Enchytraeids and nitrogen : the effects of nitrogen on enchytraeid populations and the influence of enchytraeids on nitrogen translocation in soil

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ENCHYTRAEIDS AND NITROGEN

THE EFFECTS OF NITROGEN ON ENCHYTRAEID POPULATIONS AND THE INFLUENCE OF ENCHYTRAEIDS ON NITROGEN TRANSLOCATION IN SOIL

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DECLARATION

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Abstract

Enchytraeids and Nitrogen

The background to the study was concern over the impact of atmospheric global nitrogen emissions on key soil fauna such as Enchytraeidae which are abundant in the acid soils found extensively in Britain. The project examined the effects of nitrogen and lime fertiliser on the populations of enchytraeids, and the role of enchytraeids in nitrogen translocations.

In a deep blanket peat with a gradual increase of background atmospheric N deposition over the past 40 years, there was no difference in the abundance of enchytraeids in 2002 compared to published data from 1968. However the timing of fragmentation appeared to be related to soil temperature and this occurred in August 2002 but was one month earlier in 1968.
In an acid peat soil experimental nitrogen fertilisation appeared to increase the abundance of enchytraeids whereas in an acidic brown earth soil it appeared to decrease abundance. These differences were explained by differences in the amount and timing of the N fertiliser applied and the quantity and quality of litter. Liming soil totally changed enchytraeid species composition.

Litter labelled with the stable isotope ^{15}N was contained in coarse and fine mesh litterbags in a field experiment also receiving different fertiliser treatments. Fewer enchytraeids were found in fine mesh bags and up to 88% of their tissue N was shown to be derived from bag litter whereas a maximum of 45% tissue N was found in enchytraeids from coarse mesh bags. Nitrogen fertiliser substantially increased the half-life values of nitrogen in enchytraeid tissue in both acid and neutral soils. Similar results were found for tissue carbon in a field experiment using $^{13}CO_2$ and the possible reasons are discussed.

Translocation of nitrogen into soil by enchytraeids was measured by the change (Δ) of $\delta^{15}N$ signal in soil prior to litterbag placement and after 14 months. The $\Delta\ \delta^{15}N$ in soil around coarse mesh litterbags was in the order control (~ 5‰), nitrogen treated (~4‰), lime treated (~2.5‰) and lime and nitrogen treated plots (~1 ‰).
In soil around fine mesh litterbags, control, nitrogen and lime + nitrogen treated plots had similar $\Delta\ \delta^{15}N$ values (0.5 - 1‰) whereas that of lime treated plots was 3‰. The influences of fertiliser and mesh size on results are discussed in the context of enchytraeid activity and nitrogen leaching.

Changes in $\delta^{15}N$ and %N values of litter were used to estimate both N release and incorporation over 14 months. Nitrogen fertiliser and lime appeared to adversely affect the rate of N release from litter contained in coarse and fine bags, but had no effect on N incorporation. N incorporation was less in fine litterbags than coarse bags, and the interactions between enchytraeids, microbes and their impact on N release and incorporation are discussed.

Dual labelled (^{13}C and ^{15}N) urea was successfully used to label *Agrostis capillaris* using a simple 'watering in' method which did not require specialist equipment.

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CHAPTER 1

Introduction and background.

Scientific investigations into the effects of anthropogenic pollutants on different ecosystems, plant and animal species and human health have been carried out for many years. This work has informed legislation and voluntary action which has led to the abatement of some pollutants, for example the reduction in sulphur dioxide emissions (NEGTAP 2001) and chlorofluorocarbons over the past twenty years. However, the emissions of some pollutants such as carbon dioxide (CO_2) and methane (CH_4) continue to rise (IPPC).

Nitrogen emissions are rather more complex due to the different forms that are released. Nitrogen oxides have been declining since their peak in the 1980s (850 kt-N to 500 kt-N) and expected to decline further (NEGTAP 2001) but emissions of ammonia have changed little since the mid-1980s (NEGTAP 2001). Nitrogen is considered to be both an acidifier and a nutrient of both terrestrial and aquatic ecosystems. In terrestrial ecosystems, soils that are already acid and nutrient poor are therefore more sensitive to N deposition as both an acidifier and nutrient source.

The current level of nitrogen emissions have prompted much recent research in both the UK and abroad into the sources of nitrogen and the effects of excess nitrogen on various ecological processes and semi-natural ecosystems in different geographical locations. A number of sources of nitrogen released into the atmosphere are the result of human activities. Many are associated with intensive agricultural practices (e.g. the increased use of chemically fixed fertilisers, increases in animal farming) and land use changes which are considered necessary to provide food for a growing global human

population. Others are as a direct consequence of fossil fuel burning for power and transportation. These sources are estimated to have doubled the amount of biologically available nitrogen transferred from atmosphere to land (Vitousek, 1997). Nitrogen compounds can be carried by rain, mist and snow or deposited directly onto surfaces. The scale of deposition therefore varies at both the local to the global level.

Nitrogen may be deposited as ammonium (NH_4^+) and nitrate (NO_3^-) in rain, and as occult deposition (cloud water droplet deposition) at high elevations (wet deposition). Nitrogen dioxide, nitric acid and ammonia constitute the majority of dry deposition, with smaller inputs as peroxyacetyl nitrate (PAN), nitrous acid (HONO) and particulate NH_4^+ and NO_3^- (Pitcairn *et al*, 1995).

The transformations of nitrogen which take place within the soil are part of a complex process which is still being researched. The following brief summary is based on Killham (1994) and Coleman and Crossley (1996).

The large pool of atmospheric nitrogen, which constitutes 78% of the earths' atmosphere, is unavailable in its gaseous state to plants. It must be converted to a another form before it can be utilised. This process (fixation) is the incorporation of atmospheric nitrogen in a combined form, such as ammonia (NH_3) or nitrate (NO_3). A small amount of nitrogen is fixed abiotically by processes such as lightening, but the majority is fixed biotically in the soil by nitrogen-fixing organisms which are either free-living or have a symbiotic relationship with plants (eg legumes).

Organic nitrogen is returned to the soil in the forms of above and below ground plant litter (wood, leaves, roots), animal urine and faeces, dead animals and microbes. Organic nitrogen is converted to inorganic nitrogen (ammonium and nitrate) by the process of mineralistaion. Inorganic nitrogen is then available for plant and microbial uptake. When used by organisms in this way nitrogen is immobilised.

The basic process of mineralisation within the soil is nitrification. Nitrification is the process which involves the oxidation of ammonium (or other reduced forms of nitrogen) to nitrate (or a more oxidised form of nitrogen) by the action of microbes, predominantly chemoautotrophs under aerobic conditions. For example, species of the bacteria *Nitrosomonas* carry out the following reactions:

$NH_4^+ + O_2 + H^+ \rightarrow NH_2OH$ (hydroxylamine) $+ H_2O$ and

$NH_2OH + H_2O \rightarrow NO_2^-$ (nitrite) $+ 5H^+ + 4e^-$

while *Nitrobacter* carry out the following reaction:

$NO_2^- + H_2O \rightarrow H_2O{\cdot}NO_2^- \rightarrow NO_3^-$ (nitrate) $+ 2H$.

The process of denitrification (the reduction of nitrate) is carried out by facultative anaerobes in anaerobic soils or soil microsites.

$NO_3 \rightarrow NO_2 \rightarrow NO \rightarrow N_2O \rightarrow N_2$.

In this process nitrate replaces oxygen as the electron acceptor in soil microbial respiration. As a result of this process, nitrogen is lost from the soil as N_2O and N_2.

In acid soils, for example those dominated by coniferous forest and heather moorland systems, N_2O evolution is high.

The process of volatilisation of ammonia occurs under dry, warm conditions with high air movement and alkaline soil pH. If ammonia is present in an unadsorbed state i.e. decomposing manure or hydrolysing urea fertiliser, ammonia as a gas can be lost from the soil surface. Plants can hinder the loss by competing for ammonium in the soil and the direct foliar absorption of volatilised ammonia. However, ammonia can also damage microbes, plants and soil animals as cell membranes are highly permeable to ammonia and the resulting rapid rise in cell pH is too fast for compensation mechanisms to operate.

Soils are heterogeneous with both above- and below-ground compartments, and these provide many different habitats for many different soil biota. These habitats vary both spatially and temporally and may also be ephemeral. They are influenced by abiotic factors such as moisture and temperature and also the movement and activities of the soil biota. Soils in undisturbed systems may be well stratified, whereas those subject to disturbance (particularly tillage in agricultural systems) are mixed.
Soil flora and fauna may be classified according to size, habitat or food source, and the classifications are linked to a certain extent.

The soil flora - bacteria and fungi – fall into the smallest size classification, and may therefore occupy varying habitats and are classified functionally as primary decomposers. Although neither are motile, fungi are able to move through the soil via hyphal extension. Some soil flora are generalists, being capable of the breakdown of

simple molecules such as simple carbohydrates, whereas others are more specialised, being able to break down complex molecules such as plant lignin.

Soil microfauna include protistans, rotifera and nematodes and vary from 2 – 100 μm. Soil mesofauna include animals whose size varies from 100 μm to 2 mm, and macrofauna are those animals between 2 and 20 mm.

The size of the organism and the abiotic conditions within the soil structure determine its position in the soil. For example, some soil organisms have requirements of pore size, pH, temperature and moisture, and mobile animals can migrate vertically up and down in response to microclimatological changes. However, there are animals that are found in specific soil strata. Earthworms are classified as inhabiting three main habitat types: epigeic species, which are surface active and are involved mainly with litter comminution; endogeic species, which live in mineral soil and are geophagous and anecic species, which transport material between the mineral soil and organic litter layer. The burrowing activities of earthworms directly change soil structure and aeration, create casts which are higher in nutrients than surrounding soil and move soil/litter between different soil layers.

Generally, soil food webs consist of autotrophs and heterotrophs, animals which feed on them and predators which feed on the grazers. There may also be top carnivores feeding on any of the two lower trophic levels.

At the soil surface and within the soil, material such as plant litter, faeces, urine and dead animals are deposited and the processes of decomposition and nutrient release

occur. Initially, soluble products are leached from the litter and may be immobilised by plants and/or microflora. Litter is also 'invaded' by microbes and active decomposition begins. The microbes are grazed by soil animals, and grazing may be directed (or active) grazing, or non-directed as part of the ingestion of soil and/or litter. The generalised feeding activity of many soil animals results in the comminution of litter and its pre-conditioning for further microbial colonisation, and, because of the mobility of soil animals, the distribution of litter and microbes away from the site of the feeding activity can occur. This in turn creates further opportunities for colonisation of comminuted litter by dispersed fungal spores, and therefore 'hotspots' which attract soil animals.

It is often difficult to be certain of the food sources of soil animals as they are almost impossible to observe directly. Traditional methods used to assess their food include examination of their mouthparts, gut contents, gut enzymes and food choice experiments. These do not, however, confirm specific food sources.

Some genera of soil animals are ubiquitous, but individual species may have specific biotic and/or abiotic conditions necessary for their survival. For example, many species of earthworm thrive in soils with neutral pH and co-exist with many species of enchytraeid worms, a related but much smaller genera. However, in soils with low pH, not only are many earthworm species absent, but also many species of enchytraeids. In these acid soils, the dominant enchytraeid is *Cognettia sphagnetorum*.

Enchytraeidae are a family of Clitellata related to the Lumbricidae within the phylum Annelida. Annelids are relatively large, active worms with species adapted to marine, freshwater and terrestrial habitats throughout the world. They are metamerically segmented and the 'sealed unit' segments together with circular and longitudinal muscles work on incompressible coelomic fluid as a hydrostatic skeleton. The gut is straight with well developed musculature and food is moved along by peristalsis (Pond, 1990).

Enchytraeidae are found throughout the world from the arctic to the antarctic, but their stronghold appears to be the northern hemisphere. They are smaller than Lumbricidae – 3mm - 30mm and generally classed as mesofauna. They are hermaphrodite with some parthenogenic and a few fissiparous species such as *Cognettia sphagnetorum*. Enchytraeids feed on decomposing plant material, which includes bacteria and fungi. The gut has no crop or gizzard and digestion is aided by enzymes secreted by peptonephridia and/or oesophageal glands. As in Lumbricidae (Bouche, 1984) different species occupy different spatial niches within the soil i.e. some are found mainly within the surface layers (epigeic) such as species of *Cognettia, Henlea* and *Enchytraeus*, some occupy the lower layers (endogeic) such as *Achaeta*, while others such as species of *Fridericia* burrow throughout the soil profile (anecic). The larger anecic species such as *Fridericia galba* probably have a more direct effect on the translocation of material throughout the soil profile compared to epigeic or endogeic species.

The impact of Enchytraeidae on the decomposition of plant material is partly by comminution as this increases the surface area of material available for colonisation

by bacteria and fungi. Enchytraeids have been shown to have a direct impact on microorganism populations (Hedlund and Augustsson, 1995) Whether enchytraeids digest plant material or microorganisms or the products of breakdown of plant material by microorganisms is unclear for most species.

Like Lumbricidae, most species of enchytraeids are intolerant of soil with low pH, but a few such as *Cognettia sphagnetorum, Marionina clavata* and *Mesenchytraeus sanguineous* flourish in acid peat soils. These three species and some *Henlea* and *Enchytraeus* species found in neutral conditions are associated with wet soils while most species of *Fridericia* are able to withstand low soil moisture both as adults and as eggs in cocoons. A few species such as *Mesenchytraeus sanguineous* characteristic of wet and temporarily anaerobic soils have haemoglobin in their blood. Several species have been shown to withdraw to lower levels within the soil under drought conditions (Briones *et al.*, 1997).

Some species of enchytraeids are tolerant of regular disturbance, for example *Enchytraeus buchholzi* is found in seasonally droughted soils in the Camargue and also in periodically frozen soils in Alaska (Standen, pers. comm.). This small species has a short life span, but many of the larger species live for more than 12 months. Several hundred species of enchytraeids have been recorded in the British Isles with *Fridiericia* as the most speciose genus. There is no definitive check list of UK or European species of Enchytraeidae and new species are described each year.

Observed and potential impacts of N deposition, and the scale of emissions, during the past 50 years have highlighted the fact that the impacts of anthropogenic nitrogen deposition are poorly understood and little quantified. The Natural Environment Research Council (NERC) provided funding to further our understanding through the Global Atmospheric Nitrogen Enrichment (GANE) Thematic programme, of which this project constitutes a small part. The GANE project ran for 5 years. The main areas of study were transformations and pathways of reactive nitrogen, quantification of nitrogen fluxes at large temporal and spatial scales and impacts of reactive nitrogen on semi-natural ecosystems and coastal waters. This project is concerned with the latter.

Understanding how N deposition affects wet, organic, acid soils is of direct relevance to semi-natural ecosystems of high conservation value. Upland heather moorlands fall within the EU Habitats Directive and it is estimated that the UK has about one third of the European total (Thompson *et al.*, 1995). These areas are under threat from increasing grazing pressure and sub-optimal management regimes but excessive N deposition creates another threat. For example, increased sensitivity to abiotic and biotic stresses and changes in species composition (e.g. Caporn *et al.*, 1994, Leith *et al.*, 1999, Pitcairn *et al.*, 1995, Bobbink and Roelofs, 1996) could adversely affect the *Calluna* dominated plant community.

Although acid grasslands are considered to be of lower conservation value, there is an increasing trend for improvement by liming and/or fertilisation (Chalmers, 2001). Long-term studies of acid and neutral grasslands with N additions have shown that N-responsive plants dominate with a concomitant reduction in species richness

(Silvertown, 1980). Burt-Smith (2002) found increased litter accumulation with lime and lime plus nitrogen treatments in an upland pasture where mown grass clippings were removed. Lime has been shown to affect nitrogen mineralisation by increasing microbial biomass and N assimilation (Chambers and Garwood, 1998). Effects of lime on upland pastures increased microbial activity and rates of carbon and nitrogen transformations (Isabella and Hopkins, 1994; Hopkins, 1997). Carbon loss from such soils as a result of liming is of concern when considered in the context of global carbon emissions.

The project aims were twofold to examine

1. the impact of nitrogen of populations of enchytraeid worms and
2. their role in translocation of nitrogen in acid soils at sensitive sites.

This work was to focus on *Cognettia sphagnetorum* (Vejdovsky), which is the dominant soil animal in acid soils at sensitive sites, comprising 75 – 90% of the soil animal biomass, but also to examine enchytraeid communties of a neutral soil.

The aims were met by sampling experimental field sites which had been treated with ammonium nitrate at different concentrations and plant litter labelled with ^{15}N confined in mesh bags. The field sites sampled in which *C. sphagnetorum* was the dominant enchytraeid were a peat soil with *Calluna vulgaris* (L.) as the main plant species at Ruabon, North Wales, and a brown earth acid grassland dominated by *Agrostis capillaris* (L.) and *Festuca rubra* (L.) at Sourhope, South Scotland. To supplement these experimental population studies, the abundance of *C. sphagnetorum* from a blanket peat at Moor House, in the North Pennines of England, which has

received increasing nitrogen deposition during the last 40 years, was compared to that from1968 described in the published literature.

The Sourhope experimental site included plots which had been modified by the addition of lime alone or in combination with nitrogen and the enchytraeid populations of these neutral soils were also studied.

To assess translocation of nitrogen from plant litter to soil by Enchytraeidae, pot grown *A. capillaris* was labelled with ^{15}N urea, and the leaf blade litter placed in nylon mesh litterbags at the Sourhope experimental site.

The specific objectives of the work are described in Chapter 2 – Field Experiments and Chapter 3 – The Litterbag Experiment.

CHAPTER 2

FIELD EXPERIMENTS ON POPULATIONS OF ENCHYTRAEIDS

2.1 Introduction.

The impact of nitrogen deposition sourced from anthropogenic activity was the theme of the GANE programme. In the context of GANE, this study examined the impact of nitrogen on Enchytraeidae which are considered to play an important role in the functioning of nitrogen-sensitive ecosytems (Huhta *et al.*, 1998). Acid soils at high altitude in the UK are considered to be sensitive to N inputs. It is the wet and occult deposition pathways that are of greatest concern at sites at high elevation (above 300m), particularly in the west and north of the UK (Pitcairn *et al*, 1995; NEGTAP, 2001). This is by the action of cloud droplet deposition and orographic enhancement (Hicks *et al*, 2000). These acid soils have little buffering capacity to acidic/acidifying inputs.

The concept of critical loads of acidity and N deposition have been revised during the past 10 – 15 years as more research information becomes available. In the updated report 'The Status of UK Critical Loads 2004' (CEH Edinburgh) exceedance maps for acidity and nutrient nitrogen clearly demonstrate that upland areas of the UK exceed the 5^{th} percentile for both. Areas such as the Pennines, North and South Wales, North Yorkshire, SW England, Cumbria, South West Scotland and North East Scotland exceed critical loads of nutrient nitrogen by up to 28 kg N ha^{-1} yr^{-1}, and acidity by > 2 keq ha^{-1} yr^{-1}. All of these areas are National Parks.

Ecosystems that are N sensitive may be affected in two ways. Nitrogen in excess of biological requirements can cause soil acidification if it is transformed to nitrate. Over

the course of years or a few decades biotic uptake of nitrogen by microbes and plants may prevent soil acidification but ultimately nitrate leaching in response to continued N inputs (regardless of the form of the inputs) will cause acidification (NEGTAP, 2001). Nitrate is a highly mobile anion in soils and attracts cations, and when nitrate is leached the cations are also leached. This can result in shortages of plant nutrients such as potassium, calcium and magnesium.

Excess N also provides a supply of nutrients, which, in nutrient poor acid soils, can influence plant growth and community composition. The link between increased plant uptake of deposited N and acidification of soil is dependent on whether plant biomass is harvested or not. Removal of biomass also removes the base cations that the plants have taken up from the soil, and into which they exude protons via roots, an acidifying process. Natural die-back returns the base cations to the soil, thus re-dressing the balance (NEGTAP, 2001).

Caporn *et al.*, (1994), Leith *et al.*, (1999), Pitcairn *et al.*, (1995) have shown that wet N deposition results in increased foliar N concentration, increased plant biomass and changes in species composition. Plant response to both biotic and abiotic stresses such as frost, drought and insect infestation were affected adversely. Leith *et al.*, (2001) found a strong dose-response relationship in foliar N content in ombrotrophic bog vegetation. Hicks *et al* (2000) examined the foliar nitrogen concentrations of upland vegetation to try to predict atmospheric nitrogen deposition, and these authors found that there had been increases over time in the foliar N content of typical upland plants such as heather (*Calluna vulgaris*), grasses (*Nardus stricta, Deschampsia flexuosa*) and mosses (e.g. *Hylocomium splendens*), the latter being particularly sensitive as

they are ectohydric. If the increase in foliar nitrogen in areas of high deposition is a general phenomenon, this will impact on the quality of litter available for soil organisms.

In addition to the responses described, the competitive balance between plant species may be disrupted. For example, high nitrogen deposition has been implicated in the conversion of heathland to grassland in the Netherlands (Hicks *et al*, 2000). Other affected plant communities include forest ground flora, calcareous grasslands, wetlands, upland moorlands and coastal dunes (Pitcairn *et al*, 1995). Long-term studies of grasslands with N additions have shown that N-responsive plants dominate with a concomitant reduction in species richness (Silvertown, 1980).

In the Netherlands, nitrogen deposition of 85 kg ha^{-1} yr^{-1} has had serious implications for nutrient-poor lowland heaths. Much of the *Calluna vulgaris* (heather) has been replaced by the nitrogen demanding grasses *Brachypodium pinnatum* and *Molinia caeruelea* (Bobbink et al 1988, Bobbink and Roelofs, 1996).

The direct and indirect effects of N deposition on the plant community may also have implications for the community structure and activity of soil microbes and animals. Changes in litter quality and increased N availability in soil is thought to increase initial stages of decomposition by microbes but delay later stages. Berg and Matzner (1998) suggest that this is due to suppressed lignolytic enzymes in white rot fungus which, coupled with the formation of stable compounds via the reactions of nitrate and ammonium with phenolic and lignin components, results in an overall increase in soil N accumulation with N deposition.

Decreased microbial activity in response to N additions has been reported (Kennedy *et al.*, 2004) as well as changes in microbial community structure (Ledgard *et al.*, 1998; Bardgett and McAllister, 1999; Innes *et al.*, 2004) and an increase in bacterial decomposition (Bardgett *et al.*, 1999). More available N may have detrimental effects on nitrogen fixers, particularly symbionts. In forests in the Netherlands, the occurrence of endomycchorizal fungi has declined and this has been attributed to atmospheric nitrogen pollution and/or soil acidification (Arnolds, 1991).

Soil animals also influence microbial activity, and consequently the process of decomposition and nutrient release and nutrient availability to plants. Soil food webs are complex with many trophic levels and feeding guilds (Brussaard, 1998) and the contribution of individual species is difficult to quantify. Edwards and Heath (1963) demonstrated that earthworms were responsible for removing 92% of oak litter material in a year. Exclusion studies have shown synergistic effects on soil processes when a diversity of species are included as opposed to single species (Mikola and Setälä, 1998; Bardgett and Chan 1999). On the other hand, there is some evidence that there is much functional redundancy in soil food webs (Liiri *et al.*, 2002) and that single keystone species exert the greatest influence on increased nutrient mineralisation and plant nutrient uptake e.g. Laasko and Setälä, 1999; Cragg and Bardgett, 2001; Wardle, 1999. Soil animals influence microbial biomass and activity by their grazing activity. Microbial response is regulated by both the community composition of the soil animals and their density (Hedlund and Augustsson, 1995; Bardgett *et al.*, 1993).

The low pH of many upland and plantation forest sites excludes much of the soil fauna found on more neutral soils. Earthworms do not tolerate these acid conditions (Satchell, 1955) but some species of enchytraeid worms (pot worms – also members of the Annelida) thrive in acid soils. Several species of earthworms in experiments avoided low pH (Satchell, 1955) and Standen (1984) found a negative relationship between earthworm and enchytraeid abundance and biomass in a range of different soil types. This was explained by the positive relationship between earthworms and soil pH, and the negative relationship between enchytraeids and soil pH although competitive interactions betweens the two groups cannot be ruled out.

In acid soils the dominant enchytraeid species is *Cognettia sphagnetorum*, which at one site (Moor House National Nature Reserve) accounted for 90% of enchytraeid biomass (Standen, 1973) and is often the major faunal component in such soils (Abrahamsen and Thompson, 1979). This species reproduces by fragmentation, which Standen (1973) suggested is an adaptation to the poor nutrient quality of the food available to them. The optimal pH for *C. sphagnetorum* was found to be between 3.6.and 3.8 in a blanket peat (Standen and Latter, 1977) with an upper limit of 5.6 (Healey, 1980; Yesmin *et al.*, 1995).

Enchytraeids have been described as litter transformers involved in the comminution and inoculation by micro-organisms of litter (Standen, 1978, Brussaard, 1998). Standen (1984) demonstrated that different species burrow to different depths in the soil in a similar manner to earthworms. Didden (1993) also argued that enchytraeids influence the structure of soils by faecal deposition, burrowing, and transportation of soil particles within the soil horizons in which they are active. Burrowing by

enchytraeids may also increase soil porosity and drainage and ultimately nutrient release. As well as burrowing activity, enchytraeids are able to move through the soil profile in response to environmental factors (Springett *et al*, 1970). The vertical distribution of *C. sphagnetorum* was examined in a study by Briones *et al* (1997), who showed the downward vertical migration of the species during dry periods when air temperatures were higher.

It is difficult to establish whether soil animals are primary decomposers *per se*. They may consume and digest only plant material, consume partly decomposed plant material but digest only the breakdown products due to micro-organism activity, digest micro-organisms directly, utilise microbial enzymes to enable them to digest complex organic material, or a combination of these.

Research by Latter and Howson (1978) using different food substrates including different plant species of different ages, fungi and bacteria suggests that *C. sphagnetorum* is a primary decomposer. Other authors have established that some enchytraeid species feed directly on fungi (Springett and Latter, 1977; Dash and Cragg, 1972). Kristufek *et al* (1995) found that the species *Enchytraeus cryticus* showed preference and avoidance strategies to various *Streptomyces* bacterial species and in laboratory studies some micro-organisms appeared to be toxic to some enchytraeid species (Dash and Cragg, 1972; Latter and Howson, 1978).

Despite the difficulties encountered in demonstrating the precise food source, many recent studies have highlighted the importance of *C. sphagnetorum* in the release of

nutrients in acid soils (Setälä *et al.*, 1991a,b, 1996; Setälä. 1995; Briones *et al.*, 1998a,b; Briones *et al.*, 2004; Cole *et al.*, 2000, 2002a,b; Liiri *et al.*, 2001, 2002).

The impact of nitrogen on enchytraeids (especially *C. sphagnetorum*) has been studied particularly in boreal forest regions in acidic, forest soils. Generally, applications of N fertilisers initially depress abundance regardless of the type of fertiliser (Lohm, 1977; Abrahamsen and Thompson, 1979; Huhta, 1984) but in some cases a subsequent explosive increase in abundance.

The aim of the field experiments was to assess the impact of N on populations of enchytraeids. The specific objectives were

1. to study seasonal population changes of *C. sphagnetorum* in acid soils receiving N fertiliser. This aspect was supplemented by a comparison of current populations at a site receiving long term increases in atmospheric nitrogen with that described 35 years earlier;
2. to compare the changes in populations of *C. sphagnetorum* in acid soils with other enchytraeid species in neutral soils;
3. relate the observed differences to direct or indirect effects of N deposition.

In addition to field work, a laboratory microcosm experiment was used to investigate the possible effects of raised % tissue N on enchytraeid populations under controlled conditions.

Hypothesis 1: enchytraeids will respond favourably to increases in plant tissue nitrogen in litter with an increase in abundance in N treated plots in comparison to controls in both acid and neutral soils.

Hypothesis 2: the cause of increased population, if found, will be due to increases in plant tissue N in litter.

2.2 METHODS.

2.2.1 Study sites.

Sourhope experimental field site, Kirk Yetholm, Scottish Borders, UK.

The site lies at grid reference NT 854196 and is characterised as a temperate upland grassland, series SH (Soil Survey of Scotland). The soil is a shallow, organic rich, acid brown forest soil on a bedrock of glacial till derived from andesitic lava.

It is an upland site (213 – 605 m above sea level) of rough grazing vegetation, *Agrostis/Festuca/Pteridium* (NVC U4) (Rodwell, 1991). Soil pH in control plots ranged from 4.0 to 4.5. Soil depth varied between 10 – 50 cm.

Prior to treatment, the Rigg Foot Experimental Site at Sourhope was classified as NVC community U4d consisting of *Festuca ovina (L) -Agrostis capillaris (L)-Galium saxatile* grassland with *Luzula multiflora-Rhytidiadelphus loreus* subcommunity. The site consists of 5 replicate blocks in downslope environmental gradients. Each block contains 6 main plots with treatments allocated at random. The site was set up as part of the Soil Biodiversity Programme in 1998 when a series of baseline measurements including soil and vegetation % tissue N, soil moisture, pH, plant biomass and weather data were made.

Ammonium nitrate (as Nitram) was applied annually in April and May (240 kg N ha^{-1} yr^{-1}) and lime was applied annually in April (6000 kg ha^{-1} yr^{-1}) and treatment began in 1999. The site had been grazed until 1998 when a stock proof fence was erected and

subsequently the vegetation is cut monthly (May to September) to approximately 6cm and the clippings removed. Appendix 1 shows the site layout.

Plate 1. Rigg Foot experimental site, Sourhope.

Ruabon heather moorland, Denbighshire, North Wales.

The study site was situated on an upland moor (470 m asl) near Ruabon (Grid reference: SJ 225489).The soil was peat formed over an iron pan stagnopodzol (Hiraethog series) overlying lower Palaeozoic sediments. Overall soil depth was up to 50 cm.

Vegetation cover consisted almost exclusively of *Calluna vulgaris* (L.) (British National Vegetation Classification of H12; *C. vulgaris–Vaccinium myrtillus* heath (Rodwell, 1991)). Soil pH ranged from 3.9 to 4.5.

The 2 x 2 experimental plots were set up by Dr Simon Caporn of Manchester Metropolitan University in 1998 as part of an extensive study of the effects of N deposition on vegetation and N transformations in the soil. Treatments included N alone, or N with P at different concentrations. Ammonium nitrate was watered onto the plots monthly (x8) to give N plots receiving 20, 40 and 120 kg N ha^{-1} yr^{-1} (described as low, medium and high N treatments). Four replicates of each treatment were arranged in a randomised grid design. Only N treatment plots were sampled in the present study.

Plate 2. Ruabon heather moorland.

Moor House National Nature Reserve, North Pennines, Cumbria, England.

Bog End (Grid reference NY 765329) had been sampled to study enchytraeids as part of the International Biological Project in 1968.

This is an area of blanket peat which is up to 3 m in depth. The dominant plant is *C. vulgaris*, with *Eriophorum vaginatum* (L.), *E. augustifolium* (L.), *Vaccinium myrtillus*, *Emperetum nigrum* (L.), *Rubus chamaemorus* (L.), *Sphagnum* spp and *Cladonia* spp. as associated species (NVC M19, Rodwell, 1991).

The soil pH at the Moor House-Upper Teesdale NNR ranges from 3.5 to 7.5 and in gley and podsol soils pH has increased in the O layer but decreased in lower layers during the past 20 – 30 years (NEGTAP, 2001). This area of the North Pennines is estimated to receive ~ 25 kg N ha^{-1} yr^{-1} via wet reduced deposition.

Plate 3. Moor House National Nature Reserve – Bog End.

2.2.2 Sampling procedure.

A soil corer of approximately 5cm diameter was used to take soil samples. The diameter of the corer resulted in an area of 0.001m^2, and abundances given as 'per core' can be simply multiplied by 1000 to give numbers m^{-2}. Soil samples were a minimum of 5 cm depth, and if deeper than this, the excess was removed. The cores were placed in individual labelled polythene bags and kept upright in a cool box until they were transported back to the laboratory. They were then stored at 4°C until extraction.

At Ruabon, replicate cores were taken from each plot. However, due to the small plot size and the fact that other research being carried out at the site, the decision was taken to sample at the outer edge of the plots in order to minimise damage to the central part.

At Sourhope, two cores were taken from each allocated sub-plot sampled. The protocol here was to take cores from the border area of the subplot to replace the plot core. The border core holes were filled with sand.

At Moor House, 15 cores were taken from the Bog End site. Following the 1968 study, the position of each sampling unit was determined randomly, by tossing the corer within the selected area.

2.2.3 Extracting and handling the enchytraeids.

Enchytraeids were extracted using the wet funnel technique (O'Connor, 1955). Enchytraeids from each soil core were put into Petri dishes in a small amount of water and kept at 3 - 5°C until identified.

Identification of enchytraeid worms relies on the use of high-power microscopy in order to identify external (segment and setae numbers, etc) and internal (spermatheca, sperm funnels, etc) morphological features. Eggs within the body can be seen by eye or with a hand lens, indicating a mature individual. Worms without eggs may be immature or a fragmenting species, the most common of these being *C. sphagnetorum* which is found in high densities in acid soils. Identification was according to Nielsen and Christensen (1959).

C. sphagnetorum reproduces by fission, one large individual producing two or three smaller individuals. A worm of small size therefore indicates that it is a fragment or a recently regenerated worm. It was thought that the ratio of large to small worms would give an indication of the reproductive activity of this species and the worms extracted from the soil cores were assessed for size.

The large *C. sphagnetorum* extracted from Moor House were examined under low power microscopy (x20 - 60) and segments counted. The small worms left were examined under high power microscopy (x100 - 400) to assess whether it was a fragment, and if so, whether it was a head, tail or middle fragment. At Ruabon only the ratio of large to small worms was recorded.

Soil moisture and organic content of soil.

Soil cores were weighed prior to enchytraeid extraction, and following extraction, oven dried to constant weight to assess moisture content.

Cores were then placed in a muffle furnace at 600 °C for 30 minutes, cooled and immediately weighed. Any stones present were also weighed. These data were used used to calculate the loss on ignition and therefore the organic content of the soil cores.

Laboratory microcosm experiment.

Heather litter from the field experimental plots at Ruabon had been shown to have significantly different nitrogen content (M. Pilkington, pers. comm.). Permission was given by Dr S. Caporn to collect litter from the control and high nitrogen (120kg N ha^{-1} yr^{-1}) plots. The litter collected was analysed for nitrogen – method as for stable isotope analysis, Chapter 3.

Plastic pots (4cm diameter) had 50g play sand added. A circular piece of litterbag material (200 microns) was placed on top of the sand. The sand was moistened with 20mls of tap water and excess water was removed using a plastic pipette.

The air-dried heather litter was broken into smaller pieces using a pestle and mortar and weighed to give 1g portions. These were moistened with tap water and placed on top of the litterbag material. The quantity of water varied as the absorption varied between samples. Twenty microcosms contained control litter and twenty contained high nitrogen litter.

Enchytraeids (*C. sphagnetorum*) were extracted from soil cores collected from control plots at Ruabon. The worms were identified to species level using high power microscopy. On 21st August 2003 each microcosm received 20 enchytraeid worms. These were medium to large sized worms.

The microcosms were placed randomly on a tray, covered with black plastic and kept in a controlled temperature room at 15°C. The pots were removed each week, and tap water added at a rate of 2 – 4 drops from a 3ml plastic pipette.

Movement of litter/faecal material into the sand (below the litterbag layer) was observed in some pots after 6 weeks. By 10 weeks, this was observed in all microcosms. In order to demonstrate that the movement was caused by enchytraeids and not wash-down from water addition, more identical microcosms were created. Half of the microcosms had worms added and half had no worms added. They were subjected to the same treatment regime as the main experiment.

Destructive sampling of the microcosms began in June 2004. The litter layer was lifted out on the litterbag gauze, placed in a Petri dish and water added. The sand layer was emptied onto a Petri dish and water added. Enchytraeids were removed by hand sorting with the aid of a low power microscope. The number of worms in each pot was recorded, and the number of segments of each worm noted. Individuals that had fragmented were recorded as a head, middle or tail segment. This process took about one hour for each pot. Sand and litter were dried, and the litter weighed. It was hoped that the material deposited in the sand could be removed and weighed. However, a suitable method was not found.

2.3 Results

2.3.1 Changes to soil physical factors brought about by treatments.

Changes in soil pH at Sourhope.

In August 1998 the pH of the soil was 4.6 (Burt-Smith, 2001). Subsequently the application of different treatments onto the plots at Rigg Foot changed the soil pH. There was also a certain amount of change in soil pH of the control plots with a decline in 2002. The changes in soil pH are shown in Figure 2.1.

Application of ammonium nitrate alone at 240 kg ha^{-1} yr^{-1} resulted in an increase in soil pH to a maximum of 5.5. Lime application at 6000 kg ha^{-1} yr^{-1}, increased the soil pH to almost 7.0 by March 2003 with lime combined with nitrogen having slightly (0.2 units) higher pH than lime only treatments.

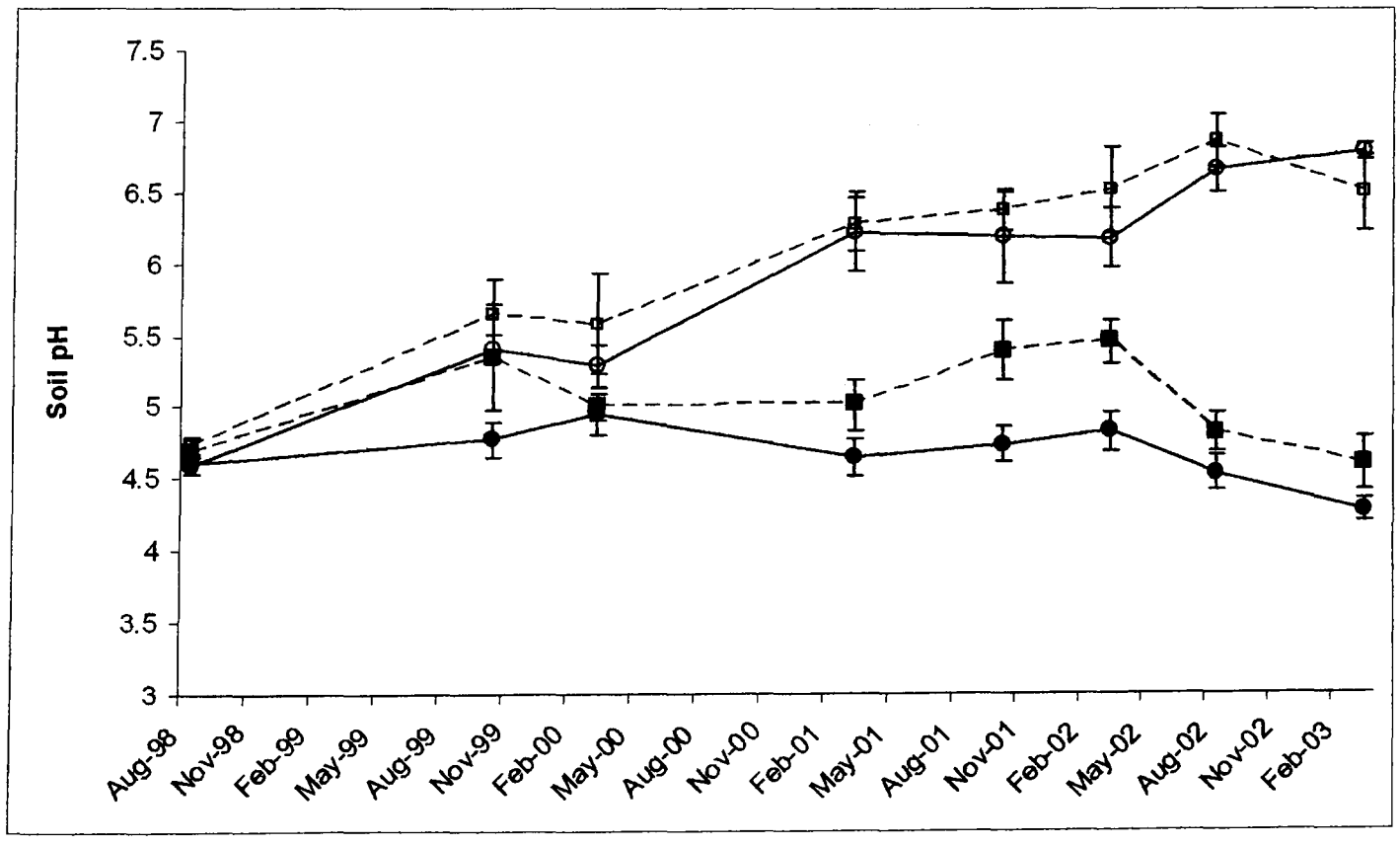


Figure 2.1. Changes in mean soil pH (± 1SE) in control (●), nitrogen (■), lime (○) and L+N (□) plots at Rigg Foot, Sourhope between August 1998 and March 2003.

There was no significant difference between pH when comparing lime and L+N plots, but control plots had significantly lower pH than nitrogen treated plots (Table 2.1).

Table 2.1. One-way ANOVA comparing changes in pH in control and nitrogen, and lime and L+N plots at Rigg Foot, Sourhope between August 1998 and March 2003.

	df	Mean Square	F	Sig.
Control and nitrogen	1,78	2.79	16.03	0.0001
Lime and L+N	1,78	0.43	0.655	0.4207

Effects of pH change on Enchytraeidae.

Increases in pH in limed plots led to the disappearance of the previously dominant enchytraeid *C. sphagnetorum* and the appearance of a more diverse community of enchytraeids. Many of these were larger worms, and the population was dominated by a number of species of the genus *Fridericia* and one species of *Henlea.* This was an effect that had been well documented (Standen, 1984, Hågvar and Abrahamsen,1980).

Although diversity is increased, the abundance of each species is low. However, total enchytraeid biomass may equal that of *C. sphagnetorum* due to the larger size of many of the *Fridericia* species even though some species are smaller.

Of the species found, only *C. sphagnetorum* reproduces by fragmentation; the others reproduce sexually and lay cocoons in the soil.

Many of the species found in limed soils live for only one season with the exception of some of the larger worms such as *F. galba, bisetosa* and *magna* which may live up to two years. It is difficult to assess such life strategies for *C. sphagnetorum*; one worm may divide into two or three fragments, growing a new head, tail or both, with

each new worm carrying the same genetic material. The only genetic difference will be that which occurs by mutation.

C. sphagnetorum has been extensively studied, particularly in the boreal forest ecotype in Scandinavia since the late 1960's. The soils are acidic and *C. sphagnetorum* is the dominant enchytraeid. The studies were in response to acid deposition, although the role of nitrogen was also examined. Other enchytraeids have not been so extensively studied; in fact it is difficult to find much literature referring to them, particularly aspects of their functional and feeding ecology.

There difference between the populations in soils with low and high pH is such that it is difficult to make comparisons between the two. For this project it was decided that the acid soils (control and nitrogen), dominated by *C. sphagnetorum*, would be studied in relative isolation to the neutral soils (lime and L+N treatments) but comparisons made wherever possible.

Changes to litter tissue nitrogen.

Ruabon.

Litter collected from the control and high N treatment plots at Ruabon were analysed for %N content which was 1.23 (± 0.015) from control plots and 1.82 (± 0.036) from high N treatment plots (n = 3). The litter from control plots contained significantly less N than that from the high N plots (T test assuming unequal variances, df 3, t statistic 14.88, $p > 0.001$).

Sourhope.

Vegetation clippings were collected from each plot, air dried and analysed for % tissue N in 1998 (prior to the start of treatments at Rigg Foot) and again in 2003. Table 2.2 shows the results of the analysis.

Table 2.2. % tissue N of grass clippings taken from the treatment plots at Rigg Foot, Sourhope in 1998 and 2003.

1999				
	Control	Nitrogen	Lime	L+N
Block1	2.307	2.639	2.578	2.534
Block2	2.257	2.473	2.585	2.641
Block3	2.403	2.648	2.160	2.705
Block4	2.294	2.213	2.047	2.134
Block5	2.581	2.630	2.623	2.489
Mean	**2.36**	**2.52**	**2.39**	**2.50**
Standard Error	0.058	0.083	0.122	0.099
2003				
Block1	1.39	2.16	1.91	1.8
Block2	1.75	1.94	1.74	2.3
Block3	1.89	2.47	1.72	2.05
Block4	2.05	2.03	1.85	1.95
Block5	1.69	2.12	1.68	1.91
Mean	**1.75**	**2.14**	**1.78**	**2.00**
Standard Error	0.110	0.089	0.043	0.084

There were no significant differences between plots in 1998, but in 2003 % tissue N of clippings was lower in control and lime plots compared to nitrogen and L+N plots. One way ANOVA showed that some of the differences were significant ($df_{3,16}$, $F = 4.75$, $p > 0.02$) and Tukey *post hoc* analysis showed that the differences were significant between nitrogen and both control and lime.

Comparing the same treatments between 1998 and 2003 with One-way ANOVA showed that in all plots % tissue N was lower in 2003 compared to 1998 (Table 2.3).

Table 2.3. One-way ANOVA results comparing % tissue N in 1998 with that of 2003 of clippings removed from the treatments at Sourhope.

		Sum of Squares	df	Mean Square	F	Sig.
Control	Between Groups	0.944	1	0.943	24.29	0.001
	Within Groups	0.311	8	0.039		
	Total	1.255	9			
Nitrogen	Between Groups	0.355	1	0.355	9.43	0.015
	Within Groups	0.300	8	0.037		
	Total	0.655	9			
Lime	Between Groups	0.957	1	0.957	22.86	0.001
	Within Groups	0.334	8	0.042		
	Total	1.291	9			
L+N	Between Groups	0.621	1	0.621	14.61	0.005
	Within Groups	0.340	8	0.042		
	Total	0.961	9			

Changes to soil moisture.

Ruabon.

Generally, soils at Ruabon remained saturated but there was substantial drying during July and August. Mean annual rainfall was 1010mm and mean monthly soil temperature at 10cm was 6.3°C in 1998/99 (Pilkington *et al.*, 2005). In July and September soil cores taken from the site were assessed for soil moisture. Figure 2.2 shows the results.

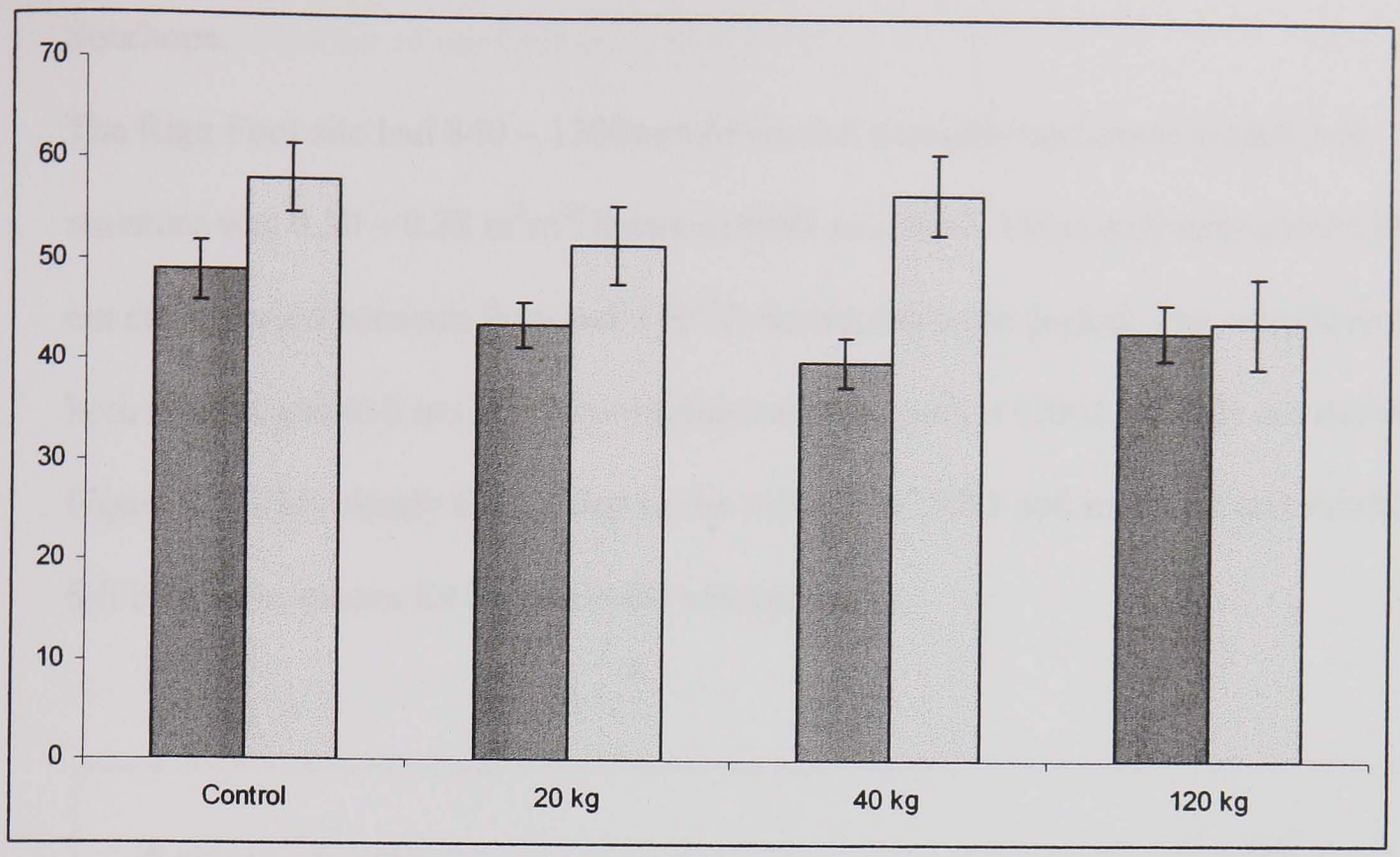


Figure 2.2. Mean soil moisture content of soil cores collected from Ruabon in July (dark shaded bars) and September (light shaded bars) 2002 ± 1SE. (n=12) 2002.

Soil moisture was lower in July than in September and the difference was significant in control and moderate N treated plots (Table 2.4).

Table 2.4. One-way ANOVA result for comparison of soil moisture for July and September (2002) in individual treatment plots at Ruabon.

	df	Mean square	F	Significance
Control	1,22	514	4.17	0.05
Low N treatment	1,22	384	3.00	0.09
Moderate N treatment	1,22	1710	12.66	0.002
High N treatment	1,22	4.950	0.029	0.87

However univariate analysis of the data showed there were no significant differences for soil moisture between treatment plots in July or in September (Table 2.5.

Table 2.5. Univariate analysis of soil moisture between treatments in July and September 2002 at Ruabon.

	df	Mean square	F	Significance
July	3,44	161.07	1.88	0.15
September	3,44	485.11	2.51	0.07

Sourhope.

The Rigg Foot site had 840 – 1200mm of rainfall annually and mean annual soil moisture was 0.30 – 0.38 m^3m^{-3} between 1999 and 2002. Mean soil temperature at 10 cm depth varied between 7.66 and 8.65°C during the same period. The variations in both rainfall and soil moisture during the sampling period (2001 – 2003) are shown in Figure 2.3. This clearly shows that in the summer of 2003 soil moisture and rainfall fell below the means for the previous two summers.

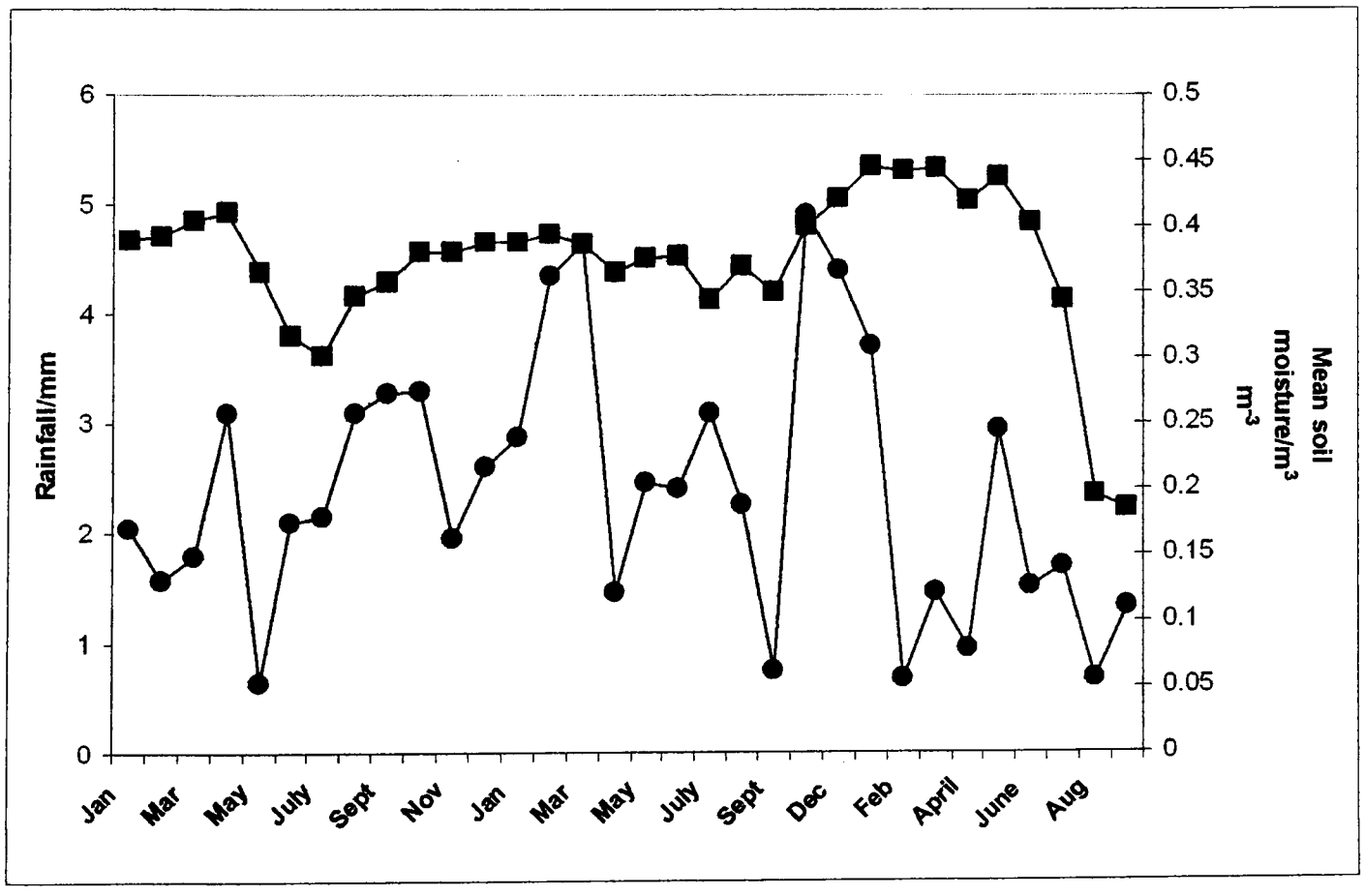


Figure 2.3. Mean monthly rainfall and soil moisture data for Rigg Foot, Sourhope January 2001 – September 2003.

2.3.2 The response of enchytraeid populations.

Moor House

i. *C. sphagnetorum* abundance in 2002.

The enchytraeid population at Moor House was predominantly *C. sphagnetorum*, with occasional *M. sanguineous* and *Cernosvitoviells briganta.* Only *C. sphagnetorum* were used for data analysis.

Figure 2.4 shows the mean numbers, and the ratio of large to small, worms per core extracted from April to September 2002.

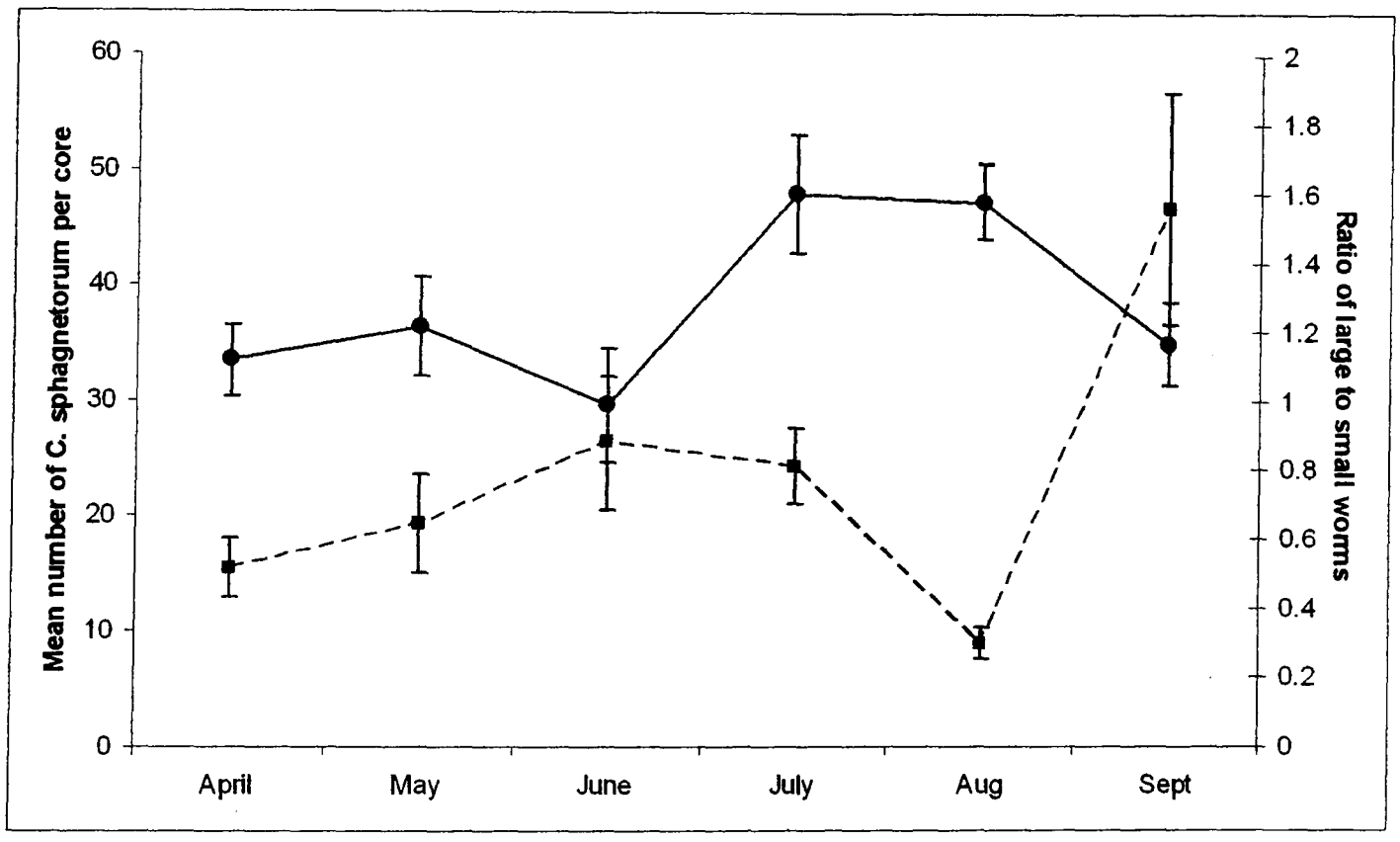


Figure 2.4. Mean number of total (●), and ratio of large to small (■), *C. sphagnetorum* per core at Moor House, April to September 2002. Bars = 1SE.

The mean number of *C. sphagnetorum* for the entire six month period was 38 ± 1.8. Abundance changed little for the first three months (mean numbers ~35) then increased in July and August to 50 per core before falling in September to the initial abundance. Abundance in June was significantly less than in July and August (One-

way ANOVA, $df_{5,84}$, F = 3.4, $p > 0.01$, *post hoc* Tukey test shown in Table 2.6) but all other values were not significantly different.

Table 2.6. *Post hoc* Tukey test results of One-way ANOVA for total abundance of *C. sphagnetorum* between April and September, 2002, Moor House.

Time		Mean difference	Significance
June	April	-3.87	0.986
	May	-6.8	0.853
	July	-18.4	0.027
	August	-17.8	0.036
	September	-5.4	0.939

The ratio of large to small worms was below 1:1 until September and thus the population was dominated by small worms (< 42 segments) until autumn. In September the population was dominated by large worms.

One-way ANOVA showed significance differences between ratios of large to small enchytraeids ($df_{5,84}$, F = 5.86, $p > 0.001$) and *post hoc* Tukey test showed that the ratio in September was greater than all other months (Table 2.7).

Table 2.7. *Post hoc* Tukey test results of One-way ANOVA for ratio of large to small *C. sphagnetorum* between April and September, 2002, Moor House.

Time		Mean difference	Significance
September	April	1.041	0.0012
	May	0.912	0.0067
	June	0.68	0.0878
	July	0.746	0.0456
	August	1.261	4.67×10^{-5}

Figure 2.5 shows the mean number of large and small *C. sphagnetorum* per core, and the number enchytraeids that were regenerating a head, tail or both. The latter excludes worms which had been damaged in the extraction process.

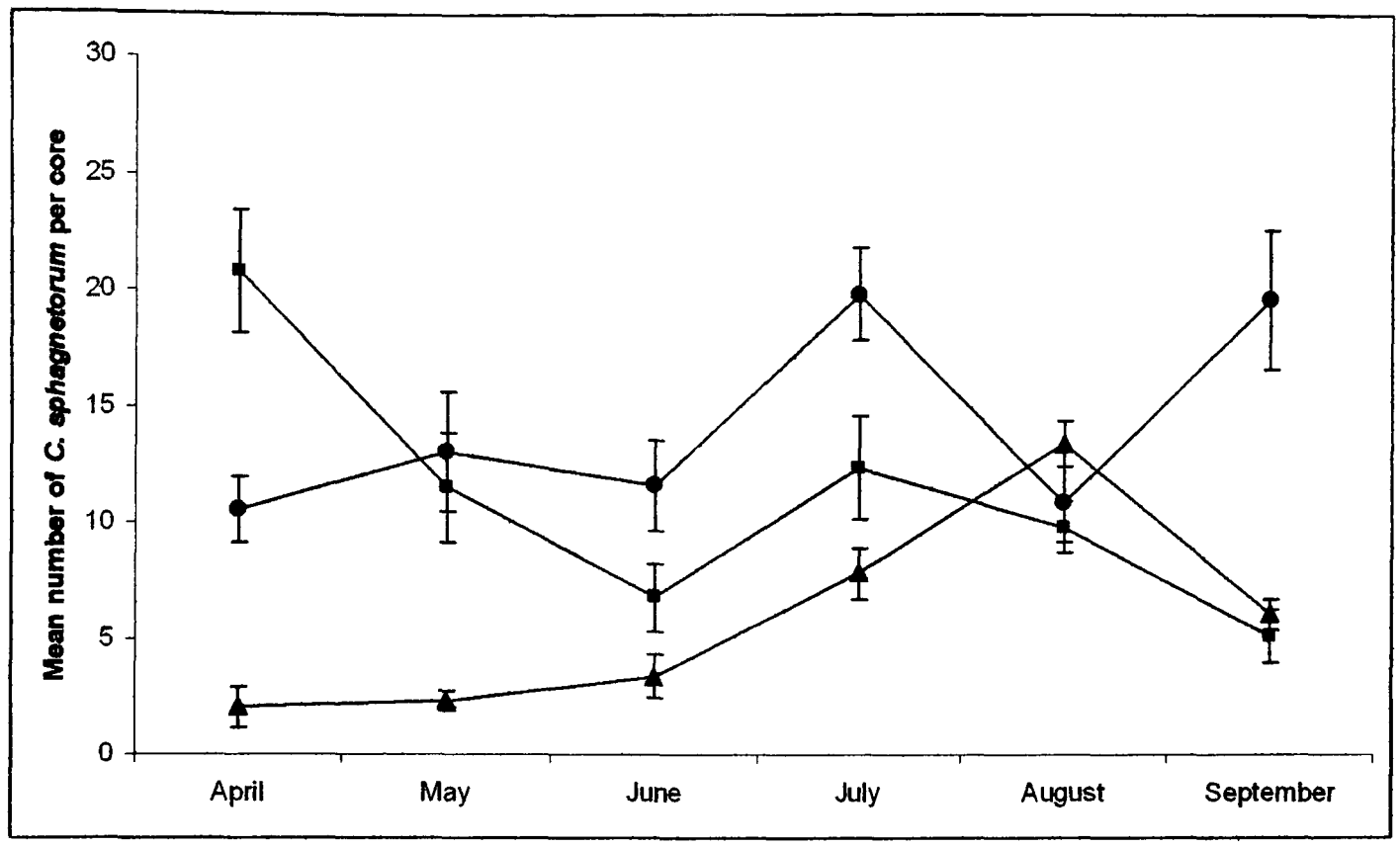


Figure 2.5. Mean numbers (± 1SE) of large (●) and small (■)and regenerating (▲)*C. sphagnetorum* per core at Moor House, April to September 2002.

Maximum numbers of large worms were found in July and September (~ 20 per core). The number of small worms increased between June and August, reaching a maximum of 20 per core and at the same time the number of regenerating worms increased to a maximum of 14 per core.

ii. C. sphagnetorum abundance in 1968.

Data collected for the abundance of *C. sphagnetorum* in 1968 as part of the International Biological Programme (Standen, 1973) were used to construct Figure 2.6. This shows the mean number of *C. sphagnetorum* per core and ratio of large to small worms between April and September 1968.

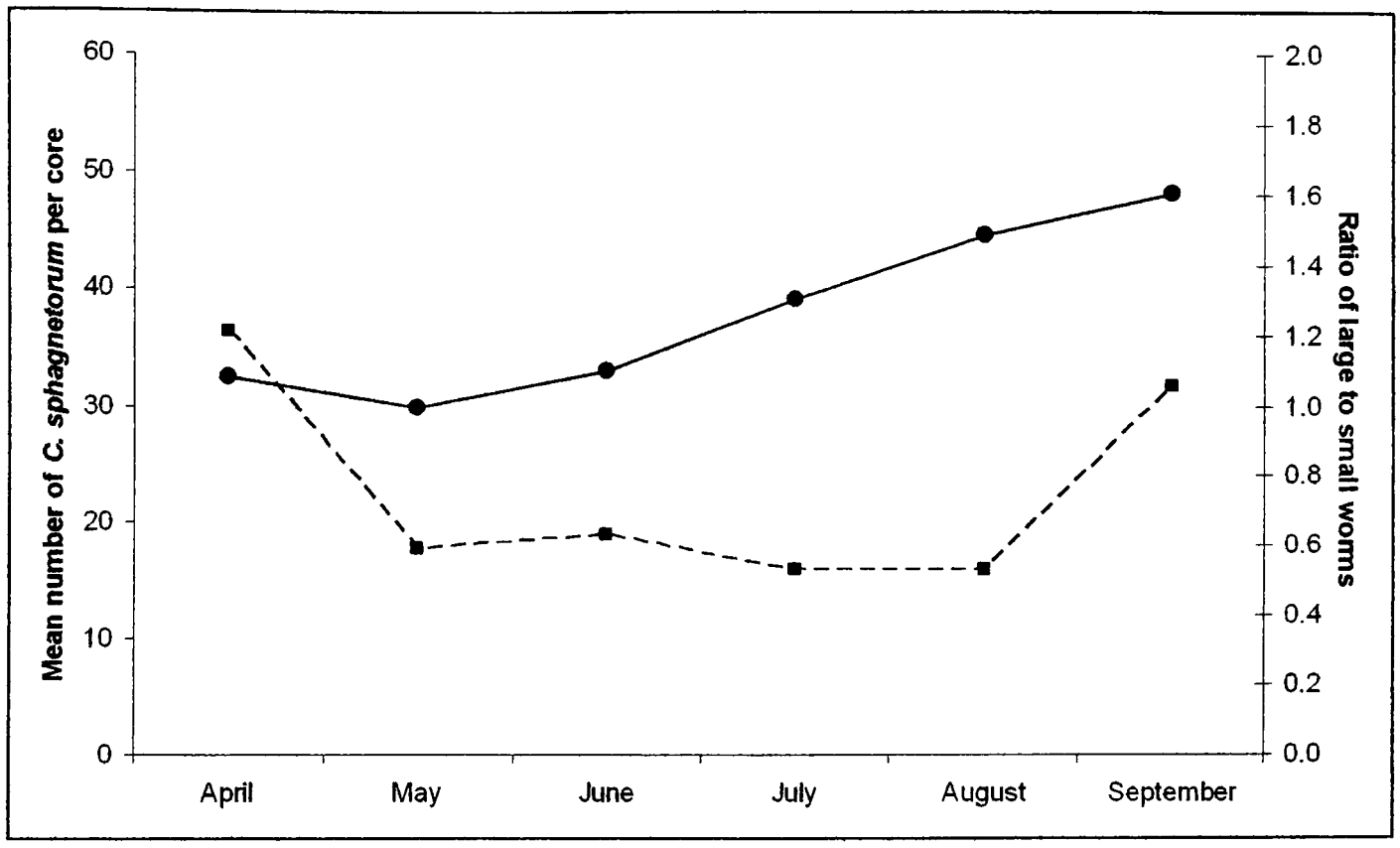


Figure 2.6. Mean number of total (●), and ratio of large to small (■), *C. sphagnetorum* per core at Moor House, April to September 1968.

Mean numbers of *C. sphagnetorum* were between 30 and 50 per core, and the population increased over time. The ratio of large to small worms > 1:1 in April 1968, fell to 0.6 between May and August and then increased in September, again to 1:1.

Figure 2.7 shows the mean number of large, small and regenerating worms. Abundance of small worms increased through the season from 10 to 25 per core, with a slight decrease in September. The abundance of large worms initially decreased, with 10 – 15 per core, and increasing in September to 25 per core. The number of regenerating fragments remained fairly steady at ~7 per core, peaking slightly in July and then dropping to <5. No SEs are included on the graphs as this information was not available.

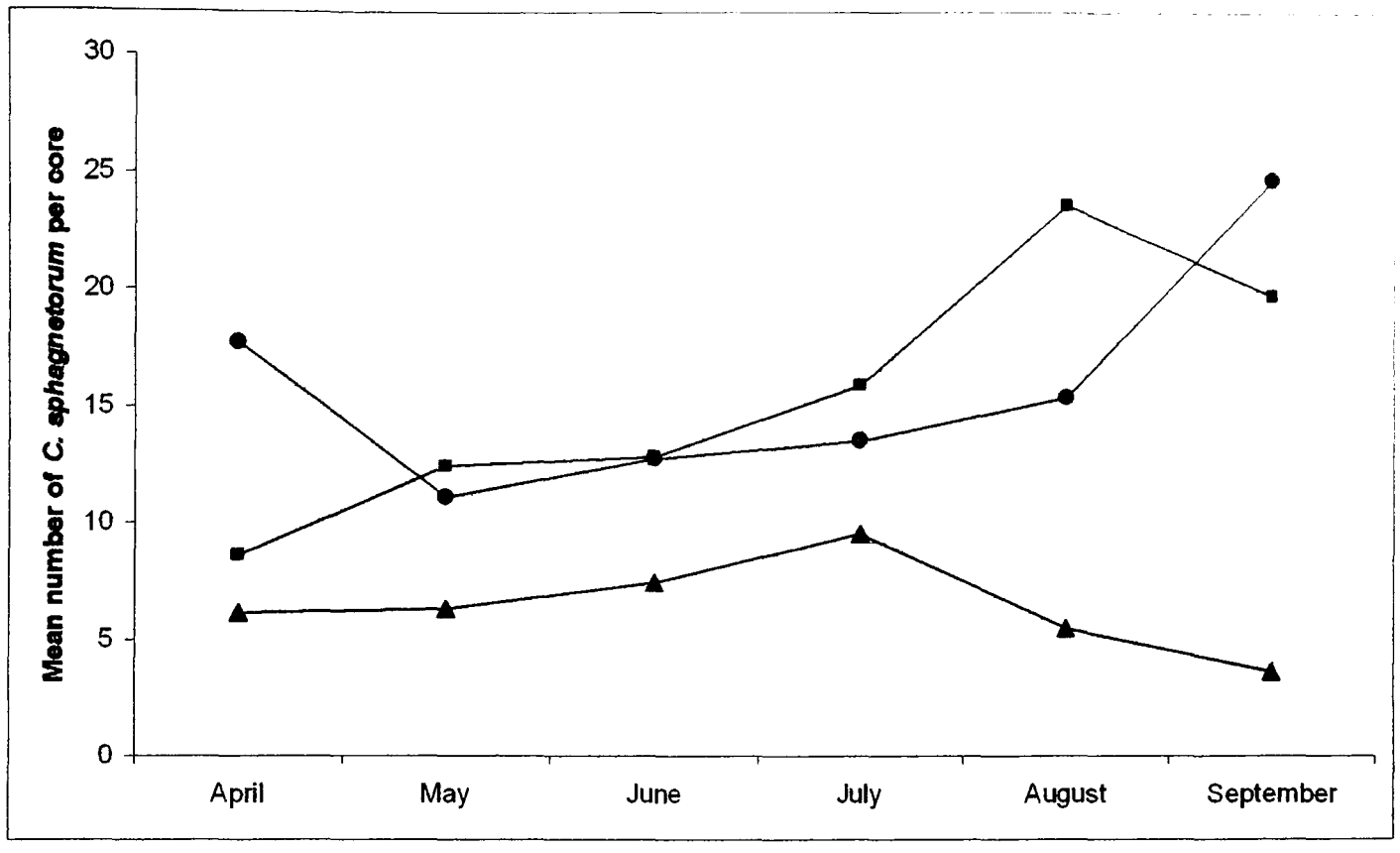


Figure 2.7. Mean numbers of large (●) and small (■) and regenerating (▲)*C. sphagnetorum* per core at Moor House, April to September 1968.

It was not possible to compare 1968 data with those obtained in 2002 as those from 1968 were mean figures with no measure of variance. However in both years numbers fluctuated between 30 and 50 per core.

Table 2.8 shows the mean monthly soil temperature at Moor House for 1968 and 2002. Mean soil temperature in 1968 was higher than that for 2002. These differences were not significantly different (One-way ANOVA). However, soil temperature was higher between May and August in 1968 whereas in 2002 it remained low until August

Table 2.8. Mean monthly soil temperatures at Moor House for 1968 and 2002. Note difference depth for temperature probes.

	1968 (-6cm)	2002 (-10 cm)
April	4.8	4.4
May	10.4	6.4
June	10.2	8.5
July	10.8	9.6
August	11.2	11.8
September	9.9	10.2
October	5.2	4.3
Mean	**8.93**	**7.89**
SE	1.02	1.10

Ruabon

C. sphagnetorum was the dominant species at Ruabon with *Mesenchytraeus sanguineous* present at very low density. Only *C. sphagnetorum* abundance was used for data analysis.

Figure 2.8 shows the population trends in the different treatment plots at Ruabon. In all plots there was a dramatic fall in the population between spring and summer 2003 followed by an increase between summer and autumn. The increase accounted for 70, 80, 112 and 81% of the spring population (in ascending order of treatment), with the population in 40 kg N ha^{-1} yr^{-1} treated plots recovering above the spring population level.

In control and the lowest treatment plots (20 kg N ha^{-1} yr^{-1}), the abundance of C. sphagnetorum remained relatively constant at 45 – 50 per core until the summer of 2003. In moderately treated plots (40 kg N ha^{-1} yr^{-1}) the population size was more variable but this was associated with large SE's. In the highest treated plots (120 kg N ha^{-1} yr^{-1}) the population rose from a mean of 20 individuals per core in autumn 2001 to 70 per core by autumn 2002. This increase was almost linear.

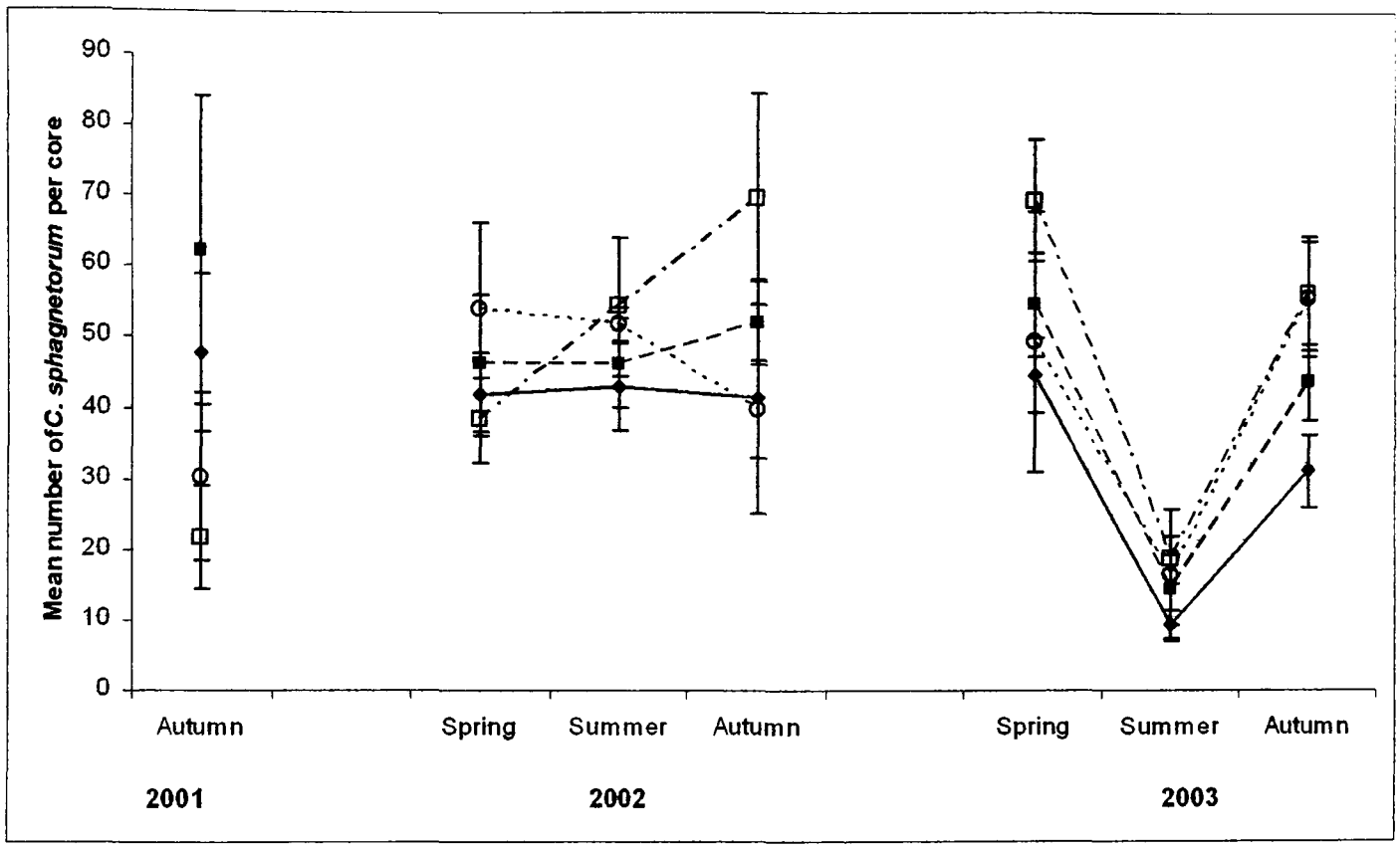


Figure 2.8. Mean numbers of *C. sphagnetorum* extracted from treatment plots at Ruabon ± 1 SE. (n=12).Control (●), 20 kg N ha^{-1} yr^{-1} (■), 40 kg N ha^{-1} yr^{-1} (○) and 120 kg N ha^{-1} yr^{-1} (□).

Univariate analysis for all treatments at each sampling time was performed on the enchytraeid abundance data. There were no significant differences between abundance in the different treatment plots in 2002 or in 2003.

The number of small (< 30 segments) and large (> 30 segments) enchytraeids was recorded and the ratio of large to small worms calculated. Ratio of > 1 means there were more large than small worms, and a ratio of < 1 indicates there were more small than large worms. A decrease in the ratio would mean either that the worms were fragmenting or that larger worms were selectively dying. An increase in the ratio would mean that small worms were growing into the large category or that small worms were selectively dying.

Figure 2.9 shows the ratios of large to small enchytraeids for all treatment types.

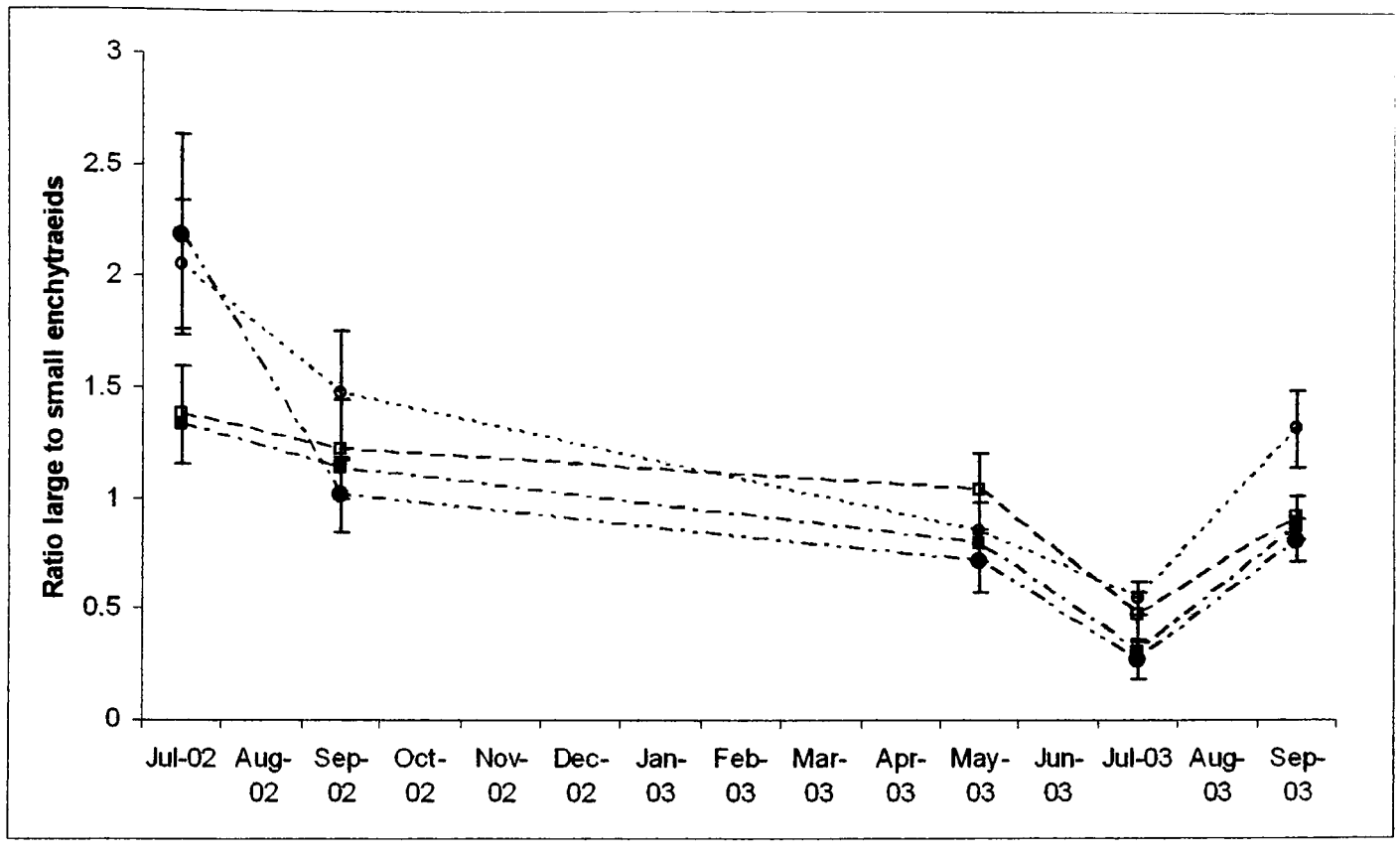


Figure 2.9. Mean ratio of large to small *C. sphagnetorum* in all treatment plots at Ruabon. Control (●), 20 kg N ha^{-1} yr^{-1} (■), 40 kg N ha^{-1} yr^{-1} (○) and 120 kg N ha^{-1} yr^{-1} (□). Means ± 1SE. (n=12).

There were striking changes in the ratio throughout the sampling period. In July 2002 the ratio was between 1.4 – 2.2 then declined in all treatment plots, remaining at a similar level over winter until May 2003. However there was no increase in the ratio in July 2003 as was expected due to growth of small individuals into larger ones, as again the ratio declined in all treatment plots. This coincided with the marked decline in the population referred to earlier. By September 2003 the ratio had risen to 1.0 – 1.5 and was greatest in the moderate N treated plots.

Univariate analysis for all treatments at each sampling time was performed on the ratios. Ratios were not significantly different except in September 2003 (ANOVA $df_{3,44}$, $F = 3.85$, $p = 0.016$). Tukey's post-hoc test is shown in Table 2.9. The ratio of large to small worms was significantly higher in moderate N treated plots compared to those in control and high N treated plots.

Table 2.9. Tukey's post-hoc test of multiple comparisons for the ratio of large to small enchytraeids in September 2003 at Ruabon.

Treatment (kg N ha^{-1} yr^{-1})		Mean difference	Significance
40	Control (0)	0.5067	0.02
	20	0.4225	0.07
	120	0.4467	0.05

Data were collected during 2002 to assess the depth distribution of *C. sphagnetorum*. Percentage abundance extracted from the top 3 – 4 cm of the soil core and the bottom 3 cm is shown in Figure 2.10. In all treatment plots percentage abundance of *C. sphagnetorum* in the lower layer of the soil cores increased as the year progressed. A higher proportion of enchytraeids were extracted from the upper layers in control plots than in N treated plots, and the lowest proportion from the upper layers were extracted from the highest N treated plots. Univariate analysis for all treatments at each sampling time was performed on the data but there were no significant differences between any of the treatment plots.

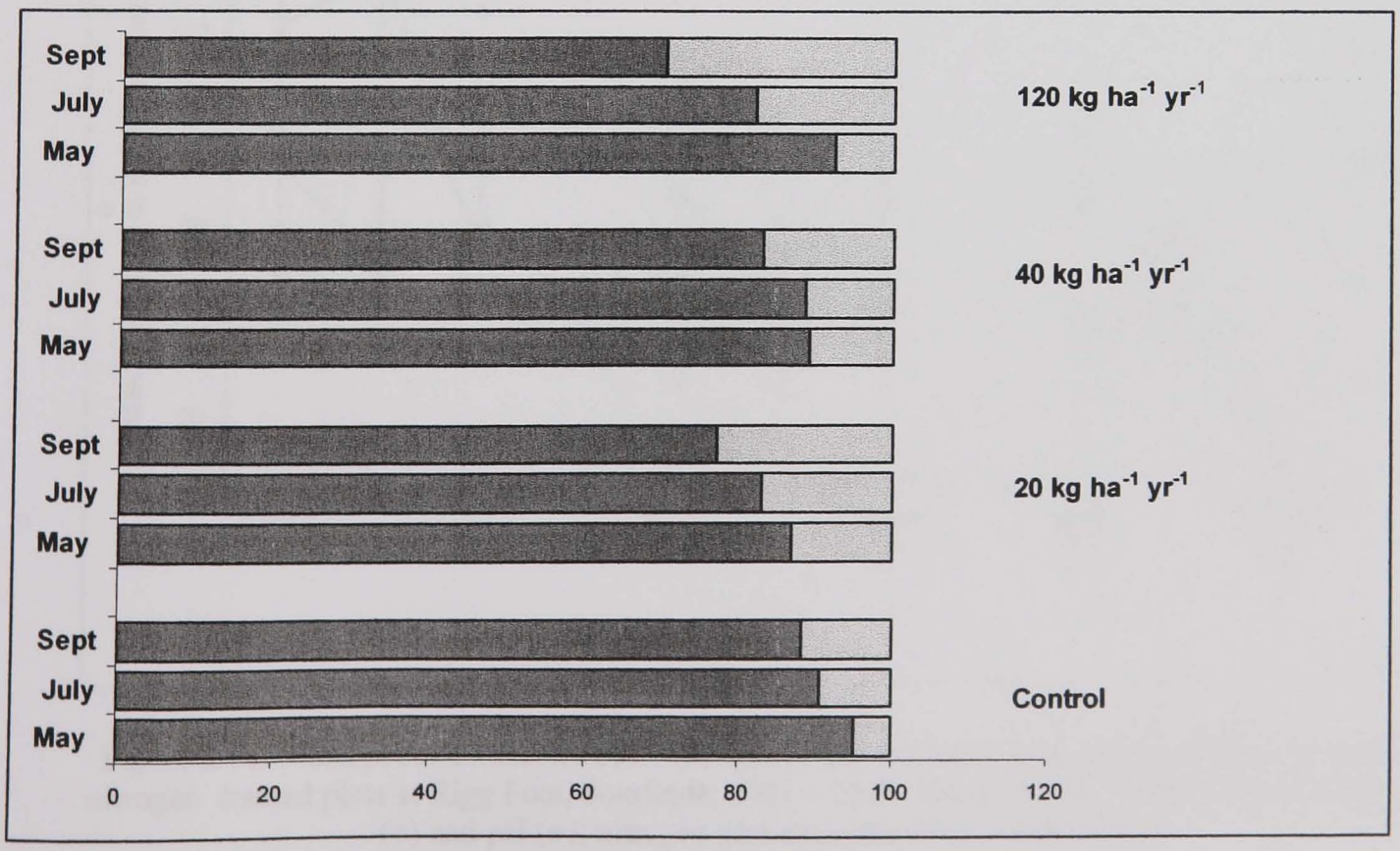


Figure 2.10. Mean % abundance of *C. sphagnetorum* in the top 3 – 4 cm (dark grey portion) and bottom 3 cm (light grey portion) of soil cores in the treatment plots at Ruabon in 2002. (n=12).

Sourhope

i. Control and nitrogen plots – abundance of *C. sphagnetorum*

There was a great amount of variability in the abundance of *C. sphagnetorum* extracted from individual soil cores resulting in large SEs for the means of the five replicate plots. A Kolmogorov-Smirnov test of goodness-of-fit provided no evidence against the null hypothesis that the sample had been drawn from a normal population therefore the data were not log transformed.

Figure 2.11 shows the abundance of *C. sphagnetorum* extracted from soil cores in control and nitrogen treated plots and the soil pH of each plot.

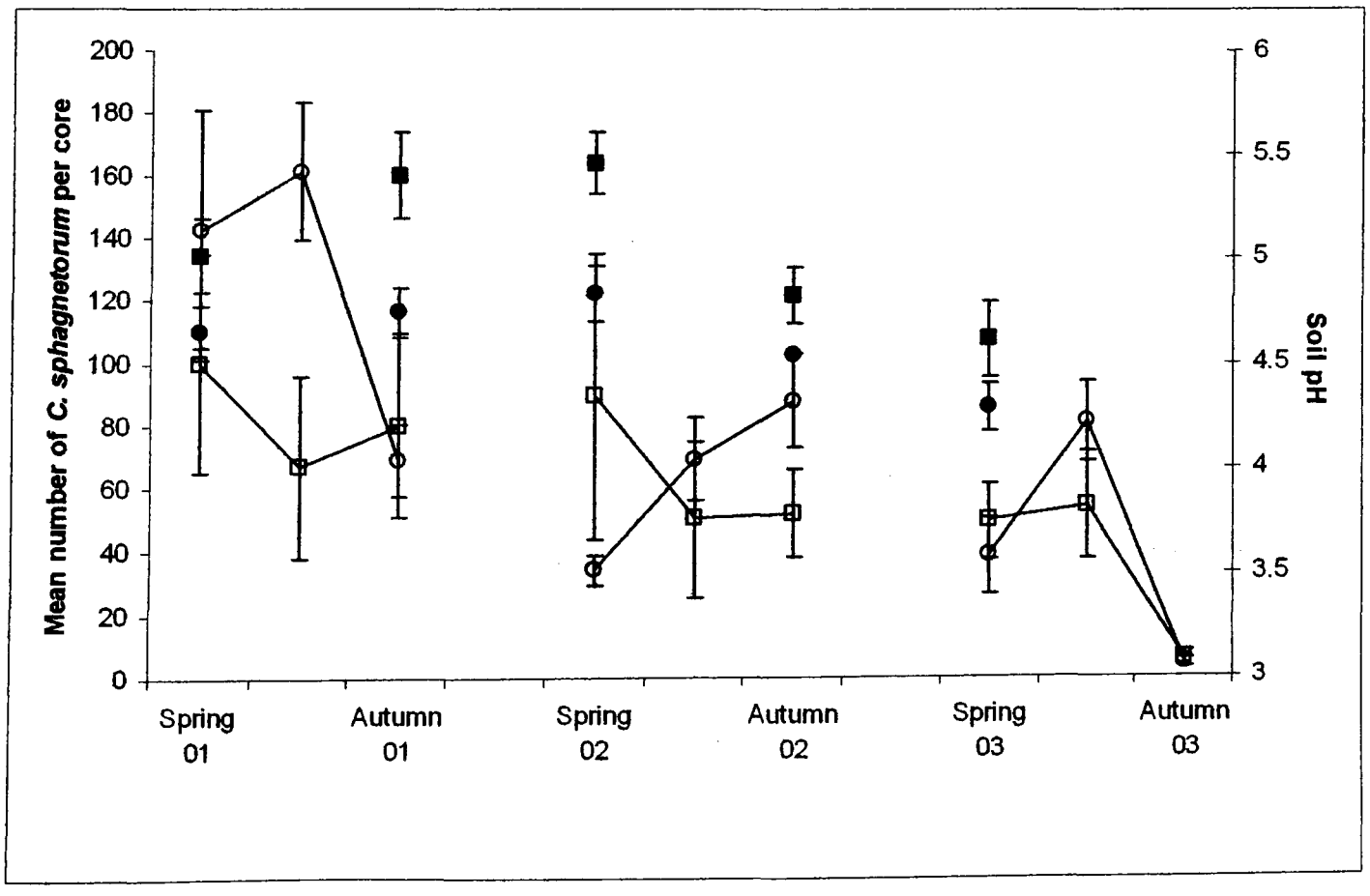


Figure 2.11. Mean pH and numbers of *C. sphagnetorum* extracted from soil cores from control and nitrogen treated plots at Rigg Foot, Sourhope, 2001 – 2003. Means ± 1SE. Control plot enchytraeids (○) and pH (●); nitrogen plot enchytraeids (□) and pH (■).

Over the three years of the experiment the mean number of enchytraeids extracted from control plots was 76 ± 17 and, from nitrogen plots, 61 ± 9 per core. The maximum number of *C. sphagnetorum* extracted from control plots was 161 per core and from nitrogen plots, 100 per core.

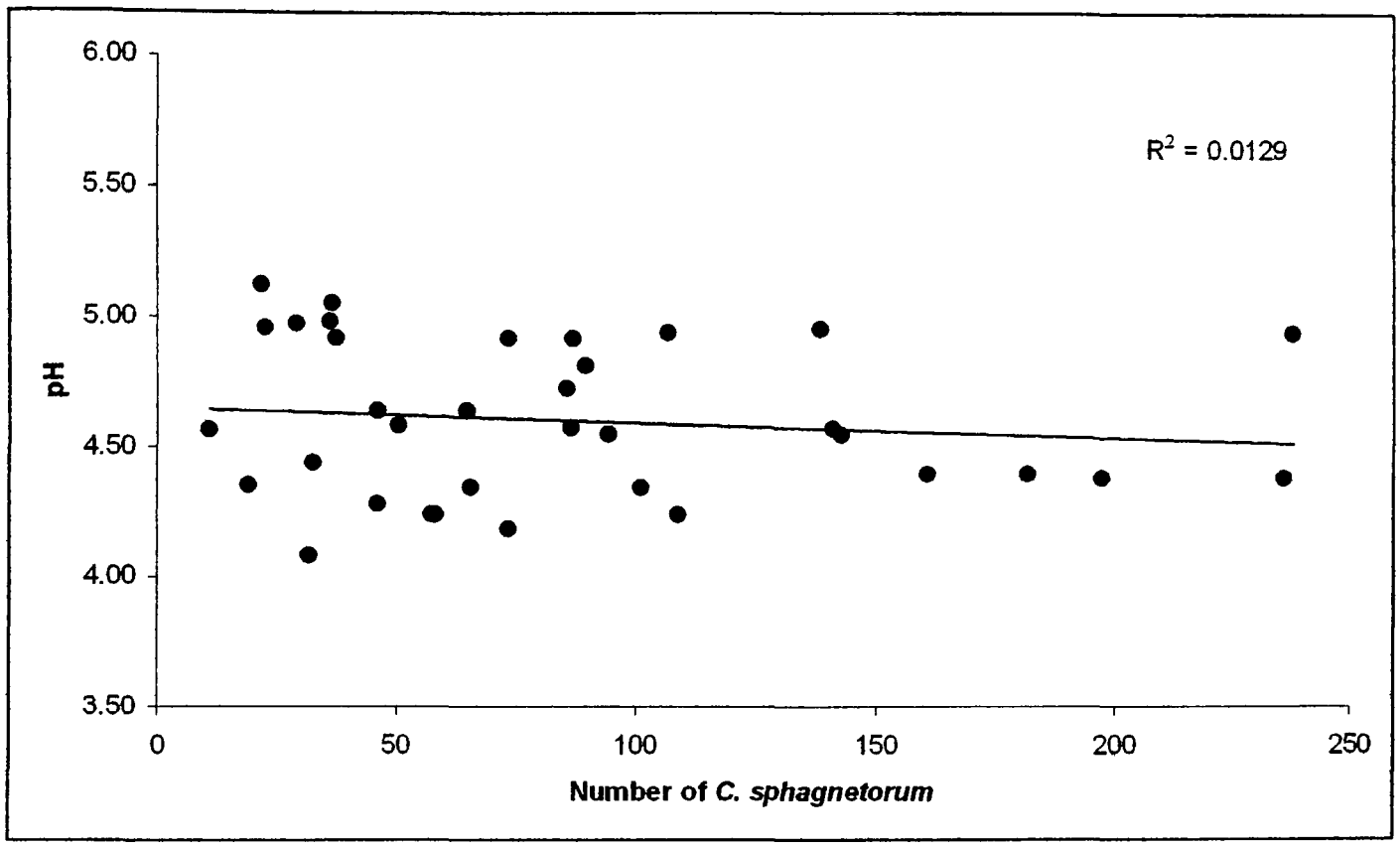
The patterns of abundance of the enchytraeids recorded over 3 years of sampling differed greatly in the two plots. In control plots, the population increased between spring and summer, whereas in nitrogen plots there was a decrease in abundance between spring and summer in 2001 and 2002, and no change in 2003. Between summer and autumn 2003 populations in both plots plunged dramatically.

.

There did not appear to be any correlation between soil pH and *C. sphagnetorum* abundance. A scatterplot with regression line of pH and number of *C. sphagnetorum* showed little association between pH and enchytraeid abundance in either control or nitrogen plots (Figure 2.12) and Pearson's correlation confirmed this (Table 2.10).

Table 2.10. Pearson correlation of number of enchytraeids and soil pH in control and nitrogen plots at Rigg Foot, Sourhope.

		Enchytraeids	pH
Control	Pearson Correlation	1	-0.112
	Significance		0.52
Nitrogen	Pearson Correlation	1	-0.209
	Significance	.	0.23



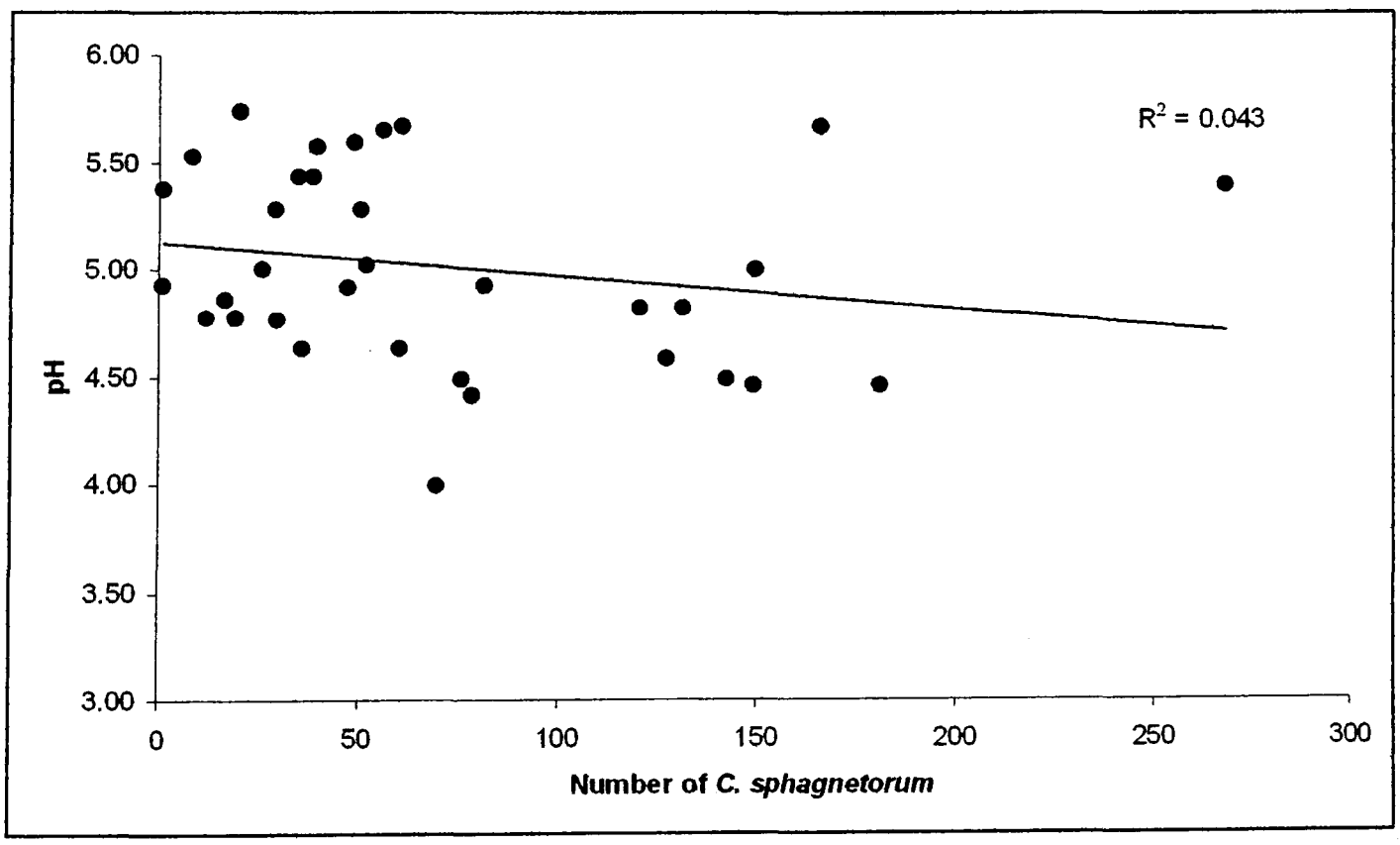


Figure 2.12. Scatterplot of enchytraeid numbers and pH in control (top) and nitrogen (bottom) plots at Rigg Foot, Sourhope. R^2 values are shown on the charts.

Univariate analysis of numbers of *C. sphagnetorum* in the two plots at each sampling date in 2001 and 2002 showed that the abundance of *C. sphagnetorum* was not different (Tables 2.10 and 2.11). However, in 2002, the interaction of time and treatment significantly influenced the abundance of *C. sphagnetorum.*

Table 2.11. Univariate analysis of numbers of *C. sphagnetorum* in control and nitrogen plots at 3 sampling occasions in 2001 at Rigg Foot, Sourhope.

	Mean square	Sum of squares	df	F	P
Time	10986	24483	2	2.538	0.088
Treatment	846569	16409	1	3.402	0.070
Time x treatment	12242	6367	2	.660	0.521
Error	16409	274927	57		

Table 2.12. Univariate analysis of numbers of *C. sphagnetorum* in control and nitrogen plots at 3 sampling occasions in 2002 at Rigg Foot, Sourhope.

	Mean square	Sum of squares	df	F	P
Time	2044	4088	2	0.575	0.566
Treatment	1.260	1.260	1	0.000	0.985
Time x treatment	16036	32071	2	4.513	0.016
Error	3553	181200	51		

Univariate analysis of numbers of *C. sphagnetorum* in the two plots at each sampling date in 2003 showed that abundance of *C. sphagnetorum* was significantly lower in autumn than in spring and summer (Table 2.13) but there were no differences in abundance between treatments.

Table 2.13. Univariate analysis of numbers of *C. sphagnetorum* in control and nitrogen plots at 3 sampling occasions in 2003 at Rigg Foot, Sourhope.

	Mean square	Sum of squares	df	F	P
Treatment	1798	1798	2	2.516	0.119
Time	21299	42598	1	29.804	<0.001
Time x treatment	2606	5212	2	3.646	0.033
Error	715	36447	51		

ii. Lime and lime and nitrogen (L+N) plots – abundance of *Fridericia* and *Henlea*.

Liming the plots caused the disappearance of *C. sphagnetorum* within three years and its replacement with a diverse community of enchytraeids at lower abundance. Table 2.14 is a species list of enchytraeids found in the limed plots. Some species occurred in only one sample, occasionally with only one individual being found.

Table 2.14. List of species extracted from soil in lime and L+N plots at Rigg Foot, Sourhope. Those marked * occurred with greatest frequency.

Cognettia sphagnetorum	*Fridericia striata*
Cognettia cognettii	*Fridericia sylvatica*
Cognettia glandulosa	*Fridericia ratzeli*
Fridericia alata	*Fridericia immature*
Fridericia christeri	*Henlea perpusilla* *
Fridericia bisetosa *	*Henlea perpusilla. immature*
Fridericia bulboides	*Henlea ventriculosa**
Fridericia bulbosa	*Mesenchytraeus flavus*
Fridericia conNata	*Mesenchytraeus sanguineous*
Fridericia galba *	*Mesenchytraeus armatus*
Fridericia leydigi	*Mesenchytraeus immature*
Fridericia magna *	*Marionina spp*
Fridericia maculata	*Enchytraeus buchholzi* *
Fridericiaparoniana *	*Buchholzia appendiculata*
Fridericia perrieri *	*Achaeta spp.*

The overall numbers of enchytraeids from limed plots and soil pH are shown in Figure 2.13. Mean number of individuals ranged from 7 – 30 per core. Mean numbers in L+N plots were lower than those in lime plots, but associated with very large standard errors.

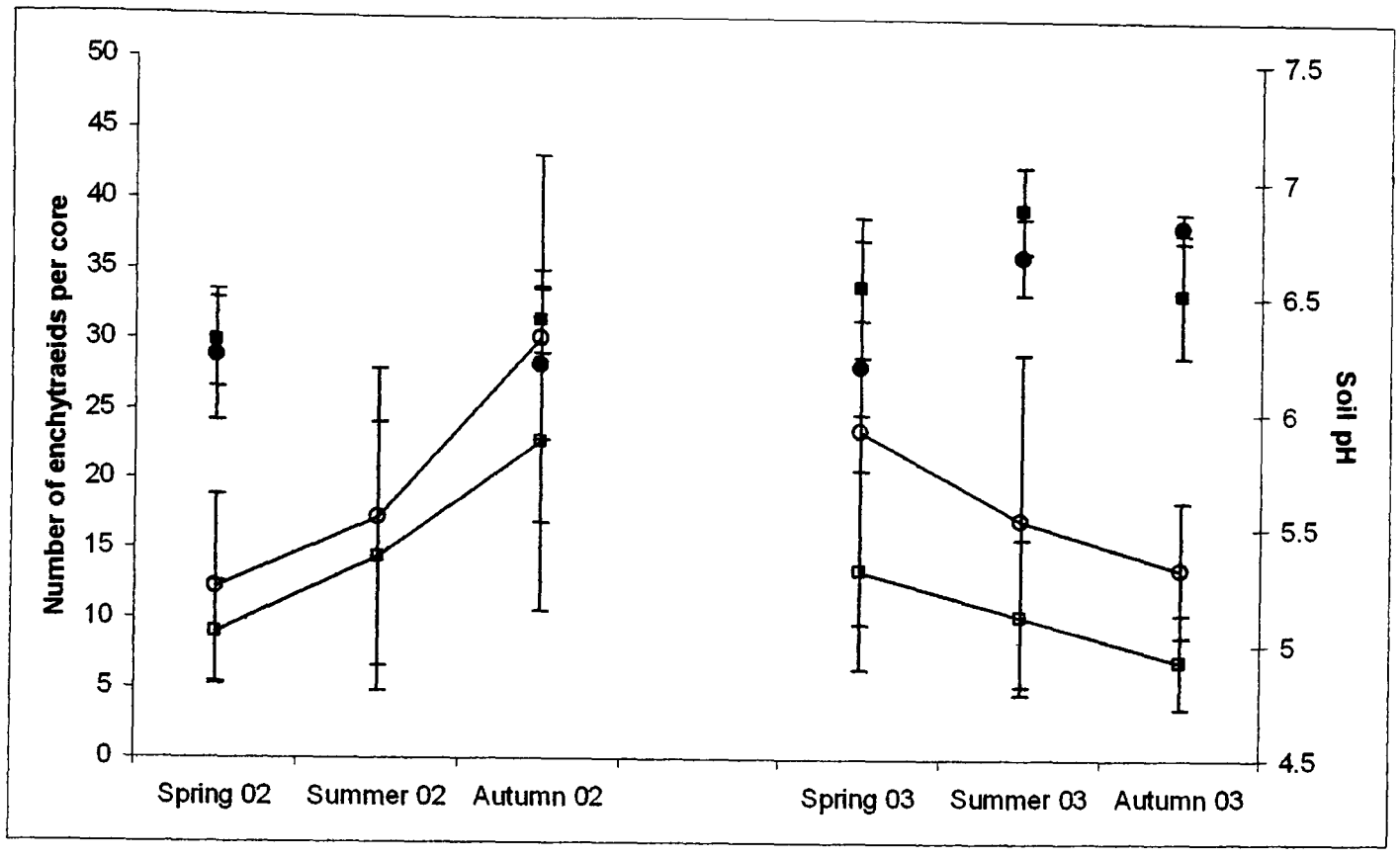
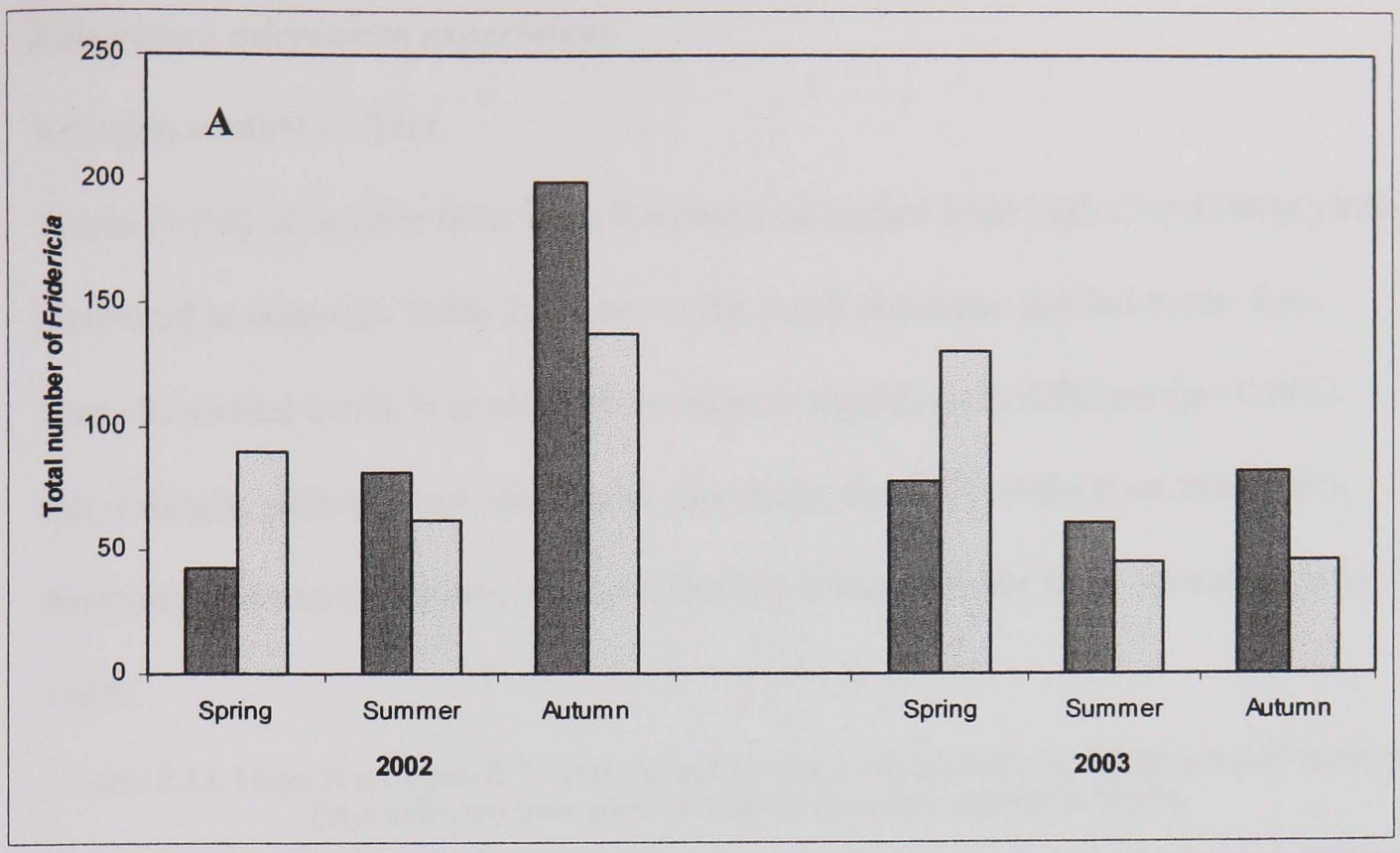


Figure 2.13. Mean pH and numbers of enchytraeids extracted from soil cores in lime and L+N treated plots at Rigg Foot, Sourhope, 2001 – 2003. Means ± 1SE. Lime plot enchytraeids (○) and pH (●); L+N plot enchytraeids (□) and pH (■).

The pattern of abundance was similar in both plots with an increase in abundance during 2002, and a decrease over winter between autumn 2002 and spring 2003. Numbers in both plots fell throughout 2003.

Two genera of enchytraeid – *Fridericia* and *Henlea*– were dominant in the lime plots and Figure 2.14 shows the total abundance of each genus. By autumn 2002 there was an increase in the abundance of *Fridericia*, with a greater increase in lime plots. In 2003, total abundance remained relatively static in lime plots but decreased in L+N plots. Abundance of *Fridericia* was not consistently different between lime and L+N plots. *Henlea* also increased in abundance in 2002 in both plots in summer and autumn, but abundance declined during 2003. There were consistently more individuals in lime compared to L+N plots.



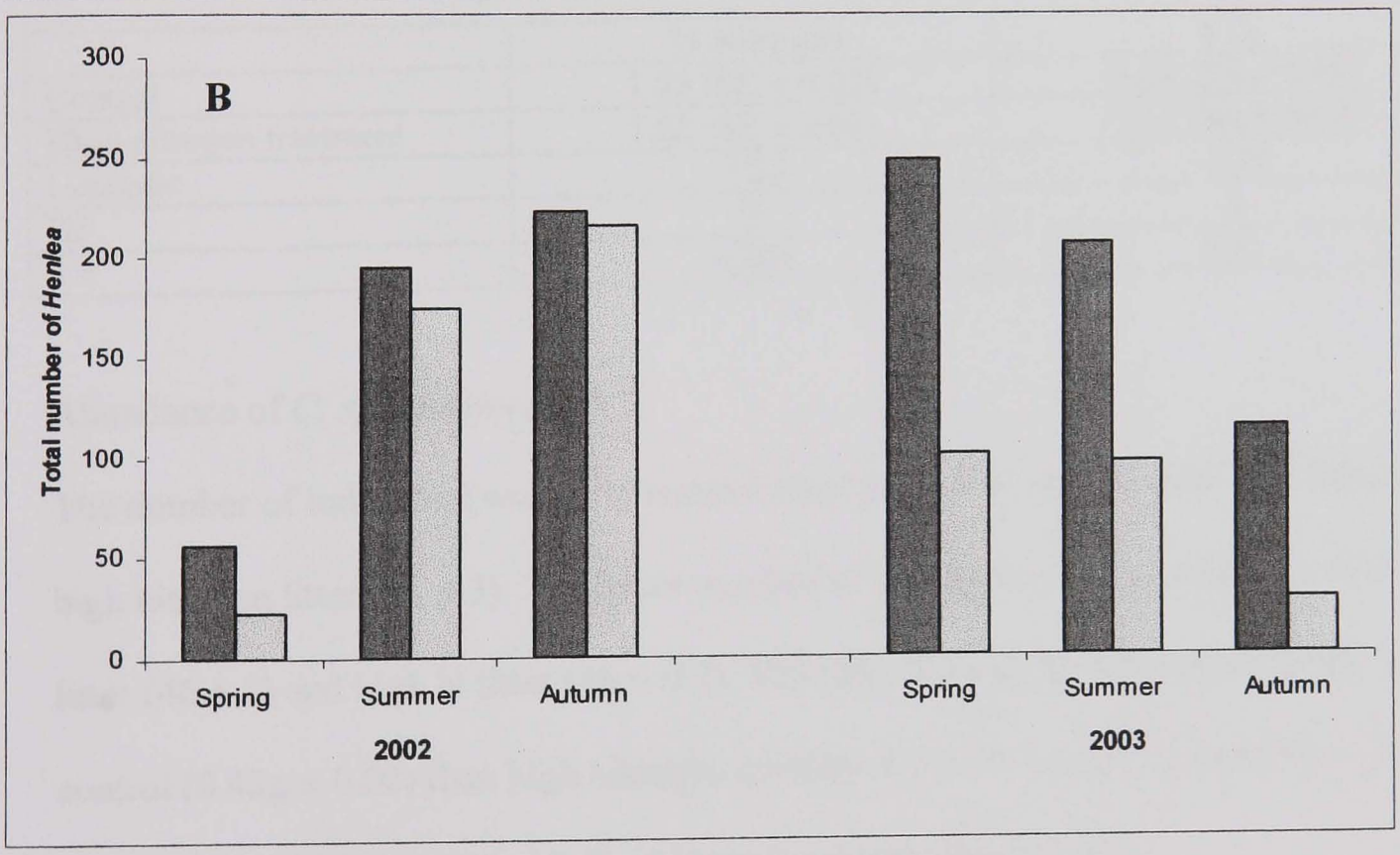


Figure 2.14. Total number of *Fridericia* (top) and *Henlea* (bottom) individuals extracted from soil cores in lime (dark grey) and L+N (light grey) plots at Rig Foot, Sourhope, 2002 and 2003.

Laboratory microcosm experiment.

Nitrogen content of litter.

Tissue N (%) of heather litter from Ruabon was higher from high N treatment plots compared to controls. Table 2.15 shows the result and t-test applied to the data. This shows that the % N content of the litter is significantly different ($p< 0.001$). Interestingly, although not relevant to this study, the $\delta^{15}N$ of the litter from plots receiving ammonium nitrate was significantly lower than for those receiving only water.

Table 2.15. Mean % nitrogen, $\delta^{15}N$ and student's t-test result of control and high nitrogen treatment litter collected from plots at Ruabon moorland experimental site.

	% Nitrogen	$\delta^{15}N$
Control	1.23 (SE ± 0.02)	-8.68 (SE ± 0.33)
High nitrogen treatment	1.82 (SE ± 0.04)	-5.51 (SE ± 0.49)
t statistic	-15.1	-5.38
Df	3	3
$p <$	0.001	0.01

Abundance of *C. sphagnetorum.*

The number of individual worms in control litter (30 ± 2.4) was slightly less than in high nitrogen litter (31 ± 3). The mean number of segments was the same in control litter (46 ± 1) and high N litter (46 ± 0.7). The litter remaining was slightly more in control (0.43g ± 0.02) than high nitrogen conditions (0.42 ± 0.02). Independent t-tests show no significant differences for any of these data (Table 2.16).

Table 2.16. Independent t-test results for enchytraeid abundance, number of segments and litter mass in control and high N litter microcosms.

	df	t statistic	p
Abundance	36	-0.45	0.33
Segment number	35	0.26	0.40
Litter mass	36	0.28	0.39

Movement of litter/faecal material.

There was no movement of material in microcosms with no enchytraeids added but deposition of litter/faecal materials was observed in microcosms with worms added (Plate 4).

Plate 4. Movement of litter/faecal material in microcosms without enchytraeids (top) and with enchytraeids (bottom).

2.4 Discussion

2.4.1 *C.sphagnetorum* populations

Populations of soil animals are distributed heterogeneously in the soil. Aggregation results in great variability within the samples taken which makes it difficult to gain statistical significance between samples (Southwood, 1978). The same problem resulting in large SEs of the mean was encountered in this study, and therefore trends of population size are discussed which generally have not been proved to be different.

Studies spanning many decades show that different soils support different abundances of *C. sphagnetorum.* Table 2.17 shows mean numbers found in different soils under different vegetation types. The population size of *C. sphagnetorum* at Sourhope is at the upper end of the range.

Table 2.17. Population density of *C. sphagnetorum* found in different soil types with different vegetation in various parts of Europe.

Site and year	Author	Dominant vegetation type	Soil type	Mean number/ 000s m^{-2}
Moor House 1970	Standen, 1972	Heather	Deep peat	30 - 50
Moor House 2002	Thesis	Heather	Deep peat	30 -50
Ruabon	Thesis	Heather	Shallow peat	45 – 50
Deepsyke	Thesis	Sitka spruce	Peat	60
Sourhope	Thesis	*Agrostis/Festuca*	Brown earth	80 – 160
Cockle Park	Standen, 1982	*Agrostis/Festuca*	Silt loam	38
Norway	Abrahamsen, 1972	Spruce	Mor humus	37 - 84
Sweden	Lundkvist, 1982	Scots pine	Iron podsol	20 - 35
Finland	Huhta, 1976	Spruce	Mor humus	40 – 60
Poland	Makulec, 1982	Oak-hornbeam Pine-oak Pine	Black earth Podsol Podsol	21 18 17

2.4.2 Moor House National Nature Reserve

Moor House National Nature Reserve (NNR) is not an experimental site but is an area in which nitrogen deposition has increased over the past 40 years. Pitcairn *et al.* (1995) quote the rates of wet nitrogen deposition in upland regions of the North of England e.g. Moor House NNR as increasing from ≈ 7 kg N ha^{-1} y^{-1} during 1960-70 to 20 – 25 kg N ha^{-1} y^{-1} for 1990. It is a sensitive area for both acidity and nutrient N deposition as it has little buffering capacity, consists of deep peat, and is N-limited. For this reason a comparison of enchytraeid abundance and population dynamics of data from 1968 was undertaken as part of this study in order to assess whether increases in N deposition had changed the abundance and reproductive activity of *C. sphagnetorum*.

Overall, the abundance of all *C. sphagnetorum* (large, small and fragments) had not changed since the study by Standen (1973). The range of abundance was 30 to 50 worms per core (30 to 50 000 m^{-2}) in both 1968 and 2002. The patterns of abundance were slightly different due to slight falls in June and September 2002, but the significance of these are difficult to ascertain in a short study such as this. They may be accounted for by seasonal differences in physical factors such as rainfall.

The ratio of large to small worms showed some differences in pattern between years again suggesting between year differences in physical factors. For example in September 1968 the ratio reached ~1:1, with equal numbers of large and small worms, while in 2002 this value was 1.6:1, with more large than small enchytraeids. This could be accounted for by differences in fragmentation or the rate at which fragments regenerated and grew, both of which Standen (1973) had shown to be temperature

dependant. In both years there were more small than large individuals but whereas there was a smooth transition in 1968 for both large and small enchytraeids, the pattern was more erratic in 2002. In 1968 soil temperatures were higher between May and July than in 2002 and fragmentation rate remained steady during this period whereas in 2002 the increase in temperature did not occur until August and September. This may explain the differences in fragmentation and growth rates between the different years.

Comparing means for the start and end of the season, the abundance increased in 1968 from 32 to 48 whereas it decreased in 2002 from 33 to 31. The scale of the differences observed at Moor House after 44 years did not suggest that *C. sphagnetorum* had responded to enhanced N deposition by increased population density at this site even though the 5th centile for critical loads for both acidity and N deposition has been exceeded ('The Status of UK Critical Loads 2004' (CEH Edinburgh)). In semi-natural ecosystems, the increases in N deposition are more gradual than at experimental sites allowing the soil animal communities to become acclimatised to the changing soil conditions.

2.4.3 The experimental sites.

Changes to physical factors brought about by treatments.

One of the aims of this project was to assess the impact of nitrogen fertiliser application on the abundance of *C. sphagnetorum* in acid soils. The fertiliser may act directly on the soil animals or may change the physical factors such as soil pH which would also impact on the animals and their food and competitors. I also hypothesised that an increase in N application would result in increased % tissue N in plant and litter material, providing a better quality of food for enchytraeids. This, in turn, would increase the fragmentation rate of *C. sphagnetorum* and therefore its population size.

The type of N fertiliser used affects soil pH in different ways. Generally, ammonium nitrate causes a fall in soil pH (e.g. Huhta, 1984 and Bardgett *et al.*, 1999) whereas urea increases soil pH (Huhta, 1984, Abrahamsen and Thompson, 1979). Ammonium sulphate also decreases soil pH in neutral to acid grasslands (Arnold *et al.*, 1976).

At Ruabon there was no change in soil pH in the top 5 cm in response to ammonium nitrate fertiliser application. At Sourhope soil pH increased in N treated plots particularly between March 2000 and March 2002, with a maximum pH of 5.5 whereas in control plots it remained relatively steady at 4.5. This was contrary to expectation but in an experiment at Whim Bog, Dr Sheppard (pers. comm.) also found that ammonium was associated with increased soil pH.

At Ruabon % tissue N of *Calluna* litter was significantly higher in high N treated plots (1.82%) compared to control (1.23%) after 4 years treatment. At Sourhope

nitrogen treated plots had significantly higher % tissue N than plots not receiving N fertiliser, but overall %N of clippings lower in all plots in 2003 compared to 1999.

Fluctuation of ***C. sphagnetorum*** *populations in acid soils.*

In control plots at Ruabon the population of *C. sphagnetorum* was very stable at 45 – 50 worms per core, showing very little change in abundance between autumn 2001 and spring 2003. At Sourhope, in control plots in all years, the population increased between spring and summer, when fragmentation rate has been shown to increase, but apart from 2002 the increase observed by Standen (1973) in early autumn at Moor House was not evident at Rigg Foot. However the overall abundance of *C.sphagnetorum* at Sourhope declined throughout the study period.

The abundance of enchytraeids is known to fluctuate widely. In addition to changes due to reproduction, *C. sphagnetorum* is particularly sensitive to soil moisture as it has no mechanism to survive drought (it does not produce cocoons). This sensitivity to low soil moisture was demonstrated by the dramatic decrease in abundance at Sourhope in autumn 2003. Figure 2.2 shows that mean rainfall and soil moisture at Sourhope declined sharply throughout 2003. Although rainfall had been low in previous years (e.g. May 2001 and September 2002), soil moisture had never previously been below 0.3 m^3m^{-3}. The fall in abundance from a maximum of 160 per core in summer 2001 to a more stable value of ~60 – 80 per core throughout the next two years suggests that either the field carrying capacity at this site declined or that environmental factors caused increased death rates and inhibited recruitment. There was also a dramatic fall in abundance of *C. sphagnetorum* in all plots at Ruabon between spring and summer 2003 which did not occur the previous summer.

Substantial drying of the soil in July and August had been noted for previous years (M. G. Pilkington, pers comm.), and the soil seemed unusually dry in 2003 but this was not quantified.

C. sphagnetorum has been shown to respond to drought and changes in soil temperature by migrating to lower depths (Springett *et al.*, 1970; Briones *et al.*,1997). In a study carried out only at Ruabon a greater proportion of *C. sphagnetorum* were found in lower soil layers as the season progressed and this was probably in response to changes in soil moisture and temperature. As soil moisture was higher in September compared to July, lower soil temperature was the most likely cause.

The influence of N fertiliser on C. sphagnetorum in acid soils.

At Ruabon the application of N fertiliser appeared to cause an increase in the abundance of *C. sphagnetorum* in the high N treatment plots where there was an almost linear increase from 20 to 70 enchytraeids per core within the first year of sampling. This was in contrast to the very stable abundance seen in the control plots. Patterns of change in abundance were more erratic in the low and moderate N treatments but following the summer drought in 2003 when enchytraeids plummeted to 10 (control) and 15 (treatments) per core, it was the enchytraeid populations in moderate and high N treatments which recovered more quickly to 55 per core, whereas that of low N treatment and control reached 45 and 30 per core respectively. The population increase observed in the high treatments was not accompanied by a distinct fall in the ratio of large to small worms (indicating fragmentation), and, given the small size of the plots, immigration into the high N treatment plots cannot be ruled out.

At Sourhope the overall abundance was lower in N treated plots compared to controls. The expected increase in abundance between spring and summer seen in the control plots did not occur. In 2001 and 2002 abundance fell by 30 per core and remained static in 2003 (40 per core).

Thus N fertiliser appeared to have different effects at the two sites which may be due to differences in the direct impact of fertiliser on the animals or to different indirect effects brought about at the two sites. The effects of nitrogen fertiliser application on enchytraeids, particularly *C. sphagnetorum*, have been explored in a number of studies, particularly with forest soils. Abrahamsen and Thompson (1979) used large one-off doses of urea (100, 400 and 1600 kg N ha^{-1}) in a Scots pine and Norway spruce forest on a podsol soil of pH 3.7 – 4.0. The highest dose of fertiliser almost destroyed the population of *C. sphagnetorum* immediately, and it took 4 years for the population to recover to its pre-treatment abundance. Thereafter the abundance increased to over 400% of that of control plots. The changes were thought to be associated with an increase in pH, direct ammonium toxicity and unfavourable osmotic conditions immediately after fertiliser application. Huhta (1984) used both urea and ammonium nitrate (200 kg N ha^{-1} yr^{-1}) in field (spruce and pine forest) experiments. In field experiments both fertilisers caused a reduction of about 50% of *C. sphagnetorum* abundance almost immediately. In urea treated plots the population began to recover after the third year, but there was no recovery after 4 years in plots fertilised with ammonium nitrate.

Huhta (1984) then went on to investigate the direct and indirect effects in a laboratory study. The aim was to try to separate the effects of the nutrient from the effect of pH. Ammonium nitrate almost destroyed the population of *C. sphagnetorum* within 2 days

of application, which he attributed to direct toxicity of the fertiliser. With urea treatment there was a gradual decrease in abundance until the end of the second month, but recovery was slow with urea treatment. At the end of the experiment (8 months) the population in the ammonium nitrate treated soils exceeded that of controls.

Ammonium nitrate is known to be toxic to many soil animals either directly as ammonia is produced (Moursi, 1962) or indirectly via changes in soil water osmotic pressure. Lohm *et al.* (1977) considered that in the longer term the effect of fertiliser on microbes (with which *C. sphagnetorum* interacts (Hedlund and Augusstson,1995) may influence both microbial activity and litter formation. There is evidence that fertiliser and raised pH change the community of microbes in favour of a higher population of bacteria (Ledgard *et al.*, 1998) which may not be an ideal food source for *C. sphagnetorum* and may even be toxic to this enchytraeid (Latter and Howson, 1978).

The studies cited above used a one-off application of fertiliser and followed enchytraeid abundance over varying time periods. At Sourhope ammonium nitrate was applied in two doses each of120 kg N ha^{-1} yr^{-1} in April and May, whereas at Ruabon each treatment of 20, 40 and 120 kg N ha^{-1} yr^{-1} was applied in 8 monthly doses. This would suggest that at Sourhope there was direct fertiliser toxicity to C. sphagnetorum, whereas at Ruabon small regular doses may have increased microbial activity sufficiently to provide a growing, high nutrient food source for the enchytraeids.

In the study carried out at Ruabon there was a trend towards a greater proportion of *C. sphagnetorum* at lower depths with increased fertiliser application. This effect on the enchytraeid distribution could have been caused by direct fertiliser effect or indirectly acting on, for example, soil moisture content, particularly of the litter layer, soil pH or the distribution of suitable food. Abrahamsen and Thompson (1979) also found the vertical distribution changed with fertiliser application, with many more found in the mineral layers than the humus layers and this was coincident with a decrease in the thickness of the humus layer, particularly at high fertiliser N concentration.

Clearly the changes to soil physical factors brought about by application of ammonium nitrate would be complex. Nevertheless these indirect effects may have influenced enchytraeid populations and some are considered further.

C. sphagnetorum is considered to be sensitive to increases in soil pH. Standen (1982) found a negative correlation between pH and abundance of *C. sphagnetorum* in a grassland, and Standen and Latter (1977) found that optimum pH was between 3.6 and 3.8 in a blanket peat. At Sourhope, pH was raised by treatment with ammonium nitrate – probably towards the upper limit of tolerance of this enchytraeid (Healy, 1980) and the abundance of enchytraeids was lower in N plots in the majority of samples. However, at Ruabon there was no change in pH in the upper 5 cm of the soil profile Thus higher pH also may explain the lower abundance in N treated plots at Sourhope.

C. sphagnetorum is also sensitive to soil moisture and at Sourhope soil moisture was significantly lower in N treated plots (Burt-Smith, 2002) compared to controls. This

may also explain the lower population abundance in these plots although synergistic effects of low soil moisture and fertiliser toxicity cannot be ruled out.

The hypothesis that increased % tissue N would provide a better quality food source and higher density of *C. sphagnetorum* was not proved. At Sourhope tissue N of litter from nitrogen plots was higher, but the abundance of *C. sphagnetorum* was lower compared to control plots. However, at this site the experimental protocol decided on was to mow plots and remove the clippings. This caused a significant decrease in tissue N in all plots between 1998 and 2003 so that % tissue N in nitrogen plots in 2003, although significantly higher than that of control plots for that year, was still lower than the mean for all plots in 1999. At Sourhope at least, results suggest that the decline in abundance of *C. sphagnetorum* in nitrogen plots between 2001 and 2003 may have been associated with decreased volume and % tissue N of litter.

Thus despite the higher % tissue N found in vegetation from N treated plots relative to the control plots in 2003, the abundance of *C. sphagnetorum* in N treated plots was lower and it must be concluded that increase in litter quality did not enhance *C. sphagnetorum* populations in the field. Further support was obtained from the microcosm experiment with heather litter with two different % tissue N concentrations and *C. sphagnetorum*. This experiment showed there were no differences in the abundance or size of the enchytraeids, nor did litter mass loss vary between the two.

The causal mechanism for a decline in field carrying capacity for enchytraeids is not known but the lack of litter input may influence the partitioning of N between

microbes and plants, despite the additions of fertiliser and lime. Although the quantity of litter input was reduced, enchytraeids may have had access to root exudate sourced nutrients via microbial immobilisation. However, if the microbial community had shifted towards one with greater dominance of bacteria (Anderson and Domsch 1994; Ledgard *et al.*, 1998 and Blagodatskaya and Anderson 1998), this may not be the ideal food source for *C. sphagnetorum* and could, in fact, be toxic (Latter and Howson, 1978).

Enchytraeidae in limed plots at Sourhope.

In limed plots at Sourhope soil pH increased substantially within three years of first application. There was a concomitant change in the enchytraeid community as *C. sphagnetorum* was replaced by a suite of enchytraeids of several genera but there was numerical dominance by individuals of *Fridericia* and *Henlea.* This is a well established effect on enchytraeids (Hågvar and Abrahamsen, 1980; Standen, 1984; Pokarzhevskii and Persson, 1995; Black *et al.*, 2002).

The pattern of enchytraeid abundance was the same in both lime and L+N plots with an increase in 2002 and a decrease in 2003, and consistently lower abundance in L+N plots. The pattern for 2002 could be considered typical of a short-lived, sexually reproducing species. Many species live for only one year whereas the larger species such as *F. galba* may live for up to two. Hatchlings emerge in spring and boost abundance during summer, when some newly deposited cocoons may also hatch and adults survive until late autumn. The lower abundance in spring reflects adult mortality during the winter.

The abundance of enchytraeids in L+N plots was lower than in lime plots. However, it was more likely to be soil moisture conditions that influenced abundance in 2003 as described for acid plots (above). Burt-Smith (2002) had reported that the limed plots contained less soil moisture than control, and L+N contained least (Control: 0.55 m^3m^{-3}; lime: 0.44 m^3m^{-3}; L+N: 0.37 m^3m^{-3}). Abundance levels did not fall to the same magnitude as seen in control and nitrogen plots with *C. sphagnetorum* and confirms that enchytraeids found in limed plots are more tolerant of dry soil conditions partly because most species are larger and also that they produce drought resistant cocoons.

The overall decline in abundance seen for *C. sphagnetorum* in acid plots was not seen for the enchytraeids in limed plots. The possible reasons suggested for that species - lower amount of litter due to removal of clippings and decline in % tissue N in all plots (although L+N plots still relatively higher than L in 2003) – applied equally to the enchytraeids of the limed plots, but did not produce the same result. This is perhaps not surprising as the species in limed plots have very different requirements and tolerances compared to *C. sphagnetorum*. The larger size and activity of most species of the enchytraeid community of neutral soils compared to *C. sphagnetorum*, may enable them to move greater distances in response to environmental factors and so reduce changes in abundance. The remarkable speed with which the community of enchytraeids developed following the first application of lime supports this suggestion to some extent.

Chapter 2

Summary.

Enchytraeid populations at two experimental and one reference site were sampled between 2001 and 2003. Soil pH at all sites was low (pH 3.4 – 4.6), and the dominant enchytraeid species was *Cognettia sphagnetorum* but the soil types varied. Ruabon, in North Wales, was a peat soil on an iron pan where vegetation consisted almost exclusively of heather and bilberry. Different concentrations of ammonium nitrate were used in this experiment. Sourhope, on the Scottish border, was an acid brown forest soil with dominant grasses – sheep's fescue and common bent with heath bedstraw, heath woodrush and moss. One concentration of ammonium nitrate was made, with and without lime. The reference site, Moor House NNR, was a deep blanket peat supporting heather, cottongrass, bilberry, cloudberry, crowberry and sphagnum mosses.

Physical changes occurred at both sites. At Ruabon, % tissue N increased in heather plants and litter, and there was drying of the soil during summer. At Sourhope, soil pH increased in plots treated with lime leading to a change in the enchytraeid community with *C. sphagnetorum* replaced by other enchytraeid species. Tissue N content of grasses decreased over the whole site – attributed to the policy of removing grass clippings after mowing - although in plots treated with nitrogen, tissue N was still higher than either control or limed only treatment. Soil moisture was stable until the late summer of 2003, when low rainfall led to low soil moisture.

The population of *C. sphagnetorum* in control and treated plots at both Ruabon and Sourhope suffered serious decline in the summer and autumn of 2003 and the most likely cause was the very low water content of soil in that year.

In control plots at Ruabon, *C. sphagnetorum* abundance remained stable whereas at Sourhope abundance declined from an initial high level. This may have been caused by the mowing and removal of grass clippings at this site which led to successive decline in litter quality and quantity.

The application of ammonium nitrate fertiliser appeared to increase the abundance of *C. sphagnetorum* at Ruabon whereas at Sourhope it had an adverse effect. In addition to the difference in soil and vegetation type, these contradictory effects may be explained by differences in management. At Ruabon, where fertilizer application was in small doses applied 8 times each year, the tissue N content of heather increased in the N treated plots and it is suggested that this improved quality led to higher *C. sphagnetorum* populations in N

treated plots at that site. At Sourhope, ammonium nitrate was applied in 2 large doses in spring and it is possible that following the high dose of fertilizer, ammonia was released which toxic to small soil animals such as *C. sphagnetorum* and this was the cause of the lower abundance in N plots. Another possible cause of this decline in abundance was the slight, but significant, increase in soil pH in N fertilised plots.

The population of *C. sphagnetorum* at Moor House NNR was monitored in 2002 and compared to the population at the same site in 1968. There was no difference in the abundance of *C. sphagnetorum* but reproduction (assessed by regenerating worms) appeared to be delayed in 2002. This may have been related to maximum soil temperature, which occurred in August and September 2002, whereas it was earlier (July and August) in 1968.

CHAPTER 3

INVESTIGATING INTERACTIONS BETWEEN ENCHYTRAEIDAE AND NITROGEN USING ^{15}N STABLE ISOTOPE – THE LITTERBAG STUDY.

3.1 Stable isotopes and their use in ecology.

A little history.

The fact that elements have different isotopes was discovered in 1913 when Fleck and Soddy found different atomic weights but identical chemical properties in radioactive series and Thompson found ions with mass-to-charge ratios of 20 and 22 which were identified in 1919 as ions of stable isotopes by Aston. Isotopes can be radioactive, stable radiogenic or stable (Galimov, 1985). Radioactive isotopes (e.g. ^{14}C, ^{32}P, ^{3}H) disintegrate in an invariant manner and are easy to measure at high precision. They have been used to define metabolic and genetic pathways by molecular biologists and biochemists. However, concern about the use of radioactive isotopes and the improvement of the precision of analytical methods for stable isotopes has seen less use of radio-isotopes and a large increase in the use of stable isotopes in relevant cases such as carbon, nitrogen, oxygen and hydrogen. The cost of analysis and of isotopically 'heavy' compounds e.g. $^{13}CO_2$, $^{15}NH_4(\pm)^{15}NO_3$ have also fallen, which make their use more accessible to a wide range of scientific study.

Definition of stable isotopes and isotopic fractionation.

'Isotopic composition is the distribution of isotopes of a given element, usually expressed in the form of the ratio of a less common isotope to the most common one: D/H, $^{13}C/^{12}C$, $^{18}O/^{16}O$ and $^{15}N/^{14}N$. The isotopic composition determines the atomic weight' (Galimov, 1985).

Isotopes are forms of an element that differ in respect of the number of neutrons in the nucleus. More neutrons increase the atomic mass of the element. The electron shell structure is the same and the isotopes are chemically the same. However, the difference in mass and nuclear spin causes the isotopes to behave slightly differently. This difference can result in the redistribution of isotopes between interacting compounds, resulting in the increase in concentration of an isotope in one compound and a decrease in the concentration in the other compound. This is isotopic fractionation. It is the consequence of the physicochemical inequality of isotopes, and can affect the rate of a process (kinetic isotope effect) or the energy state of a system (thermodynamic isotope effect).

Concentration of isotopes in the atmosphere.

The heavy isotopes of elements are rare in nature and make up only a small percentage of the abundance of an element. Table 3.1 shows the average abundance of the stable isotopes most frequently used in ecological studies and the common standards used (from Rundel *et al*, 1988). The heavy isotopes of elements are rare in nature and make up only a small percentage of the abundance of an element.

Table 3.1. Isotopic composition, abundance, ratio pair and primary standard used in their analysis by IRMS technique. These are the most commonly utilised stable isotopes used in ecological studies.

Element	Isotope	Abundance (%)	Isotope ratio	Primary standard
Hydrogen	^{1}H	99.985	$^{2}H/^{1}H$	Standard Mean Ocean Water (SMOW)
	^{2}H	0.015		
Carbon	^{12}C	98.89	$^{13}C/^{12}C$	PeeDee Belemnite (PDB)
	^{13}C	1.11		
Nitrogen	^{14}N	99.63	$^{15}N/^{14}N$	Air
	^{15}N	0.37		
Oxygen	^{16}O	99.759	$^{18}O/^{16}O$	SMOW/PDB
	^{17}O	0.037	$^{17}O/^{16}O$	
	^{18}O	0.204		
Sulphur	^{32}S	95.00	$^{34}S/^{32}S$	Canyon Diablo Troilite
	^{33}S	0.76		
	^{34}S	4.22		
	^{36}S	0.014		

Measuring stable isotopes.

There are 3 methods for the measurement of stable isotopes. Nuclear magnetic resonance and infrared spectroscopy can detect and measure stable isotope abundance but the most common method used is isotope ratio mass spectrometry (IRMS). The ratio of heavy to the light isotope is measured and compared to a standard. (For a more comprehensive description of analytical techniques see Shimel (1993) or Knowles and Blackburn (1993).)

IRMS can process 6 – 12 samples hourly, depending on the isotope in question.

Isotope ratios are usually expressed in δ notation in parts per thousand or per mil (‰)

The δ value of a sample is defined by the following equation:

$$\delta_{sample} = ((R_{sample} - R_{standard}) / R_{standard}) \times 1000\ (‰)$$

The primary standard has a δ value of zero. A minus sign of a sample indicates that the sample is depleted in the 'heavy' isotope compared to the standard, and a plus sign that the sample is enriched in the 'heavy' isotope compared to the standard (Galimov, 1985).

Sample preparation

Preparation depends on the type of sample being analysed and the isotope of interest. A review of methodology is described by Lajtha and Michener (1994).

In general, solid media have to be oven or freeze dried. Large samples need to be homogenised and a representative sample obtained. Accurate weighing of the sample is required, as the IRMS calculates the isotopic ratios on a dry weight basis.

The percentage of the element in question is also needed in order to calculate the weight of both the sample and the standards required. The accuracy of the analysis requires a minimum mass of sample. Carbon analyses generally require smaller samples than those of nitrogen due to the greater carbon content of plants, animals, and soils.

Bulk sample isotope ratios do not take into account the different individual components. A $\delta^{13}C$ value of a bulk sample is the ratio of the total $^{13}C/^{12}C$ ratio. If a researcher wanted to investigate an individual carbohydrate component, then compound specific techniques must be employed. This involves the use of liquid or gas chromatography coupled to IRMS. This technique has been used successfully to examine individual amino acids, lipids and carbohydrates. A new method of analysis has been described by Evans *et al.*, (2004).

Measuring whole body stable isotopes in small animals such as those found in soils (collembola, enchytraeids, mites) requires many individuals bulked together to provide sufficient mass for analysis. For example, the dry weight of an individual enchytraeid is about 0.03mg. For bulk (whole body) analysis and accuracy a minimum of 0.15mg sample is required for $\delta^{15}N$ so at least five individuals would be required for a single sample. Compound specific techniques have the advantage of requiring much smaller sample sizes.

Stable isotope studies.

Stable isotope studies can be broadly divided in natural abundance (NA) and isotope enrichment studies.

Natural abundance isotopic studies.

Natural abundance stable isotope signatures have been used in many different scientific fields e.g. past climates – ice core/foraminifera data (global carbon budget), pollution studies, paleodiets, food web analysis, photosynthetic pathways, plant water use efficiency, the nitrogen cycle, drugs, biochemical pathways, tracing rock sources.

Natural abundance ecological studies.

Some 'classic' work.

1. *Plants and photosynthetic pathways*

Plant studies using NA stable isotopes found that plants using different photosynthetic pathways have different isotopic signatures. C_3 plants use the Calvin cycle and have $\delta^{13}C$ values of ~ -27‰. C_4 plants use the Hatch-Slack cycle and have $\delta^{13}C$ values of ~ -14‰. CAM (Crassulacean acid metabolism) plants are at ~ -11‰ (Sternberg and De

Nero, 1984). When the $\delta^{13}C$ values are plotted against δD values, the plants using different photosynthetic pathways separate into distinct clusters.

2. *Soil studies*

Studies of $\delta^{15}N$ in soils have generally found that the litter layer is isotopically light but there is an increase in $\delta^{15}N$ value with depth (Nadelhoffer and Fry, 1994; Högberg, 1997; Ponsard and Arditi, 2000). A number of explanations for this have been offered:-

- Plant $\delta^{15}N$ is less than that of soil and plant material forms a large part of the litter layer; hence it is isotopically light.
- Fungi, particularly the caps of fruiting bodies, are isotopically heavy (Taylor *et al.*, 1997; Högberg *et al.*, 1996). Studies of ectomycorrhizal fungi have found the same (Handley *et al.*, 1996) and that caps were enriched relative to stipes and chitin (Taylor *et al.*, 1997). Fungi are important precursors of recalcitrant nitrogen in soils and recalcitrant nitrogen increases with depth.
- There might be selective *in situ* synthesis of $\delta^{15}N$-enriched compounds during decomposition.

Kerley and Jarvis (1997) examined an undisturbed and recently disturbed soil and found the same $\delta^{15}N$ increase with depth. They also found an increase in $\delta^{15}N$ with smaller particle size. The E_4/E_6 ratio of humic material (HM) (the ratio of absorbances at 465 and 665nm) was less with depth. This ratio is considered to be a measure of the complexity of molecules present, with higher ratios related to greater molecular weights. They considered this decreasing ratio and smaller soil fraction HM with depth to indicate a more complex nature of the humic content.

3. *Food web analysis.*

Food web analysis has been another successful use of NA stable isotopes, particularly the use of $^{13}C/^{12}C$ and $^{15}N/^{14}N$. Generally, *a priori* identification of the food web structure is required as the food source of the organisms in the web is required. In water and above ground this is fairly straightforward as the interactions between organisms are visible.

Some of the 'classic' frequently cited work was carried out in the 1980's. DeNiro and Epstein (1978 and 1981) analysed various animals which had been fed on monotonous diets of known isotopic composition for both $\delta^{13}C$ and $\delta^{15}N$. The importance of the diet was to ensure that the isotopic signature of a single food source fed to the animals over a sufficiently long period of time was the only contributor to the $\delta^{13}C$ and $\delta^{15}N$ found in the animal tissues. Overall there was a 1‰ enrichment of $\delta^{13}C$ and a 3‰ enrichment of $\delta^{15}N$ compared to the food source of the animals. DeNiro and Epstein (1981) analysed different tissues of mice used in the study and found a stepwise enrichment from diet → kidney → hair → liver → brain.

Mingawa and Wada (1984) studied the $\delta^{15}N$ of organisms taken from natural environments – marine, estuarine and a paddy field. The average enrichment of $\delta^{15}N$ was 3‰ per trophic level from phytoplankton → zooplankton → fish. Similarly, field animals were reared in the laboratory on a monotonous diet of known $\delta^{15}N$ content and showed the same magnitude of enrichment.

Tieszen *et al* (1983) studied gerbils whose diet was switched from C_4 corn to C_3 wheat and analysed different tissues at different time intervals. They found, again,

that different tissues had different $\delta^{13}C$ values. Hair was enriched by ~1‰ but fat was depleted by ~ 3‰ compared to the diet. This depletion was explained by the discrimination against ^{13}C during lipid synthesis.

Tieszen *et al* (1983) also calculated the half-life and tissue turnover rate of carbon in the different tissues. Half-life varied from 6.4 days in liver (a more metabolically active tissue) to 15.6 days for fat and 47.5 days for hair. They calculated that for replacement (99.99%) of carbon derived from the C_3 diet would take 84 days in liver tissue and 208 days in fat tissue.

The purpose of this study was to assess the usefulness of analysing different tissue types as indicators of animals' diet. They emphasised that tissue analysis may obscure the contributions of isotopically distinct components of an animals' diet if the diet varies over time. This was also the opinion of DeNiro and Epstein (1978) who recommended that the total body isotopic value may be a more accurate indicator of diet.

Mingawa and Wada (1984) studied the effects of age on the $\delta^{15}N$ content of animals. They used 2 species of mussels which, once attached to a substrate, remain sessile. They were divided into age class according to shell size. Initially there was a sharp increase in $\delta^{15}N$, which then dropped and levelled off to a difference of ~ 3‰ from their food source.

A study of termites by Tayasu *et al.*, (1997) using $\delta^{13}C$ and $\delta^{15}N$ natural abundance showed a distinct separation of wood feeding and soil feeding groups. Again, *a priori*

knowledge of the feeding habits of the animals was required. However, one species (*Acanthotermes acanthothorax*) which is a wood and litter feeder but also a fungus grower, had relatively depleted $\delta^{15}N$ values, suggesting that it does not feed on the fungus. No data were given for the $\delta^{15}N$ value of the fungus, but other studies have found that they are $\delta^{15}N$ enriched (Taylor *et al.*, 1997). The use of ^{13}C NMR has also clarified the transformations of organic materials which take place in the guts of termites and Diptera larvae (Hopkins et al., 1998).

The general 'rule of thumb' derived from these early studies is that the $\delta^{13}C$ value of an organism increases by 1‰ compared to that of its diet, and the $\delta^{15}N$ increase of 3‰ is indicative of the organisms' trophic position in the food web – one trophic level higher in the food web for each 3‰ increase.

Omnivory in natural ecosystems.

The omnivorous nature of organisms in various media, or the availability of spatially separated food sources, may muddy the waters for the general 'rule of thumb' described above. Organisms feeding on different substrates tend not to display such clear cut trophic or dietary positions.

Haines and Montague (1979) studied invertebrates in a salt marsh estuary using $\delta^{13}C$ values. Analysis of the potential food sources (seston, benthic algae, C_3 and C_4 plants) enabled them to classify the food sources of a range of aquatic and terrestrial invertebrates.

Vander Zanden and Rasmussen (1999) used primary consumers as the base for their study of freshwater lakes using $\delta^{13}C$ and $\delta^{15}N$ isotopes. They also applied a method for correcting variation in baseline $\delta^{15}N$. This correction factor was used hypothetically with two fish species which inhabit different zones in the lakes and obtain energy from different food chains. They exhibit a similar $\delta^{15}N$ values but are trophically separated by one unit. The correction factor worked successfully for this hypothetical situation.

Soil food webs

Soil food webs present a different challenge to soil ecologists. There are a multitude of different organisms in a multitude of different soils and frequently the diet, trophic position and function of each organism is unknown or assessed by gut contents and mouthpart assemblage. The soil and litter are heterogeneous and parts of it may, or may not, be a suitable environment for each individual organism. Added to this, each organism may have different requirements during different parts of its life cycle, with some organisms having different morphological forms in their life cycle.

Laboratory experiments may give an indication of the food source of organisms but choice experiments may present food sources that, although present in the animals' environment, are not available to them in the field (Latter and Howson, 1978). Gut content analysis gives an indication of what has been ingested but not necessarily what has been assimilated.

Switching the diet of an animal to an isotopically distinct food source has been a useful method for analysis of $\delta^{13}C$ in animal tissue. C_3 and C_4 plants have different

$\delta^{13}C$ signatures of -27‰ and -14‰ respectively so changing the animals' diet will also change the $\delta^{13}C$ signature of the animal (DeNiro and Epstein, 1978, 1981). To effect a similar change in $\delta^{15}N$, changing the diet from a non-nodulating plant to a nodulating plant such as clover, which is isotopically 'lighter', or any other food source with a distinctly different $\delta^{15}N$ signature can be used. A number of such studies on earthworms are described in the next section.

Ecological studies using diet switching.

Schmidt and Ostle (1999) used dairy cattle slurry as a manure and detected changes in the natural abundance $\delta^{15}N$ of two earthworm species after 3 applications of the slurry but not after a single application. An endogeic species (feeding on soil and organic matter within the soil profile) was consistently more enriched in $\delta^{15}N$ than an anecic species (feeding on surface litter and on soil).

Similarly, Schmidt *et al.* (1997) studied earthworm $\delta^{13}C$ and $\delta^{15}N$ in fields with wheat only and a wheat/clover bicrop. The earthworm isotopic signatures from the bicrop field reflected the isotopic depletion of clover (an N_2 fixing symbiont) showing a slight depletion in isotopic composition. In both fields the enrichment in both isotopes followed the pattern epigeic>anecic>endogeic feeders (epigeic species feed on surface litter).

However, Nielson *et al.* (2000) studied earthworms from six different habitats and their results suggested that the functional group classification may be site specific, and that increases in $\delta^{15}N$ may be indicative of habitat diversity. The data from worms from coniferous woodland and ungrazed pasture was 5.7 – 6.3‰ more enriched than

deciduous woodland or arable sites. Applying the 'rule of thumb' of a 3‰ increase per trophic level, they suggested that the former sites contain an extra trophic level and are therefore more diverse.

Briones *et al.* (2001) and Briones and Bol (2003) also studied earthworms in relation to land use changes (different cropping systems) in Britain and Spain. The original land use was grassland (C_3) which was subsequently changed to maize production (C_4). They found that the original Spanish soils were $\delta^{13}C$ enriched and $\delta^{15}N$ depleted compared to the British soils as was the NA tissue content of worms. The isotopic signature of C_3 plants is ~ -27‰ and that of C_4 plants ~ -14‰, and this was reflected in the isotopic value of litter and soils. Soil isotopic values of $\delta^{15}N$ also increase with depth (Taylor, *et al.,* 1997, Nadelhoffer and Fry, 1994; Högberg, 1997; Ponsard and Arditi, 2000), thought to be a result of isotopic fractionation during mineralisation of nutrients. As soil depth is related to age, the older the soil, the greater the NA isotopic signature. Briones *et al.* (2001) and Briones and Bol (2003) found that earthworms in the maize plots that they sampled became $\delta^{13}C$ enriched, which they interpreted as evidence that the worms were selectively feeding with a preference for fresh (C_4) residues.

Briones *et al.* (1999) also used this technique with two species of Collembola. The animals $\delta^{13}C$ was equilibrated using bakers' yeast, then added to microcosms with a C_3 soil and maize (C_4) litter. The $\delta^{13}C$ of both Collembolan species showed a preference for fresh litter but also showed no signs of resource partitioning. Chamberlain *et al.* (2004) utilised a similar methodology with two Collembolan species. They were fed with bakers' yeast and switched to C_3 labelled, then C_4

labelled yeasts which were isotopically distinct. Using a compound specific technique, the incorporation of the carbon into fatty acids and the rate of incorporation were calculated using a negative exponential equation. They also found that some fatty acids were biosynthesised *de novo.*

Whole soil food web studies, with particular reference to Enchytraeidae.

Most of the studies undertaken have concentrated on a single species or two or three members of the same genus. Soil food webs are much more complex than this, but few studies have examined the many different components of the soil. There is suspected to be much omnivory within the web, with many species utilising a number of different food resources in common. There are also spatial and temporal variations in the web. However, some authors consider that there is also a great deal of redundancy (Huhta *et al.*, 1998) and that some species may be keystone species in particular soils (Chapter 1).

Two recent studies have used natural abundance stable isotopes to examine macro-, and meso- and micro-invertebrates in different soils to try to assess trophic positions through assimilated $\delta^{13}C$ and/or $\delta^{15}N$. Ponsard and Arditi (2000) studied soil animals (including enchytraeids) in 3 temperate, deciduous forests which were within 1km of each other. One site was on a plateau and the other two on the slopes leading from the plateau. They sampled the three sites at three different time intervals – winter, spring and autumn. They sampled for macro-invertebrates which were roughly divided into detritivores and predators with some scavengers and root, sap or nectar feeders. The $\delta^{13}C$ values of detritivores and predators were homogeneous, but several per mil higher than the litter or soil – more than the 'rule of thumb' 0.4 - 1‰ increase

between an organism and its putative food source. Similar findings had been reported for earthworms (Spain and Le Feuvre, 1997). The authors suggest that this situation is not uncommon in soil detritivores and may be explained by their inability to digest ^{13}C-poor organic components such as lignin so they preferentially assimilate organic carbon which is ^{13}C rich.

Ponsard and Arditi (2000) also found that the $\delta^{15}N$ of the animals did not show distinct trophic bands but were a continuum, in all soils at all time periods. The detritivore values were such that the authors consider them to be a single trophic level. The values for carnivores also suggested that they belong to a second trophic level. However there were a few anomalously high values for spiders and a beetle. This they attributed either to artefact, a third trophic level or to predator predation. They concluded that there was a detritivorous group which fed on isotopically 'light' food (young, superficial litter or components with similar $\delta^{15}N$ values) and a carnivorous group, some of which fed on detritivores and some which fed on each other.

The second study, by Scheu and Falca (2000), was of two beech forests, one of which had a mull soil and the other a moder soil. They sampled once for meso- and macro-fauna, including enchytraeids, and analysed them for $\delta^{15}N$. Their findings were similar to those of Ponsard and Arditi (2000) – that there was a continuum rather than well-defined trophic bands. The two forests differed in their overall $\delta^{15}N$ range; the moder animals spanning 8 delta units and the mull animals 15 delta units. They classified their animals into trophic levels rather differently, dividing the detritivores

into primary and secondary decomposers. The moder forest had one predator band, and the mull forest two predator trophic bands.

Stable isotope enrichment studies.

There are many texts describing stable isotope enrichment studies, and reviews are given by Rundel *et al.* (1989), Lajtha and Michener (1992) and Unkovitch *et al.* (2001).

However, there are fewer studies utilising enriched materials in soil food webs, although there have been some studies of individual species. Utilising an enriched material as a tracer is advantageous, especially if a NA signal would be 'swamped' or difficult to trace.

Setälä and Aarnio (2002) utilised a Canadian Douglas fir stand that had been fertilised with ^{15}N urea ten years previously. It had a well stratified soil and their aim was assess whether the soil biota were vertically stratified within this soil. They found that the litter layer and microbes and animals within it (large and small detritivores, microbivores and large and small predators) had similar ^{15}N values (atom percent excess), indicating that these organisms were feeding in this layer. The F+H layer was 1.45 times more enriched in ^{15}N than the litter layer, with microbes and small detritivores having a similar ^{15}N signal to the soil. All other soil organism groups were relatively depleted in ^{15}N, and close to that of the litter layer. From this they concluded that this soil was compartmentalised and that litter microbes (fungi) do not extend into the F+H layer to sequester nitrogen. They also concluded that in the F+H layer, apart from the small detritivores (enchytraeids and dipteran larvae), the other

groups of organisms utilise the litter layer for food resources and the F+H layer for refuge.

Caner *et al.* (2004) undertook a similar study in beech forests in Germany (with mull humus) and Denmark (with moder humus) using ^{15}N labelled (highly enriched) beech leaves. The German site had beech stands of different ages (40, 70, 120 and 150 years old) and at the Danish site the beech stand was 100 years old. They traced the $\delta^{15}N$ signal from the leaves into the soil underlying litter boxes and into the soil animal community, from which they calculated the amount of nitrogen assimilated by the animals (including enchytraeids) from the labelled beech litter.

They found a number of differences at the two sites. The soil below the litter boxes varied in $\delta^{15}N$ signal after the 9 months of the experiment. At the mull site, the $\delta^{15}N$ values increased with increasing age of the stand to 120 years, then decreased in the oldest stand. At the moder site the $\delta^{15}N$ signal was greater than at the mull site in any age stand of beech.

Soil animals also varied in the amount of litter derived nitrogen they assimilated. At the mull sites the labelled litter derived nitrogen was >10% for small and 5 – 10% for variable sized fungivores, coprophages and herbifungivores with 1 – 5% for saprophages and herbifungivores. At the moder site, saprophages derived 5 – 10% and herbivores and fungivores derived 0.1 – 5% of their nitrogen from the labelled litter.

This study also showed a gradient from primary and secondary decomposers and primary and secondary predators similar to that found in the natural abundance studies of Ponsard and Arditi (2000) and Scheu and Falca (2000).

In these studies Enchytraeidae were shown to be part of the detritivore fauna of woodland soil communities but their specific role in nitrogen dynamics of litter decomposition and the translocation of nitrogen from litter to soil was not clear.

Aims and objectives

The study described here was designed to clarify the role of Enchytraeidae in litter nitrogen dynamics and translocation in an acid grassland modified by the addition of nitrogen fertilizer and also by lime. This aim was met using ^{15}N labelled *Agrostis capillaris* litter in nylon mesh bags in a field experiment at the Rigg Foot site at Sourhope. The specific objectives were to:

1. determine the contribution of enchytraeids to the decomposition and nitrogen dynamics of litter by using litterbags of two mesh sizes, the finer of which would exclude the enchytraeids;
2. assess the impacts of enchytraeids on the translocation of nitrogen from the litterbags by tracing the ^{15}N signal in surrounding soils and plants;
3. evaluate the effects of nitrogen fertilisation on these aspects of enchytraeid ecology in an acid soil and in a soil modified by the addition of lime.

Rationale of modified objectives.

Two different mesh sizes were used for constructing the litterbags. It was intended that the course mesh (200 microns) would allow free movement of enchytraeids into and out of the litterbags, allowing deposition of faecal material outside the bag in the surrounding soil. The fine mesh (20 microns) would exclude enchytraeids and could therefore be used to differentiate between enchytraeid and microbial activity.

Table 3.2 . Summary of litterbag experimental objectives.

Bag type	Enchytraeid activity	Changes inside bags	Changes outside bags
Coarse	Enchytraeids in and out	Enchytraeids feeding inside and outside bags. Deposition of faeces inside bags. Microbial activity.	Enchytraeid faeces. Leaching.
Fine	No enchytraeids in bags.	Microbial activity.	Leaching.

However, when the litterbags were destructively sampled, enchytraeid worms were found in all fine litterbags albeit in smaller numbers than in coarse bags .It was assumed that the worms had entered the bags when they were very small – after fission, for *C. sphagnetorum* or as hatchlings for other species. Observations to be discussed later indicated that once the enchytraeids were inside the bags and had grown, they did/could not get out again.

Standen (1978) had used the same litterbag technique and found no enchytraeids inside the fine bags. Her study used litter taken from the soil surface in a wet peat mire. Possibly the higher quality litter used in the present experiment attracted worms into the fine bags and the more constant moisture inside the fine bags retained them. However, another difference was that the litterbags from the 1978 study were placed on the surface, whereas here the bags were placed within the soil profile.

Table 3.3 . Revised summary of experimental objectives.

Bag type	Enchytraeid activity	Changes inside bags	Changes outside bags.
Coarse	Enchytraeids in and out	Enchytraeids feeding inside and outside bags. Deposition of faeces inside bags. Microbial activity Roots.	Enchytraeid faeces. Leaching.
Fine	Some enchytraeids in but exit restricted.	Enchytraeids feeding inside. Deposition of faeces. Microbial activity. No roots.	Leaching.

A further, unpredicted difference between the bag types was that coarse bags contained many roots whereas fine bags did not. It proved impossible to extract all root material when the bags were retrieved from the field.

3.2 Methods

3.2.1 Litterbag construction.

Nylon mesh obtained from Lockertex Ltd. was used to make fine mesh (20 microns) and coarse mesh (200 microns) litterbags. Pieces of mesh (10 x 8cm), were folded in half lengthways, and stitched using nylon thread and a very small stitch length, leaving the top open. The raw edges were sewn with an overlock stitch.

3.2.2 The litter.

The methods used to incorporate ^{15}N into *Agrostis capillaris* - the chosen food source for the enchytraeids - is described in Chapter 5.

The bags were filled with ^{15}N labelled leaf blade of *A. capillaris*. The leaf blades were roughly chopped and then 1g (weighed to 3 decimal places) was put into each bag. The tops of the bags were then stitched and overlocked with nylon thread. The filled bags had a diameter of 3 cm and were 6 cm in length and would allow a core to be

taken from around the bag with a soil corer with a diameter of 5 cm. The $\delta^{15}N$ value of the litter in each bag was recorded, as each tray of grass grown had different values, ranging from 97 to 145‰ .

Unlabelled litter was placed in coarse mesh bags, constructed in the same way. These bags would be used to assess any major fractionations that occurred in the litter and enchytraeids found in the bags.

3.2.3 Placement of litterbags in soil.

Litterbags were placed in the field on 2nd July 2002. A 1 cm diameter cork borer was used to remove a soil core, and the surface vegetation was cut horizontally for a distance of ~ 2 cm on both sides of the removed core. The litterbags were then placed in the soil with the top of the bag level with the soil surface and the coloured tag above the surface. The small cores removed were used to calculate the $\delta^{15}N$ 'natural abundance' of soil, vegetation and enchytraeids in each treatment plot.

3.2.4 Positioning the litterbags within the experimental plots.

Originally it was intended to place the litterbags in a random manner, but there were a number of constraints to this.

The length of the identifying tag had to be kept <5 cm to ensure that the bags were not accidentally removed during mowing. This would make it difficult to locate them easily.

The subplot that was being used for litterbag placement (Figure 3.1) was also being used by other researchers, and it would have been difficult for others to locate the bags and so avoid them,

The bags containing labelled litter were placed at the bottom of each subplot at intervals of 0.2 m, with alternating coarse and fine bags (Figure 3.1).

Coarse litterbags with unlabelled litter were placed at the top of Block 3 subplots at 0.5 m intervals.

3.2.5 Experimental treatments.

The treatment blocks (Appendix 1) used were control, nitrogen, lime and lime + nitrogen. There were four replicates of control and lime plots and five replicates of nitrogen and L+N plots.

3.2.6 Removal of litterbags

It was planned to remove the litterbag and the core surrounding the bag at intervals of 4, 8, 16, 32 and 64 weeks. The 32 week removal date was at the beginning of February and there was heavy frost at the site at that time. It was considered that the enchytraeids would burrow down into the soil profile during frost episodes, and that the litterbags would yield very few animals. It was decided that the bags would be removed later, when the ground would be thawed. The bags were ultimately removed at 4, 7, 16, 39 and 60 weeks (1, 2, 4, 9 and 14 months). On each occasion, a soil corer of 5 cm diameter was used to take a core of soil surrounding each litterbag. The soil cores were a minimum of 5 cm depth.

3.2.7 Sample processing

Litterbags and each surrounding soil core were removed, bagged and labelled and placed in a cool box. They were stored at 4°C until extraction, which was within 2 days of collection.

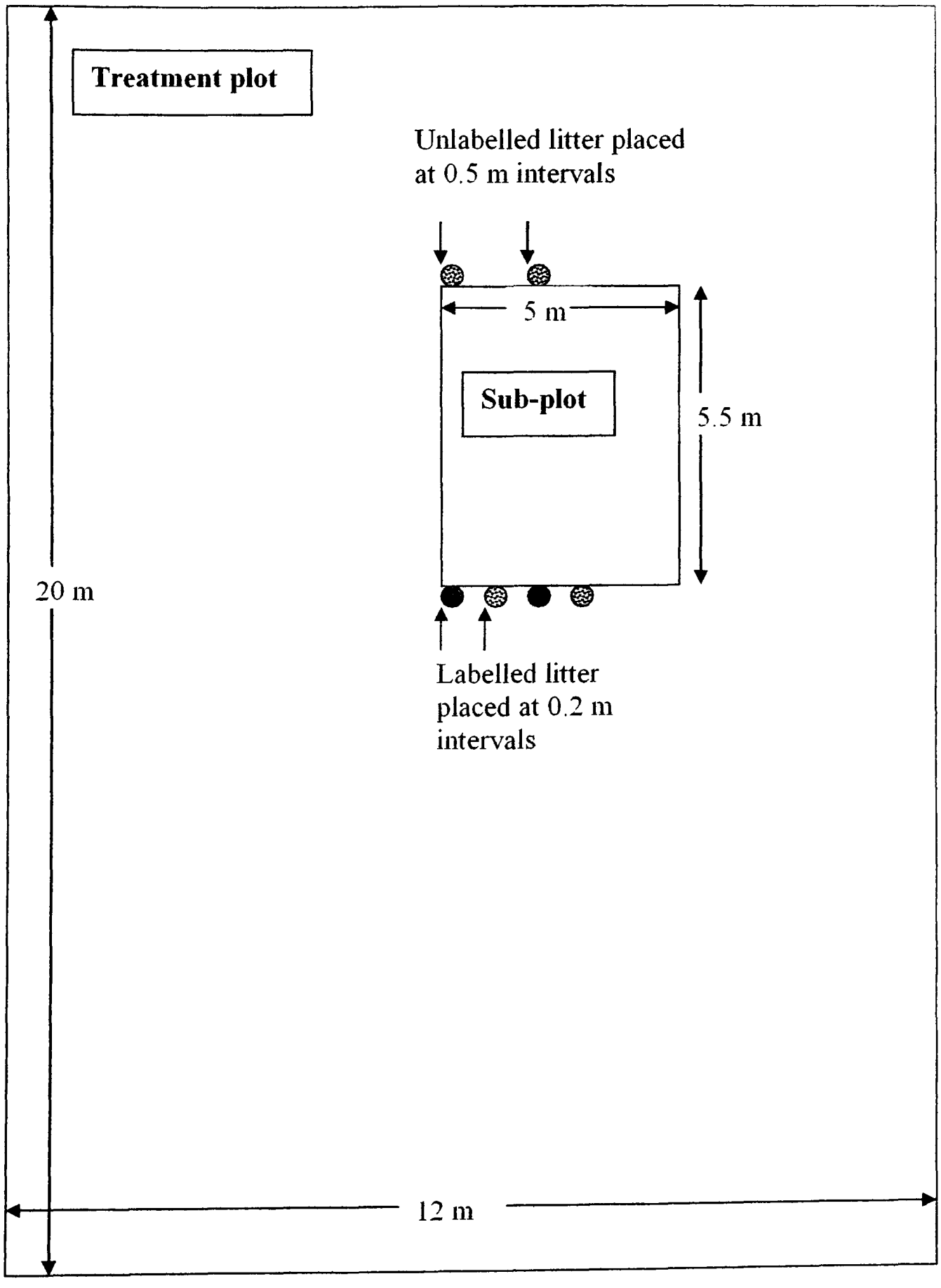


Figure 3.1. Placement of the fine and coarse litterbags within the treatment block and sub plot at Rigg Foot, Sourhope. Litterbags were placed in the ground on 2nd July 2002.

Enchytraeids.

Extraction of the enchytraeids from soil cores was carried out using a wet funnel technique (O'Connor, 1955). Enchytraeids were identified to species level using high power microscopy (Nielsen and Christensen, 1951).

Enchytraeids were extracted from litter by hand sorting for the first sample. However, in subsequent samples when numbers were higher, this became practically difficult due to the time taken; the enchytraeids would continue consuming ^{15}N labelled litter. A 'mini-extractor' wet funnel system was produced which was followed by hand sorting random litter samples to assess efficiency.

The litter from fine bags was first examined to check that they contained no animals. However, at the first time period there were a small number of enchytraeids present. and subsequently more enchytraeids were found, so they were treated the same as litter from coarse bags.

Enchytraeids were placed in Eppendorf tubes, labelled with species, plot, core or bag type and date of removal from the field. They were then placed in a freezer at -10°C, and later freeze-dried for 24 hours (CHRIST Beta 1-8). The final process was to weigh the samples and place them in tin cups for GC-IRMS.

Litter

All of the litter contained in the bags was air-dried and weighed and then bagged and labelled. A homogenous mix was produced by freeze-milling (SPEX, supplied by Glen Creston Ltd). A 10mg sub sample was weighed to 5 decimal places and placed into tin cups for GC-IRMS.

Soil, roots and shoots.

Soil cores were air dried and placed in labelled bags. Each core had a small hole in which the litterbag had been placed. The soil surrounding the hole was separated, and as much root material as possible removed. This was freeze-milled to produce a homogenous sample.

Live shoots at the top of soil cores were removed and freeze ground using a pestle and mortar and liquid nitrogen.

Roots which had infiltrated the litterbags were prepared in the same way, after removing as much soil from them as possible.

'Background' natural abundance samples for enchytraeids, vegetation and soil were collected at the time of litterbag placement and prepared and analysed in the same way.

^{15}N Analysis

Samples were weighed into tin capsules and analysed using either a Eurovector Elemental Analyser (Eurovector, Milano, Italy) coupled to an Isoprime Isotope Ratio Mass Spectrometer (GVI, Manchester, UK) or a Carlo-Erba NA1500 Elemental Analyser coupled to a modified Dennis Leigh Technology IRMS (Supplied by Provac Services, Crewe, UK).

Data handling and analysis.

Statistical analyses were carried out using SPSS V11.0 for all data except those for tissue turnover. The Kolmogorov-Smirnov test was carried out on all datasets to assess normality of data prior to analysis. One way ANOVA (with *post hoc* Tukey test where appropriate) and independent sample t-tests were performed on all data, except enchytraeid numbers for which a two-tailed t-test was performed. Factorial and mixed model ANOVA were performed using SAS v9.0 after logarithmic transformation of enchytraeid numbers (n+1) and square root transformation of %N release and incorporation (n+0.5).

Litter nitrogen dynamics followed the method used by Caner *et al.* (2004) and Zeller and Colin-Belgrand (2000). The simultaneous release of litter N and incorporation of external N was calculated according to the following equations.

$$\text{Released N} = \frac{(\text{mass } {}^{15}N_{T0} - \text{mass } {}^{15}N_{T1}) - R_{STD}(\text{mass } N_{T1} - \text{mass } N_{T0})}{R_{litter} - R_{STD}}$$

$$\text{Incorporated N} = \text{mass } N_{T1} - (\text{mass } N_{T0} - \text{mass } N_{released})$$

where $R = {}^{15}N/{}^{14}N + {}^{15}N$. R_{STD} is the ^{15}N value of unlabelled litter (n = 3) and R_{litter} is the ^{15}N value of the labelled litter at each time period (n = 4 or 5). Values given in the text are actual % release, not % release of initial N.

Nitrogen assimilated from labelled litter by enchytraeids extracted from coarse litterbags followed the method of Caner *et al.* (2004) using the following equation:

$$\text{Assimilated N} = \frac{\delta\ {}^{15}N_{T1,2..}\ \text{enchytraeid} - \delta\ {}^{15}N_{NA}\ \text{detritivore}}{(\delta\ {}^{15}N\ \text{deposited litter} + \delta\ {}^{15}N\ \text{collected litter})/2 \times 100}$$

Testing the difference between regression lines was carried out following the method by Fowler, Cohen and Jarvis (1998)

Step 1: b_1-b_2.

Step 2: standard error of the difference

$\sqrt{SE_{b1}^2 + SE_{b2}^2}$

Step 3: $t = b_1\text{-}b_2/\sqrt{SE_{b1}^2 + SE_{b2}^2}$

Tissue turnover rate was calculated from the equation $Y = a + be^{ct}$ where $Y = \delta\ ^{15}N$, a and b were related to the initial and final isotopic composition, c was the rate of incorporation of dietary nitrogen and t was the time of the experimental period. The exponential curve fitted to the data was performed using SigmaPlot V9.0 and the relevant equation f=y0+a*(1-exp(-b*x)) The half-life was then calculated using the equation *half-life* = (In 2)/c.

Graphs were plotted using either SigmaPlot v9.0 or Microsoft Excel 2003.

3.3 Results.

3.3.1 General observations on litterbags and enchytraeids.

The external appearance of litter within both coarse and fine litterbags did not change over the whole experimental period. To the naked eye, and under low power microscopy, it retained the features and colour of the grass blades that had been used. However, both coarse and fine litterbags did contain fruiting bodies of Ascomycete fungi. Although not quantified, the fine litterbags seemed to be moister than the coarse bags. The surface of the fine bags mesh generally had a growth of very fine, greenish material, assumed to be of microbial/algal origin.

The main difference between the coarse and fine bags was the presence of plant roots (and occasionally 'runners') infiltrating the coarse bags. Small quantities of very fine roots surrounded the fine bags, but there was very little penetration into the bags (Plate 3.1), although the reason for this is not known.

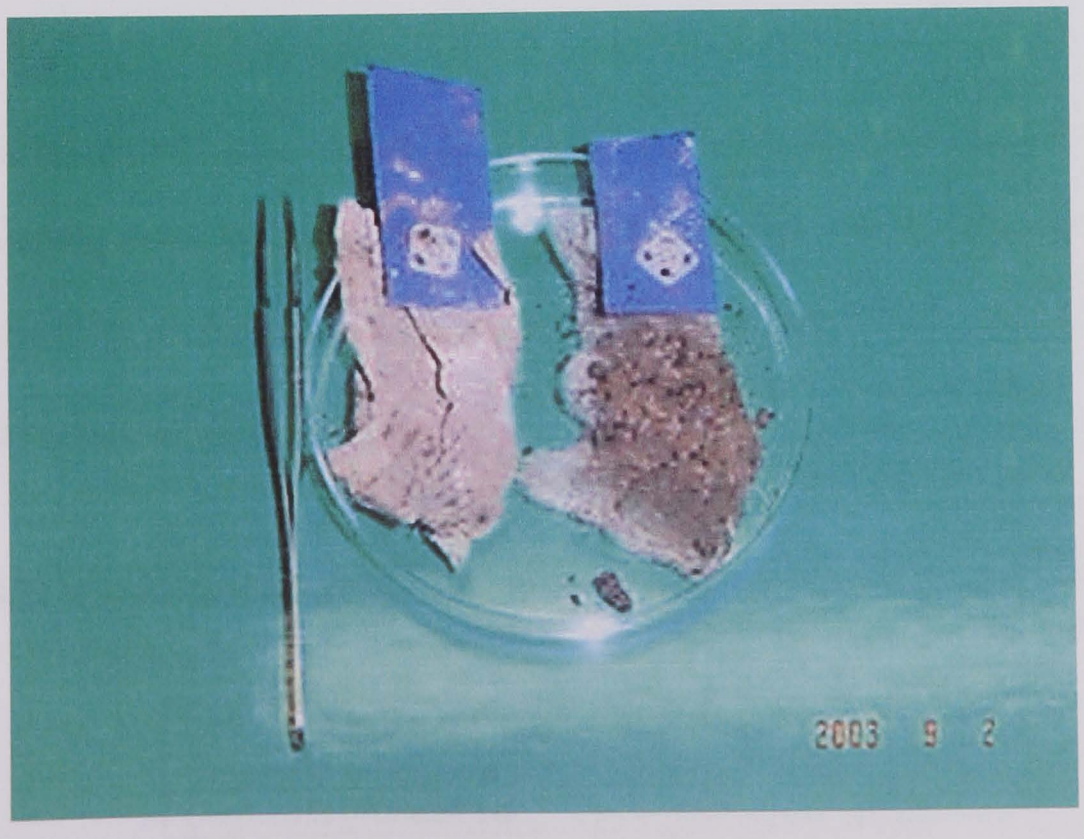


Plate 5. Coarse and fine litterbags extracted from treatment plots at Rigg Foot, Sourhope in August 2003. The coarse mesh bag on the right has many roots and soil around, and infiltrating the bag. The fine mesh bag on the left has a few fine roots around it.

One fine bag contained only diptera larvae and faeces as all the litter had been consumed. Many bags contained Collembola, and occasional bags contained mites. One bag also contained a small earthworm, and another an adult fly. The $\delta^{15}N$ values of each of these animals are given in Table 3.4.

Table 3.4. $\delta^{15}N$ values of other animals found in litterbags.

Animal	Plot	Bag type	Time	$\delta^{15}N$
Diptera larvae	L+N	Fine bag	9 months	45.14‰
Adult fly	Lime	Fine bag	14 months	125.82‰
Earthworm	Control	Coarse bag	14 months	10.16‰

The litter $\delta^{15}N$ values in bags containing diptera larvae and an earthworm were no different to the values of other bags from the same plots.

The soil conditions changed in the second year of the experiment as a consequence of low rainfall during the summer of 2003; the soil was very dry and few enchytraeids were extracted from either litterbags or surrounding cores.

Over the entire experiment, only five species of enchytraeids were found in the litterbags and surrounding cores in control and nitrogen plots (Table 3.5). Only *C. sphagnetorum* was analysed for $\delta^{15}N$, as this species was dominant in soils with low pH and accounted for 99% of all enchytraeids sampled.

Table 3.5. List of enchytraeid species found in litterbags in control and nitrogen plots at Rigg Foot, Sourhope from 2002 to 2003.

Litterbags in control plots	Litterbags in nitrogen plots
Cognettia sphagnetorum	*Cognettia sphagnetorum*
Cognettia glandulosa	*Cognettia glandulosa*
Cognettia cognettii	*Enchytraeus buchholzi*
Enchytraeus buchholzi	*Henlea perpusilla*
Fridericia. bisetosa	Immature *Fridericia*

In the limed and L+N plots, 22 species of enchytraeids were found in litterbags and surrounding cores. Only the most abundant species were analysed for $\delta^{15}N$; *Fridericia galba, F. perrieri, Henlea perpusilla.* Table 3.6 is a list of species present in the lime and L+N plots.

Table 3.6. List of species extracted from litterbags and surrounding cores from lime and L+N plots at Rigg Foot, Sourhope from 2002 to 2003.

Species	Litterbags	Cores
Buchholzia appendiculata	*	*
Cognettia glandulosa	*	*
Cognettia sphagnetorum	*	
Enchytraeus buchholzia	*	*
Enchytronia parva	*	
Fridericia bisetosa	*	*
Fridericia. bulboides	*	
Fridericia galba	*	*
Fridericia hegemon		*
Fridericia leydigi		*
Fridericia maculata	*	
Fridericia paroniana	*	
Fridericia. perrieri	*	*
Fridericia ratzelli	*	*
Fridericia regularis		*
Fridericia striata	*	*
Henlea nasuta		*
Henlea perpusilla	*	*
Henlea ventriculosa	*	
Marionina minutus	*	
Mesenchytraeus flavus		*
Mesenchytraeus sanguineous	*	

3.3.2 Colonisation of litterbags and cores by enchytraeids.

Colonisation of coarse and fine litterbags by enchytraeids are treated separately in the following two sections.

3.3.2.1 Coarse mesh litterbags and surrounding soil cores.

This section describes the numbers and biomass of enchytraeids in coarse mesh bags and soil cores in control, nitrogen, lime and L+N plots.

i. ***Numbers of C. sphagnetorum in litterbags and cores in control and nitrogen plots***

The number of enchytraeids (99% *C. sphagnetorum*) in litterbags and surrounding soil cores at each sampling time was recorded. The rate of colonisation of the coarse litterbags and the number of *C. sphagnetorum* extracted from surrounding soil cores is given in Table 3.7.

Table 3.7. Mean numbers of enchytraeids extracted from coarse litterbags and cores in control (n = 4) and nitrogen (n = 5).plots at Rigg Foot, Sourhope during the 14 months of the experiment. Figures in parentheses = ±1SE

	July	August	October	March	August	Mean number per bag
Control bags	13 (1)	45 (22)	109 (48)	9 (4)	1 (1)	36 (20)
Control cores	16 (3)	48 (15)	88 (40)	17 (4)	8 (2)	32 (15)
Nitrogen bags	9 (2)	30 (5)	17 (8)	9 (5)	0	13 (5)
Nitrogen cores	10 (3)	6 (3)	49 (29)	15 (9)	1 (1)	14 (8)

Maximum mean numbers of enchytraeids per bag were 109 at four months in the control plots, with bags from two of these treatment plots containing a total of 220 and 158 individuals. In the nitrogen plots there was a maximum mean of 30 individuals in bags after two months. In March (when the soil temperature was rising after winter), bags from both treatment plots yielded the same number of individuals. By the end of the experiment, after a very dry summer, very few enchytraeids were extracted from the bags. Numbers of

enchytraeids extracted from the soil cores surrounding the litterbags were similar to those extracted from bags.

Spearman's rank-order correlation indicated a significant positive association between number of enchytraeids in bags and cores (control - $r_s = 0.585$, d.f. = 20, $P < 0.01$, and nitrogen - $r_s = 0.61$, d.f. = 24, $P < 0.01$). This gave a ratio of ~ 1:1 in both plots.

Overall, there were half as many enchytraeids found in bags and cores in nitrogen plots as there were in control plots. The rate of colonisation for the control and nitrogen bags was not significantly different except in October when the mean number of enchytraeids was higher in control plots than nitrogen ($ANOVA_{1,7}$, F = 4.487, $p < 0.05 > 0.1$).

ii. ***Biomass of C. sphagnetorum in coarse mesh litterbags and surrounding soil cores.***

The worms from litterbags used for stable isotope (SI) analysis were freeze dried and weighed to give the mean dry weight per worm. The number of worms found in litterbags was then used to calculate the total dry biomass of all *C. sphagnetorum* found in litterbags and surrounding cores (Table 3.8).

Table 3.8. Dry weights (mg) of *C. sphagnetorum* analysed for ^{15}N stable isotope (SI) and total dry weight of the enchytraeids extracted from coarse litterbags and surrounding cores in control and nitrogen plots at Rigg Foot, Sourhope.

Plot type	Number of worms used for SI analysis	Biomass/mg	Mean biomass (mg)/worm	Mean number of worms in bags/cores	Mean biomass of worms in bags/mg	Total biomass/mg (total no. worms)
Control bags	249	6.868	0.028	36	0.99	19.6 (710)
Nitrogen bags	210	5.915	0.028	13	0.37	8.8 (312)
Control cores	350	10.824	0.031	32	0.99	32.7 (1056)
Nitrogen cores	225	6.607	0.029	14	0.41	15.4 (523)

The total biomass of *C. sphagnetorum* extracted from litterbags and cores in control plots was more than twice that of *C. sphagnetorum* from nitrogen plots.

iii. *Number of enchytraeids present in lime and L+N plots in coarse bags and surrounding cores.*

Liming the acid grassland at Sourhope has resulted in a rise in pH and is therefore treated in this study as a neutral soil. Neutral soils support diverse communities of enchytraeids, but at lower density than *C. sphagnetorum* which, if present at all, occur only in very small numbers (Hågvar and Abrahamsen, 1980; Standen, 1984).

The number and species of enchytraeids found in the litterbags and cores at each sampling time was recorded. Table 3.9 shows the rate of colonisation of coarse litterbags and surrounding cores by all species of enchytraeid.

Table 3.9. Mean number of enchytraeids extracted from coarse litterbags and cores in lime (n = 4) and L+N (n = 5) plots at Rigg Foot, Sourhope during the 14 months of the experiment.

Months of experiment	July	August	October	March	August	Mean number per bag
Lime bags	10 (3)	11 (3)	19 (8)	18 (10)	0	12
Lime cores	5 (2)	10 (5)	25 (13)	17 (10)	5 (2)	12
L+N bags	16 (8)	)19 (4)	12 (5)	5 (3)	1 (1)	11
L+N cores	3 (1)	6 (3)	7 (3)	5 (2)	6 (4)	5

Maximum numbers of enchytraeids were extracted from litterbags in lime plots in October 2002 and March 2003, but relatively large numbers of individuals were present after 1 month. In L+N plot litterbags, the highest numbers of enchytraeids were present in July and August, and numbers dwindled thereafter. The total numbers of individuals collected throughout the experimental period were similar.

The surrounding cores in lime plots yielded approximately the same number of enchytraeids as the litterbags, but only half as many from L+N cores.

The number of individuals from litterbags and cores in lime plots were plotted against each other and Spearman's rank-order correlation performed but the association was not significant.

3.3.2.2 Fine mesh litterbags and cores.

This section describes the number and biomass in fine mesh bags and cores from acid and neutral plots.

i. ***Numbers of C. sphagnetorum in litterbags and cores in control and nitrogen plots.***

The fine bags in the control and nitrogen plots contained predominantly *C. sphagnetorum* as in coarse bags but only approximately half the total numbers were extracted. Only *C. sphagnetorum* were analysed for $\delta^{15}N$.

In control plots, there were very few enchytraeids present after one month but the number of individuals increased and by March 2003 there was a mean of 29 individuals – the maximum number found in the fine bags. At the end of the unusually dry summer, a mean of 21 individuals were extracted from the fine bags. In nitrogen plots the maximum mean number of enchytraeids was reached by August 2002 – 12 worms. By the end of the experiment only a mean of only 2 worms were extracted (Table 3.10).

Table 3.10. The mean number, of *C. sphagnetorum* in fine litterbags and cores in control (n = 4) and nitrogen (n = 5) plots at Rigg Foot, Sourhope during the 14 months of the experiment. Figures in parentheses are 1 SE.

	July	August	October	March	August	Mean number of enchytraeids/bag
Control bags	1	21 (10)	18 (8)	29 (8)	21 (9)	18 (5)
Control cores	10	31 (10)	92 (50)	29 (5)	4 (2)	33 (15)
Nitrogen bags	4	12 (4)	4 (2)	8 (5)	2 (2)	6 (2)
Nitrogen cores	17	55 (38)	9 (5)	10 (4)	1 (1)	18 (9)

ii. *Biomass of C. sphagnetorum in fine mesh litterbags.*

The dry weight of enchytraeids was calculated for worms sent for analysis in the same way as for coarse litterbags. The mean dry weight of individual worms was 0.0305 mg in control and 0.0259 mg in nitrogen plots. The worms in litterbags in the nitrogen plots supported about ⅓rd of the total dry weight of the control plots (Table 3.11).

Table 3.11. Total dry weight biomass of *C. sphagnetorum* extracted from fine litterbags in control and nitrogen plots at Rigg Foot, Sourhope.

	Number of worms used for SI analysis	Biomass/ mg	Mean biomass (mg)/worm	Mean no. of worms in bags	Mean biomass of worms in bags/mg	Total biomass/mg (total no. worms)
Control	264	8.045	0.0305	18	0.548	10.849 (356)
Nitrogen	96	2.486	0.0259	6	0.155	3.859 (149)

C. sphagnetorum from surrounding soil cores were collected for analysis, but there was a technical difficulty which meant that they could not ultimately be analysed, therefore there are no biomass data for the enchytraeids in soil cores.

iii. *Number of enchytraeids present in lime and L+N plots in fine bags and surrounding cores.*

In total, the number of enchytraeids extracted from fine litterbags was about one third of the number extracted from coarse litterbags.

In lime plots, the number of individuals extracted from fine bags was roughly the same until October 2002, then decreased sharply over the winter of 2002 – 03. The pattern of

colonisation was different in L+N plots, with few enchytraeids present in July 2002 and maximum numbers of individuals present in August 2002. Thereafter fewer individuals were extracted (Table 3.12).

Table 3.12. The mean number, and total number, of enchytraeids in fine litterbags and cores in lime and L+N plots at Rigg Foot, Sourhope during the 14 months of the experiment. Numbers of replicates varied and can be seen in Figure 18.

	July	August	October	March	August	Mean number of enchytraeids/bag
Lime bags	10 (3)	12 (3)	12 (5)	12 (4)	2 (2)	9
Lime cores	-	22 (12)	18 (8)	11 (5)	3 (2)	14
L+N bags	3 (1)	20 (5)	9 (4)	3 (2)	1 (1)	7
L+N cores	-	6 (3)	19 (12)	8 (4)	6 (4)	10

3.3.3 Litter mass loss and nitrogen dynamics

The experiment was set up using coarse and fine mesh litterbags. The fine litterbags were intended to exclude enchytraeids, but unfortunately this did not happen. Once inside these bags, it appeared that as the enchytraeids grew larger they were unable to exit the bags and thus the fine mesh litterbags became almost a microcosm experiment in a field setting. They have therefore been described separately, but have also been used as a comparison with the coarse mesh bags.

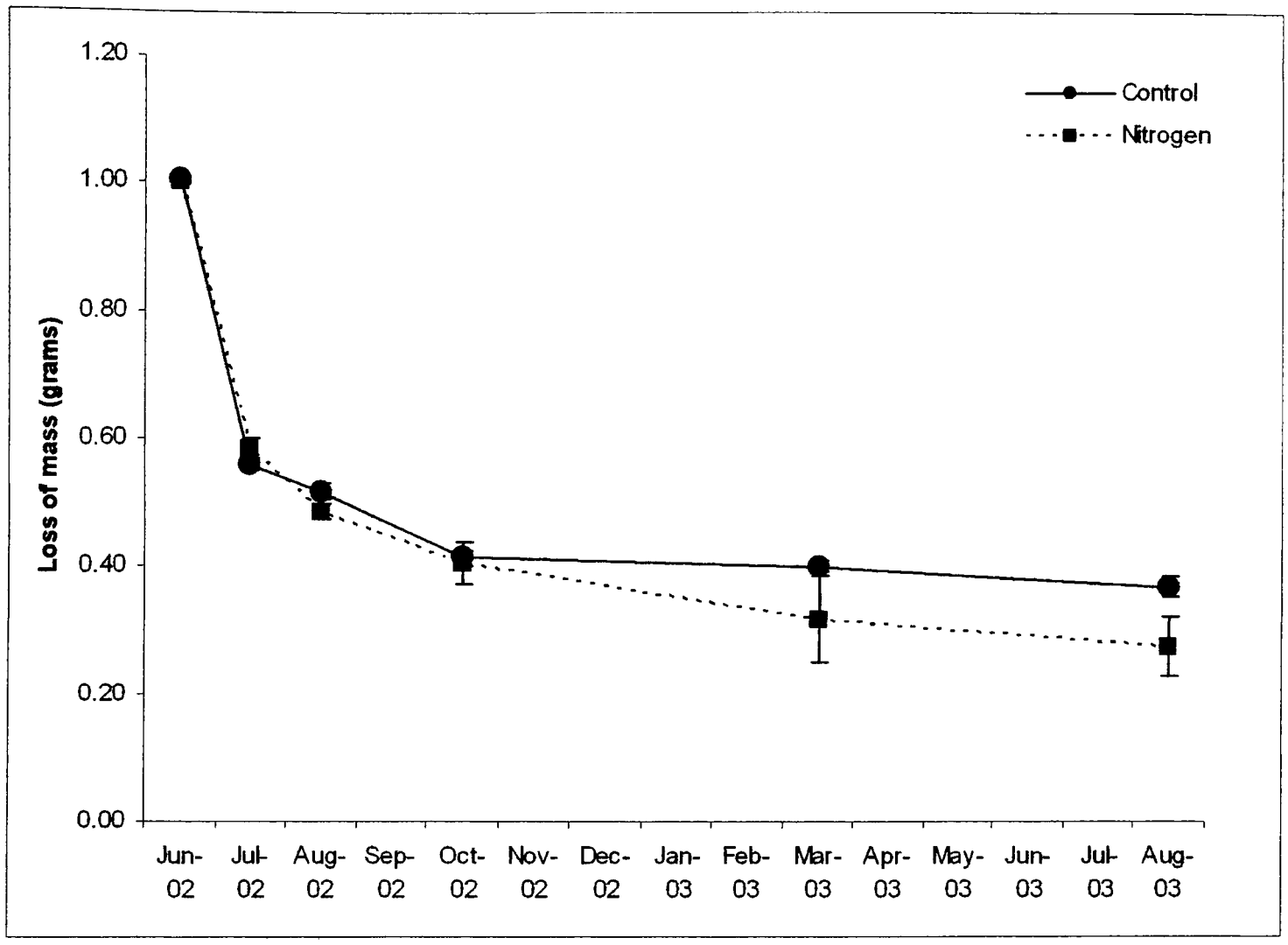
3.3.3.1 Coarse mesh litterbags

In this section litter mass loss, changes in litter C and N and N dynamics are described for treatment types.

1. Mass loss

The mass of litter at the start of the experiment was 1g in all litterbags. There was a rapid initial rapid loss of litter mass of ~ 0.4g in all treatment plots with a further mass loss of 0.2g by the end of the first summer (Figure 3.2).

In control plots there was no further mass loss – overall loss of 63.4% (± 1.7). Overall mass loss was 72.6% (± 4.6) in nitrogen, 71.4% (± 1.8) in lime and 67% (± 6.8) in L+N plots.



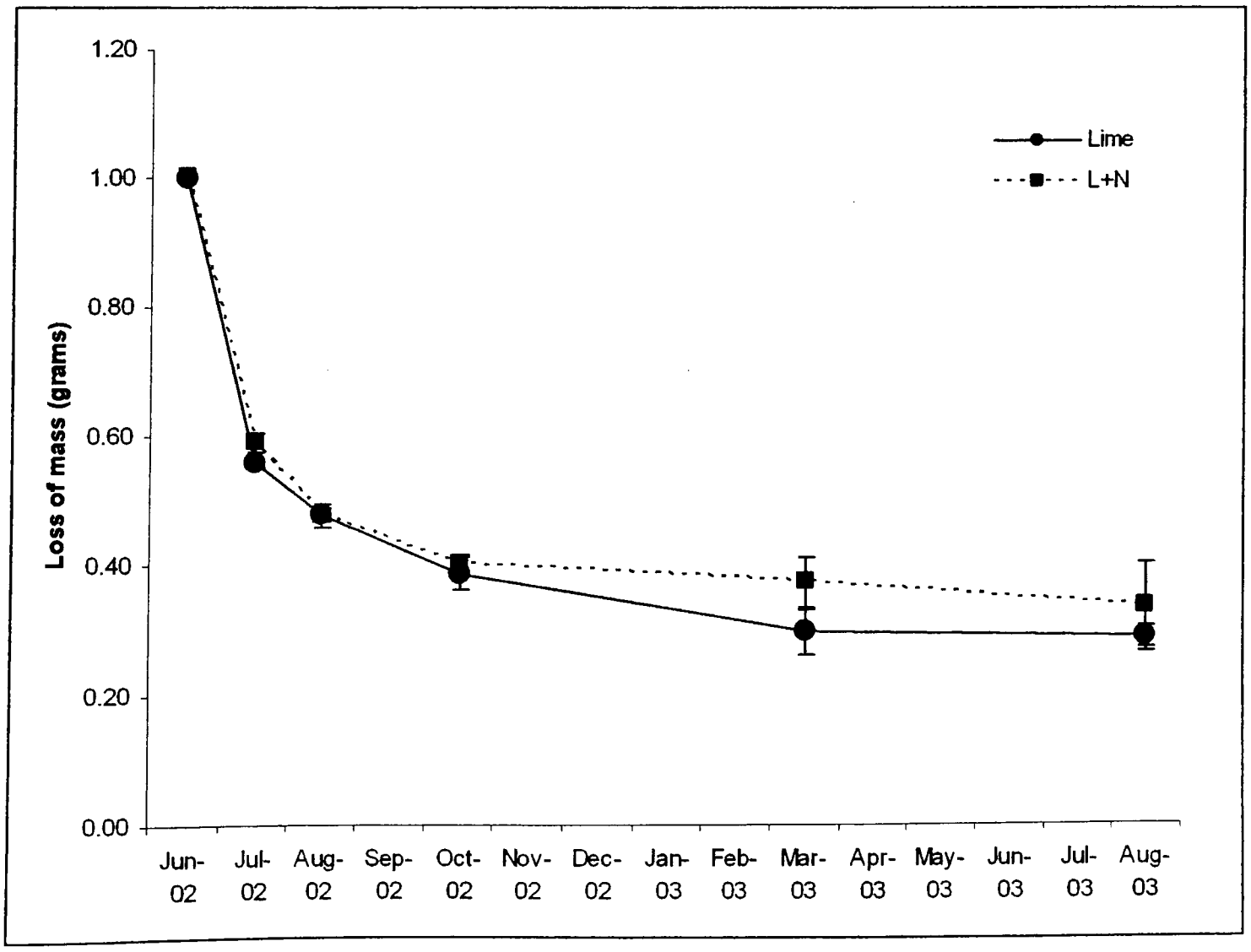
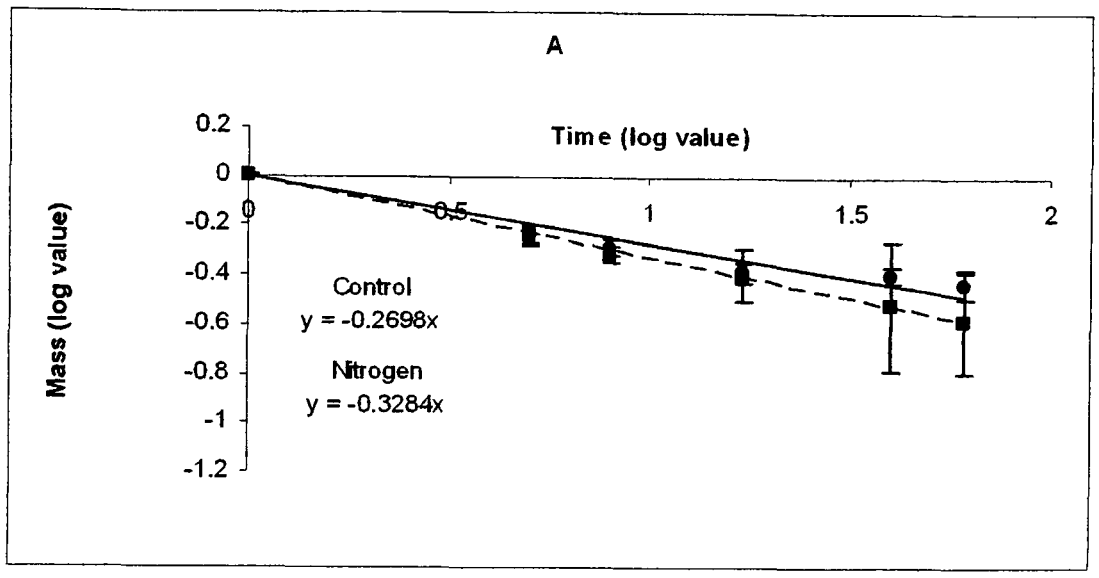
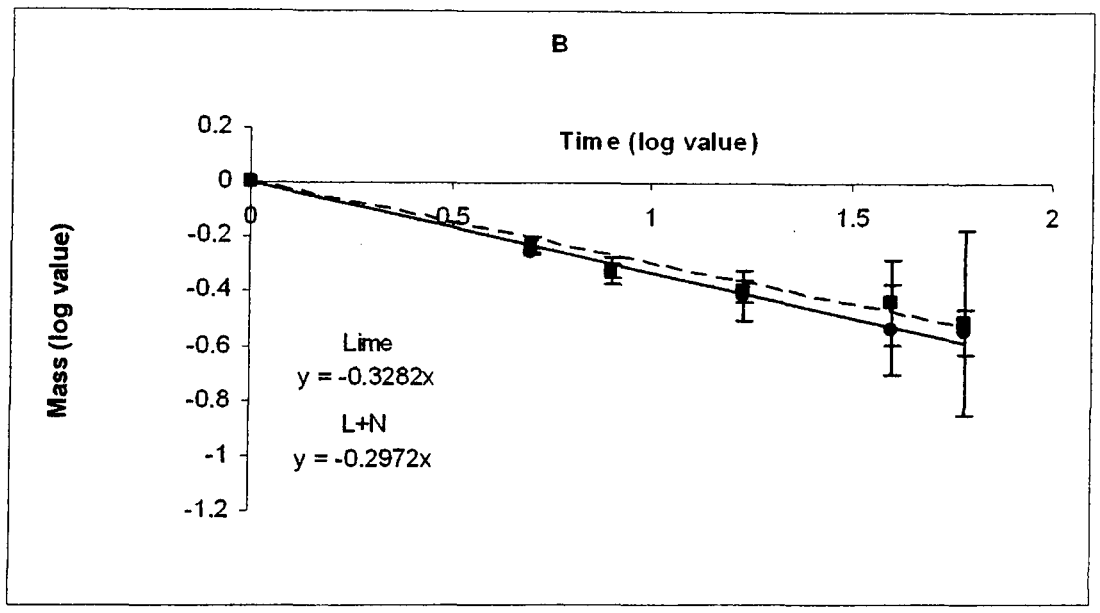


Figure 3.2. Mass loss from coarse litterbags in the treatment plots at Rigg Foot, Sourhope. Means ± 1SE. Experiment started June 2002.

Regression analysis.

The mass loss data (y-axis) and time in weeks (x-axis) were log transformed and plotted with their respective regression equations (Figure 3.3).





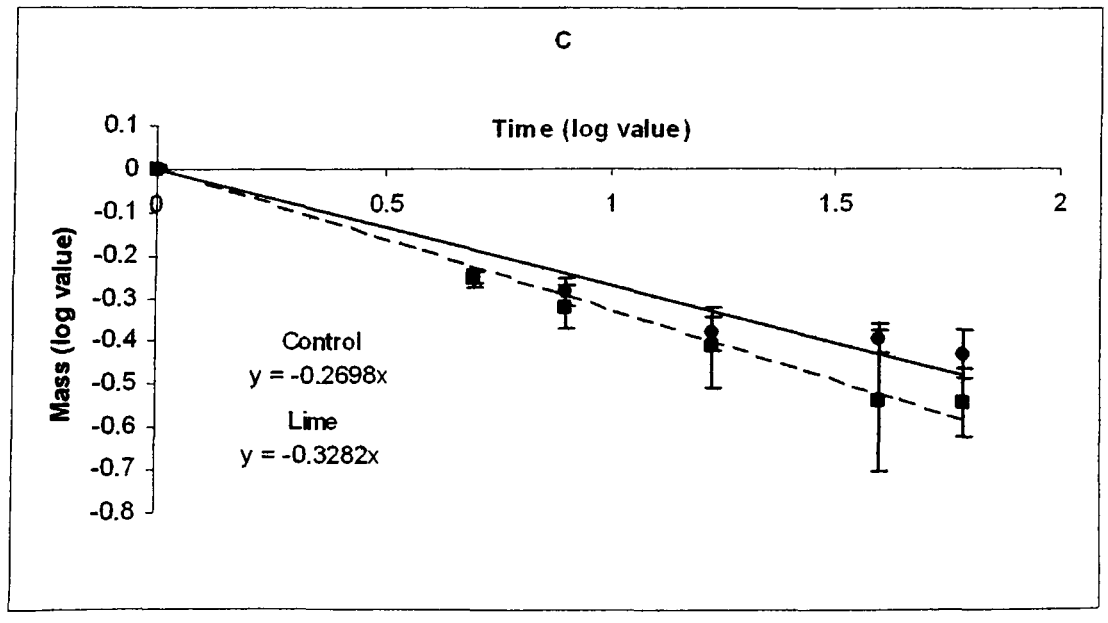


Figure 3.3. Regression lines fitted to *x* and *y* log transformed data for mass loss of litter in coarse litterbags. Geometric means ± 95% CI.

A. Control (solid line) and nitrogen (dashed line) plot litterbags.
B. Lime (solid line) and lime + nitrogen (dashed line) plot litterbags.
C. Control (solid line) and lime (dashed line) plot litterbags.

The significance of the regression lines was tested between treatments (Table 3.13). Litter in nitrogen plots lost significantly more mass than that in control plots, but mass loss was no different when comparing lime and L+N plots. Comparison of control and limed plots without addition of nitrogen showed that lime plots had a significantly greater loss of mass than control plots.

Table 3.13. Litter mass loss from coarse litterbags. Calculations of differences between regression lines, *t* critical (2-tailed) and significance level.
NB Statistics calculated on log transformed data. For calculations, see Methods.

	Step 1	**Step 2**	**Step 3**	**df**	***t* critical**	**Significance level**
Control & nitrogen coarse	0.089	0.032	2.749	48	2.011	**0.01**
Lime & L+N coarse	-0.033	0.033	-0.995	48	2.011	NS
Control & lime coarse	0.078	0.024	3.240	44	2.015	**0.005**

Coarse mesh litterbags (continued):

2. Litter carbon and nitrogen.

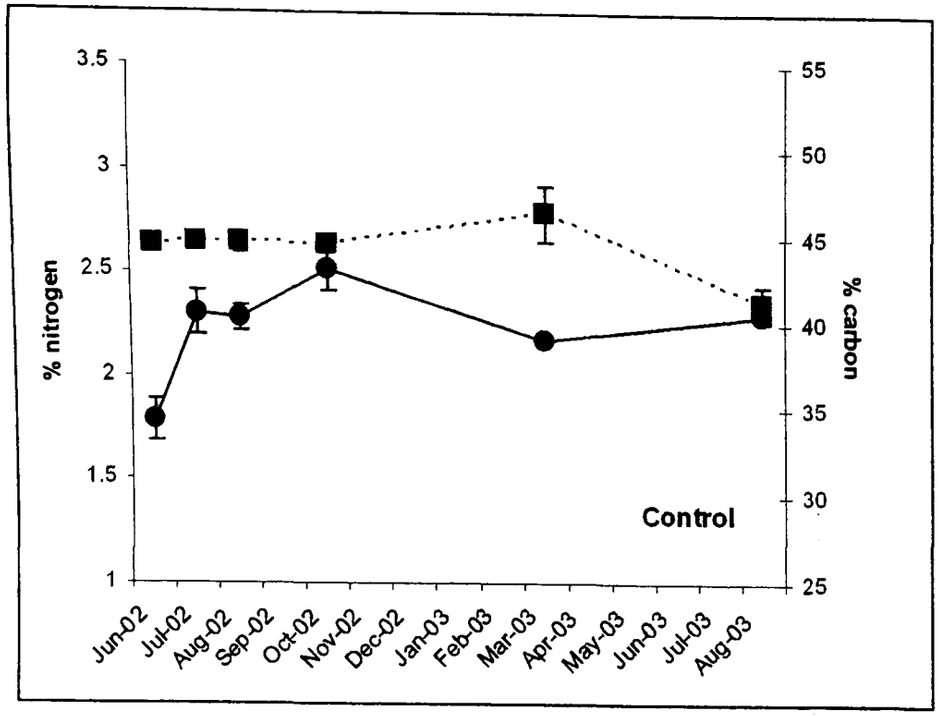
This section describes changes in % carbon and % nitrogen of litter, mass of carbon and nitrogen, change in C:N ratio, change in δ ^{15}N value of litter and change in mass of ^{15}N in coarse litterbags.

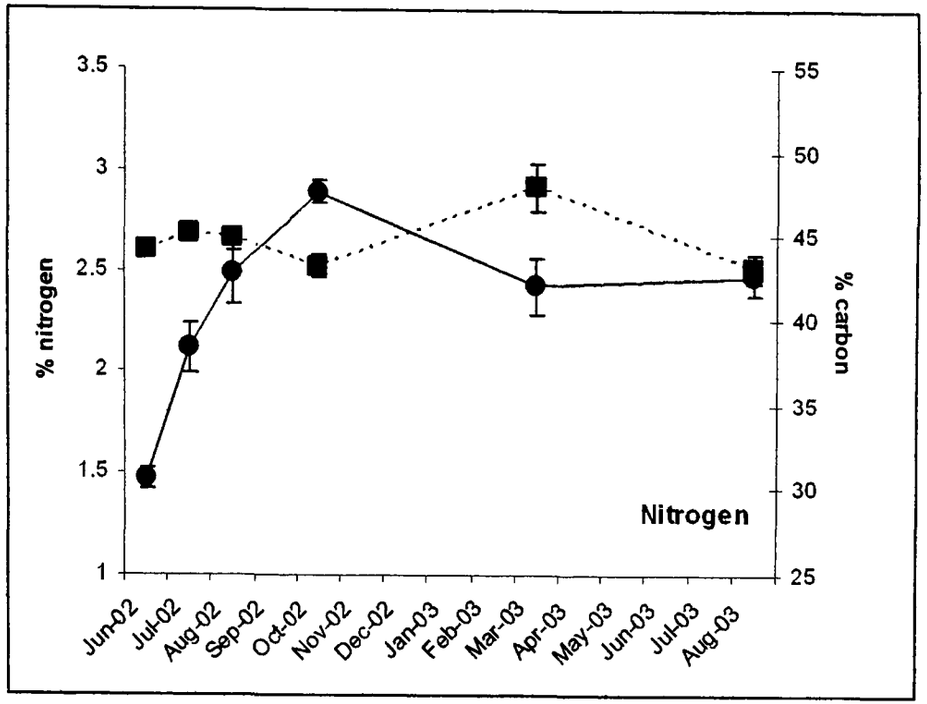
i. *% carbon and % nitrogen of the litter.*

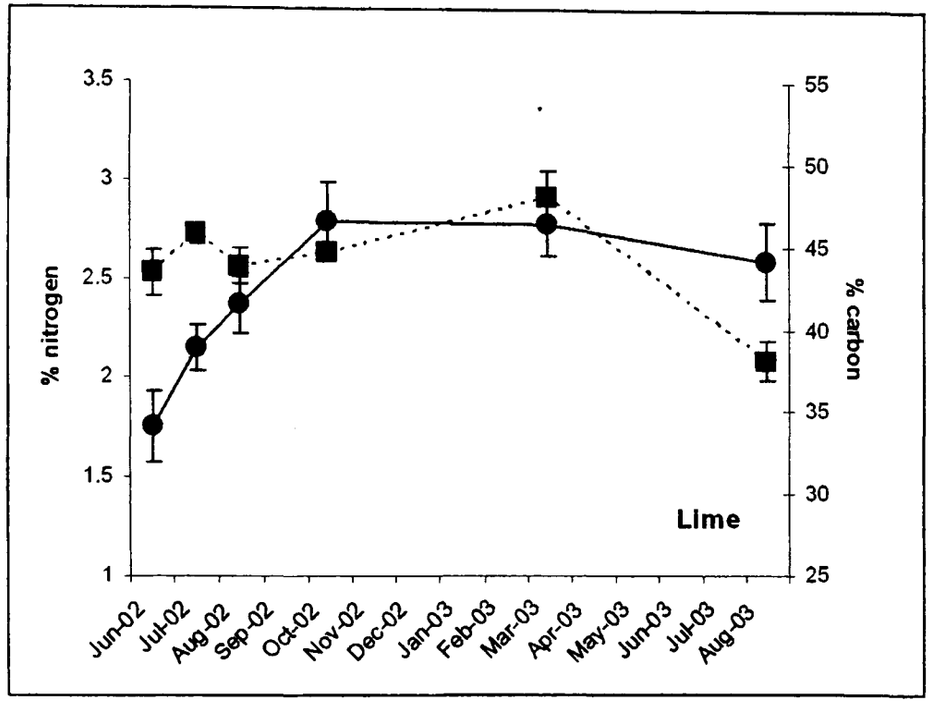
The initial % N of litter was higher in control and lime plots (~1.75%) than nitrogen and L+N plots (~ 1.5%) although these values were not significantly different (One-way ANOVA, $df_{3,14}$, $F = 2.038$, $p = 0.16$).

Figure 3.4 shows the changes that occurred in the % C and N of the litter remaining in coarse litterbags during the 14 months of the experiment. In all plots %N increased until October 2002, the increase being greatest in nitrogen and lime plots. It then declined over winter in control and nitrogen plots and, to a lesser extent, in lime and L+N plots over the second summer. %N was higher at the end of the experiment in all plots than at the start.

Carbon content varied between plots, but over the first summer it was relatively steady in control but fluctuating erratically in nitrogen, lime and L+N plots. In all plots %C fell sharply after March 2003, during the second summer.







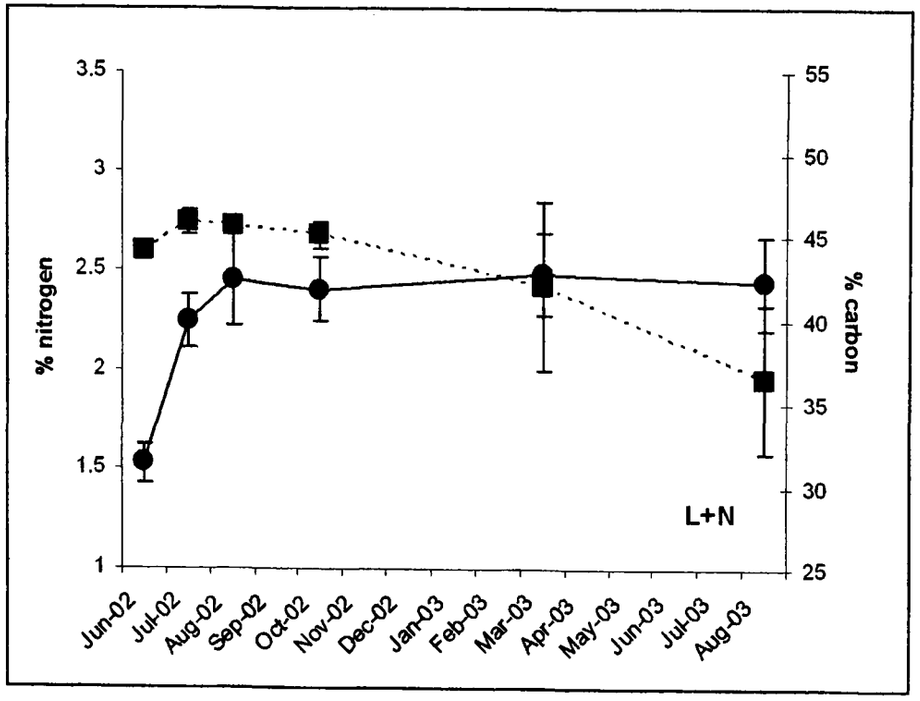


Figure 3.4. Changes in %nitroger and % carbon in litter in coarse litterbags at Rigg Foot, Sourhope Means ± 1SE. N=4 (control and lime), N=5 (nitrogen & L+N)

ii. ***Mass of carbon and nitrogen.***

Figure 3.5 shows the mean mass of litter remaining and the mass of C and N at each sampling time. Generally, all three properties followed the same pattern across all treatments although in nitrogen and lime plots N loss was almost static between July and October, with a subsequent large decrease over winter. There were no significant differences between nitrogen or carbon loss between the different treatment plots.

Error bars were omitted from the chart as the trends in loss of mass were obscured by them. Means (±1SE) are presented in Table 3.14.

Table 3.14. Means (± 1SE) for mass loss of litter, carbon and nitrogen in coarse litterbagsat each sampling date and for each treatment plot at Rigg Foot, Sourhope.

Treatment	Time	Mass loss of litter (g)		Mass loss of carbon (mg)		Mass loss of nitrogen (mg)	
		Mean	1 SE	Mean	1 SE	Mean	1 SE
Control	June	1.00	0.00	446	5	17.9	1.0
	July	0.56	0.01	250	6	12.9	0.7
	August	0.52	0.01	232	7	11.8	0.2
	October	0.41	0.01	184	4	10.3	0.2
	March	0.40	0.01	187	5	8.8	0.2
	August	0.37	0.02	153	6	8.5	0.2
Nitrogen	June	1.00	0.00	442	4	14.7	0.5
	July	0.58	0.02	263	9	12.3	0.8
	August	0.49	0.01	218	3	12.1	0.8
	October	0.40	0.03	174	11	11.7	0.8
	March	0.32	0.07	150	26	7.6	1.3
	August	0.28	0.05	120	22	6.8	1.1
Lime	June	1.00	0.00	433	13	17.5	1.8
	July	0.56	0.01	256	5	12.0	0.6
	August	0.48	0.02	209	8	11.4	1.1
	October	0.39	0.02	172	11	11.0	1.4
	March	0.30	0.04	142	20	8.2	0.8
	August	0.29	0.02	109	5	7.4	0.2
L+N	June	1.00	0.00	441	5	15.3	1.0
	July	0.59	0.01	272	11	13.2	0.7
	August	0.48	0.01	219	4	11.7	0.9
	October	0.40	0.01	182	8	9.6	0.3
	March	0.37	0.04	158	28	9.2	1.1
	August	0.33	0.07	116	21	7.7	1.4

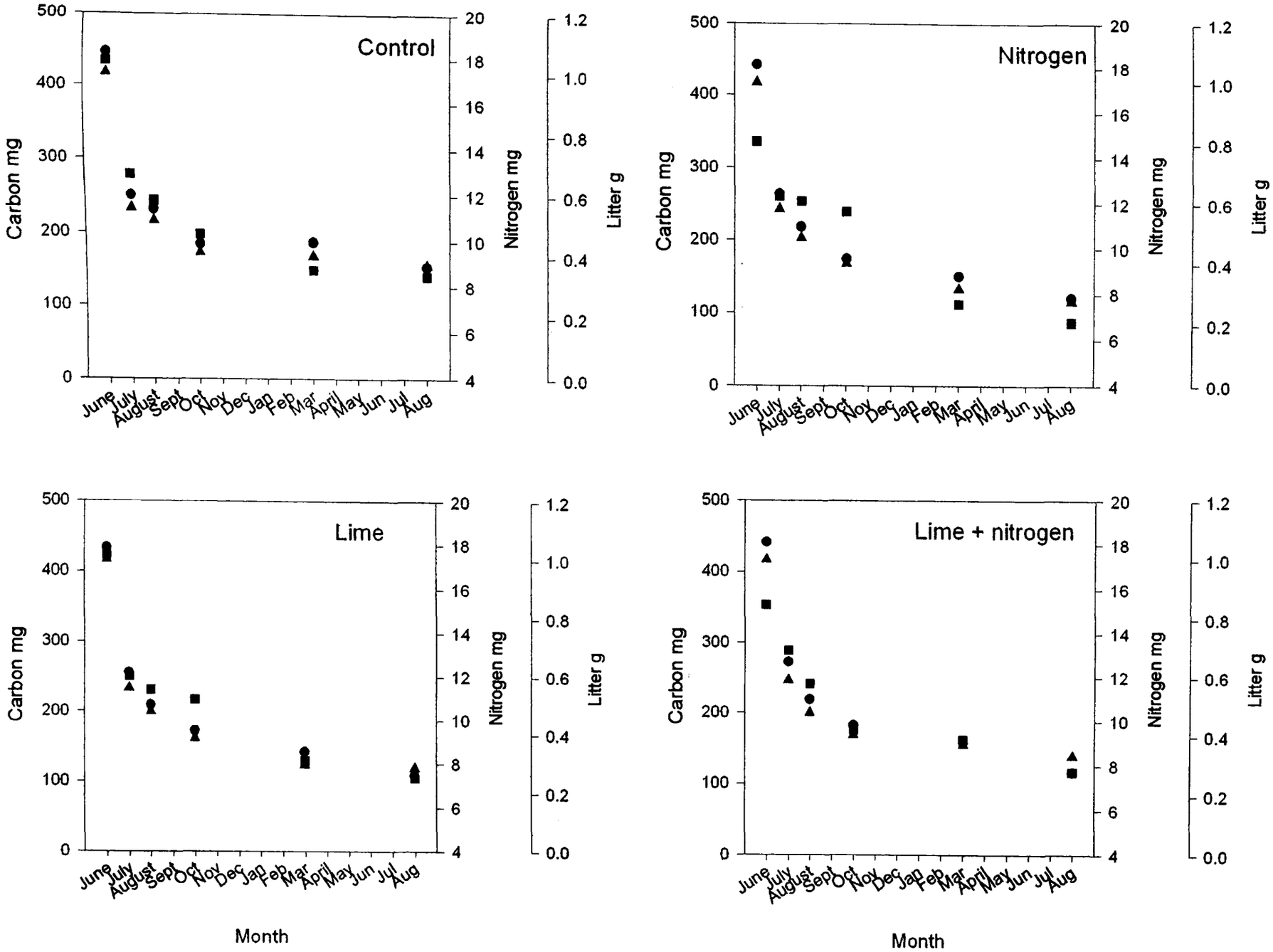


Figure 3.5. Loss of mass (mg) (▲), nitrogen (mg) (■) and carbon (mg) (●) from litter in coarse litterbags at Rigg Foot, Sourhope. Experiment started June 2002. See Table for means and SEs.

iii. *Change in carbon to nitrogen (C:N) ratio.*

The overall changes in C:N ratio are given in Table 3.15.

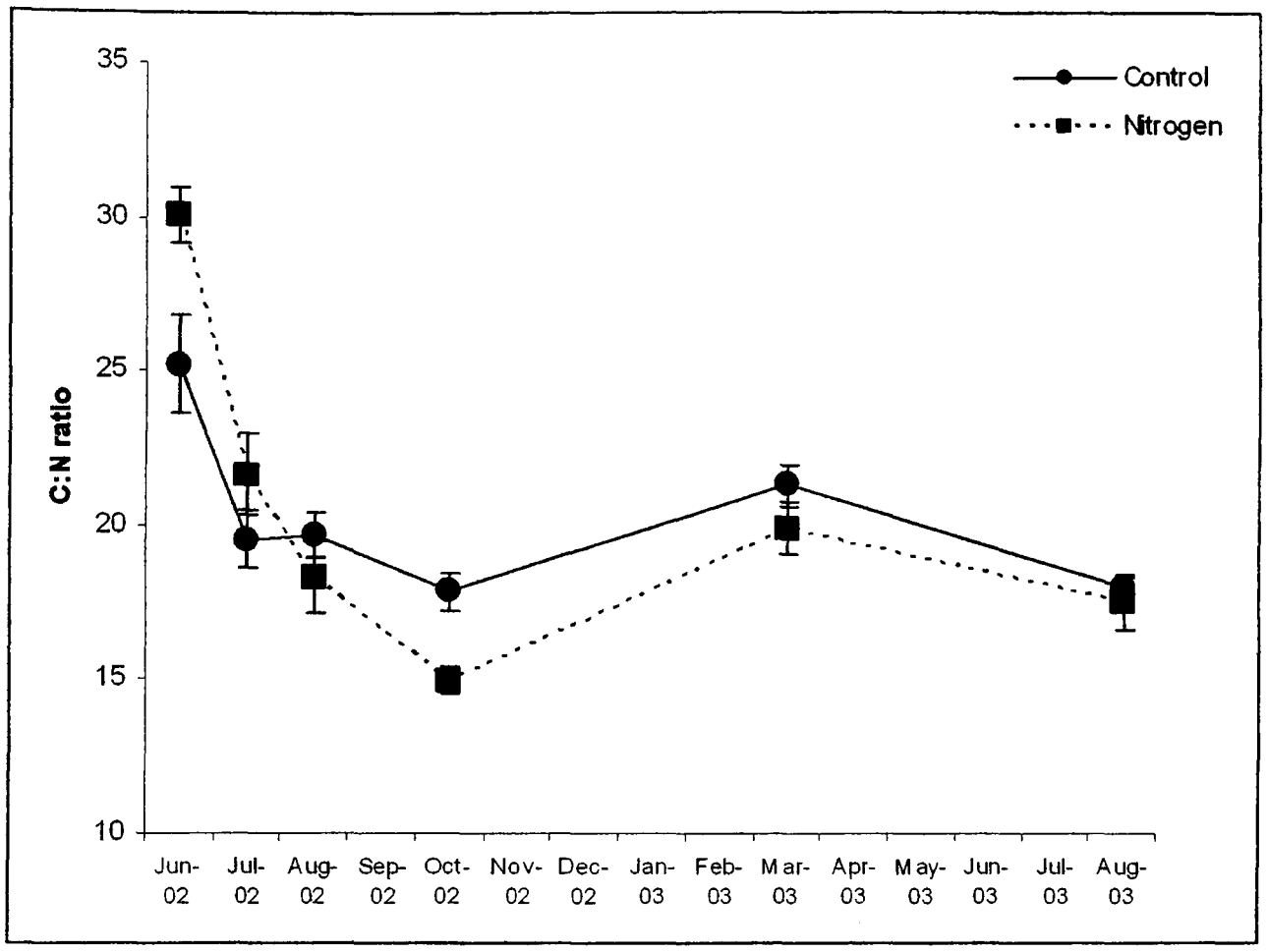
Table 3.15. Mean (±1SE) change in C:N ratio in coarse litterbags in the different treatment plots at Rigg Foot, Sourhope. The values are starting ratio and ratio at the end of the experimental period (14 months).

	C:N ratio July 2002	C:N ratio August 2003	Change in C:N ratio
Control coarse bags	25:1 (1.6)	18:1 (0.4)	7
Nitrogen coarse bags	30:1 (0.9)	18:1 (0.9)	12
Lime coarse bags	26:1 (3.3)	15:1 (0.8)	11
L+N coarse bags	29:1 (1.7)	15:1 (1.3)	14

The %N of the litter ranged from 1.15 – 2.05% and different litterbags had a different starting %N. This difference was not deliberate but reflects the different percentages of nitrogen that was assimilated into the grass tissue when it was grown in the greenhouse. The C:N ratio at the start of the experiment was significantly lower in litter placed in control plots than that placed in nitrogen plots (one-way $ANOVA_{1,7}$, $F = 8.12$, $p < 0.025$) due to the lower initial nitrogen content was lower in litter placed in nitrogen plots.

There was a relatively rapid decrease in the C:N ratio during the first month which continued to fall sharply in nitrogen and lime plots until October 2002. The decrease was much slower over the same period in control and L+N plots (Figure 3.6).

In control and nitrogen plots (and, to a lesser degree, in lime plots) there was an increase in C:N ratio over winter which was not evident in L+N plots. After 14 months, despite initial differences, the C:N ratio in control and nitrogen plots was the same (18:1), and also in lime and L+N plots (15:1).



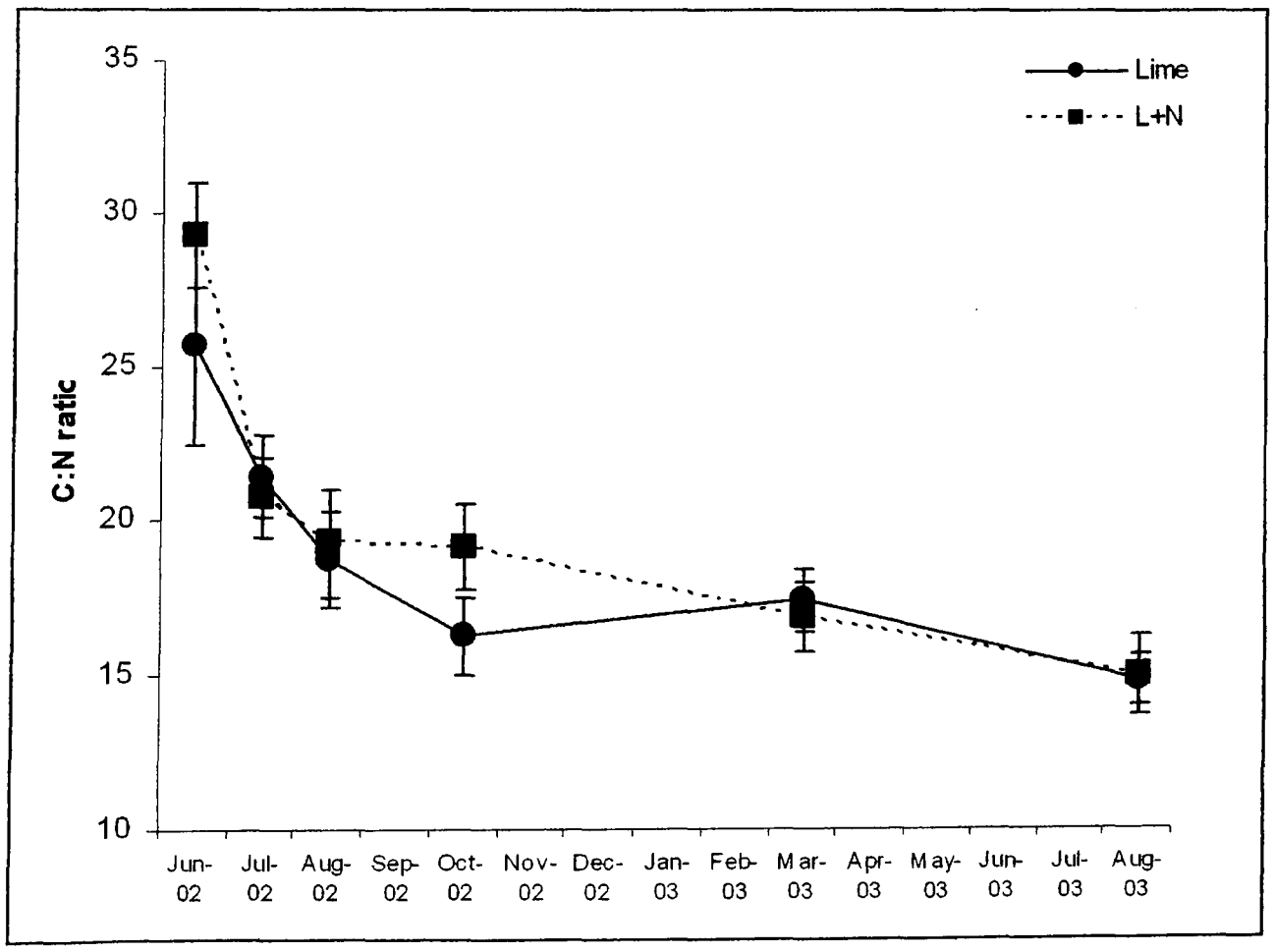


Figure 3.6. Change in C:N ratio of litter in coarse litterbags in the treatment plots at Rigg Foot, Sourhope. Mean ± 1SE. Experiment started July 2002.

There were no significant differences of the C:N ratio between treatments, but the values over time were significantly different and the interaction between time and treatment being significantly different (Table 3.16).

Table 3.16. ANOVA table of the change in C:N ratio of litter in coarse litterbags in the four different treatment plots at Rigg Foot, Sourhope.

Factor	SS	d.f.	MS	*F* ratio	*P* value
Treat	27.7	3	9.24	1.225	0.306
Time	1449	5	289.8	38.417	<0.001
Treatment * Time	206	15	13.8	1.823	0.045
Error	611	81	7.5		

The C:N ratio of litter in nitrogen and lime plots fell by a similar amount, and the difference was least in control (25:1 – 18:1) and most in L+N plots (26:1 – 15:1).

Differences in the over winter C:N ratio.

In all plots the early decrease in the C:N ratio was caused by an increase in %N until October 2002, whilst carbon remained relatively static. Then, in control and nitrogen plots over winter, nitrogen concentration decreased while there was a concomitant *increase* in carbon concentration which resulted in the rise in C:N ratio. In lime L+N plots nitrogen concentration was almost static from August 2002 to August 2003 whereas carbon concentration decreased during the same period and there was only a slight overwinter increase in C:N ratio.

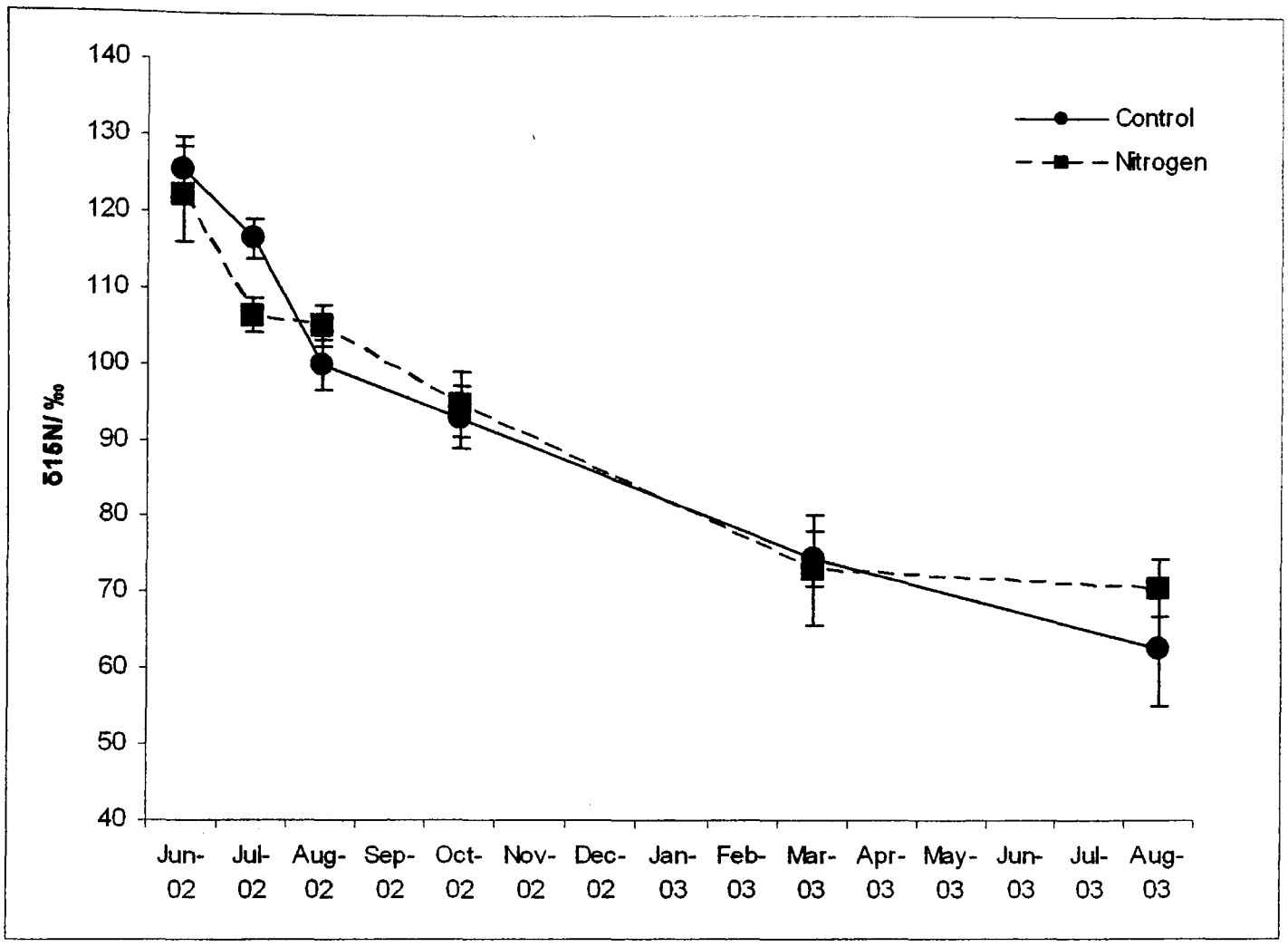
iv. ***Change in δ ^{15}N value of litter in coarse litterbags.***

There were slight variations in the starting values of δ ^{15}N in the *A. capillaris* litter in different litterbags. This was due to the different values achieved when labelling the grass with ^{15}N labelled urea.

The δ ^{15}N values of the litter in all treatment plots followed the same pattern of loss. Table 3.17 shows the initial and final values of δ ^{15}N and the change during the period of the experiment and Figure 3.7 shows the values plotted as a scatter graph.

Table 3.17. Mean initial, final and difference (± 1SE) in δ ^{15}N values of litter placed in control, nitrogen, lime and L+N plots at Rigg Foot, Sourhope. Litterbags were placed in the plots in June 2002 and the final sampling date was August 2003.

	Initial value	Final value	Difference
Control	125 (4.2)	63 (7.8)	62.61
Nitrogen	122 (6.2)	71 (3.8)	51.4
Lime	123 (8.3)	78 (7.2)	45.1
L+N	115 (4.3)	84 (8.2)	31.67



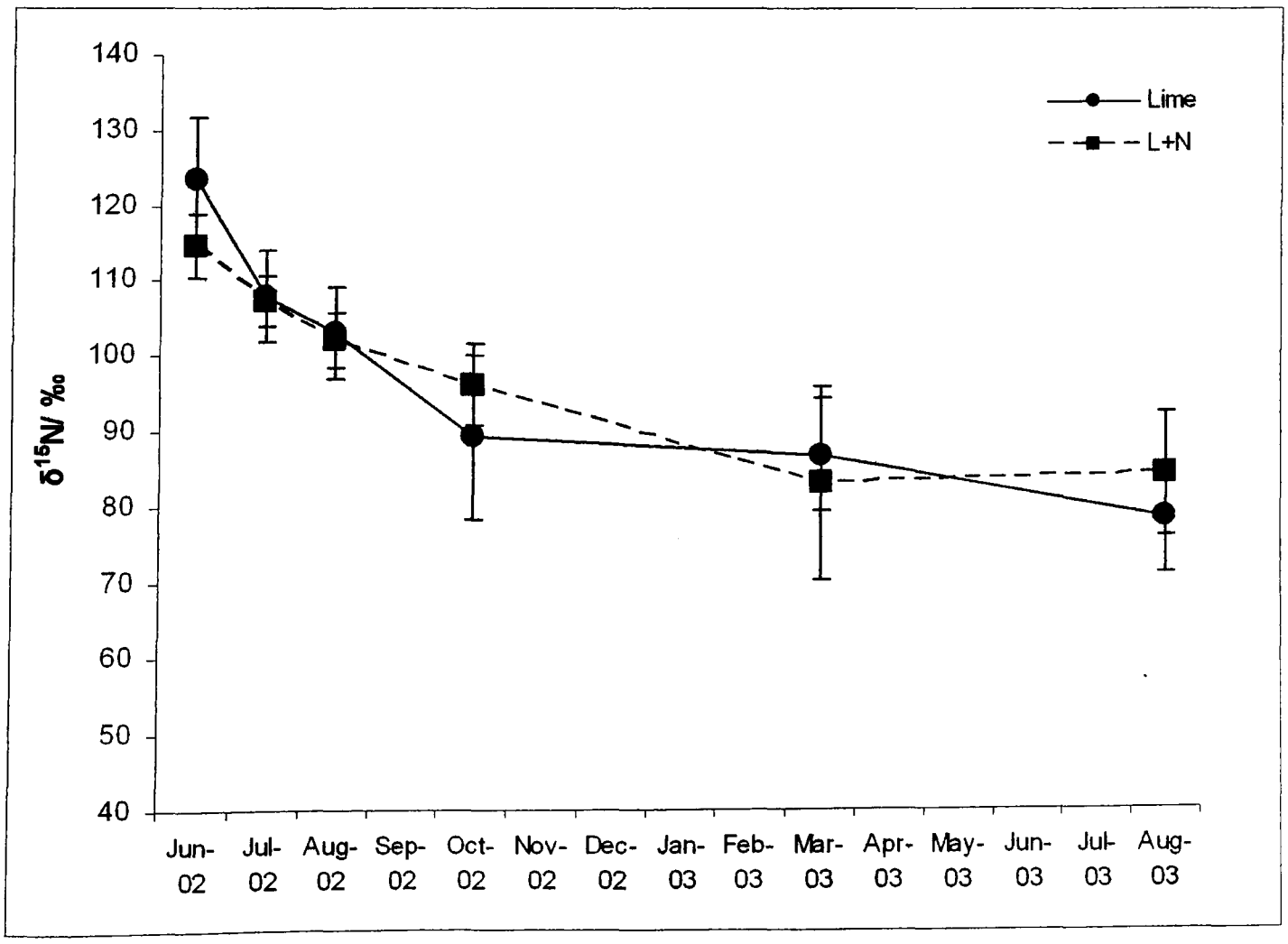


Figure 3.7. Changes in δ ^{15}N values of litter in coarse litterbags during the 14 month experimental period in control and nitrogen (top), and lime and lime + nitrogen (bottom) plots at Rigg Foot, Sourhope. Mean ± 1SE. Experiment started June 2002.

The greatest loss of $\delta\ ^{15}N$ was from litter in control plots, followed by nitrogen, lime and L+N plots. One-way ANOVA showed no significant differences between the final values in the different treatment plots ($df_{3,13}$, $F = 1.82$, $p = 0.19$).

Coarse mesh litterbags (continued):

3. Nitrogen dynamics.

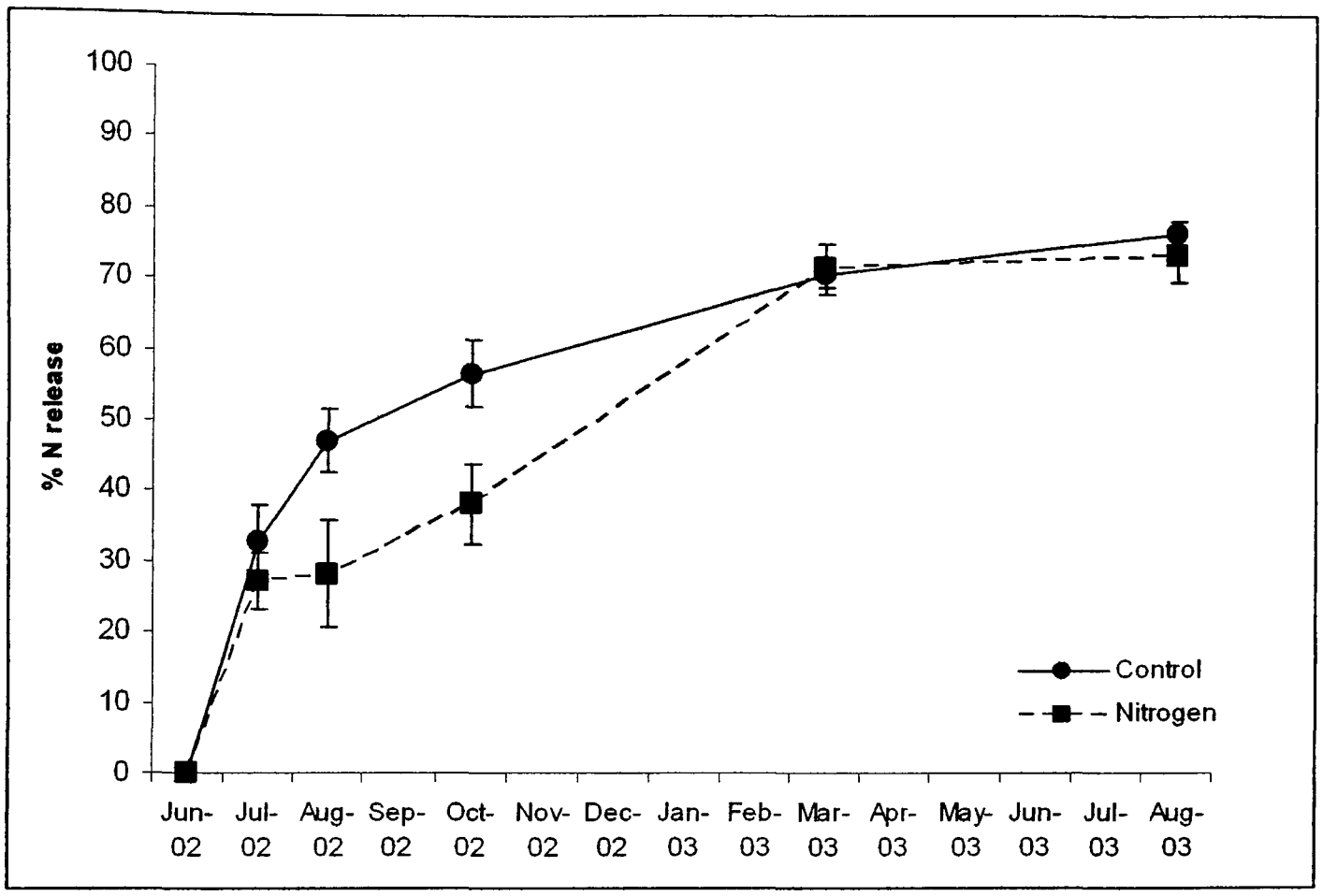
Changes in mass of litter, N content (%) and $\delta\ ^{15}N$ described in the previous section and the net result of N released and incorporated by various means in the litter was estimated using the methods of Berg (1988) and Zeller (2000).

The following section describes N release and incorporation and also their relationship to litter mass.

i. ***Nitrogen release.***

There was an initial rapid release of nitrogen from the litter during the first month of the experiment (Figure 3.18). Maximum, but not significant (ANOVA, $df_{3,14}$; $F = 3.113$, $p = 0.06$), release during the first month was from lime plots (38%), followed by control (32%), nitrogen (27%) and L+N (18%). Nitrogen was released throughout the experimental period and the overall release of nitrogen after 14 months was in the order control (76%), nitrogen (73%), lime (71%) and L+N (65%) although the final %N release in different treatment plots was not significantly different (ANOVA, $df_{3,13}$; $F = 1.422$, $p = 0.28$). Regression analysis also showed no differences between the slopes of the regression lines between treatment plots.

Despite the lack of significant differences it was noted that the pattern of release differed between treatment plots. In control plots, after the initial rapid release of N, there was a steady release throughout the experimental period. L+N plots followed a similar pattern although the initial and overall release was much lower. However, in nitrogen plots and lime plots, there appeared to be relatively little activity between



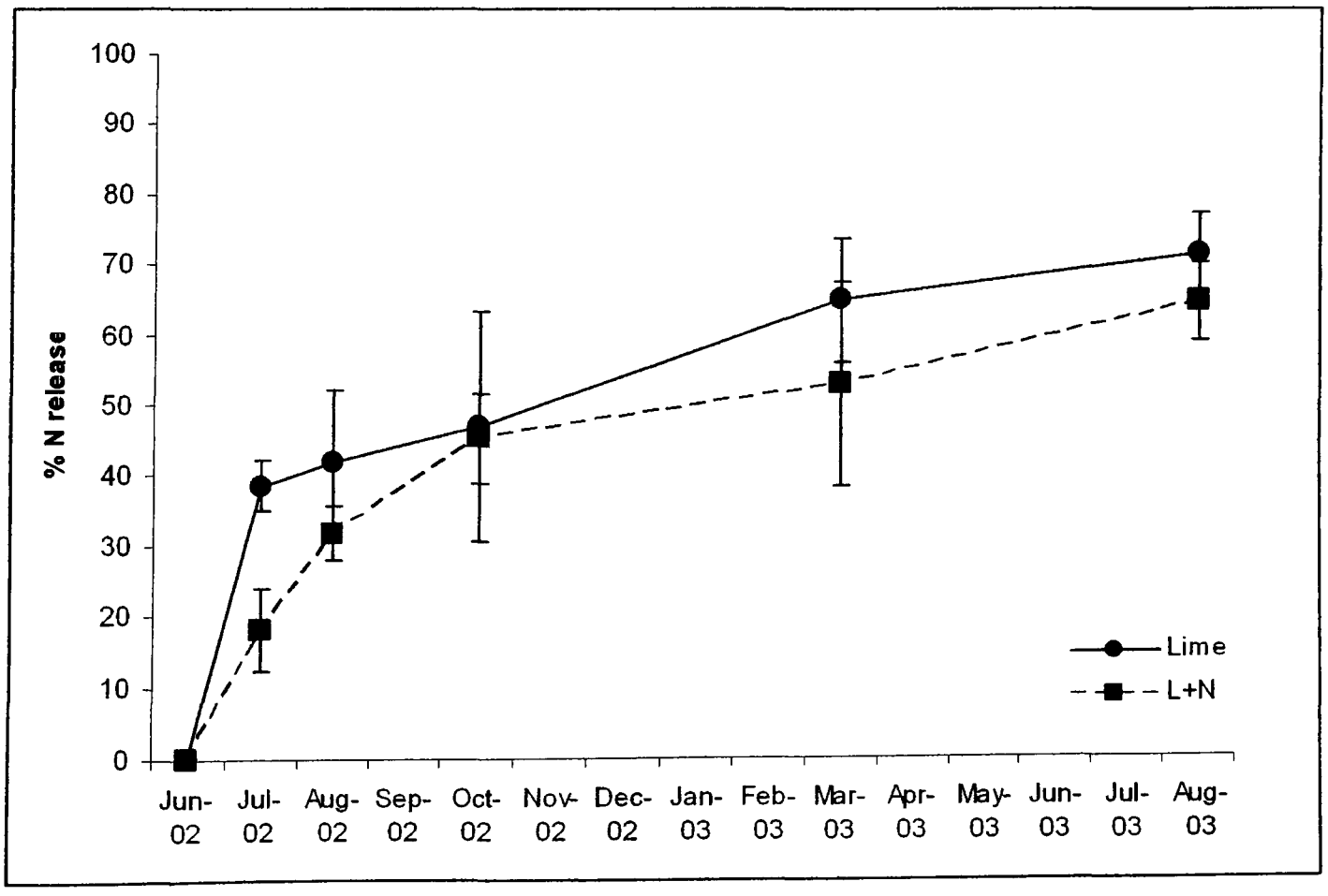


Figure 3.8. Cumulative nitrogen release (%) from litter in coarse litterbags in treatment plots at Rigg Foot, Sourhope during the experimental period. Mean ± 1SE.
Each data point calculated as T0 – T1; T0 – T2 etc.

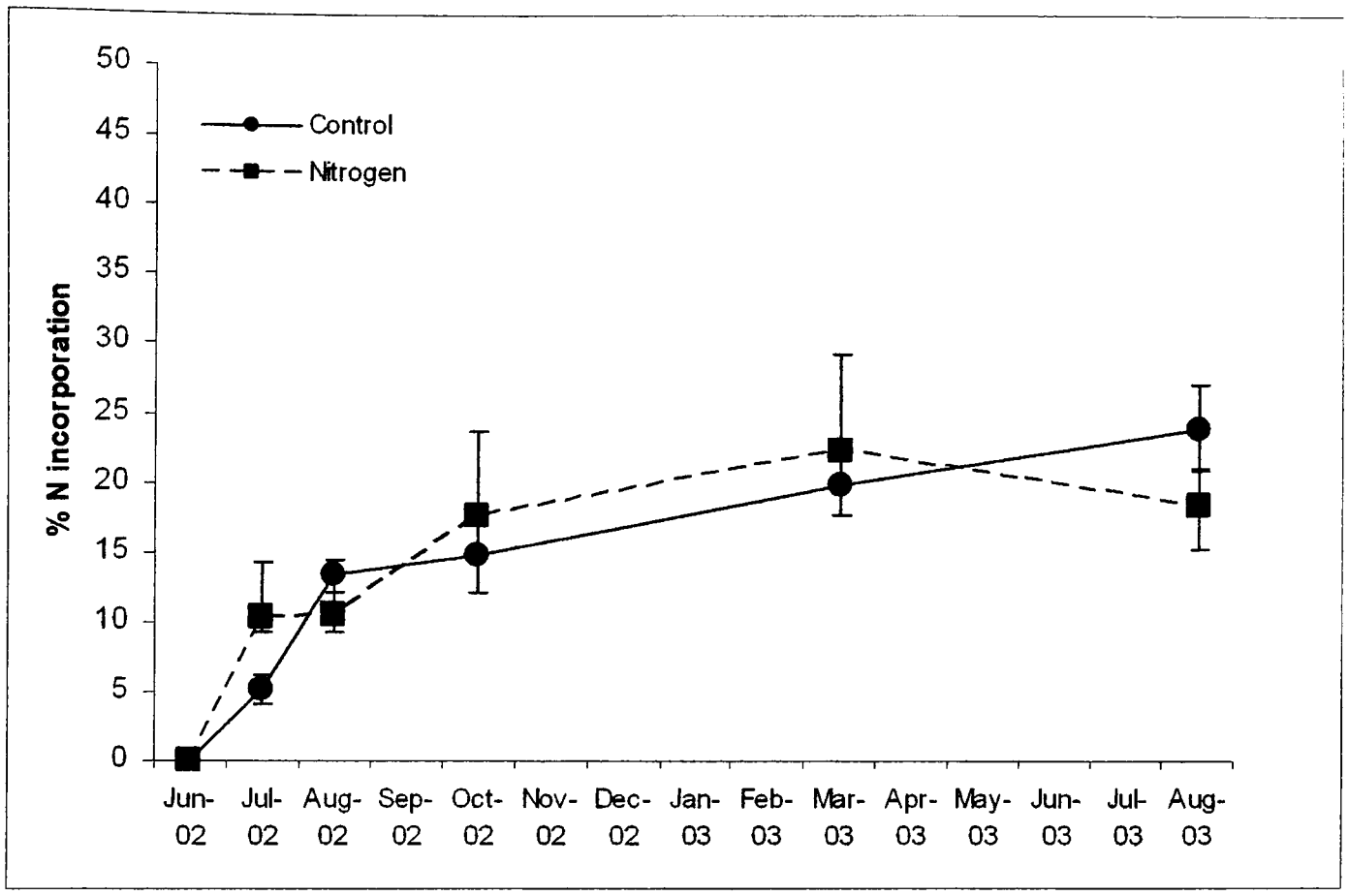
July and October, but an increase in release during the winter months, which was particularly marked in the nitrogen plots.

ii. ***Nitrogen incorporation.***

The rates of incorporation (approximately 20%) were lower than seen for N release (approximately 70%). N incorporation increased steadily in control plots throughout the experimental period to a maximum of 24%. In nitrogen plots incorporation was static between July and August, steadily increased over winter and then fell during the second summer of the experiment. Overall the pattern followed that of release until the last summer. However, variability was high.

In lime plots incorporation occurred during the first month and between August and October but it remained static for the remainder of the experiment. Incorporation in L+N plots was similar to control plots with a steady increase throughout the experimental period (Figure 3.9).

Although analysis of the regression slopes showed that the rate of incorporation in lime plots was less than that of control plots (2-tailed t-test, df 44, $t = 2.57, p < 0.001$), ANOVA of N incorporation was not significantly different between plots (ANOVA, $df_{3,13}$; $F = 1.657, p = 0.26$).



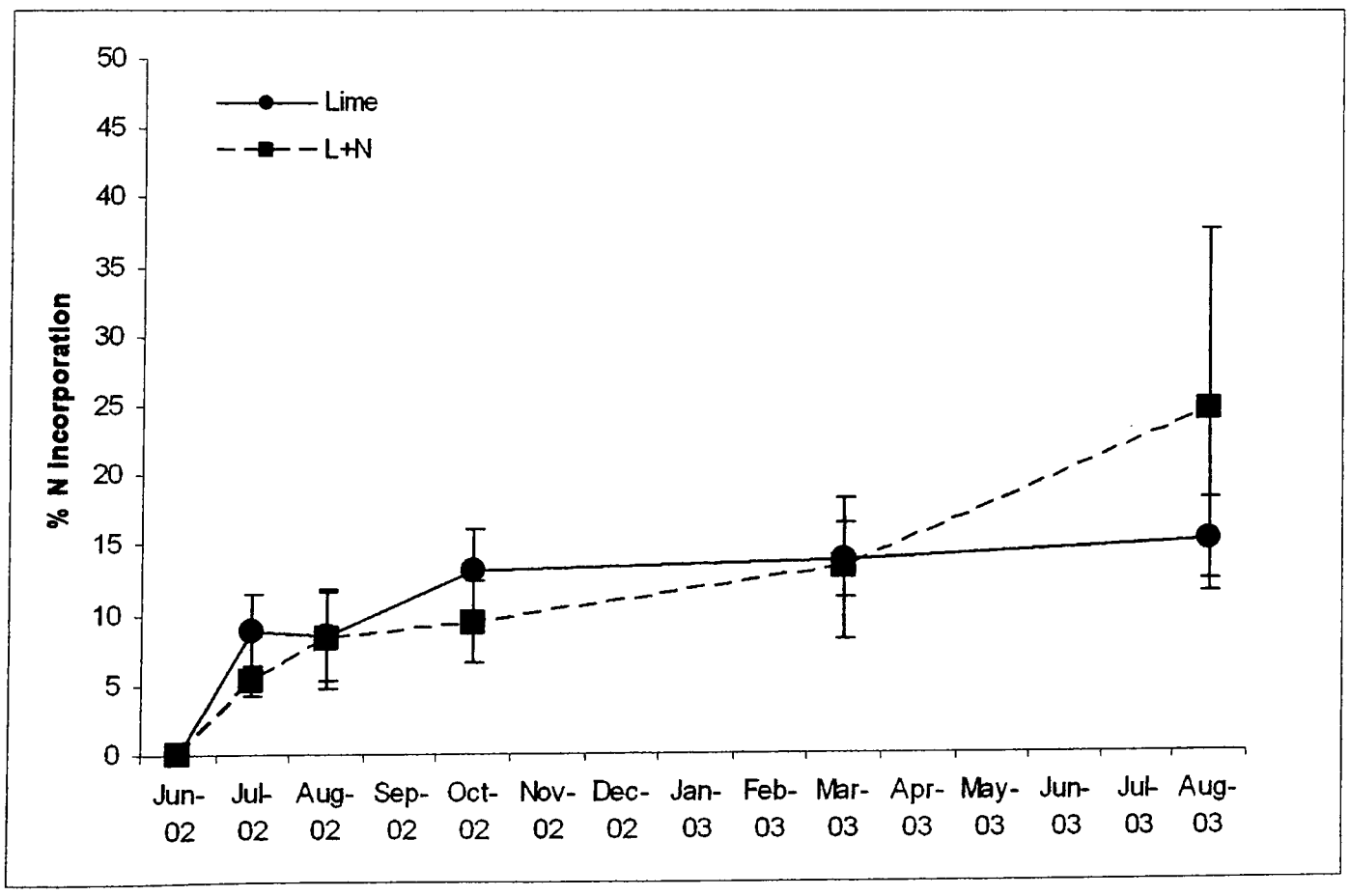


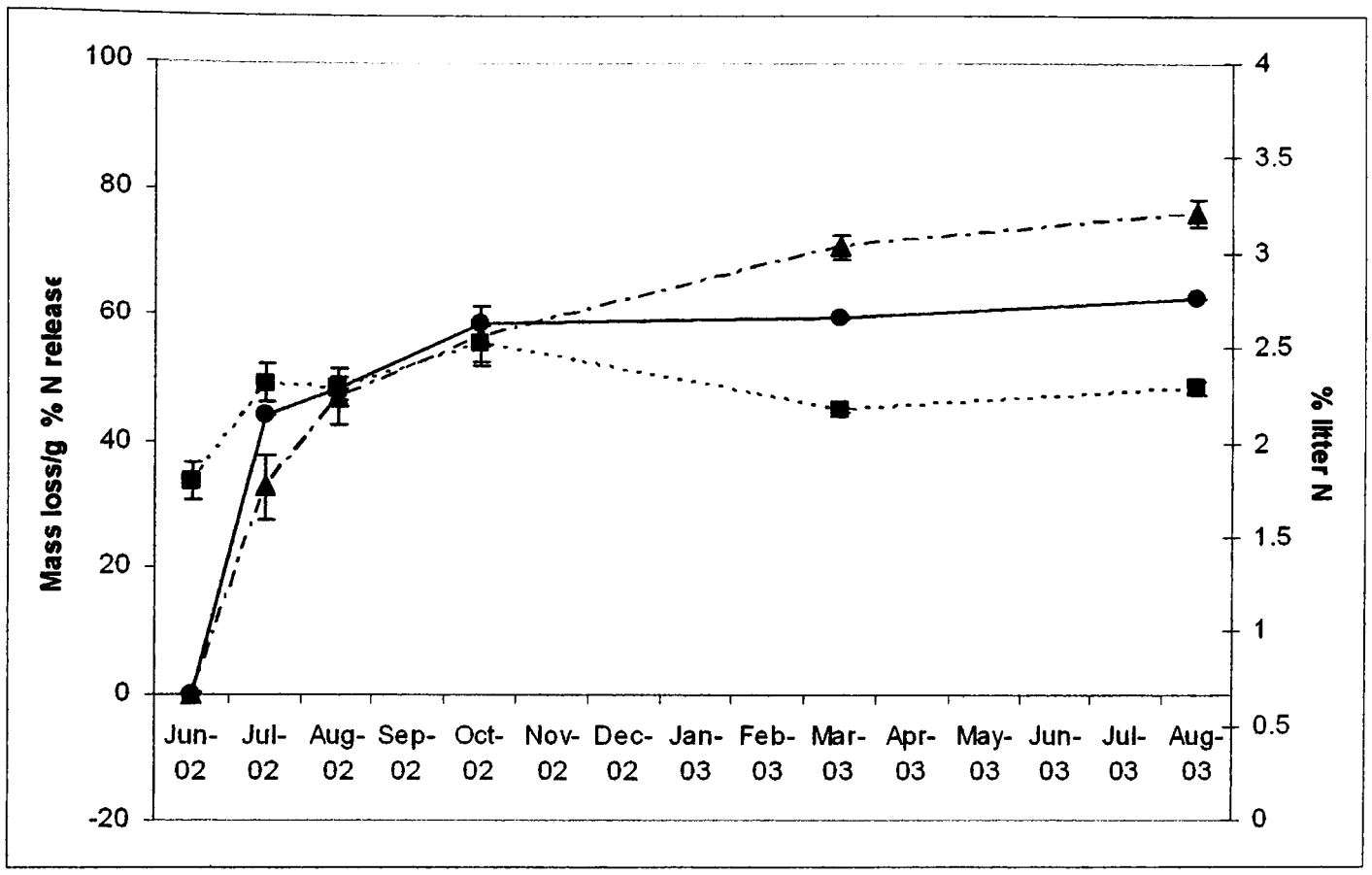
Figure 3.9. Cumulative nitrogen incorporation (%) in litter in coarse litterbags in treatment plots sampled at Rigg Foot, Sourhope during the experimental period. Mean ± 1SE.
Each data point calculated as T0 – T1; T0 – T2 etc

iii. ***Nitrogen release and % litter N in relation to loss of litter mass.***

Changes in litter mass, % litter N and % N release are shown in Figures 3.10 and 3.11.

In C and N plots % litter N declined overwinter when litter N release was 40-58% and mass loss 58 - 60%. N release overwinter was 15-25%.

In L and L+N plots % litter N declined only in the second summer when N release was 50-60% and mass loss was 60-70%. N release overwinter was only 10-15%



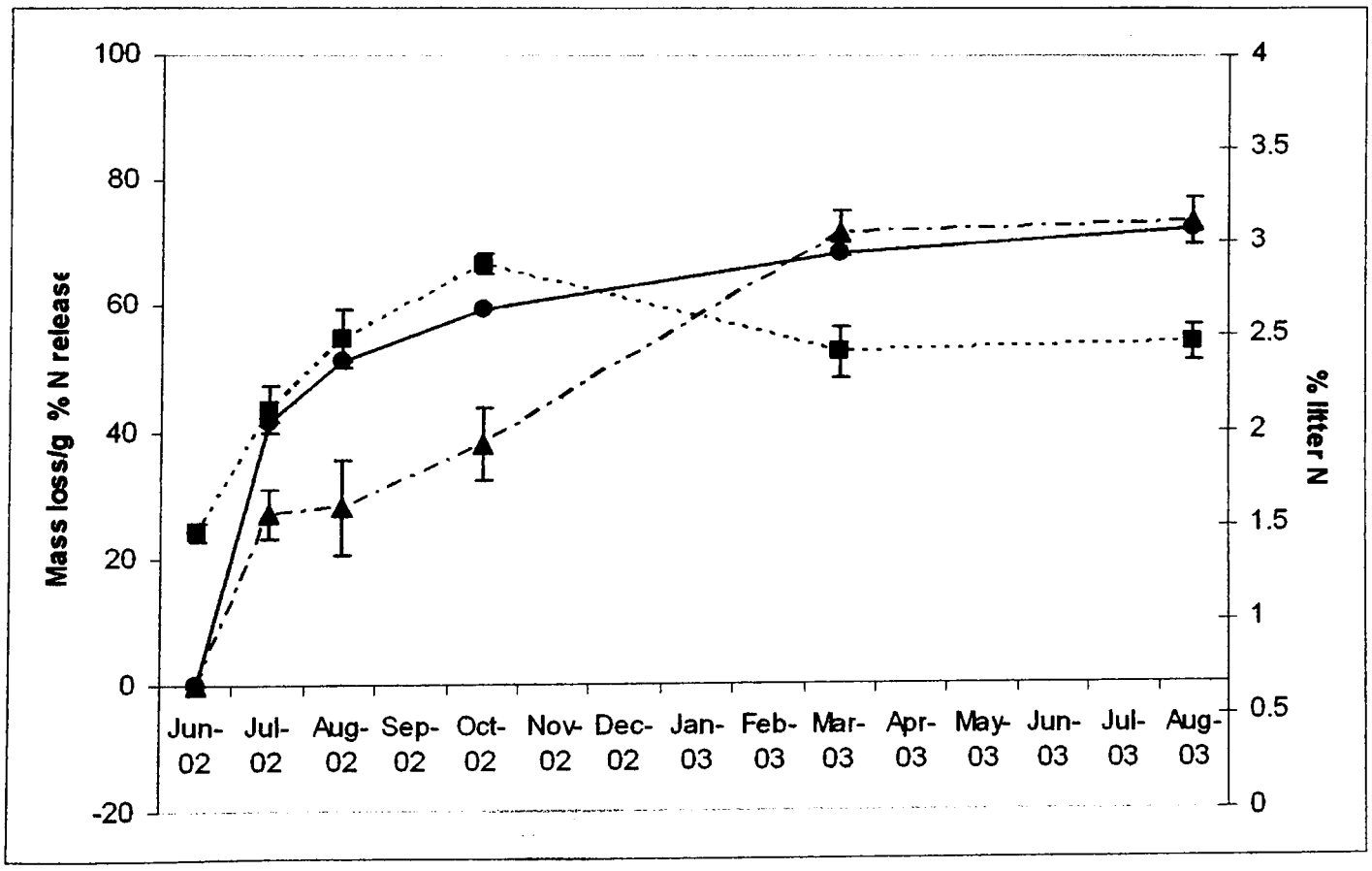


Figure 3.10. Relationship between mass loss (●), % N release (▲) and % litter N (■) of litter in coarse litterbags in control (top) and nitrogen (bottom) plots at Rigg Foot, Sourhope. Means ± 1SE.

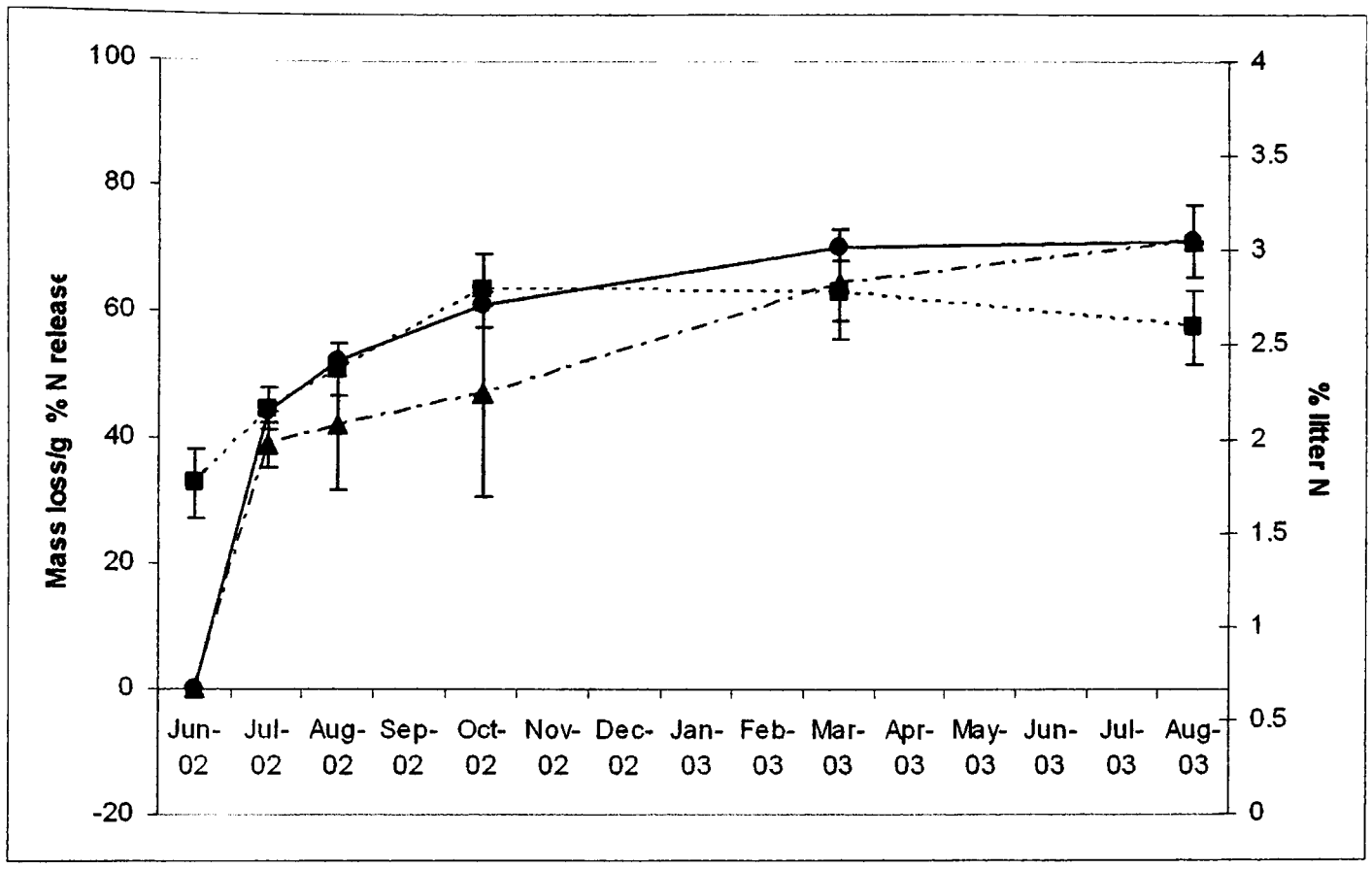


Figure 3.11. Relationship between mass loss (●), % N release (▲) and % litter N (■) of litter in coarse litterbags in lime (top) and L+N (bottom) plots at Rigg Foot, Sourhope. Means ± 1SE

3.3.3.2 Fine mesh litterbags

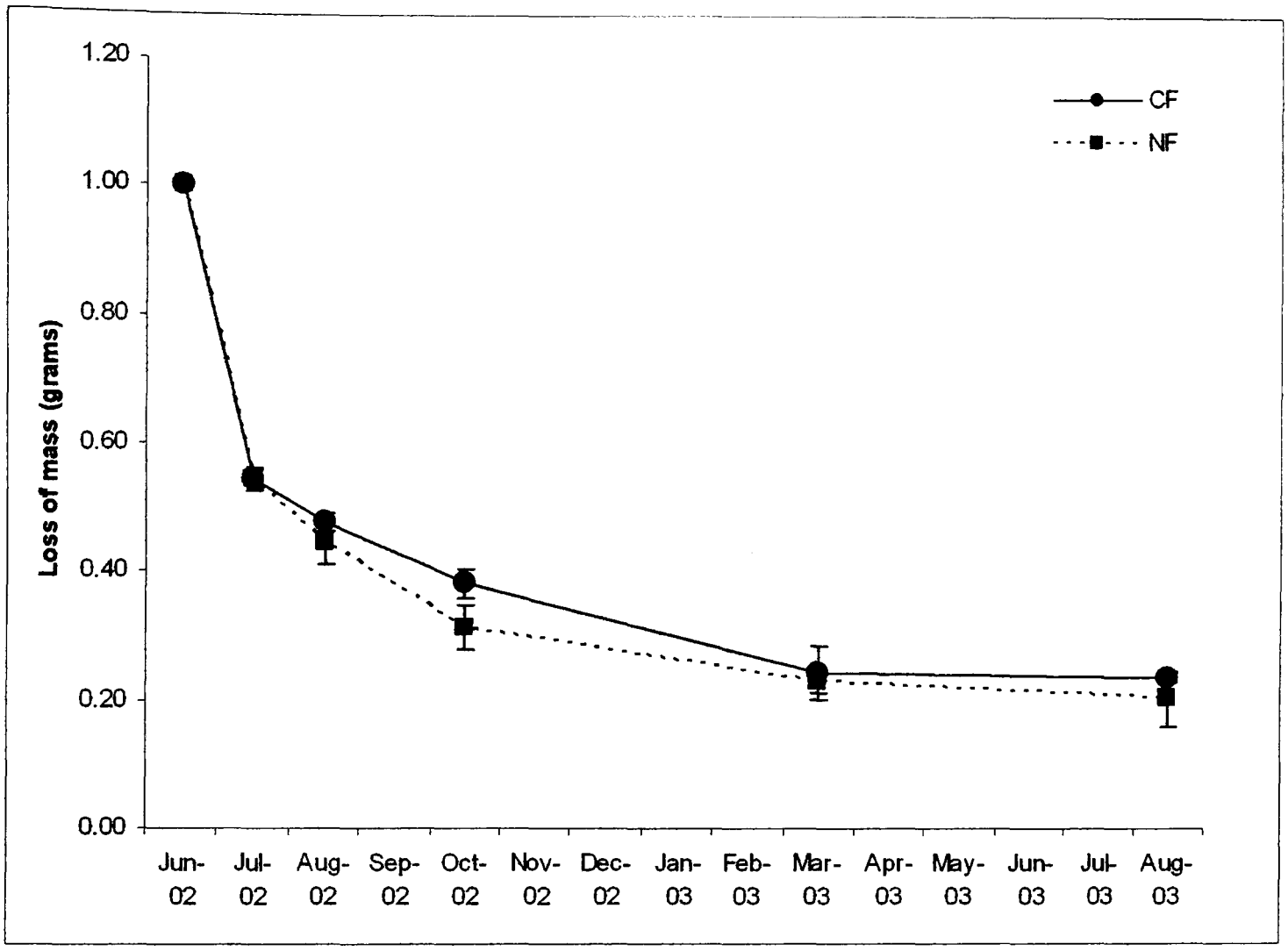
Fine mesh litterbags were intended to exclude animals – in fact they initially allowed a smaller number of small individuals to enter and appeared to restrict exit or provide more favourable conditions inside the bags than in the cores. The fine litterbags had less roots around, and infiltrating, them.

In this section changes in litter mass, carbon and nitrogen content of litter and nitrogen dynamics in fine bags are described for all four treatments.

1. Mass loss.

The mass of litter at the start of the experiment was 1 g in all litterbags

There was an initial rapid loss of mass in the first month of the experiment of ~ 0.4g in all treatment plots. There was no mass loss between March and August 2003 in control and nitrogen (acid) plots and only a slight loss in lime treated plots (Figure 3.12). The overall loss of mass was 76.5% (± 0.93) in control, 79.6% (± 4.3) in nitrogen, 77.4% (± 2.2) in lime and 75.7% (± 3.1) in L+N treated plots.



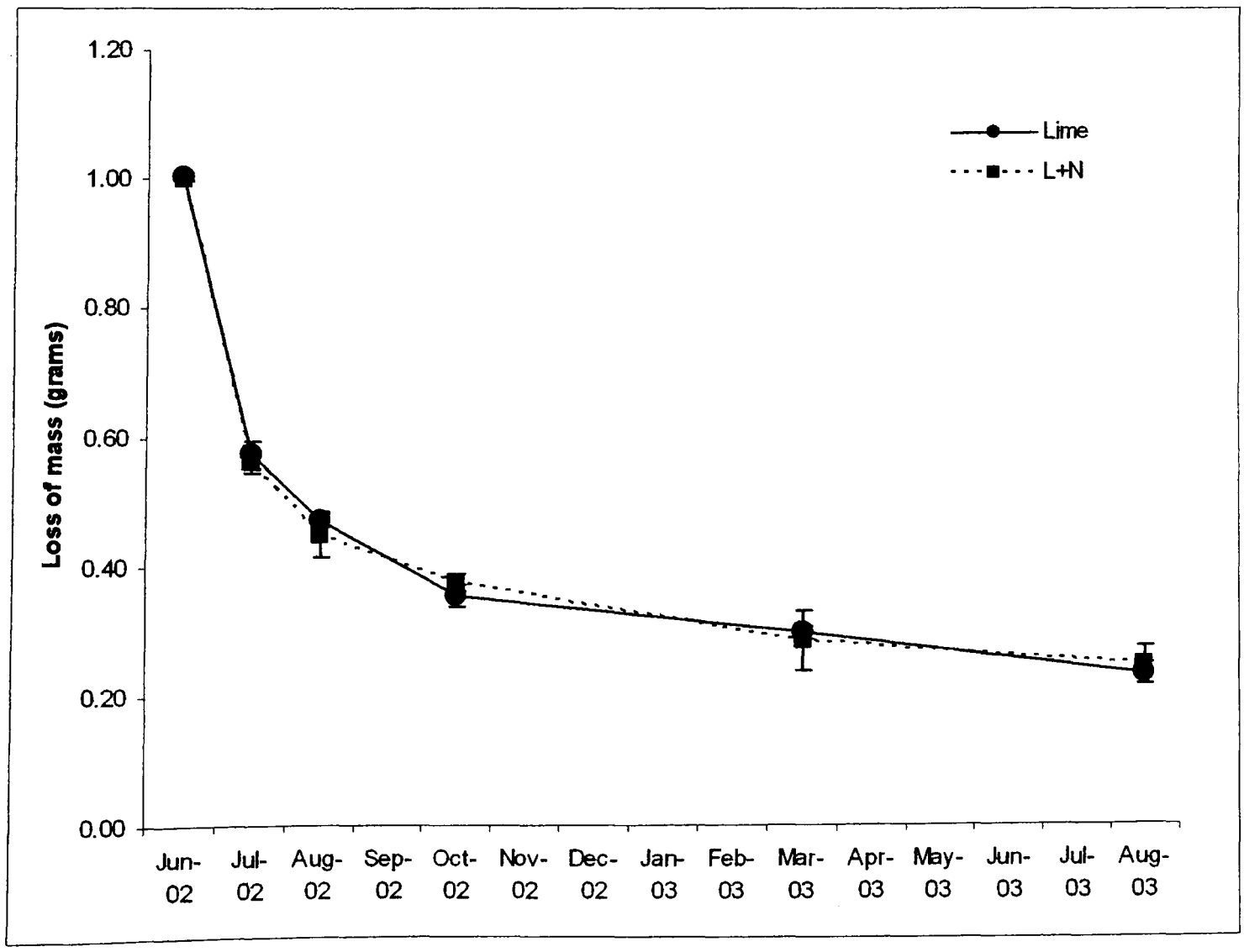
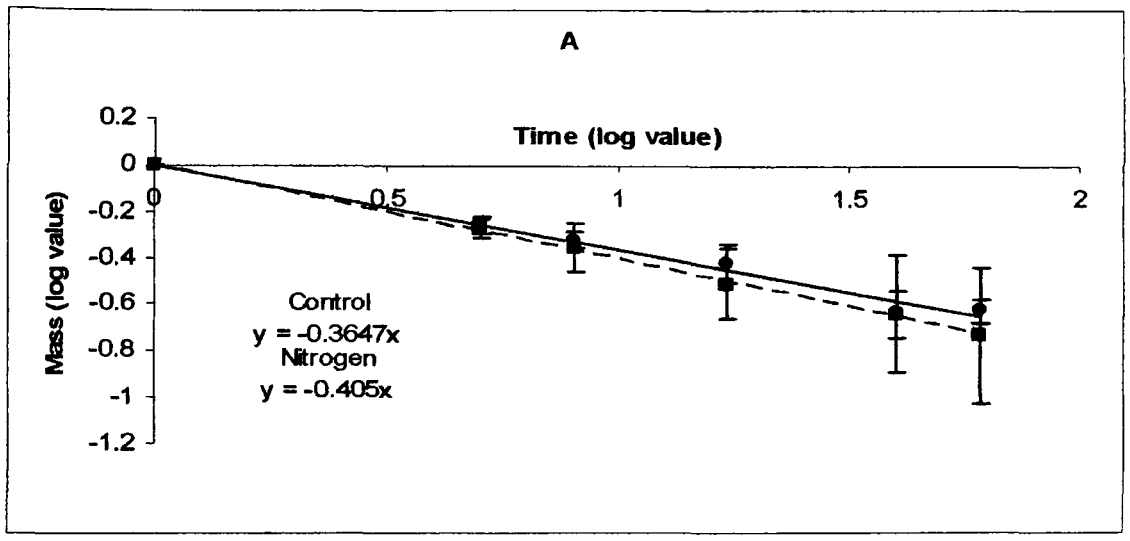
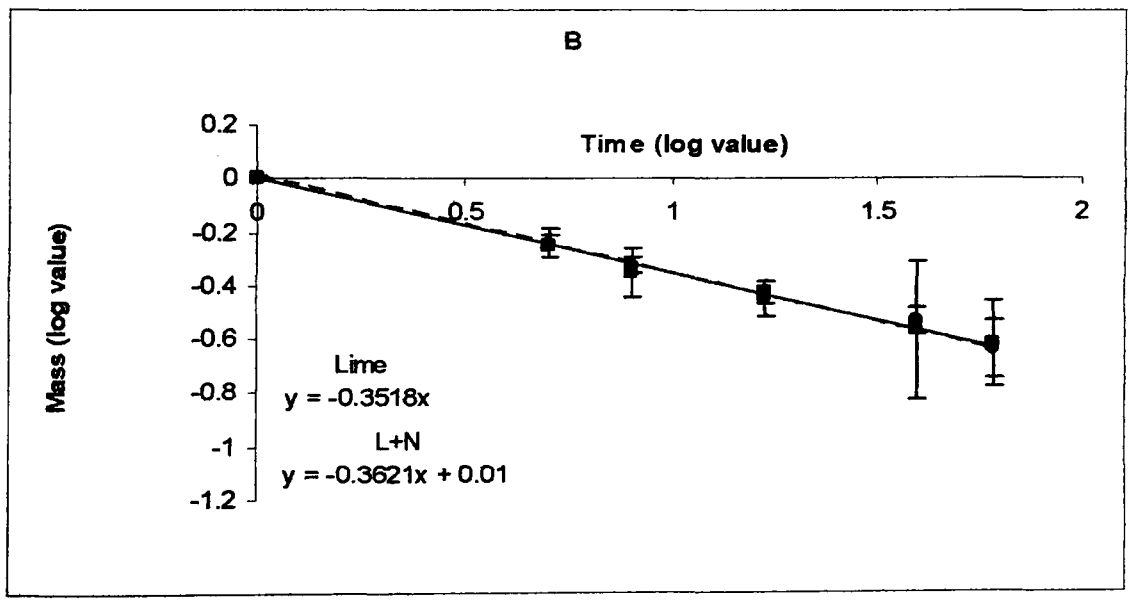


Figure 3.12. Mass loss from fine litterbags in the treatment plots at Rigg Foot, Sourhope. Means ± 1SE. Experiment started June 2002.

Regression analysis

The mass loss data (y-axis) and time in weeks (x-axis) were log transformed and plotted with their respective regression equations (Figure 3.13).





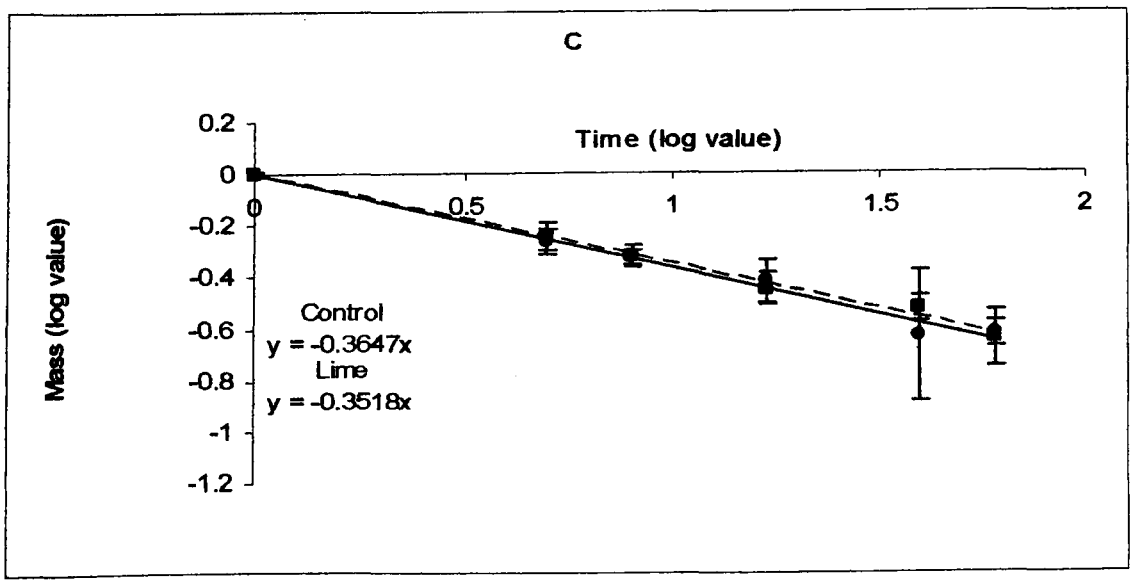


Figure 3.13. Regression lines fitted to *x* and *y* log transformed data for mass loss of litter in fine litterbags.
Geometric means ± 95% CI.

D. Control (solid line) and nitrogen (dashed line) plot litterbags.
E. Lime (solid line) and lime + nitrogen (dashed line) plot litterbags.
F. Control (solid line) and lime (dashed line) plot litterbags.

The significance of the regression lines was tested between treatments (Table 3.18). There were no significant differences between loss of mass when comparing control and nitrogen, lime and L+N or control and lime.

Table 3.18. Calculations of differences between regression lines, *t* critical (2-tailed) and significance level.
NB Statistics calculated on log transformed data. For calculations, see Methods.

	Step 1	**Step 2**	**Step 3**	**df**	***t* critical**	**Significance level**
Control & nitrogen fine	0.0465	0.0411	1.1315	48	2.011	NS
Lime & L+N fine	-0.0025	0.0275	-0.08981	48	2.011	NS
Control & lime fine	-0.015	0.0273	-0.54855	44	2.015	NS

Fine mesh litterbags (continued):

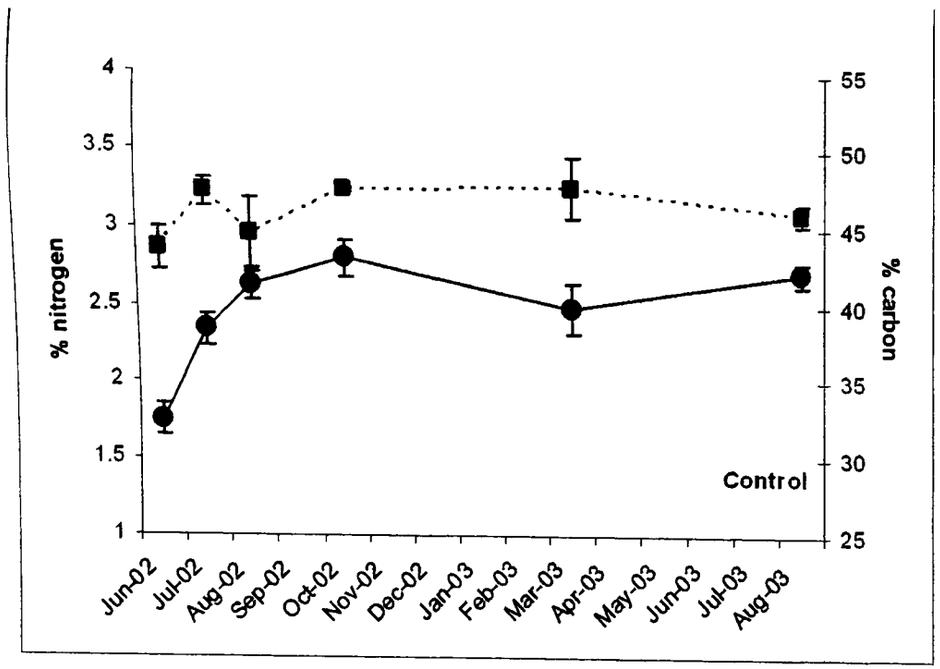
2. Litter carbon and nitrogen.

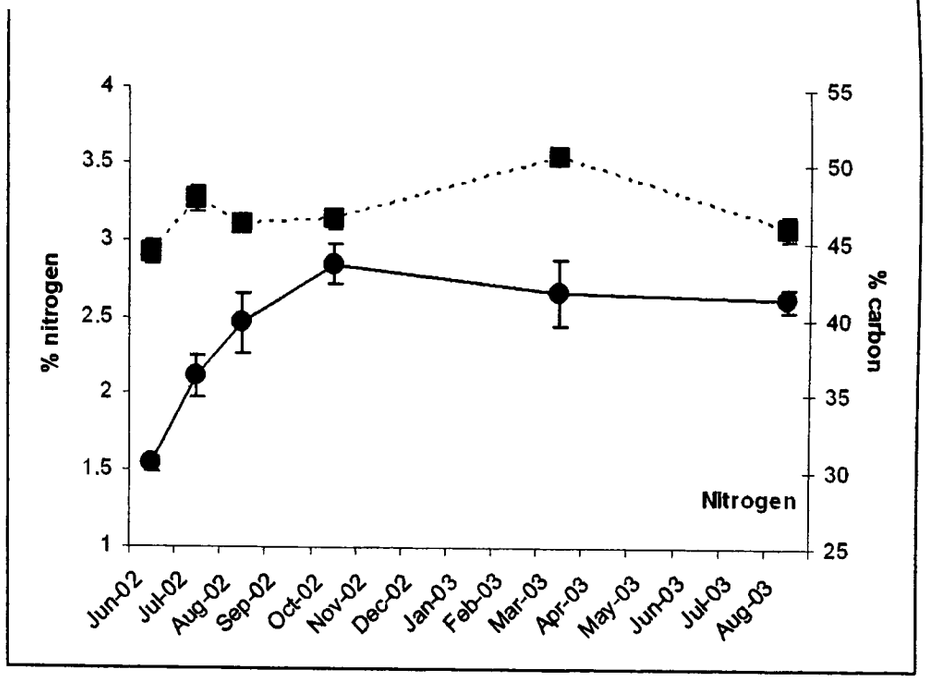
This section describes changes in % carbon and % nitrogen of litter, mass of carbon and nitrogen, change in C:N ratio, change in δ ^{15}N value of litter and change in mass of ^{15}N in fine mesh litterbags.

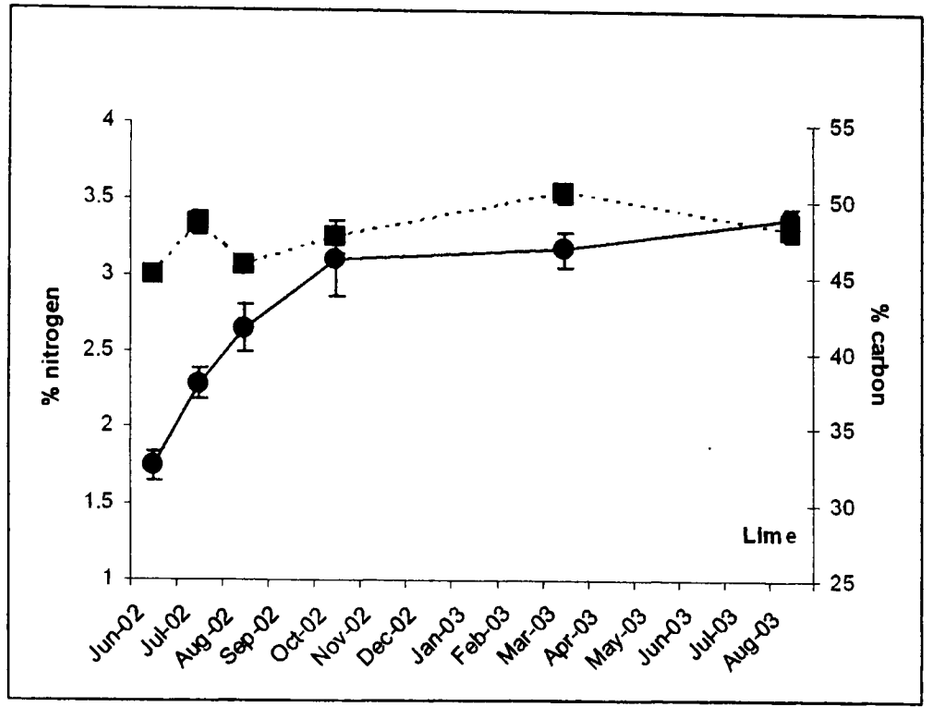
v. *% carbon and % nitrogen of the litter.*

The %N of litter was slightly higher in control and lime plots than Nitrogen and L+N plots although there was no significant difference between these values (One-way ANOVA, $df_{3,14}$, $F = 2.9$, $p = 0.072$).

Figure 3.14 shows the changes that occurred in the % C and N of the litter in fine litterbags during the 14 months of the experiment. In control and nitrogen plots, %N increased until October 2002 and then fell over winter. %C fluctuated throughout the first summer then remained relatively static. The pattern of carbon fluctuation was similar in lime and L+N plots, but %N continued to rise in these plots until the end of the experiment with no overwinter decline.







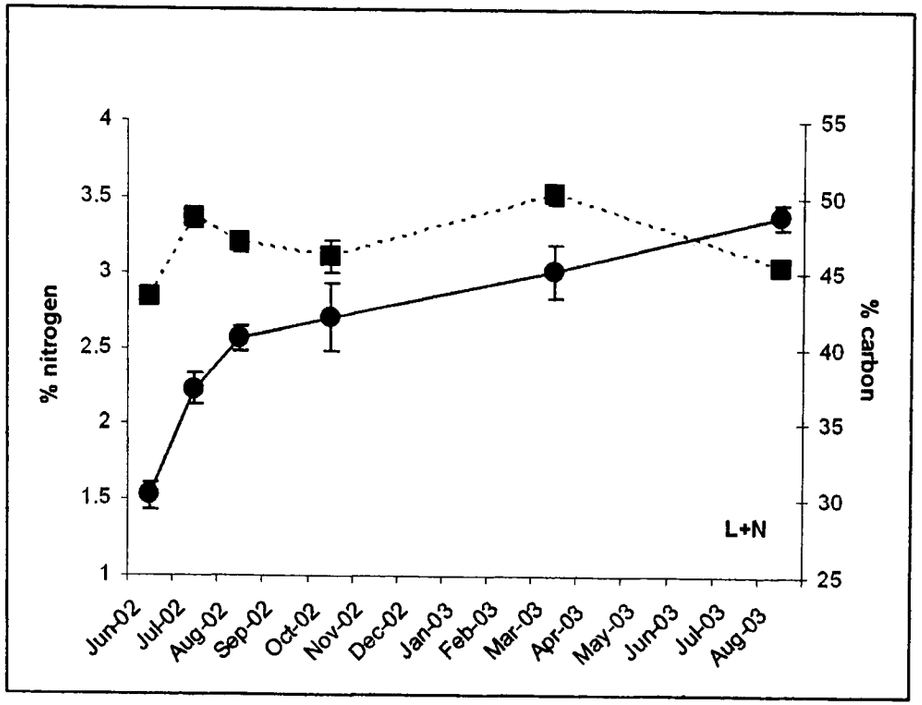


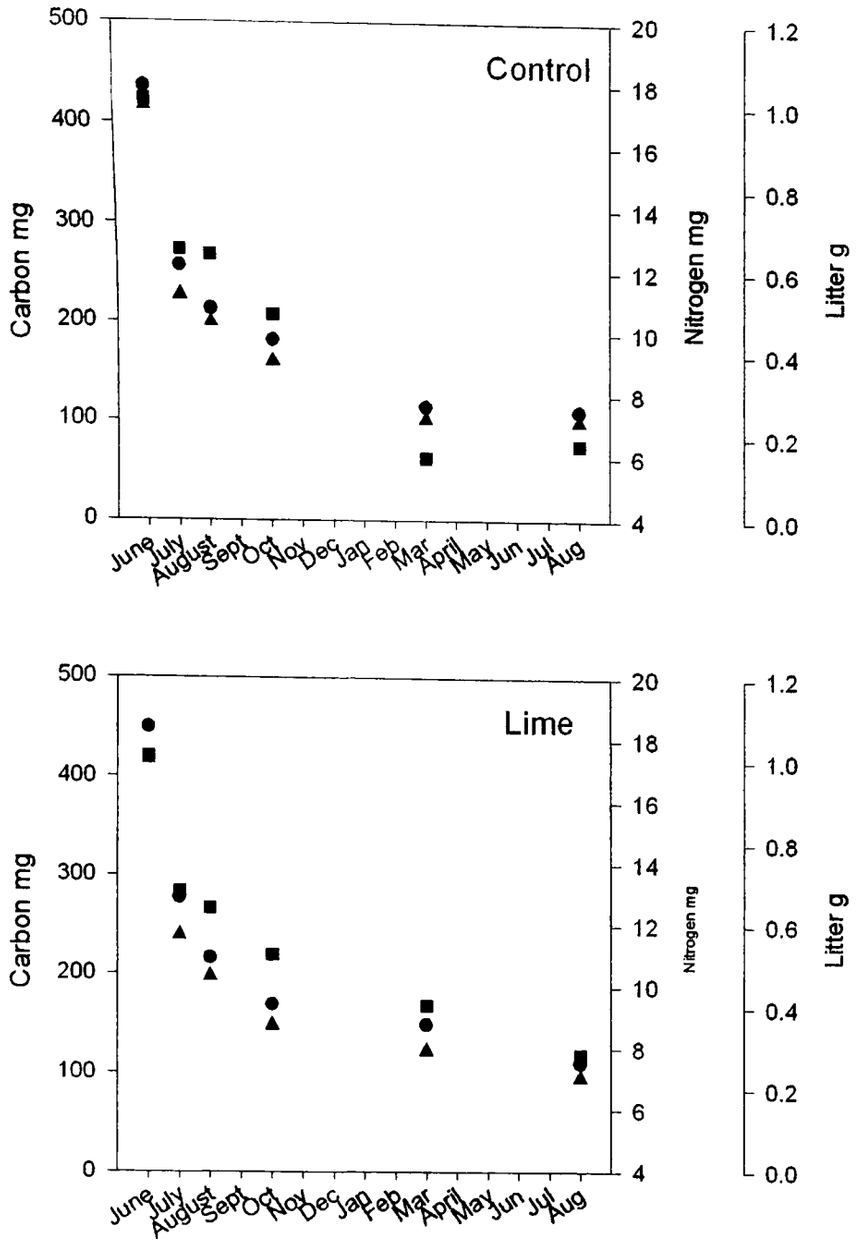
Figure 3.14. % nitrogen (●) and % carbon (■) changes in litter in fine litterbags at Rigg Foot, Sourhope during the course of the experiment (started June 2002). Mean ± 1SE

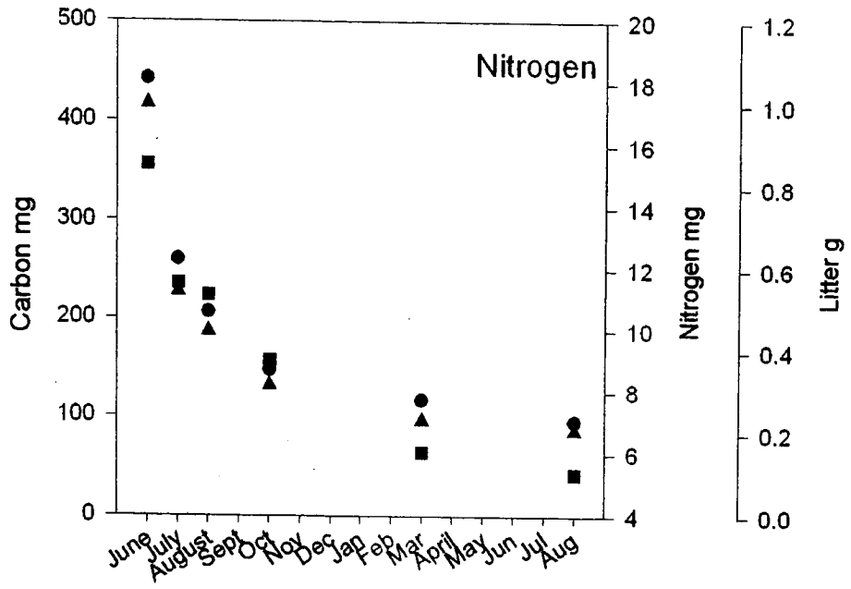
a. ***Mass of carbon and nitrogen***

Figure 3.15 shows the change in mass of remaining litter, C and N at each sampling time. In nitrogen and L+N plots, C and N closely followed mass loss, with a greater loss of N in nitrogen plots. In control and lime plots, decrease in C concentration and mass loss closely followed each other. Nitrogen loss was slow between July and August 2002 and fell sharply in control plots between October and March. N loss in lime plots was slower during the same period. Error bars were omitted from the chart as the trends in loss of mass were obscured by them. Means (±1SE) are presented in Table 3.19.

Table 3.19. Means (±1SE) for mass loss of litter, carbon and nitrogen in coarse litterbags at each sampling date and for each treatment plot at Rigg Foot, Sourhope.

Treatment	Time	Mass loss of litter (g)		Mass loss of carbon (mg)		Mass loss of nitrogen (mg)	
		Mean	1 SE	Mean	1 SE	Mean	1 SE
Control	June	1.00	0.00	435	13.6	17.53	1.07
	July	0.54	0.02	257	8.3	12.74	0.93
	August	0.48	0.01	213	14.7	12.58	0.60
	October	0.38	0.02	182	11.4	10.65	0.30
	March	0.24	0.04	114	15.7	5.99	0.97
	August	0.24	0.01	109	4.6	6.42	0.38
Nitrogen	June	1.00	0.00	442	7.8	15.38	0.43
	July	0.55	0.01	260	6.9	11.55	0.93
	August	0.45	0.04	206	16.5	11.17	1.39
	October	0.32	0.03	147	17.3	9.04	1.18
	March	0.23	0.02	116	9.2	6.04	0.12
	August	0.20	0.04	95	20.5	5.35	1.11
Lime	June	1.00	0.01	449	5.0	17.45	0.95
	July	0.57	0.02	278	9.3	13.07	0.62
	August	0.47	0.01	216	5.3	12.55	0.81
	October	0.36	0.02	169	10.0	11.03	0.98
	March	0.29	0.01	149	7.1	9.38	0.43
	August	0.23	0.02	110	8.5	7.82	0.69
L+N	June	1.00	0.00	433	3.8	15.24	0.90
	July	0.56	0.02	274	11.7	12.54	0.74
	August	0.45	0.03	213	18.1	11.45	0.80
	October	0.38	0.01	176	9.8	10.33	0.88
	March	0.28	0.05	143	24.7	8.64	1.56
	August	0.24	0.03	110	13.9	8.19	0.98





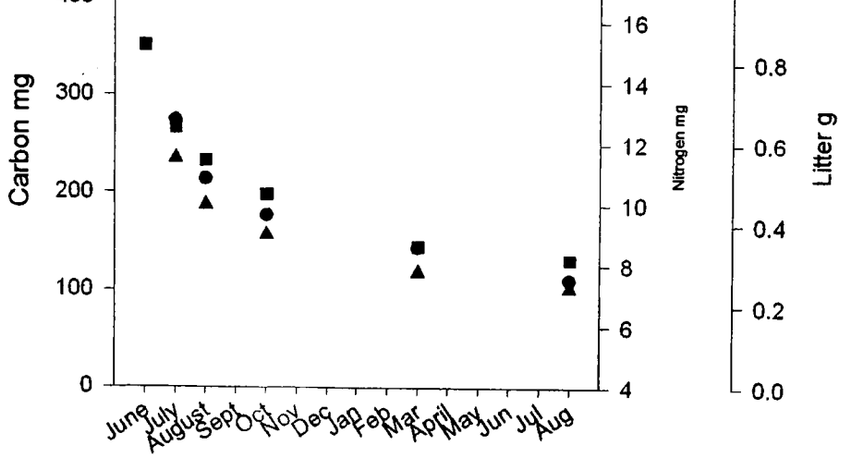


Figure 3.15. Mean loss of mass (mg) (▲), nitrogen (mg) (■) and carbon (mg) (●) from litter in fine litterbags at Rigg Foot, Sourhope. Experiment started June 2002.

vi. *Change in carbon to nitrogen (C:N) ratio.*

The overall changes in C:N ratio are shown in Table 3.20.

Table 3.20. Mean (± 1SE) change in C:N ratio of litter in fine litterbags in all plots at Rigg Foot, Sourhope during the 14 months of the experiment.

	C:N ratio July 2002	C:N ratio August 2003	Change in C:N ratio
Control fine bags	25:1 (2.1)	17:1 (0.5)	8
Nitrogen fine bags	29:1 (1.1)	18:1 (0.7)	11
Lime fine bags	26:1 (1.5)	14:1 (0.2)	12
L+N fine bags	29:1 (1.9)	13.5:1 (0.3)	15.5

The C:N ratios were slightly, but not significantly different in each treatment plots at the start of the experiment. This difference was not deliberate but reflects the different percentages of nitrogen that was assimilated into the grass tissue when it was grown in the greenhouse (range 1.15 – 2.05%).

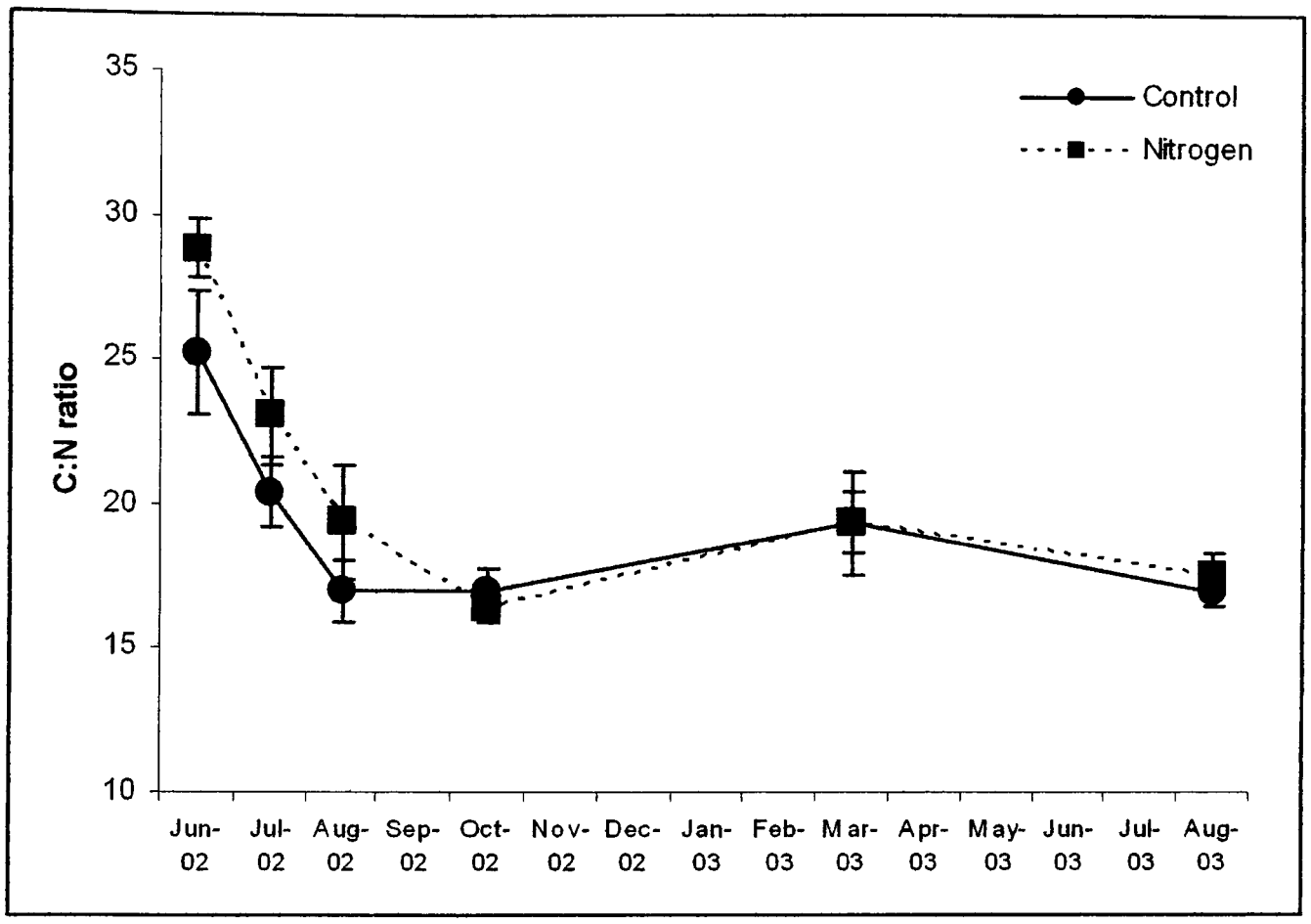
C:N ratio decreased rapidly during the first month and continued to fall in nitrogen, lime and L+N plots until October 2002. In control plots it remained static between August and October 2002. In the control and nitrogen (acid) plots there was an increase in C:N ratio over winter, whereas in the lime and L+N (neutral) plots it remained static during this period. It fell again during the second summer of the experiment in all plots (Figure 3.16).

The significance of the changes in final C:N ratio divided the treatments into acid and neutral (One way ANOVA, $df_{3,13}$, $F = 15.01$, $p > 0.001$). Post hoc Tukey's test showed the following values (Table 3.21).

Table 3.21. Tukey's post hoc test of differences and its significance between C:N ratios in the different treatment plots.

Treatment comparison	Mean difference	Significance
Control & lime	2.8	0.013
Nitrogen & lime	3.4	0.002
Control & L+N	3.6	0.002
Nitrogen & L+N	4.1	0.001
Control & nitrogen	-0.55	0.872
Lime & L+N	0.74	0.777

In nitrogen and lime plots, the C:N ratio decreased by a similar amount. The changes were least in control and greatest in L+N plots. After 14 months, despite the initial differences, the C:N ratio in control and nitrogen plots was similar (17:1 and 18:1) as was that of lime and L+N plots (14:1 and 13.5:1)



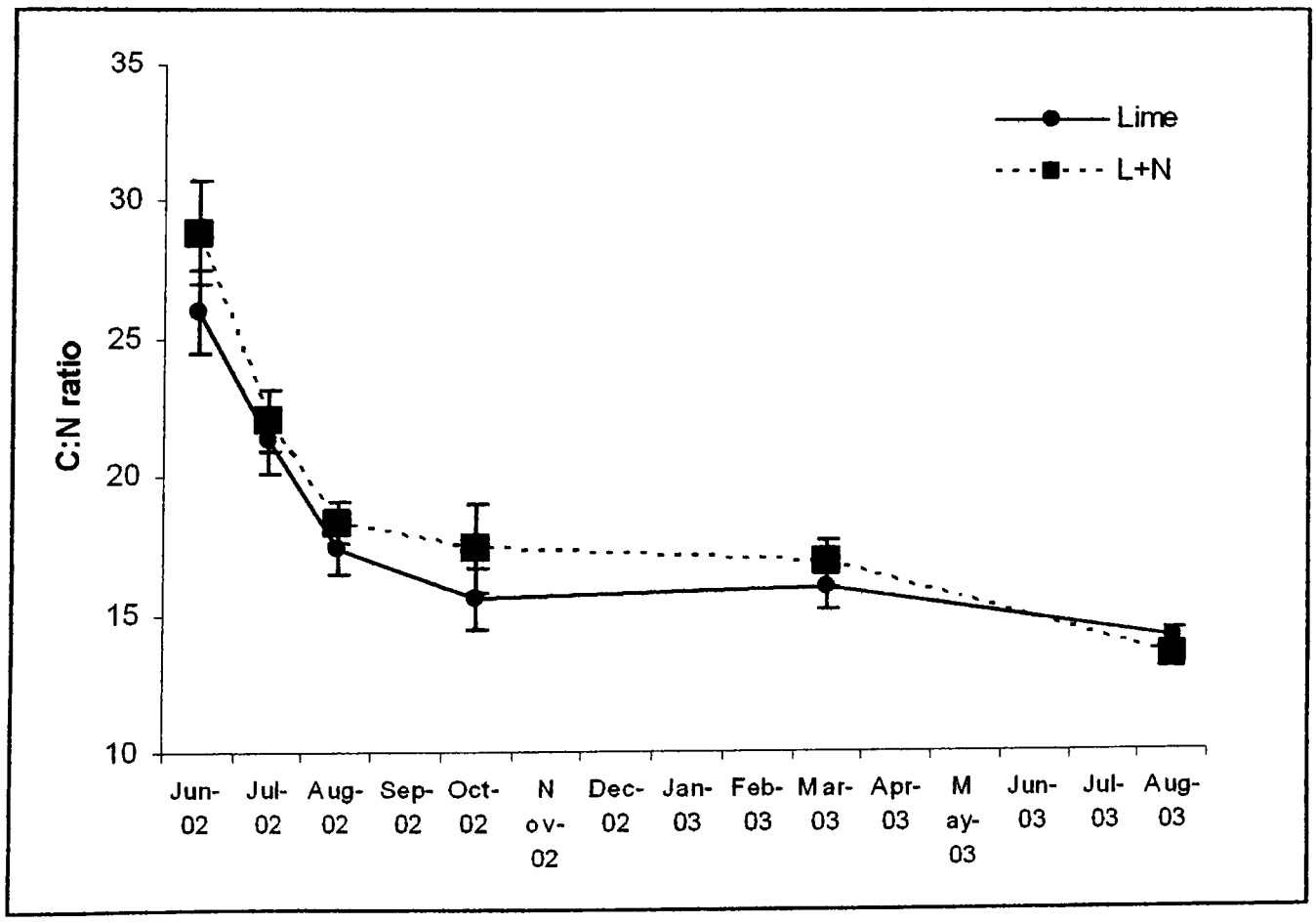


Figure 3.16. Change in C:N ratio of litter in fine litterbags in treatment plots at Rigg Foot, Sourhope. Experiment started July 2002. Mean ± 1SE.

Cause of the over winter increase in C:N ratio.

Both %N and %C increased in all plots in the first summer. The increase in %N was relatively greater causing the fall in C:N ratio in all plots. The increase in C:N ratio in Control and nitrogen plots over winter was caused by a continued increase in carbon content and a concomitant decrease in nitrogen content. However, in lime and L+N plots %N increased throughout the experiment and there was no overwinter increase in C:N ratio.

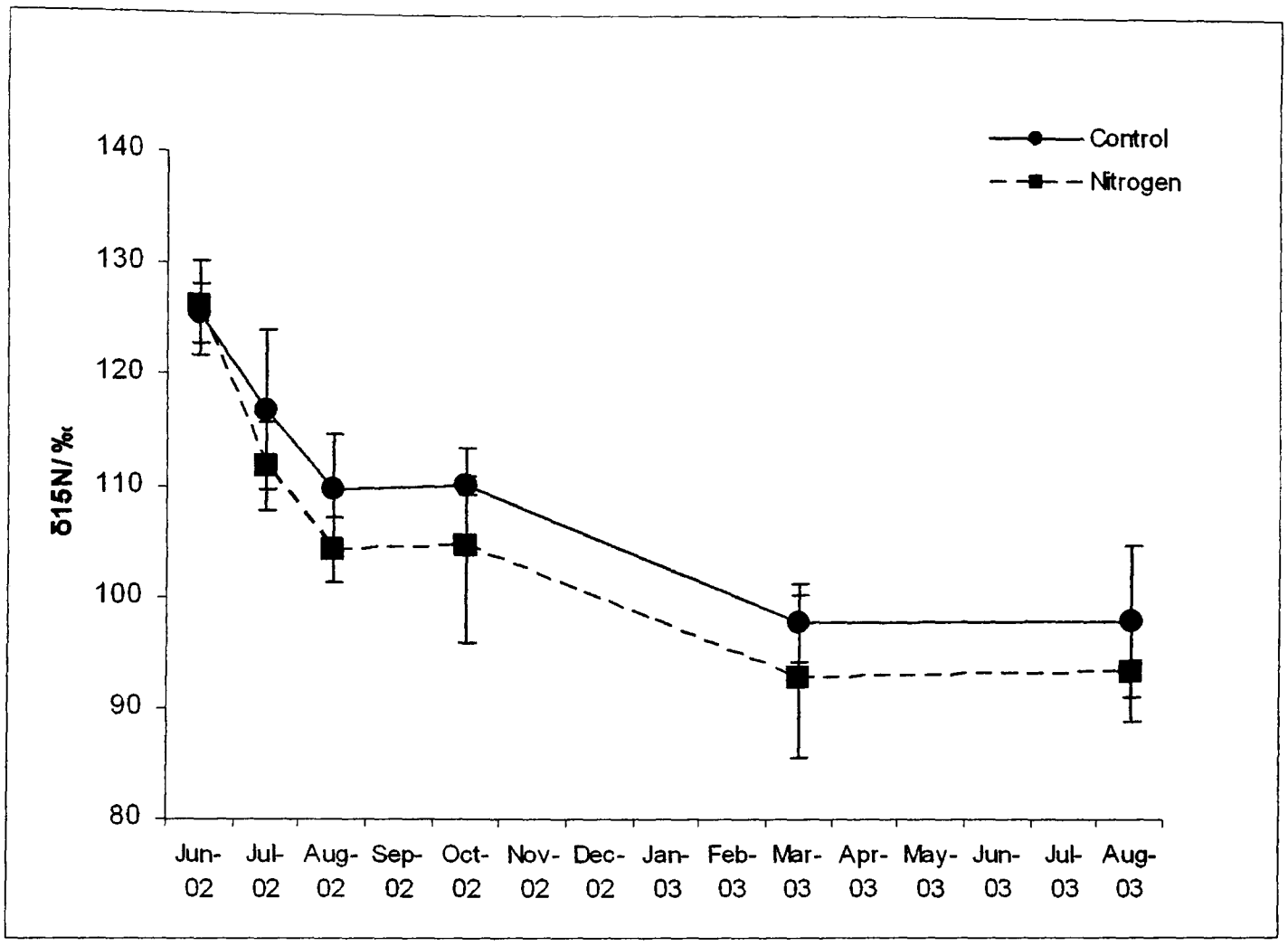
vii. *Change in $\delta^{15}N$ value of litter in fine litterbags.*

There were slight variations in the starting values of $\delta^{15}N$ in the *A. capillaris* litter in different litterbags. This was due to the different values achieved when labelling the grass with ^{15}N labelled urea.

The $\delta^{15}N$ values of the litter in all treatment plots followed the same pattern of loss. Table 3.22 shows the initial and final $\delta^{15}N$ and change during the period of the experiment, and Figure 3.17 shows the values at each sampling time.

Table3.22. Initial, final and difference in $\delta^{15}N$ values of litter placed in control, nitrogen, lime and L+N plots at Rigg Foot, Sourhope. Litterbags were placed in the plots in June 2002 and the final sampling date was August 2003. Mean (± 1SE).

	Initial value	Final value	Difference
Control	125 (2.7)	98 (6.8)	27
Nitrogen	125 (4.2)	93 (4.4)	32
Lime	123 (7.1)	107 (5.5)	16
L+N	125 (6.0)	104 (4.1)	21



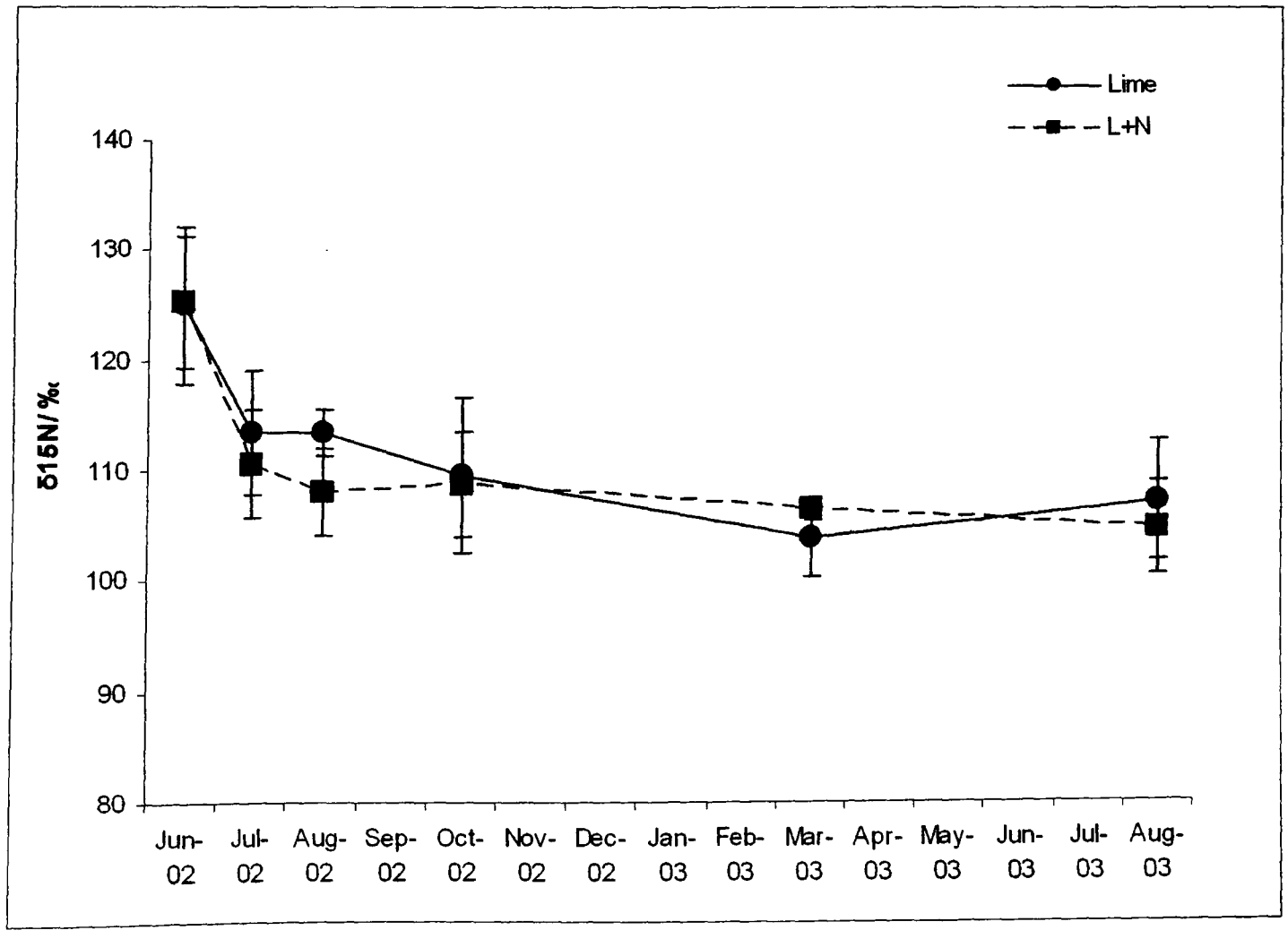


Figure 3.17. Changes in $\delta^{15}N$ values of litter in fine litterbags during the 14 month experimental period in control and nitrogen (top), and lime and lime + nitrogen (bottom) plots at Rigg Foot, Sourhope. Mean ± 1SE. Experiment started June 2002.

The greatest loss of $\delta^{15}N$ was from litter in nitrogen and control plots, with less lost from lime and L+N plots. One-way ANOVA showed no significant differences between the final values in the different treatment plots ($df_{3,13}$, $F = 1.19$, $p = 0.35$).

Fine mesh litterbags (continued):

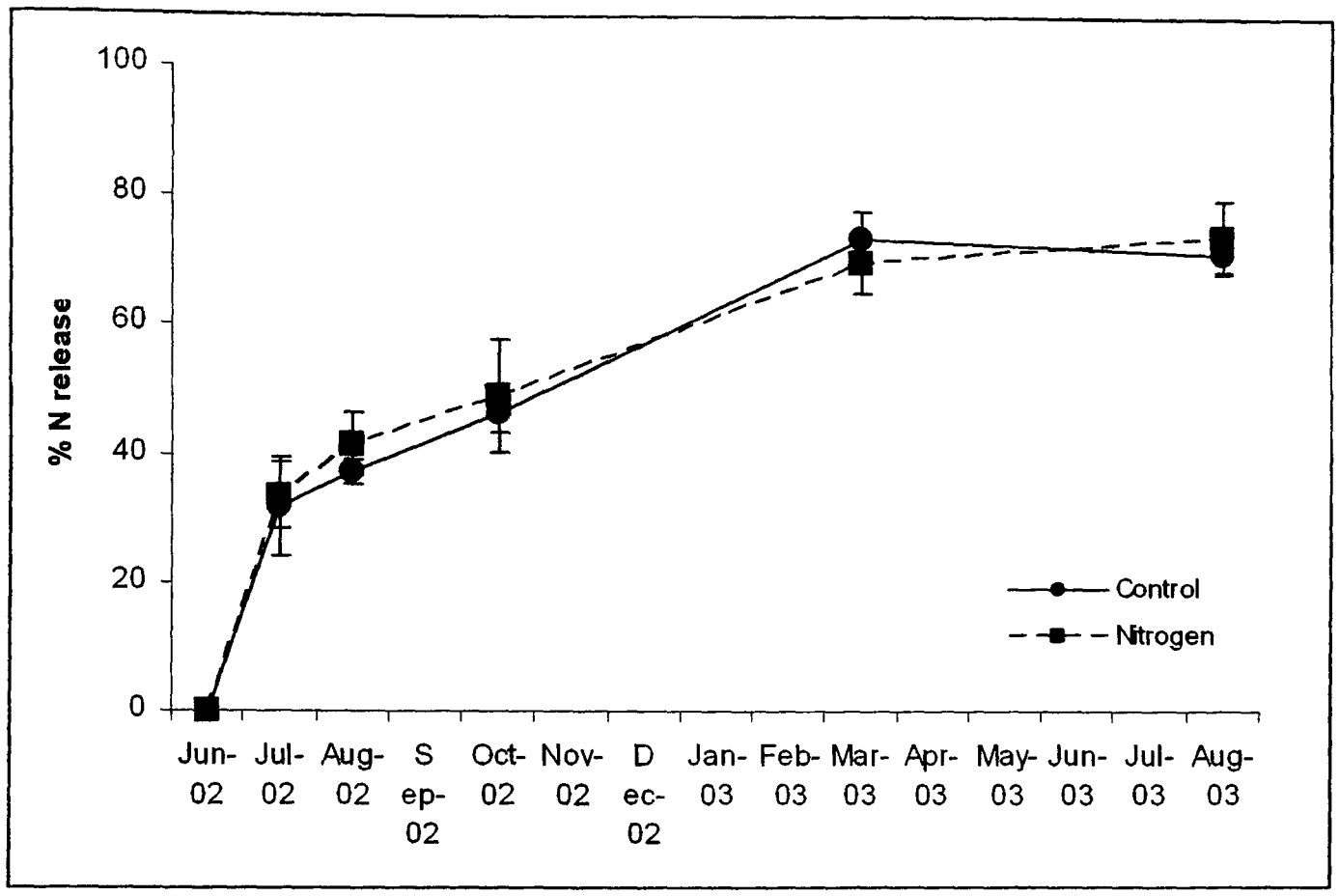
3. Nitrogen dynamics.

Changes in mass of litter, N and $\delta^{15}N$ described in the previous section and the net result of N released and incorporated by various means in the litter was estimated using the methods of Berg (1988) and Zeller (2000).

i. ***Nitrogen release.***

There was a rapid release of nitrogen from the litter during the first month of the experiment. Maximum initial release was from nitrogen, control and lime plots (33, 32 and 31% respectively) with L+N plots releasing less N (26%). Nitrogen was released throughout the experimental period and overall release of N during the experiment followed the same treatment pattern – N, C, L & L+N (74, 71, 60 and 51% respectively). The values were not significantly different from each other (ANOVA, $df_{3,13}$, $F = 2.76$, $p = 0.08$). Regression analysis also showed no differences between the slopes of the regression lines between treatment plots.

The subsequent pattern of N release was similar in control and nitrogen plots, where release slowed during the first summer but increased during the winter. In lime and L+N plots, N release was more constant (Figure 3.18) as there was no overwinter increase



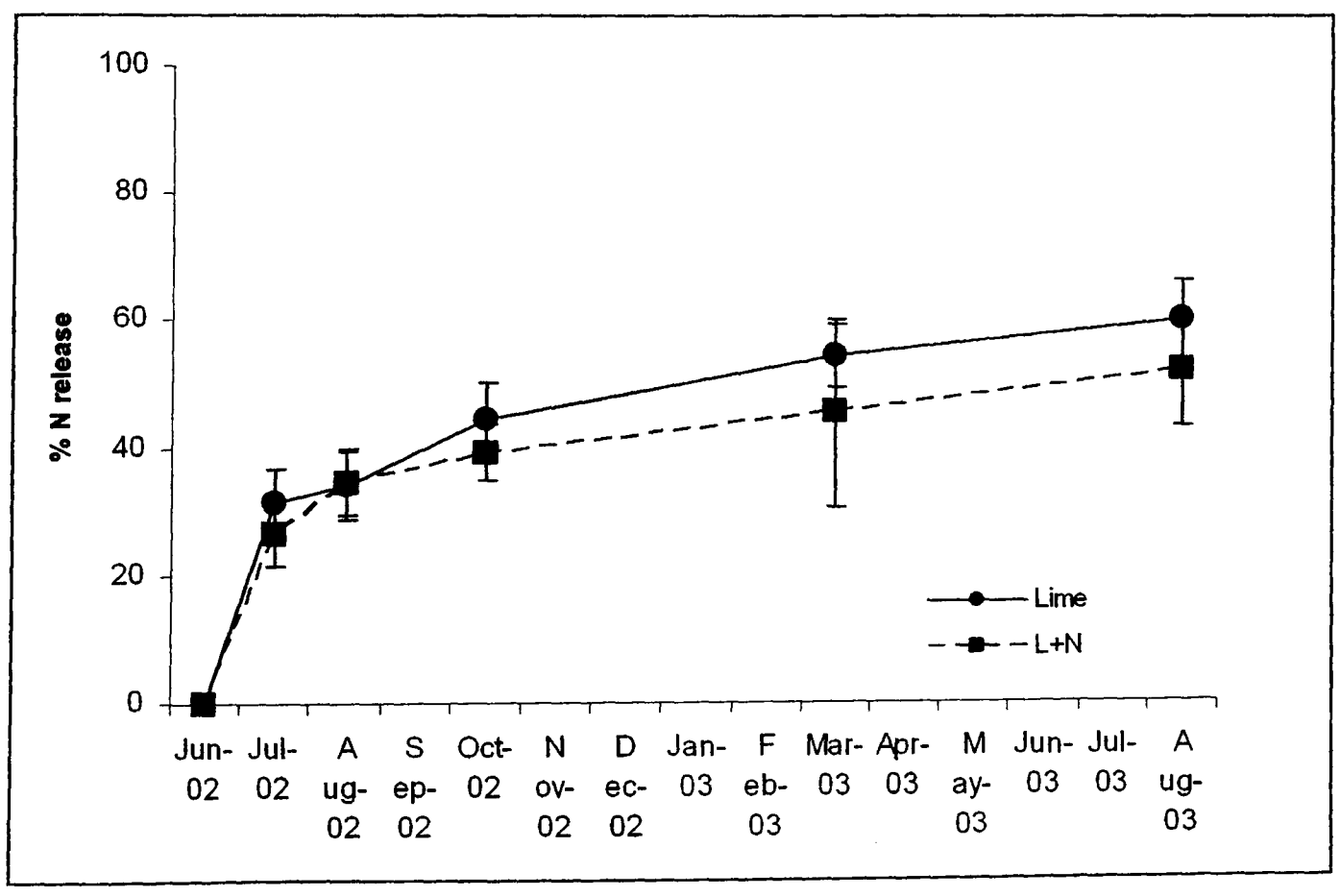
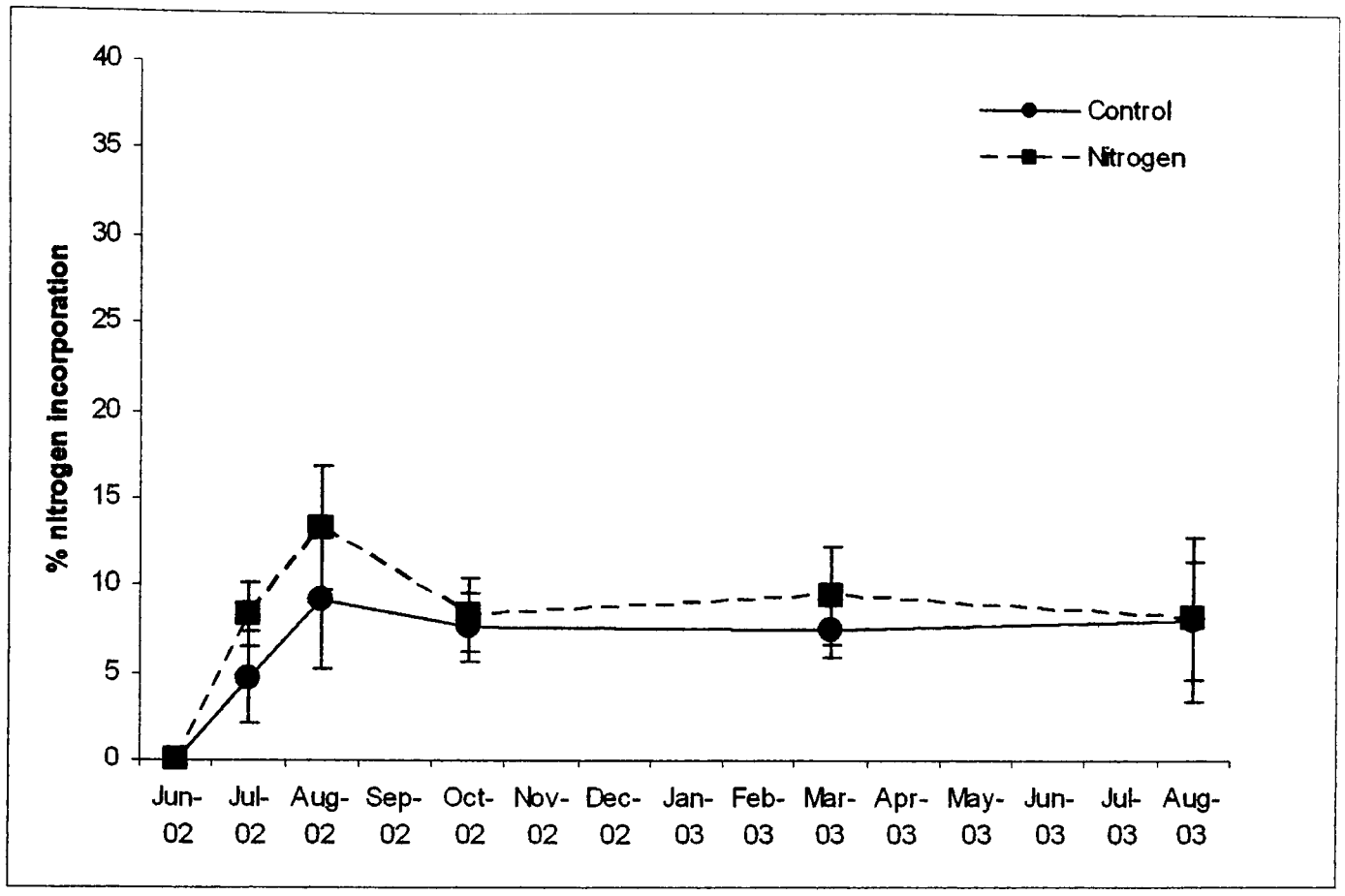


Figure 3.18. Cumulative nitrogen release (%) from litter in fine litterbags in treatment plots at Rigg Foot, Sourhope during the experimental period. Mean ± 1SE.
Each data point calculated as T0 – T1; T0 – T2 etc.

ii. ***Nitrogen incorporation.***

%N incorporation was considerably less in fine litterbags than coarse litterbags in all plots. There was initial incorporation of external N of between 5 and 9%, but following this there was no further increase or decline in incorporation (Figure 3.19). Maximum incorporated N was 13% in nitrogen plots in August 2002, but thereafter it fell to the same value as the other three plots. However these values were not significantly different from each other (One-way ANOVA, $df_{3,13}$, $F = 0.098$, $p = 0.96$).



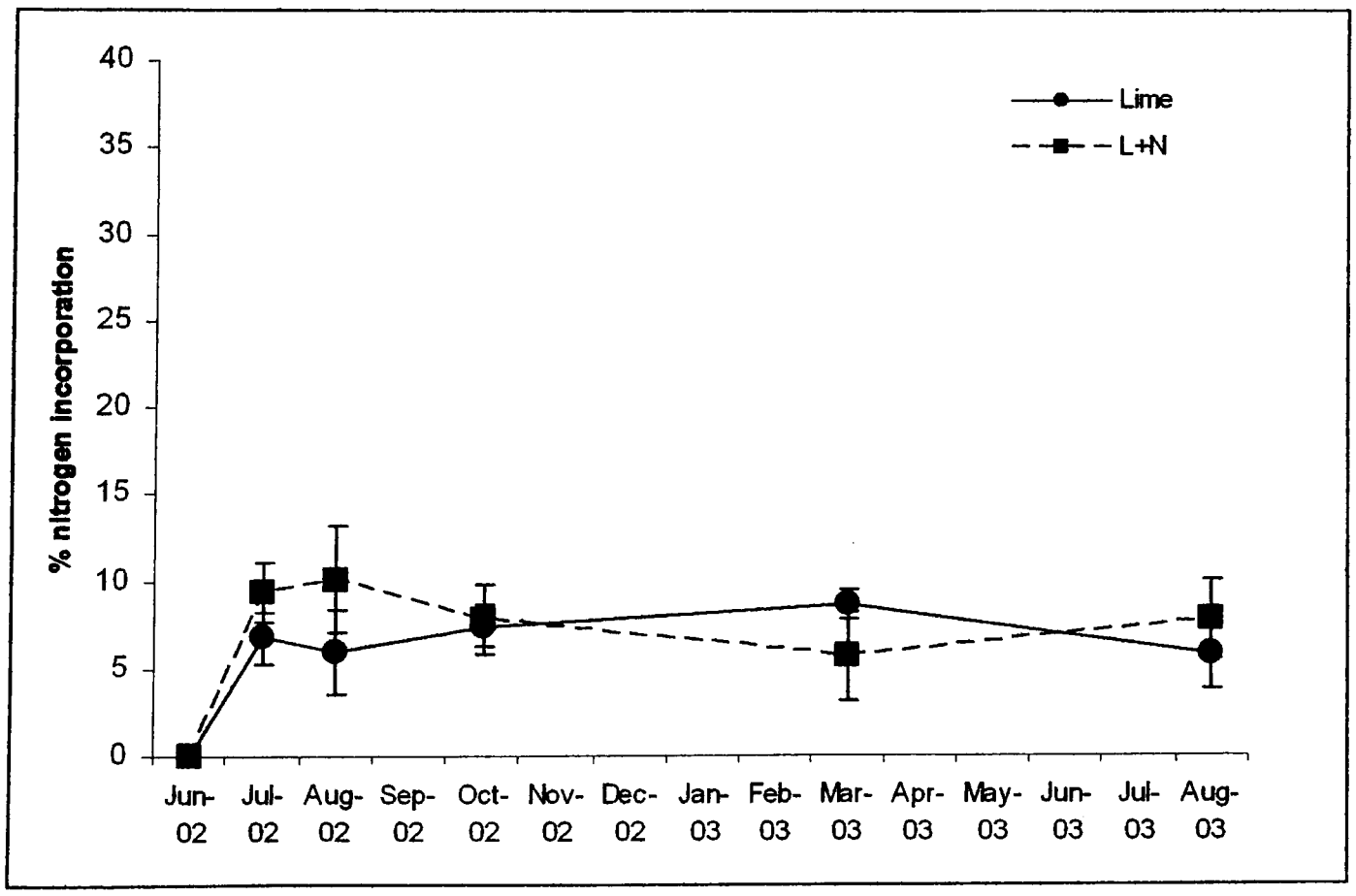


Figure 3.19. Cumulative nitrogen release (%) from litter in fine litterbags in treatment plots at Rigg Foot, Sourhope during the experimental period. Mean ± 1SE.
Each data point calculated as T0 – T1; T0 – T2 etc

iii.*Nitrogen release and % litter N in relation to loss of litter mass.*

Changes in litter mass, % litter N and % N release are shown in Figures 3.20 and 3.21.

In C and N plots % litter N declined overwinter when litter N release was 45% and mass loss was 60-65%. N release overwinter was 25%.

In L and L+N plots there was no decline in litter %N even though N release had reached 45-55% and mass loss was 75%. N release overwinter was only 10%.

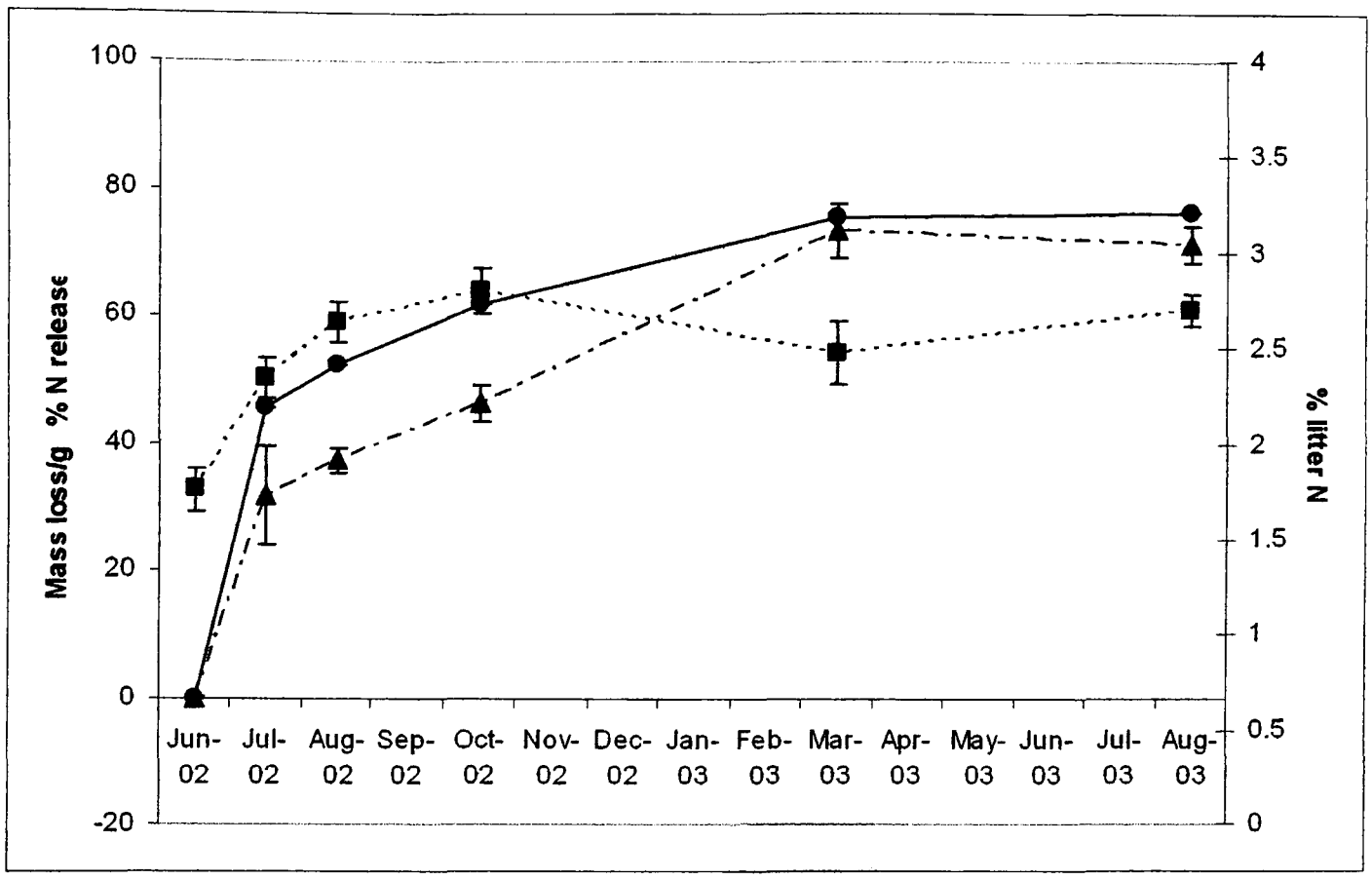
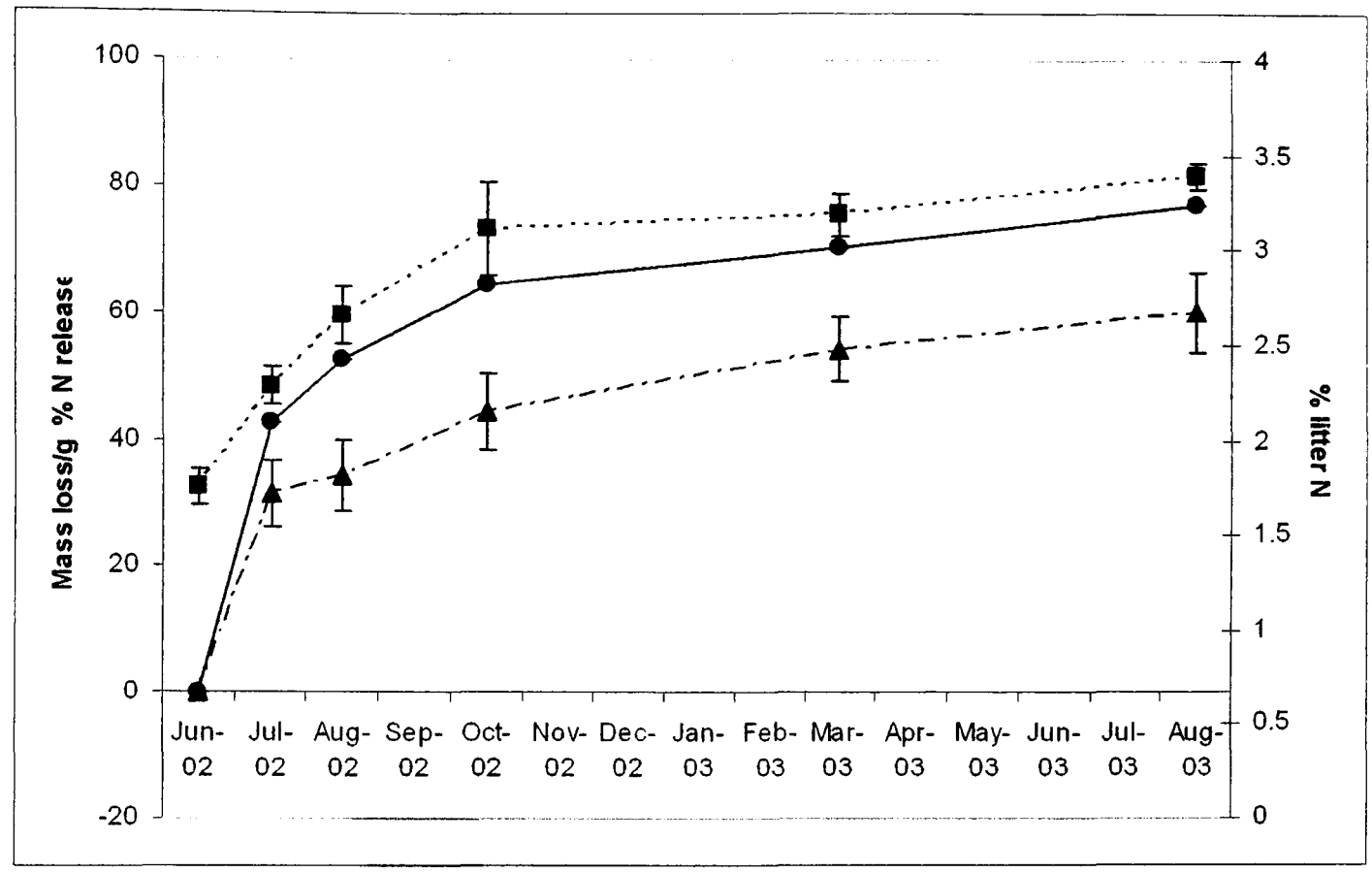


Figure 3.20. Relationship between mass loss (●), % N release (▲) and % litter N (■) of litter in fine litterbags in control (top) and nitrogen (bottom) plots at Rigg Foot, Sourhope. Means ± 1SE.



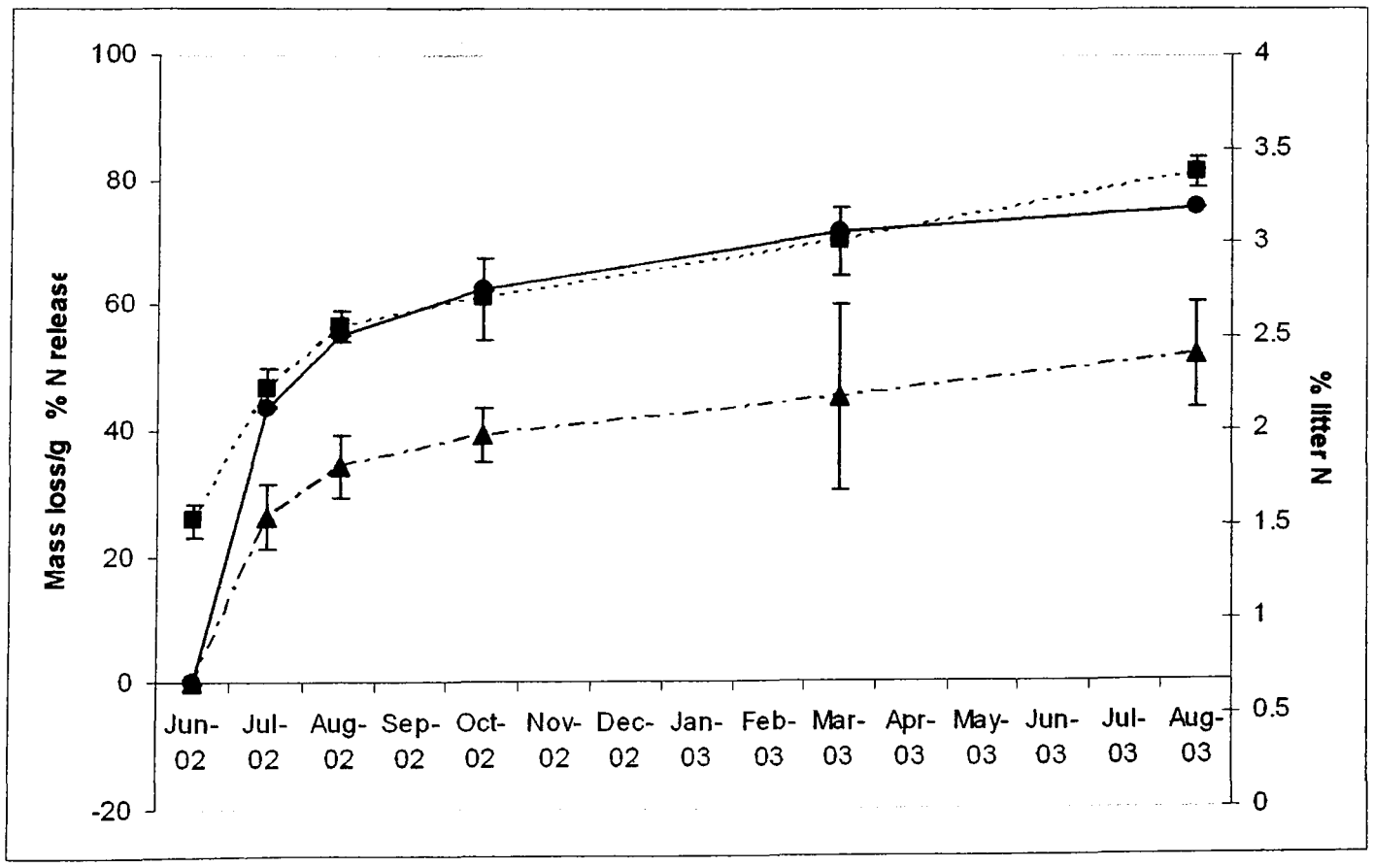


Figure 3.21. Relationship between mass loss (●), % N release (▲) and % litter N (■) of litter in fine litterbags in lime (top) and L+N (bottom) plots at Rigg Foot, Sourhope. Means ± 1SE.

3.3.4 Enchytraeids and nitrogen translocation.

3.3.4.1 $\delta^{15}N$ in enchytraeids.

1. Coarse mesh litterbags and surrounding soil cores.

It was assumed that the $\delta^{15}N$ values of enchytraeids extracted from bags and cores increased due to tissue assimilation of dietary nitrogen from labelled litter, with a proportion due to gut contents (Appendix II).

i. *Control and nitrogen plots.*

Coarse litterbags.

Only *C. sphagnetorum* were analysed for $\delta^{15}N$.

Figure 3.22 shows the patterns of $\delta^{15}N$ (‰) of the enchytraeid *C. sphagnetorum* initially and extracted at intervals from coarse litterbags and surrounding soil cores in the control and nitrogen plots. An anomalous value in control coarse bags at 9 months (<80‰) was omitted.

A maximum of ~ 40‰ (change in (Δ) ^{15}N of ~35‰ from NA) was found in worm tissue in bags in both control and nitrogen plots. This was reached by 2 months into the experiment. In the control plots, the ^{15}N value of enchytraeids was significantly higher at 1, 2 and 4 months in comparison to natural abundance (Table 3.23). In the nitrogen plots, ^{15}N in enchytraeid tissue was significantly higher than NA after one and two months. There was no difference between treatments.

Table 3.23. One-way ANOVA of ^{15}N values of *C. sphagnetorum* extracted from coarse litterbags in control and nitrogen plots at Rigg Foot, Sourhope during the 14 months of the experiment.

	Time	*p*	F	df
Control plots coarse bags	NA < 1, 2 and 4 months	0.006	10.42	3,10
Nitrogen plots coarse bags	NA < 1, 2 months	0.035	5.12	3,10
Control versus Nitrogen coarse bags		NS		

Coarse soil cores.

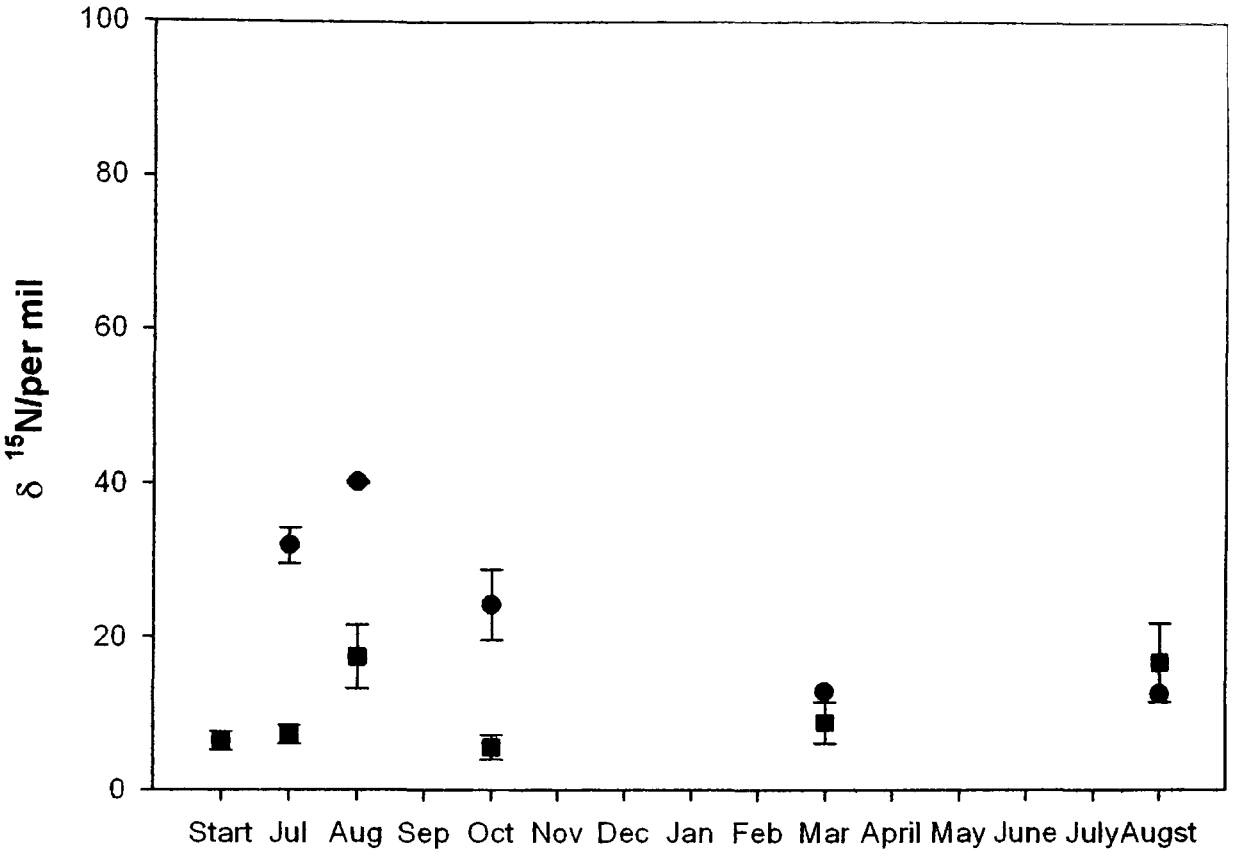
In the soil cores compared to the litterbags there was a 'lag' before any change in mean tissue $\delta^{15}N$ of the enchytraeids was detected. The $\delta^{15}N$ values of enchytraeids after one month were similar to NA, and it wasn't until two months after litterbag placement that rises in $\delta^{15}N$ were detected.

The maximum $\delta^{15}N$ found in enchytraeid tissue in the soil cores was ~ 17‰ in control plots and ~26‰ in nitrogen plots after 2 months, a $\Delta^{15}N$ of 12‰ and 20‰ respectively. In both treatment plots there was a fall in ^{15}N in October and March. In both plots, it rose to 18‰ in September 2003. However there were no significant differences between $\delta^{15}N$ in enchytraeid tissue in the control plots between any of the sampling periods or NA.

In the nitrogen plots ^{15}N values were significantly higher at some time periods compared to NA values (Table 3.24). Comparing between treatments, the mean $\delta^{15}N$ value of soil core enchytraeids in nitrogen plots after four months was greater than that in control plots. The latter had returned to a natural abundance value.

Table 3.24. One-way ANOVA of $\delta^{15}N$ values of *C. sphagnetorum* extracted from surrounding cores in control and nitrogen plots at Rigg Foot, Sourhope during the 14 months of the experiment.

	Time	*p*	F	df
Control plot coarse cores		NS		
Nitrogen plot coarse cores	2 months > NA, 1 and 4 months	0.002	16.27	3,10
Control versus nitrogen cores		NS		



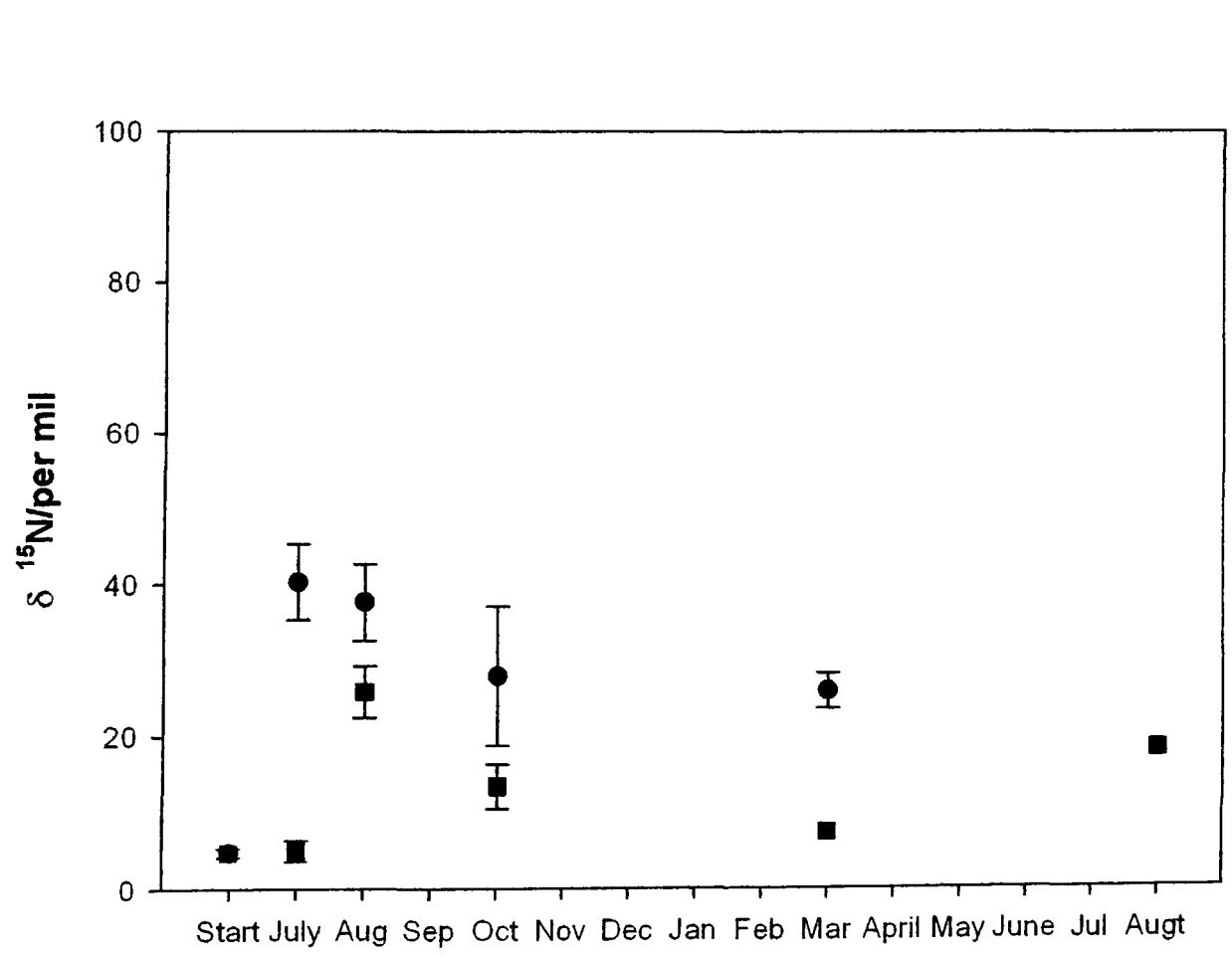


Figure 3.22. $\delta^{15}N$ values of enchytraeids extracted from coarse litterbags (●)and surrounding cores (■) in control (top) and nitrogen (bottom) plots at Rigg Foot, Sourhope. Experiment started June 2002.

ii. ***Lime and L+N treated plots.***

Many different species of enchytraeid were extracted from coarse bags in neutral plots and in order to have sufficient material for analysis 4 *Fridericia* and 1 *Henlea* species were bulked together.

Coarse litterbags.

Figure 3.23 shows the pattern of $\delta^{15}N$ of enchytraeids (*Fridericia spp* and *H. perpusilla)* initially and extracted at intervals from coarse litterbags and surrounding soil cores in the lime and L+N plots.

A maximum of ~ 57‰ (Δ ^{15}N of 52‰) was found in worm tissue in bags in lime plots, and ~34‰ (Δ ^{15}N of 26‰) in L+N plots. This was reached by 2 months into the experiment. In lime plots, the tissue ^{15}N of enchytraeids was significantly higher at 1, 2 and 4 months compared to NA (Table 3.25). There was no significant difference between tissue ^{15}N of enchytraeids in L+N plots. Enchytraeids in lime plots had significantly higher ^{15}N values than those in L+N plots.

Table 3.25. One-way ANOVA of ^{15}N values of enchytraeids extracted from coarse litterbags in lime and L+N plots at Rigg Foot, Sourhope during the 14 months of the experiment.

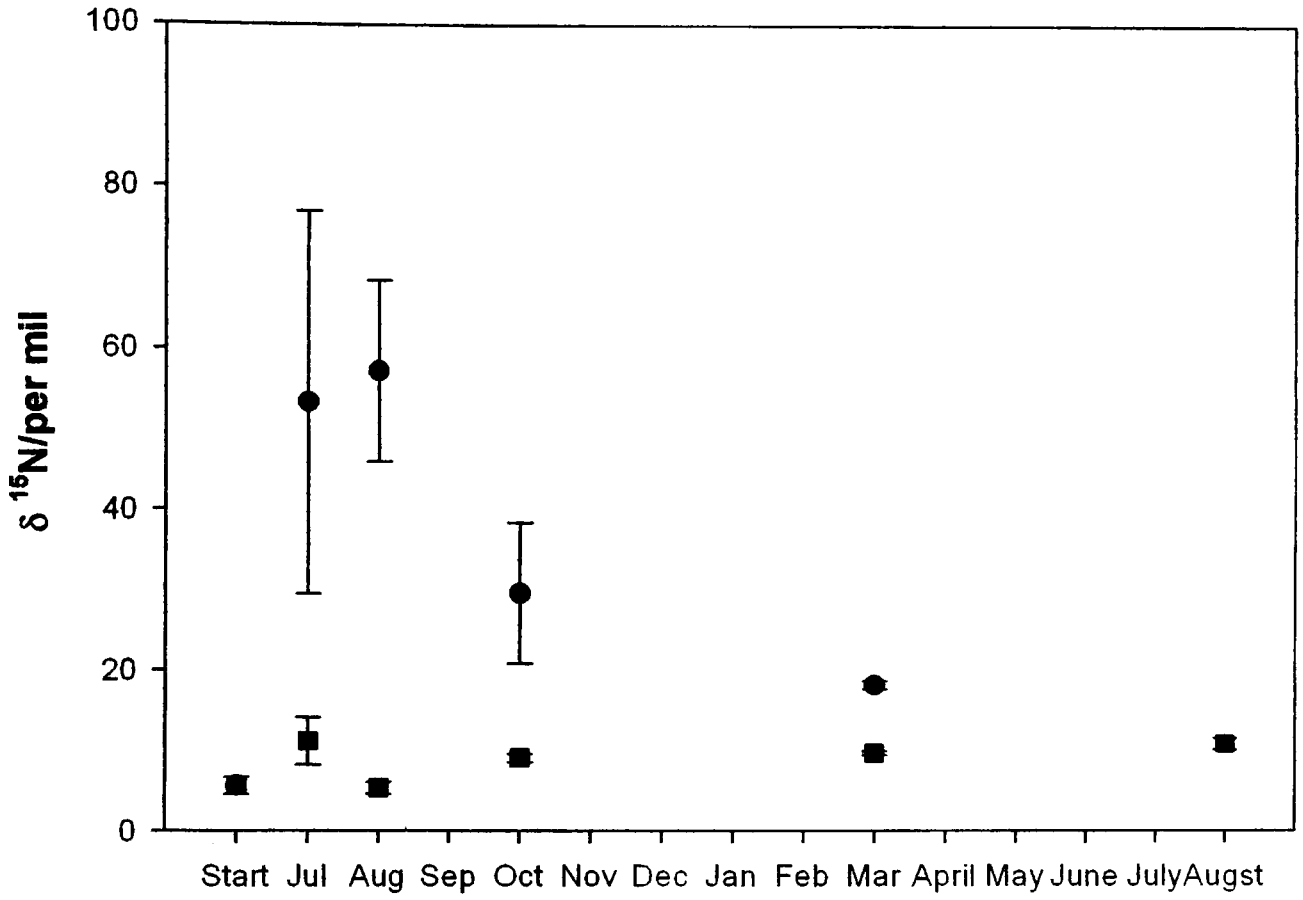
	Time	*p*	F	df
Lime plots coarse bags	NA < 1, 2 and 4 months	0.001	10.67	4,12
L+N plots coarse bags		NS		
Lime versus L+N coarse bags		0.02	3.96	3,57

Coarse soil cores.

Mean $\delta^{15}N$ values of enchytraeids extracted from coarse soil cores increased immediately, with a $\Delta\ \delta^{15}N$ of 6‰ in lime and 16‰ in L+N plots after one month – i.e. there was no 'lag' as observed in control and nitrogen plots. $\delta^{15}N$ values were slightly raised (10‰) from October 2002 until August 2003 in lime plots. Values were comparatively high in L+N plots, although in March 2003 they had returned to NA. In lime plots, the increase in $\delta^{15}N$ value after 1 month was significantly higher than NA (Table 3.25).

Table 3.25. One-way ANOVA of $\delta^{15}N$ values of enchytraeids extracted from surrounding cores in lime and L+N plots at Rigg Foot, Sourhope during the 14 months of the experiment.

	Time	*p*	F	df
Lime plot coarse cores	NA < 1 month	0.03	3.66	4,18
L+N plot coarse cores		NS		
Lime versus L+N plots		NS		



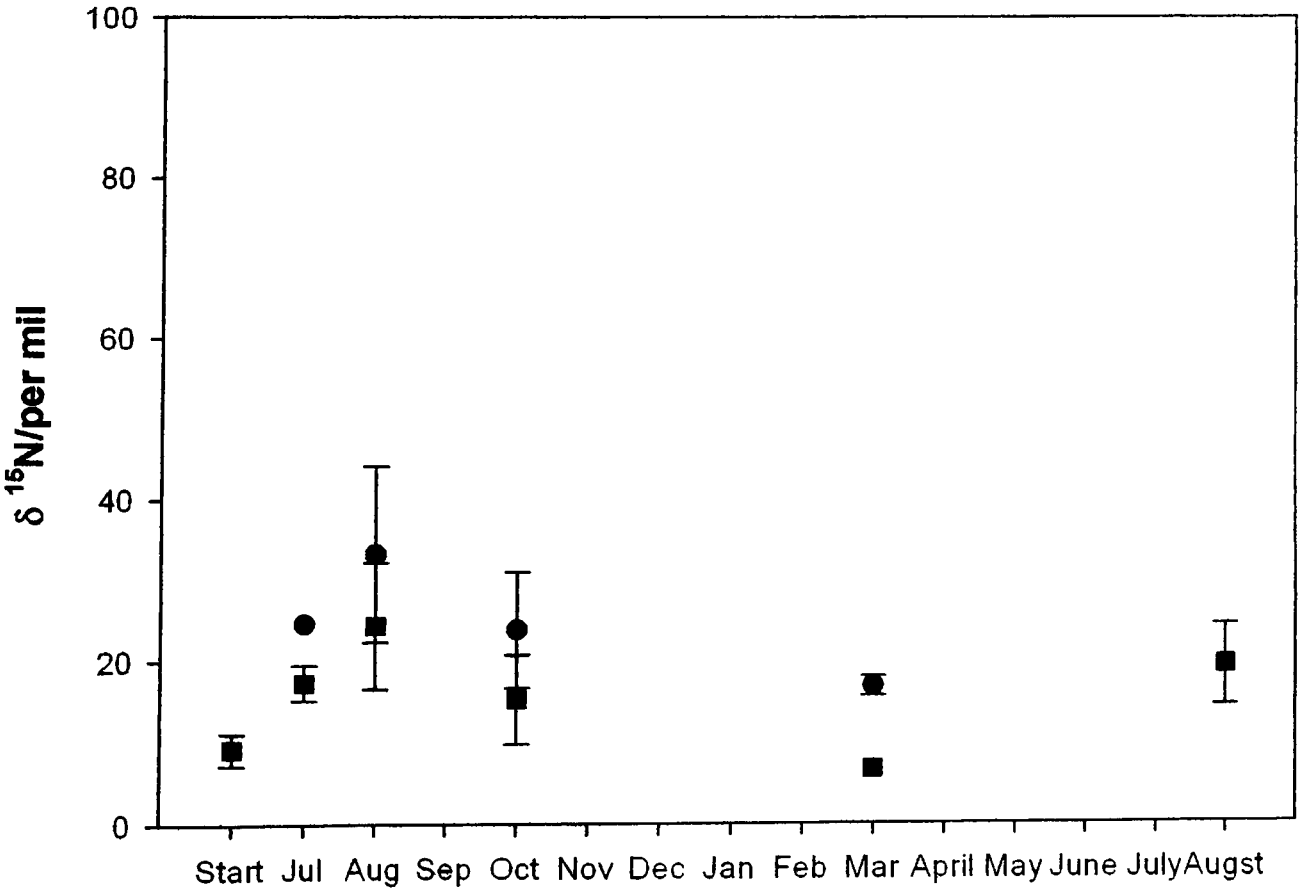


Figure 3.23. $\delta^{15}N$ values of enchytraeids extracted from coarse litterbags (●) and surrounding cores (■) in lime (top) and lime + nitrogen (bottom) plots at Rigg Foot, Sourhope. Experiment started June 2002

iii.*Comparison of control and lime treated plots.*

Coarse litterbags.

The overall pattern of $\delta^{15}N$ values of enchytraeids extracted from coarse bags in control and lime plots was very similar. However, in lime plots the greatest $\delta^{15}N$ value was higher (~ 60‰) compared to control plots (~ 40‰) after 2 – 4 months. After 14 months the $\delta^{15}N$ values were still slightly above NA values in both plots, but none of the differences were significant owing to small sample size and large S.E.'s.

Coarse soil cores.

The initial 'lag' detected in control plots enchytraeids was not apparent in lime plots, and the $\delta^{15}N$ values of enchytraeids extracted from cores in control plots (~ 18‰) was greater than lime plots (~ 10‰). In both plots, however, the $\delta^{15}N$ signal of enchytraeids in cores was quite distinctly separated from that of enchytraeids in bags. There were no significant differences between the $\delta^{15}N$ values of enchytraeids in control and lime cores.

3.3.4.1 $\delta^{15}N$ in enchytraeids (continued).

2. Fine mesh litterbags.

Results for control and nitrogen and lime and L+N treatments are described separately and then compared.

i. *Control and nitrogen plots.*

Only *C. sphagnetorum* were analysed for $\delta^{15}N$. *C. sphagnetorum* from surrounding soil cores were collected for analysis, but there was a technical difficulty which meant that they could not ultimately be analysed, therefore there are no biomass data for the enchytraeids in soil cores.

Figure 3.24 shows the mean $\delta^{15}N$ values of the enchytraeids in the fine bags in control and nitrogen plots. There were no data for the first month as there were insufficient enchytraieds for analysis. In control plots, the tissue $\delta^{15}N$ of *C. sphagnetorum* rose from NA ~5‰ to ~60‰ within two months, then to ~80‰ thereafter. In the nitrogen plots, fewer replicate samples were obtained and the pattern of ^{15}N content of enchytraeid tissue was less clear cut. However, the maximum value didn't exceed 100‰ (9 months) and appeared to stabilise at approximately 80‰ after 14 months.

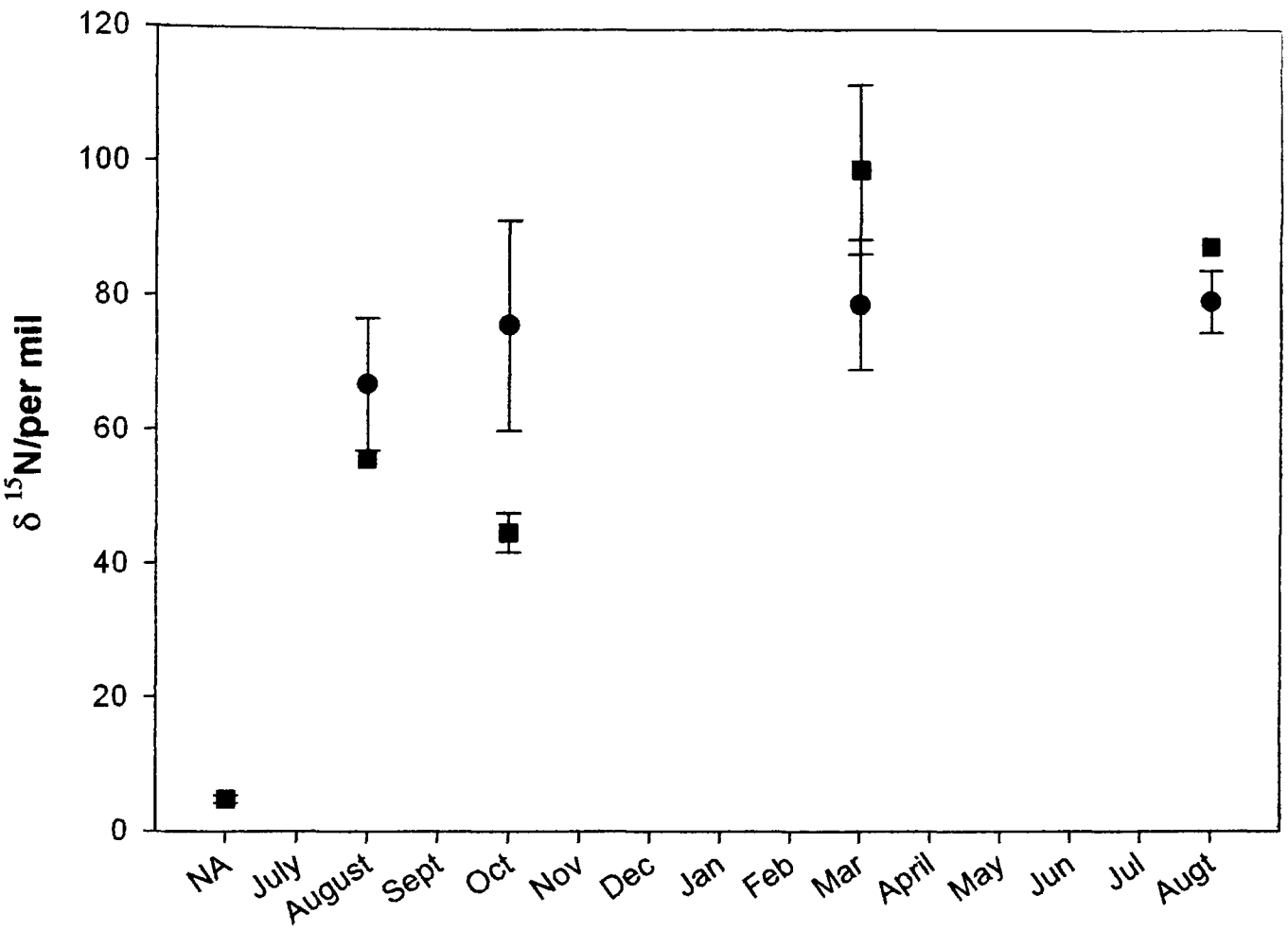


Figure 3.24. Tissue $\delta^{15}N$ of enchytraeids extracted from fine litterbags in control (●) and nitrogen (■) plots at Rigg Foot, Sourhope. Experiment started June 2002. Mean ± 1SE.

There were no significant differences between $\delta^{15}N$ values of *C. sphagnetorum* in control or nitrogen treatments (Two-way ANOVA with replication, $df_{1,20}$, $F = 0.53$, $p = 0.48$).

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ii. *Lime and L+N plots.*

The fine bags in lime and L+N plots contained many different enchytraeid species (Section). In order to have sufficient sample for analysis, it was necessary to bulk together three species of *Fridericia* and one of *Henlea*. The species present in the fine litterbags during the period of the experiment are shown in Table 3.27.

Table 3.27. List of species extracted from the fine litterbags in lime and lime + nitrogen plots at each sampling time at Rigg Foot, Sourhope. * denotes presence in bags.

	July 2002		August 2002		October 2002		March 2003		August 2003	
	Lime	L+N	Lime	L+N	Lime	L+N	Lime	L+N	Lime	L+N
Enchytraeus buchholzia	*	*	*	*	*			*		
E. buchholzia immature				*						
Enchytronia parva							*			
Fridericia perrieri			*	*	*		*	*		*
F. paroniana						*				
F. galba					*	*				*
F. bisetosa			*	*	*	*	*	*		*
F. ratzelli								*		
F. bulboides	*		*							
F. immature			*		*				*	
Henlea perpusilla	*		*	*	*	*	*		*	
H. perpusilla immature				*		*		*		
H. venticulosa				*			*			
Bucholzia appendiculata					*					

Figure 3.25 shows the $\delta^{15}N$ values of the enchytraeids in the fine bags in lime and L+N plots. There were no data for the first month as not enough enchytraeids were extracted for analysis.

In lime plots, the tissue ^{15}N rose from NA ~ 5‰ to a final value of ~ 100‰ after 14 months. In L+N plots the increase in tissue ^{15}N was similar to lime plots, with a NA value of 6‰ and a final value of 100‰ at the end of the experiment.

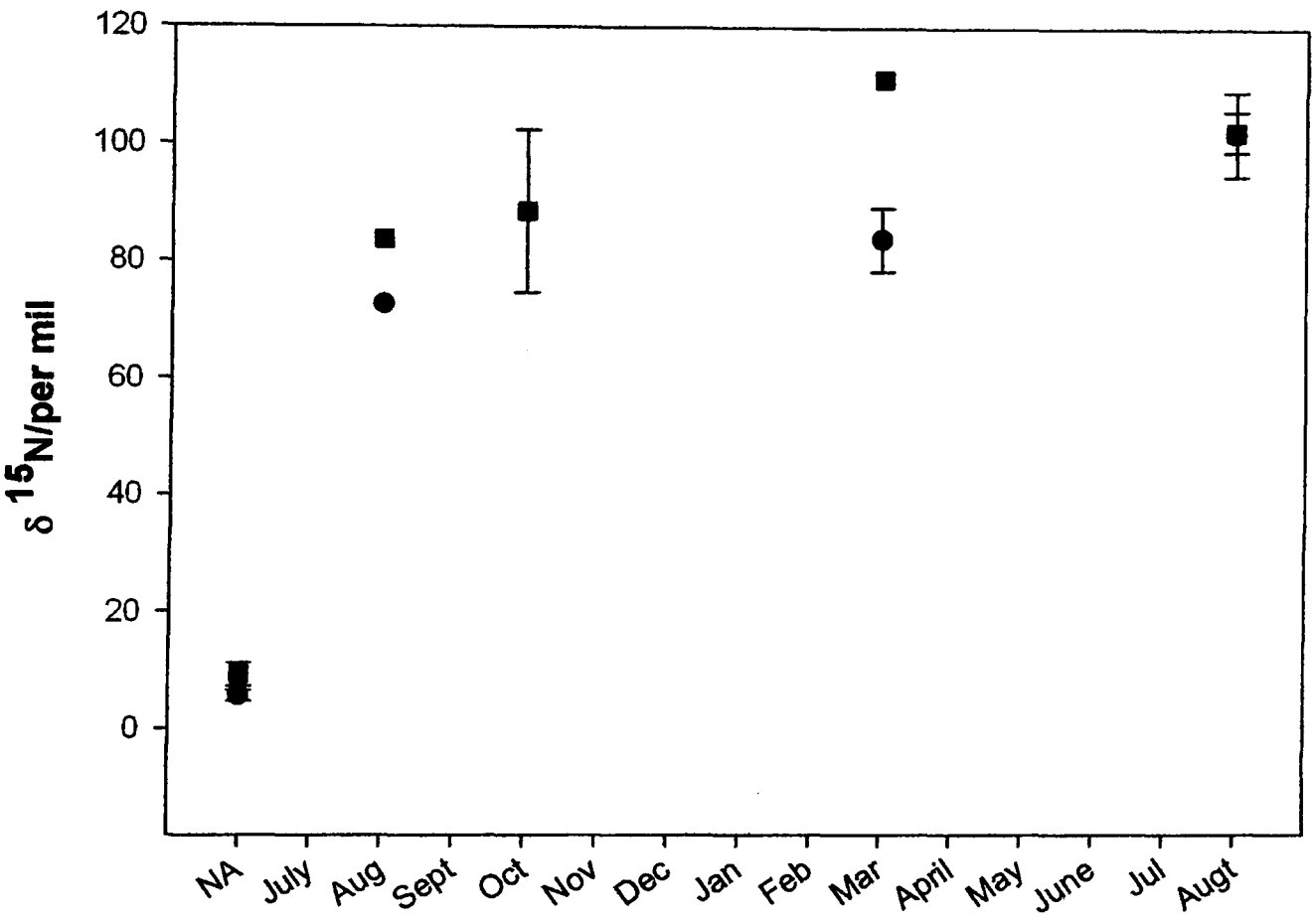


Figure 3.25. Tissue $\delta^{15}N$ of enchytraeids extracted from fine litterbags in lime (●) and lime + nitrogen (■) plots at Rigg Foot, Sourhope. Experiment started June 2002.

There were no significant differences between enchytraeid $\delta^{15}N$ values in lime and L+N plots (Two-way ANOVA with replication, $df_{1,25}$, $F = 2.28$, $p = 0.144$).

To assess whether nitrogen treatment affected $\delta^{15}N$ values of enchytraeids, values for nitrogen treatments were bulked (nitrogen and L+N), as were those for non-nitrogen treatments (control and lime). Treatment had no effect on $\delta^{15}N$ values of

enchytraeids, but time significantly affected the values. However, time and treatment had no significant interaction (Tables 3.28 and 3.29).

Table 3.28. Two-way ANOVA with replication comparing $\delta^{15}N$ values of enchytraeids in fine litterbags in non nitrogen (control and lime) and nitrogen (nitrogen and L+N) treated plots.

	Sum of Squares	df	Mean Square	F	Sig.
Time	61038	4	15259	63.77	>0.001
Treatment	176	1	176	.734	0.397
Time * treatment	1042	4	260	1.09	0.376
Error	9333	39	239		

Table 3.29. *Post hoc* Tukey test of differences between N treated and untreated plots.

		Mean Difference	Significance
60 weeks	NA	86.4549	>0.001
	7 weeks	24.2489	0.038
	16 weeks	25.2943	0.014
	39 weeks	5.2936	0.944

3.3.4.2 Estimating the half-life of nitrogen in enchytraeid tissue.

The pattern of $\delta^{15}N$ accumulation by enchytraeids in fine bags appeared to rise gradually and then stabilise. This was in contrast to the pattern for enchytraeids extracted from coarse bags and suggests that enchytraeids were resident within the bags. By contrast the coarse bag enchytraeids moved in and out of the bags so that values obtained were the mean of enchytraeids which had been feeding on labelled litter in the bags for some time and enchytraeids which had been feeding on unlabelled litter outside and had recently arrived.

i. *Control and nitrogen plots.*

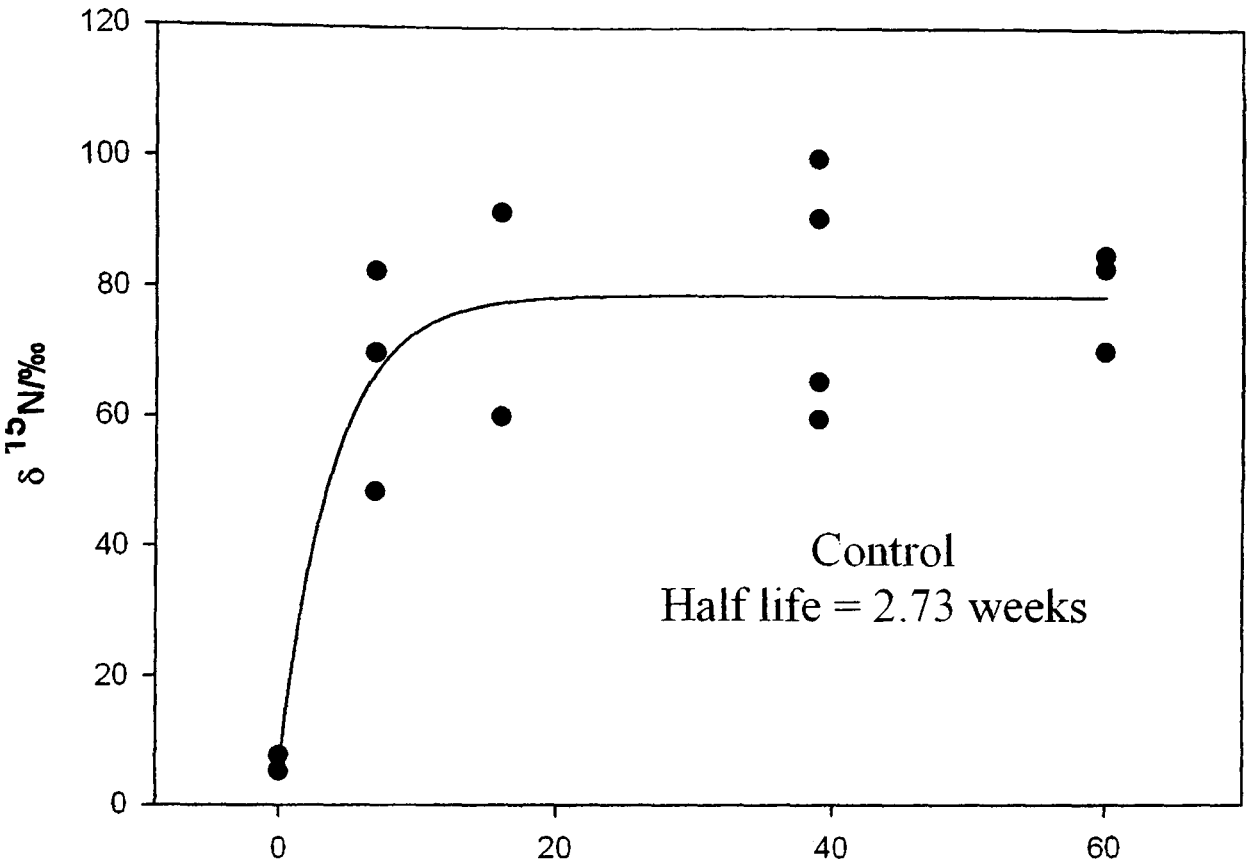
The $\delta^{15}N$ data for enchytraeids in fine bags was used to estimate the half-life of nitrogen in tissue of enchytraeids feeding on ^{15}N labelled litter in control and nitrogen plots.

The exponential equation $Y = a + be^{ct}$ was used to fit the curve of rise to maximum. This gave the values shown in Figure 3.26 and Table 3.30. The half-life of the curve was calculated from In(2)/*c* (Chamberlain *et al.*, 2004).

Table 3.30. Equation results ($Y = a + be^{ct}$) and half-life of *C. sphagnetorum* in fine litterbags in control and nitrogen plots at Rigg Foot, Sourhope.

	R^2 (Adjusted)	SE	*a*	*b*	*c*	Half-life (Weeks)
Control	0.958	14.63	6.44	72.29	0.2538	**2.73**
Nitrogen	0.931	7.45	7.45	96.38	0.0468	**14.81**

The lines of rise to maximum were tested for significance using the method described in Fowler *et al.*, 1999 and found to be significantly different (t-test; $t = 1.757$, df = 18, $p = 0.05$).



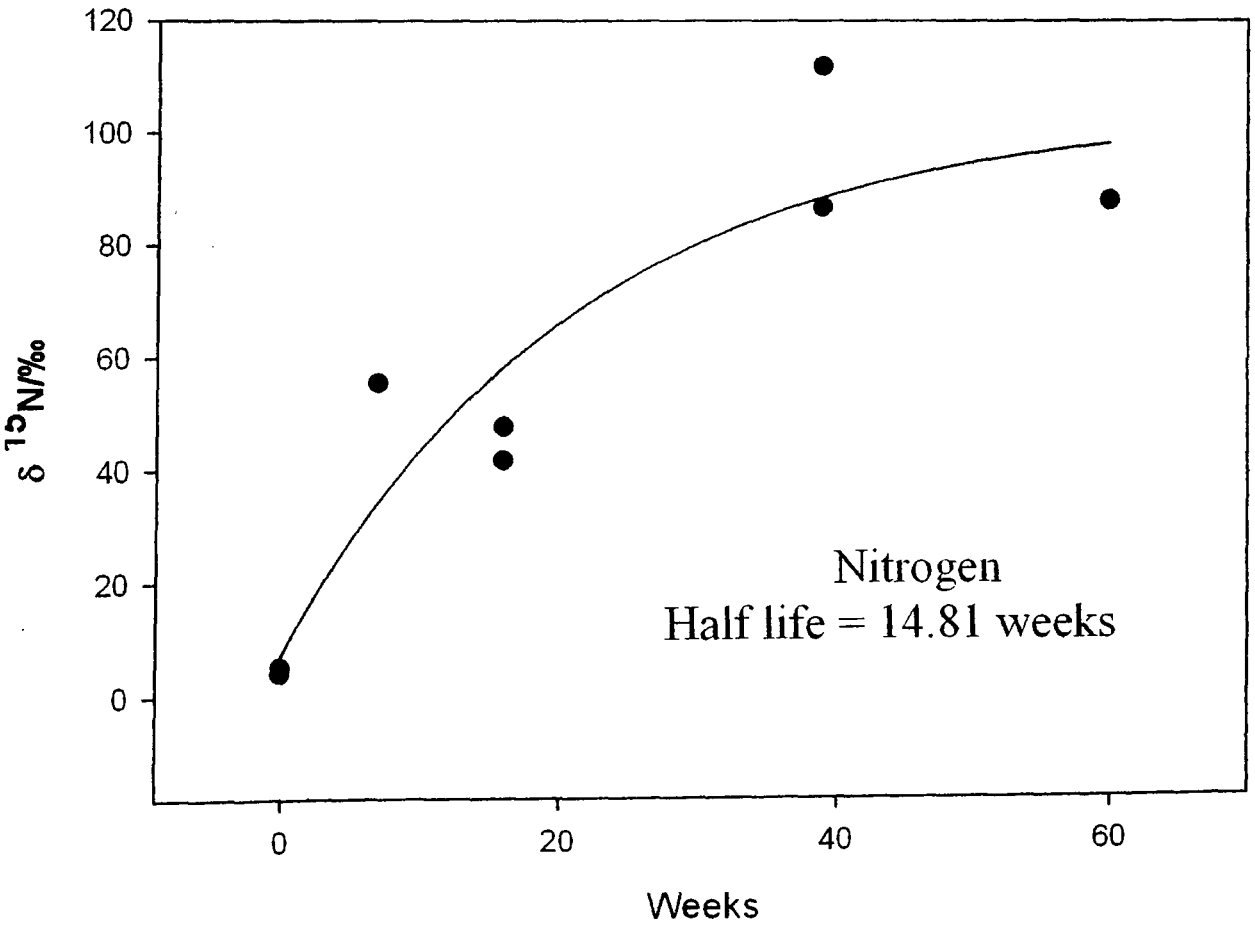


Figure 3.26. Rate of incorporation into enchytraeid tissue of nitrogen derived from ^{15}N-labelled litter in fine litterbags, and half-life value.

ii. ***Lime and L+N plots.***

The pattern of $\delta^{15}N$ accumulation by enchytraeids in fine bags appeared to rise gradually and then stabilise. This was in contrast to the pattern for enchytraeids extracted from coarse bags and suggests that the fine bags enchytraeids were resident within the bags whereas the coarse bag enchytraeids moved in and out of the bags.

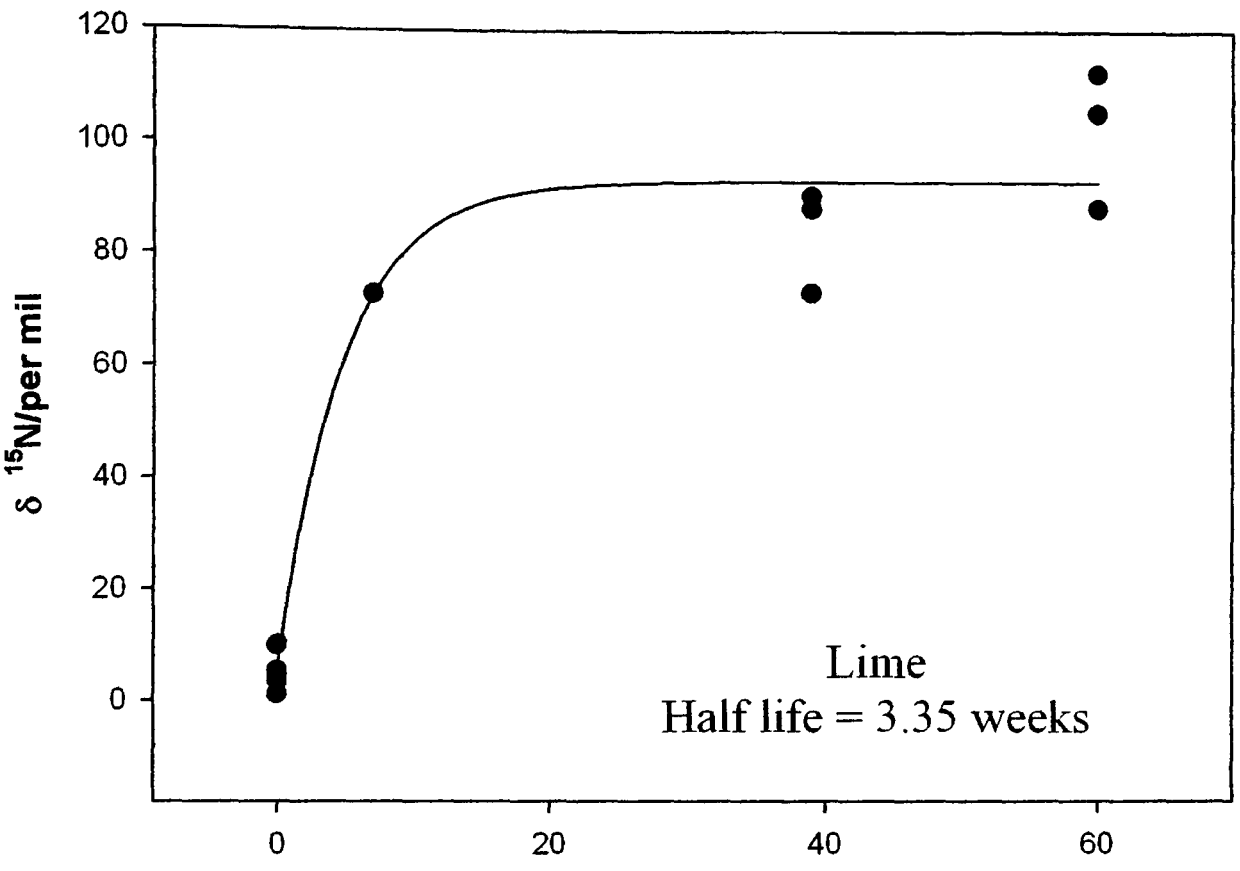
The data for fine bags were used to estimate the half life of N in tissue of enchytraeids feeding on ^{15}N labelled litter.

The exponential equation $Y = a + be^{ct}$ was used to fit the curve of rise to maximum. This gave the values shown in Figure 3.27 and Table 3.31. The half-life of the curve was calculated from In(2)/*c* (Chamberlain *et al.*, 2004).

Table 3.31. Equation results ($Y = a + be^{ct}$) and half-life of enchytraeids in fine litterbags in lime and L+N plots at Rigg Foot, Sourhope.

	R^2 (Adjusted)	SE	*a*	*b*	*c*	Half-life (Weeks)
Lime	0.978	9.32	5.61	87.35	0.207	**3.35**
L+N	0.91	22.38	11.1	91.19	0.096	**7.11**

The lines of rise to maximum were tested for significance but there was no difference between the two slopes.



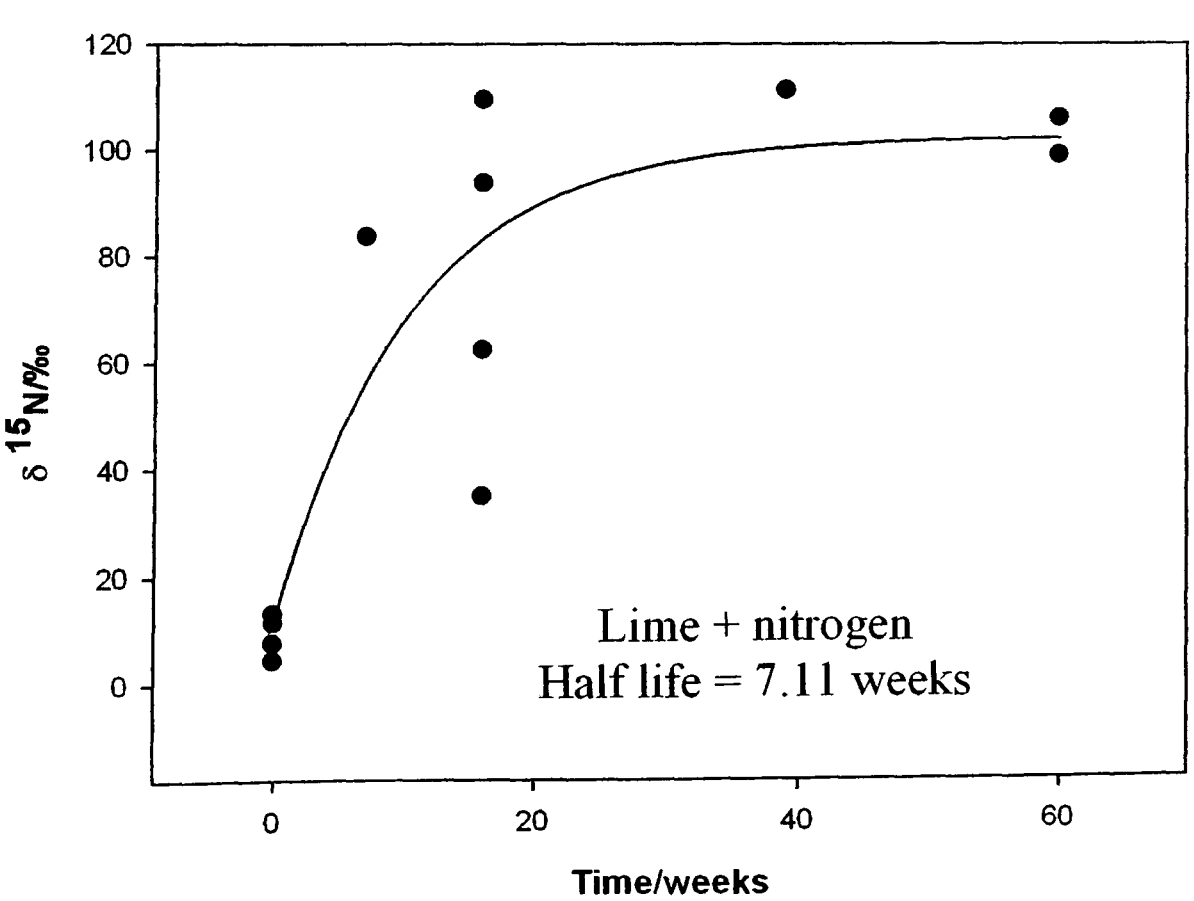


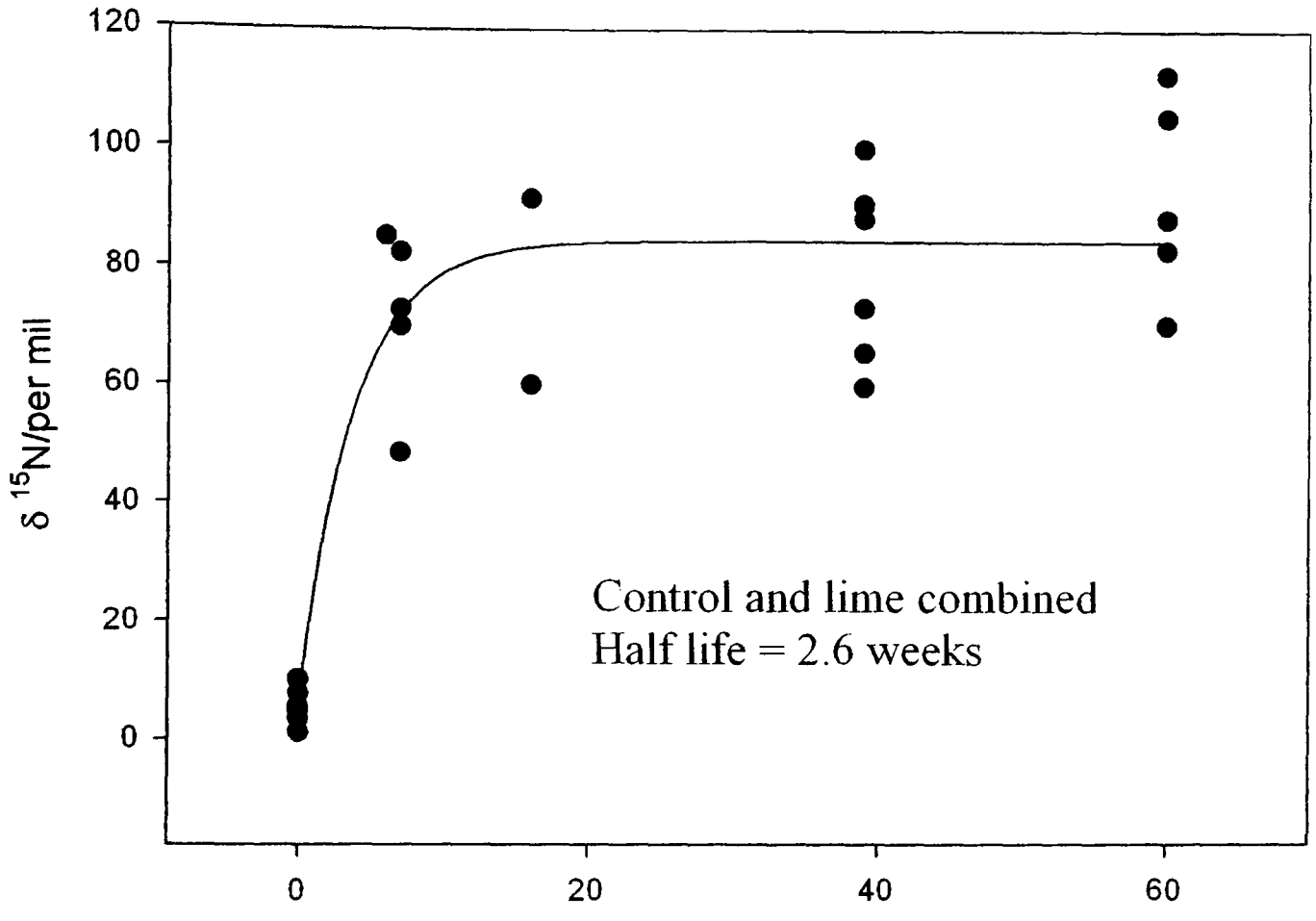
Figure 3.27. Rate of incorporation into enchytraeid tissue of nitrogen derived from 15N-labelled litter in fine litterbags, and half-life value.

The bulked control and lime, and nitrogen and L+N, values were used in the exponential equation $Y = a + be^{ct}$ to fit the curve of rise to maximum and the half-life of the curves calculated from In(2)/c (Chamberlain *et al.*, 2004). This gave the values shown in Figure 3.28 and Table 3.32.

Table 3.32. Equation results ($Y = a + be^{ct}$) and half-life of enchytraeids in fine litterbags in control and lime and nitrogen and L+N (combined values) plots at Rigg Foot, Sourhope.

	R^2 (Adjusted)	SE	a	b	c	Half-life (Weeks)
Control & lime	0.961	13.16	5.84	78.47	0.266	**2.6**
Nitrogen & L+N	0.916	20.66	10.04	90.65	0.074	**9.4**

The rise to maximum of the two slopes were tested for significance and were found to be different (t-test, t value 2.36, $df_{1,47}$, $p > 0.025$).



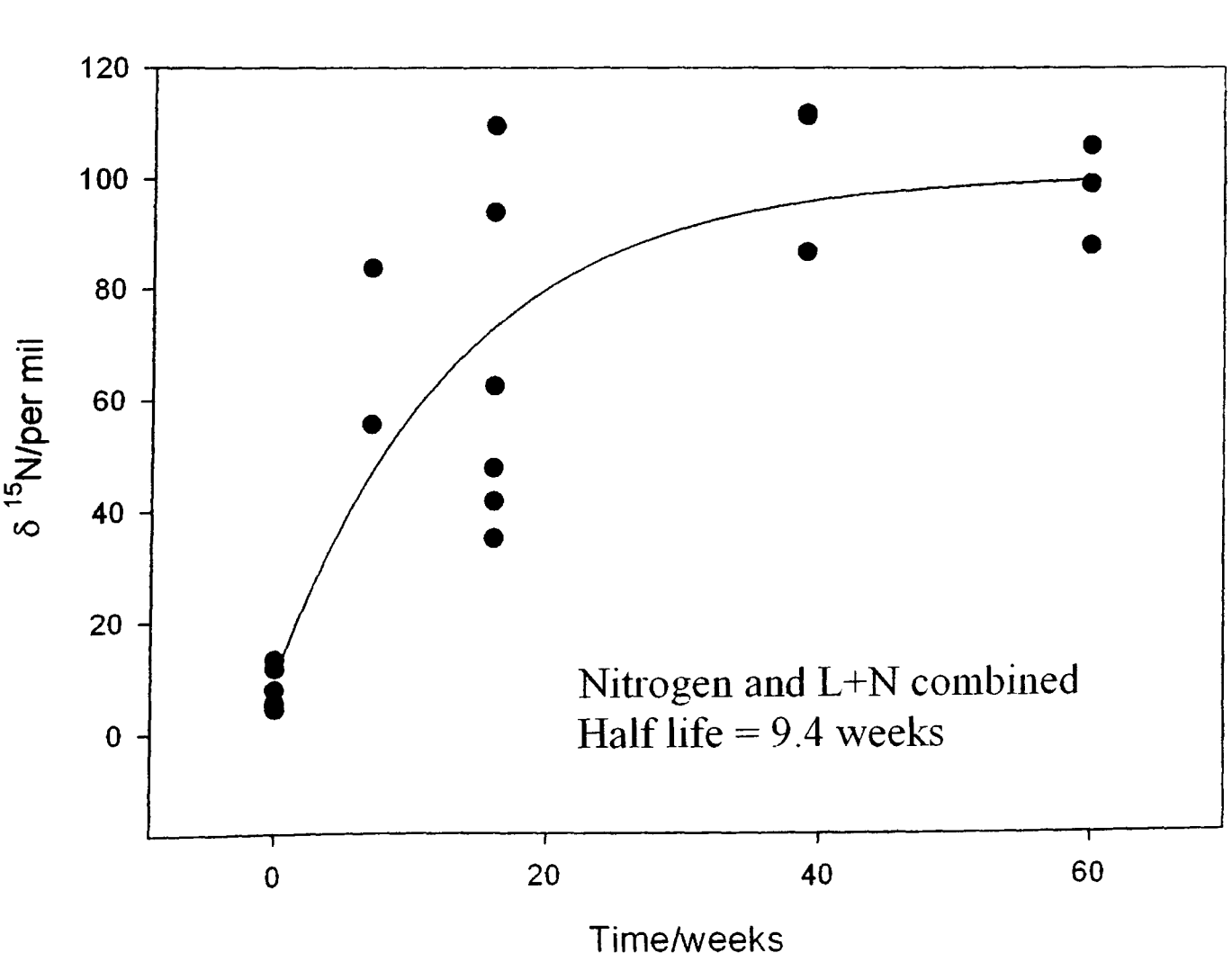


Figure 3.28. Rate of incorporation into enchytraeid tissue of nitrogen derived from ^{15}N-labelled litter in fine litterbags, control and lime (top) and nitrogen and L+N (bottom) combined and half-life values.

3.3.4.3 Derivation of tissue nitrogen from labelled litter.

i. *Coarse litterbags and cores.*

Control and nitrogen plots.

Assimilation of labelled litter-derived nitrogen was calculated for enchytraeids from acid plot coarse mesh litterbags and surrounding soil cores using the equation after Caner *et al.*, 2004. Table 3.33 gives the values obtained for enchytraeids in fine litterbags.

Table 3.33. % N assimilated by enchytraeids that was derived from labelled litter in coarse litterbags in control and nitrogen plots at Rigg Foot, Sourhope. Each value calculated from the starting values of $\delta^{15}N$ of enchytraeids and litter and that of each time period. Figures in parentheses are mean no of *C. sphagnetorum* extracted from the litterbags. There are no data for the final sampling period as there were insufficient worms for analysis.

	Control bag	Control core	Nitrogen bag	Nitrogen core
0 – 1 month	**21** (13)	**0.7** (16)	**31** (9)	**0.2** (10)
0 – 2 months	**30** (45)	**10** (48)	**29** (30)	**18.5** (6)
0 – 4 months	**16** (109)	**0** (88)	**21** (17)	**8** (49)
0 – 9 months	**6.5** (9)	**2.5** (17)	**20.5** (9)	**3** (15)
0 – 14 months		**11** (8)		**14** (1)

Maximum %N derived from labelled litter was reached within two months of litterbags placement. Generally, more N was assimilated by enchytraeids in nitrogen plots than control plots. Averaged values of %N assimilation for each individual worm verify this. However, there were many less enchytraeids extracted from litterbags and surrounding cores in nitrogen plots.

Lime and L+N plots.

Using the equation after Caner *et al.*, 2004, the following values for assimilation of nitrogen derived from labelled litter were calculated (Table 3.34).

Table 3.34. % N assimilated by enchytraeids that was derived from labelled litter in coarse litterbags in lime and L+N plots at Rigg Foot, Sourhope. Each value calculated from the starting values of $\delta^{15}N$ of enchytraeids and litter and that of each time period. Figures in parentheses are mean no of enchytraeids extracted from the litterbags. There are no data for the final sampling period as there were insufficient worms for analysis.

	Lime bag	Lime core	L+N bag	L+N core
0 – 1 month	**41** (10)	**5** (5)	**14** (16)	**7** (3)
0 – 2 months	**45.5** (11)	**0** (10)	**22** (19)	**14** (6)
0 – 4 months	**22.5** (19)	**3** (25)	**14** (12)	**6** (7)
0 – 9 months	**12** (18)	**4** (17)	**8** (5)	
0 – 14 months		**5** (5)		**10** (6)
	(58)	**(62)**	(52)	**(22)**

In lime plots, more enchytraeids were extracted from the litterbags and surrounding cores in October and March. In L+N plots, maximum numbers were extracted from litterbags in July and August. Overall, there were similar numbers of enchytraeids extracted from litterbags but only half the numbers of enchytraeid extracted from L+N cores cf lime cores. More labelled litter derived N was assimilated by enchytraeids in lime plots in litterbags, but more had been assimilated by enchytraeids in L+N cores.

ii. Fine litterbags

Control and nitrogen plots.

Assimilation of labelled litter-derived nitrogen was calculated for enchytraeids from acid plot fine litterbags using the equation after Caner *et al.*, 2004. Table 3.35 gives the values obtained for litterbags only as there were no data for surrounding cores.

Table 3.35. Percentage of nitrogen derived from ^{15}N labelled litter in *C. sphagnetorum* tissue in control and nitrogen plots at Rigg Foot, Sourhope.

	Control	Nitrogen
0 – 2 months	51	44
0 – 4 months	58	35
0 – 9 months	65	86
0 – 14 months	65	76

In control plots, the %N derived from ^{15}N-labelled litter increased rapidly during the first two months of the experiment, moderately until October 2002 and slowly during the winter and following summer.

In nitrogen plots the values vary, as did the δ ^{15}N values on which the equation is based. However, assimilation was slower during the first summer and then increased rapidly over winter. The drop during the second summer may have been an artefact (very few enchytraeids available for analysis) or an indicator that conditions in the bags had changed. Overall values were higher in nitrogen plots.

Lime and L+N plots.

Assimilation of labelled litter-derived nitrogen was calculated for enchytraeids from neutral plots using the equation after Caner *et al.*, 2004. Table 3.36 gives the values obtained for litterbags only as there were no data for the surrounding cores..

Table 3.36. Percent of nitrogen derived from ^{15}N labelled litter in enchytraeid tissue in lime and lime + nitrogen plots at Rigg Foot, Sourhope.

	Lime	L+N
0 – 2 months (August)	56	64
0 – 4 months (October)	57	56.5
0 – 9 months (March)	67	88
0 – 14 months (August)	83	81

In L+N plots the values varied, as did the δ ^{15}N values on which the equation is based In both plots there was a rapid assimilation of labelled litter derived nitrogen during the first summer, and this was greater in L+N plots. Over winter there was a slight increase in lime plots but a large increase in L+N plots; conversely, during the second summer there was a large increase in lime plots whilst the value fell slightly in L+N plots.

However, overall values were slightly higher in L+N plots.

3.3.4.4 Translocation of nitrogen.

The aim of the litterbag experiment was to assess whether the ^{15}N signal from the litter in the bags placed in the experimental plots in July 2002 was taken up by enchytraeids and transferred to the surrounding soil, roots and shoots.

$\delta^{15}N$ in soil, roots and shoots.

The assessment was made at the end of the experiment after the bags had been in the ground for 14 months. The soil surrounding each litterbag was analysed for $\delta^{15}N$, as were the roots surrounding each of the coarse litterbags and vegetation that was rooted in the core. As much soil was removed from the roots as possible, and as many roots removed from the soil as possible but there still remained some soil with the roots and roots with the soil.

This section describes the results for $\Delta\ \delta^{15}N$ in coarse and fine litterbags in all treatment plots.

i. Soil.

Figure 3.29 shows the $\Delta\ \delta^{15}N$ in soil around coarse and fine litterbags in the different treatments. The $\Delta\ \delta^{15}N$ values around coarse bags decreased in the order control, nitrogen, lime and L+N. One-way ANOVA showed that the values were not significantly different between plots ($df_{3,14}$, $F = 1.86$, $p = 0.18$). The $\Delta\ \delta^{15}N$ values of soil around fine bags were higher in lime plots than the other three treatments, and this was significantly greater (One-way ANOVA, $df_{3,11}$, $F = 3.96$, $p < 0.05$). *Post hoc* Tukey test of differences showed that only soil around nitrogen plot bags was significantly lower than that around lime plot bags.

ii. Roots.

There was only one value for the NA of roots in coarse bags, and no data for the fine bags because there were very few roots around them. Figure 3.31 shows a great deal of variability in the values associated with large S.E.'s. Roots around coarse bags in lime plots had higher $\Delta\ \delta^{15}N$ values than the other three treatments but the values were not significantly different from each other (One-way ANOVA, $df_{3,12}$, $F = 0.23$, $p = 0.88$).

iii. Shoots.

Shoot material from cores around coarse bags in lime plots were more enriched with $\delta^{15}N$ than shoots from cores in the other three treatment plots. However, the values were not significantly different to each other (One-way ANOVA, $df_{3,12}$, $F = 1.98$, $p = 0.35$).

Shoots from cores around fine bags in control plots were more $\delta^{15}N$ enriched than shoots from cores in the other three plots but the values were not significantly different (One-way ANOVA, $df_{3,10}$, $F = 1.56$, $p = 0.26$).

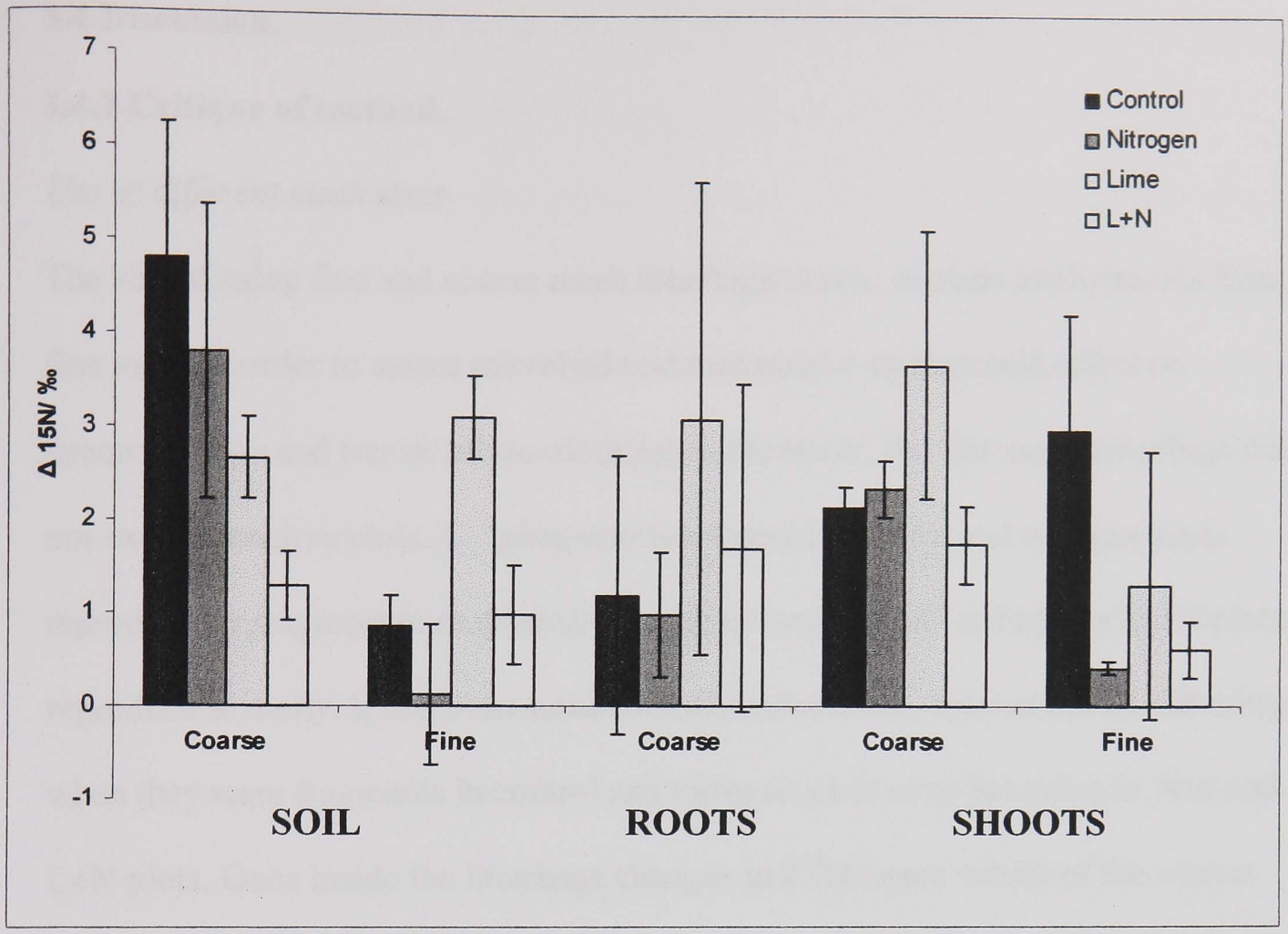


Figure 3.29. Δ $\delta^{15}N$ values of soil, roots and shoots associated with coarse and fine litterbags in all treatment plots at Rigg Foot, Sourhope after the 14 month experimental period

3.4 Discussion.

3.4.1 Critique of method.

Use of different mesh sizes.

The aim of using fine and coarse mesh litterbags was to exclude enchytraeids from fine mesh in order to assess microbial and microbial + enchytraeid effect on decomposition and translocation of nitrogen. However, the fine mesh litterbags did not exclude enchytraeids. *C. sphagnetorum* found in control and nitrogen plots reproduce by fragmentation whereas the enchytraeids found in lime and L+N plots reproduce sexually. It has been assumed that enchytraeids entered the fine litterbags when they were fragments in control and nitrogen plots or as juveniles in lime and L+N plots. Once inside the litterbags changes in $\delta^{15}N$ tissue values of the worms indicated that the enchytraeids remained in the bags either because they were unable to leave again or because they preferred the conditions inside the bags. The fine litterbags therefore acted almost as a microcosm in the field.

The main effect of the fine mesh was to reduce the numbers of enchytraeids in these bags relative to the coarse bags; in control and nitrogen plots the number of *C. sphagnetorum* was 50% lower, and in lime and L+N plots enchytraeids were reduced by 25 - 30% in fine litterbags compared to the coarse.

Different initial %N and $\delta^{15}N$ values of litter.

The *A. capillaris* was grown in a number of different trays. The trays of plants were placed randomly in the greenhouse, and the position of the trays was changed twice weekly in order to ensure a relatively randomised design. However, conditions in the greenhouse were not as well controlled as they would have been in a growth room, and each tray of plants would have been subjected to slightly different solar radiation and drought conditions. Thus, although they were all given with identical nutrient and

^{15}N labelled urea solutions in the same volume, the plants assimilated both different concentrations of nitrogen and $\delta^{15}N$ (range 1.15 – 2.05% N and $\delta^{15}N$ 97.2 – 136.73‰). *A. capillaris* is a fine leaved grass and 180 g of leaf material was needed for the litterbag experiment. It was difficult to mix this amount of roughly chopped material to achieve a homogeneous mix therefore litter from each tray, with slightly different starting %N and $\delta^{15}N$, was used to fill the litterbags. However, the source tray for each bag and the positioning of the bags in the field was recorded in order to maintain accuracy. The difference in initial %N may have had an influence on some nitrogen dynamics but the effect was temporary and appeared not to have an effect after the first year. Comparisons of changing $\delta^{15}N$ were helped by calculating change ($\Delta^{15}N$) from the initial $\delta^{15}N$ values for litter from each bag.

Extraction of enchytraeids from litterbags.

Wet funnel extraction is the usual method of removing enchytraeids from soil cores. Initially hand sorting was used to extract enchytraeids from litter but this proved to be difficult and time consuming. Time was an important factor, as the longer the enchytraeids were in the litter the greater the (potential) assimilation of N. A wet funnel system was designed (Methods) on a suitably small scale to extract the enchytraeids. After extraction, random samples were then hand sorted to assess efficiency, which was in the order of 90%.

3.4.2 Colonisation of litterbags and cores by enchytraeids.

The rate of colonisation of litter by different enchytraeid species has not been studied under field conditions. The communities in control and nitrogen plots were dominated by *C. sphagnetorum* whereas in lime and L+N plots the community was diverse, dominated by *Fridericia* and *Henlea*, but at low density. The latter community had become established within four years of the start of treatments. In a laboratory study, Hågvar and Abrahamsen (1980) investigated three different soil types with adjusted pH and found after 5 months that soil type and pH influenced both the numbers and species of enchytraeids.

1. Coarse mesh litterbags in control and nitrogen plots.

Considering colonisation of coarse mesh litterbags in control and nitrogen plots first, there were overall more *C. sphagnetorum* in and around the litterbags in control plots than nitrogen plots and the rate of colonisation was different with earlier colonisation in the nitrogen plot. Although overall numbers of *C. sphagnetorum* were not statistically significant, abundance in October 2002 was significantly different with more enchytraeids in control litterbags. It would appear that the litter was more attractive to the enchytraeids in nitrogen plots earlier and may have been related to a more rapid rate of decomposition and conditioning of the litter.

The effect of nitrogen fertiliser on the population of *C. sphagnetorum* (Chapter 1) was to depress the population. The population was smaller and possibly more patchily distributed, and *C. sphagnetorum* has been shown to move only about one metre each year which would mean that the enchytraeids in nitrogen plots either didn't detect the 'hot spot' of the litter bags or couldn't reach them. Alternately the litter in the bags may have initially attracted enchytraeids in nitrogen plots but if there was a shift of

microbial community in favour of bacteria (Ledgard *et al.*, 1998; Bardgett *et al.*, 1999), this may have proved unpalatable or even toxic (Latter and Howson, 1978).

There was no difference in the individual biomass of *C. sphagnetorum* from control and nitrogen plots which suggests that, although numbers were reduced, there were no major shifts in the population structure or dynamics of this species as a result of nitrogen additions. Therefore difference in colonisation and decomposition appear to be purely based on a reduction in numbers of *C. sphagnetorum*.

2. Coarse litterbags in lime and L+N plots

Although there were similar populations of enchytraeids in these treatments there were differences in the colonisation and decomposition of the litter. Colonisation was faster and initially higher in the L+N plots but more consistent in the lime plots. This was similar to the rate of colonisation in acidic plots treated with nitrogen and the reasons may have been similar and associated with changes in microbial community in favour of bacteria.

3. Fine litterbags in control and nitrogen plots.

Colonisation of fine bags was not expected and it is assumed that individuals entered as fragments (*C. sphagnetorum*) or hatchlings and then remained trapped as they grew. This is supported by the $\delta^{15}N$ data (Section 3.3.4.2). Although not intentional, this did in fact lead to some interesting new information on N assimilation and turnover by enchytraeids.

Control plots had more *C. sphagnetorum* in the litterbags and soil than the nitrogen plots, reflecting the general depression of the population in the nitrogen treatment. In nitrogen plots the dry weight per worm was lower although this may have been due to the lower numbers available for analysis. After August 2002 the abundance of enchytraeids in fine bags in nitrogen plots decreased and this was concomitant with a large increase in N release after October. This reduction in abundance may have been associated with the predominant nitrogen compounds (e.g. ammonia – Moursi, 1962; Huhta, 1984) or the decomposer community which may have been toxic to the enchytraeids trapped in the fine bags either via CO_2 evolution due to respiration (Ashman and Puri, 2001) or as a food source (Latter and Howson, 1978).

4. Fine litterbags in lime and L+N plots.

There were greater numbers of enchytraeids present earlier in L+N plots whereas in lime plots the numbers were more consistent. As with all plots treated with nitrogen, colonisation decreased after August but in L+N plots there was no associated increase of N release. This again may have been due to reasons discussed above.
Because of the high diversity and low numbers of enchytraeids it was not possible to obtain sufficient biomass data to evaluate the effect of nitrogen on biomass of enchytraeids in fine litterbags in lime and L+N plots.

5. The impact of liming, mesh size and fertiliser application on colonisation by enchytraeids.

Liming soil had a profound effect on colonisation in that the enchytraeid community was entirely different in the control and nitrogen compared to the lime and L+N plots, species of *Fridericia*, *Henlea* and other genera having replaced *C. sphagnetorum*. A similar effect was found by Hågvar and Abrahamsen (1980) and Abrahamsen and Thompson (1979) with increasing pH. Most Enchytraeidae, like most earthworm species, are intolerant of low soil pH (below 5.2), whilst relatively few, such as the enchytraeid *C. sphagnetorum* and the earthworms *Dendrobaena octaedra* and *Bimastos eiseni* thrive in soils with pH considerably less than 5.2. Soil pH was probably the direct cause of this change.

The attempt to exclude enchytraeids from litter by using fine mesh whilst allowing enchytraeids to colonise litter in coarse mesh bags was not successful. Standen (1973) used the same mesh with natural litter in a blanket bog where *C. sphagnetorum* did not enter the bags. The different results for the two experiments may be that in the Standen experiment litter and conditions inside the bags were no different to those outside (natural litter and surface placement) whereas in the Rigg Foot experiment, litter inside the bags and possibly humidity (in-soil placement) were more favourable than outside. The soils were also different (deep peat in the Standen experiment and brown earth in the current study), as was the litter.

Nitrogen fertiliser at Rigg Foot appeared to depress the abundance of *C. sphagnetorum* primarily by the timing of the application and the high concentration of the fertiliser applied. This was also found in field and laboratory studies by Huhta

(1984), and ammonia toxicity and unfavourable osmotic conditions (the salt effect) were considered to be important contributors to the decline of *C. sphagnetorum* abundance.

3.4.3 Litter mass loss.

Litter mass loss from coarse litterbags was less in control plots, compared to nitrogen and lime plots. Mass loss is influenced by a number of biotic and abiotic factors such as temperature, moisture, pH and litter type. However, as the experiment was carried out at the same site, many of these parameters would be similar. The major differences between the plots were treatments which influenced pH, enchytraeid community structure and abundance of enchytraeids.

Factorial ANOVA of mass loss of litter in both bags types showed that time, bag type and treatment all significantly influenced mass loss. The time element is not surprising as there was an initial large decline in mass which accounted for this. Regression analysis had shown that the rate of mass loss of litter in coarse bags in control plots was significantly less than in nitrogen and lime plots which would account for the treatment factor and contribute to the bag effect. Overall mass loss was slightly greater in fine bags. However, looking at effects in each bag type shows only time as a significant factor.

A mixed model ANOVA to investigate the effects of treatment (firstly lime and then nitrogen) and enchytraeid numbers on mass loss showed that time was the most significant factor in both bags types. However, in fine litterbags enchytraeid numbers was also a significant factor. *C. sphagnetorum* in control and nitrogen plots increased litter mass loss (combined litter mass remaining 0.219 ± 0.02) compared to that of lime and L+N (0.237 ± 0.02) which suggests that the enchytraeids in lime treatments do not increase decomposition and this must be a function of microbial activity in limed plots.

Soil in control and nitrogen plots remained acidic and retained the enchytraeid *C. sphagnetorum* as dominant. Control plots had more *C. sphagnetorum* than nitrogen plots, and litterbag colonisation rate differed between the two treatments. *C. sphagnetorum* has been shown to positively increase decomposition processes (Standen, 1973; Coulson and Butterfield, 1978). Although its precise food source remains an enigma, *C. sphagnetorum* have been shown to have microbes, particularly large quantities of fungi in their gut contents (Dash and Cragg,1972; Dash *et al.*, 1980) although Springett and Latter (1977) found poor growth on isolated fungi. Latter and Howson (1978) found that litter had to be partially decomposed to be palatable to this soil animal.

Fungal grazing is considered to be part of its feeding activity and Hedlund and Augustsson (1995) found a positive relationship between increased respiration and the presence of *C. sphagnetorum* at optimal density. Lower densities increased fungal hyphal length. Similar effects have been found with other soil animals (Visser, 1985; Seastedt, 1984; Bardgett *et al.*, 1993).

By October, there were very many *C. sphagnetorum* extracted from coarse litterbags in control plots – above field capacity – which coincided with a cessation of mass loss and therefore overgrazing of fungi in relation to mass loss cannot be excluded.

Of interest, a study by Beare *et al.*, (1992) of buried rye litter found a similar pattern of mass loss to this study but which differed from surface placed litter. That study also examined the effects of fungicide and bactericide on mass loss and found that these treatments slowed decomposition by 21% and 35% respectively. In fact, the

decomposition curves for fungicide and bactericide treatments compared to controls of their study were very similar to the differences seen in this study. In the case of this study it was the controls that resembled more closely Beare *et al. 's* treatment curves. This adds some evidence to suppression of microbial activity by enchytraeid overgrazing which resulted in an effect similar to application of treatments known to suppress microbial activity.

Application of fertilisers has been shown to have varying effects on microbial populations (Ledgard *et al.*, 1998; Bardgett *et al.*, 1999; Bardgett and McAllister, 1999; Kennedy *et al.*, 2004) but generally there is an increase in bacterial population and activity. However, plant species may also influence microbial activity directly (in the rhizosphere - Bardgett *et al.*, 1999; Kennedy *et al.*, 2004) or indirectly by changing species composition and therefore litter composition (King *et al.*, 2002; Donnison *et al.*, 2000). It may therefore have been a change in microbial community (not quantified in this study) that positively influenced mass loss in N treated plots, rather than enchytraeid feeding activity.

Mass loss in lime and L+N plots was similar to that seen in the acidic plots. These treatments at Sourhope, with pH ~ 6.5 – 7.0, did not support the enchytraeid *C. sphagnetorum*, but a much more diverse assemblage of enchytraeid species each at lower density, some of which show seasonality in abundance. These enchytraeids have not been investigated extensively in terms of function and effects on decomposition or mineralisation of nutrients, as they are found alongside earthworms whose greater biomass and individual size is thought to be more important in terms of

ecosystem engineering. Standen (1984) demonstrated a negative relationship between enchytraeid and earthworm biomass in experimental plots.

In preliminary microcosm studies using a sand substrate associated with this project, the presence of larger enchytraeids such as *Fridericia galba, F. bisetosa* and *F. magna* resulted in greater perturbation and mixing of litter/faecal material and sand. The burrowing activities of these enchytraeids were also more evident in the microcosms. These enchytraeids may produce effects through their movement and burrowing similar to earthworms, moving surface litter to deeper levels within the soil, aerating the soil via burrow formation and producing larger faecal pellets of comminuted litter for recolonisation by microbes.

Litter in fine litterbags in all plots had a consistently greater rate of mass loss than the corresponding litter in coarse mesh litterbags. This was surprising as it had been assumed that the greater activity of mesofauna entering and leaving the coarse bags would have enhanced mass loss (Setälä *et al.,* 1996).

However, these differences between bags types may have been due to the growth of plant roots into the coarse litterbags, thus adding to mass left in the bag. This did not occur to the same extent in fine litterbags.

3.4.4 Changes in carbon and nitrogen content in litter.

1. Initial nitrogen concentration of litter.

One of the aims when growing the ^{15}N labelled *A. capillaris* was to keep the nitrogen content low (< 2%) to reflect field conditions at Sourhope. This was manageable but it proved difficult to obtain litter with even N content and thus there was a certain amount of variation in the N and C content of the litter; 1.15 – 2.05% and 39.48 – 45.89%, respectively . These variations affected the initial nitrogen concentration and consequently the initial C:N ratio of the litter with initial litter N concentration lower and C:N ratio higher in nitrogen and L+N plots.

2. Changes in carbon and nitrogen content and C:N ratio of litter.

Most previous studies of changes in litter in field experiments have employed relatively coarse mesh bags.

In the Rigg Foot experiment the pattern of change in C:N ratio in litter in buried coarse mesh litterbags followed the same pattern as that found by Beare *et al.* (1992) in buried rye litter in 1.8mm mesh litterbags but not to the same magnitude. In the study by Beare *et al.* there was an initial and overall greater loss of litter mass than that seen at Rigg Foot. During the initial stages of decomposition, readily available and easily metabolised carbon and nitrogen compounds are rapidly utilised by microbes. In this study the increase in %N caused the fall in C:N ratio in the first summer.

The initial increase and subsequent decline of %N seen in acid plots has been reported elsewhere. Beare *et al.* (1992) found that there was an increase in total N by 150% of initial N in buried litterbags in CT plots for two months after litter placement, then a decline.

In a moder soil of pH 2.9, using beech litter covered by nylon net of 1.5 cm, Zeller *et al.* (2000) found a similar increase of total N (120%) during the first year, followed by a decline. Total N fell below 100% of initial N after $\geq$ 2 years. In another similar study on mull and moder soils with beech litter, Zeller *et al.* (2001) found similar results. Although the litter type in the study by Beare *et al.* (1988) was similar to that used in the present study the soil type and disturbance were different. In the studies by Zeller *et al.* (2000 and 2001) the soil types and lack of disturbance were similar but both the litter type and placement were different to the present study. However, although the time scale may differ depending on the soil and/or litter type, these studies showed that an increase in N followed by a subsequent decline is not uncommon.

The pattern of change in C:N ratio in the fine mesh bags also followed that described by Beare *et al.* (1992). Again increased %N caused a fall in C:N ratio and, as with the coarse mesh litterbags, changes after the first summer differed between acid and neutral plots.

Despite differences in initial C:N ratio between plots, the final C:N ratio of litter in both coarse and fine litterbags in control and nitrogen plots was 17 – 18:1, and in lime and L+N plots, 14 – 15:1.

In nitrogen and control plots in both bag types there was an increase in C:N ratio over the winter which was not seen in lime and L+N plots. This increase in C:N ratio is attributed to the decline of %N over the winter in the acidic plots.

Changes in $\delta^{15}N$ values of litter.

Decline in $\delta^{15}N$ in coarse litterbags followed a similar pattern to mass loss and loss of mass of carbon and nitrogen. However, there was a striking difference between coarse and fine litterbags in the decline of $\delta^{15}N$ with little further loss after July 2002 in the latter. The decline was less in lime and L+N treatments. This suggests that $\delta^{15}N$ was being recycled within the fine bags and may be attributable to re-working of faecal material and dead enchytraeids. This is particularly pertinent in lime and L+N plots which had a diverse community of enchytraeids, many of which survive only one season and die soon after laying cocoons.

3.4.5 Nitrogen dynamics.

The use of ^{15}N labelled litter allowed the dynamics of total nitrogen to be examined. The method utilised the $\delta^{15}N$ value, %N and litter mass loss to determine nitrogen release from the litter and incorporation of external N into the litter.

The mechanisms of nitrogen incorporation are uncertain but have been described by Berg (1988) and Setälä *et al.* (1996) as the result of a number of processes: fungal hyphal infiltration of the litter; throughfall of nitrogen into the litter; passive diffusion of nitrogen from the surrounding soil, and feeding and movement of soil fauna between soil and litter. To this we add the possibility of infiltration of litterbags (particularly coarse mesh bags) by plant roots in this grassland system.

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Nitrogen release occurs immediately with an initial rapid flush. The rate thereafter appears to be dependent on the litter type and perhaps placement of the litter. The import of external N also occurs from the beginning of the decomposition process. The processes of release and incorporation occurred simultaneously.

i. The release and incorporation of nitrogen in the first month.

The initial release of nitrogen from litter in the first four weeks in all litterbags was probably the readily leachable substances and rapidly metabolised compounds that required little active decomposition (Coleman and Crossley, 1996; Berg, 1988 and Zeller *et al.*, 2000), but colonisation of the litterbags by enchytraeids had already occurred and their activity may also have contributed to N release in the first month.

%N released initially appeared to be associated with the starting N concentration. In coarse bags there was slightly greater initial loss of N in control and lime plots (which had higher starting %N) compared to nitrogen and L+N plots where starting %N was lower. This would suggest that small differences in initial N content may be correlated with nutrient release in the first month. In this early stage of the process N release was similar from coarse and fine bags.

Incorporation of external N occurred from the beginning of the present experiment. It was approximately one third or less of that released and it did not appear to be associated with starting N concentration, unlike decomposition of beech litter in a study by Zeller *et al.* (2000). In this early stage of the process there was little difference between coarse and fine mesh bags.

ii. Patterns of release of nitrogen in coarse mesh litterbags..

It has been suggested that in some cases the C:N ratio must reach a 'threshold' value before N mineralisation occurs (Berg and Staaf, 1981; Blair, 1988) and prior to this N accumulation occurs until a critical level is reached. In this experiment nitrogen was released almost continuously, certainly until March 2003. However the data do not separate the N species and the initial release was probably due to the readily leachable substances and rapidly metabolised compounds that required little active decomposition. This continuous release was also observed in ^{15}N studies by Berg (1988) and Zeller *et al.* (2000 and 2001) with pine and beech litter. This suggests that the use of the stable isotope ^{15}N reveals more of the dynamics of nitrogen cycling, as reported by Davidson *et al.* (1991, 1992), than the use of non-labelled nitrogen sources.

Although the final %N release was similar in all treatment plots, the pattern of release during the 14 month experimental period differed. Nitrogen release from litter in bags in control and L+N was steady after the initial rapid first 4 week flush, although less nitrogen was released from the L+N plots. In nitrogen and lime plots, N release during the summer showed very little change after the initial rapid flush. However, the overwinter release was substantial in nitrogen and lime plots, accounting for ~35% and 20% of N total release respectively, effectively 'catching up' with control and L+N plots. There are a number of possible reasons for the more erratic pattern of nitrogen release from coarse litterbags observed in nitrogen and lime plots and these are discussed below.

Changes in microbial community.

Although not investigated in this study, it is likely that the soil microbial community has been changed by the application of nitrogen and lime treatments directly due to changes in pH and floristic composition.

The effects of N fertiliser on microbial communities has been explored by a number of studies. These have shown that the microbial community changes with the addition of N fertiliser. Ledgard *et al.* (1998), in a short-term study of a grassland with soil of pH 5.6 which had been treated with ammonium nitrate fertiliser (200 kg $ha^{-1}yr^{-1}$) over a 13 year period, found that there were more fungi than bacteria in the untreated soil compared to treated. They also found that N mineralisation increased in the treated soil which was attributed to a greater supply or quality of substrate for mineralisation.

Bardgett *et al.* (1999), in a microcosm study using soil of pH 4.7 and single species of grasses common to unimproved, semi-improved and improved upland grasslands, found no effects of N fertiliser (equivalent to 100 kg N ha^{-1} yr^{-1}) on measures of soil microbial activity or biomass. However, the microbial community structure was altered with N addition, with an increase in fungi relative to bacteria, in contrast to the study by Ledgard *et al.* (1998). Bardgett's team also found that the plant species themselves had a greater influence on soil microbial biomass and activity than N fertiliser although this was inconsistent over time. However, *A. capillaris* and *F. rubra,* which are both common species at Rigg Foot, had a beneficial effect on soil microbial biomass, and demonstrate coupling of the grasses with the soil microbial community. Although root biomass was reduced with N fertiliser, with overall shoot:root ratio increases, it was thought that root exudates would be greater due to greater plant productivity.

Kennedy *et al.* (2004) also studied microcosms with soil of pH 4.2, again planted with single species of grasses common to unimproved, semi-improved and improved upland grasslands. The authors found that addition of ammonium nitrate fertiliser reduced microbial activity and bacterial ribotype number (a measure of species richness), and *A. capillaris* also reduced microbial activity.

The greatest difference between these studies and that at Rigg Foot is the effect of ammonium nitrate fertiliser on soil pH. Reported in these studies is a fall in pH which was not seen at Rigg Foot; in fact during the 3 years of this experiment, soil pH increased in the nitrogen treated plots with a mean of 5 (maximum of 5.5) compared to a mean of 4.6 in control plots. Although slight, the increased pH may have shifted

the balance between different types of microbes in N plots towards those found in limed plots.

The findings of Ledgard *et al.* (1998) may be more relevant to this study in view of the higher pH in that soil which did not change with N addition in their study, with an increase in bacteria relative to fungi. However, as higher N concentration promotes bacterial decomposition (Bardgett *et al.*, 1999) and bacterial mediated nitrogen cycling processes such as nitrification and nitrogen fixing (Kennedy *et al.*, 2004), it must be assumed that it is the provision of ammonium nitrate fertiliser – easily available to microbes when applied, and later as root exudates and biomass – that the microbes decomposed rather than the litter placed in these plots. Only when all other sources of N were exhausted did active nitrogen cycling of litter N occur in nitrogen plots, and this happened over the winter

A unique feature of the Rigg Foot site was that the plots were mown between May and the end of September and the clippings taken off-site, so there was no input of new litter for decomposition. The decomposition of litter could therefore be monitored within the different treatments. In the IV Report (Soil Biodiversity Thematic Programme), Burt-Smith (2002) recorded significantly more litter hits in nitrogen and L+N plots than control and lime plots in a Point Analysis Survey. This indicated that litter was being decomposed in the latter plots but not in the former which supports the premise that it was the supply of N fertiliser that fuelled microbial nitrogen cycling in N treated plots.

Enchytraeid abundance and activity.

The abundance and activity of enchytraeids may influence patterns of N release directly and also indirectly through their interactions with microbes.

Control and nitrogen plots were dominated by *C. sphagnetorum* and both field population abundance and numbers extracted from bags and cores indicated that nitrogen addition adversely affected these enchytraeids. The mechanism is not known but is probably due to the increase in pH in nitrogen plots or even direct toxicity of NH_4NO_3 (Huhta, 1980, Hågvar and Abrahamsen, 1980). However, it could also be due to changes in microbial community structure, with an increase in bacteria (Ledgard *et al.*, 1998). If bacterial populations were enhanced by the addition of ammonium nitrate they may not provide (directly or indirectly) a suitable food source for *C. sphagnetorum* or may be directly toxic to this enchytraeid (Latter and Howson, 1978).

However, another relevant feature found at Rigg Foot was the relationship between treatment and soil moisture. Burt-Smith (2002) reported a negative relationship between soil moisture and increased pH. Soil moisture was 0.55 m^3m^{-3} in control, and 0.45 m^3m^{-3} in nitrogen treated plots which may have adversely affected enchytraeid movement and activity. Migration to deeper soil layers by *C. sphagnetorum* in response to changes in temperature and soil moistures has been reported by Springett (1970) and Briones *et al.* (1997). Abrahamsen and Thompson (1979) found a strong positive correlation between abundance of *C. sphagnetorum* and precipitation in a forest soil.

Enchytraeids have been shown to interact with microbial activity. Enchytraeid grazing (particularly *C. sphagnetorum*) has been shown to positively affect fungal growth at population optima (Hedlund and Augusstson, 1995). In acid plots dominated by fungi, reduced numbers of enchytraeids in N plots may not have grazed effectively and limited the extent to which fungi export N from the litterbags, although this was not quantified in this study. Again, changes in microbial community in favour of bacteria may also have influenced enchytraeid feeding either directly (as a food source) or indirectly by more efficient utilisation of available root exudates and root biomass decomposition. Downward migration in response to increasing temperature and decreasing soil moisture may have taken the enchytraeids outside the reach of microbes colonising the litter in the litterbags.

Effects of lime application.

The application of lime may have changed patterns of N release from litter. Liming of acid soils increases the soil pH, soil microbial biomass and activity, C and N transformations and plant productivity (Rangel-Castro *et al.*, 2004, Webster *et al.*, 2000; Kennedy *et al.*, 2004). Anderson and Domsch (1994) and Blagodatskaya and Anderson (1998) found that the fungal:bacterial ratio decreased with increasing pH. Also, bacteria are less tolerant of dry conditions than fungi (Holland and Coleman, 1987; Killham, 1994).

The slow rate of N release in lime plots at Rigg Foot during the first summer may have been related to the above factors. Burt-Smith (2002) reported that soil moisture was less in both lime (0.43 m^3m^{-3}) and L+N (0.36 m^3m^{-3}). Bacterial activity in soils is reduced when water potential is less than -10 MPa, although the tolerance of fungi

and actinomycetes is greater at -60 to -70 MPa. If bacteria play a more dominant role in N release in limed soils (Webster *et al.*, 2000 and Kennedy *et al.*, 2004), water stress will slow down this process. Burt-Smith (2002) observed that plants in limed plots at Rigg Foot showed signs of water stress during the summer of 2002 when large patches of vegetation showed signs of chlorosis, and this would reduce photosynthetic activity and therefore availability of carbon products via root exudates. Even with efficient C utilisation (Webster *et al.*, 2000), there may have been insufficient C supply to fuel N cycling processes.

In terms of enchytraeids, liming alters the community structure, becoming more species rich, but each species at lower abundance (Standen, 1984, Hågvar and Abrahamsen, 1980). Soil drying did not appear to negatively impact on enchytraeid abundance in lime plots, but in L+N plots enchytraeid abundance was reduced slightly. However, it is not known how this directly influences the activity of this diverse community and unfortunately little is known of their feeding ecology.

The overwinter N release.

In control and L+N plots N release continued throughout the experiment at an almost linear rate after the initial N release 'flush'. However, in lime and (particularly) nitrogen plots, where very little N was released during the first summer, there was a rapid release of N during the winter. N mineralisation is associated with temperature, and nitrification tends not to occur at temperatures below 7°C, although ammonification occurs (albeit slowly) down to freezing point, with consequential ammonium accumulation in soils in autumn and spring (Killham, 1994). At Rigg Foot, the soil temperature in November was 6°C at -2cm depth and presumably the

majority of the observed N release occurred between October and November, with slow release and soil accumulation of ammonia during the colder winter months.

iii. Patterns of incorporation of nitrogen.

In control and L+N plots %N incorporation was steady and final values were similar. The erratic pattern of N release observed in the litter in nitrogen plots was matched by a similar pattern of N incorporation, but this was not so obvious in the lime plots. Overall, litter in lime plots incorporated less external N than the other three plot types but standard errors for all treatments were very large.

Nitrogen incorporation probably best reflects the activity of microbes as litter is colonised. In order to maintain microbial C:N ratio during decomposition, a shortfall of N is supplemented by soil N (immobilisation), and N released that is excess to microbial requirements is available for plant uptake. Substrates with high C:N ratios therefore result in N immobilisation and those with low C:N ratios result in N release.

Optimal grazing of microbes by enchytraeids promotes fungal growth (Hedlund and Augusstson, 1995) and therefore incorporation of available litter N plus the balance obtained from external sources. Free movement of enchytraeids in coarse mesh bags would result in faecal deposition inside the bags and thus a dilution of the litter ^{15}N signal from which the nitrogen incorporation and release were calculated.

In the nitrogen plots possible influences on nitrogen release and enchytraeid activity have been discussed in the previous section. Between July and August 2002, N incorporation was static (as was N release). This suggests that either any N released

was immediately immobilised by microbes or that there was no N dynamics occurring at this time. Thereafter, when N release increased rapidly until March, incorporation of external N also increased in a similar fashion, but at less magnitude. Litter derived N of enchytraeids in nitrogen plots was also less during the first four months of the experiment but increased rapidly over winter, suggesting that enchytraeid feeding activity was greater after October.

iv. Patterns of release of nitrogen in fine mesh litterbags.

Final %N release was similar in control and nitrogen plots, but less in lime and L+N plots. However, patterns of release differed between these plots, with a steadier, lower overall rate in lime and L+N plots but a more erratic pattern in control and nitrogen plots, similar to that observed in coarse bags in nitrogen plots. Nitrogen release during the first summer in control and nitrogen plots appeared depressed, but followed by a rapid release overwinter.

The differences attributable to nitrogen fertilisation described earlier for litter in coarse litterbags are equally applicable to conditions for litter in fine litterbags. However, these fertiliser differences are not applicable to conditions in control plot fine litterbags, which displayed the same pattern of nitrogen release as nitrogen plot coarse and fine litterbags. However, conditions in the fine bags may have altered the pH within the bags and therefore the microbial community.

The major difference between coarse and fine litterbags was the lack of root infiltration in the latter. A combination of lower root biomass (N effect) and less root infiltration (mesh effect) may have led to a reduction in microbial colonisation of the

fine litterbags, thus affecting enchytraeid food sources. A reduction in enchytraeid fungal grazing would therefore lead to less fungal growth and affect nitrogen release and incorporation. However, why there should have been a sudden and dramatic increase in N release overwinter control and nitrogen is more difficult to explain but may have been associated with death of enchytraeids within the fine bags with release of N from decomposition of enchytraeid bodies.

Release of N was fairly steady from litterbags in lime and L+N plots, but less than 60% was released in both. This may have been related to temperature and low soil moisture adversely affecting microbes and/or enchytraeids in these plots, or the availability of alternative sources of C and N outside the litterbags.

v. Patterns of nitrogen incorporation in fine litterbags.

The most striking feature was the lack of incorporation of external N in comparison to coarse litterbags in the same plots. Lack of root infiltration may have affected both the root dilution effect of litter ^{15}N, but also the more direct involvement of roots with N uptake.

The low incorporation suggests that the N source for uptake by enchytraeids came from within the litterbags. Enchytraeids were captive in the bags once they had got in and grown. Their only food source would be what was contained within the bags – decomposing litter and microbes. The enchytraeids would have grazed the fungi, deposited faecal material which would be subject to microbial colonisation and re-ingestion by the worms. There was probably mortality initially until the litter was suitably conditioned. Enchytraeids have 10 – 12% total nitrogen in their bodies which

would have added to the N content of the bags because, being soft-bodied animals, they would have decomposed rapidly. Both N and ^{15}N could have been 'recycled' within the fine litterbags and this was likely to be the source of incorporated N.

vi. % nitrogen in relation to loss of litter mass and nitrogen dynamics.

In control and nitrogen plots in both coarse and fine litterbags, %N increased until mass loss reached 60% in October 2002, after which %N fell. In lime plots this fall in %N occurred in March 2003, when mass loss reached 70%, but this interaction was not seen in litterbags in lime fine and L+N plot coarse or fine.

This interaction was observed by Berg (1988) in decomposing Scots pine needle litter, and also by Zeller *et al.* (2000 and 2001) in beech litter. Berg attributed this to a new and more intensive process of N release.

Looking more closely at the relationship between these three aspects of decomposition, it appears that %N begins to decrease when litter mass loss and N release reach 60 – 70%.

The lower threshold was reached for litter in coarse mesh bags in control plots in October with 60% N release, in nitrogen plots between October and March with 65% N release and in lime plots in just after March at almost 70% N release. However, in L+N plots decline in %N did not occur in the course of the 14 month experiment even though N release was just less than 65%. This indicates that in lime treated plots, %N release has to reach the higher threshold of 70% before %N declines and the more intensive release process occurs.

In fine mesh litterbags in control and nitrogen plots, there was a delay in %N release during the summer with a large release overwinter, and this seemed to influence the timing of %N decline and the threshold values. In control plots, when 65% N was released between October and March, %N declined and in nitrogen plots this occurred nearer to March when almost 70% N had been released. In both limed plots, where %N release did not rise above 60%, %N continued to accumulate in the litter even though mass loss continued.

It would thus appear that %N decline – indicating intensive N release processes - is more associated with % N release than with mass loss in this particular litter type and that it is N release rather than mass loss which determines the timing of the decline in %N. This delay in N release but continued mass loss was probably associated with the entrapment and mortality of enchytraeids in the fine litterbags. Faecal pellets, mucus and dead enchytraeids would provide a more easily available N source for microbes, which continued to utilise carbon products from both the litter (hence the continued loss of litter mass) and these animal carbon and nitrogen sources.

3.4.6 Translocation of nitrogen.

The litterbag experiment was an attempt to elucidate aspects of nitrogen translocation within soil. Two different litterbag mesh sizes and plots where nitrogen and lime fertiliser applications had been made were used. The $\delta^{15}N$ values of labelled litter and enchytraeids taken from the bags were followed at intervals for 14 months. At the end of the experiment the values in soil, roots and shoots were measured.

3.4.6.1 The $\delta^{15}N$ of enchytraeids.

It is assumed that any increase above natural abundance in $\delta^{15}N$ values was due to enchytraeids feeding on the ^{15}N labelled litter. Enchytraeids were free to move in and out of the coarse bags, and the mean $\delta^{15}N$ values will be a combination value of enchytraeids that had natural abundance values (i.e. had just entered the bags) and those that had spent varying periods of time in the bags.

The potential of gut contents confounding the $\delta^{15}N$ results was considered. For *C. sphagnetorum* this was addressed by observation of the gut fullness of newly extracted enchytraeids, and whether they fully emptied their guts whilst being kept in water at 5° C. The proportion of gut to body ratio was calculated giving 94.6% of the enchytraeid mass as tissue and 6.4% gut content. A mass balance equation was used to calculate the percentage of ^{15}N that could be attributed to gut content. Appendix II shows these calculations.

There were very large differences between the values obtained for enchytraeids in coarse and fine litterbags and therefore the results are discussed separately.

1. Enchytraeids from coarse bags and cores.

The enchytraeids were free to move in and out of the coarse mesh bags so $\delta^{15}N$ values represent a mixture of worms that had, or had not, fed on labelled litter. It should be noted that the abundance of enchytraeids may have resulted in a dilution effect of the $\delta^{15}N$ values if there had been an influx of worms with NA values. For example, in July 2002 the mean number of *C. sphagnetorum* extracted from litterbags was 45 with a mean $\delta^{15}N$ value of ~40‰ whereas in October 2002 mean enchytraeid numbers were 109 with a mean $\delta^{15}N$ value of ~ 25‰.

The $\delta^{15}N$ signal of enchytraeids from litterbags was consistently higher than from cores. In the cores, compared to the bags, there was a lag time in the control and nitrogen plots with the $\delta^{15}N$ signal detected in the core enchytraeids after 2 months. This effect was not seen in the enchytraeids from lime and L+N plots where raised $\delta^{15}N$ were detected in core enchytraeids after one month. This suggests that enchytraeids in control and nitrogen plots stayed in the litterbags for longer than those in lime and L+N plots. These patterns of $\delta^{15}N$ incorporation also indicate that the litter was most attractive to the enchytraeids after 1 - 2 months.

The raised $\delta^{15}N$ values of enchytraeids from soil cores was lower than those from bags but nevertheless indicates that they had fed in the bags and then moved into the surrounding soil where they were then captured. A subsequent decrease in $\delta^{15}N$ indicates a dilution by enchytraeids from further away from the bags and surrounding core.

In the lime and L+N plots raised $\delta^{15}N$ values were detected in worms after one month (the first sample) indicating either that the litter in the bags was relatively less attractive to the worms so that they moved out immediately or simply reflecting the greater size and mobility of the *Fridericia* and *Henlea* compared to *C. sphagnetorum* in the control and nitrogen plots.

2. Enchytraeids from fine mesh bags.

There are no data for enchytraeids in soil cores surrounding fine litterbags but $\delta^{15}N$ values from enchytraeids in fine mesh litterbags was used to estimate the half-life of enchytraeid tissue nitrogen and the %N of ^{15}N labelled litter derived tissue nitrogen.

The fine mesh litterbags confined the enchytraeids, restricting their exit from the bags. In the current study, the rate of incorporation of dietary nitrogen is assumed to be the same as the incorporation of dietary $\delta^{15}N$. Other studies have used diet switching to assess this rate e.g. Tieszen *et al.*, 1983; Chamberlain *et al.*, 2004.

The $\delta^{15}N$ values of enchytraeids from fine bags reached a plateau in control and nitrogen at ~ 80‰ and those in lime and L+N plots plateaued at 90 -100‰. However the rate of increase was slower in nitrogen and L+N plots and half-lives of tissue N were estimated to be longer. The maximum %N assimilated from the labelled litter was greater in nitrogen and L+N plots. Estimates of all three values were greater in lime and L+N compared to control and nitrogen plots and those receiving N fertiliser compared to those without fertiliser.

The slower increase of $\delta^{15}N$ might be because conditions were sub-optimal for *C. sphagnetorum* in the bags in nitrogen plots and for *Fridericia* and *Henlea* in the L+N plots and that more worms died there. However factors known to influence the survival of enchytraeids, such as pH and moisture, were not quantified in the bags. Alternatively, net immigration during the first weeks of the experiment would dilute the ^{15}N signal and result in an apparent slower rate of uptake of N during the first four months of the experiment. It is possible that net immigration into bags occurred in nitrogen and L+N plots if conditions outside were less favourable than inside but total numbers inside fine bags were very low. However another explanation is that worms entered the bags but did not feed as well. The quality of the food in the litterbags in nitrogen and L+N plots might not have been as good as food sources outside the bags. Clippings from vegetation in nitrogen and L+N plots had significantly higher tissue % total nitrogen (> 2%) whereas that of the litter in bags was ~ 1.6%.

Combining values for enchytraeids from nitrogen treated and non-nitrogen treated plots slightly reduced the half-life values of enchytraeids in control and lime plots. In nitrogen plots the half-life was halved from 14.81 to 7.11 weeks, and this represented a slight increase in L+N plots of ~ 2 weeks. These values may be more representative as they provided more replicate values and therefore less variability.

Trapped in the fine bags, the enchytraeids in nitrogen and L+N plots may not have been feeding and therefore growing more slowly. The lower numbers extracted from these bags suggests higher mortality or less attraction of the litter as a food source. However, by the end of the experiment the amount of tissue nitrogen derived from

^{15}N labelled litter was greater in nitrogen and L+N plots compared to control and lime plots.

Comparable tissue turnover rates using ^{15}N enriched material in invertebrates have not been found. Schmidt *et al.* (1999) used NA stable isotopes of carbon and nitrogen in a diet switch from a C_3 to a C_4 plant food source for earthworms over a 100 day experimental period but found that the difference in δ ^{15}N between sources was too small to enable quantification.

Other studies using δ ^{13}C have demonstrated variations in half-lives of different tissue types (Tieszen *et al.*, 1983, Hobson and Clark, 1992) in birds and mammals. DeNiro and Epstein (1981 and 1984) advised the use of whole animals rather than individual tissues if assessing food sources in food web analyses, as they also found variations in the uptake of NA isotopes of carbon and nitrogen in different tissues. Of those demonstrated, the curve relating to *C. sphagnetorum* in nitrogen plots is similar to δ ^{13}C incorporation into brain tissue in gerbils (Tieszen *et al.*, 1983) and fatty acids in Collembola (Chamberlain *et al.* (2004).

It seems improbable that same species would in reality have such differing tissue nitrogen turnover rates in broadly similar environments as those seen in this experiment. It is more probable that conditions in the nitrogen and L+N plot litterbags were less suitable for enchytraeids, resulting in higher mortality and poorer feeding. This was supported by the difference in dry biomass per worm calculated for *C. sphagnetorum*, which was lower for worms from nitrogen plots than those from control plots.

3. % tissue N derived from labelled litter.

Enchytraeids extracted from bags had higher % tissue N derived from litter than those extracted from cores. Maximum values for enchytraeids from lime plots bags were almost 50% and considerably higher than values for enchytraeids from control, nitrogen and L+N plots.

% N of enchytraeid tissue N peaked after one month in nitrogen plots and two months in the remainder with the exception of lime soil cores. Thereafter it declined. Unfortunately there was no sample for litterbags at the end of the experiment (14 months), but core values were slightly higher at this time compared to those at the end of the first winter. Variances of such estimates are high but the results seem to indicate that *C. sphagnetorum* from nitrogen plots were the first to assimilate litter nitrogen and values remained comparatively high (20.5%) after 9 months.

A similar field study using highly ^{15}N enriched beech litter on mull and moder soils was carried out by Caner *et al.* (2004). Soil animals were collected after nine months. Enchytraeids extracted from inside litter baskets were estimated to have assimilated 7 and 11% and, in soil beneath litter baskets, 3 and 1% of labelled litter derived N in mull and moder soils respectively. Values for the present study at nine months for enchytraeids extracted from coarse mesh litterbags were 6.5 and 20.5%, and from surrounding soil cores, 2.5 and 3% N derived from labelled litter in control and nitrogen plots respectively. Taking average figures for all samples in the present study, these values were 17 and 26% and 5 and 9% labelled litter derived N for enchytraeids in litterbags and surrounding soil. This demonstrates the variability of labelled litter derived %N when samples are taken at different time intervals, and

movement into and out of coarse mesh enclosed litter can contribute to this variability.

3.4.6.2 Soil, roots and shoots.

The deposition of ^{15}N enriched material in the soil around the coarse bag cores is assumed to be due to the activity of enchytraeids moving out of the coarse litterbags and depositing faecal material after feeding on the ^{15}N labelled litter in the bags – translocation - and also leaching of labelled N from the bags due to microbial activity. These processes would occur in all plot types, despite the different composition of enchytraeid community in the acid and neutral soils. Deposition of enriched material around fine litterbags is assumed to be mostly due to leaching as movement of the enchytraeids was restricted.

Enrichment of soil around coarse litterbags varied from 1.5 to 5‰. The gradation of enrichment was matched by the number of enchytraeids extracted from bags and cores, with greater numbers of individuals being correlated with more $\delta^{15}N$ enrichment.

Change in $\delta^{15}N$ in soil around coarse litterbags was less in all plots at this site than changes found by Caner *et al.* (2004) in mull and moder soils using ^{15}N enriched beech litter. At the mull site, a mean increase of 7.5‰ was found and at the moder site, 13‰. However, this was found in the top 5cm of soil beneath the litter baskets, whereas in the present study only soil parallel to the litterbags was used for analysis. It is possible that leaching caused the higher values beneath litter baskets. The soil enrichment found by Caner *et al.* (2004) occurred after nine months. In the present study, subsamples of soil were analysed for $\delta^{15}N$ after nine months and only a 1‰

difference from NA was found. A study by Zeller *et al.* (2000) using ^{15}N enriched beech litter on a moder soil found a $\Delta\ \delta^{15}N$ of 20‰, four times that found in this study, after 14 months. However, enrichment of litter material in both studies cited was much higher (1200 - 2500‰) than that of this study (~125‰).

There was little $\delta^{15}N$ enrichment of soil around fine litterbags with the exception of lime treatment plots. The abundance of enchytraeids in lime plots was less than that in control and nitrogen plots and this effect could be due to a number of possible reasons.

a. The decomposer community may have been more active in lime plots and translocated the $\delta^{15}N$ signal from within the bags to the surrounding soil either via growth of fungal hyphae into the surrounding soil or by faster breakdown of litter by bacteria leading to more leaching of labelled N. As bacteria are dominant in neutral soils greater bacterial activity may account for the raised signal seen in lime plots (Kennedy *et al.*, 2004).

b. Enchytraeids that died and were decomposed in the bags would have had a higher $\delta\ ^{15}N$ content (as they have a higher N content of 10 – 12%) which could have been incorporated into microbial tissue and exported from the bags via the hyphal network or by leaching. Whereas *C. sphagnetorum* in acid plots reproduce by fissure, the enchytraeids found in limed plots reproduce sexually. Sexually reproducing individuals in the fine bags would have laid cocoons within the bag and many adults subsequently died. Only the larger species such as *F. bisetosa, galba* and *magna* survive for more than one year.

c. The decomposer community may have discriminated against the heavier isotope (^{15}N) in favour of the lighter ^{14}N (Shearer and Cole, 1986; Herman and

Rundel, 1989). Any leaching of inorganic nitrogen from litter in the bags into the surrounding soil would have been enriched in $\delta\ ^{15}N$.

Changes in $\delta\ ^{15}N$ values for roots in this study (1.2 – 3.1‰) were much lower than values found by Zeller *et al.*, (2000, 2001) at a number of forest sites but litter used by Zeller was many times more enriched than that used in this study. However, differences may be due to major differences in vegetation type (grassland cf beech forest) and turnover of root material within the soil.

Despite large variation and lack of significance it appears that the plants in all treatments were incorporating ^{15}N into shoot tissue and it was assumed that the source of this ^{15}N was the labelled litter from the mesh bags.

Chapter 3

Summary

Experimental setup.

Agrostis capillaris was labelled with ^{15}N stable isotope and the leaf litter collected for use in a litterbag experiment at Rigg Foot experimental plots, Sourhope. Two mesh sizes were used in a control plot and plots receiving nitrogen fertilizer, lime and lime and nitrogen. Both coarse and fine mesh bags were used in an attempt to distinguish the effects of enchytraeids from those of microbes. Coarse mesh allowed more enchytraeids to enter and leave the bags and also allowed more infiltration by plant roots. Fine mesh bags 'captured' enchytraeids within them. Bags were retrieved at intervals over 14 months. The labelled litter attracted enchytraeids into the litterbags in all treatment plots within 4 weeks. There were similar overall mean numbers of enchytraeids in both bags and cores, and, as the bags were smaller, the density in the bags was higher. Fewer enchytraeids were extracted from nitrogen treated plots.

Uptake of ^{15}N labelled nitrogen by enchytraeids.

Mean $\delta^{15}N$ and litter derived N values of enchytraeids collected at intervals over 14 months were raised in comparison to natural abundance values, indicating that the enchytraeids had fed inside the litterbags on the labelled litter.

Mean $\delta^{15}N$ values of enchytraeids from the surrounding soil cores were raised which indicated that earlier some of the worms had fed in the bags and then moved out into the soil. Enchytraeids extracted from all plots in fine bags had higher mean $\delta^{15}N$ values and greater litter derived N suggesting that they had fed for longer on labelled litter than enchytraeids in coarse mesh litterbags, which were free to move and feed outside. The lower dry weight and longer half life of *C. sphagnetorum* in fine bags in nitrogen plots suggests they were not feeding well, feeding on bacteria rather than fungi or adversely affected by fertiliser application.

The application of ammonium nitrate fertiliser reduced the abundance of enchytraeids in plots, litterbags and surrounding cores. The toxicity of ammonia to soil animals is well established, and *C. sphagnetorum* are also sensitive to both high pH and low soil moisture both of which were changed by the application of N fertiliser. Lower $\delta^{15}N$ values of enchytraeids in soil and higher $\delta^{15}N$ values of enchytraeids in litterbags in nitrogen fertilised plots suggests that the enchytraeids preferred conditions inside the bags to outside, which may have reduced their contribution to N translocation in these plots.

Nitrogen dynamics.

The use of labelled litter provided a method to distinguish between N released from, and N incorporated into, the litter in the mesh bags. Following initial rapid release of N in summer, there was also a substantial N release over winter in all plots and bags of both mesh size, with the percentage being generally greater in control and nitrogen treated plots.

In coarse litterbags there was less mass loss, higher C:N ratios and greater release of nitrogen in some treatment plots and greater external N incorporation into litter than in fine bags. These effects could be accounted for either by the infiltration of plant roots or by greater enchytraeid activity/interaction with microbes. It was not possible to separate these interactions but it seems likely that the greater release of N from coarse bags could be attributed to the greater abundance of enchytraeids. More enchytraeids would lead to more translocation of litter out of the bags into surrounding soil and possibly also the enhancement of microbial activity due to comminution of litter resulting in more leaching of N.

In nitrogen treated plots %N release was depressed in coarse bags during the summer, suggesting that fertiliser derived nitrogen was utilised preferentially. This may have been direct mineralisation of fertiliser N and/or increased root exudation for microbial uptake. When these sources were used up in late autumn, %N release from litter in bags increased rapidly.

Fine mesh appeared to delay the release of, and raise the threshold for, N release and thus the decline in %N in control and nitrogen plots compared to coarse mesh bags. This may have been attributable to the lack of root infiltration in the fine bags and their uptake of mineralised nitrogen, and/or lower grazing by fewer enchytraeids. Release of N from fine bags was later and lower in lime and L+N plots in comparison to control and nitrogen plots. This may have been due to lack of plant root infiltration, the lower abundance of enchytraeids in these litterbags, differences in the microbial and enchytraeid communities or the feeding activity of these enchytraeids.

Liming destroyed the original population of *C. sphagnetorum* within three years, by which time > 15 previously undetected species were occupying the lime and L+N plots. Most of these species were less abundant than *C. sphagnetorum*, but were also larger. Most available literature records changes in microbial community structure in favour of bacteria when soils are limed and it is possible that the same effect occurred at Rigg Foot.

The lower final C:N ratios in limed plots was probably related to lower %N release from litter, but it is not possible to say whether this was due to the changed enchytraeid community or to changed microbial activity.

Translocation of nitrogen.

Mean $\delta^{15}N$ values of soil, roots and shoots measured on one occasion after 14 months were raised.

Lower mean $\delta^{15}N$ values of enchytraeids and the higher $\delta^{15}N$ values of soil in coarse compared to the fine mesh bags confirm that enchytraeids moved between litter and surrounding soil and promoted the translocation of N.

Lower mean $\delta^{15}N$ value of soil surrounding the litterbags suggests that there was less effect by the neutral soil enchytraeid community on translocation of N. However, the larger enchytraeids found in the neutral plots may have been more mobile that *C. sphagnetorum* in acid plots, thus depositing faeces containing ^{15}N signal further away from the cores surrounding the litterbags.

CHAPTER 4

INVESTIGATION OF CARBON ASSIMILATION BY ENCHYTRAEIDS USING FIELD PULSING WITH $^{13}CO_2$.

4.1 Introduction.

Stable isotopes can be used as a powerful tool in biology and ecology, particularly when attempting to trace an element in a system. Many uses of both natural abundance and enriched stable isotopes have been described in Section 3.1. Although not directly relevant to the nitrogen focus of this study, the opportunity arose to take part in field pulsing studies using $^{13}CO_2$ to investigate assimilation of carbon in enchytraeids.

The rapidity of the movement of carbon from plants into fungi and soil has been demonstrated using both $^{13}CO_2$ and $^{14}CO_2$ (Johnson *et al.*, 2002; Staddon *et al.*, 2003; Ostle *et al.*, 2003) and occurs within hours of labelled CO_2 delivery. Black *et al.* (2002) used carbon pulse technique to measure carbon assimilation into cholesterol in different species of enchytraeid in different plots at Sourhope. Again, this showed the rapid movement of $\delta^{13}C$ into the soil animals.

4.2 Methods

Vegetation in control, nitrogen and lime plots was labelled with $^{13}CO_2$ using a mobile stable isotope delivery (SID) system (for full description see Ostle *et al.*, 2000). Briefly, steel collars were inserted to about 20cm depth and closed-top chambers (40cm diameter) placed over them. ^{13}C labelled CO_2 was supplied via plastic pipes to the chambers for a period of 6 hours during peak solar radiation.

Two pulse experiments were performed in September 2002 and July 2003. There were 3 replicates for each of the control, nitrogen and lime treatments.

September 2002.

Three soil cores ($0.001m^2$) to a depth of $\geq$ 5cm from each treatment were taken prior to commencement of $^{13}CO_2$ pulsing, which was done on 18th September 2002. Thereafter, samples were taken after 1, 3, 7, 16 and 31 days. Samples were placed **in** a cool box to maintain a temperature of ~ 4°C. Enchytraeids were extracted using a modified wet funnel technique immediately after sampling and identified to species using high power microscopy. Enchytraeids were placed in Eppendorf tubes, labelled with species, plot and date of removal from the field. They were then placed in a freezer at -10°C, and later freeze-dried for 24 hours (CHRIST Beta 1-8). The final process was to weigh the samples and place them in tin cups for GC-IRMS.

June 2003.

The soil appeared to be relatively dry (Figure 4.1). The day prior to pulse labelling with $^{13}CO_2$ (25th June 2003) soil cores were taken for enchytraeid extraction for NA δ ^{13}C. The plots to be used for pulse labelling (control, nitrogen and lime) were watered in order to encourage enchytraeids to move up the soil profile.

During the first 24 hours, soil cores were taken 6, 12, 18 and 24 hours post-pulse. They were extracted, identified, placed in Eppendorf tubes (as above) and frozen. Thereafter samples were taken on the third and seventh day post pulse.

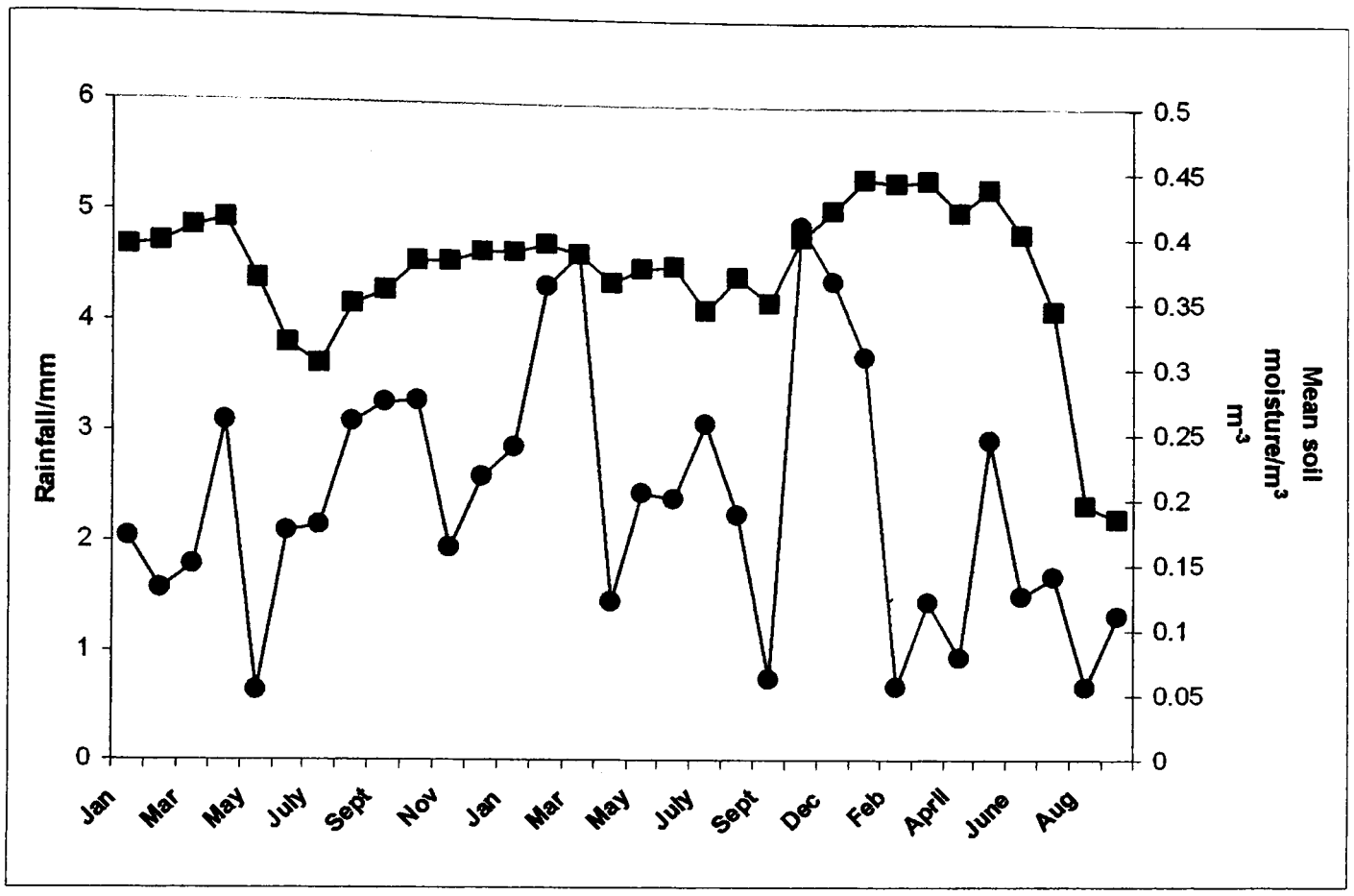

Figure 4.1. Mean monthly soil moisture and rainfall data for the Rigg Foot Site, January 2001 – October 2003.

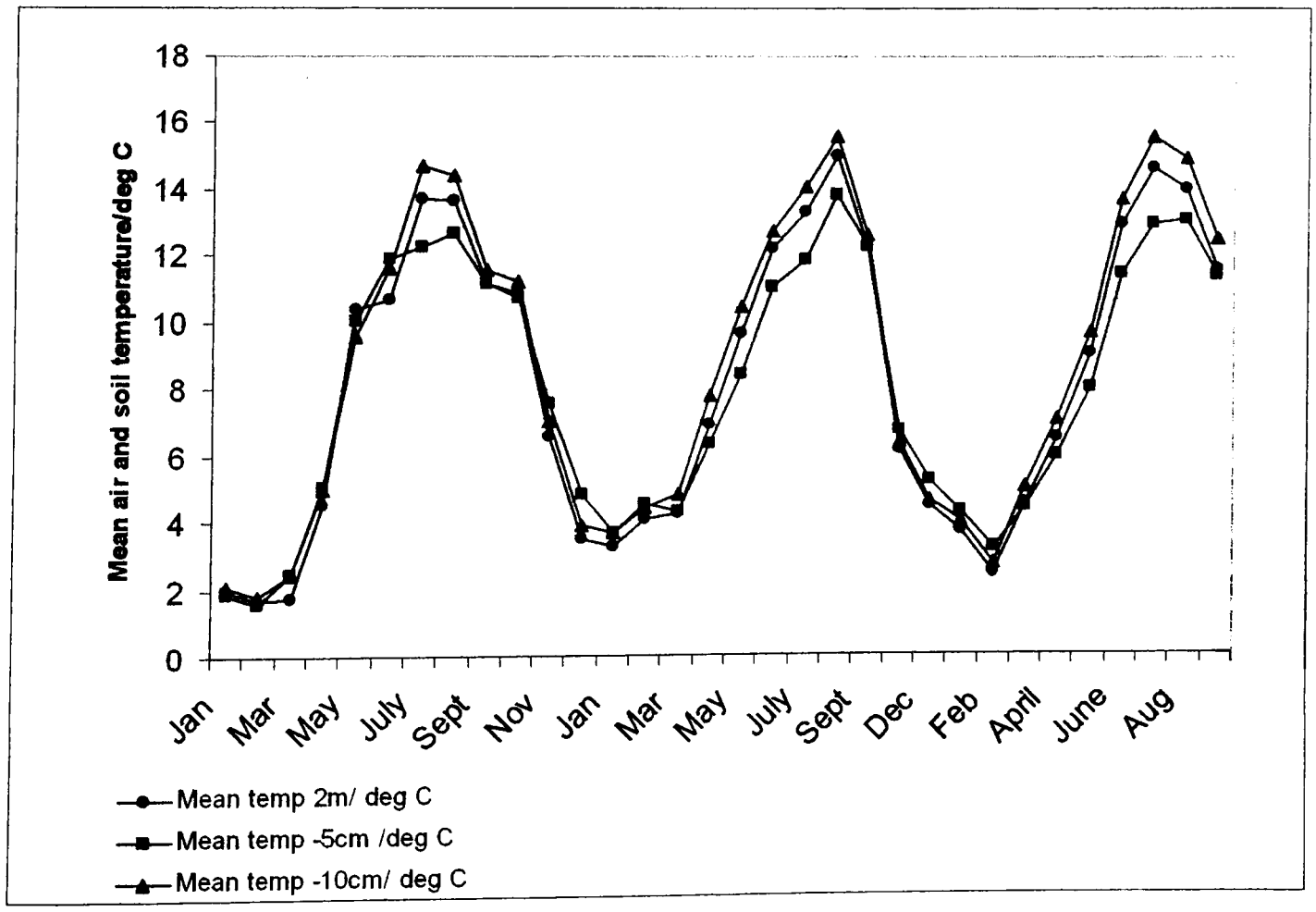

Figure 4.2. Mean monthly temperatures in air at 2 metres, and in soil at 5 and 10cm at Rigg Foot, Sourhope, January 2001 – October 2003.

4.3 Results.

September 2002.

Accumulation of ^{13}C into *C.sphagnetorum* tissue was substantial three days post-pulse in control plots (Figure 4.3A). There was an increase of 2‰ during this time period, which was almost reached within the first 24 hours and a further slight increase after seven days. Thereafter the value remained static. There was little variability in the values during the first week, indicating that the enchytraeids (*C. sphagnetorum*) had all accumulated ^{13}C at a similar rate. After sixteen and thirty-one days there was much more variation in the values suggesting signal dilution or dissipation of ^{13}C. Overall the Δ ^{13}C in the enchytraeids was 2.7‰.

In the nitrogen plots (Figure 4.3B) the starting value of ^{13}C for *C. sphagnetorum* was slightly higher than that of control plots and the overall pattern of ^{13}C uptake was similar to control plots. There was an increase of ~ 2‰ after seven days and a slight increase by the thirty-first day. However, there appeared to be a slowing of the rate of ^{13}C uptake between the first and second days. Overall the Δ ^{13}C in the enchytraeids was ~3‰.

In lime plots (Figure 4.3C) the overall pattern of accumulation by the combined enchytraeid species was similar to that of control plots, but there was much more variation in δ ^{13}C values. The starting value was between those of the control and nitrogen plots with a relatively rapid rise to a maximum after seven days. However, there was a further rise at the end of the experiment and overall there was a 3.4‰ Δ ^{13}C.

The pattern of δ ^{13}C accumulation by enchytraeids was similar to that of δ ^{15}N (Section 3.3.4.2), although at near NA levels.

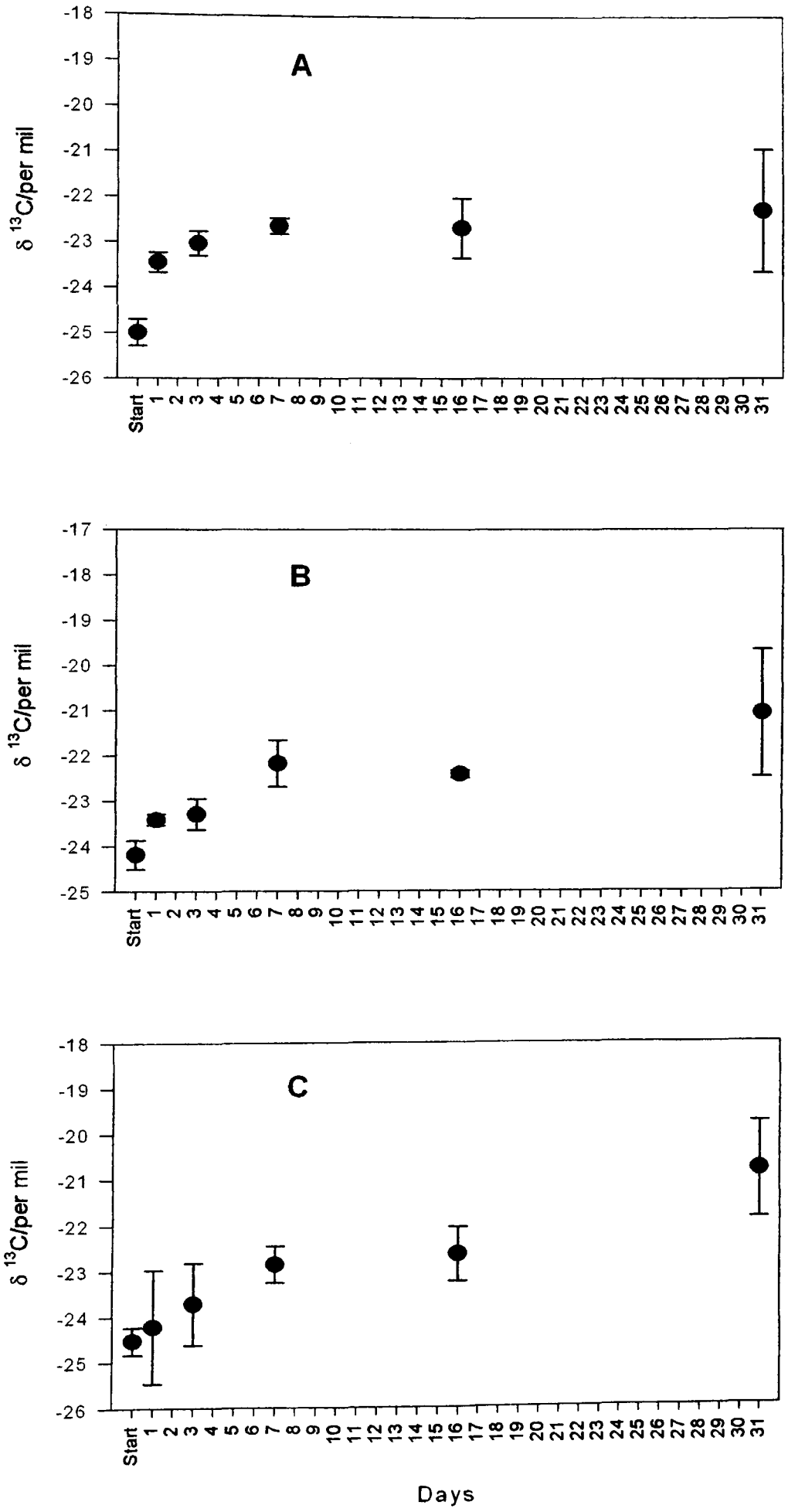

Figure 4.3. $\delta^{13}C$ values of enchytraeids extracted from soil cores from control (A), nitrogen (B) and lime (C) plots at Rigg Foot, Sourhope, September 18th – October 15th 2002.

Estimating the half-life of carbon in enchytraeid tissue.

The data were used to estimate the half-life of C in enchytraeid tissue. The exponential equation $Y = a + be^{ct}$ was used to fit the curve of rise to maximum. This gave the values shown in Figure 4.4 and Table 4.1. The half-life of the curve was calculated from In(2)/*c*.

Table 4.1. Equation results ($Y = a + be^{ct}$) and half-life of enchytraeids in control, nitrogen and lime plots at Rigg Foot, Sourhope.

	R^2 (Adjusted)	SE	*a*	*b*	*c*	Half-life (days)
Control	0.997	1.19	2.60	-24.81	0.445	1.56
Nitrogen	0.997	1.17	2.78	-23.92	0.098	7.1
Lime	0.998	1.13	5.94	-24.36	0.028	0.38

The lines of rise to maximum were tested for significance using the method described in Fowler *et al.*, 1999. There were no significant differences between treatments.

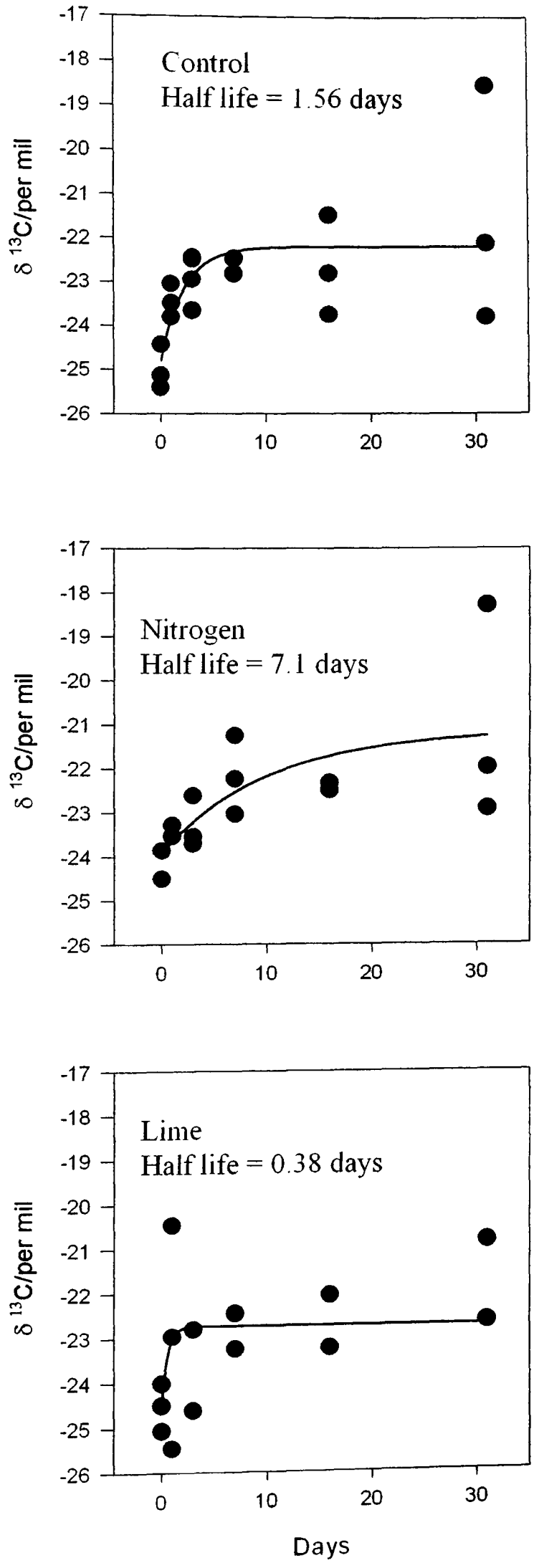

Figure 4.4. Rate of incorporation into enchytraeid tissue of carbon derived from $^{13}CO_2$ pulse and half life value in treatment plots at Rigg Foot, Sourhope. Experiment lasted for 31 days.

June 2003.

There were few *C. sphagnetorum* extracted for NA from control plots and none from nitrogen plots. In lime plots only *Henlea perpusilla* was abundant enough to collect a single sample for NA.

Figure 4.5 shows the $\delta^{13}C$ values of enchytraeids from the three treatment plots. The NA $\delta\ ^{13}C$ of *C. sphagnetorum* was higher in June (~ -23‰) than the previous September (~ 25‰), although the former was based on a single sample. $\delta^{13}C$ values fluctuated slightly over the first 24 hours. After three and seven days the $\delta\ ^{13}C$ values remained static. Overall there was no $\Delta\ ^{13}C$. There were no significant differences between any of the values in control plots, or compared to nitrogen and lime plot data.

In nitrogen plots the $\delta\ ^{13}C$ values at 24 hours appeared to have two anomalous values of 17.59 and -30.86. The remaining value was -23.43, and only this single value was used. Overall there was no $\Delta\ ^{13}C$ and was not significantly different. However, the values in the nitrogen plots were significantly lower than those in lime plots.

In lime plots, enchytraeid $\delta\ ^{13}C$ values during the first 24 hours increased and decreased by ~ 1‰, and there was a slight but steady rise during the following 7 days. Again, 2 anomalous values for the 6 hour sample have been omitted. Overall there was a slight (~ 1.5‰) $\Delta\ ^{13}C$ but the changes in values were not significant.

Table 4.2 shows the ANOVA result, and Figure 4.3 the $\delta^{13}C$ values for enchytraeids from the different treatment plots.

Table 4.2. One-way ANOVA results for each plot and between plot differences, with *pots hoc* Tukey test for differences.

	F	df	*p*
One-way ANOVA (all 3 treatments)	3.999	2,58	0.024
Tukey's Lime and nitrogen			0.05

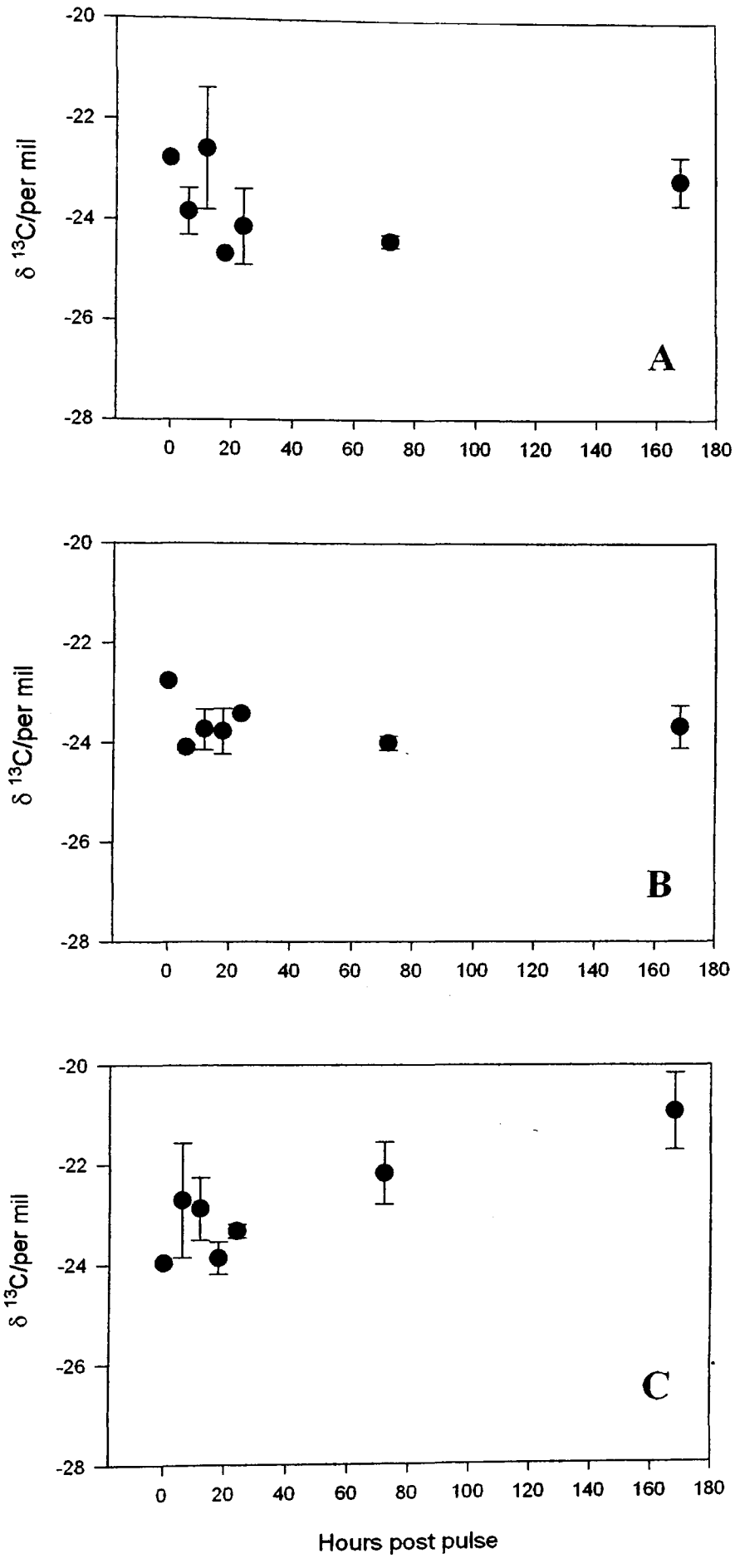

Figure 4.5. $\delta^{13}C$ values of enchytraeids extracted from soil cores from control (A), nitrogen (B) and lime (C) plots at Rigg Foot, Sourhope, June 2003.

4.4 Discussion

September 2002.

The enchytraeids appear to have incorporated ^{13}C into their body tissue from $^{13}CO_2$ that was taken up by plants. As they are purely soil animals, the ^{13}C must have entered the soil system via the plant photosynthetic pathway and translocation to the roots. A number of studies using both stable and radioisotope techniques have demonstrated the speed with which the isotope is translocated from shoots to roots and microbes.

Johnson *et al.* (2004) used $^{14}CO_2$ as a tracer using turfs from Rigg Foot. Microcosms could be manipulated to include or exclude arbuscular-mycorrizal (AM) fungi. They found that in the presence of AM fungi more $^{14}CO_2$ was respired from the soil, and also calculated that 3.4% of the ^{14}C initially fixed by plants was allocated to AM fungi over the 70 hour experimental period.

Treonis *et al.* (2004) used $^{13}CO_2$ pulse labelling combined with microbial community phospholipid fatty acid (PLFA) analyses in order to identify microbes actively involved in assimilation of root derived carbon. The experiment was carried out at Rigg Foot and utilised control and lime plots. After 4 and 8 days post-pulse they found that fungal and Gram-negative biomarkers had most ^{13}C enrichment and that the turnover rates of these microbes was also the most rapid. This was indicative that the microbes were assimilating root carbon that had been recently photosynthesised.

Black *et al.* (2002) detected enrichment of 1 - 2‰ ^{13}C in enchytraeid cholesterol 11 days post pulse and this rose to 8 - 14‰ 50 days post pulse. This was a more accurate assessment of ^{13}C assimilation as cholesterol is synthesised biochemically from

dietary carbon sources (Chamberlain *et al.*, 2003). In the present study the $\delta^{13}C$ represented bulk (whole body) values and did not take into account contributions from gut contents. However, this study showed that in control (and to a lesser extent, nitrogen and lime) plots there was a clear increase in $\delta^{13}C$ value within the first 24 hours post pulse, with a slight increase on the third and fifth days to a plateau thereafter. This suggests that within 24 hours of pulsing, ^{13}C had been translocated from the plant leaves via the roots into the soil where enchytraeids accumulated enriched carbon either via soil microbial grazing or directly from the soil.

Comparing the control and nitrogen plots, the $\delta^{13}C$ values of enchytraeids from nitrogen plots result in a rather flattened curve compared to control plots. This was a similar pattern to that of the $\delta\ ^{15}N$ of fine litterbag enchytraeids (Section 3.3.4.2), suggesting that the enchytraeids are either not feeding or are in some way negatively affected by nitrogen fertilisation, either directly or indirectly.

In lime plots the half-life value of < 1 day appears rather short. However, Chamberlain *et al.* (2004), studying Collembola lipids, recorded half-life values from 1.5 days. In the current study, 4 different species of *Fridericia* and one species of *Henlea* from lime plots were used for stable isotope analysis, all relatively large enchytraeids. The presence of gut contents contributing to the $\delta^{13}C$ values from which the half-life is calculated cannot be excluded, and the larger the animal, the greater the potential gut content.

The $\Delta\ ^{13}C$ increase in the enchytraeids in control plots during the first 24 hours was relatively rapid. A further $^{13}CO_2$ pulse planned for June 2003 was used to try to assess how rapidly this was achieved during the first 24 hours.

June 2003

The patterns detected in the September 2002 pulse experiment were not apparent in the 2003 experiment. Although there appeared to be some variability in the $\delta\ ^{13}C$ values of enchytraeids in control plots, the values were not significantly different. In nitrogen plots there was no change in $\delta\ ^{13}C$ values during the 7 days of the experiment, and a slight increase in lime plots.

It would appear that in the acid plots *C. sphagnetorum* were not incorporating labelled carbon. Possible reasons for this are - enchytraeids were not feeding because of some environmental factor; enchytraeids extracted were from deeper levels in the soil and had not fed on $\delta\ ^{13}C$ labelled exudates in the rhizosphere; the enchytraeids were regenerating after fragmenting and were therefore unable to feed.

C. sphagnetorum thrives in cold, wet, acid soils. The vertical distribution of *C. sphagnetorum* was examined in a study by Briones *et al.* (1997), who showed the downward vertical migration of the species during dry periods when air temperatures were high. Figure 4.2 shows the mean monthly temperature of air at 2 meters above ground level and soil at 2 and 5 cm below ground level. The temperature at -5 cm is 1.2°C lower than air temperature and > 0.6°C lower than at -2 cm. At -10 cm the soil temperature was higher than air temperature, so it reasonable to assume that in June, as temperatures were rising toward the July maximum, that *C. sphagnetorum*, would be found at lower levels within the soil profile. The low numbers found and the possibility that individuals were only active at lower levels may mean *C.sphagnetorum* could not access the rhizosphere, where the ^{13}C signal would have been strongest. Furthermore in a laboratory study, Standen (1973) monitored activity of *C. sphagnetorum* at 5, 10 and 15°C. The worm was passive at 5 and 15°C and

active at 10°C. The soil temperature during June and July (the experimental period) was approaching 15°C and the *C. sphagnetorum* would therefore have been passive and not feeding.

C. sphagnetorum reproduces by fission, which occurs more frequently in the early summer and autumn but also in response to environmental stress (Standen, 1973). During the regeneration period the enchytraeid does not feed. If fragmentation had occurred in this population many would not have been feeding for the 7 days of the experiment.

In lime plots, there was evidence that the enchytraeids had accessed ^{13}C from the pulse. These enchytraeids were a diverse community of species each at low density. This community of enchytraeids is acclimatized to dryer soil conditions in more neutral soils. These enchytraeids reproduce sexually and reproduction occurs during the warmer summer months. Cocoons can over-winter and hatch during late spring, so at the time of the pulsing experiment relatively small hatchlings could access smaller soil pores. This also applies to the smaller species of enchytraeid such as *Enchytraeus buchholzi* and *Henlea perpusilla*. This ability to avoid the worst consequences of drought may have given them access to the rhizosphere and microbes, and so to ^{13}C labelled food sources. Although the overall increase in $\delta\ ^{13}C$ value was not significantly different during the experimental period, there was a trend of increasing ^{13}C accumulation. It was apparent that the enchytraeids in the neutral soils were more active and feeding, although the rate of C accumulation was slower possibly because adverse environmental conditions such as drought influenced their behaviour.

Chapter 4

Summary.

Rigg Foot Experimental Site at Sourhope was used for the $^{13}CO_2$ pulse experiment. Treatments pulsed and sampled were control, nitrogen and lime. The first experiment was carried out in September 2002 when abiotic factors such as rainfall, soil moisture and temperature were typical for the site, and the second experiment was carried out in June 2003, when conditions were much drier.

Sampling intervals for September 2002 were 1, 3, 7, 16, and 31 days post-pulse. The majority of uptake of $\delta^{13}C$ occurred within 3 days of the pulse. Assuming that the $\delta^{13}C$ detected in enchytraeid tissue was $^{13}CO_2$-pulse derived, the half-life of carbon was calculated to be ~ 1.5, 7 and < 0.5 days for control, nitrogen and lime plots, respectively. This suggested that the enchytraeids were feeding close to the rhizosphere, as other research had shown that ^{13}C was detected here within 24 hours of the $^{13}CO_2$ pulse.

Sampling intervals for June 2003 were 0.25, 0.5, 0.75, 1, 3 and 7 days post-pulse. Assuming that the $\delta^{13}C$ detected in enchytraeid tissue was $^{13}CO_2$-pulse derived, there was no uptake of carbon by enchytraeids in control and nitrogen plots. In lime plots carbon uptake appeared to occur by the end of the experiment. This was attributed to the dry soil conditions during this period which adversely affected the feeding activity of *C. sphagnetorum*.

CHAPTER 5

INCORPORATING ^{13}C AND ^{15}N INTO *Agrostis capillaris.*

5.1 Introduction.

Upland soils generally have low pH, are frequently wet, or even waterlogged and temperatures are low. These factors influence the type of soil organisms that can tolerate these conditions, decomposition rate of plant material and also the plant community that grows on the soil.

The importance of the enchytraeid worm *C. sphagnetorum* in nitrogen (and other nutrient) cycling has already been discussed. In order to assess whether nitrogen mobilisation was influenced by this enchytraeid, it was decided to carry out a litterbag experiment. This required growing plant material into which ^{15}N and possibly also ^{13}C had been incorporated, which would be used as the litter. The aim was to use the labelled litter as a tracer from the litterbags into enchytraeids and subsequently into the surrounding soil.

The plant species used

At Sourhope (an acid grassland), where the litterbag experiment was to take place, *Agrostis capillaris* was the dominant grass species. It was the plant of choice for this experiment as it grows easily and quickly under laboratory conditions and is a typical acid upland plant.

The field experiment required tissue nitrogen content (< 2%) to simulate field conditions, and the incorporation of ≈ 200‰ ^{15}N and ^{13}C. Carbon is more abundant in plants and animals than nitrogen and it was hoped to detect carbon in soil even if nitrogen was not detected.

The usual method of incorporation of ^{13}C is by enclosing the plants in an airtight chamber and providing $^{13}CO_2$ to ensure uptake of ^{13}C during photosynthesis. The equipment required is relatively costly and not widely available (e.g. Berg *et al.*, 1991). A new method had been described by Schmidt and Scrimgeour (2001) using ^{13}C- and ^{15}N-labelled urea. Urea had been sprayed onto maize plants and both isotopes were successfully incorporated.

Trial 1. The spray-on was attempted using *A. capillaris* and unlabelled urea, to assess whether nitrogen entered the tissues, and whether the tissue nitrogen content could be kept <2%. The harvested stems were washed in de-ionised water to remove any trace of urea from them, and the washings analysed to ensure a steady drop in N. It proved to be impossible to demonstrate that the N content of the plant material was within the plant and not on the surface and therefore the spray-on method was abandoned.

Trial 2. Pilot studies were then undertaken which used urea as a nitrogen source, watered into the base of the pots, not sprayed on, in order to keep tissue N <2%. These experiments showed that a 0.25M urea solution was required.

5.2 The method used to provide the labelled litter.

The field litterbag experiment required 200g dry weight of plant material, as each litter bag required 1g dried shoot material, and 200 litter bags were required. A pilot study had yielded a mean of 1.5g dry shoot mass per pot (V.Standen, pers comm.). The pots used were 13cm in diameter and there were 5 replicates. From this, it was estimated that 2 m^2 would yield 200g of dry shoot material needed.

Growing conditions.

Square plastic plant pots (7.15cm x 7.5cm) were placed in propagator trays (50cms x 30cms). Twelve trays were used with 24 pots in each tray. The pot bases were covered with filter paper to prevent loss of growth medium. They were filled to within 1 – 1.5cms of the rim of the pot with horticultural sand. It was necessary to trim the edges of the pot to ensure a good fit within the propagator. Water was poured into the tray and allowed to soak up into the sand to completely wet the sand, and *A. capillaris* seeds (Emorsgate Seeds) sprinkled on top. Black plastic sheets were placed over the trays to aid germination. The trays were placed in a constant temperature growth room at 22 – 25^{0}C with an 18/6 hour light/dark cycle. Germination was evident by 7 days and the plastic covers removed.

Due to space constraints and the growth of fungus on the sand surface, the trays of plants were moved to a greenhouse, average temperature 12^{0}C.

Experiment 1 – labelling with ^{15}N and ^{13}C.

Calculation of concentration of ^{13}C- and ^{15}N-labelled urea to add to ambient urea.

To achieve 200 ‰ enrichment of ^{15}N urea for a 1 molar solution of urea requires 45mg ^{15}N-labelled urea to 59.955g unlabelled urea, and 200‰ enrichment of ^{13}C urea requires 133mg ^{13}C-labelled urea to 59.867g unlabelled urea.

The urea solution to be used is 15mg L^{-1}, or 0.25M.

Therefore it requires (45/4) = 11.25mg ^{15}N and (133/4) = 33.25mg ^{13}C urea to 14.9555g ambient urea.

Labelled urea (Cambridge Isotope Laboratory, Massachusetts, USA) was purchased with ^{13}C and double-labelled ^{15}N urea as separate chemicals. The calculated weights were added to 1 litre of deionised water and formed the stock solution.

Plant feeding.

The ratio of urea to $^{1}/_{5}^{th}$-strength Long Ashton solution to be used was 1:160. Five litres of plant nutrient solution was made in a batch. From the urea stock solution 31.25mls were added to a 5-litre vessel, the appropriate quantities of LA stock solutions (Appendix III) and this was made up to 5 litres with deionised water. Initially, the plants were fed 3 times weekly, with 15mls per pot (equivalent). This was watered into the tray, and the propagator lids fixed firmly in place, and taped to the bases in places to ensure that they were not accidentally displaced. It was not known whether the urea entering the pots by the base would be taken up by the plant, or whether urease from microbial sources would begin the hydrolysis of urea. In either case it is likely that CO_2 would be the excretory product and plants are known to discriminate against ^{13}C in favour of ^{12}C therefore the lid vents were closed as it was necessary to exclude as much atmospheric carbon as possible.

It was planned to give each tray of plants ~ 350 mls of feed stock solution weekly. This had been calculated from the previous trial, based on the pot area. However, the sand substrate became saturated and, when the propagator lids had been added over the first weekend, some pots had a heavy fungal growth. The trays were drained and smaller quantities of food stock solution added on alternate days. The propagator lids were added after each 'feed' for 24 hours, then removed until the next feed solution was added. However, by the fifth week it became apparent that the plants were receiving insufficient nutrient solution, therefore the nutrient concentration was doubled. The double dose continued until the end of the experimental period.

Experiment 2 – labelling with ^{13}C.

The first experiment did not successfully incorporate ^{13}C (see Results), and therefore a second experiment was designed to try to establish a more suitable method for future use. Apart from the nutrient solution the materials used were the same as those used for the previous experiment. Three replicates of control (unlabelled ^{13}C urea) and three replicates of ^{13}C-labelled urea were set up. The trays were placed in a temperature-controlled room, with a temperature range of 22 - 25°C, and a 16-hour light, 8-hour dark cycle.

Long Ashton nutrient solution with unlabelled urea as the nitrogen source was watered into the base of the pots, as with the previous experiment. ^{13}C-labelled urea (800mg) was then added to the nutrient solution on the final day, and added to three trays of plants. The propagator lids were added to all six trays, the vents closed and the lids were taped in place. The plants were left lidded for 48 hours, after which the lids were removed. No further nutrient solution was added, but the plants were given deionised water. The control plants were removed from the room to prevent cross-contamination with circulating ^{13}C but otherwise treated in the same way.

Half of the plants from all six trays were harvested after 7 days, and the remainder harvested on the 14th day.

Harvesting and analysis of material - Experiments 1 and 2.

The plants were allowed to die back over a 7 -14-day period. The leaves were then cut off just above the shoot base, air-dried and placed in paper bags. The harvest from each tray was kept in a separate, labelled bag. A representative sample of each tray was sent for stable isotope analysis (Method as Section 3)

5.3 Results.

Experiment 1.

Table gives the stable isotope values and % of carbon and nitrogen achieved by this experiment. The tissue content was kept below 2% (1.59 ± 0.07) and $\delta^{15}N$ successfully incorporated (122.39 ± 3.23 ‰). However, the ^{13}C values are typical of natural abundance concentrations and it was concluded that the ^{13}C from the urea supplied was not incorporated into the grass tissue.

Table 5.1. % nitrogen and carbon and δ ^{15}N and ^{13}C incorporated into leaf tissue of *A. capillaris* by watering into base of pots.

	% nitrogen	delta ^{15}N (‰)	% carbon	delta ^{13}C (‰)
Sample				
1	1.71	126.91	44.88	-28.97
2	1.6	131.19	41.78	-29.28
3	2.05	123.77	39.48	-29.02
4	1.42	105.2	43.91	-29.25
5	1.75	117.06	43.56	-28.74
6	1.91	136.75	43.11	-29.52
7	1.39	119.23	43.61	-28.66
8	1.99	144.35	45.33	-29.53
9	1.64	130.15	43.75	-28.62
10	1.26	111.89	43.66	-28.71
11	1.53	127.07	45.89	-29.14
12	1.54	131.95	44.95	-29.22
13	1.41	121.68	44.38	-29.99
14	1.15	97.2	43.4	-29.39
15	1.55	109.93	43.49	-29.58
Mean	**1.59**	**122.29**	**43.68**	**-29.18**
SE	0.07	3.23	0.4	0.11

Experiment 2.

Table 5.2 shows the ^{13}C, % C and %N values of the control and experimental plants. Nitrogen content of the plants was within the parameters required (< 2%). ^{13}C was successfully incorporated into the plants - ~ 80‰ after 7 days and 60‰ after 14 days. A t-test comparing control and experimental plant results for ^{13}C on day 7 and day 14 showed significant differences (7 days $t = -13.05$, $p < 0.01$; 14 days $t = -3.89$, $p < 0.05$).

Table 5.2.δ ^{13}C, % C and % N incorporated into leaf tissue of *A. capillaris* by watering into base of pots (n = 3).

	Control		Experimental	
	7 days	14 days	7 days	14 days
%N	1.73	1.57	1.81	2.01
	1.59	1.43	1.86	2.00
	1.70	1.41	2.01	1.65
Mean	**1.67**	**1.47**	**1.89**	**1.89**
SE	0.04	0.05	0.06	0.12
%C	45.41	44.93	44.85	43.41
	45.35	45.13	45.10	45.91
	45.86	44.91	46.69	46.11
Mean	**45.54**	**44.99**	**45.55**	**45.14**
SE	0.16	0.07	0.58	0.87
δ ^{13}C	-25.11	-25.74	51.53	54.80
	-23.85	-27.91	47.51	40.90
	-22.76	-26.80	67.31	3.28
Mean	**-23.91**	**-26.82**	**55.45**	**32.99**
SE	0.68	0.63	6.04	15.39

5.4 Discussion.

The first experiment was successful for nitrogen isotope incorporation but not for carbon. The labelled carbon was added gradually over the 6 week course of the experiment, the plants enclosed for short periods only and their exposure to atmospheric carbon was much greater than to the ^{13}C urea. These factors are likely to be the reason for the lack of ^{13}C in the plant tissue. Furthermore, Schmidt and Scrimgeour (2001) used 1g of ^{13}C-labelled urea in their experiment which achieved ^{13}C enrichment, whereas in this experiment only 33 mg l^{-1} of the isotope was used.

The second experiment where 0.8g of 99 atom% ^{13}C-labelled urea was applied in one dose on the final day of the experiment, achieved enrichment in the plant tissue of 80‰ and 60‰ after 7 and 14 days. This single dose was divided between the three trays of experimental plants so that each tray received 0.27 g ^{13}C-labelled urea. To achieve greater enrichment a greater quantity of labelled urea would have to be provided.

Schmidt and Scrimgeour (2001) used 1g of ^{13}C labelled urea in their spray on experiment. Extrapolating from the present study, using 1g labelled urea with the watering in method thus would have given ~ 240 - 180‰ enrichment after 7 and 14 days respectively. The watering-in method represents a simple and cost-effective method to incorporate ^{13}C into plant tissue using basic equipment.

The material used in the litterbag experiment was that produced in the first experiment i.e. the material was labelled only with ^{15}N.

Chapter 5

Summary.

Labelling *A. capillaris* with ^{13}C and ^{15}N with urea using Scmidt and Scrimgeours spray-on method was unsuitable for the fine-bladed grass.

The first 'watering-in' experiment successfully incorporated ^{15}N into the plant tissue at ~ 125‰. ^{13}C was not incorporated, and this was probably due to the low concentration of ^{13}C labelled urea used. The N content of *A. capillaris* was successfully kept below 2%.

For the second experiment, ^{13}C labelled urea of ~ 0.27g per tray of plants was added to the nutrient solution and applied in a single dose, watered in, at the end of the experiment. This achieved a mean enrichment of 80‰ after 7 days and 60‰ after 14 days. These time periods were chosen to allow the grass to die down and be more palatable to the enchytraeids.

Extrapolating from this, 1g ^{13}C labelled urea would produce enrichments of ~ 240 - 180‰ after 7 and 14 days respectively.

CHAPTER 6

GENERAL DISCUSSION

In the context of the GANE programme, different approaches were made to the basic questions raised by a study of enchytraeids and nitrogen - how does nitrogen deposition affect enchytraeid abundance and activity, how do enchytraeids influence nitrogen dynamics of litter decomposition and are these dynamics affected by nitrogen deposition? The enchytraeid species *Cognettia sphagnetorum* is considered to be a key species in decomposition processes in acid soils because it is very abundant. Because acid soils are common in areas of high N deposition, the main impetus of the work was on *C. sphagnetorum* in acid soil supplemented by studies of the enchytraeid community in an acid soil changed by liming.

The effects of atmospheric N deposition on Enchytraeidae populations were first addressed by re-examining a site (Moor House NNR) in an area which has received high background N deposition for 30 years. No difference in abundance was detected, but the time of reproduction was changed and appeared to be related to changes in soil temperature. However the uncertainty in knowing exactly how much N had been deposited reduced the usefulness of this study. Furthermore, Adamson *et al.* (1998) studied precipitation, soil solution and drainage water at Moor House and concluded that the majority of deposited N was immobilised in the peat.

The two other sites sampled were experiments where known quantities of N fertilizer had been applied. The analogy between this type of experiment and actual background deposition is crude, but nevertheless the experiments gave important insight into the impact of nitrogen on enchytraeid abundance.

The results of the field experiments were equivocal, with an increase in *C. sphagnetorum* abundance in a peat soil, but an apparent depression of the population in a brown earth soil. Abrahamsen and Thompson (1979) and Huhta (1984) had reported an initial depression of *C.sphagnetorum* abundance after application of nitrogen fertilizer followed by an increase in abundance. The population increase was seen in the high N treatment plots at Ruabon - from 20 – 70 000m^{-2}. Generally ammonium nitrate fertiliser causes a fall in pH but this did not occur in the upper 5cm in this peat soil and, unless the pH decrease was dramatic to <2.5, it is unlikely to affect *C. sphagnetorum*, which thrives in soils with low pH (Standen and Latter, 1977; Healy, 1980; Yesmin *et al.*, 1995). Also the application of fertiliser at this site more closely resembles 'natural' deposition in that it is applied in eight doses through the year rather than in one or two large doses as in the studies referred to above and so the drastic effects of N fertilizer application may have been avoided. Another factor at this site relates to the amount and quality of litter produced. Lee and Caporn (1998) describe increased foliar N in *Calluna* and almost doubling of litter mass as a result of initial increased plant growth and flower production, as well as increased mycorrhizal biomass and bacterial activity.

At Sourhope the population of *C. sphagnetorum* appeared depressed in comparison to those of control plots. The expected spring to summer increase in abundance which was apparent in control did not occur in the nitrogen plots; in fact there was a decline in abundance in N treated plots between spring and summer in 2001 and 2002. This may have been related to an increase in pH which occurred during this period in N treated plots, as *C. sphagnetorum* has a strong negative response to increased pH (Standen, 1984). The direct toxic effects of N fertiliser application may also have

been a contributory factor (Moursi, 1963; Huhta, 1984) as at Sourhope the nitrogen fertiliser was applied in two large doses in April and May, when this enchytraeid would usually be reproducing rapidly. Although litter %N was higher in N plots, litter quantity was low as clippings were removed. *C. sphagnetorum* is also sensitive to decreased soil moisture and Burt-Smith (2002) reported that soil moisture in nitrogen treated plots had fallen. However, by 2003 abundance in both plots was low and the population crashed in autumn 2003 in response to the extremely dry soil conditions.

The more diverse community of enchytraeids in limed plots at Sourhope showed some decline in abundance during 2003 but not to the same extent as *C. sphagnetorum*, confirming the sensitivity of the latter to adverse soil conditions. However, abundance of this diverse community was slightly less with nitrogen addition. Soil moisture was less in L+N treated plots (Burt-Smith, 2002) and, although these enchytraeids appear to tolerate drier soil conditions, these factors suggests that soil ammonium toxicity and lower soil moisture were affecting all enchytraeids.

Evidently there is no straightforward description of the impact of N deposition on enchytraeids. Much appears to depend on soil and vegetation type, quantity of litter available for decomposition, changed physical factors such as pH and possible changes brought about by treatment to the soil microbes. How the factors discussed influence the population dynamics of *C. sphagnetorum* is not known. Bacteria are not thought to be the best food source for this enchytraeid (Latter and Howson, 1978), but increases in bacterial activity may release nutrients available for other soil biota on which the enchytraeid feeds. Increased litter accumulation may physically provide a

more suitable habitat by retaining moisture, thus avoiding the necessity of vertical migration in warm, dry conditions (Springett *et al*, 1970; Briones *et al*. 1997).

Field experiments are by their nature subject to uncontrolled influences. The experiments described here, although by no means conclusive, point the way to useful further work.

The role of enchytraeids in nitrogen dynamics under different experimental conditions was explored in the litterbag experiment. This was designed to follow the translocation of litter nitrogen by enchytraeids within the soil profile. In addition the rate at which nitrogen was assimilated into enchytraeid tissue and the % tissue N which was derived from litter was calculated. A further interesting outcome of the litterbag experiment was that it provided information which allowed calculation of the rates of incorporation and release of nitrogen from litter. The experimental plots at Sourhope were used so as to investigate how the role of enchytraeids might be influenced by the deposition of nitrogen in the natural acid soil and in the same soil modified by liming.

The litterbag experiment confirmed that all species of enchytraeid translocated nitrogen from its food source into the soil and that some of this nitrogen was taken up by plants. There were increases in $\delta^{15}N$ signal in enchytraeids, soil and plant material. Greater soil ^{15}N signal in control plots may be related to the greater numbers of enchytraeids found in these plots. However, many of the enchytraeid species found in limed plots are larger than the dominant acid soil enchytraeid (*C. sphagnetorum*) and may have deposited faecal/litter material out of range of the soil core taken.

The effects of nitrogen on these processes is clearly demonstrated when the total Δ ^{15}N signal in the different media are examined. In nitrogen treated plots, a total change of 7.7 and 6.3‰ were found but in plots not receiving nitrogen these values were 11.9 and 13.8‰. This suggests that fertiliser N was utilised by microbes first before the litter N was used, that enchytraeids were not influencing nitrogen mineralisation as efficiently or that more nitrogen was leached away in these plots. There were also nitrogen impacts on litter nitrogen release, with a 'dampening' effect on N release in nitrogen plots during the first summer which again suggests that external sources of N were utilised before litter N.

However, an insight into the impact of nitrogen on enchytraeid physiology was demonstrated with the unexpected finding that fine mesh did not exclude enchytraeids but contained them. This provided an opportunity to assess the nitrogen half life in these animals. The half life values for enchytraeids in nitrogen treated plots – different species in an acid and neutral soil – were much lower than for their counterparts not receiving nitrogen fertiliser treatment. This indicates *either* that there was more external N being brought into the bags and so providing a non-^{15}N labelled food supply for the worms *or* that the worms were not feeding well as the incorporation of external N was very low in all treatments in fine litterbags. The conclusion is that enchytraeid feeding behaviour was disrupted in some way. The same depression of the half life curve and the half life value was observed in the $^{13}CO_2$ pulsing experiment in nitrogen plots. These experiments suggest that nitrogen adversely affects both *C. sphagnetorum* in acid soil plots and the *Fridericia* and *Henlea* species in limed plots. No causal mechanism is known but changes in feeding behaviour is implicated.

If enchytraeid grazing influences microbial growth and activity (Hedlund and Augustsson, 1995; Cole *et al.*, 2002a), adverse effects on feeding behaviour would be expected to reduce effects on microbes. However, nitrogen treatment also influences microbial activity and this seems to be dependant on soil and vegetation types (Ledgard *et al.*, 1998; Lee and Caporn, 1998). In the latter study, there was an increase in bacterial utilisation of organic substrates and in an acid soil, sustained stimulation of N mineralisation.

In coarse litterbags – where enchytraeids could enter and leave the bags freely – there were no differences in the overall release of nitrogen from litter, although the patterns of release varied between the treatments. In fine litterbags there was no nitrogen effect but there was an effect attributable to liming, with nitrogen release lower in lime treated plots. If nitrogen was limiting for microbial activity/growth increased incorporation of external N would be expected but in fact there were no differences between nitrogen incorporation in different treatments. Thus it can be said that any interference of enchytraeid feeding behaviour caused by nitrogen treatment did not affect nitrogen dynamics and it must be concluded that other soil animals and/or microbial activity were more influential than enchytraeids.

There are few comparable studies, and those cited in the text have had rather different aims to the current study. Caner *et al.* (2004) reported labelled litter-derived nitrogen in enchytraeids of up to 11%, despite using highly $\delta^{15}N$ enriched litter. However, the litter used (beech leaves) are more resistant to decomposition than the grass species used here. Litter-derived nitrogen in enchytraeids in this study varied between

treatments and time, but ranged between ~ 6.5 and 45% from coarse and 35-88% from fine bags.

The litterbags experiment using ^{15}N labelled litter did not meet all the original requirements in that it failed to exclude enchytraeids and so separate enchytraeid and microbial activity, but it provided other opportunities for observations of enchytraeid physiology. The original objective of following translocation of litter N by enchytraeids in soil was successfully achieved.

This study has revealed some information on the effects of enchytraeids on nitrogen dynamics and effects of nitrogen on enchytraeid abundance and behaviour. It did not take into account the effects of global carbon emissions and warming. A laboratory study by Briones *et al.* (2004) showed increased biomass of *C. sphagnetorum* and CO_2 flux from soils particularly at higher temperatures (15°C). The study at Moor House NNR showed no change in abundance of this enchytraeid, which suggests that there have been no positive effects on enchytraeids to date. Most importantly in the context of nitrogen deposition is the prediction that recovery of acid-sensitive soils to acid inputs are likely to take decades and for some upland areas, soil base saturation may never return to previous values i.e. prior to the onset of acid deposition (NEGTAP, 2001).

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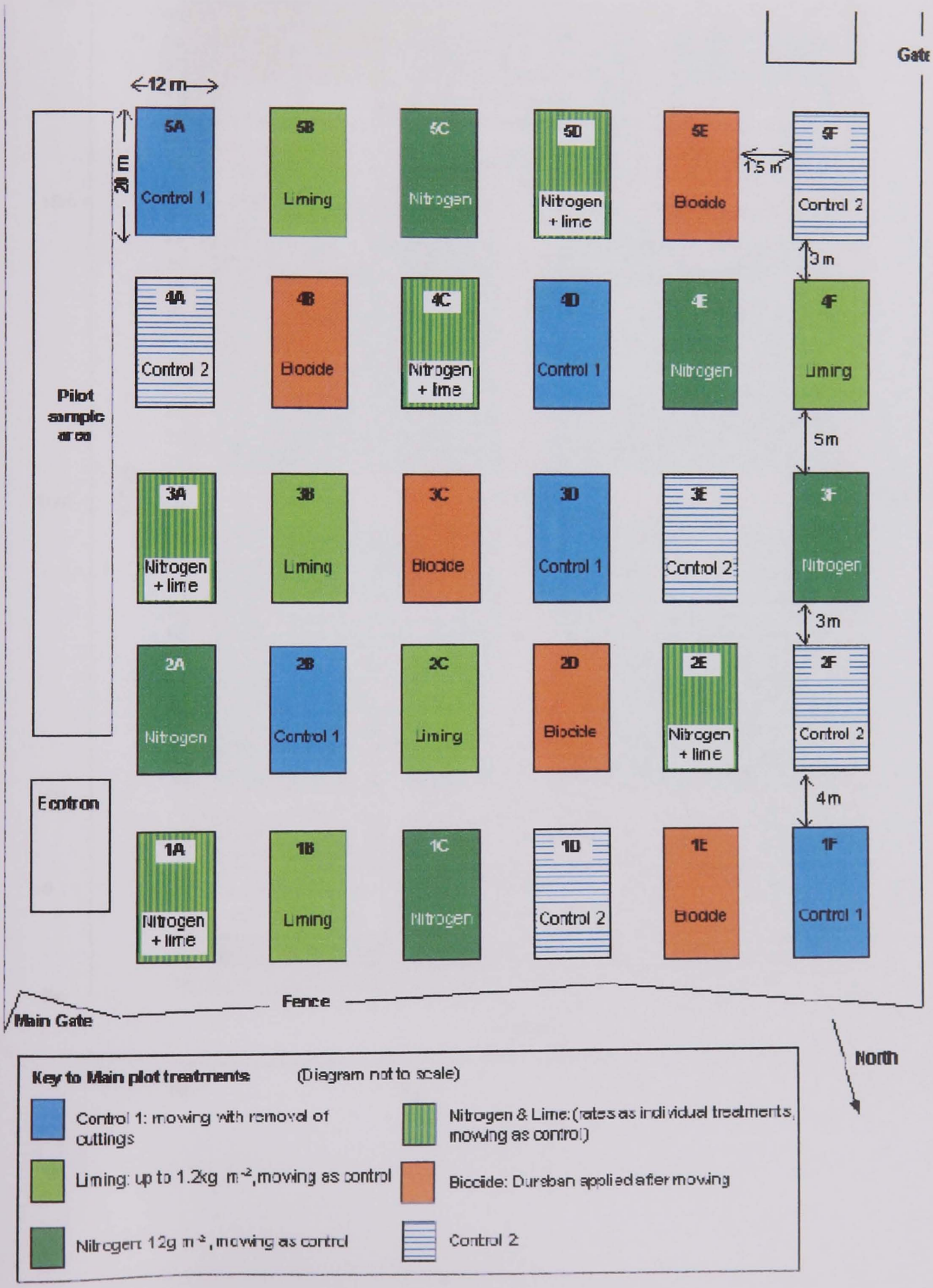
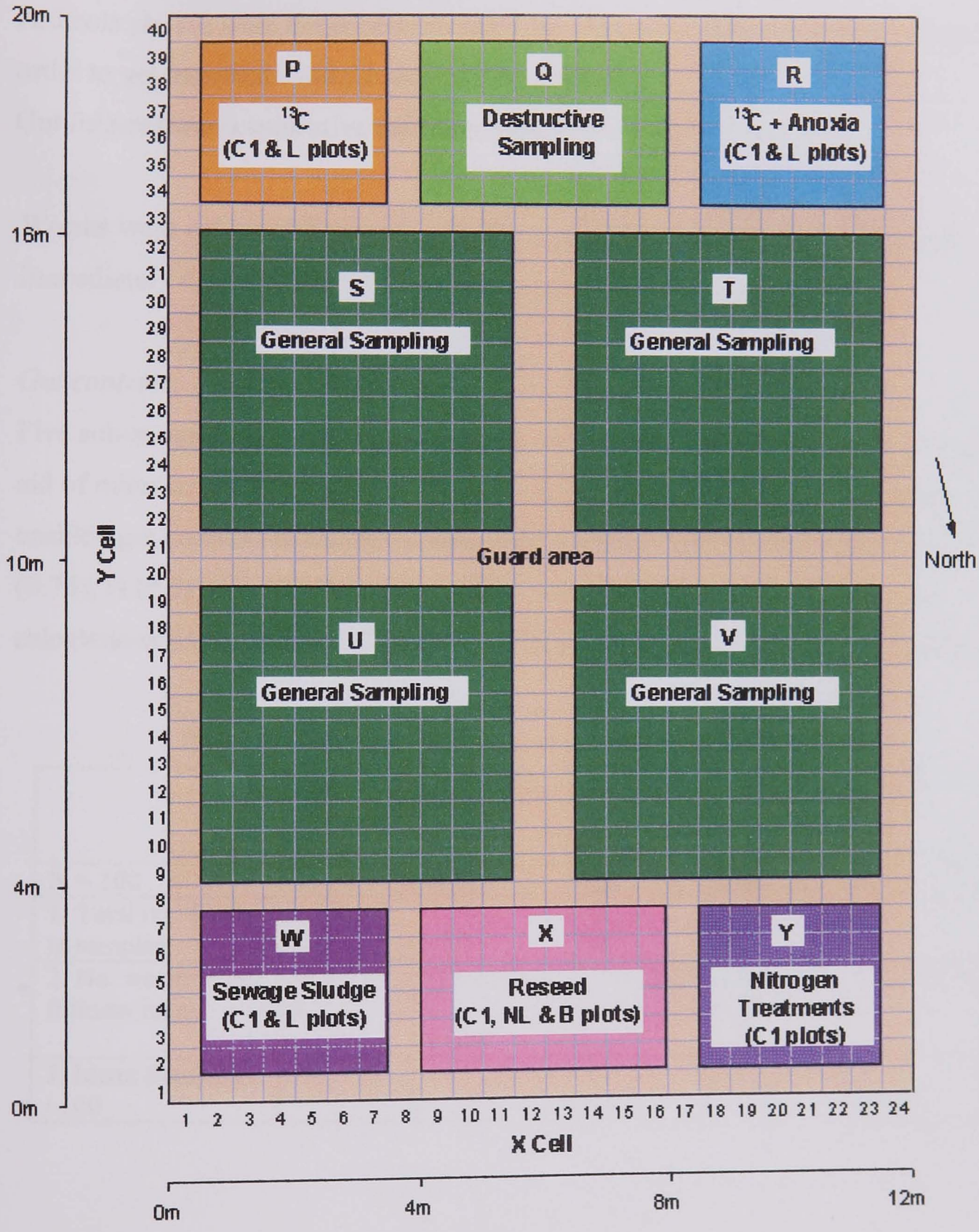
Appendix Ia – Soil Biodiversity site, Sourhope. Map showing orientation of site and treatments allocated to each plot



Appendix Ib Plot size and subplots with treatments allocated



APPENDIX II

Calculation of mean gut contents and proportion of gut to body of *Cognettia sphagnetorum.*

Animals chosen were large/medium sized, as chosen for stable isotope analysis (in order to get maximum weight required for analysis).
Gut fullness was a subjective measure; very few worms had full guts.

Worms were extracted from soil cores from an upland peat soil site. They were immediately decanted into Petri dishes and placed in the fridge overnight.

Gut contents.

Five sub-samples of twenty large/medium worms were randomly chosen without the aid of microscopy. They were examined using a low power microscope, which enabled gut contents to become visible. Gut contents were categorised as full, ¾ (0.75), ½ (0.5) or ¼ (0.25) full, and empty (0). The following table shows the calculations performed to estimate the mean gut fullness of these 100 enchytraeids.

Table 1: Calculation of mean gut contents of *Cognettia sphagnetorum.*

	Gut fullness index				
N = 100	**1**	**0.75**	**0.5**	**0.25**	**0**
1. Total no. worms in samples	4	17	26	25	28
2. No. worms x gut fullness index	(4 x 1) = 4	(17 x 0.75) = 12.75	(26 x 0.5) = 13	(25 x 0.25) = 6.25	(28 x 0) = 0
3. Mean (total (2)) / 100					**0.365**

∴ the mean gut content of each worm is 0.37 or 36.5% (±0.03).

Ratio of body to gut.

Five subsamples of 20 large worms were selected from the Petri dishes.

Each worm was placed on a microscope slide in a drop of water, covered with a cover slip and placed under a high power microscope (x 10 magnification). The central portion of the worm (the plumpest part of the enchytraeid) was identified and measured across the body wall. The gut area was identified and measured from inside the gut wall.

	Body diameter/mm	Gut diameter/mm	Ratio
N =100			
Mean	2.48	1.04	2.39
SE	0.1	0.06	
Range	1.7 – 3.1	1.5 – 0.6	

Calculation of body and gut areas.

A. Body area: $\pi*(1.24^2) = 4.83$

B. Gut area: $\pi*(0.52^2) = 0.85$

C. Actual body area = A – B = 4.68

D. % gut = (B/A)*100 = 17.6%

Mean gut contents of worm = 0.365 and 17.6% of the worm is gut ∴% of δN due to food is (17.6*0.365) = 6.4% and 93.6% of δN value is tissue δN.

APPENDIX III

Chemicals required to make Long Ashton solution.

Salt	Stock solution g/L	Vol. of stock (ml) for 1L strength L.A.
$MgSO_4.7H_2O$	184	1
$NaH_2PO_4.2H_2o$	208	1
Fe EDTA	37.3	1
NH_4NO_3 *	160	1
K_2SO_4	87	1
$CaCl_2.6H_2O$	438	1
Monosodium Complex (see table below)		0.2

Salt	Monosodium complex (g/500ml)
$MnSO_4.4H_2O$	11.15
$CuSO_4.5H_2O$	1.25
$ZnSO_4.7H_2O$	1.45
H_3BO_3	15.5
NaCl	29.25
$Na_2MoO_4.2H_2O$	0.6

*Omitted and replaced with ^{15}N labelled urea.