCGCCCCTCGTGGG
CGTTTCGAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCAT
CGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAG
TGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGC
ATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGGTCGGCGT
TGGGGATCGGCCCTTTACGGGGCCGGCCCCGAAATACAGTGGCGGTCTCGC
CGCAGCCTCTCCTGCGCAGTAGTTTGCACACTCGCATCGGGAGCGCGGCGC
GTCCATTGCCGTAAAACACCCAACTTTCTGAAATGTTGACCTCGGATCAGGT
AGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAA

> Fungal isolate: 27 >GenBank accession number: JF303874
>Seq20 [organism=Penicillium simplicissimum strain PS1] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
TCTGGGTCACCTCCCACCCGTGTTTATTTACCTTGTTGCTTCGGCGGGCCCGC
CTCACGGCCGCCGGGGGGCACCCGCCCCCGGGCCCGCGCCCGCCGAAGACA
CCATTGAACTCTGTCTGAAGATTGCAGTCTGAGCGATTAGCTAAATCAGTTA
AAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCG
AAATGCGATACGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTG
AACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTC
ATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCCCCGCCCCCCGGTCCCGG
GGGGCGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTAT
GGGGCTTCGTCACCCGCTCTGTAGGCCCGGCCGGCGCCCGCCGGCGACCCC
CAATCAATCTATCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAAC
TTAAGCATATCAATAAGCGGAGGGAAAAGAAA

> Fungal isolate: 28 >GenBank accession number: JF303875
>Seq21 [organism=Hypocrea viridescens strain HV1] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
CCCATGTGAACCATACCAAACTGTTGCCTCGGCGGGGTCACGCCCCGGGTG
CGTCGCAGCCCCGGAACCAGGCGCCCGCCGGAGGGACCAACCAAACTCTTT
CTGTAGTCCCCTCGCGGACGTTATTTCTTACAGCTCTGAGCAAAAATTCAAA
ATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGA
ACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATC
GAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTC
CGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGTCCGGCGTTGGGGATC
GGGAACCCCTAAGACGGGATCCCGGCCCCGAAATACAGTGGCGGTCTCGCC
GCAGCCTCTCATGCGCAGTAGTTTGCACAACTCGCACCGGGAGCGCGGCGC
GTCCACGTCCGTAAAACACCCAACTTCTGAAATGTTGACCTCGGATCAGGTA
GGAATACCCGCTGAACTTAAGCATATCAATAAGCGGGGGAAAAAGAAA

> Fungal isolate: 29 >GenBank accession number: JF303876

>Seq22 [organism=Fusarium cerealis strain FC2] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
CCTGTGACATACCTTATGTTGCCTCGGCGGATCAGCCCGCGCCCCGTAAAAA
GGGACGGCCCGCCGCAGGAACCCTAAACTCTGTTTTTAGTGGAACTTCTGA
GTATAAAAAACAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTG
GCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAAT
TCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGC
GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCAGCTTGGTGTTGG
GAGCTGCAGTCCTGCTGCACTCCCCAAATACATTGGCGGTCACGTCGAGCTT
CCATAGCGTAGTAATTTACATATCGTTACTGGTAATCGTCGCGGCCACGCCG
TTAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTG
AACTTAAGCATATCAATAAGCGGAGGAAAAGAAA

> Fungal isolate: 30 >GenBank accession number: JF303877
>Seq23 [organism=Cladosporium cladosporioides strain CC1] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
GTCTACCACCGGGATGTTCATAACCCTTTGTTGTCCGACTCTGTTGCCTCCG
GGGCGACCCTGCCTTCGGGCGGGGGCTCCGGGTGGACACTTCAAACTCTTG
CGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATTAAAACTTTTAACAA
CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGT
AATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG
CCCCCTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAA
GCCTCGCTTGGTATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCG
GCTGGGTCTTCTGTCCCCTAAGCGTTGTGGAAACTATTCGCTAAAGGGTGTT
CGGGAGGCTACGCCGTAAAACAACCCCATTTCTAAGGTTGACCTCGGATCA
GGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGGAAAAGA
AA

> Fungal isolate: 32 >GenBank accession number: JF303878
>Seq24 [organism=Trichoderma longipile strain TL2] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
CTCCAACCCATGTGAACGTTACCAAACTGTTGCCTCGGCGGGATCTCTGCCC
CGGGTGCGTCGCAGCCCCGGACCAAGGCGCCCGCCGGAGGACCAACCAAA
ACTCTTTATGTATACCCCCTCGCGGGTTTTTTACAATCTGAGCCATCTCGGCG
CCCCTCGTGGGCGTTTCGAAAATGAATCAAAACTTTCAACAACGGATCTCTT
GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATT
GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGT
ATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCG
GGGGGTCGGCGTTGGGGATCGGCCCTTTACGGGGCCGGCCCCGAAATACAG
TGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACACTCGCATCGG
GAGCGCGGCGCGTCCATTGCCGTAAAACACCCAACTTTCTGAAATGTTGAC
CTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAG
GAAAAGAAA

> Fungal isolate: 33 >GenBank accession number: JF303879

>Seq25 [organism=Hypocrea viridescens strain HV2] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
AACCCATGTGAACCATACCAAACTGTTGCCTCGGCGGGGTCACGCCCCGGG
TGCGTCGCAGCCCCGGAACCAGGCGCCCGCCGGAGGGACCAACCAAACTCT
TTCTGTAGTCCCCTCGCGGACGTTATTTCTTACAGCTCTGAGCAAAAATTCA
AAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAA
GAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCA
TCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTG
TCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGTCCGGCGTTGGGGAT
CGGGAACCCCTAAGACGGGATCCCGGCCCCGAAATACAGTGGCGGTCTCGC
CGCAGCCTCTCATGCGCAGTAGTTTGCACAACTCGCACCGGGAGCGCGGCG
CGTCCACGTCCGTAAAACACCCAACTTCTGAAATGTTGACCTCGGATCAGGT
AGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAAGAAA

> Fungal isolate: 34 >GenBank accession number: JF303880
>Seq26 [organism=Fusarium oxysporum strain FO1] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
CAACCCCTGTGACATACCACTTGTTGCCTCGGCGGATCAGCCCGCTCCCGGT
AAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTAATATGTAA
CTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCTCTTGG
TTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGC
AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATT
CTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCACAGCTTGGT
GTTGGGACTCGCGTTAATTCGCGTTCCTCAAATTGATTGGCGGTCACGTCGA
GCTTCCATAGCGTAGTAGTAAAACCCTCGTTACTGGTAATCGTCGCGGCCAC
GCCGTTAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCC
GCTGAACTTAAGCATATCAATAAGCGGGGGAAAAAGAAAA

> Fungal isolate: 35 >GenBank accession number: JF303881
>Seq27 [organism=Fusarium cerealis strain FC3] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
CTGTGACATACCTTATGTTGCCTCGGCGGATCAGCCCGCGCCCCGTAAAAAG
GGACGGCCCGCCGCAGGAACCTTAAACTCTGTTTTTAGTGGAACTTCTGAGT
ATAAAAAACAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGC
ATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTC
AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGG
GCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCAGCTTGGTGTTGGGA
GCTGCAGTCCTGCTGCACTCCCCAAATACATTGGCGGTCACGTCGAGCTTCC
ATAGCGTAGTAATTTACATATCGTTACTGGTAATCGTCGCGGCCACGCCGTT
AAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGA
ACTTAAGCATATCAATAAGCGGAGGAAAAGAAA

> Fungal isolate: 36 >GenBank accession number: JF303882
>Seq28 [organism=Fusarium cerealis strain FC4] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal

transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
AACTCCAACCCCTGTGACATACCTTATGTTGCCTCGGCGGATCAGCCCGCGC
CCCGTAAAAAGGGACGGCCCGCCGCAGGAACCTTAAACTCTGTTTTTAGTG
GAACTTCTGAGTATAAAAAACAAATAAATCAAAACTTTCAACAACGGATCT
CTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGA
ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCA
GTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCAGC
TTGGTGTTGGGAGCTGCAGTCCTGCTGCACTCCCCAAATACATTGGCGGTCA
CGTCGAGCTTCCATAGCGTAGTAATTTACATATCGTTACTGGTAATCGTCGC
GGCCACGCCGTTAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGG
AATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAA

> Fungal isolate: 37 >GenBank accession number: JF303883
>Seq29 [organism=Fusarium sporotrichioides strain FS2] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
CACTCCAAACCCCTGTGACATACCTTTATGTTGCCTCGGCGGATCAGCCCGC
GCCCCGTAAAACGGGACGGCCCGCCGCAGGAAACCCTAAACTCTGTTTTTA
GTGGAACTTCTGAGTATAAAAAACAAATAAATCAAAACTTTCAACAACGGA
TCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATG
TGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCG
CCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCC
AGCTTGGTGTTGGGATCTGTGTGCAAACACAGTCCCCAAATTGATTGGCGGT
CACGTCGAGCTTCCATAGCGTAGTAATTTACACATCGTTACTGGTAATCGTC
GCGGCCACGCCGTTAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTA
GGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGGAAAAGAAA

> Fungal isolate: 38 >GenBank accession number: JF303884
>Seq30 [organism=Cladosporium cladosporioides strai CC2] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
GGTCTACCACCGGGATGTTCATAACCCTTTGTTGTCCGACTCTGTTGCCTCC
GGGGCGACCCTGCCTTCGGGCGGGGGCTCCGGGTGGACACTTCAAACTCTT
GCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATTAAAACTTTTAACA
ACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAG
TAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGC
GCCCCCTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCA
AGCCTCGCTTGGTATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACC
GGCTGGGTCTTCTGTCCCCTAAGCGTTGTGGAAACTATTCGCTAAAGGGTGT
TCGGGAGGCTACGCCGTAAAACAACCCCATTTCTAAGGTTGACCTCGGATC
AGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGGAAAAG
AAA

PUBLICATIONS

- **Crotty, F. V.,** Blackshaw, R. P., and Murray, P. J. (2011) Tracking the flow of bacterially derived ^{13}C and ^{15}N through soil faunal feeding channels. *Rapid Communications in Mass Spectrometry* **25:** 1503-1513 **(Chapter 5)**
- **Crotty, F. V.,** Blackshaw, R. P., and Murray, P. J. (2011) Differential growth of the fungus *Absidia cylindrospora* on $^{13}C/^{15}N$ labelled media. *Rapid Communications in Mass Spectrometry* **25:** 1479-1484 **(Chapter 7)**
- **Crotty F. V.** First Prize in the Agriculture, Food, Diet and Health category in the BBSRC science photo competition (Feb-10). Featured in Science **327**, (2010) p1183.
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