# Impact of Green Manure on Soil Organisms

**With Emphasis on Microbial Community Composition and Function**

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# Abstract

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Green manure is used as a nitrogen and carbon source in crop production, especially in organic cropping systems without access to farmyard manure. Decomposition and release of nutrients from the green manure is mediated by soil organisms. Consequently, increased understanding of how the biomass, activity and community composition of soil organisms are influenced by this resource input may contribute to improved plant nutrient management.

The influence of different green manure forms and application methods on soil organisms, with emphasis on microbial community composition (described by phospholipid fatty acid analysis) and soil enzyme activity, was investigated in field trials. The green manure systems investigated were various red clover-based green manure forms (biogas slurry, compost, surface mulch and direct incorporation of a red clover ley) and long-term timothy grass amendments. In addition, C routes from decomposing green manure and the living plant roots into soil food webs were investigated using $^{13}C$ labelling, which allowed differentiation between the two C sources.

It was found that red-clover derived biogas slurry and mulch could provide alternatives to direct incorporation, enabling farmers to remove the green manure crop from their crop rotation and to adjust timing of application to crop nutrient requirements. Fresh green manure forms (direct incorporation, mulch) stimulated microbial growth and soil enzyme activity more than processed green manure forms (biogas slurry, compost), and also influenced microbial community composition differently. Long-term green manuring enhanced microbial biomass and soil enzyme activity and stimulated a different microbial community composition compared with other fertilizer regimes. *In situ* $^{13}C$ labelling identified microbial groups specialised on decomposing green manure or rhizosphere C, respectively, and other microbial groups that were strongly linked to both C sources, indicating a general competitive ability. Different soil faunal taxa (Collembola, mites, enchytraeids and earthworms) were more linked to C from the green manure than the roots of the growing crop.

To conclude, green manuring can be a useful management practice for enhancing microbial biomass and soil enzyme activity, but the green manure quality and application method needs to be considered.

*Keywords:* acid phosphatase, arylsulphatase, cropping system, long-term, organic amendments, phospholipid fatty acid (PLFA), protease, soil fauna, stable isotope

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# Contents

# Appendix

**Papers I-IV**

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. Båth, B. & Elfstrand, S. Use of red clover-based green manure in leek cultivation. (Manuscript submitted to *Biological Agriculture and Horticulture)*.

II. Elfstrand, S., Båth, B. & Mårtensson, A. 2007. Influence of various forms of green manure amendments on soil microbial community composition, enzyme activity and nutrient levels in leek. *Applied Soil Ecology* 36, 70-82.

III. Elfstrand, S., Hedlund, K. & Mårtensson, A. 2007. Soil enzyme activities, microbial community composition and function after 47 years of continuous green manuring. *Applied Soil Ecology* 35, 610-621.

IV. Elfstrand, S., Lagerlöf, J., Hedlund, K. & Mårtensson, A. Carbon routes from decomposing plant residues and living roots into soil food webs, studied with pulse labelling of $^{13}C$. (Manuscript).

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Contribution of Sara Elfstrand to the papers included in this thesis was as follows:

I. Performed field sampling, laboratory and data analysis concerning microbial abundance and wrote corresponding part in manuscript.

II. Planned the experiment together with co-authors. Participated in practical field work and performed field sampling, laboratory work and data analysis concerning enzyme activity and PLFAs. Did most of the writing, with contributions by Båth and Mårtensson.

III. Performed laboratory work concerning enzyme activity and substrate utilisation, performed PLFA analysis together with Hedlund. Data analysis and writing were mainly carried out by Elfstrand, with contributions by Mårtensson and Hedlund.

IV. Planned the experiment together with co-authors. Performed field sampling, laboratory work and statistical analysis of microbial and faunal data together with Hedlund and Lagerlöf. Did most of the writing, with contributions by Lagerlöf, Hedlund and Mårtensson.

# Introduction

Green manuring means using a crop primarily as a soil amendment and a nutrient source for subsequent crops. Since they represent a local source of nitrogen (N) and carbon (C), leguminous green manures have the potential to reduce the dependency on external N sources and to increase soil organic matter content. As with other organic amendments, the nutrient release from green manure is difficult to synchronise with plant requirements, but by using forms and application methods other than direct incorporation, the nutrient use efficiency may be enhanced. The decomposition and release of nutrients bound in the green manure is mediated by soil microorganisms and fauna. Due to their key role in decomposition of organic amendments and plant nutrient cycling, it is important to understand how resource inputs regulate the biomass, activity and community composition of soil organisms. The amount and quality of resources entering the soil regulate the response of soil organisms and ultimately decomposition rate and nutrient turnover. However, there is a need for increased knowledge on organism responses to different forms and application methods of organic amendments.

This study formed part of two projects with broader concepts, one focusing on the use of green manure in vegetable production and the other on long-term evaluation of different fertiliser regimes. The aim of the inter-disciplinary project "The ecology of the cropping system - Green manure as a multifunctional 'tool' in vegetable production" was to develop locally adapted vegetable production systems by using green manure for integrated management of plant nutrients, pests and product quality, and also to evaluate the agronomic and economic performance of vegetable production systems. The aim of the long-term fertiliser evaluation experiment was to investigate how continuous inputs of either different mineral N fertilisers or different organic amendments, including green manure, influenced soil organic matter development and crop yields.

## Objectives

This thesis investigates the influence of different green manure forms and other types of organic amendments, on soil microbial communities. Different alternatives to direct incorporation of a green manure ley, *i.e.* surface mulching, biogas residue and compost, and their respective influence on microbial biomass and crop development were investigated during a growing season (Paper I). The response of the microbial community to these different green manure forms was investigated further by studying microbial community composition and soil enzyme activity, and the relationship between soil enzyme activity and nutrient levels in the crop (Paper II). The long-term influence of green manuring on microbial biomass, community composition, soil enzyme activity and substrate utilisation potential was compared with the influence of other organic amendments (Paper III). Finally, a stable isotope technique was used to differentiate between C originating from decomposing green manure residues or from the living roots of

the cash crop. Different soil microbial and faunal communities could then be linked to these two C sources (Paper IV).

The green manure amendments were expected to stimulate microbial activity and growth and potentially cause changes in community composition. The hypotheses were:

- Amendment with fresh green manure stimulates microbial biomass and soil enzyme activity more than processed forms of green manure.
- Differences in quality of the fresh and processed green manure amendments, and also different application methods, stimulate different microbial communities.
- The long-term influence of green manure on soil microbial biomass, composition, substrate utilisation and soil enzyme activity differs from that of other organic amendments. These changes are correlated to changes in soil chemical properties.
- C from green manure and the growing crop is allocated differently into different microbial and faunal groups of the soil food web.

# Background

## Green manure

Green manuring is an old practice of using crops primarily as a soil amendment and a nutrient source for other crops, which lost importance as the use of mineral fertilisers became widespread. However, it is now expected to regain importance as a result of an increased interest in organic food production (Thorup-Kristensen, Magid & Jensen, 2003; Dahlin *et al*., 2005; Cherr, Scholberg, & McSorley, 2006). Leguminous green manure crop species, grown in pure stands or mixed with other legume or nonlegume crops, are commonly used due to their symbiosis with N-fixing bacteria (*Rhizobium*). Green manure from legumes is thus a local N source that has the potential to reduce the dependence on external N inputs. However, the amount of N fixed and the availability of this N for subsequent crops is difficult to predict. The N-fixing capacity of the associated *Rhizobium* varies between crop species, but also as a response to soil properties such as soil texture and nutrient concentration, climatic factors and management practices (Suhr, Thejsen & Thorup-Kristensen, 2005 and references therein). Red clover, which is used as the green manure crop in Papers I, II and IV in this thesis, has been reported to contain between 23 and 197 kg N $ha^{-1}$ in temperate climates (Wetterlind *et al*., 2005), of which on average 80% comes from fixation (Karlsson & Huss-Danell, 2003). The amount and timing of N release from a green manure crop varies with

the quality of the green manure, which is influenced by crop species (Kirchmann & Marstorp, 1991; Gunnarsson, 2003) and plant age (Kirchmann & Bergquist, 1989), soil properties, climatic factors and management (Dahlin *et al*., 2005 and references therein). As for other organic fertilisers, losses through leaching, denitrification and ammonia volatilisation (Janzen & McGinn, 1991; Larsson *et al*. 1998; Torstensson, Aronsson & Bergström, 2006) further complicate the prediction of nutrient delivering capacity and are of importance for water quality. Consequently, a question of great concern is how release of N from green manure can be synchronised with crop demand and how losses, through leaching or gaseous emissions, can be minimised. The N use efficiency of green manures can be improved through manipulation of the chemical composition of fresh or pretreated plant materials and animal manures, and through the timing of incorporation (Båth, 2000; Dahlin *et al*., 2005).

Satisfactory supply of N, the most limiting nutrient for plant growth, is often regarded as a 'keystone' property for green manure use (Cherr, Scholberg, & McSorley, 2006). However, the incentives for using green manure is not solely to supply N, but also the potential delivery of multiple services, which could include pest and weed control (for example through allelopathy), increase in soil organic matter, reduction of soil erosion or agrochemical losses or creation of habitats or resources for beneficial organisms (Suhr, Thejsen & Thorup-Kristensen, 2005). The green manure crop may also be used as a catch crop in order to prevent nitrate leaching in high input systems (Thorup-Kristensen, Magid & Jensen, 2003). A factor that is particularly important in vegetable cropping systems, and especially in vegetable crops with poor root systems, is the effect on soil structure, which enhances root growth and consequently the ability of the crop to utilise soil resources (Stirzaker & White, 1995). Good soil structure also enhances the water holding capacity of the soil (Haynes & Naidu, 1998). In systems where N is less limiting, these functions may be a stronger incentive for green manure use (Cherr, Scholberg, & McSorley, 2006).

The green manure crop can be a crop, or crop-derived fertiliser, that is incorporated directly *in situ* or brought from a distance (mobile green manure). It can be grown during parts of the growing season, throughout the whole year or for several years, depending on cropping system and the purpose of the green manure (Table 1), but the characteristic that all the different forms of green manure have in common is that they are non-commercial crops (Suhr, Thejsen & Thorup-Kristensen, 2005).

Many studies have been carried out on the functions of green manure, but the possibilities are still to a large extent unexplored and there is a need for increased knowledge on the extent of these functions (Thorup-Kristensen, Magid & Jensen, 2003). For example, although use of green manure is routinely credited for its ability to increase soil organic matter (SOM) and microbial biomass pools, the actual extent of such changes depends on management and environment as well as green manure biomass accumulation and there is little information on how these properties are affected by long-term green manuring practices (Cherr, Scholberg & McSorley, 2006).

Table 1. *Some green manuring practices, their possible benefits in cropping systems and those studied in this thesis*

| Green manure form | Practice | Main benefits | Studied in Paper/s |
|---|---|---|---|
| **Whole-year crop** | An annual or perennial ley is established by undersowing in a cereal or is sown after an early crop | Improves soil structure, control weeds, functions as catch crop | I, II |
| **Part-year crop** | The green manure crop is established in the spring or under-sown in a cereal and incorporated in the autumn or early spring next year | Enables fallow periods, optimises biomass production and incorporation time, functions as catch crop | |
| **Intercrop (live mulch)** | The green manure crop is grown together with the cash crop for parts or all of the cropping season | Reduces weed and pest problems | |
| **Mobile green manure** | Harvested crop residues are used in biogas production, composting, silage or fresh in another field | Enables the green manure crop to be moved out of the crop rotation, storage of nutrients and ability to optimise time of application | I - IV |

Sources: Båth, 2000; Ögren, 2003; Suhr, Thejsen & Thorup-Kristensen, 2005; Wetterlind *et al*., 2005.

## Soil organisms and decomposition of organic amendments

The soil food web is composed of a diverse community of organisms, which can be grouped according to body width; microorganisms *i.e.* bacteria (<2 μm) and fungi (2-100 μm), microfauna *i.e.* nematodes, protozoa and rotifers (<100 μm) mesofauna *e.g.* mites, springtails and enchytraeids (0.1-2 mm) and macrofauna *e.g.* earthworms and millipedes (>2 mm) (Swift, Heal & Anderson, 1979). The soil organisms are responsible for decomposition of the organic resources that enter the soil, and thereby the cycling of nutrients bound in these resources.

Bacteria, which are mobile and active in the aqueous pore spaces within the soil, and fungi, which are sessile and intimately linked to the resource base, are the dominating primary decomposers (Swift, Heal & Anderson, 1979). During the decomposition of complex organic matter there is a succession of microorganisms as the substrate is degraded, and the number of organisms capable of degrading the litter decreases with increasing complexity of the substrate (Atlas & Bartha, 1998). In the initial stages, opportunistic species of fungi and bacteria, which utilise soluble substrates such as sugars and amino acids, dominate (Begon, Townsend & Harper, 2006). Fungi are regarded as being more capable than bacteria and actinomycetes, which can constitute 10-30% of bacterial biomass in soil, of degrading the sugars and polysaccharides of the primary resources (Atlas & Bartha, 1998; Lavelle & Spain, 2001), whereas many bacteria prefer N-rich substrates (Miller, 1993). The more complex carbohydrates, such as cellulose and

non-cellulosic polysaccharides, and lignin that remain at later decomposition stages are degraded by extracellular enzymes and fungi are in general better adapted to utilise these resources (Miller, 1993; Lavelle & Spain, 2001). However, each succession is unique and influenced by the quality of the material, its environment and interactions with organisms at other trophic levels.

It has been estimated that about 10% of the energy input from litter is used for growth and respiration by the soil animals, but their influence on decomposition is larger through the indirect effects on soil microbial activity (Killham, 1994). In terrestrial ecosystems, protozoa and microbial feeding nematodes (microfauna) are important grazers of microorganisms, especially in arable soils where bacteria tend to dominate over fungi. As a result of grazing, nutrients temporarily locked up in microbial biomass are mineralised (Clarholm, 1985), although the resulting mineralisation is dependent on the quality of the prey (Griffiths, 1994). Plant growth has been shown to increase by 30-80% due to the presence of protozoa (Griffiths, 1994; Bonkowski *et al*., 2000). This plant growth promoting effect is attributed both to an enhanced nutrient availability and altered root architecture due to the production of hormonal substances by the grazed microbial communities (Bonkowski, 2004).

The meso- and macrofauna initiate and accelerate the decomposition of organic matter through mixing of organic matter within the soil profile, fragmenting the organic matter and thus giving the microorganisms a larger surface area to colonise and by facilitating dispersal of microorganisms (Wardle, 2002). The mesofauna is dominated by microarthropods and enchytraeids. Springtails (Collembola) and mites (Acari) are the most numerous soil microarthropods (Hansson *et al*., 1990). Most microarthropods and enchytraeids are part of the decomposer community, feeding on litter or microorganisms colonising the litter, and only a few feed on live plants (Hansson *et al*., 1990). Soil fauna are dependent on the enzymatic action of microorganisms. Faunal activities enhance mineralisation through the 'external rumen mutualism' (faecal pellets) with microbes (Swift, Heal & Anderson, 1979), whereas larger soil invertebrates such as earthworms (macrofauna) have mutualistic associations with bacteria in their gut cavities (Wardle, 2002).

## Influence of green manure amendments on soil organism communities

In arable soils, C can be supplied to belowground communities both from the growing crop and through organic amendments. The inputs of C resources to soil can be grouped in two main pathways, regulated partly by different mechanisms and utilised by different subsets of the soil organism community: (1) the dead organic matter, which is used as a resource by the decomposer and detritivore community: and (2) the living plant, which is used as a resource by associated microorganisms and root feeders within the vicinity of roots (rhizosphere), also referred to as the grazer community. One implication of using dead rather than live plant material, and a major difference between the decomposer and the grazer

pathways, is that there are no direct consumer-producer feedback mechanisms in the decomposer pathway (Wardle, 2002). The decomposer food chain has therefore been described as being mainly donor controlled (Pimm, 1982), whereas consumers in the grazer pathway directly interact with, and influence, the live plant through release of hormones or other molecular signals (Phillips *et al*., 2003; Bonkowski, 2004), by root feeding or by competing for resources (Wardle, 2002). However, in the longer-term perspective there is an indirect consumer-producer interaction also in the decomposer system, since plant production is influenced by plant nutrient supply from decomposing resources (Wardle, 2002).

The arable crop contributes to both the decomposer and grazer pathways; through litter fall and root turnover and through rhizodeposition, the latter being typically around 20% of the C assimilated by photosynthesis (Nguyen, 2003), but may account for up to 40% of assimilated C (Paterson *et al*., 1997). Thus, substantial energy transfer occurs from annual crops to soil biota during a growing season. In a cropping system where on average 7-15% of net assimilation is allocated to rhizodeposition, including root decay, this amount of C has been found to be twice that derived from roots left at crop harvest (Swinnen *et al*., 1995). As a consequence, microbial and invertebrate numbers are higher in the rhizosphere than in the bulk soil (Foster & Rovira, 1976; Lussenhop & Vogel, 1991).

Organic amendments, such as green manure crops or animal manures, influence soil biota both immediately, through increased food supply, and indirectly, by changes in soil chemical and physical variables (Kautz, López-Fando & Ellmer, 2006). Direct effects of litter inputs include an increase in biomass compared with the biomass supported by living plants alone and changes in the trophic structure and dynamics of the decomposer food webs, whereas indirect effects such as alterations in habitats may also alter community structures by supporting some species and inhibiting others (Moore *et al*., 2004). Green manure or crop residue inputs have been shown to increase the size and activity of soil microbial communities (Bolton *et al*., 1985; Martens, Johanson & Frankenberger, 1992; Kirchner, Wollum & King, 1993; Fauci & Dick, 1994; Kautz, Wirth & Ellmer, 2004; Manici, Caputo & Babini, 2004). Although only few studies exist on the specific effects of catch crops and green manures on soil fauna (Thorup-Kristensen, Magid & Jensen, 2003), these inputs can be expected to increase the abundance of soil fauna. Enchytraeids have been shown to increase in abundance in response to organic matter additions (Lagerlöf, Andrén & Paustian, 1989). Microarthropod (Aagard-Axelsen & Thorup-Kristensen, 2000; Kautz, López-Fando & Ellmer, 2006) and earthworm abundance (Schmidt *et al*., 2001) have been found to be significantly increased as a response to catch crops and green manures.

Organic resources entering the soil can be categorised by their chemical composition and are composed of ‘a complex mixture of compounds including specialised polymers associated with the cell walls of plants (*e.g.* cellulose, hemicellulose, lignin), and fungi (*e.g.* chitin, tannin, melanin), as well as universal biomolecules such as fats, nucleic acids, proteins, other polysaccharides, and the

monomeric constituents of these polymers: sugars, amino acids, nucleotides and nucleiosides, fatty acids and other aliphatics, and aromatics' (Moore *et al*., 2004). Timothy (*Phleum pratense* L.) and red clover (*Trifolium pratense* L.), the green manure crops studied in this thesis, has been reported to contain approximately 10% soluble substances (organic acids, amino acids, simple sugars), 5-10% protein, 25% cellulose, 13-19% hemicelluloses and 13-16% lignin of dry matter (Gunnarsson, 2003) and the rate and extent of decomposition decrease in the following order: soluble C > proteins and hemicelluloses > cellulose > lignin (Begon, Townsend & Harper, 2006). Properties of litter that reflect chemical composition, such as C:N ratio, lignin and nutrient contents, are useful predictors of decomposition of newly deposited litter and of whether net mineralization or net immobilization of nutrients retained in organic matter will occur (Swift, Heal & Anderson, 1979; Moore *et al*., 2004). As decomposition of the litter proceeds, the diversity of materials is reduced as labile constituents are consumed and recalcitrant constituents remain (Moore *et al*., 2004). Similarly, pre-treatment of fresh plant residues before use as green manure alters the availability of the residual organic material for soil organisms and consequently their decomposition and nutrient cycling rates, and possibly their influence on soil microbial community composition. Changes in substrate quality can affect the relative abundance of bacteria and fungi, where materials of high C:N ratios favour colonisation by fungi, while more labile materials with low C:N ratios favour bacteria, and with these changes come shifts in the abundances of the consumers of bacteria and fungi (Moore *et al*., 2004). Soil microfauna respond indirectly to litter quality via the microflora that they consume, whereas meso- and macrofauna to a greater extent ingest litter directly and have therefore been suggested to be more strongly influenced by differences in litter quality (Wardle, 2002).

## Soil biota and soil fertility

The quality or fertility of soils is often discussed in relation to the sustainability of agricultural practices. Soil quality has been defined as 'the continued capacity of soil to function as a vital living system, within ecosystem and land use boundaries, to sustain biological productivity, promote the quality of air and water environments, and maintain plant, animal and human health' (Doran & Parkin, 1994). Soil fertility is an integral part of soil quality focusing more on the productivity of the soil, and has been defined by Persson & Otabbong (1994) as 'the sustainable capacity of a soil to produce good yields of high quality on the basis of chemical, physical and biological quality factors'. Different components of soil fertility that are central for crop production are *e.g.* water retention, the capacity of the soil to anchorage plants, suppress weeds, diseases and pests and to supply nutrients to crops (van Bruggen & Semenov, 2000; Stockdale, 2002). The ability to deliver nutrients depends on the size of the nutrient pools in the soil, the processes (and their rates) which transform and transfer nutrients between these pools and the potential for losses of nutrients (Stockdale, 2002). Biological indicators are often used to assess soil quality or fertility, due to their central role in nutrient transformations and disease suppression and a rapid response to changes in management practices (Powlson, 1994; van Bruggen & Semenov,

2000). Soil biological properties that have been proposed as indicators are general biological and biochemical properties such as microbial biomass (C) (Doran & Parkin, 1994; Brookes, 1995), ecophysiological quotients (Anderson & Domsch, 1993), specific biochemical properties such as activity of hydrolytic soil enzymes related to C, N and P cycles (Dick, 1997) and composition of the microbial community (Verstraete & Mertens, 2004) and soil fauna (Killham & Staddon, 2002). However, there is a problem in selecting a few or single indicators of soil fertility due to the multi-factorial nature of this concept (Burns, 1982; Doube & Schmidt, 1997).

Due to the key role of soil biota in nutrient cycling and pest control, it has been argued that the build-up of a large and active soil microbial biomass is critically important for sustaining the fertility of soils, especially in organic farming systems (Tu, Ristaino & Hu, 2006). However, there is a lack of understanding of how microbial community composition is related to diversity, and how these are linked to the function of microbial communities (Nannipieri *et al*., 2003). Partly, this can be ascribed to difficulties in studying organism diversity, species identity or their functions in soil. The ability to remain in a dormant state under less favourable conditions and to become active as conditions improve is a general feature of soil organisms, which means that they may be present, but not active or detectable in soil. There is some experimental evidence that changes in decomposer communities have implications for litter decomposition. For example, Cookson, Beare & Wilson (1998) showed that continuous use of a particular residue altered microbial community composition and litter decomposition rates, with significantly faster decomposition of wheat (*Triticum aestivum* L.) residues compared with barley (*Hordeum vulgare* L.) and white lupin (*Lupinus albus* L.) residues in soil previously amended with wheat residues. But most studies in soil suggest that there is a considerable redundancy among decomposer organisms (Moore *et al*., 2004), which can explain why changes in microbial communities can occur without associated changes in decomposition (Degens, 1998). However, functions such as stability or resilience of ecosystems may be influenced by the complexity of the community (Scheu & Setälä, 2002).

To summarise, the complex microbial and faunal communities in soil are fundamental for many ecosystem processes on which crop production depends, although the relationship between soil biodiversity or community composition and biological processes is not well understood. Green manure amendments can be expected to increase the biomass and activity of decomposer organisms, whereas the influence on decomposer community composition and function is more uncertain. Against this known background, we wanted to assess the influence of different green manure cropping systems on the composition and function of decomposer communities, with emphasis on microbial community composition, soil enzyme activity and substrate utilisation profiles, as well as allocation of C from green manure amendments and the growing crop into decomposer and grazer communities in soil.

# Materials and methods

## Field experiments

The field experiments in this thesis were performed at two different sites, Krusenberg and Ultuna, both located outside Uppsala, (59º49´N, 17º43´E) in south-central Sweden. Krusenberg is located 10 km south of Uppsala and has been one of the Swedish University of Agricultural Sciences experimental field stations since 1996. The Ultuna Long-term Soil Organic Matter Experiment is located close to the Ultuna Campus, south of central Uppsala. Some properties of the soils at the two sites and of the green manure systems are presented in Table 2, which shows a range with the lowest to the highest value in the selected treatments of the Ultuna long-term experiment. The nutrient status and pH of the Krusenberg soil were generally in the lower or middle part of the range of the Ultuna long-term experiment.

Table 2. *Green manure crops, application methods and soil characteristics at the two field sites. Soil chemical properties at Ultuna are presented as the range lowest- highest*

| **Site** | **Krusenberg** | **Ultuna** |
|---|---|---|
| **Green manure** | Red clover (*Trifolium pratense* L.) | Timothy (*Phleum pratense* L.)[1] |
| **Application methods** | Direct incorporation, mulching, compost, biogas slurry or incorporation of fresh shoots and roots in spring | Incorporation of dried shoot residues in the autumn |
| **Subsequent crop** | Leek (*Allium porrum* L.) | Maize (*Zea mays* L.) |
| **$C_{tot}$ (%)**[2] | 1.4 | 1.2-2.1 |
| **$N_{tot}$ (%)**[2] | 0.13 | 0.11-0.19 |
| **$P_{tot}$ (%)**[3] | 0.06 | 0.08-0.11 |
| **$S_{tot}$ (%)**[3] | 0.02 | 0.02-0.04 |
| **pH ($H_2O$)** | 6.1 | 6.2-6.9 |
| **Clay (%)** | 34 | 37 |
| **Silt (%)** | 66 | 41 |
| **Sand (%)** | 0 | 23 |

[1]In 2003 the green manure crop was timothy (*Phleum pratense* L.), but this varied during the experimental period

[2]LECO

[3]ICP, $HNO_3$-extracted, except $P_{tot}$ at Krusenberg which was HCl-extracted

There follows a short description of the design of the experiments included in this thesis. For more detailed information about experimental and sampling procedures, the reader is referred to Papers I-IV.

### *Green manure experiment at Krusenberg (Papers I and II)*

In this study, different forms of mobile green manure, harvested on fields separate from those in this trial, were tested as an alternative to direct incorporation of a red clover ley on-site. The field experiment was performed at Krusenberg in 2004 and included the following treatments:

(1) Direct incorporation of a red clover ley in spring.
(2) Mulching (surface placement) of fresh red clover shoot residues.
(3) Incorporation of composted red clover.
(4) Incorporation of biogas slurry from fermented red clover.
(5) Incorporation of mineral fertiliser.
(6) Unfertilised.

The three mobile green manure forms (mulch, compost and biogas slurry) were applied as equal amounts of total N (~ 217 kg N $ha^{-1}$), total C (~ 3 600 kg C $ha^{-1}$) and available N as in the shoots and roots of the red clover crop in direct incorporation system. This resulted in three separate treatments of each of the mobile green manure forms. In the mulch treatment, equal N and C amounts could be supplied in one treatment and an extra treatment based on a practically appropriate mulch application was therefore included. Soil was sampled for analysis of microbial properties in treatments receiving equal amounts of C and N on four occasions during the cropping season. All treatments had four replicates (plot sizes 12 $m^2$), randomly placed within blocks.

*Long-term experiment at Ultuna (Paper III)*

The purpose of this study was to compare the long-term influence of green manure on soil microbial properties with that of other organic amendments and to investigate possible correlations between soil microbial and soil chemical properties. Sampling was carried out on two occasions (June and September 2003) in the Ultuna Long-term Soil Organic Matter Experiment which was first established in 1956 in order to study the effects of different N fertilisers and manures on crop yields and soil organic matter. Five treatments were sampled:

(1) Unfertilised.
(2) Mineral N ($Ca(NO_3)_2$).
(3) Green manure.
(4) Farmyard manure (FYM).
(5) Sawdust (coniferous) + mineral N ($Ca(NO_3)_2$).

80 kg N $ha^{-1}$ was added annually as calcium nitrate in the mineral N and the sawdust treatments. The different organic amendments have been added at equal C rates, corresponding to 4 t C $ha^{-1}$, in alternate years, with the latest amendment being applied 19-22 months prior to sampling. All treatments included in the present study had been fertilised with 20 kg P $ha^{-1}$ as superphosphate and 38 kg K $ha^{-1}$ as potassium chloride every year. All treatments had four replicates, randomly placed in blocks, with plot sizes 2 by 2 $m^2$. More information about the experiment can be found in Kirchmann, Persson & Carlgren (1994) and Kirchmann *et al*. (2004).

Fig. 1. *The field trials at Krusenberg (to the left) and Ultuna (to the right).*

### *Stable isotope study at Krusenberg (Paper IV)*

This experiment was set up to study the allocation of C from decomposing green manure residues and the growing crop into different microorganisms and fauna in the soil food web. This was achieved by $^{13}C$-labelling of the green manure material and the growing crop. The experiment was carried out at Krusenberg in 2004 and consisted of three treatments:

(1) $^{13}C$-labelled green manure and unlabelled leek.
(2) $^{13}C$-labelled leek and unlabelled green manure.
(3) Unlabelled green manure and leek.

Red clover green manure was grown and labelled with $^{13}C$ in a greenhouse before incorporation in the field prior to planting of the leek crop. The leek crop was labelled by repeated pulse-labelling with $^{13}C$-enriched $CO_2$ on three occasions during the cropping season. Sampling was done on four (for microbial analysis) and three (for soil fauna analysis) occasions during the cropping season. All treatments had four replicates, laid out in a randomized block design. Plot sizes were 0.8 by 0.8 $m^2$.

## Characterisation of soil organism communities

The methods by which organisms or their functions in soil are studied will define what part of the community that is described and influence interpretations of the results. The methods used to characterize soil microbial and faunal communities that have been utilized in this thesis and some of their benefits and constraints will be presented here. For a detailed description of the analytical procedures, the readers are referred to the specific papers (I-IV).

### *Phospholipid fatty acid analysis for determining microbial biomass and community composition*

Phospholipids are major components of the membranes of all living cells (Tunlid & White, 1992). They consist of a polar, hydrophilic end and long, non-polar

hydrophobic fatty acid chains, and these properties cause them to form a bilayer in cell membranes. Phospholipid fatty acids (PLFAs) can be used as indicators of microbial biomass, to classify microbial groups and evaluate their physiological conditions (Tunlid & White, 1992). PLFAs are valuable biomarkers since they make up a relatively constant proportion of the biomass of organisms and their persistence in soil after cell death is short (White *et al*., 1979). The total PLFA content correlates well with other methods for estimating microbial biomass such as substrate induced respiration (SIR) and ATP (Klamer & Bååth, 2004), but contrasting results has also been reported (Böhme, Langer & Böhme, 2005). It is also important to note that if no conversion factors are used, the estimates of total microbial biomass and biomass of different microbial groups (Paper I, II and III) should be employed only as relative measures of biomass (Olsson *et al*., 1999; Klamer & Bååth, 2004).

PLFA analysis of microbial communities provides a cultivation-independent, broad-scale approach to monitor changes in community composition (Papers I-IV) since different groups of microorganisms have different signature PLFAs in their membranes (Tunlid & White, 1992). PLFA analysis is particularly useful to indicate shifts in microbial community composition, since it provides both a qualitative and a quantitative measure. However, the PLFA composition of microbial cell membranes varies not only due to taxonomy, but also due to stress factors such as toxicity, exposure to solvents or starvation (White, Stair & Ringelberg, 1996). Thus, changes in PLFA profiles can depend on both taxonomic and physiological changes (membrane alterations) within a population. However, as pointed out by Frostegård (1995), in both cases changes in PLFA composition indicate that the microbial community has been affected. Signature fatty acids overlap between organism groups, *i.e.* various fatty acids can be regarded as signature fatty acids for the same taxonomic group, which is another constraint that restricts the definitive interpretation of shifts in community composition (White, Stair & Ringelberg, 1996). However, analysis of neutral lipid fatty acids (NLFA) can be included to improve the resolution, since these are only produced by fungi (Bååth, 2003). For example, the PLFA and NLFA 16:1ω5 are both markers for arbuscular mycorrhizal (AM) fungi, but since PLFA 16:1ω5 also occurs in bacteria, this fatty acid does not always give a reliable estimate of AM fungi in environmental samples (Olsson *et al*., 1999). The NLFA might be a better marker for AM fungi in natural systems where a large contribution of bacterial PLFA 16:1ω5 can be expected (Hedlund, 2002).

Although the PLFA analysis has mainly been used to study microbial communities, PLFAs have also been used as indicators of soil microfauna (Frostegård, Petersen & Bååth, 1997). In recent reports, fatty acid composition has been used to determine the feeding preferences of soil microarthropods, based on the observation of trophic transfer of fatty acids between microorganisms and grazing soil fauna (Ruess *et al*., 2005; Chamberlain *et al*., 2005).

The PLFAs identified in this work and the organism groups in which they can be found are described in Table 3. PLFA nomenclature follows the pattern of A:BωC where ‘A’ indicates the total number of C atoms in the fatty acid chain,

'B' the number of double bonds and 'ωC' the position of the double bond (*i.e.* 'C' is the number of the C atom, counted from the aliphatic 'ω' end, where the double bond is located). This is followed by 'c' for cis- or 't' for trans- configuration. The prefixes 'i' and 'a' stand for iso- and anteiso- branching and 'br' for methyl branching at an unknown position. Mid-chain branching is denoted by 'me', and cyclopropane fatty acids are designated as 'cy'. For example, 18:2ω6,9 has 18 C atoms and two double bonds which are located on the $6^{th}$ and $9^{th}$ C from the aliphatic end.

Table 3. *Signature lipids for different microbial groups as used in Papers II-IV*

| **Microbial group** | **Signature fatty acid** |
|---|---|
| Gram-negative bacteria | 16:1ω5, 16:1ω7, 18:1ω7, *cy*17:0, *cy*19:0 |
| Gram-positive bacteria | *i*15:0, *a*15:0, *i*16:0, *a*16:0, *i*17:0, 10Me16:0 |
| Fungi | 18:2ω6,9, 18:1ω9 |
| Actinomycetes | 10Me18:0 |
| AM fungi | 16:1ω5, NLFA 16:1ω5 |

Sources: Tunlid & White, 1992; Frostegård *et al*., 1993; Frostegård & Bååth, 1996; Olsson, 1999

### *Soil enzyme activity*

Soil enzymes are produced by microorganisms, plants and soil animals (Dick, 1997). There are two categories of enzymes that are involved in the cycling of macronutrients (N, P, S) – intracellular enzymes that oxidise or reduce inorganic N and S and extracellular enzymes that recover N, P and S from organic substrates. The extracellular enzymes are thus those that catalyse the rate limiting steps of decomposition and nutrient cycling (Sinsabaugh, 1994).

In the case of N, the extracellular enzymes include chitinases, proteases, peptidases, amidases, deaminases and nucleases. For P they consist of phosphomonoesterases (mainly phytases), phosphodiesterases and phospholipases, and for S sulphatases (Sinsabaugh, 1994). In the work presented in this thesis, the activity of proteases (Kandeler, 1996), acid phosphatase (Sjöqvist, 1993) and arylsulphatase (Tabatabai, 1994) was determined (Papers II and III).

Proteases take part in the hydrolysis of proteins to ammonium ions ($NH_4^+$) and thus have a key function in N cycling in soil, since one third of all N in soil can be found in proteins (O´Sullivan *et al*., 1991). Proteolytic activity has been shown to be induced by exposure of microorganisms to protein and C, N, or S starvation (Sims & Wander, 2002 and references therein).

Acid phosphatase belongs to the phosphomonoesterase hydrolases, which is the most studied phosphatase group (Turner & Haygarth, 2005). Phosphatases hydrolyse and mobilise inorganic phosphorous (P) from monoester soil organic phosphates, which have been estimated to account for 30-80% of total P in agricultural soils (Dakora & Phillips, 2002). Phosphatases have two pH optima; pH 6.5 (acid phosphatases) and pH 10-12 (alkaline phosphatases) (Speir & Ross, 1978). The acid phosphatase assay was chosen over the alkaline phosphatase assay

in the studies presented in this thesis, since the surveyed soils were weakly acidic and acid phosphatase has been shown to dominate under such conditions (Eivazi & Tabatabai, 1977). P-limited conditions (Olander & Vitousek, 2000) and organic P compounds (Gressel & McColl, 1997) can stimulate phosphatase activity, whereas orthophosphate inhibits the activity of phosphatases, either through inhibition of its activity or its synthesis (Olander & Vitousek, 2000; Colvan, Syers & O'Donnell, 2001).

Up to 95% of the total S in soils may be in organic form (Scherer, 2001). Traditionally, organic sulphur (S) speciation in soils has been differentiated by wet chemical extraction, but the accuracy of this methodology has been questioned (Solomon *et al*., 2005). Organic S has been divided into sulphate esters and ethers, representing 30-70% of total organic S in soils (Tabatabai & Bremner, 1972), and C-bonded S, *e.g.* amino acids, accounting for 10-20% of total organic S in soil (Havlin *et al*., 1999). Mineralisation of S from these two sources has been referred to as biochemical and biological mineralisation, respectively (McGill & Cole, 1981). The biochemical mineralisation, *i.e.* the release of sulphate from sulphate esters is mediated by several different sulphatases in nature (Tabatabai & Bremner, 1970), of which arylsulphatase (arylsulphatase sulphohydrolyse, EC 3.1.6.1) is the most extensively studied. The activity of sulphatases is regulated by the availability of $SO_4^{2-}$ and S-containing amino acids (Scherer, 2001), and also by soluble orthophosphate in soil (Al-Khafaji & Tabatabai, 1979), whereas the biological mineralisation is a response to microbial demand for C (McGill & Cole, 1981).

The substrate-enzyme feedback mechanisms regulating soil enzyme activity could be used to indicate nutrient limitations (Sinsabaugh *et al*., 1993), since high enzyme activities could indicate substrate deficiency while low activities indicate synthesis suppression when substrate is available in high concentrations (Dilly & Nannipieri, 1998). However, several factors confound such interpretation in a complex environment such as soil. In addition to the substrate-specific regulation of extracellular enzyme activity, *i.e.* interactions with the substrate (litter, detritus or minerals) through inhibition, adsorption, stabilisation and humification, the activity is also regulated by site-related factors such as temperature, moisture and nutrient availability, directly and indirectly through control of microbial activity and consequently enzyme synthesis (Sinsabaugh, 1994). For example, even if a resource is limiting and microbes would benefit from producing enzymes to obtain it, enzyme synthesis can be constrained by the availability of C and N required for enzyme synthesis (Allison & Vitousek, 2005). Regulating mechanisms and short-term changes in microbial enzyme synthesis in soil may also be overshadowed by the activity of stabilised extracellular enzymes (Sinsabaugh, 1994) *i.e.* the adsorption of enzymes to organic or mineral particles, which stabilises their activity (Burns, 1982). Another problem, as pointed out by Olander & Vitousek (2000), is that if background levels of an end-product are high, enzyme activity will already be repressed and further additions of the nutrient will not result in additional suppression of enzyme activity. Additionally, an increase in enzyme activity can reflect both induction due to altered substrate availability and microbial growth (Sims & Wander, 2002). The nature of the standard enzyme

assays further complicate interpretation, since the assay is generally carried out with an artificial substrate and under optimised reaction conditions (Whalen & Warman, 1996; Dilly & Nannipieri, 1998). As a result, the potential enzyme activity is estimated and not the actual activity in the field.

Consequently, one challenge for increased understanding of soil enzyme activity dynamics in soil has long been to understand the extent to which the measured activity reflects the activity of proliferating microorganisms or activity that is not related to microbial growth, either substrate-induced or derived from abiotic, extracellular enzymes (Burns, 1982; Sinsabaugh, 1994; Dilly & Nannipieri, 1998; Sims & Wander, 2002). The combined approach of analyses of functional gene expression and classical soil enzyme assays or flux rates may further resolve this issue (Emmerling *et al*., 2002).

### *Substrate utilisation potential*

Substrate utilisation potential was determined photometrically over a period of five days using the commercial Biolog EcoPlate (Paper III). Biolog, originally a bacterial identification system based on utilisation patterns of 95 single C sources, was first used by Garland & Mills (1991) to assess the functional diversity of microorganisms from environmental samples (Insam & Rangger, 1997). A soil inoculum is added to the wells of a 96-well microtitre plate and the response of the microbial community to the different substrates is measured as a colour development. The assay indicates the potential activity of the community, *i.e.* the ability of the microbial community to adapt metabolism and/or the relative composition and size of the community to the specific microclimate and available substrates (Preston-Mafham, Boddy & Randerson, 2002). Thus, the Biolog approach resembles that of other culturing techniques, with the drawback of selecting for a fast growing subset of the initial microbial community (Smalla *et al*., 1998). The EcoPlate, which contains three replicate sets of 31 substrates in one plate, was developed to include more ecologically relevant substrates and more replicates (Insam & Rangger, 1997), but with the same discriminatory power (Classen, 2003). Although not suitable for community characterisation, the Biolog assay is useful for comparison of soil communities from different sites or management histories, since it provides a potential insight into the functional ability of the community (Preston-Mafham, Boddy & Randerson, 2002).

### *Stable isotope techniques in soil ecology - tracing C in the soil food web*

Stable isotope techniques have recently shed light on several key areas of interest for current research within soil ecology; namely linking different groups of soil organisms to particular soil processes (Hanson *et al*., 1999; Pelz *et al*., 2001), quantifying the use of soil and plant-derived C by soil organisms (Albers, Schaefer & Scheu, 2006; Kramer & Gleixner, 2006; Leake *et al*., 2006; Williams, Myrold & Bottomley, 2006) and identifying food sources and trophic positions of organisms, as well as food chain lengths, within soil food webs (Schmidt *et al*., 2004; Schneider *et al*., 2004; Chahartaghi *et al*., 2005; Albers, Schaefer & Scheu, 2006).

The stable isotope approach within soil ecology is based both on the naturally occurring stable isotopes and their relative abundance and the use of tracers that are either enriched or depleted with the heavier isotope relative to the natural abundance. Within microbial ecology, recent advances in linking identity and function by this approach can mainly be attributed to the use of added tracer compounds (but see Kramer & Gleixner, 2006), for example through pulse-labelling (Fig. 2), in combination with compound-specific isotope analysis, which allows the analysis of isotopic ratios of specific compounds such as biological markers (Boschker & Middelburg, 2002). In the case of PLFAs, this is carried out by gas chromatography-continuous flow-combustion-isotope ratio mass spectrometry (GC-cf-IRMS) (Paper IV) and can be performed on samples containing nanogram quantities of C in the compound of interest (Staddon, 2004). The combined approach of biomarkers and stable isotope analysis provides the possibility to directly link microbial identity (biomarker), biomass (concentration of the biomarker) and activity or function (isotope assimilation) (Boschker & Middelburg, 2002).

Analyses of isotopic ratios of soil fauna have mainly been performed by conventional isotope ratio mass spectrometry (IRMS) on whole animals (Paper IV), but the use of compound-specific stable isotope analysis may further develop the use of stable isotope techniques in soil faunal ecology, since the problem with too small sample sizes of small fauna can then be avoided (Evans *et al*., 2003). The abundance of the stable isotopes $^{15}N$ and $^{13}C$ reflects the isotopic signature of the diet, but $^{15}N$ also functions as a trophic-level indicator since it is enriched in the soil food web (Ponsard & Arditi, 2000).

*Fig. 2.* Pulse labelling of leek with $^{13}C$-enriched carbon dioxide (treatment B) in the experiment at Krusenberg.

### *Summary of the methods used*

The methods used to characterise soil organisms and functions in the work presented in this thesis are summarised in Table 4.

Table 4. *Summary of methods used in this thesis and the part of the soil organism community or function described*

| Method | Community | Function | Paper | | | |
|---|---|---|---|---|---|---|
| | | | I | II | III | IV |
| PLFA analysis | Total viable microbial biomass or | | √ | | √ | |
| | community composition | | | √ | √ | √ |
| $^{13}$C-labelling, GC-cf-IRMS (PLFA) | Active, viable microbial biomass | C assimilation | | | | √ |
| Biolog EcoPlate | Culturable bacteria | Substrate utilisation potential | | | √ | |
| Enzyme assays | | Mineralisation potential | | √ | √ | |
| Soil extraction, $^{13}$C labelling, IRMS | Soil fauna | C assimilation | | | | √ |

PLFA = phospholipid fatty acid analysis
GC-cf-IRMS = gas chromatography-continuous flow-combustion-isotope ratio mass spectrometry
IRMS = isotope ratio mass spectrometry

## Characterisation of soil, crop and organic amendments

Structural carbohydrate and lignin contents were determined in the different green manure forms and other organic amendments (Papers II and III) by sequential chemical digestion as described by Goering & van Soest (1970).

Total C concentrations in the green manure and other organic amendments, as well as in soil and crop samples, were determined by combustion on a LECO analyser (LECO, CNS 2000) (Papers I-III). Total N in the red clover, timothy grass, FYM, sawdust, soil and crop samples was also determined by combustion on a LECO analyser, whereas total N in the red clover-based biogas slurry and compost was determined by Kjeldahl analysis. Total concentrations of P and S in all organic amendments, soil and crop samples were determined on an inductively coupled plasma spectrometer (Perkin Elmer Optima 3000 DV) after nitric acid digestion (Papers I-III). Inorganic N ($NO_3^-$ and $NH_4^+$) in the red-clover derived compost and slurry (Paper II) was determined by Kjeldahl analysis after extraction with sulphuric acid. Water soluble nitrate, phosphate and sulphate concentrations were determined by ion chromatography (Dionex) in the different green manure forms in Paper II. Reduced S forms in the red clover-derived biogas slurry were determined as described by Canfield *et al*. (1986) (Paper II).

The phospholipid fatty acid (PLFA) profile of the different green manure forms in Paper II was characterised according to Frostegård & Bååth (1996). Soil pH was determined in water suspension at a soil/water ratio of 1:2.5 (Papers I-IV).

## Statistical methods

Treatment or time (sampling occasion) effects were evaluated using analysis of variance (ANOVA), followed by a post-hoc test (Tukey's or t-test) when

significant differences were found ($p < 0.05$). Transformation of data was performed when needed to fulfil assumptions on normality and equal variances.

Data on microbial communities (PLFA) (Papers II, III and IV), substrate utilisation potential (Paper III) and correlations between soil enzyme activity and crop nutrient concentration (Paper II) were analysed by Principal Components Analysis (PCA). PCA is a multivariate method that shows differences and similarities between samples by reducing a multivariate data set into a small number of principal components that in turn account for a diminishing proportion of the variation in the data (Eriksson *et al*., 2001). PLFA data were normalised by dividing the concentration of individual fatty acids by the total PLFA concentration in a sample. Similarly, Biolog well-absorbance values were normalised by dividing the value of an individual well by the average well colour development of a sample before subjecting the substrate utilisation potential data to multivariate analysis. PCA on microbial community data was followed by ANOVA of the principal component (PC) scores to describe differences in soil community composition between treatments. Partial least square (PLS) analysis, which in contrast to PCA includes descriptors (Eriksson *et al*., 2001), in this case soil chemical data, was used to investigate the relationship between soil microbial community composition and soil chemical parameters.

Pearson correlation was used to analyse any linear relationships between soil enzyme activity and crop nutrient concentrations (Paper II) and between soil microbial and chemical properties (Paper III).

All statistical analyses were performed using Minitab Release 14 (2000) or Simca P 10.5 (Umetrics).

# Results and discussion

## Influence of green manure amendments on soil microbial communities

### *Fresh and processed forms of red clover-based green manure*

When amendments with different green manure forms and application methods were investigated in a leek cropping system, it was found that the leek crop had a higher initial growth rate in the direct incorporation treatment, and to some extent also in the compost (equal N) amended treatment, than in the other treatments (Fig. 2 in Paper I). This was despite the soil mineral N content being significantly lower ($p < 0.001$) than in the other, still unamended treatments at planting in June (Fig. 3). It is likely that a positive effect of the red clover ley and compost on soil structure stimulated root development and initial crop growth in these treatments. We also postulated that the timing of incorporation and the subsequent immobilisation-mineralisation pattern in the direct incorporation treatment was

better adjusted to the nutritional requirements of the leek crop, and that this could have contributed to the better growth rate in this treatment compared with the others.

This assumption was based on the observation that the lower soil mineral N content in the direct incorporation treatment at planting, two weeks after incorporation of the red clover ley, coincided with a higher microbial biomass ($p < 0.001$) in the direct incorporation treatment than in the unamended soil (Fig. 3). Thus, it is possible that N and other nutrients had been immobilised in proliferating microbial biomass in the direct incorporation treatment. However, microbial biomass is a labile source of N in soil (Myrold, 1987; Bonde *et al*., 1988) and studies with isotope labelling of the microbial biomass have found that a significant proportion of the nutrients present in microbial tissues are rapidly taken up by plants (Lethbridge & Davidson, 1983; Schnürer & Rosswall, 1987). Consequently, as the microbial biomass declined in the direct incorporation treatment, immobilised nutrients were likely to be readily mineralised and available for uptake by the leek crop.

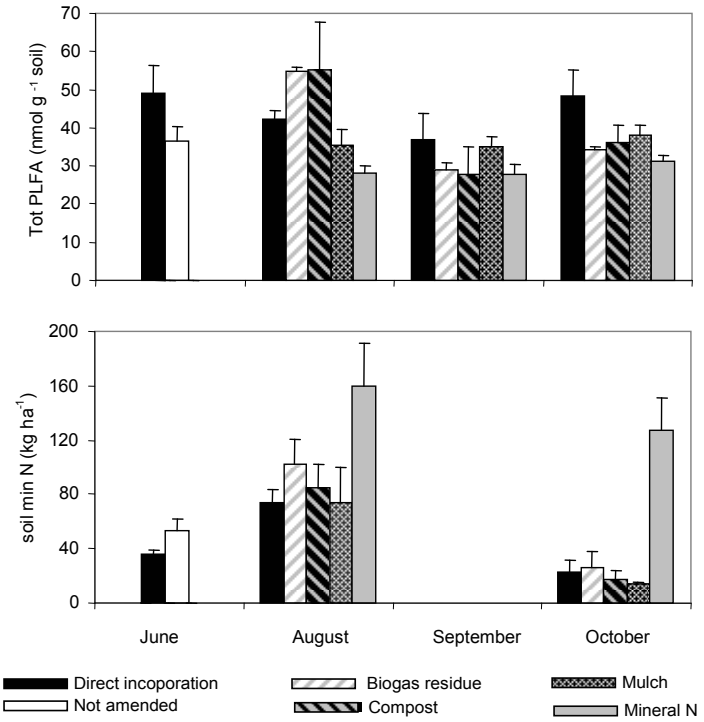


*Fig. 3.* Soil microbial biomass (total PLFA) and mineral N content at 0-30 cm depth in the Krusenberg field experiment. Mean values (n=4 for direct incorporation treatment, n=16 for unamended plots in June) of treatments amended with equal amounts of N and standard deviations. Soil min N was not sampled in September.

It is also possible that the low soil N level on the first sampling occasion was due to temporal separation of C and N mineralisation. Red clover residues with a similar C/N ratio to the red clover used for direct incorporation have been reported to initially induce a short period (4 d) of high C mineralisation along with immobilisation of soil mineral N by microorganisms, followed by a period of degradation of N-rich compounds and net N mineralisation (Gunnarsson, 2003). Those results are not directly comparable with this study, since the mineralisation was studied at 20 ºC and the time course was shorter than in this experiment (2 weeks). However, the low content of mineral N in soil in the direct incorporation treatment at planting could indicate that N-rich compounds in the red clover residues had not yet been mineralised to any large extent. Indeed, the microbial community had a relatively higher abundance of fungi in the direct incorporation treatment (Fig. 4) compared with the unamended soil on this sampling occasion ($p<0.001$), which could be indicative of carbohydrate decomposition as fungi can proliferate initially due to the presence of soluble carbohydrates (Lavelle & Spain, 2001).

Despite the initially rapid leek growth in the direct incorporation and compost treatments, these treatments were not able to promote higher crop growth during the second half of the growing season and at harvest there were no differences in final leek yield at equal N rates (Fig. 1 in Paper I). However, leek yield as a function of the area of red clover used for green manure supply to the leek differed between the different manure systems. The lowest application rates of biogas slurry or mulch were equal to, or better than, direct incorporation (Fig. 4 in Paper I). It can be concluded that biogas slurry and mulch are promising alternatives for farms wishing to remove green manure crops from their crop rotation. This would enable farmers to grow green manure crops on fields that are less suitable for cash crop production. Biogas slurry also has the advantage that timing of application can be adjusted to crop nutrient requirements, and the biogas production can compensate for the cost of investment in digestor equipment and operation, as well as for transport to and from the field (Hansson & Christensson, 2005).

The different green manure systems influenced microbial community composition, as indicated by fungal:bacterial ratios and overall community profile, and activity of protease, acid phosphatase and arylsulphatase (Paper II). In confirmation of our hypotheses, fresh red clover residues, and especially direct incorporation of a red clover crop, generally enhanced bacterial and fungal biomass and soil enzyme activity more during a cropping season than did processed green manure forms, *i.e.* biogas slurry and compost. The direct incorporation treatment stimulated both an initial, short-term pulse in microbial biomass and a long-term enhancement of microbial biomass and soil enzyme activity as a response to resource addition, which is indicative of C limitation (Anderson & Domsch, 1978) and a bottom-up control of microbial biomass (Wardle, 2002).

As seen in Fig. 4, fungal:bacterial ratios differed between green manure systems in the beginning of the cropping season, with a larger proportion of fungi in the

direct incorporation system. A shift from bacterial to fungal dominance has been suggested to be a common response to intercropping and organic amendments (Nakamoto & Tsukamoto, 2006), and our results show that this effect also differs as a response to different forms and application methods of organic amendments. It seems likely that the beneficial influence of the direct incorporation treatment on soil fungi was due to the continuous crop cover in the preceding year, since the initial differences in fungal:bacterial ratios levelled out during the cropping season and there were no significant differences at the two later sampling occasions ($p > 0.05$).

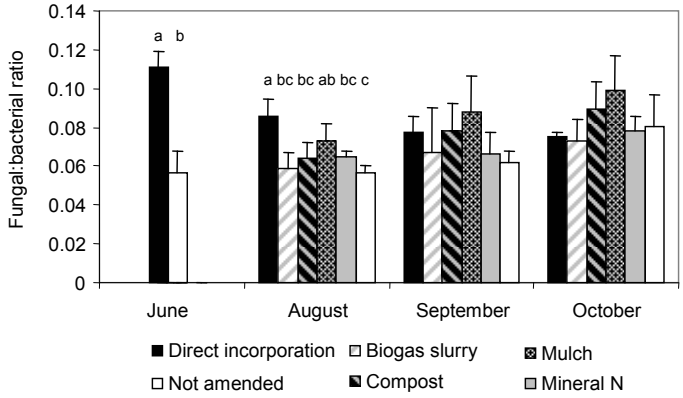


*Fig. 4.* Fungal:bacterial ratios in the different green manure systems at Krusenberg. Mean values (n=4 except for unamended soil in June where n=28) and standard deviations. An average of the different rates (equal N and C) is presented for the biogas slurry and compost treatments, since there was no significant difference due to application rate. Different letters indicate significant differences at $p < 0.05$ (Tukey's test) within sampling dates.

The mulch treatment showed a significant increase in arbuscular mycorrhizal (AM) fungal biomass or build-up of storage products at harvest (Fig. 5). It is possible that the mulch treatment provided a more favourable environment for AM fungal growth, since mulching generally increases soil moisture (Teasdale & Mohler, 1993) and since there was no mechanical disturbance due to weeding in this treatment. Disturbance of the top-soil by spade has been shown to reduce the density of spores, species richness and the lengths of extraradical mycelium of AM fungi compared with undisturbed soil (Boddington & Dodd, 2000). The mycorrhizal symbiosis is an important means for plants to improve their nutrient uptake and compete with saprophytic microorganisms for nutrients in the rhizosphere (Scheu & Setälä, 2002). This symbiosis has been suggested to be of greater importance for nutrient acquisition of crops with a poor root system, such as the leek crop in our study. It has previously been shown that crops with relatively high specific root lengths, *i.e.* cereal crops, were less affected by a reduction in AM fungal infection than crops with low specific root lengths (Ryan & Graham, 2002). However, vegetable crops are often grown on more fertile areas of land, and the significance of mycorrhizal symbiosis for crop growth under such conditions can be questioned (Ryan & Graham, 2002). On the other hand, a higher

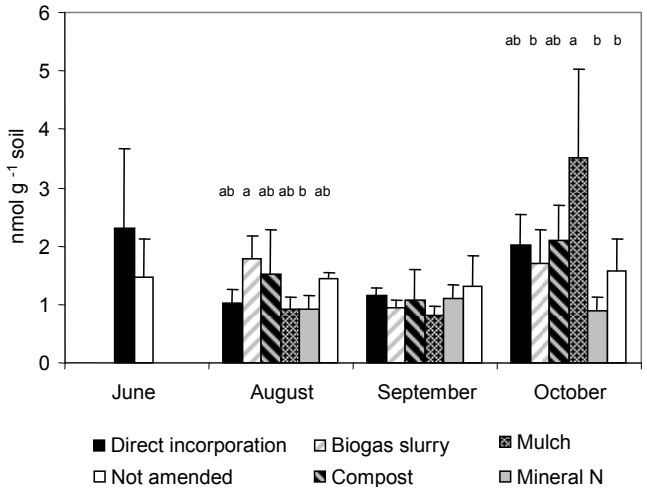


*Fig. 5.* Concentration of the AM fungal marker NLFA 16:1ω5 in the different green manure systems at Krusenberg. Mean values (n=4 except for unamended soil in June where n=28) and standard deviations. An average of the different rates (equal N and C) is presented for the biogas slurry and compost treatments, since there was no significant difference due to application rate. Different letters indicate significant differences at $p < 0.05$ (Tukey's test) within sampling dates.

proportion of nutrients may be organically bound in organic cropping systems, thus rendered less available for plant uptake, and a high proportion of AM fungal infection could be advantageous, especially early in the season when mineralisation of organic P may still be slow.

Differences in overall community composition between treatments were subtle, but there was a tendency for the treatments amended with fresh green manure to differ from the treatments amended with processed green manure (Fig. 3a-d in Paper II). When treatments were grouped according to green manure form and application method (*i.e.* ignoring amount) and analysed on the three sampling dates after amendments with all types of green manure (August-October), there was a significant effect ($p < 0.001$) of both time and green manure form on the soil microbial community composition (Fig. 6). In the ANOVA of the PC scores, the two fresh green manure forms differed significantly from the two processed forms of green manure on the two first components, with the exception of the biogas treatment on PC 1. These differences were not major, as indicated by the quite low separation of treatments and sampling times in the score plots and the low percentages of variance explained by the first two axes (Fig. 4). However, it is interesting that there was a different effect of quality of the green manure amendments on community composition already during the first cropping season, since changes in community composition can be regarded as more profound than physiological changes and are often more slowly responding (Svensson, 2002). There were no significant differences in the influence of the two fresh forms or the two processed forms, respectively, on community composition ($p > 0.05$).

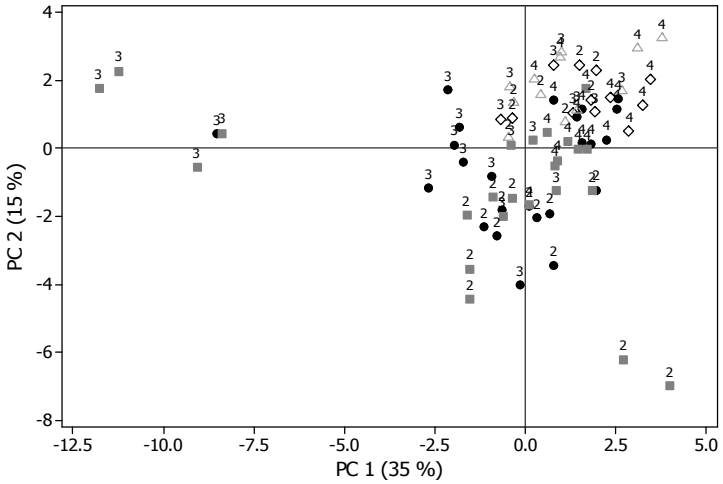


*Fig. 6.* PLFA profile in the experiment investigating different green manure forms at Krusenberg. Open diamonds (◇) represent direct incorporation, open triangles (Δ) mulching, closed circles (●) biogas residue and closed squares (■) compost. Numbers indicate time of sampling: 2 = August, 3 = September, 4= October.

Arylsulphatase activity was negatively correlated with leek S concentration at harvest (Fig. 5 in Paper II). This relationship was only visible when the mineral fertilised treatment was included in the correlation analysis, and was due to a high S content of the leek crop and a low arylsulphatase activity in the mineral fertilised treatment (Fig. 7). We postulated that this was an indication of a negative feedback mechanism, *i.e.* that the enzyme synthesis was down-regulated due to a high availability of sulphate in soil. Since sulphatases have been suggested to be more strongly regulated by the availability of S than microbial demand for C (McGill & Cole, 1981; Scherer, 2001), short-term fluctuations in activity may be more strongly regulated by the substrate feedback mechanisms than by microbial biomass. This would make arylsulphatase activity less suitable as a biological indicator of microbial biomass, but rather an indicator of soil nutrient status.

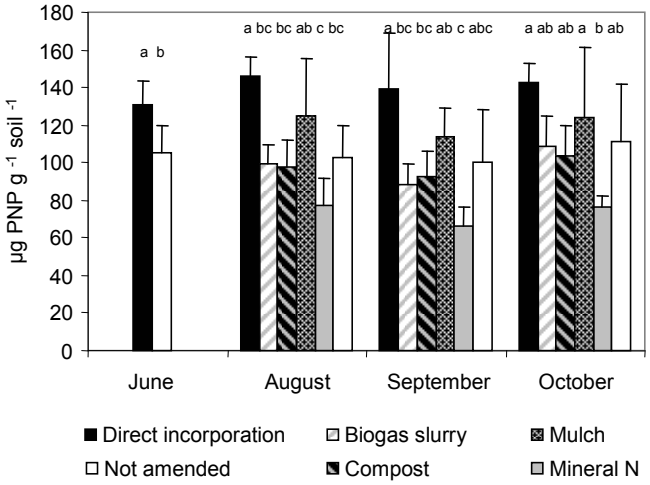


*Fig. 7.* Arylsulphatase activity in the experiment investigating different green manure forms at Krusenberg. Mean values (n=4 except for unamended soil in June where n=28) and standard deviations. An average of the different rates (equal N and C) is presented for the biogas slurry and compost treatments, since there was no significant difference due to application rate. Different letters indicate significant differences at $p < 0.05$ (Tukey's test) within sampling dates.

### *Long-term green manuring in comparison with other organic amendments*

It was evident from the short-term green manure experiment at Krusenberg that green manure amendments had a quantitative and qualitative impact on soil microbial communities. The quantitative effect was generally larger after fresh green manure additions, and especially after direct incorporation of a green manure ley, which suggests that the changes can partly be attributed to the supply of an easily available substrate for microbial growth. Thus, easily available C and nutrients enhances biomass and production of enzymes, the latter as a result of microbial demand for nutrients associated with microbial growth (Allison & Vitousek, 2005). However, manure additions can also influence microbial community composition and function indirectly through altered soil chemical and physical conditions. These properties are generally responding more slowly to management practices, and thus needs to be investigated in fields where there has been a long-term influence of a certain management. We sampled a long-term field trial where different mineral or organic fertilizers had been supplied since 1956, and in which different soil physical, chemical and biological properties have developed (Witter, Mårtensson & Garcia, 1993; Kirchmann, Persson & Carlgren, 1994; Kirchmann *et al.*, 2004). The last amendment with organic fertilizers had been done 19 months before sampling, which ensured that the microbial community was likely to reflect long-term management induced differences rather than a direct effect of resource inputs.

Continuous green manure amendments were found to have a stimulating effect on soil microbial biomass (total PLFA) and activity of protease, acid phosphatase and arylsulphatase (Tables 3 and 4 in Paper III). The changes in soil microbial and

enzyme activity levels were most strongly correlated to soil C contents, but in June also to soil total N, P and S contents, soil C/N ratio and to each other (Table 5). The different levels of microbial biomass and enzyme activity built up in the different treatments indicate the capacity of the soil microbial biomass to occupy different equilibria as a response to different long-term management regimes (Anderson & Domsch, 1989).

Factors other than soil total C content also influence the microbial community composition. Bacterial and fungal biomass was significantly higher in the green manure treatment in June compared with the other treatments when expressed on basis of soil C content (Fig. 1 in Paper III), and as in the direct incorporation treatment in Paper II, the fungal:bacterial ratio indicated a shift to fungal dominance in the green manure treatment early in the season. This is partly in line with Marstorp, Guan & Gong (2000), who found significantly higher fungal-to-total biomass ratio in the green manure treatment compared to the FYM treatment of this experiment. Thus, quality of the amendment, soil chemical properties apart from soil total C or other unknown factors altered the abundance of bacteria and fungi in the green manure treatment. Fungal:bacterial ratios have been shown to be higher in soils from undisturbed grassland ecosystems, grasslands lacking long term fertilization and reduced tillage agroecosystems (Thiet *et al*., 2006). Shifts in fungal:bacterial ratios will influence the composition of higher trophic levels in the soil food web (Bongers & Bongers, 1998; Scheu & Setälä, 2002) and have been suggested to affect soil decomposition and mineralisation rates (Wardle, 2002; Nakamoto & Tsukamoto, 2006). Slower soil organic matter turnover and enhanced soil C sequestration in fungal-dominated systems have been ascribed fungal alteration of soil physical properties and fungal physiology, but the underlying mechanisms are not fully understood (Thiet *et al*., 2006).

The analysis of overall microbial community composition indicated a clear separation in microbial community composition between the different treatments, although the differences were less prominent in September compared with in June (Fig. 2 in Paper III). The microbial community in the green manure treatment was dominated by fungi, some Gram-positive and some Gram-negative PLFA markers on the first sampling occasion and Gram-positive and Gram-negative markers on the second sampling occasion. In June, there was a clear gradient in the PCA score plot, with treatments amended with organic manures to the left in the score plot, and the unamended and mineral fertilised treatments to the right. Consequently, there was a strong correlation between soil total C, N and S and C:N ratio and the overall microbial community composition on this sampling occasion (Table 5). As for bacterial and fungal biomass, there were differences also in overall community composition when analysed on the basis of soil C content (Fig. 8). PLS analysis showed that soil total N and S were the most important soil chemical variables related to the microbial community composition on the basis of soil C content in June, and that the separation along the second axis was best correlated to soil pH.

Table 5. *Correlation matrix of soil chemical and biological properties in the Ultuna long-term study on two sampling occasions. Pearson r-values calculated from four replicates of each treatment (n=20). Significance levels of correlations: * p<0.05; ** p<0.01; *** p<0.001; no asterisk p>0.05*

| **Sampling** | **Enzymes** | | | **PLFA** | | | **Biolog** [a)] | |
|---|---|---|---|---|---|---|---|---|
| **occasion** | Protease | Phosphatase | Arylsulphatase | Total [b)] | PC 1 | PC 2 | AWCD[c)] | PC 2 |
| ***June 2003*** | | | | | | | | |
| Soil $C_t$ | 0.93 *** | 0.71 *** | 0.90 *** | 0.95 *** | -0.86 *** | 0.29 | -0.36 | 0.61 ** |
| pH | 0.38 | -0.21 | 0.32 | 0.09 | -0.16 | 0.77 *** | 0.15 | 0.30 |
| C/N ratio | 0.45 * | 0.48 * | 0.70 ** | 0.64 ** | -0.79 *** | 0.30 | -0.21 | 0.20 |
| Soil $N_t$ | 0.91 *** | 0.61 ** | 0.70 ** | 0.80 *** | -0.56 * | 0.16 | -0.32 | 0.68 ** |
| Soil $P_t$ | 0.50 * | 0.51 * | 0.34 | 0.47 * | -0.21 | -0.08 | -0.20 | 0.22 |
| Soil $S_t$ | 0.89 *** | 0.64 ** | 0.71 ** | 0.80 *** | -0.55 * | 0.24 | -0.25 | 0.52 * |
| Protease | | 0.58 ** | 0.81 *** | 0.85 *** | -0.72 *** | 0.38 | -0.22 | 0.66 ** |
| Phosphatase | | | 0.71 *** | 0.82 *** | -0.65 ** | -0.22 | -0.50 * | 0.33 |
| Arylsulphatase | | | | 0.89 *** | -0.77 *** | 0.28 | -0.31 | 0.59 ** |
| Tot PLFA | | | | | -0.88 *** | 0.07 | -0.33 | 0.57 ** |
| PLFA PC 1 | | | | | | | 0.19 | -0.43 |
| PLFA PC 2 | | | | | | | -0.03 | 0.26 |
| ***September 2003*** | | | | | | | | |
| Soil $C_t$ | 0.76 *** | 0.88 *** | 0.94 *** | 0.78 *** | 0.39 | 0.75 *** | -0.12 | 0.28 |
| pH | 0.32 | 0.09 | 0.48 * | 0.07 | 0.04 | 0.58 ** | 0.20 | 0.26 |
| C/N ratio | 0.41 | 0.40 | 0.70 ** | 0.54 * | 0.41 | 0.36 | 0.25 | 0.16 |
| Soil $N_t$ | -0.34 | -0.16 | -0.14 | -0.12 | 0.37 | -0.18 | -0.23 | -0.40 |
| Soil $P_t$ | -0.43 | -0.22 | -0.31 | -0.23 | 0.18 | -0.30 | -0.40 | -0.54 * |
| Soil $S_t$ | 0.76 *** | 0.69 ** | 0.65 ** | 0.87 *** | 0.10 | 0.68 ** | -0.38 | 0.11 |
| Protease | | 0.69 ** | 0.73 *** | 0.72 *** | 0.23 | 0.72 *** | 0.76 *** | 0.48 * |
| Phosphatase | | | 0.79 *** | 0.75 *** | 0.42 | 0.71 *** | 0.88 *** | 0.37 |
| Arylsulphatase | | | | 0.94 *** | 0.35 | 0.84 *** | 0.00 | 0.28 |
| Tot PLFA | | | | | 0.32 | 0.60 ** | -0.38 | 0.28 |
| PLFA PC 1 | | | | | | | -0.25 | 0.14 |
| PLFA PC 2 | | | | | | | -0.05 | 0.33 |

[a)] Only correlations for principal component (PC) 2 and 3 are shown since there were no significant correlations for PC 1
[b)] Total sum of all phospholipid fatty acids (PLFAs) detected, [c)] Average well colour development

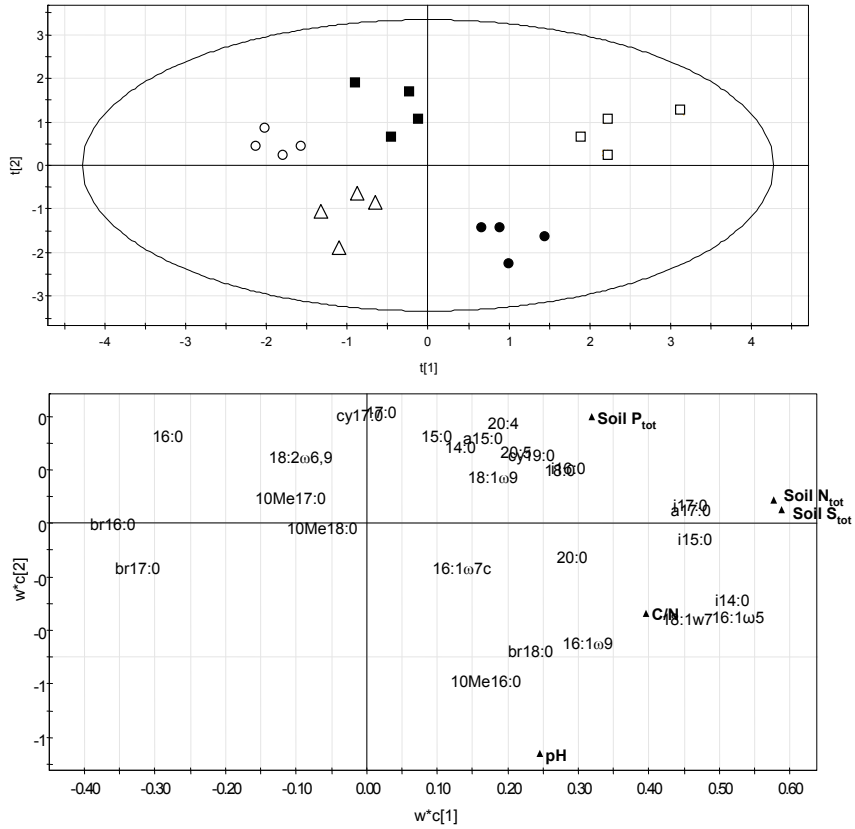


*Fig. 8.* PLS plots showing the correlation between soil microbial community composition and soil chemical variables (soil total N, P and S content, pH and soil C/N ratio as indicated by closed triangles (▲)) in the different treatments of the long-term study at Ultuna in June. The green manure treatment is represented by closed squares (■), the unfertilised control by open circles (○), the mineral fertilised treatment by open triangles (Δ), the farmyard manure treatment by open squares (□) and the sawdust treatment by closed circles (●).

Other recent studies on this field experiment using molecular fingerprinting techniques have also detected differences between treatments with respect to community structure of bacteria, AM fungi and ammonia oxidizers (Toljander *et al.*, 2006; Enwall *et al*., 2007), albeit not all studies included the green manure treatment, which confirms our findings that the microbial community has been altered by the management practices. However, although shifts in microbial biomass and community composition were found in this and other studies, there were no differences in substrate utilisation potential (Figs. 3 and 4 in Paper III). There are two possible underlying principles that can explain this; either the PLFA analysis, or the community fingerprinting techniques used in the other studies, is more sensitive to community shifts than Biolog, *i.e.* the contrasting results are artefacts caused by the methodology, or there were no associated changes with respect to this specific function. PLFA analysis has been reported to have a strong relationship to environmental context and a better discriminatory power to detect changes in microbial community composition than the Biolog analysis (Ramsey *et*

*al*., 2006). However, functional redundancy is suggested a common feature among heterotrophic soil organisms especially for the easily available substrates that dominate in the Biolog plate (Preston-Mafham, Boddy & Randerson, 2002; Waldrop & Firestone, 2004). Consequently, the observed changes in microbial community composition may not necessarily lead to a change in function, due to a plasticity allowing some microbial groups to perform the functions of others.

Green manure can be a substitute for farmyard manure (FYM) on farms without livestock, and this practice is especially common on organic farms specialized on crop production. Our results show that green manuring had a similar, stimulating influence on microbial biomass and enzyme activities as FYM, although the biomass and activity levels were generally slightly lower in the green manure amended treatment. However, the microbial community composition was influenced differently. Interestingly, the clear separation of microbial communities present at the beginning of the cropping season was not visible at harvest. Possibly, the input of rhizodeposits and root turnover by the maize crop had evened out some of the long-term induced differences in microbial community composition. Maize aboveground biomass did not differ between treatments, except for a considerably lower plant biomass in the unfertilized treatment ($p<0.001$, data not shown), which could imply that also belowground plant biomass and the quantity of C derived from roots were similar in the organic amended treatments.

## C routes from green manure and a growing crop into the soil food web

The studies reported in Papers I-III clearly demonstrate that changes in microbial community composition and soil enzyme activity occur as a response to different green manure systems or compared with other organic amendments. However, it is not known which of the different taxonomic groups identified with the PLFA analysis that are actively metabolizing the organic amendments supplied to the soil, or to what extent. In addition, the contribution of the growing crop to the development of the specific soil microbial profile cannot be separated from the influence of the green manure material. By enriching the two main potential C sources for heterotrophic organisms (the green manure material and the growing crop) with the stable isotope $^{13}C$, actively metabolising organisms in the decomposer and grazer communities could be targeted which can reveal how C is allocated into the soil food web.

The $^{13}C$-enriched red clover green manure that was incorporated before planting of the leek crop had a $\delta^{13}C$-value (*i.e.* isotopic ratio relative to a standard) of on average 522‰ in the shoots, 438‰ in the stubble fraction and 317‰ in the roots. The unlabelled red clover that was incorporated as green manure in the labelled leek treatment and the unlabelled control treatment had a $\delta^{13}C$-value of -26‰ (average of shoots, stubble and roots). The $\delta^{13}C$-values of the pulse-labelled leek crop ranged from on average 423‰ in the young leaves shortly after the pulse-

labelling occasions to 64‰ in the roots at harvest. The unlabelled leek crop had a similar $\delta^{13}$C-signal to the red clover, on average -27‰ (Table 1 in Paper IV).

The flow of C was traced into microbial lipids and soil animals *in situ*. PLFA biomarkers, the AM fungal marker NLFA 16:ω5, soil microarthropods, enchytraeids and earthworms were analysed for their $\delta^{13}$C-values on several occasions during the cropping season. In general, the stronger signal in the labelled green manure treatment was evident as a stronger enrichment in the taxa analysed in this treatment compared with those in the labelled leek treatment. In order to compensate for this, δ-values of fatty acids were expressed relative to the δ-values of the generally occurring PLFA 16:0.

Our results provided evidence that the C-flow from the two sources was allocated somewhat differently in the soil microbial community, and that different taxonomic groups differed in their C utilization strategies. Most lipids were enriched with $^{13}$C from both green manure and the leek rhizodeposits, but the degree of enrichment varied (Fig. 1 in Paper IV). The actinomycete marker 10Me18:0 and lipids characteristic of Gram-positive bacteria (i15:0, a15:0, i16:0 and 10Me16:0) were relatively more enriched with green manure-derived $^{13}$C, indicating specialisation in decomposing green manure material, whereas the neutral lipid marker of AM fungi was clearly more linked to C derived from the growing leek crop (Fig. 1-3 in Paper IV).

In contrast, markers for Gram-negative bacteria, mainly 16:1ω7c and 18:1ω7, were the most enriched in $^{13}$C in both labelled treatments (Fig. 1-3 in Paper IV), indicating a general dominance irrespective of C source Several studies have suggested that Gram-negative bacteria proliferate as a response to organic amendments and increased resource availability in soils (Bossio & Scow, 1998; Bossio *et al*., 1998; Zelles *et al*., 1995; Böhme, Langer & Böhme, 2005; Larkin *et al*., 2006). No change in Gram-positive:Gram-negative ratio was observed due to the short-term green manure amendments investigated in Paper II (data not shown), although some Gram-negative markers were dominating in the freshly amended treatments (direct incorporation and mulch) at the end of the cropping season (Fig. 3c,d in Paper II). However, in the long-term study, the green manure treatment had a lower Gram-positive:Gram-negative ratio compared with the other treatments in June, although significantly different only from the FYM and sawdust treatments (Table 3 in Paper III). Treonis *et al*. (2004) showed that Gram-negative bacterial markers had a faster turnover than markers for Gram-positive bacteria. A fast turnover and an ability to proliferate rapidly as substrate availability is increased may explain the high enrichment in our study and why Gram-negative bacteria are commonly observed to increase after resource additions.

The high number of species that co-exist in soil without extensive niche differentiation makes it difficult to ascribe soil animal species to trophic levels or feeding guilds (Chahartaghi *et al*., 2005). In this study, the natural abundance of both $^{13}$C and $^{15}$N indicated occupation of separate trophic niches by the sampled arthropods in May, before the field trial was initiated. The average natural $\delta^{15}$N-

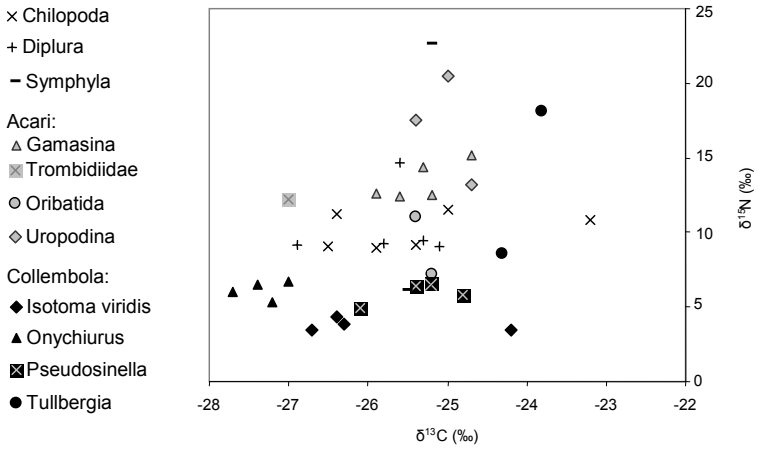


*Fig. 9.* Natural abundance $\delta^{13}C$ and $\delta^{15}N$ signatures (‰) of meso- and macrofauna in May 2004. Individual replicates may consist of several pooled individuals.

signatures spanned over 13 δ-units (from 3.7‰ to 17.7‰) with the Collembola *Isotoma virides* having the lowest and Uropodina (Acari) the highest average $\delta^{15}N$- signature (Fig. 9). Similarly wide ranges have been interpreted as an indication of trophic niche differentiation of the organisms studied (Chahartaghi *et al*., 2005). Assuming a shift in $\delta^{15}N$ of +3.4‰ per trophic step (Albers *et al*., 2006) would imply that the sampled arthropods spanned over four trophic levels with three Collembola species, *Isotoma viridis*, *Pseudosinella* and *Onychiurus* at the basal level. Interestingly, although *Onychiurus* and *Pseudosinella* had similar $\delta^{15}N$-values, indicating occupation of the same trophic level, their $\delta^{13}C$-values differed significantly, possibly indicating specialisation on different food sources.

Considering the utilisation of the two enriched C sources by soil fauna, most soil fauna seemed to derive their C directly or indirectly from the decomposing plant material and rhizosphere C seemed to be of secondary importance in this agroecosystem. However, it was difficult to draw reliable conclusions regarding specialisation of the fauna studied, due to a considerable variability in isotopic ratios within taxa. Some individuals were probably not actively feeding during the period, had recently migrated into the plots or were feeding on older plant material in soil, which could explain large variations in isotopic ratios within taxa.

General features of soil predators appear to be a generalist feeding behaviour, including polyphagy, omnivory, and intraguild predation (Scheu & Setälä, 2002), which allows switching diets according to resource availability (Chahartaghi *et al*., 2005). Dominance towards the decomposer pathway may also be explained by the fact that representatives of taxa that are known to be specialist feeders on living roots, such as protozoa and root-feeding nematodes, were not included in the study. Soil animals that are too small for hand-picking or that require large numbers to be bulked in order to get large enough sample sizes are difficult to integrate by this approach. The use of biomarkers is an interesting option for these

groups, since the problem with too small sample sizes of small animals can be avoided (Boschker & Middelburg, 2002; Evans *et al*., 2003). Fatty acids from protozoa and nematodes are included in C18 and C20 fatty acids (Frostegård *et al*., 1997; Ruess *et al*., 2005), although their relative contribution to the pool of these fatty acids in natural systems is not known.

# Conclusions

This thesis demonstrates how different green manure systems influence microbial community composition and function in temperate arable soil. The work showed that the effects of green manuring differed depending on manure form and application method.

In relation to the initial hypotheses formulated, it can be concluded that

- Fresh red clover green manure, and especially direct incorporation, stimulated microbial growth and soil enzyme activity more than processed green manure forms during a cropping season (Papers I and II).

- Fresh green manure forms tended to differ in overall microbial community composition (PLFA profile) compared with the processed green manure forms. Some differences were also apparent between the two fresh green manure forms, which could be attributed to different application methods, *i.e.* a higher fungal:bacterial ratio in the direct incorporation treatment early in the cropping season and a stimulation of AM fungi in the mulch treatment late in the season (Paper II).

- Long-term green manuring had a similar, but not as strong, stimulatory influence on build-up of soil microbial biomass and soil enzyme activity levels as farmyard manure and sawdust. Overall microbial community composition (PLFA profile) differed as a response to the different long-term amendments and the green manure treatment had a higher fungal:bacterial ratio than the farmyard manure treatment. There were however no differences in substrate utilisation potential. Both quantitative (biomass, enzyme activity) and qualitative (community composition) changes were closely correlated to soil chemical properties. However, the long-term induced differences appeared to be weakened by the influence of a cropping season (Paper III).

- Stable isotope labelling in combination with PLFA analysis indicated that actinomycetes and Gram-positive bacteria were more specialised in decomposing green manure material, whereas AM fungi were more linked to C from the growing leek crop. Markers for Gram-negative bacteria were the most $^{13}$C-enriched PLFAs in both labelled treatments, indicating a general competitive ability irrespective of C source. The soil

animal groups analysed were more dependent on the fresh, dead organic matter than on the growing crop as a primary food source (Paper IV).

As this thesis shows, green manuring can be an effective management practice for enhancing microbial biomass and soil enzyme activity, but the qualitative and quantitative response of microbial communities differed due to manure form and application method. It was found that biogas slurry and mulching could be possible alternatives to direct incorporation of a green manure ley for growers who for different reasons may want to remove the green manure ley out of the crop rotation (Paper I). However, when the green manure ley is removed from the crop rotation, its beneficial impact on soil microbial biomass and soil enzyme activities is lost and a shift in microbial community composition can be expected. Similarly, replacing farmyard manure with green manure can be expected to influence microbial community composition in the long-term.

Although various techniques are now available that allow characterisation of microbial community composition or function, *e.g.* PLFA analysis, molecular fingerprinting and Biolog plates for substrate utilisation profiles, these techniques present difficulties in addressing the question of soil fertility because of the gap in knowledge concerning these organisms and their functional roles. Therefore, the practical value for management is not clear. The work in this thesis shows that stable isotope probing has potential to bridge this gap between soil organism community composition and functional roles.

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