Disentangling microbial decomposition networks

Linking detritus-based soil microbial food webs to ecosystem processes

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Contents

Chapter 1

General Introduction

Amber Heijboer

1

1.1 A changing world

Our planet has been in motion for over 4 billion years. During this incomprehensible amount of time, the Earth has undergone numerous changes with respect to atmosphere, environment, climate and life, and these global changes continue today. During the Holocene, the last 11,700 years of Earth's history that is characterized by the development of human civilization, the Earth's environment has been relatively stable. However, over the last century, the speed of environmental change has increased dramatically due to the activities of humans and more specifically the industrial revolution. The fact that these rapid changes exceed the pace of natural variation in Earth's history is alarming (Wuebbles *et al.*, 2017). However, global change itself is not necessarily a bad thing. As we live in a continuously changing world, the focus not always needs to be to keep our environment as it is or was. However, the most important challenge facing mankind is to ensure a sustainable future for our planet, the humans that inhabit it, and the diverse organisms with whom we coexist.

As humans we depend on the Earth via the planet's ecosystem services; the benefits people obtain from ecosystems (Millennium Ecosystem Assessment, 2005). Life on Earth would not be possible without environmental provisions of food, fresh water and energy as well as the natural regulation of climate and biogeochemical cycles. Human-induced global change directly affects these ecosystem services, and recent events like the water scarcity in Cape Town, threatening wildfires in California and destructive hurricanes in the Caribbean show once more how dependent life is on a well-functioning environment. For a number of ecosystems services, we have actually already crossed critical planetary boundaries, which means that we have deviated from the safe operating space for humanity to safeguard these specific ecosystem services (Rockström *et al.*, 2009). Currently, we are we facing a tremendous loss of genetic diversity due to extinctions, and severe effects of intensive agriculture on global nutrient flows (Steffen *et al.*, 2015). In addition to this, we face the risk of crossing planetary boundaries for climate change due to rising atmospheric CO_2 concentrations and human modification of our ecosystems by land use modification, with possibly disastrous consequences for the human population as well as our planet (Steffen *et al.*, 2015). Due to these important threats on the functioning of our planet, it is now more important than ever to not only realise the importance of our ecosystems, but to understand how they function. Only with such understanding in hand we can devise informed strategies to help manage our planet in a sustainable way.

1.2 Soil: the basis of our living environment

Many ecosystem services are connected via a common natural resource: the soil. Healthy soils are crucial for all life on Earth, either directly or indirectly, and they are key to sustaining ecosystem functioning. Soils play for example an important role in agricultural food production, carbon sequestration, water purification and nutrient

cycling. It is for this reason that soils have gained significant recognition over the last years e.g. by playing an important role in a large number of the United Nations Sustainable Developments Goals (Keesstra *et al.*, 2016), the establishment of the Intergovernmental Technical Panel on Soils (ITPS) in 2013, and the UN declaring 2015 the International Year of Soils (Wall *et al.*, 2015). The attention for soils is not without reason, since a number of soil ecosystem services are currently under considerable threat due to e.g. soil degradation, soil fertility depletion, atmospheric nitrogen deposition, land use change and climate change. A good understanding of the mechanisms underlying soil ecosystem functioning and their relation to global change is therefore an important area of today's soil ecology research (Adewopo *et al.*, 2014; Eisenhauer *et al.*, 2017), so that we can mitigate global change and make sure we sustain and optimize the use of our natural soil resources for human well-being.

1.3 The importance of soil biodiversity

The functioning of soils is to a large extent governed by the huge biodiversity of soil life (Bardgett & van der Putten, 2014). Soils are one of the most biodiverse habitats on Earth, harbouring millions of microbial and animal species that are pivotal for soil ecosystem services and human health (Wall & Six, 2015). Soil organisms are an integral part of landscape processes by interacting with aboveground diversity and ecosystem functions (De Deyn & van der Putten, 2005; Bardgett & Wardle, 2010). Soils are also critical to the resilience of terrestrial ecosystem services to global change e.g. by playing an important role in the global carbon and nitrogen cycle through decomposition of organic compounds (Bardgett & van der Putten, 2014; Isbell *et al.*, 2015). Perhaps the most important ecosystem process driven by soil organisms is the decomposition of plant residues and soil organic matter. Via the decomposition of organic material, soil organisms determine the critical balance between sequestration and mineralization of carbon and nutrients, affecting soil CO_2 emissions to the atmosphere and the availability of nutrients for plants (Gessner *et al.*, 2010). There has been a large amount of attention devoted to research exploring the relationship between biodiversity loss and ecosystem functioning (Cardinale *et al.*, 2012; Tilman *et al.*, 2014; Isbell *et al.*, 2015), and a number of studies have already pointed out the potentially disastrous consequences of soil biodiversity loss for the process of decomposition (Hooper *et al.*, 2012; Handa *et al.*, 2014). However, soil biodiversity data is still under-represented in many biodiversity databases (Phillips *et al.*, 2017).

1.4 The (microbial) soil food web and impacts of land use

The interactions among the diversity of soil organisms can be depicted in the form of a soil food web: a model that describes the feeding relationships and interactions among species or groups of species that live (part of their life) in the soil (Figure 1.1). Such soil food webs depict flows or organic matter through the soil community via

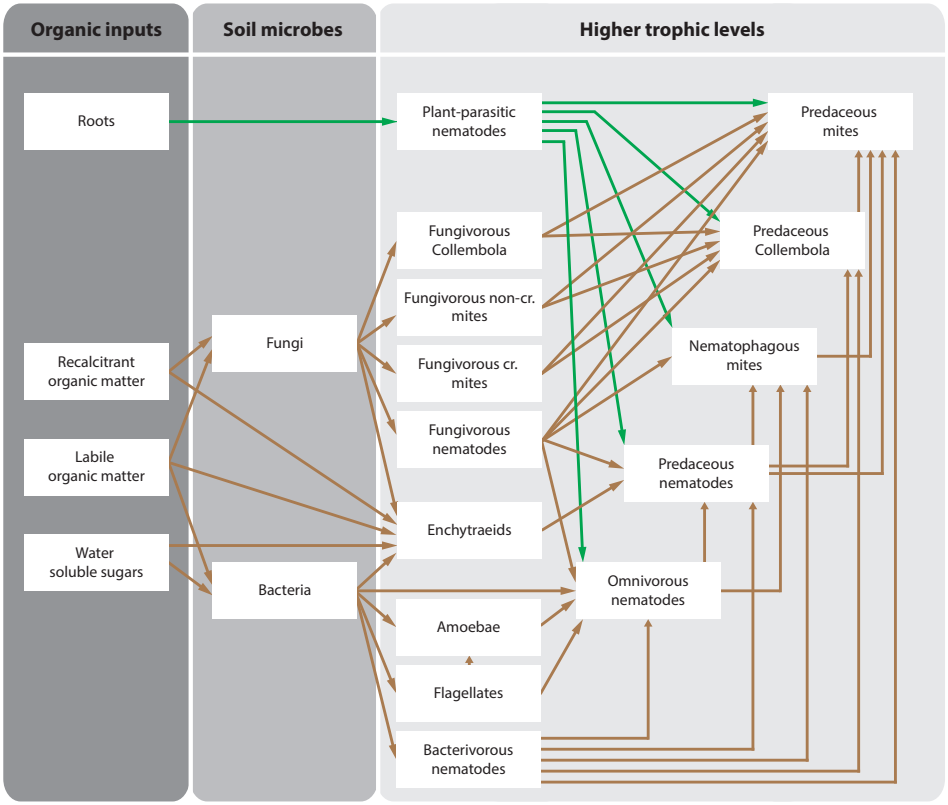

Figure 1.1 Soil food web diagram with boxes representing feeding guilds, a group of species sharing the same feeding behaviour (with cr. = cryptostigmatic), and arrows representing the direction of matter and energy flows. Green and brown arrows visualize, respectively, the plant-based soil food and the detritus-based soil food web. Microbes form the first detritus-consuming level and make up 90% of the biomass of the complete soil food web (figure adapted from Holtkamp *et al.* 2008).

two organic sources: plant root herbivory (plant-based soil food web or green soil food web) and via detritus (detrital-based food web or brown food web), as shown in Figure 1.1. The food web model approach provides an ecologically relevant perspective to analyse the relationship between community structure (biomass, stability, community composition) and ecosystem functioning, for instance in response to environmental change, by describing transfer rates of energy and nutrients. The first soil detrital food web was described by Hunt *et al.* (1987) and since then, many other studies have used this soil food web model. Soil food webs have for instance been used to study the role of soil organisms in nutrient cycling with changing land use, revealing the strong response of soil food web topology to changes in land use (de Ruiter *et al.*, 1994; Holtkamp *et al.*, 2008; de Vries *et al.*, 2013).

An important role in soil food webs is set aside for soil microbial organisms: bacteria, fungi and protozoa. Microbes form the first detritus consuming trophic level in soil

food webs (Figure 1.1), and have been shown to make up more than 90% of the total belowground biomass (e.g. Holtkamp *et al.*, 2008). Soil microbes also carry out an important task in terms of ecosystem functioning since they are the main influencers of soil organic C and N dynamics by being responsible for the majority of soil mineralization (de Ruiter *et al.*, 1994). In this way, microbes form the 'eye of the needle' in the soil through which all organic matter must pass (Jenkinson, 1977). It is thus logical that soil microbial communities are largely influenced by changes in the composition and size of organic pools, factors known to be significantly affected by land use change (Degryze *et al.*, 2004; Leifeld & Kögel-Knabner, 2005).

Soil food web models have proven to be highly useful in the study of the long-term consequences of land use on soil communities and associated ecosystem functioning. Nevertheless, microbial organisms in such models, as shown in Figure 1.1, are not yet defined beyond the level of 'bacteria' and 'fungi' (Hunt *et al.*, 1987; de Ruiter *et al.*, 1993a; Holtkamp *et al.*, 2008). In fact, these models consider only two main detritus-based energy channels: the bacterial energy channel and the fungal energy channel. The bacterial energy channel is defined as decomposing labile organic compounds twice as fast as the fungal channel, while the fungal channel in turn decomposes recalcitrant organic compounds twice as fast as the bacterial channel (e.g. Hunt *et al.*, 1987; Moore & Hunt, 1988; Moore *et al.*, 2004). The relative shift in the importance of the bacterial versus fungal energy channel is often taken as a proxy for the effects of land use change on soil food web functioning (Wardle *et al.*, 2004; Rooney *et al.*, 2006; Holtkamp *et al.*, 2008; de Vries *et al.*, 2013). Although this broad concept has advanced our ability to examine ecosystem responses to land use change, recent evidence suggests that it represents a drastic oversimplification. It has now becoming increasingly clear that microbial resource partitioning of soil organic matter takes place on a more detailed level than the broad bacterial and fungal energy channels (Paterson *et al.*, 2008; Drigo *et al.*, 2010; Kramer *et al.*, 2016).

1.5 Empirical methods to study soil microbial food webs

How can we increase the level of detail in the microbial part of the soil food web to improve soil food web modelling? In discussions concerning the future of soil research, there is an urgent call for interdisciplinary research (Eisenhauer *et al.*, 2015; Keesstra *et al.*, 2016). Extending the insights of soil microbial food webs is one of the research topics where the combined advancements of two soil disciplines can be used to explore this new field of research. On the one side, there is the field of soil food web ecology, using soil food web models to describe and quantify flows of matter, energy and nutrients. It is important to emphasize that there is nothing wrong with these models as they are able to answer a multitude of important research questions on e.g. ecosystem stability by depicting a simplified model of reality (de Ruiter *et al.*, 1995). However, the existing models are not able to answer a number of emerging priority research questions (Eisenhauer *et al.*, 2017) on the microbial level e.g.: How temporally stable are soil microbial communities, in terms of both

taxonomic and functional community structure, and which community members are active at any one time? How do soil diversity and ecological interactions in soil contribute to multiple ecosystem services, and what are the effects of land use change on the trait and species composition of soil communities? Alongside the research field of soil food web ecology, there is the research field of soil microbial ecology. This empirical field of research traditionally focussed on the isolation and identification of microbial species. However, with the introduction of molecular, and more recently high-throughput sequencing, techniques, recent research has focussed much more on the description of *in situ* microbial communities via cultivation-independent approaches. Using the upcoming insights of soil microbial ecology to incorporate microbial processes into existing soil food web models is an promising example of interdisciplinary soil science that could largely increase our understanding of soil functioning in a changing world.

One of the reasons that detailed information of soil microbial communities is not included in classical soil food web models is because it was simply not possible to follow the detailed fate of organic C and N flows through the soil microbial communities. A type of analysis that has been used in the past to identify and quantify the microbial biomass, is Phospholipid Fatty Acid (PLFA) analyses (Frostegård & Bååth, 1996). PLFAs are the primary membrane lipids of the microbial cell that serve as useful microbial biomarkers since different groups of bacteria and fungi have specific lipids. Since PLFA analysis is a rather cheap and easy method to apply, this type of analysis is still often applied to study soil microbial community responses to land use change (e.g. de Vries *et al.*, 2013; van Leeuwen *et al.*, 2017). Especially in combination with analyses targeting microbial community functioning (e.g. mineralization measurements, community level physiological profiling), this type of information can be very useful to increase our insights in the microbial functioning of soil food webs. Over the last decennia, new empirical advances have been made that have enabled us to study flows of organic matter through soil microbial communities in even more detail, and to directly link soil microbial community structure with functioning. One of these promising empirical techniques is Stable Isotope Probing (SIP), an approach that combines the tracing of stable isotopes (e.g. ^{13}C or ^{15}N) with the use of molecular techniques (Radajewski *et al.*, 2000). By adding a stable isotope-labelled compound to a community, one can detect the active consumers by following the incorporation of compound-derived stable isotopes into biomarkers like e.g. PLFA (Boschker *et al.*, 1998; Ruess & Chamberlain, 2010), DNA and RNA (Radajewski *et al.*, 2000; Manefield *et al.*, 2002b). In this way, SIP can for instance be used to trace the fate of contrasting types of labelled organic matter through the soil microbial food web by detection of stable isotope incorporation in microbial organisms over time. This type of information gives the possibility to directly link the identity of organisms with their functioning, a type of information that is crucial to gain deeper insight into the effects of microbial community shifts on ecosystem functioning.

1.6 Research aim: Unravelling the microbial soil food web

Although it is known that microbes occupy an important position in the soil food web, due to the reticulated form of microbial soil food webs, they are hard to study. Detailed information on the flow of contrasting types of organic compounds through the microbial part of the soil food web is generally lacking. Such information is crucial in order to have a full understanding of the consequences of land use change on soil ecosystem functioning. In this thesis, emerging empirical techniques were used to advance our knowledge on microbial processes and concomitant ecosystem functioning with changing land use. To this end, the following research objectives were formulated:

1. To explore emerging empirical techniques that can be used to disentangle soil microbial soil food webs.
2. To assess soil microbial C flows, derived from contrasting types of soil organic matter substrates, through the soil food web, as affected by land use.
3. To increase our insight in the link between soil microbial community structure and function in response to land use.

1.7 Thesis outline

In **chapter 2**, I discuss how state-of-the-art empirical techniques can be used to collect trophic information to construct different types of empirically-based food webs: connectedness webs, semi-quantitative webs, energy flow webs and functional webs. In this chapter, it is explained what types of information is needed from molecular and biogeochemical studies to create such food web models. Following on that, a comprehensive overview is given of the available empirical techniques with respect to the type of information they can provide and how they can be combined with different types of food web models.

In **chapter 3**, I examined litter-derived carbon flows through the soil microbial food web in ex-arable soils. In an incubation experiment, I compared recent and long-term abandoned soils that were amended with ^{13}C-labelled plant litter. Over a period of 56 days, I traced the fate of litter-derived carbon flows through the soil microbial community by using PLFA-SIP. In this way, it was revealed that after litter addition there is a clear succession of microbial decomposers that was similar across all examined fields. These analyses revealed that soil microbial communities become less efficient in decomposing litter-derived carbon with increasing time after land abandonment, most likely due to a net shift from organic matter-derived C towards root-derived C input in the soil microbial food webs after land abandonment.

In **chapter 4**, I continue assessing C flows through the soil microbial food web of ex-arable soils in more detail by tracing the fate of contrasting types of organic substrates to further assess soil microbial resource partitioning. Recent and long-term abandoned soils were incubated for 28 days after the addition of glycine,

cellulose and vanillin. In each of the treatments one or none of these compounds was ^{13}C-labelled, to trace the fate of a specific organic compound. PLFA-SIP and RNA-SIP analyses allowed me to 1) quantify the C flows through the soil microbial food web and 2) assess soil microbial resource partitioning beyond the concepts of the bacterial and fungal energy channels. These analyses revealed that a specialized microbial community for the decomposition of recalcitrant material develops in soils from long-term abandoned fields. Furthermore, it is shown that there is an intra-kingdom microbial decomposer succession and microbial resource partitioning on the taxonomic level of both fungal and bacterial classes. These results further enhance the view that the understanding of soil microbial decomposition goes beyond the concepts of bacterial and fungal energy channels.

In **chapter 5**, I show how soil microbial community structure is affected by contrasting types of organic amendments, to assess the effects of organic input on microbial biomass, activity and community structure and subsequently ecosystem services like nitrogen mineralization, microbial N immobilization and plant growth and nutrient uptake. The experiment revealed that a number of ecosystem services were directly related to soil microbial activity, while microbial N immobilization was mostly dependent on the soil microbial community structure. These outcomes further support the idea that soil microbial community structure is important to take into account when assessing the effects of the soil organic inputs on soil ecosystem functioning.

In **chapter 6**, I assess the spatial scale of drivers of both the soil microbial community structure and functional capacity as related to soil management efforts designed to optimize biogeochemical functioning. In the previous chapters, I show that soil microbial community structure and functioning are not always linked to one another, and this chapter takes this notion a step further by assessing the drivers on two spatial scales (landscape and local scale) of both soil microbial community structuring and functioning, separately. It is shown that soil microbial community structure is driven on the landscape level by phosphorous related variables, whereas soil microbial functioning is driven locally through vegetation patterns. This is important knowledge to take into account when designing management strategies targeting desired biogeochemical functioning of soils.

In **chapter 7**, I discuss the implications of the research presented in this thesis, and I give my personal vision on the future of soil microbial food web research in the light of soil ecosystem functioning and management.

Chapter 2

Empirical methods of identifying and quantifying trophic interactions for constructing soil food web models

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2.1 Introduction

Food web models, which depict the trophic relationships between organisms within a community, form a powerful and versatile approach to study the relationships between community structure and ecosystem functioning. Although food web models have recently been applied to a wide range of ecological studies (Memmott, 2009; Sanders *et al.*, 2014), such approaches can be greatly improved by introducing high resolution trophic information from empirical studies that realistically describe topological structure and energy flows (de Ruiter *et al.*, 2005). Over the last decades major technological advances have been made in empirically characterizing trophic networks by describing, in detail, the connectedness and flows in food webs. Existing empirical techniques, such as stable isotope probing (SIP) (Layman *et al.*, 2012), have been refined and new approaches have been created by combining methods, e.g. combining Raman spectroscopy or fatty acid analysis with SIP (Ruess *et al.*, 2005b; Li *et al.*, 2013). These empirical methods can provide insight into different aspects of food webs and together form an extensive toolbox to investigate trophic interactions. It is crucial to recognize the potential and limitations of empirical approaches in order to choose the right method in the design of empirically-based food web studies.

Empirically-based food webs are generally classified according to the type of input information that is required. In the following lines we will provide an overview of four types of food web models: the connectedness webs, semi-quantitative webs, energy flow webs and functional webs. Paine (1980) introduced three of those webs, which are widely accepted and applied in food web studies across ecosystems. We propose to add a fourth type of empirically-based food web, the semi-quantitative web. All of these food webs have the same basic structure, but the conceptual webs differ in the type of trophic information they describe and represent (Figure 2.1). *Connectedness webs* (Figure 2.1a) define the basic structure of a food web by describing the food web connections *per se*. The food web consists of species connected by arrows, visualizing the direction of matter and energy flows. Due to the complexity of interactions, taxa are often lumped into feeding guilds, whose members have a similar trophic level and diet, and comparable function in the food web. *Semi-quantitative webs* (Figure 2.1b) differ from connectedness webs in that they contain quantitative information on the abundances and/or biomass of the food resources and the consumers. Additionally, they provide a semi-quantitative measure to feeding relationships such as the frequency of feeding interactions between (groups) of species, which can provide a good proxy for trophic interaction strength (Baker *et al.*, 2014). *Energy flow webs* (Figure 2.1c) aim to assess nutrient flows quantitatively. They thus contain quantitative biomass information, and the feeding relationships are fully quantified by vectors summarizing both the direction and the amount of material and energy flows. Finally, *Functional webs* (Figure 2.1d) are characterized by experimental manipulation (e.g. species removal, resource exclosure or amendment) to assign the functional role of species. In this chapter, we examine how empirical trophic information can be used to construct such empirically-based food webs.

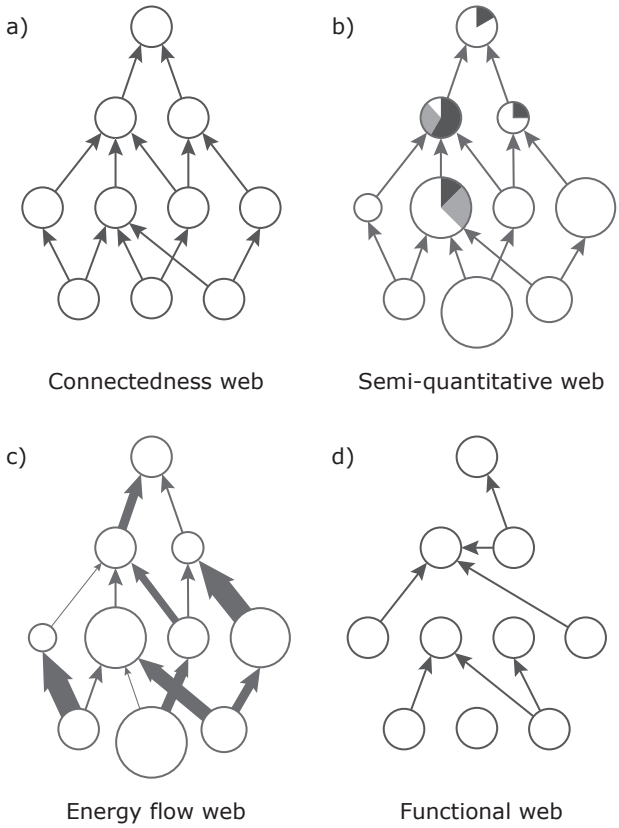

Figure 2.1 Four different types of empirically-based food webs, three of them (a, c & d) as defined by Paine (1980). a) Connectedness web visualizing qualitative feeding relationships; feeding guilds of species connected by arrows, visualizing the direction of matter and energy flows. b) Semi-quantitative web visualizing the abundances/biomasses of species (groups) and the frequency of feeding interactions. c) Energy flow web visualizing the biomasses of species (groups), connected by vectors visualizing the amount of material and energy flow. d) Functional web visualizing the effect of species manipulation on the population size of other species in the food web, highlighting the functional role of species including non-trophic effects (adapted figure from Paine (1980) and Selakovic *et al.* (2014)).

There is an increasing demand for empirical data and experiments to improve empirically-based food webs in e.g. the field of ecosystem engineering (Sanders *et al.*, 2014) and applied ecology (Memmott, 2009). Especially in the field of soil food web ecology, trophic interactions are still poorly understood, since it is difficult to define the trophic roles of belowground organisms. Ideally, a food web would be described at high taxonomic resolution, representing the present species and their interspecific trophic interactions. However, species-level approaches are often difficult and/or impractical since the assignment of trophic interactions to specific soil organisms, and especially soil microbes, is hard due to their small size, difficult extraction from their habitat and huge taxonomic and functional diversity (Eggers & Jones, 2000).

These facts particularly hamper the determination of microbial-faunal food web interactions. To circumvent this limitation, ecologists aggregate groups of species into feeding guilds based on diet and life-history characteristics. This approach has been especially applied to soils, the class of ecosystems on which this chapter will focus. New techniques introduced in this chapter provide better information on biological diversity and functionality within the food web, and in this way improve significantly the level of detail, and realism in empirically-based food webs.

Molecular and biochemical techniques that have been developed over the last decades have opened new windows in (soil) food web ecology to study which food sources sustain specific soil heterotrophs and to assess small-scale activity and trophic links in food webs. These empirical techniques offer the great opportunity to further unravel feeding guilds and to create predictive models that are able to provide answers on specific soil organisms and their role in trophic networks. However, a clear overview of what type of information empirical methods can offer for soil food web modelling is missing. This chapter provides an up-to-date overview of molecular and biochemical methods applicable in trophic soil studies. We start from a theoretical perspective of soil food webs to present the rationales and requirements of empirically-based food web modelling. Thereafter, an overview is provided on existing and upcoming empirical techniques and the research fields where they can be applied. The chapter concludes with recommendations on how to use the outcomes from empirical techniques to improve and fully exploit the use of empirically-based soil food web models as well as suggestions for future research priorities. Our goal is to provide an overview of state-of-the-art empirical approaches that can be used to create and improve food web models described in other chapters of this book, with a special focus on food web structure and flows.

2.2 From empirical data to food web models

Transferring the outcomes of empirical studies into useful data for soil food web modelling remains one of the main challenges when combining empirical and theoretical food web research. This paragraph provides an overview of the types of data resulting from molecular and biochemical trophic studies that are essential to create soil food web models. The information is categorized by the different types of food webs as introduced above: connectedness web, semi-quantitative web, energy flow web and functional web.

2.2.1 Connectedness webs

Connectedness webs are the basic form of food webs, often visualized by the use of a simple box-and-arrow diagram (Figure 2.1a). The boxes and arrows represent respectively (groups of) species and the direction of their trophic interactions. This type of information can give important insights with respect to the stability and complexity of a food web model. What does an empirically-based connectedness

web require? The construction of a connectedness web from empirical data requires at least information on the presence of organisms (binary) and the direction of feeding interactions: 'who eats whom?' arranged per feeding guild, but preferably on a more highly resolved taxonomic level. By constructing a connectedness web, valuable information on horizontal (i.e. within a trophic level) and vertical (i.e. between trophic levels) food web diversity is gained.

2.2.2 Semi-quantitative webs

Where connectedness webs only provide information on which species and feeding relationships are present, semi-quantitative webs also give information on the frequency of the trophic interactions (f_j) and the population sizes of present taxa on both the prey and consumer side (B_j) (Figure 2.1b). Frequency of interaction is based on the proportion of predators that contain remains of a specific prey, and can be calculated as:

$$f_j = \frac{F_{tot}}{N} \tag{2.1}$$

In this equation, F_{tot} refers to the total number of interactions detected and N is the total number of consumers for which the average number of interactions is calculated. This type of semi-quantification is not often used in soil ecology, but has shown great potential in aquatic food webs and host-parasitoid webs. In the past, semi-quantitative measurements of detection frequency have been criticized as a rough way of quantification, since it gives no information on the biomass of prey eaten by a predator (Hyslop, 1980). Yet it gives a robust and interpretable description of the dietary composition of organisms, as reviewed by Baker *et al.* (2014). Determining the frequency of interactions makes it possible to gain quantifiable data from presence/absence data of the dietary composition that can be relatively easily gained compared to empirical methods that are necessary to quantitatively describe energy and carbon flows.

2.2.3 Energy flow webs

While the links in connectedness and semi-quantitative webs provide a measure of food web complexity and structure, the arrows and boxes in energy flow webs are weighted in terms of population sizes and the rate with which material is transferred from the resources to the consumers (Figure 2.1c). To construct energy flow webs, information is therefore required on the biomass of resources and soil organisms, as well as the rates of flows of matter among the resources and species within the food web (Moore & de Ruiter, 2012).

Energy flux rates can be calculated by means of the Detrital Food Web Model (DFWM), originally proposed by O'Neill (1969) and subsequently applied to

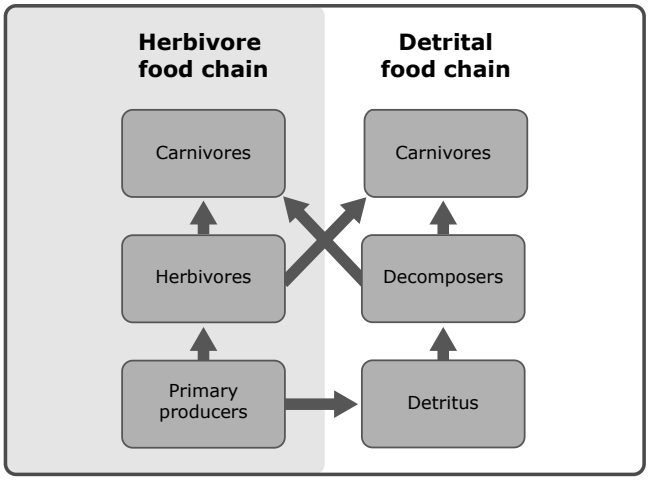

Figure 2.2 The two major soil food chains: left) the herbivore food chain (primary production-based); right) the detrital food chain (decomposition-based).

various food webs from native (Hunt *et al.*, 1987; Berg *et al.*, 2001; Schröter *et al.*, 2003) and agricultural soils (de Ruiter *et al.*, 1993a). The steady-state assumption underlies the DFWM model, i.e. that the production of a population balances the rate of loss through natural death and predation. The model was originally applied to decomposition-based (detrital) food web models, relying on dead organic matter or detritus as a source of energy. However, the model can also be applied well to primary production-based (herbivory) food chains in soil, where carbon and nutrients are originating from living plant biomass. Figure 2.2 combines those two important food chains in soil, showing a great potential of linking them together via soil food web modelling.

Using the steady-state assumption, the feeding rates can be calculated as:

$$F_j = \frac{d_j B_j + M_j}{e_j} \tag{2.2}$$

In this equation, F_j refers to the feeding rate of group j ($kg_{Carbon} ha^{-1} yr^{-1}$), d_j to its specific death rate (yr^{-1}), B_j to the average annual population size ($kg_{Carbon} ha^{-1}$), M_j to the death rate due to predation ($kg_{Carbon} ha^{-1} yr^{-1}$), e_j to the energy conversion efficiency. For predators feeding on more than one prey type, the feeding rate per prey type (F_{ij}) is calculated assuming that the predator feeds on a prey type according to the relative abundance of this prey type and on prey preferences:

$$F_{ij} = \frac{w_{ij} B_i}{\sum_{k=1}^{n} w_{kj} B_k} * F_j \tag{2.3}$$

In this equation, w_{ij} refers to the preference of predator *j* for prey *i* over its other prey types and *n* is the number of trophic groups. k is the numerator of the summation over all (n) trophic groups. The model calculates the feeding rates in a top-down sequence. It starts with the top predators, for which only natural death is assumed, i.e. M_j=0. Hence, in this step all necessary parameters values are available. Then the model proceeds working backwards to the lowest trophic levels. The M_j values then become available through the calculations in the former steps.

What does this DFWM approach require in terms of input data? In addition to the obtained values for population biomasses, only the preferences w_{ij} and the energy conversion efficiencies e_j are required. The energy conversion efficiency is known for most species of soil organisms, but the species diets and preferences are still largely unknown on soil species level. New techniques (introduced below), combined with controlled laboratory experiments, will provide information on the relative preferences w_{ij} of predator *j* for prey *i* up to a higher taxonomic resolution. Such detailed information will make it possible to construct energy flow web models with an increased level of detail and realism.

2.2.4 Functional webs

Where energy flow webs focus on the biomasses of feeding guilds and their interconnecting rates of energy transfer, functional webs originally describe the influence of the species manipulation (not only feeding interactions) on the population sizes of the remaining species (MacArthur, 1972; Paine, 1992) (Figure 2.1d). The relative impact of one species on another within functional webs is described by the interaction strength of a species relationship, comprising both trophic-and non-trophic interactions. Where the removal of a weakly interacting species will not have large consequences for food web structure, the removal of a strongly interacting species can have severe consequences. There are, however, multiple definitions of interactions strengths, and these vary between empiricists and theoreticians (Berlow *et al.*, 2004; Moore & de Ruiter, 2012). In this chapter, we will restrict our discussion to one of the theoretical approaches of interaction strengths by showing how the use of only trophic information can provide interaction strengths, by making use of a community (Jacobian) matrix as was first formulated by May (1974).

The strength of the trophic links is described by the interaction strength. In the theoretical approach, interaction strengths are defined as the 'per capita' (or 'per biomass') effects of the populations upon one another (May, 1972; Pimm, 1982). These values of interactions strength are used as the entries of Jacobian matrix representation of the food webs, which can be used to analyse the stability of the food webs. This procedure is based on the Lotka-Volterra approach, in which the dynamics of the trophic groups are described as:

$$\frac{dX_i}{dt} = -d_i X_i - \sum_{j=1}^{n} c_{ij} X_i X_j + \sum_{h=1}^{n} e_i c_{hi} X_h X_i \qquad (2.4)$$

In this equation, X represents the population sizes of the trophic groups, c_{ij} the coefficient of interaction between group i and group j, and d and e have a similar meaning as in equation (2.2). Sometimes, modifications of this equation are used, for example the equation describing the dynamics of soil organic matter (Moore *et al.*, 1993). From these Lotka-Volterra equations, one can derive interaction strengths as the partial derivatives of the differential equations near equilibrium:

$$\alpha_{ij} = \left(\frac{\delta \dot{X}_i}{\delta X_j}\right)^* \tag{2.5}$$

Here α_{ij} denotes the interaction strength imposed by group j on group i, $\dot{x}_t = \frac{dX_i}{dt}$, and * denotes at equilibrium. Hence, if we obtain empirically-based values for α_{ij} we can build a Jacobian community matrix, analyse stability and evaluate the importance of food web components to food web stability. What is required to obtain empirically-based values for the interaction strengths α_{ij}?

If we take the partial derivatives of equation (2.2), we obtain the following formulas for interaction strength:

$$\alpha_{ij} = -c_{ij} X_i^* \tag{2.6}$$

for the *per capita* effect of predator j on prey i and

$$\alpha_{ji} = e_j c_{ij} X_j^* \tag{2.7}$$

for the *per capita* effect of prey i on predator j.

Values of c_{ij} are difficult to obtain, given the measure of this parameter expressed as 'per amount per time'. However, we can rewrite the formula's for interaction strengths by using the following substitution, i.e. replace the terms $c_{ij}X_i^*X_j^*$ in the Lotka-Volterra equation by the feeding rates, F_{ij}, as estimated by the DFWM, and assuming that the equilibrium population sizes, X_i^*, X_i^*, are represented by the observed population sizes, B_i and B_j. Then we obtain:

$$\alpha_{ij} = -c_{ij} X_i^* = -\frac{F_{ij}}{B_j} \tag{2.8}$$

for the *per capita* effect of predator j on prey i

$$\alpha_{ji} = e_{ij} c_{ij} X_j^* = \frac{e_j F_{ij}}{B_i} \tag{2.9}$$

for the *per capita* effect of prey i on predator j.

From these reformulations of interaction strengths, we see that they can be directly derived from observed biomasses, calculated feeding rates and known energy conversion efficiencies. Then we are able to evaluate the stability of the Jacobian matrix and hence of the food web. This, however, does not provide an empirically-based functional web. To fill in this gap, Neutel *et al.* (2002) proposed to look at food web stability in terms of lengths and weights of trophic interaction loops. A trophic interaction loop describes a pathway of interactions (i.e., not feeding rates) from a species through the web back to the same species without visiting the species more than once; hence a loop is a closed chain of trophic links. An example of such a loop is the soil microbial loop (e.g. Bonkowski, 2004), where carbon is allocated from plant roots to rhizosphere bacteria, which are linked to micro-faunal predators, mainly protozoa. Grazing of protozoa on soil bacteria causes nutrients to be released, which are taken up by plants. These processes are running mainly between bacteria and protozoa with no higher trophic food web levels involved, thereby forming a trophic interaction loop. Such loops may vary in length; the *loop length* being the number of trophic groups visited, and in weight, the *loop weight* being the geometric mean of the interaction strengths in the loop, defined as the per capita effects of the Jacobian matrices. The maximum of all loop weights is an indicator of food web stability. Looking at the weights of trophic loops has a twofold meaning. First of all, it allows us to better understand the patterns in interaction strengths underlying stability. Secondly, it identifies food web components that are key to food web stability, which is close to the functional webs derived from manipulation experiments (*sensu* Paine 1980, 1992), while they can be calculated by using the obtained values for interaction strength.

To summarize, all required parameters for creating empirically-based soil food web models are compiled in Table 2.1. Not all of those parameters are necessary to measure and qualify in empirical experiments, since some of the parameters can be derived as explained above. The following paragraphs will therefore focus on those parameters (f_j, F_j, B_j and w_{ij}) that cannot be derived from formula's (such as parameters M_j, c_{ij} and α_{ij}) or are already known on species level (such as parameters e_j and d_j), but need real-world quantification in order to create empirical-based soil food web models.

2.3 Incorporating empirical information into food web models

The empirical design of food web research largely depends on the questions addressed, and correspondingly the type of food web model one wishes to construct. We therefore provide a comprehensive overview of the available empirical techniques in function of the type of information they can provide and how they can be combined with different theoretical models.

2.3.1 Connectedness webs

Soil food webs usually consist of diverse communities of species arranged along the

Table 2.1 Overview of parameters used to create a connectedness, semi-quantitative, energy flow or functional web. Each food web model is described by the given parameters for that specific type of model, including all of the above. Units are based on their frequent use in detrital-based food web models. Not all parameters need to be measured empirically, since some parameters (e.g. coefficient of interactions and interactions strength) can be derived from equations.

	Name	Description	Unit
Connectedness web		Presence of organisms	[a]
		Feeding relationships: 'who eats whom'	[a]
Semi-quantitative web	f_j	Frequency of interaction	[a]
	B_j	Population size	$kg_{Carbon}ha^{-1}$
Energy flow web	F_j	Feeding rates	$kg_{Carbon}ha^{-1}yr^{-1}$
	d_j	Specific death rate	yr^{-1}
	M_j	Death rate due to predation [b]	$kg_{Carbon}ha^{-1}yr^{-1}$
	e_j	Energy conversion efficiency	[c]
	w_{ij}	Prey preferences	[a]
Functional web	c_{ij}	Coefficient of interaction [d]	$kg_{Carbon}^{-1}\ yr^{-1}$
	α_{ij}	Interaction strength [d]	[a]

Note: units are based on their frequent use in detrital-based food web models. Not all parameters need to be measured empirically, since some parameters (e.g., coefficient of interactions and interactions strengts) can be derived from equations.

[a] Dimensionless

[b] Derived from Eqs. 2.1 and 2.2 (assuming $M_j=0$ for top predators)

[c] Dimensionless if units for prey and consumer population sizes are the same

[d] Derived from Eqs. 2.7 and 2.8

subterranean herbivore and detrital food chains (Scheu, 2002). Disentangling the feeding interactions in these complex communities is not easy as the opaque habitat makes it impossible to observe who is feeding on whom directly. Furthermore, the small sizes of the interacting species, liquid feeding and extra oral digestion complicates the use of a morphology-based assessment of trophic interactions. Soil food web research has thus greatly benefited from the development of techniques that overcome these hurdles, such as molecular biological methods and biomarker approaches.

DNA-based techniques on dietary samples

DNA-based approaches can identify DNA of food remains at high specificity and sensitivity, thereby opening up new possibilities to examine feeding relationships and how organisms are trophically connected in natural communities (Pompanon *et al.*, 2012; Traugott *et al.*, 2013). In brief, food DNA is extracted from the dietary sample, specific short fragments of it are amplified using PCR and the resulting PCR

products are identified either by sequencing or by their length which is indicative for a specific taxon (i.e., diagnostic PCR see below). Molecular techniques can be used for analyzing almost all of the trophic links expressed within soil food webs, including trophic interactions between mesofauna (e.g. Heidemann *et al.*, 2014a) and macrofauna (Juen & Traugott, 2007; Lundgren & Fergen, 2014) - the only requirement is that amplifiable food DNA is present in the dietary sample. Moreover, these techniques are not restricted to analyzing the consumption of fresh food, but it is also possible to detect the DNA of scavenged prey (Juen & Traugott, 2005) and decaying plant material which has been consumed (Wallinger *et al.*, 2013). It is important to point out that this approach does not take into account what has been metabolized by a consumer but merely detects what has been consumed.

A variety of sample types have been used for the molecular study of feeding interaction. The simplest way is to identify food remains, either directly taken from the consumer (e.g. masticated prey from wasps; Kasper *et al.*, 2004) or collected in the environment, is by examining the DNA present within them. In most cases, however, DNA of consumed food present within either feces, the gut content in the consumer and regurgitates is examined. Feces are usually employed to study vertebrate food choice while in invertebrates typically whole-body DNA extracts are used to retrieve food DNA from the gut content (King *et al.*, 2008). As such, gut content analysis is lethal to the consumer. In situations where these post-mortem approaches are not appropriate (i.e. in rare or protected species or when multiple feeding events of individual consumers are of interest) fecal pellets (Boyer *et al.*, 2011) and regurgitates (Waldner & Traugott, 2012) provide a means to obtain dietary samples from invertebrates non-invasively. Fecal pellets of invertebrate decomposers can also be used to assign them to their producer at the species level using PCR-techniques (Seeber *et al.*, 2010), extending the possibilities for molecular profiling of soil faunal communities (Andújar *et al.*, 2015).

Aside from analyzing dietary samples for trophic information, molecular methods can also be extremely valuable to identify the consumer via diagnostic PCR or DNA barcoding (Wirta *et al.*, 2014), providing food webs which are taxonomically highly resolved on both the consumer/host and food/parasitoid sides. The molecular methods used for analyzing trophic interactions can be classified into two basic approaches of 1) diagnostic PCR and 2) sequence-based identification (Traugott *et al.*, 2013). In the former, taxon-specific primers are employed to amplify short fragments of food DNA followed by electrophoretic separation and visualization. The amplification of these specific fragments diagnoses the presence of the targeted DNA in the sample. The level of taxonomic identification can be set according to the needs of the study, i.e. earthworm prey can be identified either generally on a family level using earthworm-group specific primers (e.g. Harper *et al.*, 2005) or down to species and even lineage level (King *et al.*, 2010). Primer pairs for different prey taxa can be mixed together in multiplex PCRs allowing to test dietary samples for several targets in parallel, strongly increasing the efficiency of the analysis (Harper *et al.*,

2005; Sint *et al.*, 2012). In sequence-based food identification, primers are employed that target short DNA fragments of a wide range of food taxa (Pompanon *et al.*, 2012). The resulting PCR products are subjected to high throughput sequencing using next generation sequencing (NGS) techniques. After quality checking and sorting these sequences, they can be assigned tentative identities using either public sequence databases and/or specific reference sequences (Pompanon *et al.*, 2012).

While diagnostic PCR is ideally suited for rapid and low-cost screening of a large numbers of samples, this approach will only allow one to detect the *a priori* selected taxa targeted by the primers. Although several multiplex PCR assays can be used in parallel to detect several tens of taxa within the consumers' diet, this approach becomes inefficient when a broad range of dietary items needs to be examined. In such cases, sequence-based food detection via NGS is advantageous as it allows to explore the diet spectrum of generalist consumers or to obtain dietary information on a population level using a pool of individual dietary samples (Deagle *et al.*, 2009). Sequenced-based diet identification, however, can be hampered by potentially poor coverage of sample sequences in public data bases. Other problems include excessive co-amplification of consumer DNA, which requires the application of blocking primers (Vestheim & Jarman, 2008), the lack of suitably conserved regions for primer bindings sites to allow for amplification of fragments suitable for barcoding a broad range of target taxa (Deagle *et al.*, 2014), and the comparably high costs for testing large numbers of individual samples. The decision of which approach to use largely depends on the nature of the research project and the questions addressed: diagnostic PCR is typically employed for assessing the detection frequency via individual-based dietary analysis using larger numbers of samples which would currently be too costly to be processed by NGS. NGS-based food identification, on the other hand, is most efficient for pooled dietary/consumer samples, allowing to obtain an in-depth picture of the diet of a specific consumer on a population level. Moreover, it is important to consider that the detection of food DNA in dietary samples does not necessarily confirm that the specific food taxon was digested and metabolized into the consumer's tissue. For example, nematodes can have a short bacterial residence time in the intestine which means that not all prokaryote cells are digested (Ghafouri & McGhee, 2007). We advise the reader to consult the latest reviews such as King *et al.* (2008), Symondson (2012), Pompanon *et al.* (2012), Traugott *et al.* (2013) and Clare (2014) for more detailed information.

Lipid analysis

Feeding interactions are generally drawn from primary producers via herbivores to carnivores, suggesting that population development at any given trophic level is limited by populations in the trophic level below. Such bottom-up control is widespread in soil food webs, as decomposers lack influence on the amount of organic matter, e.g. litter, feces, or necromass, available as basal resource. In soil, the bacterial decomposition pathway is predominantly resource controlled, while the

fungal pathway faces greater top-down effects mainly mediated by micro-arthropods (Scheu *et al.*, 2005). A useful way to assess the carbon flux in food webs is the analysis of lipids, namely phospholipid fatty acids (PLFAs) and neutral lipid fatty acids (NLFA). This *in situ* method allows one to assign animal diets and carbon transfer in cryptic systems such as soil food webs (Ruess & Chamberlain, 2010; Traugott *et al.*, 2013), providing information on feeding relationships.

In soil, total fatty acid analyses have successfully been used as a qualitative measure for carbon assimilation in primary and secondary decomposers. To date, most such feeding habits studies have focused on micro- and mesofauna, i.e. nematodes (Chen *et al.*, 2001; Ruess *et al.*, 2002, 2004) and Collembola (Ruess *et al.*, 2004, 2005a; Chamberlain *et al.*, 2005). Only recently have higher trophic levels of the soil food web been considered, such as by taking into account lipid patterns in centipedes and spiders (Haubert *et al.*, 2009; Pollierer *et al.*, 2010; Ferlian *et al.*, 2012). An underlying assumption related to the use of lipids as trophic biomarkers is a concept referred to as "dietary routing", which denotes the transfer of fatty acids from the diet into consumer tissue without modification. This process is well known in vertebrates and used e.g. in food chemistry to assign the origin of dairy products (Molkentin & Giesemann, 2007). Moreover, it was applied frequently in the herbivore food chain of marine ecosystems to monitor predator-prey interactions and carbon flow between phytoplankton and zooplankton (e.g. Müller-Navarra *et al.*, 2000; Stübing *et al.*, 2003; Pond *et al.*, 2006). The basic principle of this approach is that organisms at the base of the food web are capable of synthesizing specific fatty acids, which do not occur in the metabolism of organisms at higher trophic levels, and therefore can be used as biomarkers. Two general types of marker fatty acids have to be distinguished: 1) absolute markers the consumer cannot synthesize, and only appear in the lipid profile when it has fed on the respective diet, and 2) relative markers that are components of consumer metabolism but are additionally highly accumulated from the diet (Ruess *et al.*, 2005a). Ruess & Chamberlain (2010) provide a useful review on method application, advantages and drawbacks in fatty acids as a tool in soil food web analysis.

Table 2.2 provides an overview of fatty acid biomarkers for respectively the herbivore and detrital food chain (Figure 2.2) based on current knowledge. However, future research is likely to reveal additional biomarkers. In plant and algal tissues marker fatty acids occur in both the phospholipid and neutral lipid fractions (Ruess *et al.*, 2007; Buse *et al.*, 2013), yet for soil microbes and fungi these are predominantly found in the phospholipids of membranes (White *et al.*, 1996; Zelles, 1999). One exception is the arbuscular mycorrhiza fungi (AMF), as the marker 16:1ω5 is common in phospholipids of bacteria and fungi, whereas in the neutral lipids it is exclusive to AMF (Ngosong *et al.*, 2012). There is one notable difference in fatty acid transfer, i.e. the direction of feeding interactions: in the classical trophic cascade of the herbivore food chain it is unidirectional, whereas marker fatty acids derived from litter or debris of primary producers also fuel the detrital food chain. An

additional blur between both food chains arises from cross-feeding at higher trophic levels. Generally, tritrophic transport occurs as shown for bacteria-based (bacteria – nematodes – Collembola) and fungal-based (fungi – nematodes – Collembola) food chains (Ruess *et al.*, 2004; Chamberlain *et al.*, 2005) up to top predators such as centipedes (Pollierer *et al.*, 2010). Thus, a marker fatty acid can indicate feeding on a specific diet and/or predation on prey also feeding on this diet. On one hand, this can hamper assignment of a binary link, but on the other hand it allows one to follow the feeding relationships across multiple trophic levels of the food web.

One additional fact makes the application of fatty acids in soil food web studies particularly attractive: after feeding and ingestion of the diet, marker fatty acids are predominantly routed into the neutral lipids of consumers (Ruess *et al.*, 2004; Haubert *et al.*, 2006). As only some actinobacteria possess neutral lipids to a significant extent (Alvarez & Steinbüchel, 2002), the detection of microbial marker fatty acids in consumer storage fat enables distinguishing between viable microbes, in the gut or on the body surface, and the microbial tissue assimilated by the animal grazer. That goes beyond the detection of a bacterial DNA via gut content analysis, as it assigns bacterial carbon allocated in consumer biomass. This is a great advantage in decomposer systems, where bacteria form a basal resource, as it offers the possibility to link microbial and faunal food webs.

2.3.2 Semi-quantitative webs

Molecular prey detection, as discussed earlier in this chapter, usually provides an absence/presence matrix for the food DNA detected within a sample. With the use of these data, one can establish a detection frequency of specific food taxa (f_j) as a proxy for the strength of trophic interactions, which is necessary to construct a semi-quantitative food web. With the use of detection frequency of feeding relationships, it will allow to assess the most important diet resources for the consumer assuming that frequently consumed foods are more important for sustaining the consumer than rarely consumed ones (King *et al.*, 2008; Heidemann *et al.*, 2014b). Moreover, it is important to consider that the analysis of a dietary sample provides a snap-shot picture of the recently consumed food. Therefore, the quality and robustness of the trophic data generated is positively correlated with the number of dietary samples analyzed. Factors such as food and consumer identity (Greenstone *et al.*, 2007; Waldner *et al.*, 2013; Wallinger *et al.*, 2013) can affect post-feeding food DNA detection intervals and need to be considered when analyzing and interpreting molecularly derived trophic data (for reviews see King *et al.*, 2008; Symondson, 2012; Pompanon *et al.*, 2012; Traugott *et al.*, 2013; Greenstone *et al.*, 2014).

Although quantitative PCR (qPCR) allows one to estimate the number of food DNA molecules present within a sample (Zhang *et al.*, 2007), it is of little help for quantifying the number of prey items consumed or estimating the meal size from gut content samples. This is because a small and a big meal digested for a short and a long

Table 2.2 Fatty acid biomarkers useful for determination of carbon flows in respectively the herbivore and the detrital food chain.

Herbivore food chain	Detrital food chain
Plants	**Bacteria**
18:1ω9,	iso/anteiso – Gram positive
18:3ω3,6,9	cyclopropyl – Gram negative
18:3ω6,9,12	
Algae	**Fungi**
16:2ω6,9	16:1ω5 – Arbuscular Mycorrhiza
16:3ω3,6,9	18:2ω6,9 – Ectomycorrhiza and Saprotrophs

time, respectively, can easily provide a similar number of food DNA molecules (King *et al.*, 2008). As it is usually unknown in a field-collected consumer when a feeding event occurred before it was caught, the number of food DNA molecules cannot be used to estimate meal size or number of prey consumed. However, in feces, which are an end-product of digestion, qPCR can provide a semi-quantitative estimate of diet composition (Deagle & Tollit, 2007).

2.3.3 Energy flow and functional webs

Biomarker and molecular-based techniques are not only useful to qualify feeding interactions, but also to quantify those interactions in terms of energy flow. The following paragraphs give an overview of state-of-the-art techniques that are currently used to gather energy flow data for soil ecosystems, complemented with future perspectives on cutting edge techniques to study energy food web models empirically. Table 2.1 shows that the construction of functional webs does not require additional empirical measurements compared to energy flow webs, since the necessary parameters can be derived from equations as introduced in subchapter 2.2. Discussed techniques in this subchapter will therefore provide data for both the construction of energy flow and functional webs.

Stable Isotope Probing

Stable Isotope Probing (SIP) is one of the empirical approaches that has been upcoming in food web ecology over the past decade. SIP combines the use of molecular techniques with the detection of stable isotopes (e.g. ^{13}C and ^{15}N), making it possible to trace flows of matter in food webs on the smallest scale in all trophic levels of the food web. The main idea of SIP is that organisms feeding on a specific stable isotope enriched substrate can be traced by probing the fate of these stable isotopes into cellular biomarkers of active consumers. The big advantage of SIP is the possibility to observe the link between identity and (metabolic) functioning *in situ*,

which can give important information about the structure and flows in food webs.

The term 'Stable Isotope Probing' was used for the first time by Radajewski *et al.* (2000), describing the tracing of a ^{13}C enriched carbon source into microbial DNA. However, the labelling of metabolically active organisms can be followed by tracing labelled biomarkers as DNA, RNA and fatty acids (i.e. DNA-, RNA-, and FA-SIP). Lipids were among the first compounds to be measured after labelling due to the ease in their GC analysis. First approaches using stable isotope labelled substrates was done by the use of microbial lipid analysis (Boschker *et al.*, 1998). Boschker *et al.* (1998) traced the fate of ^{13}C enriched acetate and methane incorporated into PLFAs to link specific environmental processes to the identity of microbial groups involved. In the context of food web studies, PLFA and NLFA extraction in combination with stable isotope probing can give important information about the rate (F_j) and fate (w_{ij}) of feeding interactions and is highly complementary to other culture-independent methods (Maxfield & Evershed, 2011). Moreover, as the $\delta^{13}C$ values of a specific fatty acid is dependent on to the carbon pool it is derived from, i.e. *de novo* synthesis or dietary routing, a ^{13}C label introduced into a food web can be used to examine the route of the ^{13}C-pulse through the web by FA-SIP (Ruess & Chamberlain, 2010).

The DNA-based stable isotope probing (DNA-SIP) technique separates stable isotope labelled 'heavy' DNA from unlabeled 'light' DNA by density gradient centrifugation, after which the DNA can be identified both on a high taxonomic resolution. The possibility to derive high quality taxonomic information regarding the labelled community is the main advantage of DNA-SIP over FA-SIP. On the other hand, DNA-SIP is less sensitive because low DNA synthesis rates limit the enrichment. Therefore, a higher level of stable isotope enrichment compared to FA-SIP is required in order to get sufficient labelling. The low sensitivity of DNA-SIP applies to a lesser extent for the method of RNA-based stable isotope probing (RNA-SIP). RNA-SIP was reported for the first time by Manefield *et al.* (2002a), who studied the degradation of ^{13}C labelled phenol in an industrial bioreactor. RNA synthesis rates are higher compared to DNA, which strengthens the sensitivity of RNA-SIP. Another advantage of RNA is the large amount of information it can give on both the phylogenetic (rRNA) and the functional (mRNA) gene diversity of the labelled organism. Therefore, a combination of RNA-SIP (i.e. identity & functional gene diversity) with FA-SIP (i.e. carbon flux) is ideal to link identity and function of key biota in the food web.

Over the last decade, SIP was mainly used to trace defined microbial groups responsible for the primary degradation of specific substrates. However, labelling occurs also through secondary assimilation of labelled substrates. This so called 'cross-feeding' already occurred during the DNA-SIP experiments of Radajewski *et al.* (2000). Cross-feeding was often seen as an issue since organisms that are not involved in the primary assimilation of a substrate get labelled. However, the phenomenon cross-feeding has great potential to increase insight in trophic interactions among multiple trophic levels in the same habitat (Friedrich, 2011). Experiments designed in time

series provide an excellent opportunity to capture the dynamic nature of carbon flow through soil food webs by studying cross-feeding patterns (Drigo *et al.*, 2010).

Since its first introduction, SIP has been combined with different molecular techniques, and new applications continue to be introduced (Abraham, 2014). Huang *et al.* (2007) studied the incorporation of ^{13}C in microbial cells by combining stable isotope Raman microscopy with fluorescence *in situ* hybridization (FISH), called Raman-FISH. This method can gain increased insight into the incorporation of carbon in organisms on the cellular level, increasing the detailed understanding of energy flows in food webs. Also, the combination of SIP with Raman spectroscopy have led to increased inside in the carbon flows of food webs on the individual microbial level, by achieving a better understanding of energy flows and metabolic pathways in the context of complex food webs (Li *et al.*, 2013). One of the upcoming applications of SIP is to determine both identity and function by targeting organisms with the use of so-called Chip-SIP. This approach uses phylogenetic microarrays and Secondary Ion Mass Spectronomy (NanoSIMS) as a high-sensitivity and high-throughput method to test genomics-generated hypotheses about biogeochemical function in any natural environment (Mayali *et al.*, 2012). Although the use of Chip-SIP is still in its developing phase, it is definitely a promising tool to study the functioning of energy flows within food webs.

Quantitative fatty acid signatures (QFASA)

A prospect for the future in soil food web analysis is the application of quantitative fatty acid signature analysis (QFASA), which makes it possible to assign feeding rates (F_j) to predator diets. QFASA was recently developed as a tool to estimate predator diets in marine mammals such as grey seals, polar bears and seabirds (Iverson *et al.*, 2004; Thiemann *et al.*, 2008; Williams & Buck, 2010). At present, its wide scale application is hampered by the lack in information on specific (lipid pathways) and current metabolism (life cycle, starvation) for most soil animal grazers and predators. Nevertheless, for Collembola, metabolism and pattern of lipids are well known in regard to food quality, environmental factors and biotic constraints such as life cycle and starvation (e.g. Holmstrup *et al.*, 2002; Haubert *et al.*, 2004, 2008; van Dooremalen & Ellers, 2010), which offers a start point for establishment of QFASA in soil food webs. Other promising areas for further investigations using fatty acids are comparison of resources and fecal profiles to assign both consumption on, as well as propagation of, dietary organisms (Buse *et al.*, 2014) or to disentangle trophic from mutualistic processes in plant-microbe-fauna interactions (Ngosong *et al.*, 2014).

Nematode community analysis

The empirical method of nematode faunal analysis uses nematode assemblages to assign conditions to the soil micro-food web such as major decomposition pathways, nutrient status or disturbance (Ferris *et al.*, 2001). Exploring the soil nematode

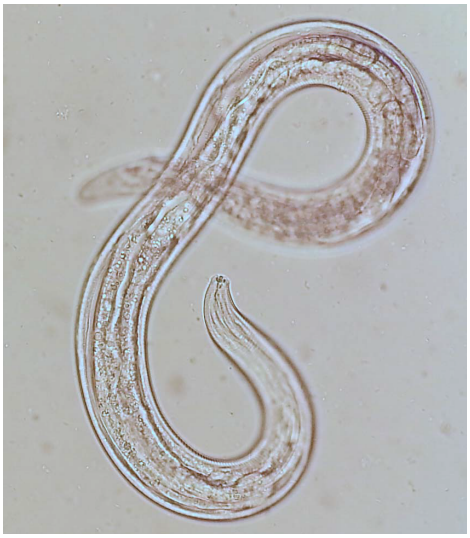

Figure 2.3 *Acrobeloides buetschlii* an opportunistic nematode species and common bacterial feeder in the soil (picture by Veronika Bartel).

community structure provides an empirical method within the framework of feeding guilds and can be used to determine the interaction of the component species within a guild but also to ask questions regarding the interactions between the various guilds that compose the larger community. Thus, nematode communities can serve as a model for general processes in the soil food web and as tool to link microbial and faunal food webs. The latter is particularly important as food web models still lack sufficient quantitative empirical data on carbon and energy flux between microbes and fauna.

The soil micro-food web consists of basal organic resources derived from photoautotrophs (e.g. plant litter, root exudates), the microflora (bacteria and fungi) and the micro- and mesofauna which feed upon the microflora or on each other (Wardle *et al.*, 1998). Within this web, nematodes (Figure 2.3) are the most abundant and diverse multi-cellular organisms with millions of individuals and up to 200 species per square meter (Yeates, 2010). Moreover, nematodes have established functional groups at each trophic level and feed on bacteria, fungi, algae or roots as well as on other microfauna (Yeates *et al.*, 1993). Due to these diverse biological interactions, nematodes hold a central position in both bottom-up and top-down controlled food webs (Ferris, 2010a; Yeates, 2010). In particular those nematodes that graze on bacteria and fungi play important roles in influencing soil microbial biomass, activity and mineralization processes (Bardgett *et al.*, 1999; Griffiths *et al.*, 1999). Thus although nematodes represent only a small amount of biomass in the soil, their key position in the micro-food web impacts on ecosystem-level processes such as energy flow and nutrient cycling (e.g. Yeates *et al.*, 2009; Neher, 2010; Ferris, 2010a).

Nematode abundance (B_j), diversity and effect on soil processes make nematode assemblages useful indicators of food web conditions. By addressing the changes

in horizontal as well as vertical diversity the nematode faunal analysis concept allows determining structure and function of the food web (Table 2.3). Nematode community indices based on life history traits and/or trophic groups are applied to assign soil decomposition pathways (Channel Index) as well as enrichment and structure (Enrichment and Structure Index) of food webs in grassland, arable and forest soil (Ferris *et al.*, 2001; Ruess, 2003; Ruess & Ferris, 2004). In particular the Channel Index is a useful tool to determine the major fluxes of carbon and energy through the soil food web in terms of feeding rates (F_j). Plant effects, i.e. aboveground impact, are expressed in the plant parasite index (PPI, Bongers, 1990), whereas the maturity index is a measure for disturbance and successional stage (MI, Bongers, 1990). Moreover, the establishment of functional groups in both primary production-based (herbivore) as well as decomposition-based (detrital) food chains can allow for a linkage between these two fundamental pathways (Figure 2.2).

Only recently, metabolic footprints of nematodes were introduced as metrics for the magnitude of services provided by feeding guilds in the soil food web (Ferris, 2010b). This approach takes advantage of the standardized morphometric characteristics used in nematode taxonomic description. This comprehensive database facilitates assessment of body volume and weight, which can be converted to carbon metabolism by prescribed coefficients. Until now, soil food web models generally apply abundance data as a proxy, partly combined with values on respiration or functional response. However, this does not take into account the relationship between prey and predator body sizes, which was reported to systematically differ across habitats and consumer types (Brose *et al.*, 2006). Body size relationship is an important factor for interactions strength pattern in food webs and thus affects resilience and stability (Jonsson, 2014). Including metabolic footprints into soil food web analysis, e.g. by making the energy conversion efficiency (e_j) body size dependent, provides an opportunity for a more detailed interpretation of energy flow webs and improves the accuracy of quantitative models. Overall, nematode faunal analysis provides a useful tool for assessing the importance of the different energy channels (i.e. bacterial, fungal, plant), as well as food chains (i.e. herbivore, detrital), in soil food webs and thus for determining food web functioning and energy flux.

Controlled laboratory experiments

Since soil food webs are often difficult to tease apart because of the large number of intertwined and potentially confounding effects, controlled laboratory experiments offer an ideal complement to field experiments. Controlled small-scale experiments offer the possibility to follow in detail single interactions under defined conditions (von Berg *et al.*, 2012). The results can be then straightforwardly connected to genetic and physiological data on the studied organisms (Brose *et al.*, 2008; Brose, 2010; Neidig *et al.*, 2011). In connection with empirical and theoretical predictions (Baiser *et al.*, 2010), laboratory experiments can serve as a basis for parameterizing more complex food webs (Brose *et al.*, 2008; Brose, 2010). Novel approaches of molecular

Table 2.3 Nematode faunal analysis and the respective community indices according to Bongers (1990), Freckman (1988) and Ferris *et al.* (2001) used to assign food web conditions.

Food web condition	Nematode faunal analysis
Bottom up effect of resources	Density and biomass of trophic groups
Decomposition pathways and energy flux	Channel Index, fungal to bacterial feeder ratio
Enrichment and structure	Enrichment Index, Structure Index
Disturbance and/or maturity	Maturity Index
Natural/managed conditions	Plant Parasite Index

marking of food items also hold great promise for experiments in such controlled environments, including the quantification of food consumption in functional response experiments. For example, Mora *et al.* (2014) showed that silica particles containing encapsualted DNA can be used to label food items with the label being detectable for several days post consumption. Moreover, the label gets transferred across trophic levels and includes the possibility to quantify prey uptake using real-time PCR.

Laboratory feeding choice experiments are commonly used to study feeding preferences of soil organisms (w_{ij}). Even generalist predators show distinct feeding preferences. Selective feeding depends on several parameters such as body mass ration, total and relative abundance (Kalinkat *et al.*, 2011). Further, prey properties such as the presence of defensive structures, and active selection processes by the predators can influence which prey will be consumed first (Jousset, 2012). Prey selection has profound impacts on the stability and evolutionary dynamics of the whole community. In controlled systems, it is possible to mix preys at different relative and total abundances and thus accurately determine under which conditions predators will eat which prey. Microcosms can be set up to mimic several environment types such as plant root systems (Jousset *et al.*, 2009), lakes (Jürgens & Simek, 2000) or litter (Vuvic-Pestic *et al.*, 2010). Prey abundance in the diet can be tracked with a vast array of available methods. For instance, prey staining and imaging allow to count remaining, unconsumed preys (Jousset *et al.*, 2009), or even vacuole content for protozoa (Jezbera *et al.*, 2005). Experimental work can subsequently be combined with DNA-based methods and field data, for example for determination of active predation versus scavenging of dead preys (Heidemann *et al.*, 2011).

2.4 Discussion & Conclusions

Soil food web ecologists have just started to exploit (molecular and biochemical) empirical tools to study subterranean feeding networks. Ten years ago, Winemiller & Layman (2005) concluded that empirical food web research is lagging behind theoretical research. The overview provided in this chapter illustrates many of the advancements that have been made over the last decade in empirically testing soil food

Table 2.4 Overview of empirical methods discussed in this chapter. For each method it is specified what type of necessary data the method can provide in terms of empirically-based soil food web modelling.

Methods	Type of data
DNA-based techniques on dietary samples	Presence/absence of specific organisms
	Frequency of interaction (f_j)
qPCR on fecal samples	Frequency of interaction, potential diet composition (f_j)
Lipid analyses	Frequency of interaction (f_j)
Fatty acid analyses on fecal pellets	Frequency of interaction (f_j), assimilation efficiency
Quantitative fatty acid signatures (QFASA)	Feeding rates (F_j)
Stable Isotope probing (SIP)	Feeding rates (F_j), Prey preferences (w_{ij})
FA-SIP	*Carbon flux*
DNA SIP	*Identity*
RNA-SIP	*Phylogenetic (rRNA) / functional (mRNA) gene diversity*
Nematode community analyses	Feeding rates (F_j)
	Population size (B_j)
Food choice experiments	Prey (substrate) preferences (w_{ij})

web models. However, a big challenge remains in bringing theoretical and empirical food web scientists together to take full advantage of the range of possibilities that empirical methods offer for food web modelling. Table 2.4 provides an overview of discussed methods and the type of information each can provide to empirically-based soil food web models. The choice for specific empirical methods will largely depend on the type of questions asked in empirical studies, as well as the properties of food webs that one wishes to obtain. It is thereby especially important to note that the properties of food webs vary depending on the techniques used to reconstruct a food web model (Wirta *et al.*, 2014). It is essential to have a good consideration of multiple empirical techniques, as displayed in the conceptual diagram of Figure 2.4.

Figure 2.4 provides an overview of the type of empirical methods that are suitable to study specific trophic levels, or the soil food web as a whole. Most presented methods allow for the detection of trophic connections in most of the trophic levels of the soil food web. Exceptions are PLFA-SIP analyses (focussing on the microbial part of the soil food web), but combined with different types of lipid analyses, lipids could be traced further into the soil food web. Nematode community analyses focus mainly on the higher trophic levels of the soil food web, acting as 'connectors' between the unexploited microbial part of the soil food web and the faunal food part of the food web that has been described in much more detail. Identifying food remains with the help of DNA-based techniques offers a high specificity and sensitivity and opens entire new possibilities to examine trophic interactions. Especially in combination with controlled feeding experiments, this method is of great value to determine exact feeding interactions, as well as feeding preferences; a combination of results that is of high value for establishing empirically-based soil food web models.

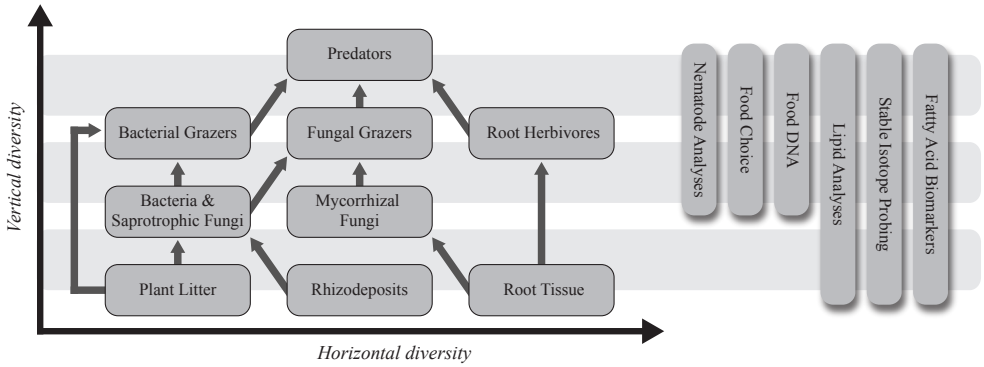

Figure 2.4 Conceptual diagram of a soil food web, showing main feeding guilds and major pathways of carbon and energy. Horizontal diversity refers to diversity within trophic levels and vertical diversity refers to diversity between trophic levels. The right sight of the diagram displays the proposed empirical methods over the range of trophic levels they can be applied to.

In the history of soil food web modelling, there has been a strong divergence between soil food web models that were based upon primary producers (herbivory-based) and models that relied on dead organic matter (detrital-based) (Figure 2.2). Those two types of food webs have been studied in separate areas of research due to large differences in empirical approaches. We expect that emerging methods as described in this chapter will yield large advances in bringing production-based and detrital-based food chains closer together and even link the two fields of research. Not only are the new arising empirical methods able to link different types of food webs, the snap-shot dietary information provided by e.g. DNA-based methods is also ideally suited for assessing the temporal dynamics in soil food webs, a topic which remains largely unexplored. Existing soil food web models could also be further improved by including host-parasitoid relationships. The discussed molecular techniques also offer an effective way to study endoparasitism by detecting for instance the DNA of parasites and parasitoids within the host sample (Agustí *et al.*, 2005; Gariepy *et al.*, 2008; Traugott *et al.*, 2013; Hrček & Godfray, 2015). Pooling empirical techniques into combined detrital- and herbivory-based food webs with host-parasitoid food webs, has therefore a great potential to better understand detailed interactions within soil food webs, as well as the functioning of soil food webs as a whole. Only recently, combining stable isotope analysis of bulk tissues as well as fatty acids gave new insight into allocation and transfer of plant-derived carbon through a food web in an arable soil. The study of Pausch *et al.* (2016) revealed that not bacteria but saprotrophic fungi are most active in these processes, challenging previous views on the dominance of bacteria in root C dynamics in arable soil.

Recent advances in empirical methods will open up new possibilities to study important areas of food web model research e.g. the link between microbial diversity and the functioning of soil food webs or the link between nematode diversity and their impact on soil food web structure. Emerging empirical techniques, as described

in this chapter, can bring a much higher resolution into food web models that will certainly revolutionize our view of soil food webs. The high specificity at which trophic links can be identified raises the characterization of trophic niches for soil invertebrates to a completely new level, allowing a critical evaluation of the commonly used grouping of specific species into feeding guilds. Although empirically-based soil food web models that make use of feeding guilds have proven their value and utility (e.g. Hunt *et al.*, 1987; de Ruiter *et al.*, 1993a; Berg *et al.*, 2001; Schröter *et al.*, 2003) an increased level of detail will be of great value for predictive models that focus on spatial and temporal patterns, as well as models that highlight the importance of specific parts of the soil food web, like the soil microbial community, by bringing in greater phylogenetic resolution.

Chapter 3

Time since land abandonment affects soil microbial litter decomposition efficiency, but not the temporal dynamics within the soil microbial detrital food web

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Abstract

Soil microbial communities impact soil organic matter (SOM) dynamics by influencing litter decomposition, but a detailed understanding of how litter-derived carbon (C) flows through the microbial portion of the soil food web is missing. Information that is necessary to facilitate predictions of soil C cycling and sequestration in response to a changing environment, as for example land use change. To examine the flow of litter-derived C through the soil microbial food web and it's response to land use change, we carried out an incubation experiment with soils from six ex-arable fields, three recently abandoned and three long term abandoned fields. In these soils, the fate of ^{13}C-labelled plant litter was tracked by phospholipid fatty acid (PLFA) analysis over a period of 56 days. The litter-amended soils were subjected to regular sampling to measure $^{13}CO_2$ and mineral N dynamics. Microbial ^{13}C-incorporation patterns revealed a clear succession of microbial groups during litter decomposition. Fungi were first to incorporate ^{13}C-label, followed by G^- bacteria, G^+ bacteria, actinomycetes and micro-fauna. The order of microbial group responses to litter decomposition was similar across all the fields examined, with no clear distinction between recent and long-term abandoned soils. Although the microbial biomass was initially higher in long-term abandoned soils, the net amount of ^{13}C-labelled litter that is incorporated by the soil microbial community was ultimately comparable between recent and long-term abandoned fields. In relative terms, this means there was a higher efficiency of litter-derived ^{13}C-incorporation in recent abandoned soil microbial communities compared to long-term abandoned soils, most likely due to a net shift from SOM-derived C towards root-derived C input in the soil microbial food webs following land-abandonment.

3.1 Introduction

Soil microbial communities play an important role in the global carbon (C) cycle by determining the balance between C respiration and sequestration of C inputs in terrestrial ecosystems. Soils receive their C via two major pathways: the 'green' pathway via readily available plant root exudates and the 'brown' pathway via slowly decomposing dead plant material in the form of litter (Moore *et al.*, 2005). Since most of the soil C input is originating from the plant detritus production, litter fall has a large influence on the soil food web in terms of mineralization processes, nutrient cycling and the formation and sequestration of organic matter (Gessner *et al.*, 2010; van der Wal & de Boer, 2017). Soil microbes (bacteria, fungi, protozoa) have been recognized to function as the 'eye of the needle' through which eventually all organic matters passes (Jenkinson, 1977), thereby determining to a large extent the C partitioning between mineralization (CO_2 evolution) and sequestration. Therefore, it is important to understand how litter-derived C flows through the microbial portion of the soil detrital food web and what the relative timing and activity is of different microbial groups in the overall litter decomposition process. Such details on microbial decomposition would further our understanding of the processes that dictate soil C sequestration and nutrient mineralization.

The input of organic material in soils is known to have consequences for the soil microbial biomass, composition and activity (Wardle, 1992; Heijboer *et al.*, 2016). However, it is still largely unknown what the role is of soil microbial functional groups at different phases of litter decomposition, and the timing and relative contribution of their activity to the overall decomposition process. The introduction of stable isotope probing (SIP) into studies of microbial decomposition affords the opportunity to follow the fate of labelled litter through the soil food web (Morriën, 2016). SIP was for instance used to show that G^- bacteria typically are associated with the consumption of easily degradable substrates (Treonis *et al.*, 2004; Creamer *et al.*, 2016), while G^+ bacteria appear to be more important in the decomposition of SOM (Kramer & Gleixner, 2006, 2008). Interestingly, SIP-based experiments have also suggested saprotrophic fungi as major players in the rapid processing of easily available root C (Pausch *et al.*, 2016). Even though there is information about the functional roles of different microbial groups in litter decomposition, a full analyses of the relative timing and activity of different microbial groups and C flows during the decomposition of a complex realistic substrate like litter is generally missing (Moore-Kucera & Dick, 2008). Information on decomposition processes can be of great value in understanding the effects of different types of environmental change on global C cycling, and will help to improve existing soil food web models with the ultimate aim of predicting soil function and C turnover.

In this study, decomposition was examined in six ex-arable soils, recent and long-term abandoned soils, representing a common form of environmental change: land use change. These ex-arable fields are known to differ in mineralization rates and microbial community structure (van der Wal *et al.*, 2006; Holtkamp *et al.*, 2011;

Morriën *et al.*, 2017). For the selected fields, Morriën *et al.* (2017) have shown that a tightening of the belowground soil food webs following land abandonment is associated with increased nutrient cycling and carbon uptake. This effect is argued to be caused by a shift in activity and composition of the fungal community that is active in the processing of 'green' (rhizosphere-derived) C. The majority of C input in these ex-arable fields is however via the 'brown' pathway (detritus-derived) (Holtkamp *et al.*, 2008). Therefore, the objective of this study was to examine how soil microbial communities differ between recent and long-term abandoned soils in response to litter addition and to examine which functional groups of the soil microbial food web are actively involved in the decomposition of organic matter at different phases of the decomposition process. We hypothesized that litter decomposition activity of the soil microbial community would increase after land abandonment, because of a relative increase in the activity and efficiency of soil fungi, with fungi being better equipped to decompose complex litter. We further hypothesized that the order in which different components of the microbial community are most involved in litter decomposition would remain the same across different land abandonment stages, with G^- bacteria responding more quickly to litter addition than G^+ bacteria.

We conducted a laboratory stable isotope probing (SIP) experiment where six different ex-arable soils were supplemented with a mixture of native plant-derived ^{13}C-labelled litter. The fate of the ^{13}C-labelled litter was followed during the different phases of decomposition by tracing $^{13}CO_2$-evolution and the immobilization of ^{13}C in microbial biomass, which was analysed by phospholipid fatty acid analysis with stable isotope probing (PLFA-SIP). By identifying litter-derived ^{13}C-incorporation patterns in different groups of the microbial community by means of PLFA-SIP, we were able to show that despite initial differences in microbial community structure and a higher microbial biomass in long-term abandoned soils, there are no discernible differences in the absolute amounts of litter processed by the soil microbial communities from different land abandonment stages. However, when looking at the relative amounts of ^{13}C processed by the microbial food web, we observed that recently abandoned soils have a relatively higher ^{13}C incorporation in their microbial biomass compared to long-term abandoned soils, caused by a higher microbial uptake efficiency of

Table 3.1 Overview of ex-arable field sites where soil samples were taken, including information on the exact location, land abandonment stage and time of abandonment of agricultural practices.

Site	Location	Land abandonment	Abandoned since
TW - Telefoonweg	N 52°00'9 E 5°45'8	Recent	2009
RK - Reijerskamp	N 52°1'0 E 5°46'21	Recent	2005
OR - Oud Reemst	N 52°2'27 E 5°48'34	Recent	2005
DK - Dennenkamp	N 52°1'43 E 5°48'2	Long-term	1982
MV - Mosselse Veld	N 52°4'23 E 5°44'13	Long-term	1985
BB - Boersbos	N 52°3'44 E 5°59'57	Long-term	1982

litter-derived C in recently abandoned soils. In addition, we demonstrated that litter decomposition followed a clear succession in time between different microbial groups of the microbial food web, with the following order of activity: fungi > G^- bacteria > G^+ bacteria ≥ actinomycetes > micro-fauna, and this pattern was consistent across all soils included in our study.

3.2 Material and methods

3.2.1 Site description and soil sampling

Soil samples were collected from six ex-arable fields, all located on the same glacial sandy soil deposits in the centre of the Netherlands (Veluwe). All fields had a similar previous crop-rotation history and are part of a well-described chronosequence of ex-arable fields (Kardol *et al.*, 2005; van der Wal *et al.*, 2006; Holtkamp *et al.*, 2008; Morriën *et al.*, 2017). Three of the sampled fields are referred to as recently abandoned fields, since they have been abandoned from agricultural practices for 5-9 years. The other field sites have been abandoned for 29-32 years and are therefore referred to long-term abandoned fields. Details of these study sites are given in Table 3.1. Soil sampling took place in October 2014 by collecting 8 soil samples (10 cm deep, 6 cm diameter) per ex-arable field. Soil samples were taken at least 20m from the edge of a field, and at least 10m apart. Aboveground vegetation was removed prior to transportation. Soil samples were transported and stored at 4°C prior to further treatment.

3.2.2 Production of ^{13}C-labelled litter

In the summer of 2012, ten soil cores (12 cm diameter, 20 cm deep) were collected per field site, including standing vegetation. Nine of those cores were transported to the laboratory and labelled with 99.99 atom% $^{13}CO_2$ (Cambridge Isotope Laboratories, Andover, MA, USA) for 13 hours; for details see (Morriën *et al.*, 2017). The remaining core served as a non-labelled control core. Aboveground biomass was harvested, freeze dried and ground (Retsch ZM100, Haan, Germany). The $\delta^{13}C$ value of the ground plant material was measured on an elemental analyser (Flash2000, Thermo) attached to an isotope ratio mass spectrometer (IRMS, Thermo) and revealed an average $\delta^{13}C$ of 904.0 and -25.7 for respectively ^{13}C-labelled and unlabelled control plant material. The labelled plant material was used as a ^{13}C-labelled substrate in the incubation experiment (see below) and is further referred to as ^{13}C-labelled litter.

3.2.3 Experimental design: ^{13}C incubation experiment

Soil samples were sieved (2 mm) to remove roots and stones and subsequently pooled into one composite soil sample per field site. For each of the six field sites, a total of 30 glass bottles of 333 ml were filled with an equivalent of 50 grams of dry weight

soil. Three out of every five bottles were supplemented with 0.5 gram of ^{13}C-labelled litter, one out of every five bottles received 0.5 gram of unlabelled litter (positive control, C^+) and the remaining bottles received no litter (negative control, C^-). C inputs corresponded to 10-15% of the total organic matter C in the fields used in our study (Holtkamp *et al.*, 2008). MilliQ water was added to the composite soil/litter samples until a WHC of 60% was reached. The bottles were closed with a cotton ball to avoid contamination, while permitting gas exchange and incubated in the dark at 17.5°C. One-sixth of all bottles was destructively sampled at each of the following time points: 1, 3, 7, 14, 28 and 56 days. Per field site, this corresponded to three labelled samples (technical replicates), plus a positive and negative control sample. A subsample of each soil (equivalent to 5g dry weight of soil) was stored at 4°C prior to the determination of extractable nutrients. Remaining soil was stored in the freezer at -80°C prior to further analyses.

3.2.4 $^{(13)}CO_2$ respiration rates

During the entire experimental period of 56 days, $^{13}CO_2$ flux measurements were performed. The 30 bottles that were only harvested on day 56 were subjected to gas sampling throughout the entire experiment. On day 1, 3, 7, 14, 28 and 55 (because of destructive sampling on day 56), these bottles were closed with a lid with a rubber septum. Directly after closure, an air sample of 15 ml was taken from the headspace (t=0) using a 25 ml analytical syringe (SGE Analytical Science, Australia), and stored in a 12 ml Labco Exetainer® 12 ml vial. The headspace air pressure-drop was compensated by injecting 15 ml of N_2 gas. A second gas sample was taken after ±4.5 hours (t=1). Gas samples were analysed on a Thermo Trace Ultra GC interfaced with a methanizer-FID or a Thermo Scientific Delta V IRMS to determine respectively CO_2 and $\delta^{13}CO_2$ values (Rt-Q-BOND column; 30m, 0.32 mm id, 10 µm film thickness). The CO_2 concentration and $\delta^{13}CO_2$ values at t=0 and t=1 were used to calculate the CO_2 and $^{13}CO_2$ respiration rates on each sampling date.

3.2.5 Extraction and analyses of PLFA

Phospholipid fatty acids (PLFAs) were extracted from 4g of each soil sample following the procedure of Frostegård *et al.* (1993) and Hedlund (2002), based on the method of Bligh & Dyer (1959) and White *et al.* (1979). PLFA extractions were analysed on a gas chromatograph (GC-FID, 7890A, Agilent technologies, Delaware, USA) to determine the abundance of PLFA biomarkers. The $\delta^{13}C$ value for each PLFA biomarker was determined by analysing PLFA extractions on a Thermo Trace Ultra GC, interfaced with a Thermo Scientific Delta V IRMS. For both GC analyses an Agilent HP-5MS UI column (60m, 0.25 mm id, 0.25 µm film thickness) was used. PLFA biomarkers were used to characterize different microbial groups: fungi (18:2ω6), G^- bacteria (16:1ω7, cy17:0, 18:1ω7), G^+ bacteria (a15:0, a17:0, i15:0, i16:0, i17:0), actinomycetes (10Me16:0, 10Me17:0, 10Me18:0) and microfauna (20:4,

Wilkinson *et al.*, 2002). A total number of 24 PLFA biomarkers were used to study soil microbial community composition and to calculate total microbial biomass. The actual $\delta^{13}C$ value of each PLFA biomarker was calculated as described by Boschker (2004):

$$\delta^{13}C_{PLFA} = ((n+1) * \delta^{13}C_{FAME} - 1 * \delta^{13}C_{methanol})/n \quad (3.1)$$

where n is the number of C atoms in the PLFA biomarker. The $\delta^{13}C_{PLFA}$ of labelled and unlabelled control samples was used to calculate the actual excess amount of ^{13}C in each PLFA biomarker (Boschker, 2004).

3.2.6 Extractable nutrient analysis

Within 24h after sampling, soil samples were extracted with 100 ml 0.2M KCl. Samples were shaken for 1h at 120 rpm on a rotary shaker (Laboshake, Gerhardt, Germany). Samples were centrifuged for 4 minutes at 4000 rpm (Megafuge 1.0R, Heraeus Instruments, Germany) and filtrated with glass fibre filters (Whatman GF/C, Whatman, Germany). All samples were analysed spectrophotometrically for NO_3-N and NH^{4+}-N on a segmented flow analyser (Skalar SanPlus, Skalar Analytical, the Netherlands). Total available N content was calculated by summing NO_3-N and NH_4^+-N.

3.2.7 Calculations of litter-derived C

To calculate the relative amounts of PLFA and CO_2 that were litter-derived, the following formula was used:

$$\%C_{litter} = \frac{\delta^{13}C_{sample} - \delta^{13}C_{reference}}{\delta^{13}C_{litter} - \delta^{13}C_{soil}} * 100 \quad (3.2)$$

where $\delta^{13}C_{sample}$ refers to the tested sample, $\delta^{13}C_{reference}$ to the corresponding control sample without litter addition and $\delta^{13}C_{litter}$ and $\delta^{13}C_{soil}$ respectively, to the added litter and soil at the start of the experiment.

3.2.8 Data analysis & Statistics

Results were analysed using R version 3.1.0 (R Core Team, 2014). Mineral N, CO_2 and PLFA abundances, excess^{13}C and $\%C_{litter}$ differences between land abandonment stages and over time were tested using linear-mixed effect modelling (*nlme* package, Pinheiro *et al.*, 2017), combined with the *car* package (Fox & Weisberg, 2011) for type II sum of squares with 'Field' as a fixed factor. Shapiro's test was used to check normality of residuals, Levene's test (*car* package) to test for heterogeneity of variances, and data was log or power transformed where necessary.

Abundances, ^{13}C-excess and %litter-derived of individual PLFA biomarkers were used as input values for the principal component analysis (PCA) to check for differences in (functional) microbial community structure between different fields and the two land abandonment stages, whereby indicative microbial groups were plotted in a PCA diagram.

To visualize the sequential order of microbial groups throughout the first stages of litter decomposition, the excess amount of ^{13}C in different microbial groups was normalized over time using the following formula:

$$Normalized\ Excess^{13}C = \frac{Excess^{13}C - mean(Excess^{13}C)}{sd(Excess^{13}C)} \qquad (3.3)$$

where $Excess^{13}C$ is the excess amount of ^{13}C in the PLFA of a specific microbial group at a specific time point, with mean($Excess^{13}C$) and sd($Excess^{13}C$), respectively, the average and standard deviation of the excess amount of ^{13}C for that same microbial group over the complete incubation time. Linear-mixed-effect modelling was used to test whether normalized $Excess^{13}C$ patterns differed between microbial groups over time, with 'Field' as a fixed factor. Absolute value transformation was applied to meet homogeneity and normality requirements.

3.3 Results

3.3.1 Soil microbial community development following land abandonment

Comparison of all control samples (no litter addition) based on abundance of phospholipid fatty acid (PLFA) biomarkers revealed an effect of land abandonment stage on the composition of the soil microbial community (Figure 3.1). Long-term abandoned fields exhibit a large degree of variation, although much of this is along the second PCA axis, while the first axis explains the vast majority of the total variation (82%). The microbial communities of the long-term abandoned fields, Dennenkamp (DK) and Mosselse Veld (MV), are strongly characterized by a relatively high abundance of G^- bacteria and fungi, while the abundance of micro-fauna, G^+ bacteria and actinomycetes was more pronounced in soil microbial communities of all long-term abandoned fields. When testing the total abundance of microbial PLFA biomarkers, we observed that there is a significantly higher microbial biomass in long-term abandoned soils compared to recently abandoned soils (χ^2=8.5307, p=0.003**). For specific microbial groups, there is a trend of higher abundances in long-term abandoned soils, compared to recently abandoned soils, however these differences are only significant for G^+-specific PLFA biomarkers (χ^2=8.5639, p=0.003**) and PLFA biomarkers specific to actinomycetes (χ^2=5.6971, p=0.017*). The abundance of PLFA biomarkers specific to fungi, G^- bacteria and micro-fauna, as well as the fungal/bacterial and G^-/G^+ bacterial ratio, were not significantly different between the two land abandonment stages (see Table 3.2 for a complete overview). When looking at

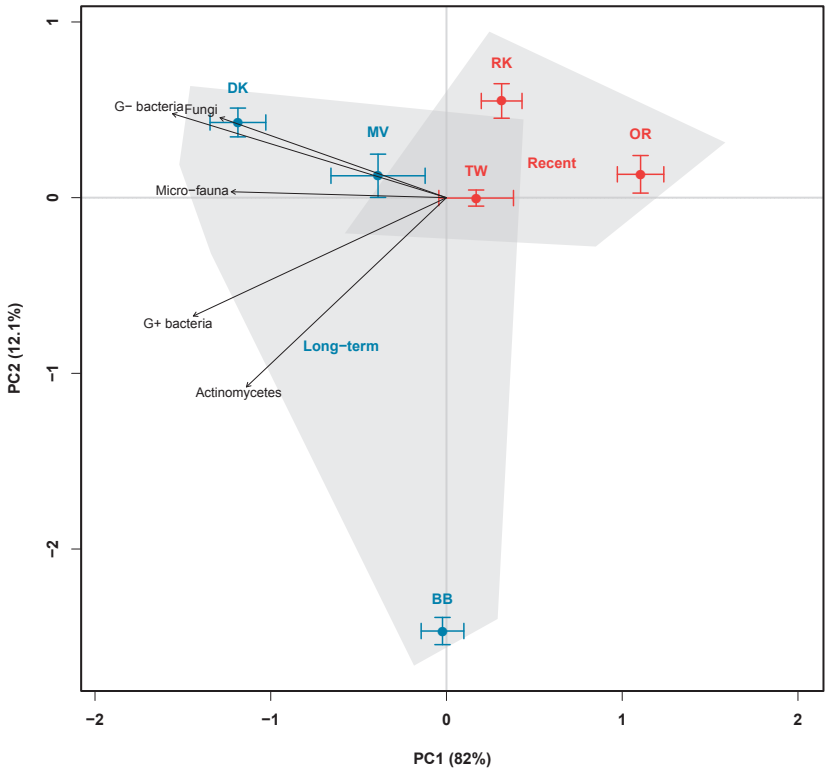


Figure 3.1 Principal component analysis (PCA) of the relative abundance of soil microbial PLFA biomarkers to characterize soil microbial community composition. All six fields are indicated by mean ± SE, where red represents recently abandoned fields and blue represents long-term abandoned fields. Arrows show indicative microbial groups (R^2 with $p<0.05$).

Table 3.2 Soil microbial community abundances and ratios based on PLFA biomarkers for each land abandonment stage (recent and long-term). The first row shows mean ± SE of the total abundance of PLFA biomarker in the soil, subsequent rows show the mean ± SE abundance of PLFA specific biomarkers for different microbial groups. χ^2 and p-values are the result of linear-mixed modelling (*** $p<0.001$, ** $p<0.01$, * $p<0.05$, · $p<0.1$).

	Land abandonment stage				
	Recent	*Long-term*	χ^2	*p*	
Total PLFA (nmol C g soil^{-1})	872.33 ± 35.11	1177.36 ± 37.50	8.4286	**0.004**	**
Fungal PLFA (nmol C g soil^{-1})	18.29 ± 1.34	22.08 ± 1.57	0.8014	0.371	
G^- bacterial PLFA (nmol C g soil^{-1})	182.4 ± 10.01	254.94 ± 16.43	1.7844	0.182	
G^+ bacterial PLFA (nmol C g soil^{-1})	190.23 ± 6.75	261.58 ± 9.09	8.5639	**0.003**	**
Actinomycete PLFA (nmol C g soil^{-1})	70.71 ± 3.23	110.16 ± 5.99	5.6971	**0.017**	*
Micro-faunal PLFA (nmol C g soil^{-1})	9.37 ± 0.66	13.03 ± 0.60	2.801	0.094	.
Fungal/bacterial PLFA ratio	0.041 ± 0.002	0.035 ± 0.002	2.209	0.137	
G^+/G^- PLFA ratio	1.07 ± 0.03	1.10 ± 0.09	0.098	0.754	

the relative abundances for individual biomarkers, more than half of all biomarkers showed a (marginally) significant higher abundance in long-term abandoned soils compared to recently abandoned soils (Supplementary Table S3.1).

3.3.2 C and N mineralization patterns following litter addition

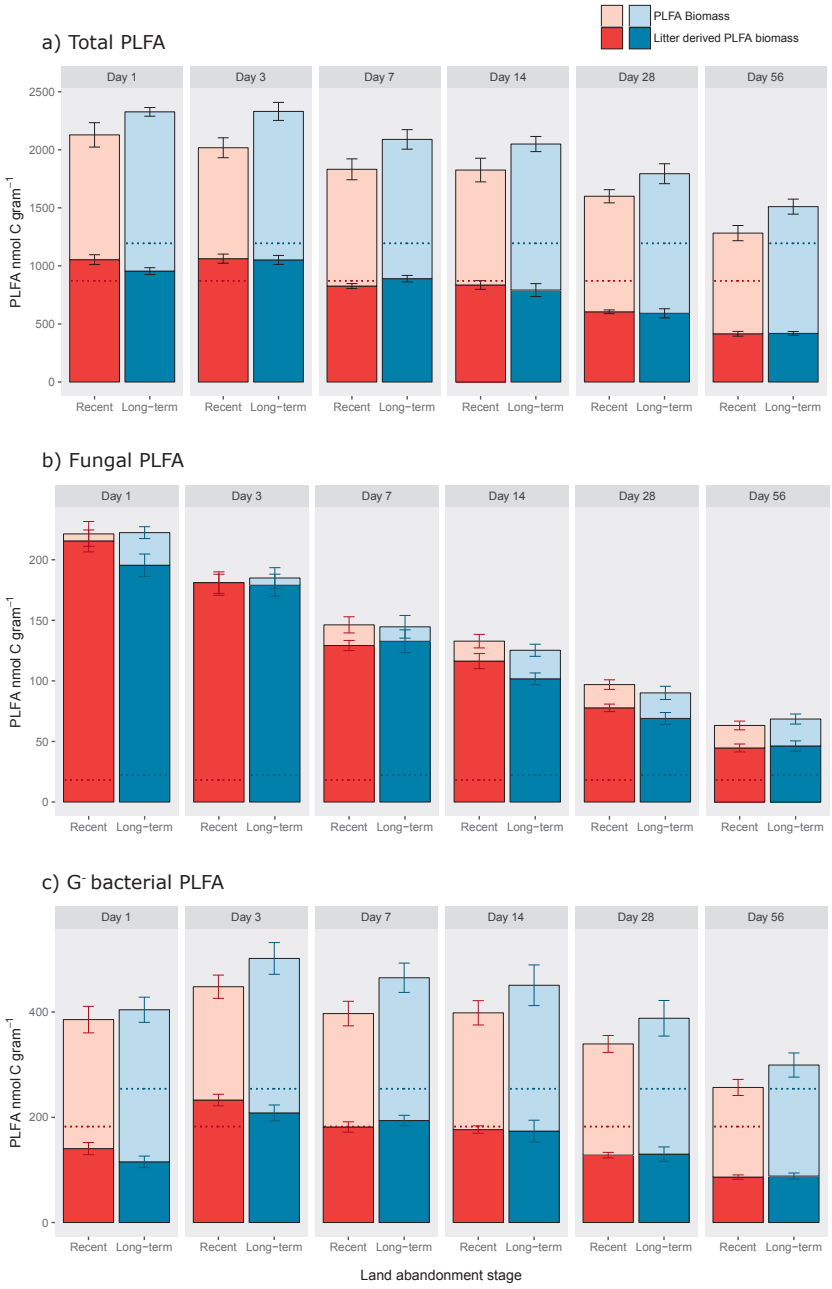
One day after the addition of ^{13}C-labelled litter there was more than a tenfold increase in the total amount of respired CO_2, which gradually decreased back to the control level over the course of the experiment (Table 3.3). Overall, long-term abandoned soils showed a marginally significant higher CO_2 respiration compared to recently abandoned soils (χ^2=3.2936, p=0.070˙). The absolute and relative amounts of litter-derived CO_2 were not significantly different between the different land abandonment stages, although there were significant interaction effects between land abandonment stage and time. The total amount of mineral N in recently abandoned soils decreased compared to the control treatment until day 7, while the mineral N content for long-term abandoned soils showed an initial increase followed by a similar trend as observed for recently abandoned soils (Table 3.3). After day 7, the amount of mineral N increased up to the end of the incubation experiment (Table 3.3). No significant differences were found in mineral N dynamics between recent and long-term abandoned soils.

3.3.3 Soil microbial community response to litter addition

Directly one day after the addition of ^{13}C-labelled litter, the total amount of PLFA in both recent and long-term abandoned soils increased from approximately 1000 to more than 2000 nmol C per gram soil, whereby the newly build up PLFA biomass corresponds to the amount of PLFA biomass that was litter-derived (Figure 3.2a). After day 3, the total amount of PLFA biomass gradually decreased, whereby at day 56 the amount of PLFA biomass was still slightly increased compared to the situation without litter addition. Overall, there was only a marginally significant higher biomass in long-term abandoned soils compared to recently abandoned soils (χ^2=2.814, p=0.093). The higher microbial biomass in long-term abandoned soils was mainly due to a relatively higher increase in bacterial biomass in response to litter addition, compared to recently abandoned fields, reflected by a significantly higher fungal/bacterial ratio for recently abandoned fields (0.186 ± 0.009) compared to long-term abandoned fields (0.158 ± 0.008) over the course of the experiment (χ^2=4.2319, p=0.039). No significant differences in the absolute amounts of litter-derived PLFA biomass (χ^2=0.2295, p=0.632) were found between land abandonment stages. However, when comparing the relative amounts of litter-derived PLFA biomass (as a percentage of PLFA biomass) between recent and long-term abandoned soils, there was a significant interaction effect between land abandonment stage and time (stage*time: χ^2=11.9256, p=0.036), with a relatively higher percentage of litter-derived PLFA in recently abandoned soils. Similar patterns were observed when examining

Table 3.3 Carbon and nitrogen mineralization parameters for recent and long-term abandoned ex-arable soils during incubation at each respective sampling moment. Control measurements refer to samples without litter addition and were excluded from linear mixed modelling from which χ^2 and p-values are shown in the latter two columns (*** p<0.001, ** p<0.01, * p<0.05, · p<0.1).

				Time (days)									
Abiotic measurement	*Unit*	*Land abandonment stage*	*Control*	*1*	*3*	*7*	*14*	*28*	*56*		χ^2	*p*	
Carbon mineralization	µg C g soil^{-1} day^{-1}	Recent	7.177	89.095	52.6	38.347	25.563	16.333	8.762	Stage:	3.2936	0.070	.
		Long-term	8.647	96.569	59.423	39.151	28.939	19.616	6.68	Stage*Time:	7.1933	0.207	
Litter-derived $^{13}CO_2$	µg ^{13}C g soil^{-1} day^{-1}	Recent		45.851	18.15	9.191	2.89	1.369	0.404	Stage:	1.3163	0.251	
		Long-term		50.572	20.407	8.87	4.161	1.804	0.414	Stage*Time:	20.796	**<0.001**	***
Litter-derived CO_2	%	Recent		51.093	34.457	23.805	11.213	8.286	4.583	Stage:	0.2719	0.602	
		Long-term		52.729	34.282	22.355	14.354	9.082	4.245	Stage*Time:	31.578	**<0.001**	***
Mineral nitrogen	nmol g soil^{-1}	Recent	316.73	243.50	118.70	105.36	240.24	506.94	833.49	Stage:	1.5005	0.221	
		Long-term	164.43	281.90	110.90	118.70	319.08	714.07	898.55	Stage*Time:	0.1335	0.715	


PLFA Biomass
Litter derived PLFA biomass
a) Total PLFA
b) Fungal PLFA
c) G- bacterial PLFA
Day 1
Day 3
Day 7
Day 14
Day 28
Day 56
PLFA nmol C gram-1
Recent
Long-term
Land abandonment stage

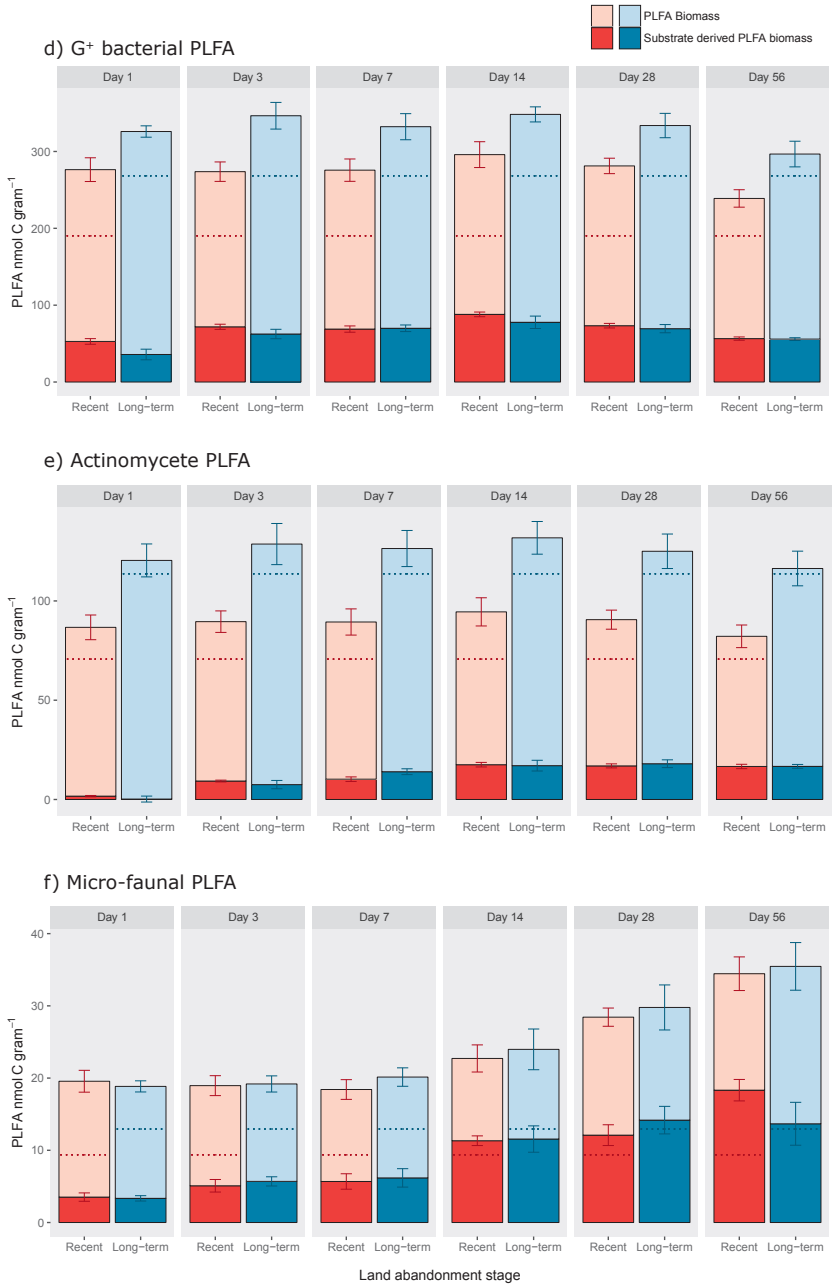


Figure 3.2 Means (± SE) PLFA biomass for different groups of PLFA biomarkers at respective sampling times, where the amount of litter-derived biomass is highlighted (± SE). Panel a) represents the total amount of PLFA biomarkers found, whereby the remaining panels show biomass and labelling patterns for specific PLFA biomarkers that were appointed to the microbial groups b) fungi, c) G- bacteria, d) G+ bacteria, e) actinomycetes and f) microfauna. Dotted lines show the corresponding control PLFA biomass without litter addition.

individual PLFA biomarkers, where only a few biomarkers were significantly affected by land abandonment stage during the incubation experiment when testing for PLFA biomass (Supplementary Table S3.2) and the absolute amount of litter-derived PLFA biomass (Supplementary Table S3.3). The relative amounts of litter-derived PLFA biomass was significantly affected by land abandonment stage for nine out of the 24 individual PLFA biomarkers analysed (Supplementary Table S3.4).

To visualize the effects of land abandonment stage and time on PLFA biomass and the absolute and relative amount of litter-derived PLFA, the information for all 24 PLFA biomarkers was combined in a principal component analysis (PCA) based on PLFA biomass (Figure 3.3a), the absolute amount of litter-derived PLFA (Figure 3.3b) and the relative amount of litter-derived PLFA biomass (Figure 3.3c). These analyses reveal a strong time effect during the incubation experiment for all three microbial community properties, whereby only the relative amount of litter-derived PLFA was clearly affected by land-abandonment stage (Figure 3.3c). The PCA revealed that some indicative microbial groups change with time, with fungal PLFA being relatively abundant and enriched at the start of the experiment (Figure 3.3), with the enrichment of G^+ bacteria and actinomycetes being indicative for later stages of ^{13}C-litter decomposition (Figure 3.3b, c).

The microbial community properties (PLFA biomass, absolute and relative amount of litter-derived PLFA) are shown with respect to specific microbial groups: fungal PLFA (Figure 3.2b), G^- bacterial PLFA (Figure 3.2c), G^+ bacterial PLFA (Figure 3.2d), actinomycete PLFA (Figure 3.2e) and micro-faunal PLFA (Figure 3.2f). For PLFA biomass, there was only a significant higher amount of PLFA biomarkers for G^+ (χ^2=4.612, p=0.032) and actinomycete PLFA biomarkers (χ^2=3.869, p=0.052) in long-term abandoned soils. There were no significant effects of land abandonment stage on the absolute amount of litter-derived PLFA biomass for any of the microbial groups. When testing the fungal/bacterial ratio of excess ^{13}C there is no effect of land abandonment stage (χ^2=0.0176, p=0.894), however there was a significant interaction effect (time*stage: χ^2=11.474, p=0.043). Thus, for most microbial groups there was a (marginally) significant interaction effect (time*stage) when looking at the percentage of litter-derived PLFA biomass (Table 3.4), with only the fungal PLFA showing no detectable effect of land abandonment stage.

3.3.4 Succession of soil microbial groups during litter decomposition

When examining the build-up of litter-derived PLFA biomass in Figure 3.2, we found that each microbial group had its own distinct incorporation pattern. This observation is apparent in Figure 3.3 where indicative microbial groups change over time during litter decomposition. This is also shown when looking at the ^{13}C excess patterns over time for individual PLFA biomarkers, with distinct patterns for different PLFA biomarkers (Supplementary Figure S3.1). To be able to compare these ^{13}C incorporation patterns between microbial groups, the amount of excess

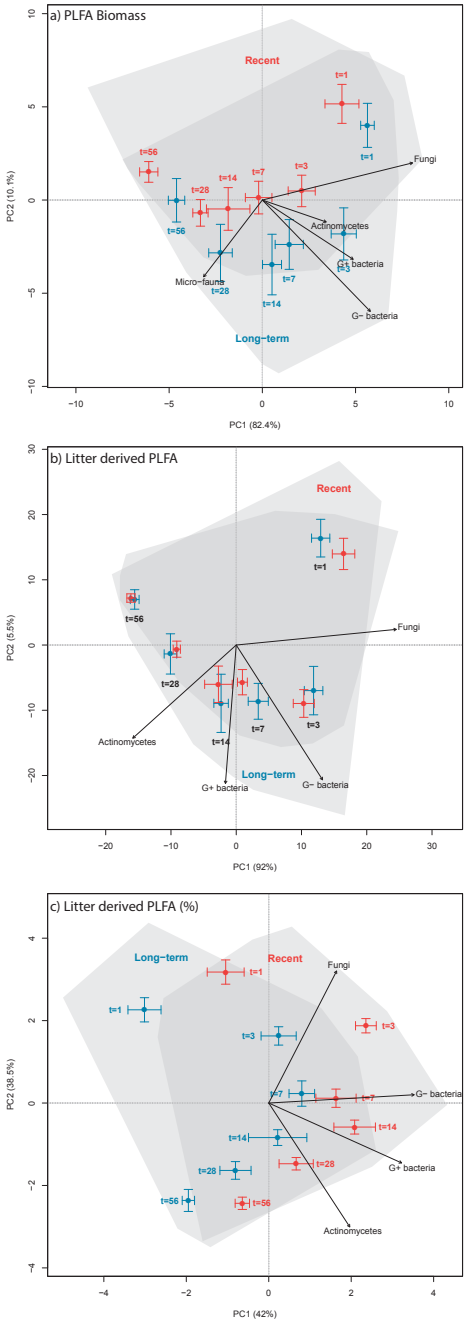


Figure 3.3 Principal component analyses of phospholipid fatty acids (PLFA) biomarkers to characterize soil microbial community structure in ex-arable soils (recent abandonment = red vs long-term abandonment = blue) and at different time points after ^{13}C-labelled litter addition. PCA a) is based on the relative abundance of 20 different PLFA biomarkers. PCA b) is based on the absolute amount of each PLFA biomarker that was litter-derived. PCA c) is based on the relative amount of each PLFA biomarker that was litter-derived. Arrows show indicative microbial groups (R^2 with $p<0.05$).

Table 3.4 Relative amount PLFA biomarker abundance for specific microbial groups that was litter-derived (%). Time indicates the total time after ^{13}C-labelled litter addition for both recent and long-term abandoned soils. χ^2 and p-values are the result of linear-mixed modelling (*** p<0.001, ** p<0.01, * p<0.05, · p<0.1).

		Time (days)									
	Land abandonment stage	*1*	*3*	*7*	*14*	*28*	*56*		χ^2	*p*	
Total PLFA	Recent	50.02	52.85	45.72	46.31	38.13	32.52	Stage:	5.759	**0.016**	*
	Long-term	41.07	45.22	42.78	38.45	32.87	27.82	Stage*Time:	11.926	**0.036**	*
Fungal PLFA	Recent	97.79	101.03	89.17	87.62	80.60	70.38	Stage:	2.056	0.152	
	Long-term	87.83	96.83	91.82	81.31	76.20	66.78	Stage*Time:	7.450	0.189	
G^- bacterial PLFA	Recent	37.15	52.21	46.13	45.02	38.13	33.87	Stage:	7.425	**0.006**	**
	Long-term	28.37	41.42	41.88	37.64	33.05	29.69	Stage*Time:	9.569	0.088	.
G^+ bacterial PLFA	Recent	19.42	26.56	25.33	30.27	26.32	23.77	Stage:	2.434	0.119	
	Long-term	11.25	18.44	21.67	22.56	21.01	19.16	Stage*Time:	14.569	**0.012**	*
Actinomycete PLFA	Recent	1.95	10.53	12.02	18.73	18.80	20.29	Stage:	0.578	0.447	
	Long-term	0.65	6.47	12.03	13.91	15.29	15.39	Stage*Time:	25.612	**<0.001**	***
Micro-fauna PLFA	Recent	17.42	17.51	30.67	51.18	43.98	52.95	Stage:	0.574	0.449	
	Long-term	17.80	29.24	30.71	46.29	46.00	37.83	Stage*Time:	13.016	**0.023**	*

^{13}C was normalized for each microbial group, and all group-specific temporal patterns were subsequently plotted together for recent (Figure 3.4a) and long-term abandoned soils (Figure 3.4b). Figure 3.4 shows that fungi have their highest amount of ^{13}C incorporation already 1 day after the addition of ^{13}C-labelled litter, with a steep decrease in the amount of incorporated ^{13}C directly afterwards. G^- bacteria also accumulate ^{13}C rapidly, but reach their peak in ^{13}C incorporation at day 3. G^+ bacteria and related actinomycetes both reach their maximum level of litter-derived ^{13}C incorporation at day 14. However, after this peak, the amount of excess ^{13}C remained relatively stable in actinomycetes in contrast to the rapid decrease observed for ^{13}C in G^+ bacteria. The PLFA biomarker specific to micro-fauna reaches its maximum amount of ^{13}C incorporation only after 28 days, after which it remains generally stable. When testing the normalized amounts of ^{13}C excess, there is a significant interaction effect between microbial group and time (time*microbial_group: χ^2=207.845, p<0.001), confirming distinct ^{13}C incorporation patterns between microbial groups. The ^{13}C incorporation patterns found for microbial groups over time are unaffected by land abandonment stage (stage*time*microbial_group; χ^2=20.702, p=0.415).

3.4 Discussion

In this study, we sought to examine how soil microbial communities and soil microbial C flows in ex-arable soils respond to litter addition and subsequent litter decomposition in both recent and log-term abandoned soils, with the ultimate goal of providing quantitative litter-derived C flows through the soil microbial community in response to a common form of land-use change. Litter-derived C flows followed a clear succession of microbial groups in the detrital microbial food web (fungi > G^- bacteria > G^+ bacteria ≥ actinomycetes > micro-fauna). This pattern was remarkably comparable across all soils, regardless of land abandonment stage. Furthermore, we also observed that in terms of absolute energy flows there are no differences between microbial communities of different ex-arable soils. However, microbial communities showed a higher uptake efficiency of litter-derived C in recently abandoned soils as compared to long-term abandoned soils.

3.4.1 Influence of litter addition and land-abandonment stage on the soil microbial community

We hypothesized that long-term abandoned soils would have a higher capacity for decomposition of added litter, as compared to recently abandoned soils. Our results show that long-term abandoned soils had an initial higher microbial biomass soils and that this difference persisted after litter addition. This might have caused a higher (litter-derived) C mineralization rate after litter addition in long-term compared to recently abandoned soils. Even though microbial parameters are often not statistically differing between secondary states, due to a low number of replicates, there is a clear trend toward higher soil microbial biomass and activity in long-term

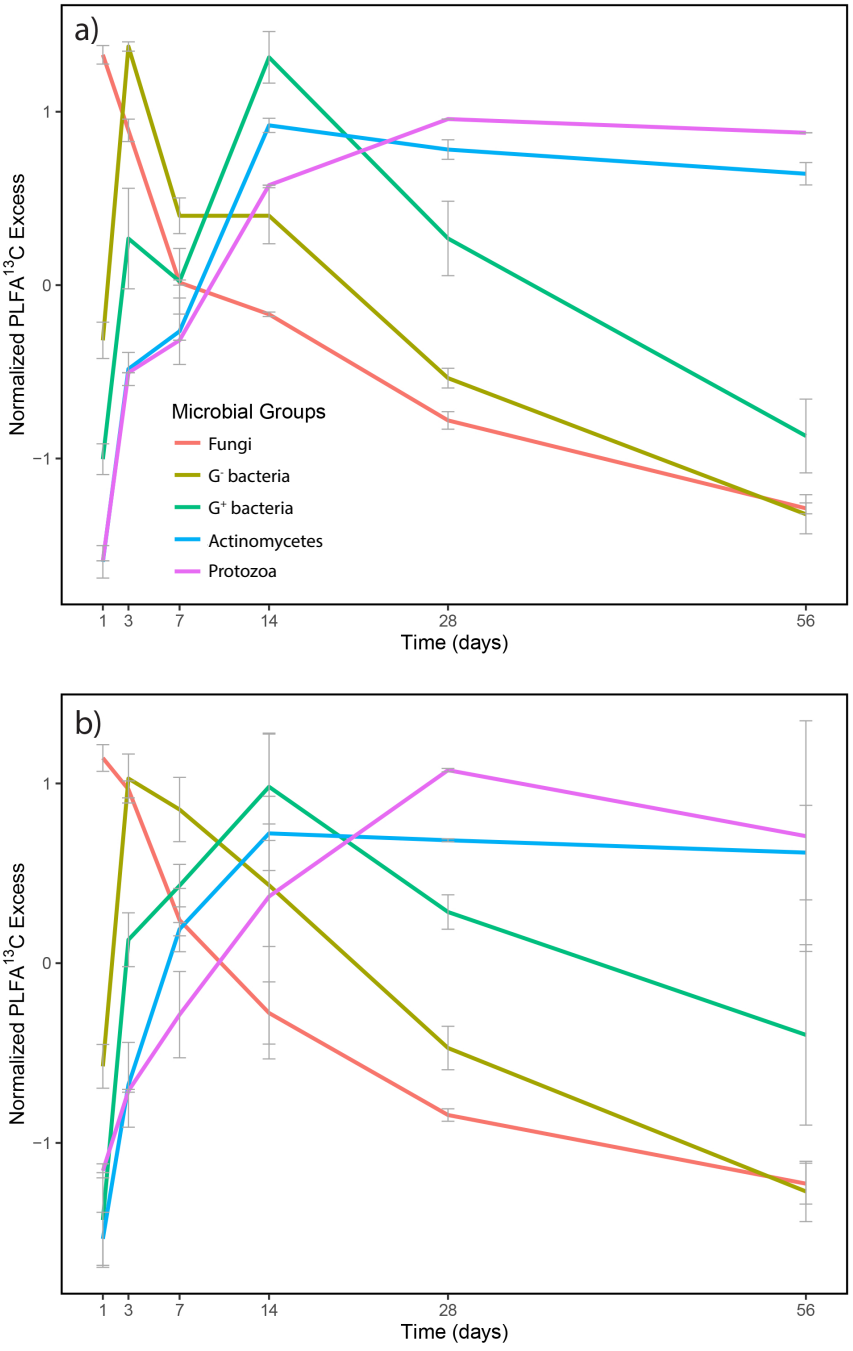


Figure 3.4 Mean normalized amounts of ^{13}C excess (± SE) in different present microbial groups as measured by the amount of ^{13}C incorporated in PLFA biomarkers over time for a) recent and b) long-term abandoned soils.

abandoned soils. This is in accordance with the research of Holtkamp *et al.* (2011) in the same study area, who show an increase in C mineralization by the soil community following land-abandonment, as well as an increase in soil microbial biomass. The higher soil microbial biomass in long-term abandoned soils was mainly caused by an increase in G^+ bacteria and actinomycetes in long-term abandoned soils, both before and after litter addition. A possible explanation for the higher abundance of these microbial groups in long-term abandoned soils, is the larger fraction of recalcitrant SOM in the C pool of long-term abandoned soils as opposed to recently abandoned soils (Kramer & Gleixner, 2006, 2008; Holtkamp *et al.*, 2008).

The fungal community showed a large increase in biomass in response to litter addition, regardless of land abandonment stage. This response may be related to the fact that, via their use of exploratory hyphae, fungi can rapidly access added litter and transport soil inorganic N to stimulate the production of fungal biomass (Frey *et al.*, 2000, 2003). In this way, they can play an important quantitative role in litter decomposition. All other microbial groups examined also showed an increase in biomass after litter addition, although effects were less pronounced compared to the fungal response. G^+ bacteria and actinomycetes retain a higher biomass after litter addition in long-term abandoned soils, whereby initial differences between ex-arable soils in terms of microbial community structure are still detectable. This suggests that, while litter addition caused a total increase of soil microbial community biomass and fungal/bacterial ratio, the initial differences in soil microbial community structure between the two types of ex-arable soils are not affected by litter addition. It could be possible that important community shifts occurred within the broad microbial groups represented by the analysed PLFA biomarkers, such as recently shown within the fungal community (Morriën *et al.*, 2017). It would therefore be of interest in future studies to analyse community dynamics during the flow of 'brown' C in soil from different land abandonment stages at a higher level of functional and taxonomic detail.

3.4.2 Litter-C uptake (efficiency) of soil microbes following land abandonment

We hypothesized that litter decomposition would be higher in long-term abandoned fields due to an increased activity and efficiency of the soil fungal community. Fungi have long been considered to be mainly responsible for decomposing labile and recalcitrant SOM in soil food web ecology (de Boer *et al.*, 2005; Holtkamp *et al.*, 2008). We however, did not find a build-up of fungal biomass or a community shift towards fungal dominated microbial communities in long-term abandoned soils, which is in line with other recent studies (Creamer *et al.*, 2016; Morriën *et al.*, 2017). In addition, we also did not observe an increased activity or efficiency of the fungal community in long-term abandoned soils when comparing the absolute and relative amounts of litter-derived C uptake. This is contrast to what was previously found by Morriën *et al.* (2017), who traced C flows entering the soil system via the

green pathway by ^{13}C pulse-labelling. Our results showed a lower litter-C uptake efficiency of the microbial community in long-term abandoned soils (Table 3.4). The counteracting trends of increasing bacterial biomass following land-abandonment, together with a decreased bacterial C uptake efficiency, resulted in a comparable net C flow for bacterial communities in both recent and long-term abandoned soils. These results are somewhat in contrast with the recent view that increased C uptake efficiency by the soil food web in long-term ex-arable fields is due to a tightening of soil food webs (Morriën *et al.*, 2017). It seems that long-term land abandonment has a positive influence on rhizosphere C uptake efficiency, but not on detritus-derived C uptake efficiency. This is in agreement with previous observations for the transition from grassland to woodlands, where it was suggested that soil microbes in long-term abandoned soils appear to be less efficient in converting organic matter C to microbial biomass (McCulley *et al.*, 2004; Liao & Boutton, 2008). A possible explanation is that root C biomass increases relatively more compared to the amount of C in SOM pools in long-term abandoned fields, which causes a shift from SOM-derived C towards root-derived C input in the soil microbial food web as reflected in the efficiency of the microbial community in processing these C flows.

3.4.3 Litter-derived C incorporation in the soil microbial food web

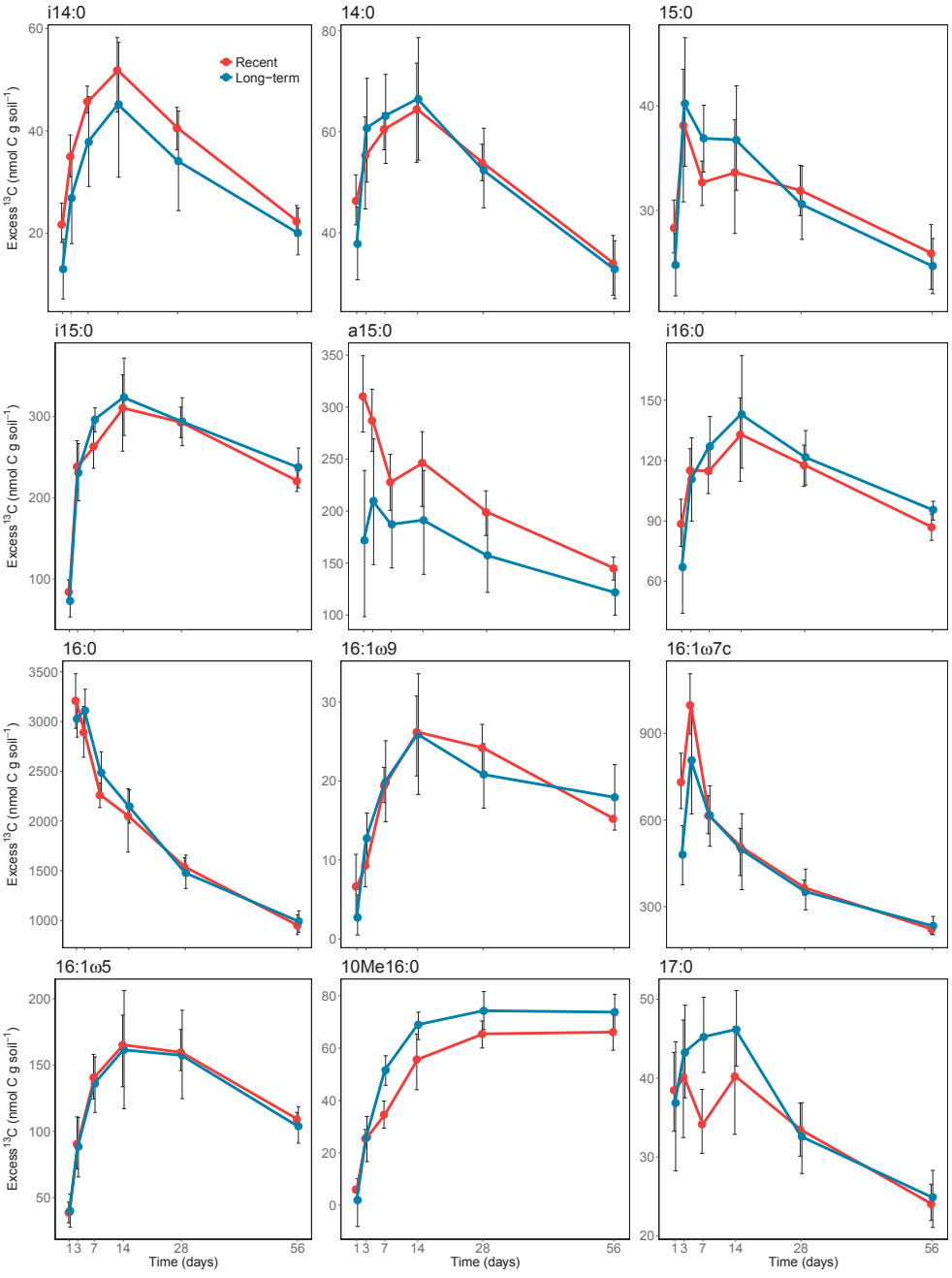
A few remarks are appropriate when interpreting litter-derived ^{13}C incorporation patterns in the soil microbial food web. First, it should be noted that the different fractions of plant litter may have contained different levels of ^{13}C enrichment. This might have affected the labelling of the microbial groups within our incubation experiment. If it would be the case that the consumed litter would have a higher ^{13}C enrichment compared to the overall litter, the amount of litter-derived C in the microbial community would have been overestimated. However, from our results it seems that this is not the case since the litter-derived biomass corresponds well to the build-up in microbial biomass after litter addition (Figure 3.2). Future experiments in which different fractions of the organic amendment are separately labelled would provide additional insight in the timing and quantity of microbial activities in relation to specific organic matter fractions. Our study also used a relative short time frame of 8 weeks with an estimated litter decomposition around 15-20% (unpublished data from litter bag experiment, in accordance with results of Cornelissen & Thompson, 1997). Therefore, it is also possible that much of the litter-derived C in this study came from the easy decomposable parts of plant remains, and that truly recalcitrant substrate decomposition fell to some degree outside the framework of our experimental design.

Interestingly, each soil microbial group examined in our study found its own 'temporal window' in terms of having a maximum amount of litter-derived C incorporation (Figure 3.3b and 3.4), giving more insight in the structure of the soil microbial detrital food web. Overall, there was a clear succession of microbial groups incorporating litter-derived C in their biomass both in time (1-56 days) and quantity (high-low), in the order of fungi > G^- bacteria > G^+ bacteria ≥ actinomycetes > micro-fauna.

With respect to the bacteria, our findings support the hypothesis that G^- bacteria are more associated with the initial decomposition of easily decomposable materials, while G^+ bacteria (and related actinomycetes) are later involved in the decomposition of more recalcitrant organic matter (Kramer & Gleixner, 2006, 2008). The fast and high decomposition activity of the saprotrophic fungal community has recently been described for the root-derived C flow in soils (e.g. Ballhausen & de Boer, 2016; Pausch *et al.*, 2015), and this patterns also appears to hold for litter-derived C flows. This results in a large build-up of fungal biomass after application of litter, which then again rapidly decreases over time. While fungi have a net loss of litter-derived C directly afterwards, bacterial groups are still building-up their biomass on litter-derived C inputs up to 14 days after litter addition. A possible explanation could be that fungal hyphae are a C source for the bacterial community (Ballhausen & de Boer, 2016). Remarkably, soil microbial ^{13}C-incorporation patterns were very similar across all the fields examined, both in terms of the timing and absolute values of C incorporation. These similarities suggest that the observed soil microbial detrital food web structure is relatively stable over a range of biotic and abiotic conditions, but would be necessary to examine such soil microbial food web dynamics across a wider range of soil systems before this can be generalized further.

3.5 Conclusions

This study gives a first quantitative insight in how litter-derived C flows through the detritus-based soil microbial food web in soils from different stages of land abandonment. By following litter-derived ^{13}C incorporation patterns in the microbial community, we found that there is a clear succession of microbial groups, irrespective of land abandonment stage, during litter decomposition (fungi > G^- bacteria > G^+ bacteria ≥ actinomycetes > micro-fauna). The decreased efficiency of the soil microbial community to transform SOM-derived C pools suggests that following land abandonment, a shift occurs from SOM-derived to root-derived based soil food webs, caused by a relative change in the importance of C input sources following land abandonment. Information of quantitative C flows through soil microbial groups can help to improve existing soil food web models, by giving structure to the "detritus black box", which currently lumps all microbial groups and their inherent traits. This perspective may lead to additional insights regarding the role and importance of the diverse microbial community in ecosystem processes like C and N mineralization, with the ultimate aim to improve predictions of ecosystem structure and important ecosystem services like C sequestration.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i15:0
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i16:0
16:0
16:1ω9
16:1ω7c
16:1ω5
10Me16:0
17:0
Recent
Long−term
Excess ^{13}C (nmol C g soil^{-1})
Time (days)
1 3 7 14 28 56

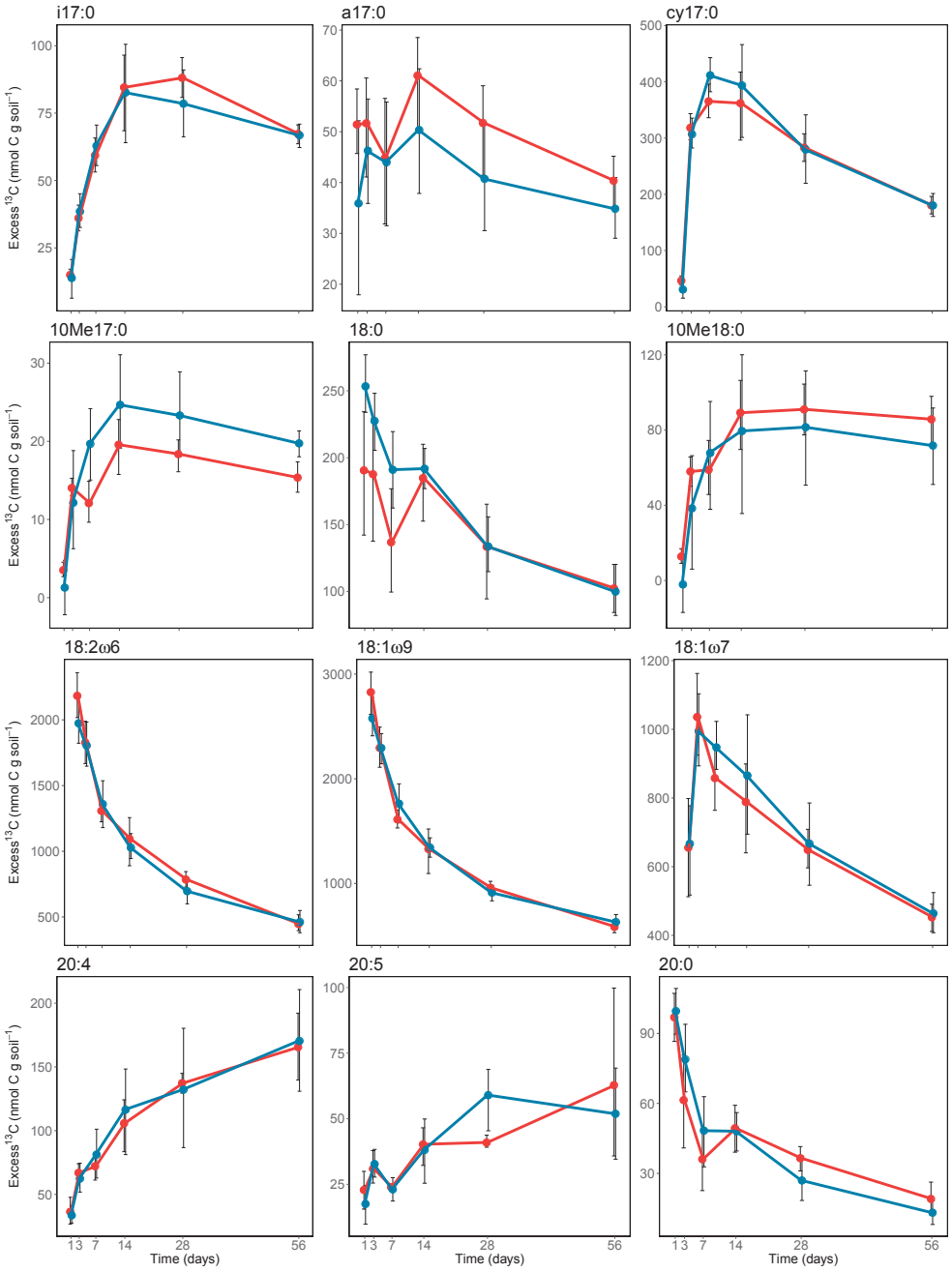

Figure S3.1 The amount of excess ^{13}C in different PLFA biomarkers over time since ^{13}C-labelled litter addition. Lines represent means of recently abandoned soils (red) and long-term abandoned soils (blue) ± SE.

Table S3.1 Soil microbial community abundances for specific PLFA biomarkers (nmol g soil^{-1}, mean ± SE) in recent and long-term abandoned soils. χ^2 and p-values are the result of linear-mixed modelling (*** p<0.001, ** p<0.01, * p<0.05, · p<0.1).

	Land abandonment stage				
PLFA marker	*Recent*	*Long-term*	χ^2	*p*	
i14.0	6.690	7.912	1.1172	0.291	
14.0	13.405	16.112	0.577	0.448	
15.0	8.877	11.124	0.8603	0.354	
i15.0	80.546	110.960	3.6654	0.056	.
a15.0	45.174	53.812	7.7192	**0.005**	**
i16.0	29.844	49.224	8.2789	**0.004**	**
16.0	193.321	248.907	1.1057	0.293	
16.1ω9	6.796	8.686	4.519	**0.034**	*
16.1ω7c	60.891	81.662	1.2551	0.263	
16.1ω5	40.462	49.692	2.6685	0.102	
10Me16.0	37.465	58.984	10.204	**0.001**	**
17.0	6.237	8.203	3.6888	0.055	.
i17.0	20.195	28.513	4.0489	**0.044**	*
a17.0	14.476	19.076	9.1756	**0.002**	**
cy17.0	28.628	37.282	4.048	**0.044**	*
10Me17.0	8.446	15.643	3.9016	**0.048**	*
18.0	35.134	44.516	1.2908	0.256	
10Me18.0	24.804	35.528	10.798	0.001	
18.2ω6	18.289	22.084	2.9288	0.087	.
18.1ω9	76.986	103.919	0.8014	0.371	
18.1ω7	92.886	135.992	8.8574	**0.003**	**
20.4	9.374	13.032	1.1302	0.288	
20.5	3.111	3.749	2.8009	0.094	.
20.0	10.301	12.750	0.232	0.630	

Table S3.2 Mean abundance of specific PLFA biomarkers (nmol g soil^{-1}) over time after ^{13}C-labelled litter addition for both recent and log-term abandoned soils. χ^2 and p-values are the result of linear-mixed modelling (*** p<0.001, ** p<0.01, * p<0.05, · p<0.1).

		Time (days)									
PLFA marker	*Land abandonment stage*	*1*	*3*	*7*	*14*	*28*	*56*		χ^2	*p*	
i14.0	Recent	10.20	10.90	12.68	13.80	12.32	8.42	Stage:	0.052	0.820	
	Long-term	10.79	11.97	12.38	13.53	12.42	9.20	Stage*Time:	0.027	0.869	
14.0	Recent	19.15	19.78	20.83	21.62	19.97	16.46	Stage:	6.023	**0.014**	*
	Long-term	22.33	24.26	23.17	24.73	22.90	19.84	Stage*Time:	0.008	0.928	
15.0	Recent	13.94	14.48	14.35	13.96	13.84	13.06	Stage:	5.540	**0.019**	*
	Long-term	16.51	16.98	16.06	15.93	15.45	14.99	Stage*Time:	0.361	0.548	
i15.0	Recent	98.48	104.93	109.28	119.08	115.26	98.02	Stage:	3.926	**0.048**	*
	Long-term	129.76	143.38	139.64	147.77	142.81	126.43	Stage*Time:	0.477	0.490	
a15.0	Recent	86.42	78.14	74.26	76.08	68.88	57.11	Stage:	0.027	0.870	
	Long-term	78.60	78.99	71.20	72.98	68.93	59.38	Stage*Time:	1.778	0.182	
i16.0	Recent	45.32	44.77	44.23	47.48	43.70	36.59	Stage:	4.062	**0.044**	*
	Long-term	63.72	67.51	65.45	67.50	62.20	55.61	Stage*Time:	0.180	0.672	
16.0	Recent	543.54	495.10	449.03	437.31	373.71	296.02	Stage:	2.471	0.116	
	Long-term	592.72	566.09	490.09	478.87	401.78	338.12	Stage*Time:	0.338	0.561	
16.1ω9	Recent	8.25	8.74	9.60	10.48	10.36	8.11	Stage:	0.715	0.398	
	Long-term	9.84	9.79	11.53	11.84	11.75	9.04	Stage*Time:	0.337	0.562	
16.1ω7c	Recent	160.91	176.55	136.34	128.67	106.46	77.62	Stage:	0.413	0.521	
	Long-term	156.98	185.38	156.24	143.18	120.64	91.34	Stage*Time:	3.639	0.056	
16.1ω5	Recent	50.70	51.33	55.11	64.13	66.12	50.26	Stage:	0.287	0.592	
	Long-term	55.82	59.61	60.54	67.81	69.06	51.48	Stage*Time:	2.406	0.121	
10Me16.0	Recent	43.60	43.32	42.29	45.29	44.95	41.74	Stage:	2.958	0.085	
	Long-term	62.78	65.56	64.25	67.37	65.61	61.51	Stage*Time:	0.160	0.689	
17.0	Recent	13.23	12.94	12.45	12.45	11.52	9.87	Stage:	2.785	0.095	
	Long-term	15.28	15.04	14.17	14.52	12.32	11.84	Stage*Time:	0.104	0.747	
i17.0	Recent	23.41	23.92	26.62	30.73	31.33	28.24	Stage:	4.296	**0.038**	*
	Long-term	29.33	31.67	32.59	35.55	36.35	34.45	Stage*Time:	0.717	0.397	
a17.0	Recent	22.70	21.96	21.31	22.52	22.03	18.92	Stage:	1.165	0.280	
	Long-term	24.57	24.94	23.36	24.47	23.46	20.78	Stage*Time:	0.207	0.649	
cy17.0	Recent	35.89	62.12	70.23	74.28	63.51	47.50	Stage:	0.913	0.339	
	Long-term	38.62	69.12	79.72	81.92	68.91	53.37	Stage*Time:	0.003	0.960	
10Me17.0	Recent	11.40	11.31	11.30	11.78	10.63	9.45	Stage:	3.215	0.073	
	Long-term	18.77	19.83	19.19	20.18	17.61	16.83	Stage*Time:	0.365	0.546	
18.0	Recent	62.43	56.84	54.97	57.07	53.60	47.33	Stage:	0.868	0.090	
	Long-term	71.34	68.03	62.23	64.39	58.34	53.76	Stage*Time:	1.267	0.260	
10Me18.0	Recent	31.69	34.93	35.81	37.42	34.98	30.99	Stage:	1.694	0.193	
	Long-term	38.85	43.25	42.94	44.22	41.78	38.00	Stage*Time:	0.026	0.871	
18.2ω6	Recent	221.31	179.33	146.25	132.80	96.94	63.21	Stage:	0.002	0.961	
	Long-term	222.38	184.92	144.64	125.27	90.09	68.51	Stage*Time:	0.012	0.911	
18.1ω9	Recent	388.05	311.50	252.08	225.87	179.44	135.99	Stage:	1.349	0.245	
	Long-term	408.05	348.18	284.91	251.59	199.08	161.72	Stage*Time:	0.088	0.767	
18.1ω7	Recent	188.85	209.45	190.55	195.55	169.43	131.56	Stage:	0.653	0.419	
	Long-term	208.72	247.40	229.23	225.82	198.64	154.59	Stage*Time:	0.452	0.501	
20.4	Recent	19.56	18.95	18.42	22.72	28.43	34.45	Stage:	0.019	0.891	
	Long-term	18.85	19.19	20.14	23.97	29.78	35.46	Stage*Time:	0.038	0.846	
20.5	Recent	8.45	7.80	7.14	8.25	8.13	9.53	Stage:	0.329	0.566	
	Long-term	7.14	6.84	6.61	7.13	7.61	9.38	Stage*Time:	0.058	0.810	
20.0	Recent	20.90	18.66	16.90	16.24	14.36	12.10	Stage:	7.262	**0.007**	**
	Long-term	24.97	22.59	19.30	19.01	16.48	14.85	Stage*Time:	0.609	0.435	

Table S3.3 Mean ^{13}C excess (pmol g soil^{-1}) of specific PLFA biomarkers over time after ^{13}C-labelled litter addition for both recent and long-term abandoned soils. χ^2 and p-values are the result of linear-mixed modelling (*** p<0.001, ** p<0.01, * p<0.05, · p<0.1).

		Time (days)									
PLFA marker	*Land abandonment stage*	*1*	*3*	*7*	*14*	*28*	*56*		χ^2	*p*	
i14.0	Recent	21.7	35.0	45.7	55.3	40.5	22.3	Stage:	7.332	0.392	
	Long-term	13.0	26.9	37.8	45.2	34.1	20.1	Stage*Time:	0.357	0.550	
14.0	Recent	46.3	55.3	60.5	69.0	53.8	33.9	Stage:	0.014	0.905	
	Long-term	37.8	60.7	63.1	66.5	52.4	32.8	Stage*Time:	0.002	0.97	
15.0	Recent	28.3	38.1	32.7	36.1	31.9	25.9	Stage:	0.006	0.936	
	Long-term	24.8	40.2	36.9	36.8	30.6	24.7	Stage*Time:	0.285	0.593	
i15.0	Recent	84.3	238.3	263.0	334.1	292.6	220.5	Stage:	0.059	0.809	
	Long-term	73.1	230.7	295.9	323.4	293.8	237.5	Stage*Time:	0.100	0.752	
a15.0	Recent	310.3	287.0	228.0	264.4	199.2	145.0	Stage:	1.767	0.184	
	Long-term	172.1	209.8	187.3	191.3	157.5	121.9	Stage*Time:	11.486	**<0.001**	***
i16.0	Recent	88.6	115.2	114.8	143.5	117.7	86.8	Stage:	0.001	0.975	
	Long-term	67.0	110.7	127.0	143.0	121.7	95.6	Stage*Time:	1.219	0.270	
16.0	Recent	3206.8	2891.2	2260.8	2194.1	1537.1	950.0	Stage:	0.207	0.650	
	Long-term	3027.4	3109.7	2486.9	2147.7	1479.6	994.2	Stage*Time:	0.031	0.860	
16.1ω9	Recent	6.6	9.3	19.4	28.1	24.2	15.2	Stage:	0.537	0.464	
	Long-term	2.7	12.7	19.9	25.9	20.8	18.0	Stage*Time:	1.355	0.244	
16.1ω7c	Recent	730.9	997.7	615.3	546.6	365.1	221.7	Stage:	0.548	0.459	
	Long-term	480.3	807.1	616.4	497.8	352.5	233.5	Stage*Time:	6.999	**0.008**	**
16.1ω5	Recent	38.6	90.4	140.7	178.2	159.6	109.1	Stage:	0.072	0.788	
	Long-term	39.9	88.5	136.1	161.5	157.4	103.9	Stage*Time:	0.010	0.922	
10Me16.0	Recent	5.9	25.4	34.6	60.3	65.5	66.1	Stage:	2.820	0.093	.
	Long-term	1.9	26.0	51.6	69.0	74.3	73.8	Stage*Time:	0.236	0.627	
17.0	Recent	38.5	40.1	34.2	43.3	33.4	24.0	Stage:	0.268	0.605	
	Long-term	36.9	43.2	45.2	46.2	32.6	24.9	Stage*Time:	0.912	0.340	
i17.0	Recent	15.0	36.1	59.3	91.4	88.1	67.3	Stage:	0.010	0.921	
	Long-term	13.8	38.5	62.8	82.7	78.6	66.8	Stage*Time:	NA	NA	
a17.0	Recent	51.4	51.7	44.9	65.9	51.8	40.4	Stage:	0.651	0.420	
	Long-term	35.9	46.3	44.0	50.4	40.7	34.8	Stage*Time:	0.024	0.878	
cy17.0	Recent	46.7	318.6	365.3	391.7	283.0	180.3	Stage:	0.011	0.917	
	Long-term	31.0	306.8	411.5	393.9	279.8	180.3	Stage*Time:	NA	NA	
10Me17.0	Recent	3.5	14.0	12.1	21.2	18.4	15.4	Stage:	0.961	0.327	
	Long-term	1.3	12.1	19.7	24.7	23.3	19.8	Stage*Time:	0.080	0.778	.
18.0	Recent	190.6	187.7	136.9	198.3	133.6	102.4	Stage:	3.679	0.055	
	Long-term	253.4	227.6	191.0	191.8	133.7	100.0	Stage*Time:	5.453	**0.020**	*
10Me18.0	Recent	12.8	58.0	58.9	96.3	91.0	85.7	Stage:	0.053	0.818	
	Long-term	-2.0	38.4	67.8	79.5	81.5	71.7	Stage*Time:	0.740	0.390	
18.2ω6	Recent	2183.7	1827.7	1308.4	1178.2	785.9	447.3	Stage:	0.568	0.451	
	Long-term	1973.6	1804.3	1359.8	1029.0	697.3	462.8	Stage*Time:	0.204	0.652	
18.1ω9	Recent	2827.6	2296.8	1613.3	1424.8	958.7	586.4	Stage:	0.024	0.877	
	Long-term	2576.7	2295.3	1762.0	1344.0	912.3	632.1	Stage*Time:	0.365	0.546	
18.1ω7	Recent	655.8	1036.2	858.1	850.5	648.9	452.9	Stage:	0.292	0.589	
	Long-term	667.3	994.9	947.6	865.4	667.6	464.4	Stage*Time:	NA	NA	
20.4	Recent	36.4	67.0	72.2	114.9	137.3	165.6	Stage:	0.010	0.922	
	Long-term	33.7	62.5	81.3	116.5	132.2	170.5	Stage*Time:	0.172	0.678	
20.5	Recent	22.9	31.0	23.9	43.6	41.0	62.8	Stage:	0.387	0.534	
	Long-term	17.5	32.8	23.1	38.1	59.0	51.9	Stage*Time:	0.069	0.793	
20.0	Recent	96.9	61.4	36.1	52.6	36.5	19.1	Stage:	0.007	**0.934**	
	Long-term	99.6	78.9	48.3	48.1	27.0	13.2	Stage*Time:	1.284	0.257	

Table S3.4 Mean ^{13}C excess (pmol g soil^{-1}) of specific PLFA biomarkers over time after ^{13}C-labelled litter addition for both recent and long-term abandoned soils. χ^2 and p-values are the result of linear-mixed modelling (*** p<0.001, ** p<0.01, * p<0.05, · p<0.1).

		Time (days)									
PLFA marker	*Land abandonment stage*	*1*	*3*	*7*	*14*	*28*	*56*		χ^2	*p*	
i14.0	Recent	20.80	30.97	36.21	39.93	32.58	26.84	Stage:	1.937	0.164	
	Long-term	11.10	21.04	28.69	31.02	26.95	22.42	Stage*Time:	1.611	0.204	
14.0	Recent	23.67	27.41	29.53	31.60	26.91	20.93	Stage:	0.976	0.323	
	Long-term	17.03	25.01	26.74	26.91	23.69	17.16	Stage*Time:	0.153	0.70	
15.0	Recent	19.57	26.30	23.24	24.72	23.01	20.35	Stage:	0.887	0.346	
	Long-term	15.13	23.53	22.59	23.06	20.75	16.89	Stage*Time:	0.090	0.765	
i15.0	Recent	8.04	22.57	23.97	27.95	25.14	22.85	Stage:	10.487	**0.001**	**
	Long-term	6.13	16.52	21.53	22.19	21.02	19.03	Stage*Time:	NA	NA	
a15.0	Recent	35.54	36.78	30.49	34.95	28.66	25.63	Stage:	3.800	0.051	
	Long-term	19.26	24.78	25.25	24.97	22.24	20.37	Stage*Time:	12.396	**<0.001**	***
i16.0	Recent	19.03	25.51	25.78	29.98	26.58	24.22	Stage:	1.976	0.160	
	Long-term	11.61	17.52	20.73	22.65	21.05	18.51	Stage*Time:	0.506	0.477	
16.0	Recent	58.13	57.82	50.06	49.74	40.69	32.61	Stage:	4.819	**0.028**	*
	Long-term	50.35	54.30	49.58	44.15	36.84	29.47	Stage*Time:	0.374	0.541	
16.1ω9	Recent	9.17	11.62	22.95	23.77	NA	NA	Stage:	0.312	0.576	
	Long-term	3.36	12.59	21.00	20.29	21.69	20.06	Stage*Time:	28.091	**<0.001**	***
16.1ω7c	Recent	44.66	55.95	44.73	42.44	34.06	28.97	Stage:	4.581	**0.032**	*
	Long-term	29.72	41.92	38.49	33.07	28.85	25.51	Stage*Time:	9.814	**0.002**	**
16.1ω5	Recent	7.81	17.80	25.94	28.56	24.25	22.54	Stage:	0.859	0.354	
	Long-term	7.12	14.20	21.54	22.35	22.52	20.23	Stage*Time:	0.053	0.817	
10Me16.0	Recent	0.85	5.83	8.54	13.71	14.33	15.71	Stage:	0.868	0.351	
	Long-term	0.43	4.32	8.90	10.98	12.22	12.85	Stage*Time:	1.359	0.244	
17.0	Recent	27.69	30.33	28.62	33.99	28.92	25.21	Stage:	0.266	0.606	
	Long-term	23.68	28.38	31.88	32.08	26.93	21.22	Stage*Time:	0.522	0.470	
i17.0	Recent	5.58	15.16	22.59	29.45	27.74	24.46	Stage:	1.433	0.231	
	Long-term	5.13	12.18	19.42	22.91	22.24	19.76	Stage*Time:	0.687	0.407	
a17.0	Recent	21.89	24.00	20.58	28.91	23.36	21.47	Stage:	1.664	0.197	
	Long-term	13.66	18.12	18.79	20.27	17.49	17.23	Stage*Time:	0.167	0.683	
cy17.0	Recent	12.49	50.97	51.68	52.17	44.07	38.15	Stage:	3.824	0.051	.
	Long-term	7.76	43.76	51.21	46.68	39.64	33.88	Stage*Time:	NA	NA	
10Me17.0	Recent	2.57	11.59	11.81	17.42	17.83	18.70	Stage:	0.348	0.555	
	Long-term	1.35	7.01	11.36	14.01	14.55	13.49	Stage*Time:	4.625	**0.032**	*
18.0	Recent	30.05	32.13	24.44	34.29	23.89	21.69	Stage:	0.179	0.672	
	Long-term	33.93	33.03	30.12	29.50	22.99	18.97	Stage*Time:	0.815	0.367	
10Me18.0	Recent	3.39	16.44	16.54	25.33	25.59	27.72	Stage:	0.734	0.392	
	Long-term	-0.20	7.94	15.32	16.81	19.13	19.39	Stage*Time:	6.903	**0.009**	**
18.2ω6	Recent	97.79	101.03	89.17	87.63	80.60	70.38	Stage:	2.091	0.148	
	Long-term	87.73	96.83	91.82	81.31	76.20	66.78	Stage*Time:	0.093	0.763	
18.1ω9	Recent	71.74	72.98	63.98	62.72	53.18	43.47	Stage:	5.415	**0.020**	*
	Long-term	62.28	65.26	60.73	53.18	45.53	38.91	Stage*Time:	0.872	0.350	
18.1ω7	Recent	35.48	49.33	45.07	44.02	38.45	35.23	Stage:	3.340	0.068	.
	Long-term	31.40	40.34	41.30	37.63	33.65	30.86	Stage*Time:	4.648	0.199	
20.4	Recent	17.42	34.43	38.51	51.18	49.11	52.95	Stage:	0.524	0.469	
	Long-term	17.80	37.34	43.03	46.29	50.10	47.64	Stage*Time:	0.163	0.686	
20.5	Recent	21.89	36.11	45.78	53.10	47.77	53.90	Stage:	1.043	0.307	
	Long-term	22.00	40.23	43.99	53.23	56.27	54.58	Stage*Time:	0.478	0.489	
20.0	Recent	45.22	32.28	24.45	31.34	24.88	15.40	Stage:	2.637	0.104	
	Long-term	39.43	34.18	23.61	24.94	16.40	9.53	Stage*Time:	NA	NA	

Chapter 4

Soil microbial resource partitioning of diverse detrital substrates in recent and long-term abandoned soils

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Abstract

Soil microbial carbon (C) mineralization of detritus is an important driver of the global C cycle and has long been assumed to be divided over distinct bacterial and fungal energy channels. Shifts in the relative prevalence of the bacterial versus fungal energy channel are commonly used as indicators for functional shifts following land use change. Recent soil microbial studies indicate that soil microbial resource partitioning likely goes beyond the concept of bacterial and fungal energy channels. However, we still lack knowledge of how specific detrital components are processed by soil-borne communities, and how this carbon flow is impacted by global change drivers such as land use change. In this study, soils from recent (6-10 years) and long-term (30-33 years) abandoned fields were compared in an experiments in which soils were supplemented with a combination of detrital substrates: namely glycine, cellulose and vanillin. The experiment was designed to allow us to track the fate of each of these substrates. To this end, a series of incubations were performed in which all soils received the same total substrate mixture, but with only one (or none) of the substrates labelled with ^{13}C. Soils were subjected to regular sampling up to 28 days, to trace the fate of ^{13}C-labelled substrates through the microbial part of the soil food web by Phospholipid Fatty Acid - Stable Isotope Probing (PLFA-SIP) and rRNA-SIP. These analyses revealed that, although recent and long-term abandoned ex-arable soils were comparable in terms of the biomass of different microbial groups, the G^+ bacteria of long-term abandoned soils were less efficient in the incorporation vanillin-derived C as compared to recently abandoned soils. More detailed rRNA-SIP results demonstrated that the long-term abandoned fields harboured a more specialized microbial community responsible for the decomposition of vanillin, with an important contribution of the G^- bacteria *Pseudomonas* and *Caulobacter*. Ternary plot analyses further revealed intra-kingdom microbial resource partitioning for both fungal and bacterial classes, and apparent intra-kingdom succession within the bacterial decomposer community during substrate degradation (Betaproteobacteria/Gammaproteobacteria followed by Acidobacteria and Alphaproteobacteria). Our results highlight the impact of different detrital substrates on carbon flow in soil microbial communities and demonstrate succession within complex bacterial and fungal communities in the incorporation of these substrates. We therefore propose that advancing our perception of soil microbial decomposition beyond the simplified concepts of bacterial and fungal energy channels will help improve our understanding of the functioning of soil microbial food webs.

4.1 Introduction

Soil organisms play a crucial role in the global carbon (C) cycle by decomposition, mineralization and stabilization of soil organic matter, a C stock that contains more C than the Earth's vegetation and atmosphere combined (Ciais *et al.*, 2013). For decennia, soil food web models have been used to study the function and importance of different soil organisms in carbon and nutrient cycling (e.g. Hunt *et al.*, 1987; de Ruiter *et al.*, 1993b). Studying the structure and functioning of these soil food webs in response to e.g. soil management and land use change can provide important information about the potential long-term effects on soil ecosystem functioning (Morriën, 2016). Soil food web models have for instance been used to study the structure and functioning of the detritus-driven soil food web and concomitant mineralisation processes after land abandonment to identify and develop soil management strategies that enhance the restoration of heathlands (Holtkamp *et al.*, 2008).

Soil microbes are central to soil food webs by being responsible for the primary decomposition of soil organic matter, and by forming the 'eye of the needle' in soils, through which all organic matter must pass (Jenkinson, 1977). In this way, soil microbes are responsible for the delicate balance between, on the one side, the turnover of organic material into mineral, plant-accessible nutrients and, on the other side, the sequestration of organic matter in soils. Both processes are crucial for ecosystem functioning, and sustainable management strategies require a thorough understanding of soil microbial C flows. Classical soil food web models rely on the decomposition of detritus via two main energy channels; the bacterial and fungal energy channel. In such models, the bacterial energy channel is designated as assimilating labile substrates twice as fast as the fungal channel, and the fungal channel uses recalcitrant substrates twice as fast as the bacterial channel (Hunt *et al.*, 1987). The size of the bacterial and fungal energy channel, and the ratio between these channels, is often examined as a proxy of the effects of land use on soil food web typology and concomitant ecosystem services (Wardle *et al.*, 2004; Holtkamp *et al.*, 2008; de Vries *et al.*, 2013).

The fundamental principles of these soil food web energy channels, and the value of the energy channel concept to assess the effects of land use change on soil ecosystem functioning, has recently been questioned by a number of studies (e.g. de Vries &K Caruso, 2016; Geisen *et al.*, 2016; Lehmann & Kleber, 2015; Pausch *et al.*, 2016). The concept of bacterial and fungal energy channels gives little room to study diversification within the soil microbial community and soil organic matter in soil food web models, factors that have shown to be affected by land use and have impacts on soil functioning (e.g. Bissett *et al.*, 2011; Heijboer *et al.*, 2016; Lauber *et al.*, 2013). However, the apparent reticulated and progressive nature of soil microbial networks made them difficult to study.

The empirical technique of stable isotope probing (SIP) offers the possibility to

study detritus C fluxes in the soil beyond the concepts of bacterial and fungal energy channels (Kramer *et al.*, 2016; Morriën, 2016; Heijboer *et al.*, 2018). The application of SIP to trace the fate of specific ^{13}C-labelled organic substrates through the soil microbial community, has already provided evidence of microbial resource partitioning (Paterson *et al.*, 2008; Drigo *et al.*, 2010; Pausch *et al.*, 2016) and microbial consumer succession (Kramer *et al.*, 2016). These results enhance the idea that soil food webs are not restricted to purely bacterial and fungal energy channels, and that a closer understanding of soil microbial C flows is needed to improve our understanding of soil food web functioning following land use changes.

A form of land use change that has been extensively studied to assess the effects of land use change on soil food web topology, is the restoration of ex-arable fields into heathlands (van der Wal, 2007; Holtkamp, 2010; Hannula *et al.*, 2017; Morriën *et al.*, 2017). Studies on a chronosequence in the Veluwe, in the centre of the Netherlands, have shown that even though carbon and nitrogen mineralization increased up to 22 years after land abandonment (Holtkamp *et al.*, 2011), changes in the relative importance of the fungal compared to the bacterial energy channel were only apparent in the first two years (Holtkamp *et al.*, 2008). Recent studies indicate that intra-energy channel changes, apparent by shifts in fungal and bacterial composition and activity, affect the efficiency of carbon flow and nutrient cycling (Hannula *et al.*, 2017; Morriën *et al.*, 2017). Earlier studies in these ex-arable fields have focussed on the root energy channel (Hannula *et al.*, 2017; Morriën *et al.*, 2017) or a single type of substrate degradation (chapter 3). However a complete assessment of detrital carbon flows in response to land use change is still missing for this model system.

In this study, a SIP incubation experiment was performed to track the fate of contrasting types of ^{13}C-labelled organic substrates in recent and long-term abandoned soils that are part of a well-studied chronosequence of ex-arable fields. The aim of the experiment was to get a more complete picture of the detrital microbial carbon flows in recent and long-term abandoned soils, thereby moving beyond the concept of bacterial and fungal energy channels, and to use these results to assess the necessary level of detail required to obtain a useful and representative view of microbial C flow patterns in soil. To study this, recent and long-term ex-arable soils were amended with a mixture of three organic substrates differing in decomposability: ^{13}C-labelled glycine (a simple compound common in plant exudates), ^{13}C-labelled cellulose (a representative compound of plant biomass), and ^{13}C-labelled vanillin (a model substance to indicate lignin degradation), and incubated for 28 days. At different time points during the incubation process, C flows were assessed using PLFA-SIP, and rRNA-SIP was used to identify key microbial groups that are active in the decomposition of specific detrital substrates. We hypothesized that in soil from recently abandoned fields, microbial communities would be rather unspecialized and capable of degrading a wide array of detrital substrates, while microbial communities in long-term abandoned soils would consist of a specialized microbial community for the degradation of more recalcitrant organic matter. We further hypothesized that

the decomposition of specific organic substrates is not limited to or dominated by the bacterial or fungal energy channel and that intra-kingdom soil microbial resource partitioning takes place in both the bacterial and fungal communities.

4.2 Material and methods

4.2.1 Site description and soil sampling

Soil samples were collected from a well-studied chronosequence of ex-arable fields located on glacial sandy soil deposits located in the same geographical region in the centre of The Netherlands (Kardol *et al.*, 2005; van der Wal *et al.*, 2006; Holtkamp *et al.*, 2008; Morriën *et al.*, 2017). In June 2015, a total number of 6 fields were sampled; 3 recently abandoned soils (6-10 years since land abandonment) and 3 long-term abandoned soils (30-33 years since land abandonment). Details on sampled fields can be found in Table 4.1. For each ex-arable field 10 soil samples were taken (10 cm deep, removal of vegetation layer). There was at least 10 m between each soil sample, and samples were taken at least 20 m from field edges. Soil samples were transported and stored at 4°C prior to further treatment.

4.2.2 Experimental design: ^{13}C incubation experiment

Soil samples were sieved using a 2 mm sieve to remove roots and stones, and subsequently pooled into one composite sample per field site. A total of 360 glass bottles (315 ml) were filled with an equivalent of 25 g dry weight of soil. This resulted in 60 glass bottles per field site. Bottles were divided over different treatments to follow the fate of a specific substrate: Glycine, Cellulose, Vanillin, positive control (C^+), and negative Control (C^-). Soil samples in all bottles were supplemented with a fixed combination of all three substrates: glycine, cellulose, and vanillin at a quantity of 0.25 mg C/g DW soil per substrate, except for the C^- treatment, which did not receive any substrates. The C^+ treatment received these three substrates in unlabelled form. For the other treatments, one of the substrates was replaced by a ^{13}C labelled version of this specific substrate. In this way, all amended treatments received the same mixture of substrates, with specific treatments dedicated to tracking the fate of the specific labelled substrate. An overview of all treatments and the corresponding substrates can be found in Table 4.2. Soil moisture was adjusted to 55% of water holding capacity by addition of MilliQ water, after which all bottles were closed with a cotton ball to prevent contamination while permitting gas exchange. Bottles were placed in an incubator (Cooled Incubator, EB1, Snijders Scientific, The Netherlands) at 20°C in the dark one quarter of the bottles was destructively sampled on days 3, 7, 14 and 28. From each unique treatment, a subsample (equivalent to 5 g dry weight of soil) was stored at 4°C prior to the determination of extractable nutrients. The remaining soil was stored in the freezer at -80°C prior to further analyses.

Table 4.1 List of ex-arable fields where soil sampling took place, including information on exact location, land abandonment stage and the year agricultural practices were abandoned.

Site	Location	Land abandonment	Abandoned since
TW - Telefoonweg	N 52°00'9 E 5°45'8	Recent	2009
RK - Reijerskamp	N 52°1'0 E 5°46'21	Recent	2005
OR - Oud Reemst	N 52°2'27 E 5°48'34	Recent	2005
DK - Dennenkamp	N 52°1'43 E 5°48'2	Long-term	1982
MV - Mosselse Veld	N 52°4'23 E 5°44'13	Long-term	1985
BB - Boersbos	N 52°3'44 E 5°59'57	Long-term	1982

Table 4.2 Overview of ^{13}C labelling treatments and the substrates they received (marked with an x). Below the specifications for each of the substrates are given.

Treatment	Abbreviation	Added substrates					
		^{13}C-labelled Glycine[1]	*^{13}C-labelled Cellulose*[2]	*^{13}C-labelled Vanillin*[3]	*Glycine*[4]	*Cellulose*[5]	*Vanillin*[6]
Glycine		x				x	x
Cellulose			x		x		x
Vanillin				x		x	x
Positive control	C^+				x	x	x
Negative control	C^-						

4.2.3 $^{(13)}CO_2$ respiration rates

At each sampling day, CO_2 flux measurements were performed on the subset of bottles that was destructively sampled at day 28. Because of destructive sampling on day 28, the last CO_2 flux measurement took place on day 27. Air sampling and analyses were performed as described in chapter 3. In short, two air samples were taken from a lid-closed bottle with an interval of four hours, after which air samples were analysed for CO_2 concentration and $\delta^{13}CO_2$ value on respectively a Thermo Trace Ultra GC interfaced with a methanizer-FID or a Thermo Scientific Delta V IRMS. These results were used to calculate CO_2 and $^{13}CO_2$ respiration rates on each sampling date.

4.2.4 Extraction and analyses of PLFA

From each unique Field-Substrate-Time combination, three technical replicate soil samples were subjected to phospholipid fatty acid (PLFA) analysis, as were single samples for each unique C^- and C^+ treatment. From each of these 264 soil samples, PLFAs were extracted from 4 grams of soil and further analyses are performed as described in chapter 3. PLFA biomarkers were used to characterize different microbial groups: fungi (18:2ω6), G^- bacteria (cy17:0, 18:1ω7), G^+ bacteria (a15:0, a17:0, i15:0, i16:0, i17:0), actinomycetes (10Me16:0, 10Me17:0, 10Me18:0). The actual $\delta^{13}C$ value

of each PLFA biomarker was calculated as described by Boschker (2004):

$$\delta^{13}C_{PLFA} = ((n+1) * \delta^{13}C_{FAME} - 1 * \delta^{13}C_{methanol})/n \quad (4.1)$$

where n is the number of C atoms in the PLFA biomarker. The $\delta^{13}C_{PLFA}$ of labelled and unlabelled control samples was used to calculate the actual excess amount of ^{13}C in each PLFA biomarker (Boschker, 2004).

4.2.5 Calculations of substrate-derived C

Relative amounts of substrate-derived CO_2 were calculated using the following formula:

$$\%C_{\text{substrate}} = \frac{\delta^{13}C_{sample} - \delta^{13}C_{reference}}{\delta^{13}C_{substrate} - \delta^{13}C_{soil}} * 100 \quad (4.2)$$

where $\delta^{13}C_{sample}$ refers to the tested sample, $\delta^{13}C_{reference}$ to the corresponding control sample without substrate addition, $\delta^{13}C_{substrate}$ and $\delta^{13}C_{soil}$ to respectively the added litter and soil at the start of the experiment.

4.2.6 RNA extraction and Stable Isotope Probing

Total RNA was extracted from a selection of soil samples (2g) using the RNA Powersoil Total RNA Isolation Kit (MoBio) and stored at -80°C. Sample selection was based on PLFA labelling patterns so that there was no effect of selection on statistical differences between recent and long-term abandoned soils. This resulted in a selection of 4 fields (2 recent and 2 long-term abandoned ex-arable fields: TW, OR, BB, MV) and three sampling moments (day 3, 7, 28). Total RNA was quantified on a NanoDrop (Denovix, DS-11, Wilmington, USA). The ^{13}C-enriched RNA was separated from unlabelled RNA by density-gradient centrifugation using an adapted protocol of Manefield *et al.* (2002b). For each sample 500 ng RNA was loaded in a 1.8 ml ultracrimp polyallomer tube (Kendro laboratory products, Newtown, USA) on a gradient buffer consisting of 1.441 ml CsTFA and 75 ul deionized formamide and added up to 1.8 ml with RNAse free water, with an overall density of 1.80 g/ml. Sealed tubes were centrifuged on an ultracentrifuge (Sorvall Discovery M120 SE, 48h, 64,000 rpm, S120 VT vertical rotor) and subsequently fractionated in 18 fractions of 100µl. A control gradient, without RNA, was included in each centrifugation run to determine the buoyant density of the gradient over different fractions. The density of each fraction was determined using a digital refractometer (AR200, Reichert Inc., Depew, USA). RNA was subsequently isolated from the fractions by precipitation with RNAse free isopropanol.

4.2.7 Sequencing of 16S and 18S rRNA

For all gradient fractions, cDNA synthesis was prepared using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Samples were diluted 20 fold prior to quantitative PCR analyses (qPCR) and library preparation for high throughput sequencing. Based on qPCR results of gradient fractions, peak fractions were selected per gradient for both control samples as well as labelled samples and subjected to 16S rRNA and 18S rRNA amplicon sequencing. Sequencing libraries targeting the V3 and V4 regions were prepared using the protocol of 16S Metagenomic Sequencing Library Preparation, Illumina. The sequencing library of the 18S V9 region was prepared using the same protocol as the 16S library, except using the Amplicon PCR primers presented in Table 4.3. Thermal cycle conditions were: 95˚C for 3 min, 26 cycles of 95˚C for 20 sec, 55˚C for 30 sec, 72˚C for 30 sec. 16S Amplicon primers were used to create a 460 bp PCR fragment covering the V3 and V4 regions. The 18S Amplicon primers yielded a 260 bp PCR fragment spanning the V9 region. Samples were divided over 4 runs (2x300bp) and sequenced on an Illumina MiSeq instrument at the Utrecht Sequencing Facility in The Netherlands, with separate sequencing runs for 16S rRNA and 18S ribosomal RNA amplicon pools. 20 % PhiX was spiked to the library to improve the complexity.

4.2.8 Bioinformatics

Sequencing reads were processed using MOTHUR 1.38.1 software, according to the MiSeq Standard Operating Procedure pipeline (Schloss *et al.*, 2009; Kozich *et al.*, 2013). Initial reads were assigned to specific samples based on their barcodes, after which barcode and primer sequences were removed. Only sequences longer than 300 bp (16S rRNA) or 80 bp (18S rRNA) without ambiguous nucleotides were selected for further analyses. The remaining sequences were aligned to a SILVA database (v123) and pre-clustered using the pseudo-single linkage-clustering algorithm to further de-noise sequences (Huse *et al.*, 2010). The resulting sequences were screened for chimeric sequences using UCHIME in the de novo mode (Edgar *et al.*, 2011). After a first assignment, using the SILVA database, non-bacterial sequences (Archaea-Chloroplast-Mitochondria-unknown) and non-protist sequences (Bacteria-Archaea-unkown) were removed from the 16S and 18S rRNA datasets, respectively. For 18S rRNA gene fragments, Metazoa sequences were additionally removed after

Table 4.3 Primer information of Illumina primers used for amplicon sequencing.

Amplicon PCR primers	Primer sequence
16SforNGS	*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG*
16SrevNGS	*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC*
18SforNGS	*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTACACACCGCCCGTC*
18SrevNGS	*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGATCCTTCTGCAGGTTCACCTAC*

assignment using the Protist Ribosomal Reference (PR2) database (Guillou *et al.*, 2013). Remaining high-quality reads were clustered into operational taxonomic units (OTUs) at a 97% similarity level. OTUs represented by 10 or fewer sequences were removed from further analyses. The taxonomic assignments were performed using the SILVA database (v123) for 16S rRNA genes and PR2 database for 18S rRNA.

4.2.9 Data analysis & statistics

Data analyses were performed using R version 3.1.0 (R Core Team, 2014). Excess ^{13}C differences between recent and long-term abandoned soils and over time were tested using *nlme* package (Pinheiro *et al.*, 2017) for liner-mixed effect modelling combined with the *car* package (Fox & Weisberg, 2011) for type II sum of squares with 'Field' as a fixed factor. Normality of residuals was checked using Shapiro's test and heterogeneity of variances was checked using Levene's test (*car* package). Data was power transformed when necessary. If these assumptions were not met, stage of abandonment and treatment effects were tested using the non-parametric Kruskal-Wallis test.

OTU reads served as input values for the principal component analysis (PCA) to check for differences in microbial community structure between fields and the two stages of abandonment, using the R package *vegan* (Oksanen *et al.*, 2015). PCA's were based on the top 250 sequences 16S rRNA OTUs and the top 250 sequenced 18S rRNA OTUs. Treatment effects on the OTU responses on genus level, relative to control, were assessed using principal response curves (PRC) (Van den Brink & Braak, 1999). Ternary plots were applied to examine the involvement of top 100 OTUs (both 16S rRNA and 18S rRNA) in the decomposition of different substrates and at different times points after substrate addition, using the R packages *vegan* (Oksanen *et al.*, 2015) and *ggtern* (Hamilton, 2016).

4.3 Results

4.3.1 Carbon mineralization rates and microbial biomass following substrate addition

Comparison of ^{13}C-labelled treatments revealed no significant interaction effect between treatments and time since abandonment on overall C mineralization rates (Supplementary Figure S4.1a, χ^2=1.590, p=0.451). Looking at the main effects, one can observe a significant effect of time since abandonment (χ^2=13.763, p<0.001***), but not of substrate treatment (χ^2=5.972, p=0.050). At the beginning of the incubation period, there is a higher C mineralization in long-term abandoned soils compared to recently abandoned soils, a difference that levels out over the course of the incubation. The specific substrate-derived ^{13}C mineralization rates (Supplementary Figure S4.1b), clearly show a significant effect of treatment (χ^2=31.506, p<0.001***), but not of time since abandonment (χ^2=0.002, p=0.961). For both recent and

long-term abandoned soils, we initially observed the highest ^{13}C substrate-derived mineralization for glycine, followed by vanillin and then cellulose. Microbial biomass patterns, as measured by PLFA analyses for fungal, G^+, G^- and actinomycete biomass, show only an effect of incubation time and no effects of treatment and time since abandonment (Supplementary Figure S4.2).

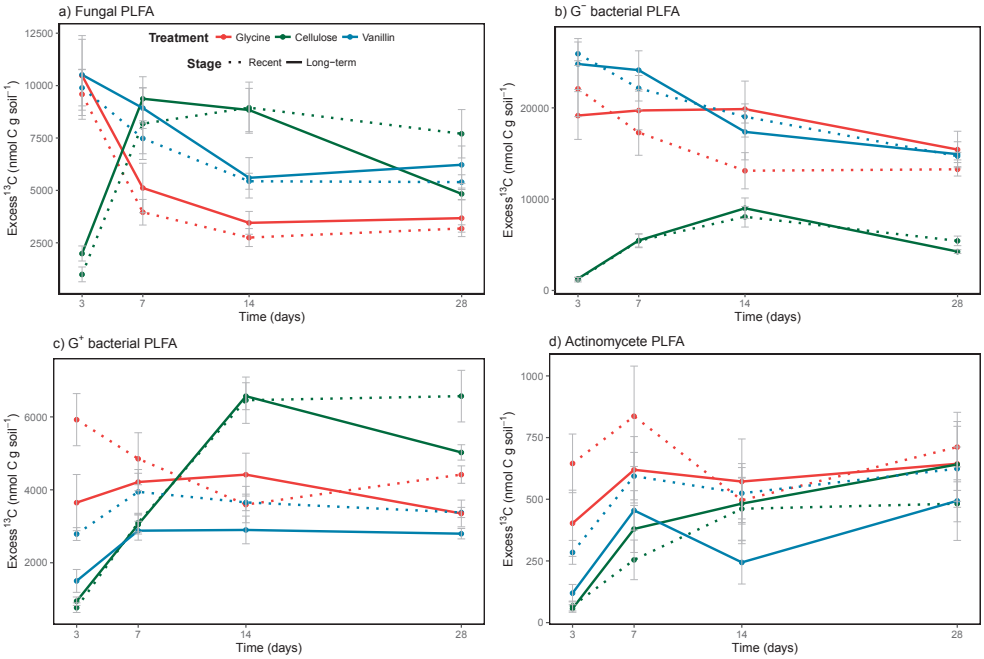


Figure 4.1 The amount of excess for different groups of PLFA biomarkers at respective sampling times during incubation, for both recent and long-term abandoned soils and for different treatments. Lines represent means ± SE. The panels show labelling patterns for specific PLFA biomarkers that were appointed to the microbial groups a) fungi, b) G^- bacteria, c) G^+ bacteria and d) actinomycetes.

Table 4.4 Statistical summary of p-values from the two factor RM-ANOVA for the amount of ^{13}C excess in different groups of PLFA biomarkers. Stage refers to time since abandonment (recent versus long-term) and time refers to time after ^{13}C-labelled substrate addition (3,7 and 28 days), where p-values are the result of linear-mixed modelling (*** p<0.001, ** p<0.01, * p<0.05, · p<0.1).

	Glycine			Cellulose			Vanillin		
PLFA biomarker	Stage	Time	Stage x Time	Stage	Time	Stage x Time	Stage	Time	Stage x Time
Fungi	0.687	<0.001***	0.830	0.775	<0.01 **	0.068	0.687	<0.001***	0.909
G^- bacteria	0.674	<0.01**	0.438	0.955	<0.001***	0.489	0.910	<0.001***	0.985
G^+ bacteria	0.050 ·	0.096 ·	0.457	0.926	<0.001***	0.278	<0.01**	<0.01 **	0.330
Actinomycete	0.637	0.570	0.364	0.645	<0.001***	0.886	0.183	<0.001***	0.472

4.3.2 PLFA Stable Isotope Probing

Comparing ^{13}C excess patterns in PLFA biomarkers following ^{13}C-labelled substrate addition yielded a distinctive ^{13}C incorporation pattern for each of microbial groups examined (Figure 4.1). When focusing on the type of labelled substrate added to the soils, all microbial groups show similar incorporation patterns for ^{13}C-glycine and ^{13}C-vanillin, while the incorporation of ^{13}C-cellulose shows a distinct pattern. Table 4.4 shows that none of the microbial groups show an interaction effect between incubation time and treatment. ^{13}C excess patterns are in most cases significantly affected by the duration of the incubation experiment (time), but only G^+ bacteria in the soils treated with ^{13}C-labelled vanillin are significantly affected by stage (Table 4.4). When analysing specific PLFA biomarkers, it can be seen that in the labelled glycine and cellulose treatment there is only a minimal number of PLFA biomarkers affected by time since abandonment; respectively 1 and 0 out of 22 PLFA biomarkers show a significant difference in ^{13}C-excess patterns between soil microbial communities in recent versus long-term abandoned soils (Supplementary Table S4.1). In contrast, the ^{13}C-vanillin treatment yielded nine PLFA biomarkers that were significantly different in ^{13}C excess patterns when comparing between recent and long-term abandoned soils (Supplementary Table S4.1). These differences are mainly found in the ise/anteiso methyl and 10-methyl branched PLFA biomarkers. For a complete overview of ^{13}C excess patterns of each individual PLFA biomarker, see Supplementary Figure S4.3.

4.3.3 Soil microbial community structure following substrate addition

Principal component analysis (PCA) revealed that there is wide variation in bacterial (Figure 4.2a) and fungal (Figure 4.2b) community composition following substrate addition. When comparing the overall bacterial community composition, it can be seen that there are large differences among the recently abandoned soils and in particular among the long-term abandoned soils (Figure 4.2a). The variation in bacterial community structure is mainly driven by incubation time, with a large degree of variability after 3 days of incubation and less variation after 7 and 28 days. Fungal-based PCA also showed a wide variation in soil microbial community structure for both recent and long-term abandoned soils, with no clear effect of incubation time or stage of abandonment (Figure 4.2b). For both the bacterial and fungal microbial community composition there is no clear distinction between soil microbial communities of recent and long-term abandoned soils.

4.3.4 rRNA stable isotope probing

Principal response curves (PRC) were created to compare the labelled microbial community fractions of different treatments after the addition of ^{13}C labelled substrates (Figure 4.3). Comparisons were made for both fungal (Figure 4.3, top) and bacterial community (Figure 4.3, bottom) composition as well as recent (Figure 4.3,

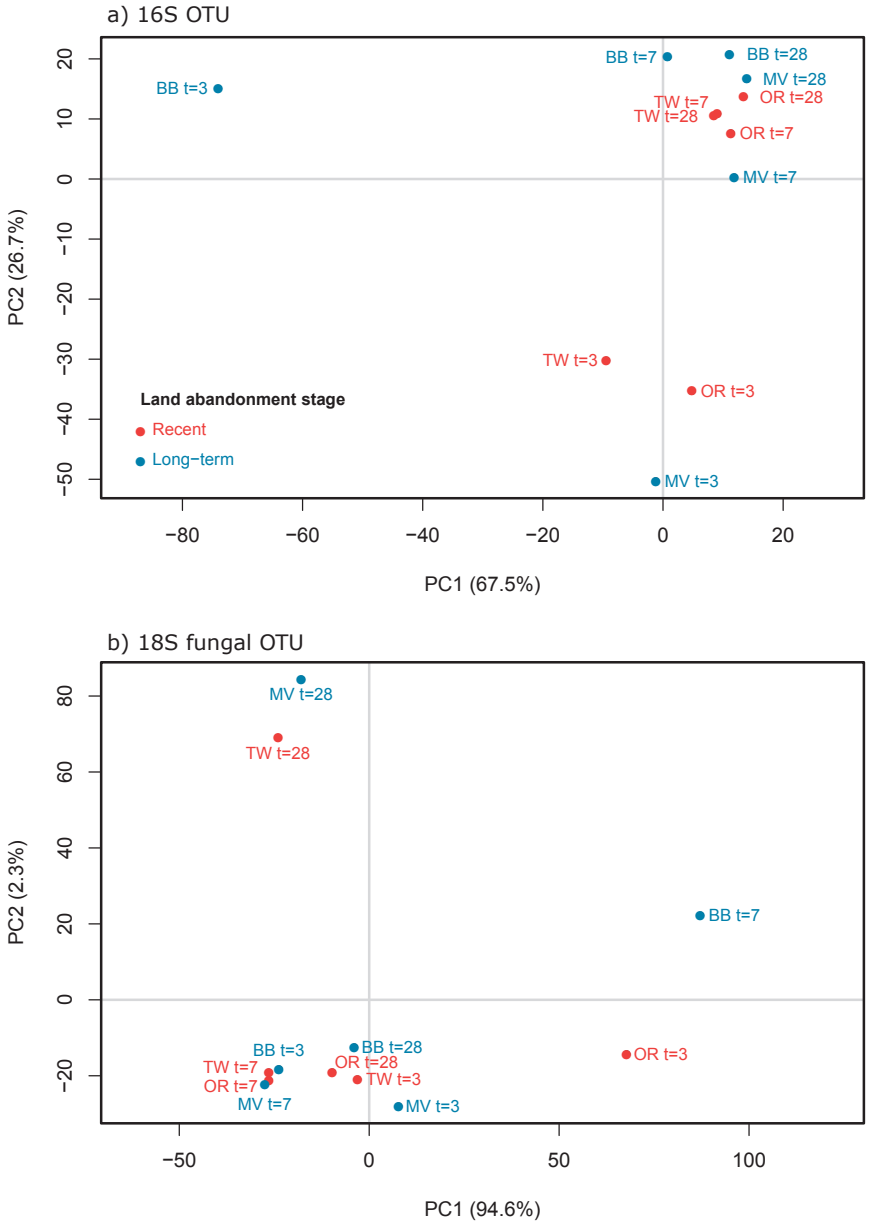
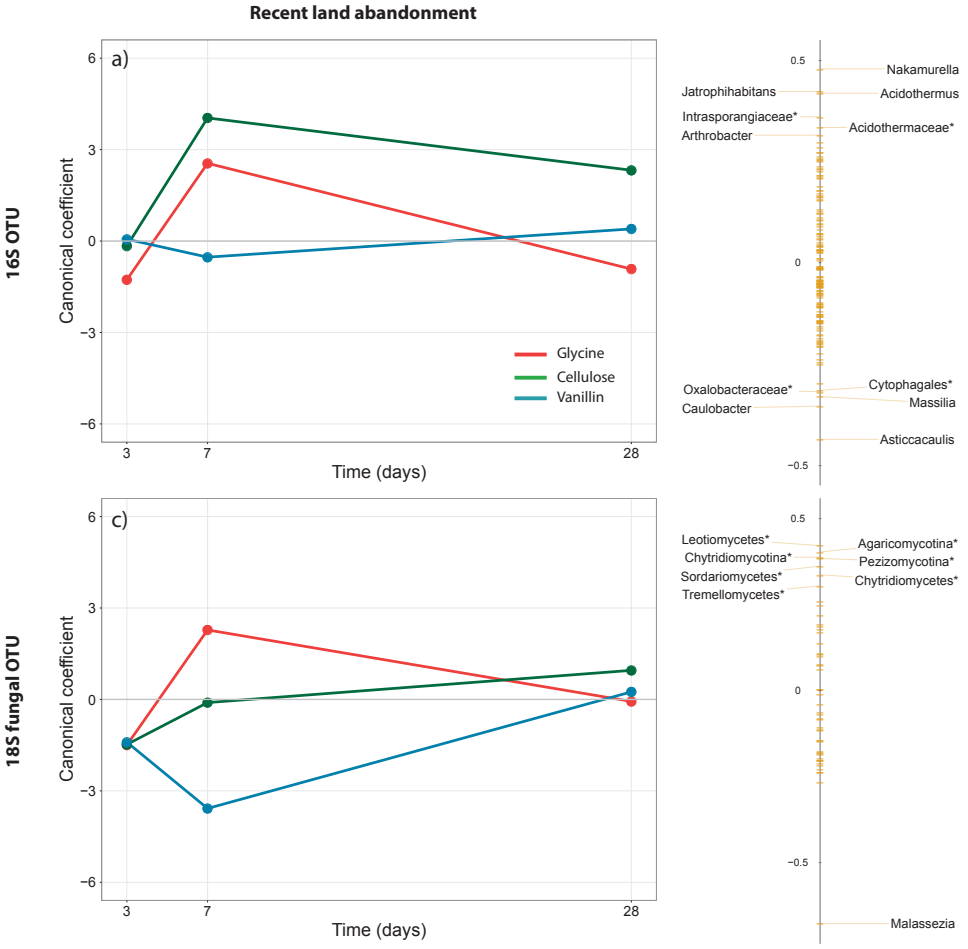


Figure 4.2 OTU based principal component analyses (PCA) to characterize soil microbial community in different ex-arable soils (recent=red vs long-term = blue) and at different time points (in days) after substrate addition. Each point represents an individual positive control sample from a specific field (recently abandoned fields: TW and OR; long-term abandoned fields: BB and MV) and at a specific time (in days) after substrates were added. a) PCA based on the OTU abundance of the top 250 sequenced 16S rRNA OTUs. b) PCA based on the OTU abundance of the top 250 sequenced fungal 18S rRNA OTUs.

left) and long-term abandoned soils (Figure 4.3, right). PRC reveal that the labelled fraction of the communities treated with ^{13}C-labelled glycine or ^{13}C-labelled cellulose are comparable for both bacterial and fungal microbial community composition in both the recent and long-term abandoned soils. The labelled fraction of the microbial communities enriched with ^{13}C-labelled vanillin show a distinctive pattern compared to the other two treatments. The divergence between treatments is mainly caused by differences in the microbial composition of the active community 7 days after substrate addition, a pattern that becomes more pronounced in long-term abandoned soils (Figure 4.3b, d). Comparing the enriched microbial communities with the baseline microbial community (x-axis), we see that in recently abandoned soils the vanillin-labelled bacterial communities are most similar to the baseline community, while in the fungal community this is true for the cellulose-labelled community. In long-term abandoned soils, the glycine- and cellulose-labelled microbial fractions were similarly close to the baseline microbial community.

The species scores on the right side of the PRC show the most indicative microbial genera responsible for the observed patterns. In recently abandoned soils, there is no clear distinction between the bacterial communities in each of the different treatments. In the fungal communities of these soils, the ^{13}C-glycine-labelled community is characterized by a number of fungal genera like Letoiomycetes, Agaricomycotina, while the ^{13}C-vanillin-labelled community is characterized by a high abundance of Malessezia. For bacterial communities in long-term abandoned soils, the ^{13}C-vanillin-treated microbial communities where most influenced by the differential representation of *Pseudomonas* and *Caulobacter* (Figure 4.3b). Indicative bacterial genera for the bottom side of the PRC were *Acidothermaceae, Solirubrobacterales* and *Acidothermus*. For 18S OTU, the pattern was less distinctive between treatments, with *Agaricomycetes, Glomeromycetes, Tremellomycetes* and *Eurotiomycetes* representing the most indicative fungal genera after 7 days.

In order to visualize potential soil microbial resource partitioning 7 days after substrate addition, ternary plots were created for both 16S (Figure 4.4a) and 18S OTU data (Figure 4.4b). The ternary plots reflect the relative abundance of OTUs in the labelled parts of the microbial community in different treatments, compared to the baseline microbial community (in control substrate amended soils). 7 days after addition of substrates, the ^{13}C-cellulose or ^{13}C-glycine amended soils appeared to have similar ^{13}C-labelled bacterial populations, with for instance strong labelling of the Acidobacteria. Labelling of bacterial groups in the ^{13}C-vanillin treated soils was more distinct, with specific labelling of for instant the class Gammaproteobacteria (Figure 4.4a). For the fungal community, it can be seen that fungal classes found their own niche in resource partitioning. Where Ascomycota were enriched in both cellulose and glycine treated soils, Glomeromycota (*Glomeromycotia* spp.) are well represented in the ^{13}C-vanillin treated soils. Basidiomyceta were underrepresented in the ^{13}C-cellulose treatment, but well- represented in the ^{13}C-glycine treatment. Ternary plots at all sampling times (3, 7 and 28 days) can be found in Supplementary


Recent land abandonment
16S OTU
a)
Canonical coefficient
6
3
0
−3
−6
3
7
28
Time (days)
Glycine
Cellulose
Vanillin
0.5
0
−0.5
Nakamurella
Jatrophihabitans
Acidothermus
Intrasporangiaceae*
Acidothermaceae*
Arthrobacter
Oxalobacteraceae*
Cytophagales*
Caulobacter
Massilia
Asticcacaulis
18S fungal OTU
c)
Canonical coefficient
6
3
0
−3
−6
3
7
28
Time (days)
0.5
0
−0.5
Leotiomycetes*
Agaricomycotina*
Chytridiomycotina*
Pezizomycotina*
Sordariomycetes*
Chytridiomycetes*
Tremellomycetes*
Malassezia

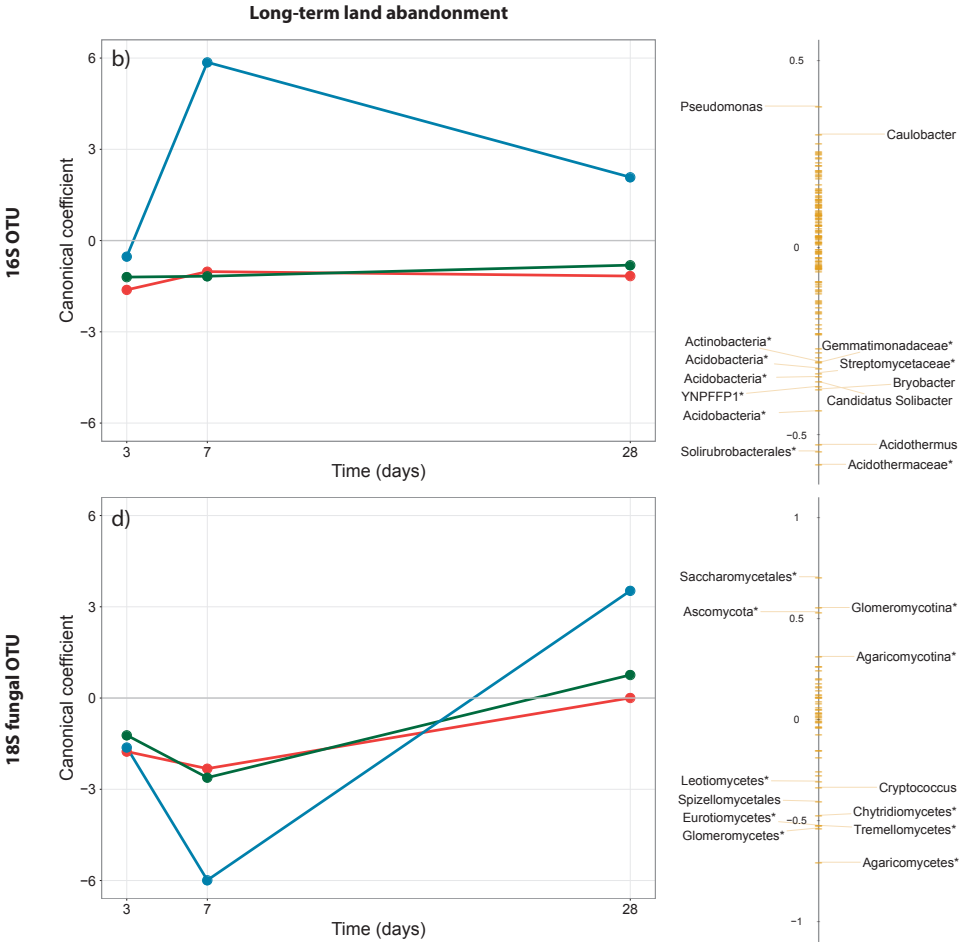


Figure 4.3 Principal response curves (PRC) for microbial genera (a-b: bacterial genera; c-d: fungal genera) involved in the decomposition of a specific substrates (red: glycine, green: cellulose, blue: vanillin). PRC are shown for recently abandoned soils (left page) and long-term abandoned soils (right page) with species score estimates on right side of the curves in orange. Only genera with an absolute species score estimate above 0.3 are labelled (genera indicated with an * are unclassified on genera level, and therefore the highest known taxonomic classification is named).

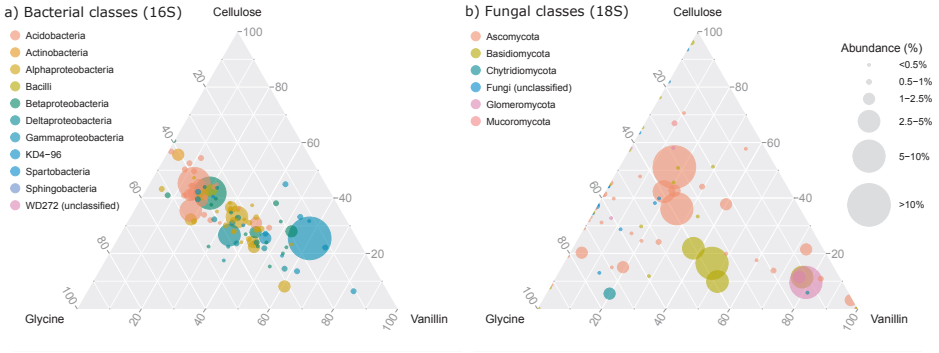


Figure 4.4 Ternary plots of the relative abundance of a) the top 100 sequenced 16S rRNA OTUs and b) the top 75 sequenced fungal 18S rRNA OTUs, 7 days after substrate addition. Each circle represents one OTU, whereby the size of the circle represents the relative abundance (%). The position of each circle is determined by the relative abundance in the labelled gradient fraction of each of the three substrate treatments compared to the positive control sample. Circles are coloured in respect to a) bacterial or b) fungal taxonomical class.

Figure S4.4, showing that day 7 (similar to Figure 4.4) shows the most distinction between both bacterial and fungal classes in terms of resource partitioning. From these plots it can also be seen that after 28 days, bacterial OTUs converge again, while fungal OTUs show further diversification.

To visualize potential time-bound patterns in microbial decomposition, ternary plots of the labelled bacterial community were also made per substrate treatment (Figure 4.5). We observed that Betaprotebacteria and Deltaproteobacteria are most pronounced after 3 days in all treatments. In contrast Alphaproteobacteria and Acidobacteria are relatively more enriched in later stages of substrate decomposition (after 7 and 28 days). Where the patterns for ^{13}C-glycine and ^{13}C-cellulose amended soil bacterial communities are relatively similar (Figure 4.5a,b), the ^{13}C-vanillin amended soil bacterial community shows distinctive patterns (Figure 4.5c), with a smaller abundance of Acidobacteria and a separation of Gammaproteobacteria (relatively unaffected by time) and Betaproteobacteria, the latter being mainly enriched after 3 days. For 18S labelled rRNA there were no clear temporal patterns found for either of the substrate treatments (Supplementary Figure S4.5).

4. Discussion

4.4.1 Organic substrate-derived microbial C flows

With this study, we investigated which microbial groups are actively involved in the decomposition of a range of organic C substrates in recent and long-term abandoned soils. Where previous SIP studies examined the impact of amendment of

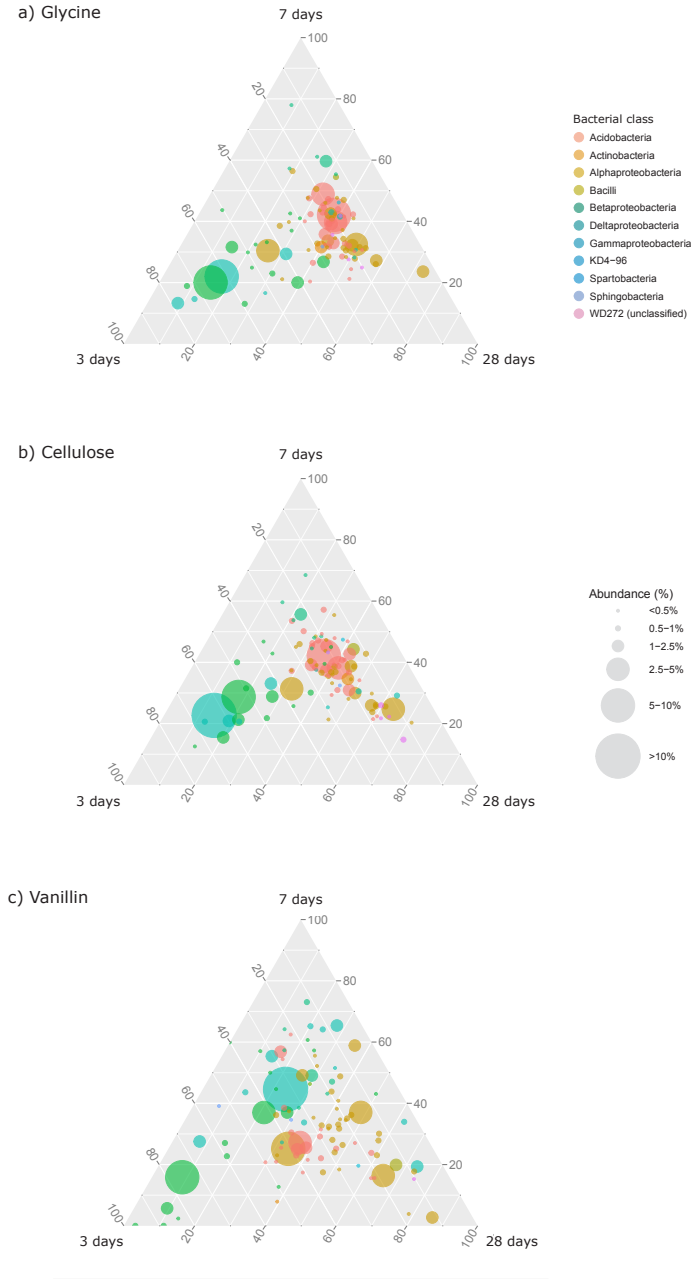


Figure 4.5 Ternary plots of the relative abundance of the top 100 sequenced 16S rRNA OTUs in the ^{13}C-enriched gradient fraction of the soils amended with a) ^{13}C-labelled glycine, b) ^{13}C-labelled cellulose and c) ^{13}C-labelled vanillin. Each circle represents one OTU, whereby the size of the circle represents the relative abundance (%). The position of each circle is determined by the relative abundance in the labelled gradient fraction at different time points after incubation (3, 7 and 28 days). Circles are coloured in respect to the bacterial taxonomical class.

different singular substrates (e.g. Kramer *et al.*, 2016; Rinnan & Bååth, 2009; Torres *et al.*, 2014), this experiment had a unique set-up by supplementing all treatments with the same set of substrates, of which only one substrate was ^{13}C-labelled. This allowed us to track the fate of specific substrates through the soil microbial food web under comparable conditions. The similarly of all substrate-amended treatments was confirmed by the fact that overall C mineralization rates (Supplementary Figure S4.1a) and the biomasses of microbial groups as measured by PLFA analysis (Supplementary Figure S4.2) were comparable between treatments throughout the incubation experiment. Following substrate addition, we show that overall C mineralization rates were initially higher in long-term abandoned soils, which is in line with earlier studies of the same chronosequence of ex-arable fields (Holtkamp *et al.*, 2008, 2011). The divergent C flow patterns observed for the cellulose-derived C flow patterns, as revealed by PLFA-SIP, may have been due to the form in which cellulose was added to the soil, namely as fine small fibres, as opposed to powered as was the case for glycine and vanillin. This might explain the delay in decomposition of cellulose, and subsequent C flow patterns, compared to the other two substrates.

4.4.2 Microbial C flows following land abandonment

Comparable C flows are shown between recent and long-term abandoned soils for most microbial groups, except for vanillin-derived C incorporation patterns in the G^+ bacteria. This group of bacteria is known to play an important role in SOM degradation (Kramer & Gleixner, 2006, 2008), but the relative decrease in vanillin-derived ^{13}C-excess suggests that their contribution to the decomposition of recalcitrant material becomes less in long-term abandoned soils. Since the biomass of this microbial group did not decrease, this indicates that G^+ bacteria in long-term abandoned soils are less efficient in decomposing recalcitrant SOM, compared to recently abandoned ex-arable soils. This matches well with the findings in chapter 3 on the same chronosequence, that indicated a decreased bacterial efficiency of decomposing SOM with time since abandonment. This may support the idea that fungi gain a more prominent role in decomposition over bacteria with time after land abandonment, even though this is not necessarily reflected in a build-up of fungal biomass (van der Wal *et al.*, 2006; Hannula *et al.*, 2017; Morriën *et al.*, 2017).

The fact that the most pronounced differences between recent and long-term abandoned soils can be found in the decomposition of recalcitrant material are further supported when looking to SIP results at the genus level, as this shows the emergence of a microbial community that is specialized in the decomposition of recalcitrant material, in this case vanillin. This supports our hypothesis of a microbial community that is specialised in the decomposition of recalcitrant organic matter in long-term abandoned soils. This apparent specialization is visible both within the bacterial and fungal kingdom, confirming the findings of Kramer *et al.* 2016, who suggest the existence of intra-kingdom niche partitioning instead of general bacterial and fungal energy channels (Kramer *et al.*, 2016). Where previous studies in these

ex-arable fields have focussed on the role of fungi (van der Wal *et al.*, 2006; Hannula *et al.*, 2017), this study shows that there is also a specialised bacterial community involved in the decomposition of recalcitrant organic matter. These results point out that soil food webs should not only be revisited because of the role for fungi in labile C consumption (de Vries & Caruso, 2016), but also because of the emerging evidence for bacterial interference in recalcitrant C decomposition.

4.4.3 Soil microbial resource partitioning and consumer succession

The apparent resource partitioning between bacterial and fungal classes further strengthens the idea that resource partitioning is not restricted to the division of fungal and bacterial energy channels (Kramer *et al.*, 2016). Acidobacteria, a general, diverse and abundant bacterial group in bulk soil (Fierer *et al.*, 2007), seem to occupy a key position for the rather unspecialised decomposition of both glycine and cellulose. The G^- bacterial classes Alphaproteobacteria and Gammaproteobacteria are e indicated as degraders of recalcitrant organic matter. Especially the bacterial genera *Pseudomonas* and *Caulobacter* were found to be relatively active in the decomposition of vanillin of long-term abandoned soils, compared to the rest of the microbial community. Even though *Pseudomonas* is well-known for the decomposition of easily soluble materials, their role in lignin degradation has been previously recognised (Ahmad *et al.*, 2010; Bugg *et al.*, 2011; Goldfarb *et al.*, 2011). Also the active role of *Caulobacter* in the decomposition of plant-derived polymers has been previously described (Hottes *et al.*, 2004; Eichorst & Kuske, 2012). The enrichment of these G^- bacterial groups in ^{13}C-vanillin treated soils could interfere with the reduced efficiency of G^+ bacteria with land abandonment, suggesting that G^- play a more prominent role in SOM degradation than previously assumed (Kramer & Gleixner, 2006, 2008).

In the fungal kingdom, a clear distinction was found between glycine-, cellulose- and vanillin-degrading communities, that remained apparent to up to 28 days. Most OTU's assigned to the *Ascomycota* were found to be active in the decomposition of glycine and cellulose, but specific genera in this class were also found to in long-term abandoned soils to play a role in the degradation of vanillin, confirming an earlier study indicating *Ascomycota* as possible litter degraders (Voriskova & Baldrian, 2013). The enrichment of *Glomeromycotina spp*. also further supports the insight that *Glomeromycotina* are not necessary only symbionts with plants (Hempel *et al.*, 2007), but that they also play a role in litter decomposition (Voriskova & Baldrian, 2013).

The observed succession in bacterial activities during litter decomposition, as shown by bacterial classes that follow up on each other over the course of the incubation experiment (Figure 4.5), confirms the findings of chapter 3 on the same ex-arable soils. These results are also in line with earlier findings of intra-kingdom microbial consumer succession in the degradation of cellulose (Kramer *et al.*, 2016), highlighting Beta- and Gammaproteobacteria as primary decomposers, and Alphaproteobacteria, Actinobacteria and Acidobacteria as secondary decomposers. Furthermore, this study

shows that the relative activity of active microbial degraders, but not the sequence, is differing over treatments, indicating that microbial consumer succession is unrelated to the type of organic substrate.

4.5 Conclusions

This study provides novel insights into how different detrital substrates are consumed and flow through the soil microbial food web in recent versus long-term abandoned ex-arable soils. We conclude that 30 years after land abandonment, the soil microbial food web becomes less efficient in decomposing SOM, but also partly develops into a specialized microbial community dealing with the decomposition of rather recalcitrant organic matter. Shifts were apparent in intra-kingdom bacterial and functional resource partitioning at the microbial class level, driven by substrate recalcitrance, and bacterial (but not fungal) consumer succession that is independent of substrate quality. The fact that these shifts in soil microbial community functioning are not apparent in the overall microbial community structure, nor in the relative importance of fungal versus bacterial energy channels, further supports the need to revise the concept of soil microbial food webs. We propose that incorporation of these new insights into soil microbial resource partitioning and consumer succession will be vital to improving existing soil food web models, thereby enhancing our ability to understand soil microbial communities responses to changing environmental conditions.

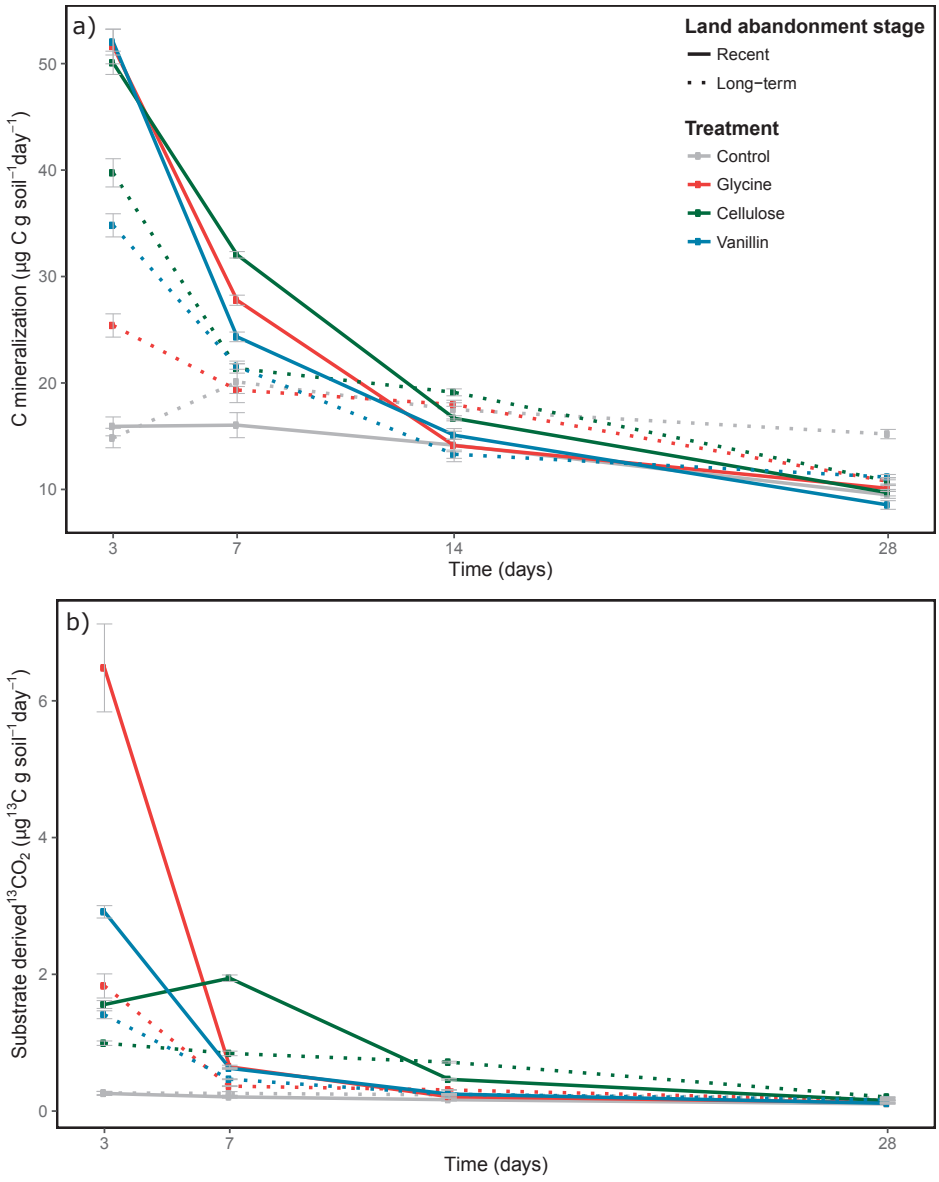


Figure S4.1 Carbon mineralization rates during incubation at each respective sampling moment, for recent and long term abandoned soils and for different treatments. All treatments received the same set of substrates with one specific ^{13}C labelled substrate (Glycine, Cellulose or Vanillin), or no substrate at all (Control). The upper panel (a) shows total C mineralization rates and the lower panel (b) shows ^{13}C-labelled substrate-derived C mineralization rates. Lines represent means of recently abandoned soils (straight) and long-term abandoned soils (dashed) ± SE.

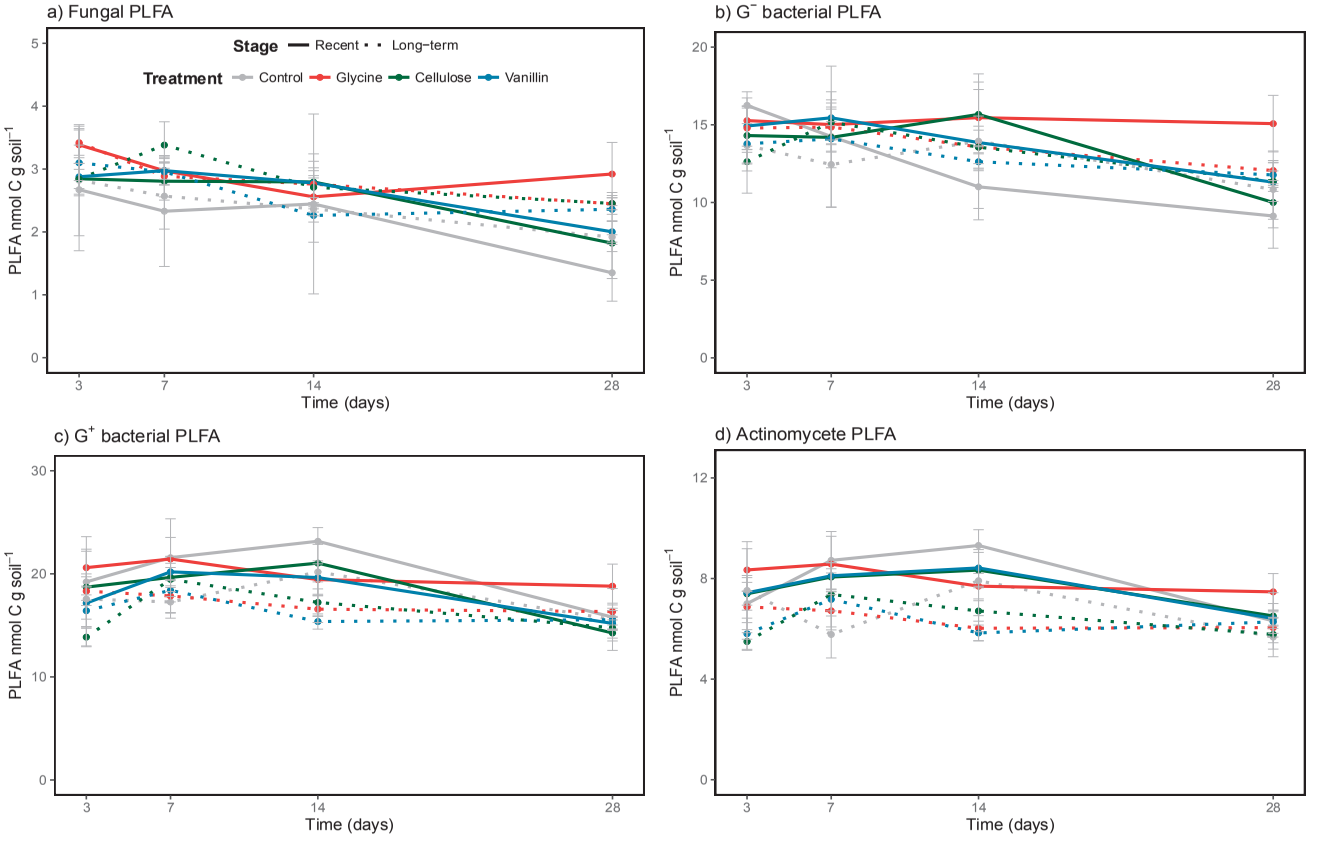


Figure S4.2 The biomass of different groups of PLFA biomarkers at respective sampling times during incubation, for both recent and long-term abandoned soils and for different treatments, whereby the control treatment represents the treatment without substrate amendments. Lines represent means ± SE. The panels show labelling patterns for specific PLFA biomarkers that were appointed to the microbial groups a) fungi, b) G^- bacteria, c) G^+ bacteria, d) Actinomycetes. No significant interaction effects were found, so the p-values of the main effects on the labelled treatments are given per microbial group.

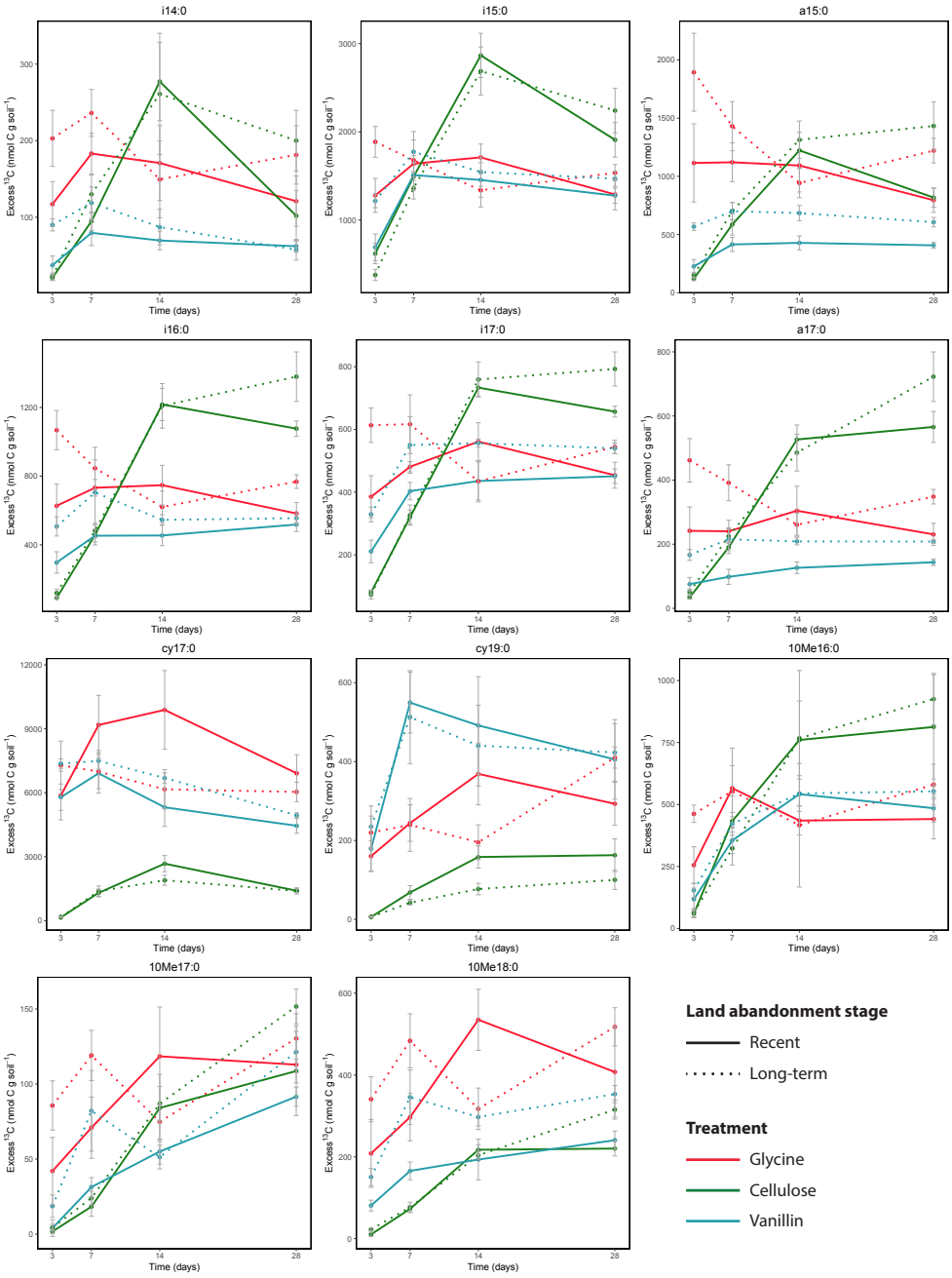


Figure S4.3a The amount of excess ^{13}C in different PLFA biomarkers during incubation at each respective sampling moment, for recent and long-term abandoned soils and for different treatments. Lines represent means ± SE.

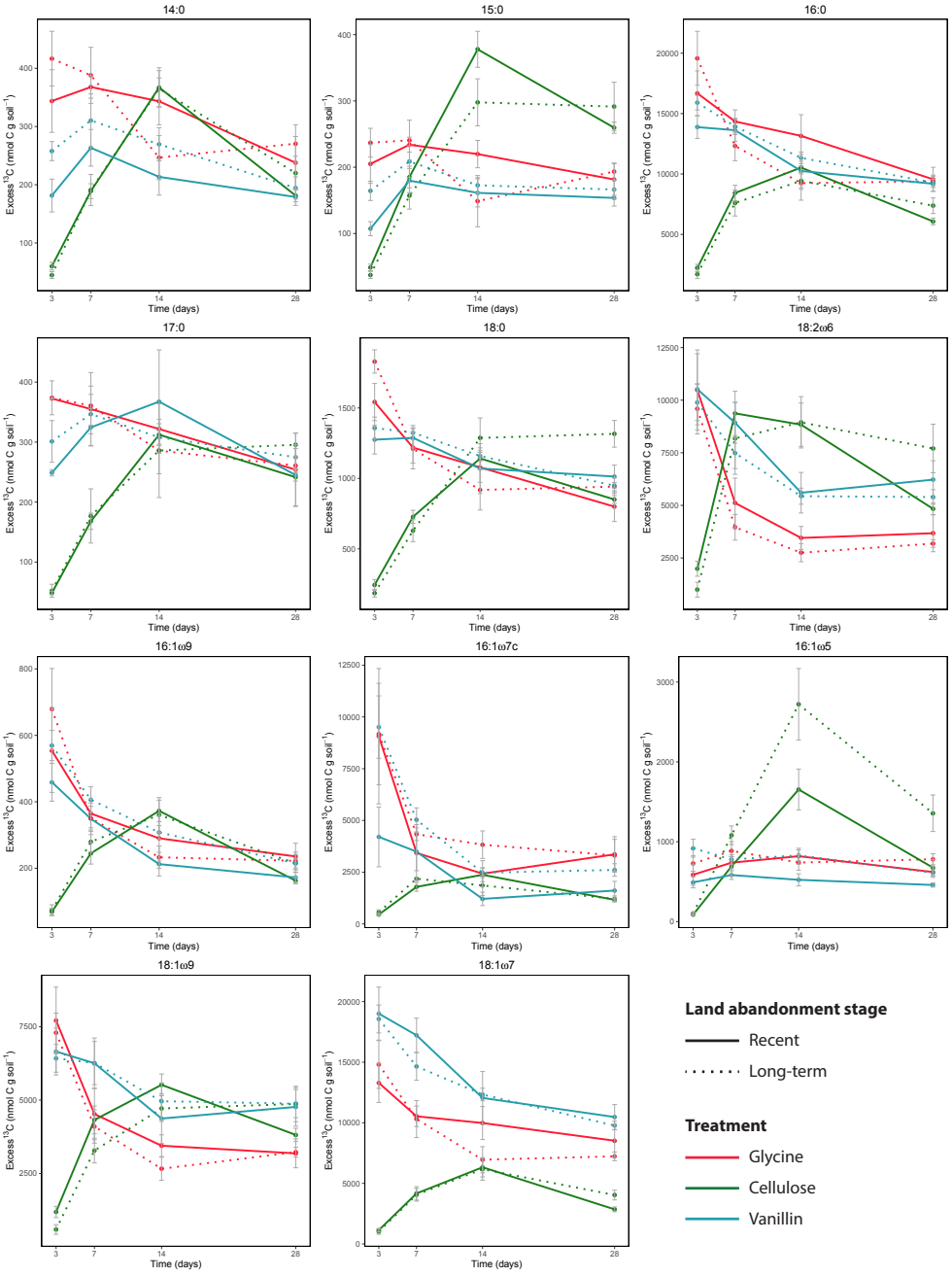


Figure S4.3b The amount of excess ^{13}C in different PLFA biomarkers during incubation at each respective sampling moment, for recent and long-term abandoned soils and for different treatments. Lines represent means ± SE.

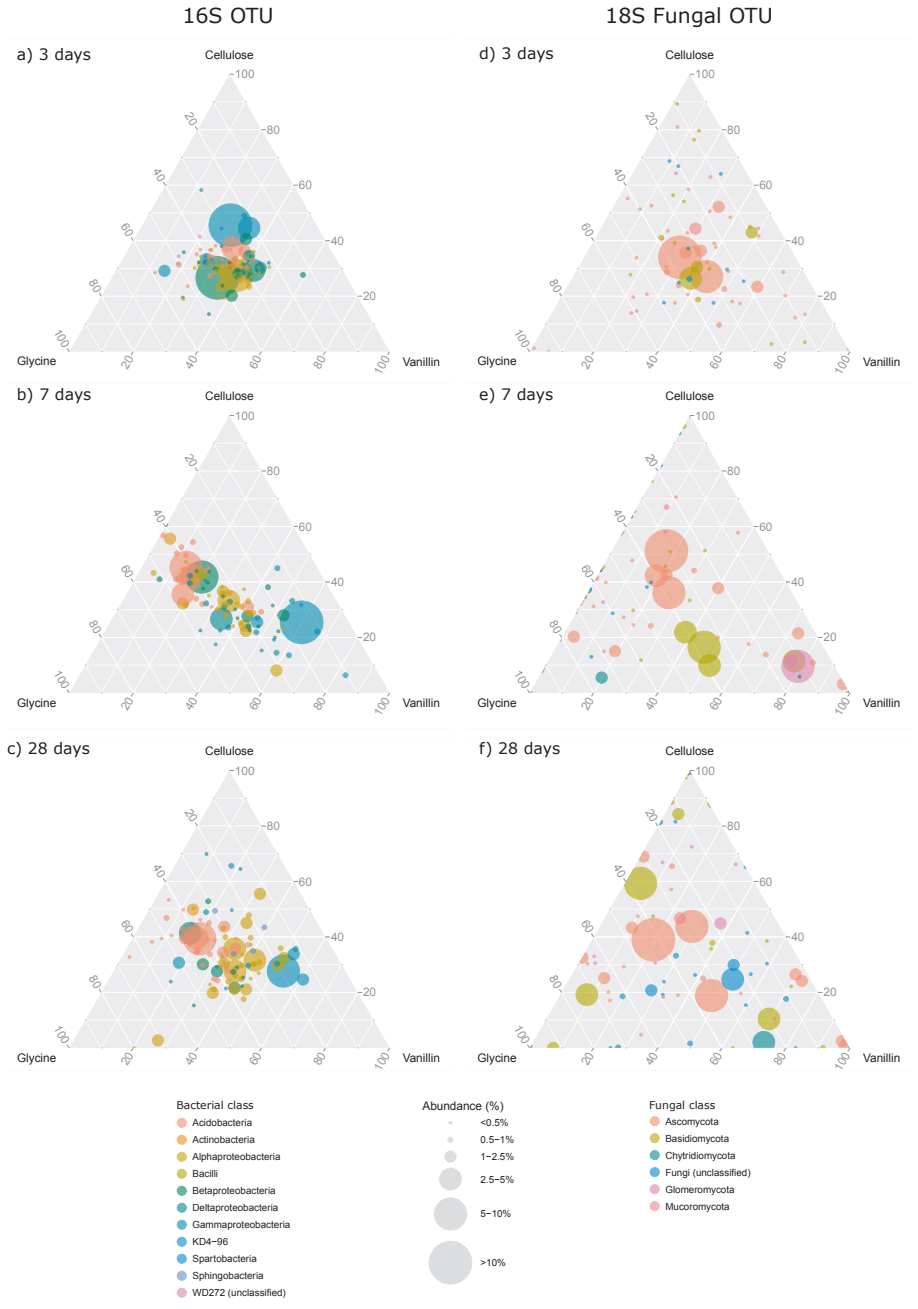


Figure S4.4 Ternary plots of the relative abundance of the top 100 sequenced 16S rRNA OTUs (a-c) and the top 75 sequenced fungal 18S rRNA OTUs (d-f); 3, 7 and 28 days after substrate addition. Each circle represents one OTU, whereby the size of the circle represents the relative abundance (%). The position of each circle is determined by the relative abundance in the labelled gradient fraction of each of the three substrate treatments compared to the positive control sample. Circles are coloured in respect to the bacterial (a-c) or fungal (d-f) taxonomical class.

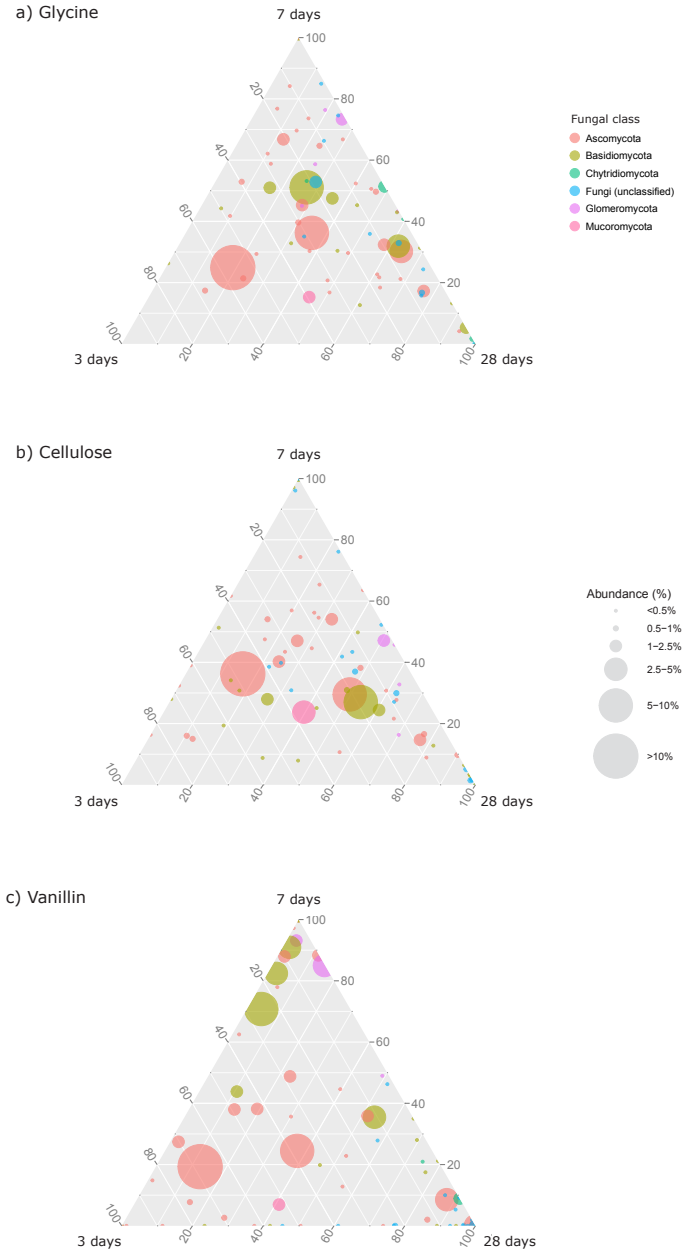


Figure S4.5 Ternary plots of the relative abundance of the top 75 sequenced 18S rRNA OTUs in the ^{13}C-enriched gradient fraction of soils amended with a) ^{13}C-labelled glycine, b) ^{13}C-labelled cellulose and c) ^{13}C-labelled vanillin. Each circle represents one OTU, whereby the size of the circle represents the relative abundance (%). The position of each circle is determined by the relative abundance in the labelled gradient fraction at different time points after incubation (3, 7 and 28 days). Circles are coloured in respect to the fungal taxonomical class.

Table S4.1 Statistical summary of p-values from the two factor RM-ANOVA for the amount of ^{13}C excess in different PLFA biomarkers. Stage refers to time since land abandonment (recent versus long-term) and time refers to time after ^{13}C-labelled substrate addition (3,7 and 28 days), where p-values are the result of linear-mixed modelling (*** p<0.001, ** p<0.01, * p<0.05, · p<0.1).

	Glycine			Cellulose			Vanillin		
	Stage	Time	Stage x Time	Stage	Time	Stage x Time	Stage	Time	Stage x Time
Ise/anteiso methyl branched									
i14:0	0.158	0.353	0.710	0.349	<0.001***	0.701	0.087 ·	0.345	<0.05*
i15:0	0.291	0.190	0.523	0.685	<0.001***	0.221	<0.05*	0.093 ·	0.540
a15:0	0.235	0.132	0.514	0.127	<0.001***	0.424	<0.001***	0.106	0.255
i16:0	<0.05*	0.073 ·	0.365	0.361	<0.001***	0.993	<0.01**	<0.01**	0.135
i17:0	0.126	0.719	0.202	0.466	<0.001***	0.639	<0.001***	<0.001***	0.949
a17:0	0.074 ·	0.612	0.211	0.256	<0.001***	0.619	<0.001***	<0.001***	0.328
Cyclopropyl ring									
cy17:0	0.733	0.692	0.454	0.651	<0.001***	0.870	<0.05*	<0.001***	0.171
cy19:0	0.994	<0.01**	0.484	0.290	<0.001***	0.096 ·	0.654	0.138	0.861
10-methyl branched									
10Me16:0	0.144	0.322	0.805	0.881	<0.001***	0.456	0.499	<0.001***	0.667
10Me17:0	0.652	<0.05*	0.467	0.414	<0.001***	0.229	<0.05*	<0.001***	0.693
10Me18:0	0.443	<0.01**	0.903	0.148	<0.001***	<0.01**	<0.05*	<0.001***	<0.05*
Straight-chain saturated <19									
14:0	0.792	<0.001***	0.634	0.819	<0.001***	0.326	0.164	0.063 ·	0.600
15:0	0.627	0.050 ·	0.721	0.238	<0.001***	0.448	0.079 ·	0.586	0.336
16:0	0.767	<0.001***	0.698	0.617	<0.001***	0.304	0.328	<0.001***	0.243
17:0	0.697	<0.01**	0.856	0.948	<0.001***	0.409	0.793	0.292	0.877
18:0	0.333	<0.001***	0.815	0.441	<0.001***	<0.05*	0.689	<0.001***	0.388
Poly-unsaturated ω6 family									
18:2ω6	0.807	<0.001***	0.779	0.775	<0.01**	0.068 ·	0.758	<0.001***	0.967
Monoenoic									
16:1ω9	0.799	<0.001***	0.346	0.578	<0.05*	0.693	0.283	<0.001***	0.567
16:1ω7c	0.876	<0.001***	0.753	0.541	<0.05*	0.675	0.235	<0.001***	<0.05*
16:1ω5	0.296	0.935	0.950	0.158	<0.001***	0.386	<0.05*	<0.01**	0.082 ·
18:1ω9	0.892	<0.001***	0.660	0.305	<0.001***	0.068 ·	0.971	<0.001***	0.655
18:1ω7	0.677	<0.001***	0.536	0.850	<0.01**	0.300	0.197	<0.001***	0.896

Chapter 5

Plant biomass, soil microbial community structure and nitrogen cycling under different organic amendment regimes; a ^{15}N tracer-based approach

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Abstract

Sustainable agriculture requires nutrient management options that lead to a profitable crop yield with relatively low nitrogen (N) losses to the environment. We studied whether the addition of contrasting organic amendments together with inorganic fertilizer can promote both requirements simultaneously. In particular we studied how the chemical composition of organic amendments affects the biomass, activity and composition of the soil microbial community and subsequently carbon (C) and N mineralization, microbial N immobilization and plant growth and nutrient uptake. In a pot experiment, Brussels sprouts (*Brassica oleracea, cvar. Cyrus*) were grown on arable soil, mixed with ^{15}N-labelled mineral fertilizer and different kinds of organic amendments (cattle manure solid fraction, maize silage, lucerne silage, wheat straw) differing in C:N ratio and lignin content. After 69 and 132 days, destructive sampling took place to assess the effects of the different treatments on soil microbial biomass (microscopic measurements), microbial community composition (phospholipid fatty acid profiles), soil microbial activity (^{14}C-leucine incorporation), C and N mineralization, plant biomass and ^{15}N retrieval in soil pools, microbial biomass and plant biomass. Addition of organic amendments increased soil microbial biomass, activity and fungal/bacterial ratio and created distinct microbial community compositions, whereby high C:N ratio organic amendments had stronger effects compared to low C:N ratio amendments. Structural equation modelling showed that higher values of soil microbial activity were associated with increased N mineralization rates, increased plant biomass and plant ^{15}N uptake, while microbial ^{15}N immobilization was associated with soil microbial community composition. The outcomes of this study highlight the importance of the chemical composition and the amount of the organic amendments for finding a balance between plant N uptake, microbial N immobilization and N retention in labile and stable soil pools through the effects on the composition and activity of the soil microbial community. The results provide insights that can be used in designing combined input (nutrient and organic) nutrient management strategies for a more sustainable agriculture.

5.1 Introduction

Agricultural intensification has powered the increase in food production of the last century, but has to a large extent been fuelled by the application of large amounts of mineral fertilizer to meet the nutrient requirements of high productivity croplands. For example, between 1960 and 2000, the global application of nitrogen (N) fertilizers increased from 10 to 80 million tonnes (Fixen & West, 2002). Such N additions may reduce natural soil functions like microbial decomposition of soil organic matter and concomitant nutrient mineralization (Giller *et al.*, 1997; Thiele-Bruhn *et al.*, 2012).

Intensive agriculture may also lead to significant amounts of N being lost through NH_3 volatilization, denitrification and leaching of mineral N to ground and surface water (Neeteson, 1990). The leaching of reactive N has devastating implications for the environment, as it lowers air and water quality, but also impacts biodiversity and human health (Schlesinger, 2009; Robertson & Vitousek, 2009). Current levels of reactive N inputs in the environment are seen as beyond the 'planetary boundaries' for a steady Earth system (Rockström *et al.*, 2009; Steffen *et al.*, 2015), which asks for a reduction of N application levels by 75% to avoid important Earth subsystems from shifting into new states, with potentially dramatic consequences (Rockström *et al.*, 2009). However, the problems are expected to increase in the coming decades, as it is predicted that global N inputs will exceed 200 million tonnes by 2050 (Tilman *et al.*, 2001).

To restrict the amounts of N ending up in the environment, it is important that farm systems optimize their N management (Robertson & Vitousek, 2009), by ensuring an efficient use of N for food production, while not allowing N inputs to impact other ecosystems (Goulding *et al.*, 2008). In order to improve nitrogen use efficiency (NUE), agricultural N management strategies, and associated research dealing with NUE, have focused on synchronizing the N availability in soil with N-demand by the crop, in both time and space (Campbell *et al.*, 1992). Although such approaches have led to increased yields, they have not had large effects on reducing N leaching (Cassman *et al.*, 2002).

The low efficiency of N use in agricultural soils may to some extent be caused by a decoupling of the carbon (C) and N cycles, as mineral N applications take over the role of the microbial community in soil organic matter decomposition and N mineralization (Drinkwater & Snapp, 2007). The use of organic amendments instead of mineral fertilizer can then have the potential to optimize agricultural N management by restoring this microbial link between soil C and N processing (Drinkwater *et al.*, 1998; Nosengo, 2003). Recent attention is given to combining organic amendments with mineral fertilizer, allowing for both improved yield as well as a greater N retention capacity (Vanlauwe *et al.*, 2001; Chivenge *et al.*, 2011). It is known that the application of mineral N is an important factor controlling decomposition of organic material, however the net effect depends on the quality of the organic pool and the time scale of the decomposition process, as reviewed by i.e. Fog (1988), Mary

et al. (1996) and Knorr *et al.* (2005). The design of a successful fertilization strategy that combines organic and mineral inputs requires therefore a better understanding of microbial decomposition of organic matter, the concomitant mineralization and immobilization of nutrients and plant nutrient uptake and plant biomass. It is known that addition of organic substrates, whether or not in combination with mineral fertilizers, stimulates the microbial biomass and activity (Wardle, 1992). However, the precise effects of different kinds of organic amendments on plant nutrition and nutrient mineralization and immobilization depends on the chemical composition of the organic matter and the potential of the soil microbial community to decompose particular organic compounds (Grandy *et al.*, 2013).

The aim of the present study is to obtain insight in the effect of chemically contrasting types of organic amendments on the biological composition of soil microbial community, and how that influences C and N mineralization rates, and subsequently the ecosystem services microbial N immobilization, plant growth and N uptake. C and N mineralization could also have been referred to as soil ecosystem services, however in this article the term ecosystem service is restricted to benefits that have a direct social value in terms of food production and the minimization of N losses, where we believe N immobilization plays a central role (Millennium Ecosystem Assessment, 2005). The study was carried out by means of a full factorial pot experiment with ^{15}N-labelled mineral fertilizer and four different kinds of organic amendments, differing in C:N ratio and lignin content (i.e. cattle manure solid fraction, lucerne silage, maize silage and wheat straw). The addition of ^{15}N-labelled mineral fertilizer allowed to measure mineral fertilizer uptake by the plant, as well as fertilizer-derived microbial N immobilization and soil N retention. The soil microbial community was examined in terms of biomass (microscopic measurements), activity (^{14}C-leucine incorporation) and composition (phospholipid fatty acid profiles). Structural equation modelling (SEM) was used to link the different kinds of organic amendment to the composition and activity of the soil microbial community and subsequently to C and N mineralization, microbial N immobilization, plant growth and plant N recovery. We expected that application of all organic amendments will result in increased biomass and activity of the microbial community, and increased microbial mineralization rates; and that the organic amendments with a high C:N ratio will have an increasing effect on the fungal/bacterial ratio, on N immobilization and consequently on the N storage capacity of the soil microbial community as compared to low C:N ratio amendments. Impacts of amendments on plant N uptake and growth are the outcome of the balance between immobilization and mineralization, and we had no a priori expectation on the evolution of this outcome relative to plant N demand dynamics.

5.2 Material and methods

5.2.1 Experimental design

The pot experiment was carried out at Wageningen University and Research Centre, the Netherlands, starting in September 2011 and ending in January 2012. The soil used in the experiment was a fine sand textured topsoil from the Vredepeel experimental farm (51˚32′ N, 5˚52′ E; altitude 27 m a.s.l.) from a field where no organic fertilization was applied during the previous 10 years (Langeveld *et al.*, 2005). The soil had a soil organic matter (SOM) content of 3.6%, total organic carbon (TOC) content of 2.0%, a total N content of 0.9% and a pH-H_2O of 6.4. The experiment consisted of five treatments which represented different forms of organic amendments, including a control treatment that did not receive organic amendments. The used organic amendments were chosen for their different C:N ratios and lignin contents. The organic amendments consisted of the solid fraction of cattle manure (low C:N, high %lignin), lucerne silage (low C:N, low %lignin), maize silage (high C:N, low %lignin), and wheat straw (high C:N, high %lignin). Chemical properties of the organic amendments are listed in Table 5.1.

In September 2011 10L pots were filled with 10 kg of dry soil that was pre-mixed with finely cut fresh organic amendments (5-10 mm). Ammonium sulphate ($(NH_4)_2SO_4$) was applied as mineral N fertilizer, labelled with the stable isotope ^{15}N at 10% enrichment (Isolife BV, Wageningen, The Netherlands). Every treatment received an equal amount of organic N and mineral N (both 0.98 g N/pot), except for the control treatment which only received 0.98 g N/pot mineral N fertilizer (hence the amount of ^{15}N added was equal in all treatments including control). The N rate of 0.98 g N/pot is similar to 200 kg N/ha, respectively, based on soil surface area in the pots. In addition, all pots received 3.27 g triple super phosphate $Ca(H_2PO_4)_2$ and 4.90 g KCl dissolved in water which was spread over the soil surface to allow its even infiltration. Micronutrients (Mn, B, Zn, Cu, Mo) were added regularly, dissolved in irrigation water. Two days later, one 14-day old seedling of Brussels sprouts (*Brassica oleracea, cvar. Cyrus*) that emerged on commercial potting soil (Lentse potgrond), was planted in the pots without adhering soil. There were eight replicate pots for each treatment. Of these, four replicates were harvested in November (t=69d), the remaining four replicates were continued until harvest in January 2012 (t=132d). Pots were first kept outdoors under a rain shelter (allowing full daylight), and after six weeks they were moved to the greenhouse until harvest.

At harvest times, bulk soil was collected and sieved through a 4mm sieve. Fresh soil samples were used for soil biology and plant available soil N analyses. Soil samples for PLFA analyses were stored at -80˚C prior to further analyses. For chemical and isotope analyses soil was dried at 105˚C. All aboveground plant biomass was dried at 70˚C to determine aboveground plant biomass, and plant chemical and isotope analyses.

Table 5.1 Properties of organic amendments used in the pot experiment, expressed as percentage of dry weight organic material.

Organic amendment	Total C	Total N	C:N ratio	Sugars, proteins, fats, etc.	Starch	Hemi-cellulose	Cellulose	Lignin
	%	%		%	%	%	%	%
Cattle manure	46.3	2.4	19	28.6	0.0	16.1	32.7	22.7
Lucerne silage	41.4	3.5	12	59.6	0.5	6.8	23.9	9.1
Maize silage	53.6	1.3	41	17.1	39.3	19.0	22.0	2.7
Wheat straw	52.9	0.4	132	15.0	0.0	33.5	34.1	17.4

5.2.2 Microbial biomass, community composition & activity

Bacterial and fungal biomasses were determined by direct microscopic counts using fluorescently stained soil smears (Bloem & Vos, 2004). Fungal hyphal length was measured using the grid intersection method. Fungal biomass (C) was estimated assuming a mean hyphal diameter (width) of 2.5 µm and a specific C content of $1.3x10^{-13}$ g C μm^{-3}. Bacterial numbers and cell volumes were measured by confocal laser scanning microscopy (Leica TCS SP2) and automatic image analysis (Leica Qwin pro) (Bloem *et al.*, 1995). Bacterial biomass (C) was estimated from bio volume using a specific C content of $3.2x10^{-13}$ g C μm^{-3}. Phospholipid fatty acid (PLFA) analysis was used as another measure of microbial biomass, as well as to examine microbial community composition. PLFAs were extracted from 4 gram of (fresh) soil per pot using the procedure as described by Frostegård *et al.* (1993) and Hedlund (2002) based on the method of Bligh & Dyer (1959) and White *et al.* (1979) and analysed by gas chromatography (Hewlett-Packard, Palto Alto, CA, USA). PLFAs i15:0, a15:0, 15:0, i16:0, 16:1ω9, i17:0, a17:0, cy17:0, 18:1ω7 and cy19:0 were chosen to represent bacterial biomass and marker 18:2ω6 was used as an indicator of saprotrophic fungal biomass (Hedlund, 2002). In total a number of 26 PLFA biomarkers were used to study shifts in microbial community composition due to treatment effects using PCA. A part of those biomarkers were grouped according to the classification as shown in Table 5.2, to study the relative importance of specific types of PLFA biomarkers in principal component analyses. Microbial activity was determined by ^{14}C-leucine incorporation into microbial proteins during a short (1h) incubation period (Michel & Bloem, 1993). For a more detailed description see de Vries *et al.* (2006).

5.2.3 Potential C and N mineralization

Potential C and N mineralization rates were determined by incubating 200 g of fresh soil at 20°C for six weeks in a pot closed with a septum lid (Bloem *et al.*, 1994). Headspace O_2 and CO_2 were measured weekly using a Thermo TraceGC Ultra gas chromatograph (Thermo Fisher Scientific Inc., Waltham, MA, USA). The accumulation of CO_2 in the headspace between week 1 and 6 was taken as a

Table 5.2 Classification of PLFA biomarkers plotted in the PCA analyses, adapted from (Ruess & Chamberlain, 2010).

Fatty acid type	PLFA biomarkers	Predominant origin	References
Ise/anteiso methyl-branched	i14:0, i15:0, a15:0, i16:0, i17:0, a17:0	G^+ bacteria	Zelles (1997, 1999)
Cyclopropyl ring	cy17:0, cy19:0	G^- bacteria	Zelles (1997, 1999)
10-methyl-branched	10Me16:0, 10Me17:0, 10Me18:0	Sulphate reducing bacteria and/or Actinomycetes	SRB: Dowling *et al.* (1986); Kerger *et al.* (1986)
Straight-chain saturated < 19	15:0, 16:0, 17:0, 18:0	Non-specific	
Poly-unsaturated ω6 family	18:2ω6	Sapotrophic fungi	Frostegard & Baath (1996); Zelles (1999)
Mono-unsaturated double bond C5	16:1ω5 (NLFA)	Arbuscular Mycorrhiza	Olsson *et al.* (1995, 2003); Sakamoto *et al.* (2004)

measure for respiration. Results of the first week were not used to avoid the impact of disturbance. N mineralization was calculated from the increase in mineral N (nitrate and ammonium) between week 1 and week 6, reflecting net mineralization of plant-available N.

5.2.4 N pools and ^{15}N isotope analyses

To determine plant ^{15}N uptake, ground plant material was analysed by the Stable Isotope Facility, UC Davis, California. Isotope ratios were determined in the sample by EA-IRMS (Elemental Analysis / Isotope Ratio Mass Spectronomy) using a PDZ Europa ANCA-GSL elemental analyser interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK).

Labile soil ^{15}N retention in the soil was determined by exposing soil samples to a 0.5 M K_2SO_4 extraction. Extracted labile N was destructed using persulfate to convert all N to NO_3^-. The destruct was transferred to a plastic vial, after which acid traps were added in the form of a filter paper disc amended with K_2SO_4 and wrapped in Teflon tape. Addition of Nevarda reagent and MgO caused that all present N was transformed to NH_4^+ and diffused in the acid trap. Acid traps were removed after 48 h, rinsed, dried and wrapped in tin cups. Subsequently, ^{15}N isotope ratio in the acid traps was analysed similar to plant samples.

Microbial biomass N was measured by chloroform fumigation followed by a 0.5 M K_2SO_4 as described by Brookes *et al.* (1985). Extracted microbial N was divided by a K_N factor of 0.54 to calculate microbial biomass N. Microbial ^{15}N immobilization was determined by applying a similar destruction to the extracted microbial N as described above for labile ^{15}N. Since the microbial N extracts contain both labile soil N and microbial N, the previous determined labile N pool was subtracted from the latter to calculate immobilized microbial N.

Remaining soil ^{15}N retention was determined by subjecting bulk soil samples to ^{15}N analyses comparable to ^{15}N analysis for plant samples, only now using a Finnigan elemental analyser (Thermo Finnigan GC Combustion III, Bremen, Germany) and Delta V Advantage IRMS (Thermo Scientific, Bremen Germany). Microbial and labile N pools were subtracted from bulk soil N values to determine remaining soil N.

5.2.5 Statistical analyses

Statistical analyses were conducted using R version 3.1.0 (R Core Team, 2014). Differences in microbial and plant parameters, C and N mineralization and ^{15}N retrieval between fertilization strategies were analysed using a one-way ANOVA with a Tukey's post hoc test. All data was tested for normality prior to statistical analyses using the Shapiro-Wilk Normality test ('stats' package), and log-transformed if necessary. The assumption of homogeneity was tested using the Levene's test ('car' package). If these assumptions were not met, fertilizer treatment effects were tested using the non-parametric Kruskal-Wallis test, combined with the Mann-Whitney test with Bonferroni correction for multiple comparisons.

Abundances of individual PLFAs were used as input values in principal component analysis (PCA) to explore the effect of amendment treatments and time on soil microbial community composition ('vegan' package). A Hellinger transformation was applied to the abundance of PLFA biomarkers (nmol/g soil), whereby indicator PLFA biomarkers were plotted in a PCA diagram for both harvest times combined. When representing PLFA biomarker data in separate PCA's a normal transformation was applied to the data. To interpret the ordination axes the relative abundance of microbial groups (see Table 5.2 for classification) were projected in the ordination (when $p<0.05$ for the R^2).

Path analysis, a subset of structural equation modelling (SEM; 'lavaan' package; Rosseel, 2012), was used to study the relative strengths of the effects of organic amendments on the microbial biomass (microscopic count, bacterial + fungal biomass), activity (^{14}C-leucine incorporation) and community composition (PLFA profiles), and subsequently the relative effects of these microbial community parameters on the ecosystem processes C and N mineralization, and services plant biomass, plant ^{15}N uptake, and microbial ^{15}N immobilization. The input data for 'microbial community composition' in the structural equation model was the first axis of a PCA on natural transformed abundances of PLFA biomarkers. Data was tested for multivariate normality using Mardia's multivariate normality test ('MVN' package). The hypothesized models were tested using observed data, by computing a lack-of-fit chi-square statistic using maximum likelihood estimation. If the consequent p-value is larger than 0.05, the observed data support the tested hypothesis.

5.3 Results

5.3.1 Effects of organic amendments on soil microbial biomass and community composition

Soil organic amendments with a high C:N ratio (i.e. maize and straw) led to a significantly higher amount of total microbial PLFA compared to application of only mineral fertilizer (control) after 69 days in the experiment. In the straw treatment this effect lasted until day 132 (Table 5.3). Within the microbial community, these high C:N ratio organic amendments greatly increased fungal biomass at both harvest times, as determined by microscopic counts and PLFA, compared to application of only mineral fertilizer (control). However, the increasing effect of the maize treatment on soil fungal biomass was only significantly detectable after 132 days using PLFA analyses. Bacterial biomass was also increased by organic amendments according to the PLFA measurement, although the microscopic counts did not show significant differences (Table 5.3). The increases in fungal biomass were larger than that of the bacteria as appeared from the fungal/bacterial (F/B) ratios, showing shifts towards a higher F/B ratio when applying high C:N ratio organic amendments. Hence, organic amendments with a high C:N ratio (i.e. high C inputs) affected both microbial biomass and community composition. Organic amendments with a low C:N ratio had no significant effect on either of those soil microbial parameters (Table 5.3).

Principal component analysis showed two separated pairs of treatments that were quite similar after 69 days in terms of microbial community composition, i.e. the control and manure treatment and the maize and straw treatment, with the lucerne treatment in the middle (Figure 5.1a). After 132 days the microbial community compositions in the control and manure treatment were still relatively similar, and the microbial community composition in the lucerne treatment became more similar to the control and manure treatment. The maize and straw treatment were still clearly separated from the other treatments, but the maize treatment became more distinguishable from the straw treatment (Figure 5.1a). Hence, the high C:N ratio treatments (straw and maize) stayed separated from the control and the manure treatments up to 132 days, while the lucerne treatment tended to become more similar to the manure and control treatment throughout the experiment.

When we relate the relative abundance of indicative microbial groups to the PCA, it appeared that the high C:N ratio amendments stimulated the relative abundance of fungal PLFAs after both 69 and 132 days (Figure 5.1b,c). The control and manure treatments were characterized by a relatively high amount of Actinomycete and straight-chain PLFA biomarkers, also after both 69 and 132 days. After 132 days, the lucerne treatment exhibited a relatively high proportion of G^+ bacteria in the soil microbial community (Figure 5.1c).

Table 5.3 Soil microbial community parameters for each fertilizer treatment after both 69 and 132 days. Values correspond to the means of 4 pots per treatment and those treatments that do not share the same letter are significantly different ($p<0.05$; Tukey post-hoc test). P-values are the results from ANOVAs.

			Treatment					
Microbiological property	**Unit**	**Time (days)**	**Control**	**Manure**	**Lucerne**	**Maize**	**Straw**	**p-value**
Bacterial biomass	µg C g^{-1}	69	20.10	11.50	17.80	23.50	20.15	0.274
(microscopic count)		132	16.68	27.70	18.47	21.43	26.30	0.5505
Fungal biomass	µg C g^{-1}	69	20.03 a	15.50 a	24.55 a	52.25 a	101.78 b	<0.001***
(microscopic count)		132	18.52 a	25.28 ab	29.80 ab	59.68 ab	128.47 b	0.006**
F/B ratio		69	1.014 a	1.852 a	1.582 a	2.443 ab	6.232 b	0.013*
(microscopic count)		132	1.160 a	0.994 a	1.619 ab	3.109 bc	4.890 c	<00001***
Microbial PLFA	nmol g^{-1}	69	36.61 a	41.73 ab	42.91 ab	51.14 b	49.70 b	0.021*
		132	39.73 a	38.65 a	48.21 a	46.04 a	59.08 b	0.001***
Bacterial PLFA	nmol g^{-1}	69	16.37 a	18.14 ab	19.18 ab	22.38 b	21.76 ab	0.025*
		132	17.26 a	17.09 a	21.06 ab	19.97 a	24.88 b	<0.001***
Fungal PLFA	nmol g^{-1}	69	0.58 a	0.80 ab	1.54 ab	2.59 ab	3.22 b	0.005**
		132	0.78 a	0.72 a	1.26 a	1.99 b	4.22 c	<0.001***
F/B ratio (PLFA)		69	0.036 a	0.043 ab	0.075 abc	0.115 bc	0.145 c	0.002**
		132	0.045 ab	0.042 a	0.060 b	0.100 c	0.170 d	<0.001***
Microbial Nitrogen	µg N g^{-1}	69	12.05 a	11.77 a	14.21 a	23.79 b	35.89 c	<0.001***
		132	7.64 a	2.24 a	17.56 b	28.04 c	17.29 b	<0.001***
Leucine incorporation	(pmol g^{-1} h^{-1})	69	264.23 a	240.55 a	291.50 a	524.50 b	673.27 c	<0.001***
		132	440.25 a	485.50 a	641.67 b	853.25 c	818.67 c	<0.001***
Potential C mineralization	mg C kg^{-1} wk^{-1}	69	1.60 a	4.83 b	17.30 c	33.10 cd	72.75 d	<0.001***
		132	3.43 a	11.08 b	18.13 c	11.98 b	54.60 d	<0.001***
Potential N mineralization	mg N kg^{-1} wk^{-1}	69	1.40 a	0.69 ab	1.69 ac	3.22 ad	-3.16 ae	<0.004**
		132	1.47 a	1.60 a	2.33 b	2.27 b	0.03 c	<0.001***

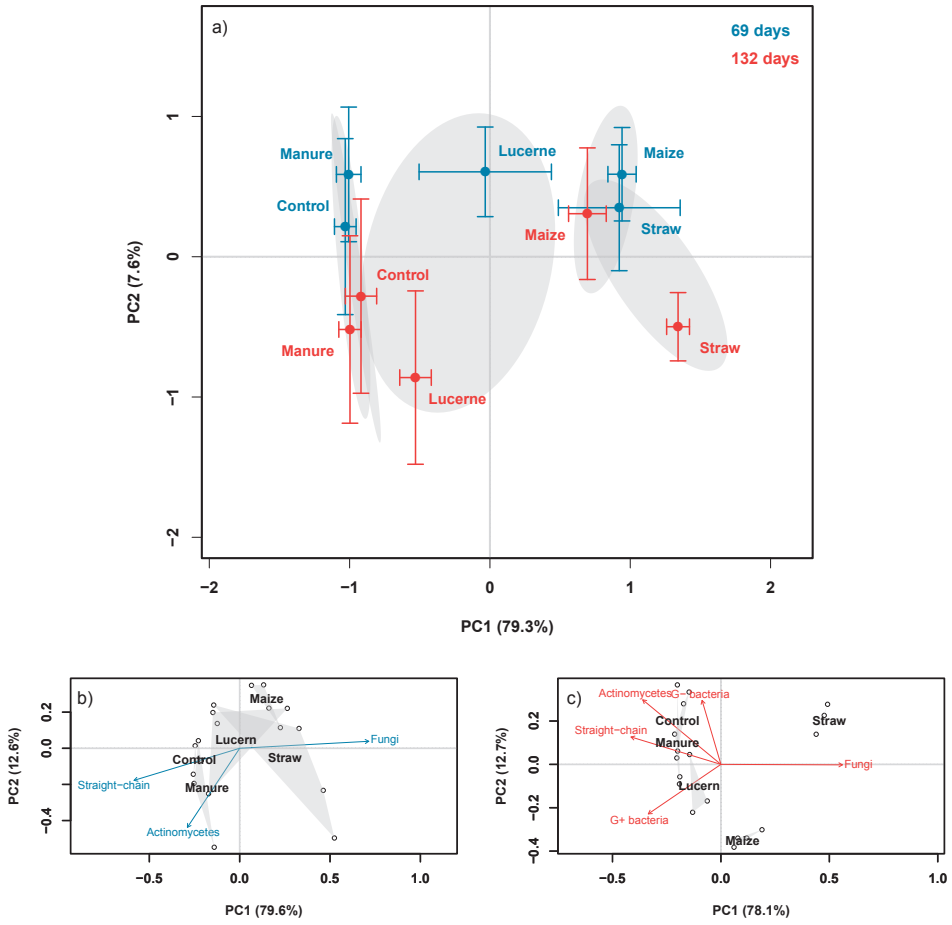

Figure 5.1 Top: Principal component analyses (PCA) of soil microbial biomarker data (phospholipid fatty acids; PLFAs) used for examination of the soil microbial community composition in response to different organic amendment treatments (a). Treatments are indicated as the mean ± SE of four replicates. Microbial community composition was assessed both 69 and 132 days after application of organic amendments. Bottom: PCAs showing the microbial community composition at time point 69 days (b) and 132 days (c) for different treatments together with indicative microbial groups (R^2 with $p<0.05$).

5.3.2 Effects of organic amendments on soil microbial activity

Addition of organic amendments with a relatively high C:N ratio, i.e. maize and straw, resulted not only in a higher soil microbial biomass, but also in higher microbial growth rates as indicated by significantly higher rates of ^{14}C-leucine incorporation (protein synthesis) after 69 days (Table 5.3). After 132 days not only the maize and straw treatment, but also the lucerne silage amendment (low C:N, low lignin content) had a significantly higher activity of the soil microbial community as compared to the control treatment.

5.3.3 Effects of organic amendments on C and N mineralization

Potential C mineralization rates showed a higher respiration of the microbial community in all organic amendment treatments compared to the control treatment, whereby the strongest effect was seen for the amendment of straw (Table 5.3). After 69 days, potential C mineralization rates were well related to C:N ratio of the applied organic materials; the higher the C:N ratio (and thereby the amount of applied organic C) the higher potential mineralization rates. Potential N mineralization rates were more linked to the lignin content of the organic amendments (Table 5.3); the higher the percentage of lignin (manure and straw) the lower the potential N mineralization rates after 69 (both) and 132 days (only straw). Application of maize and lucerne silage showed significantly higher potential N mineralization rates after 132 days, compared to the control treatment.

5.3.4 Effects of organic amendments on ecosystem services: microbial ^{15}N immobilization, plant biomass and plant ^{15}N uptake

Organic amendments with a low C:N ratio resulted in a significantly higher plant biomass of Brussels sprouts, while high C:N ratio amendments (maize and straw) had a negative effect, compared to the control, on plant biomass after both 69 days ($F_{4,14}$=74.93, p<0.001) and 132 days ($F_{4,13}$=223.88, p<0.001) (Figure 5.2). Figure 5.3 shows that most of the applied mineral ^{15}N was taken up by the plant. The applied mineral fertilizer ^{15}N in this experiment was successfully taken up by the plants with a recovery of 71-78% (after 69 days) and 55% (after 132 days) for low C:N ratio organic amendments, compared to respectively 58% and 41% in control treatments. Plant ^{15}N uptake was lower in treatments with high C:N ratio organic amendments compared to low C:N ratio organic amendments, with 8-45% after 69 days and 13-40% after 132 days. The amount of ^{15}N in the plants was directly related to the plant biomass at both measuring points (t=69 days: R^2=0.8562 p<0.001***; t=132 days: R^2=0.8593 p<0.001***).

Microbial ^{15}N immobilization was found in all treatments after 69 days (2-14%), with increasing immobilization levels for treatments that received high C:N ratio amendments (and thereby large doses of C). After 132 days the ^{15}N immobilized in the soil microbial community in the control and manure treatment was fairly small (<1%), while lucerne, maize and straw addition still showed a significant pool of microbial immobilized mineral ^{15}N (4-7%). Microbial immobilized ^{15}N was related to the microbial biomass (t=69 days: R^2=0.3469 p<0.005**; t=132 days: R^2=0.3235 p<0.008**). The amount of ^{15}N, which was not recovered in the plant or microbial biomass, was mostly found in the bulk soil. The labile soil ^{15}N pools in the control soil and low C:N treatment soil were rather small (<1%), while high C:N ratio treatments showed increased levels of labile ^{15}N up to 11% in the straw treatment after 69 days. In most treatments the labile soil N values consisted mainly of organic N; only in the straw treatment (at both harvest times) and the manure treatment (after 132 days), more than half of the labile N consist of NH_4^+ (Supplementary Table S5.2).

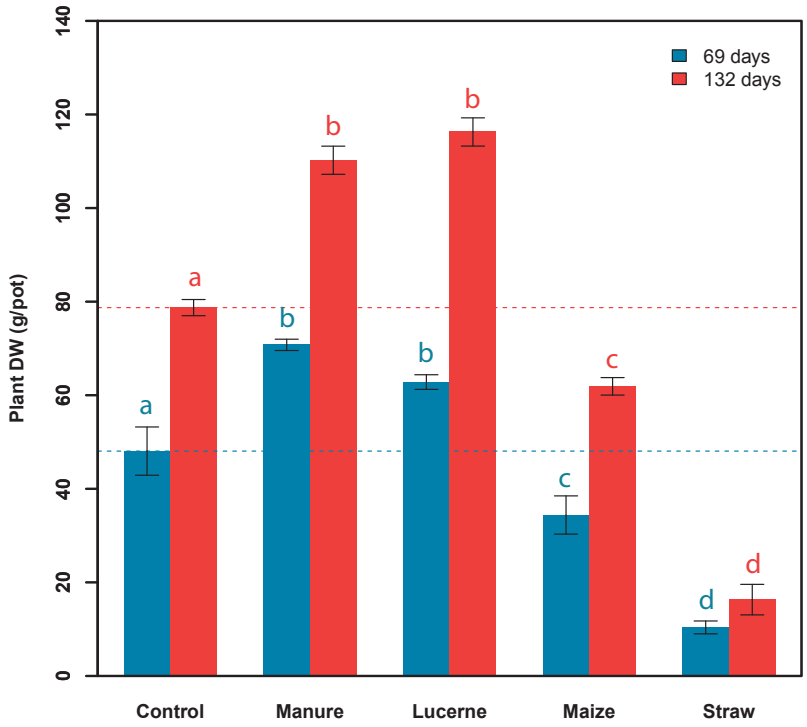

Figure 5.2 Aboveground dry weight of Brussels sprouts (*Brassica oleracea, cvar. Cyrus*), 69 and 132 days after application of different types of organic amendments. Values are the means of 4 replicates and error bars represent the SEM. Different letters within each measuring point indicate significant differences ($p<0.05$).

5.3.5 Structural equation modelling: Linking soil microbial community to microbial ^{15}N immobilization, plant biomass and plant ^{15}N uptake

Structural equation modelling was used to describe and quantify the direct and indirect effects of the different kinds of organic amendment on the biomass, activity and composition of the soil microbial community, and subsequently on soil ecosystem processes (C and N mineralization) and services (microbial N immobilization, plant growth and plant N uptake).

Structural equation modelling showed that it were the organic amendment induced changes in the soil microbial community composition that most strongly affected microbial N immobilization rates. The tested SEM was successful in predicting ^{15}N immobilization by the microbial community 132 days after organic amendment (Figure 5.4a). In the accepted model, the C:N ratio and lignin content of the organic amendments respectively increased or decreased the soil microbial biomass, activity and contrasts in microbial community composition. Microbial activity indirectly stimulated ^{15}N immobilization via N mineralization, while microbial community composition had a strong direct effect on microbial ^{15}N immobilization.

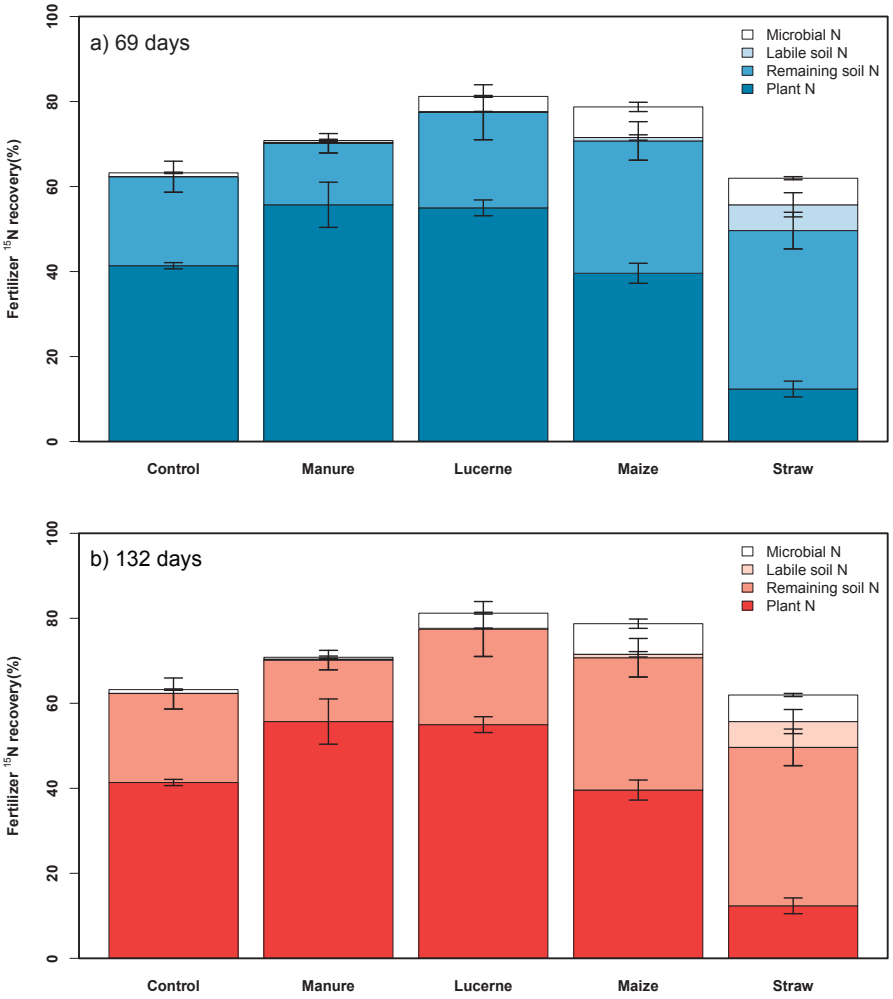

Figure 5.3 Recovery of the stable isotope ^{15}N originating from applied ^{15}N-labelled mineral fertilizer after 69 days (a) and 132 days (b). Bars shows means ± SE of four replicates for ^{15}N recovered in the microbial community, labile soil pool, remaining soil pool and Brussels sprouts. Related statistics can be found in Supplementary Table S5.1.

Linear regression showed that ^{15}N uptake by the plant was strongly positively related to plant biomass. This relationship was supported by the results of structural equation modelling, where the tested and accepted model showed that plant ^{15}N uptake was positively correlated to plant biomass 132 days after the start of the experiment (Figure 5.4b). Plant biomass itself was affected by processes of C and N mineralization driven by microbial biomass and activity, as well as the quality of the applied organic amendments in terms of C:N ratio and lignin content.

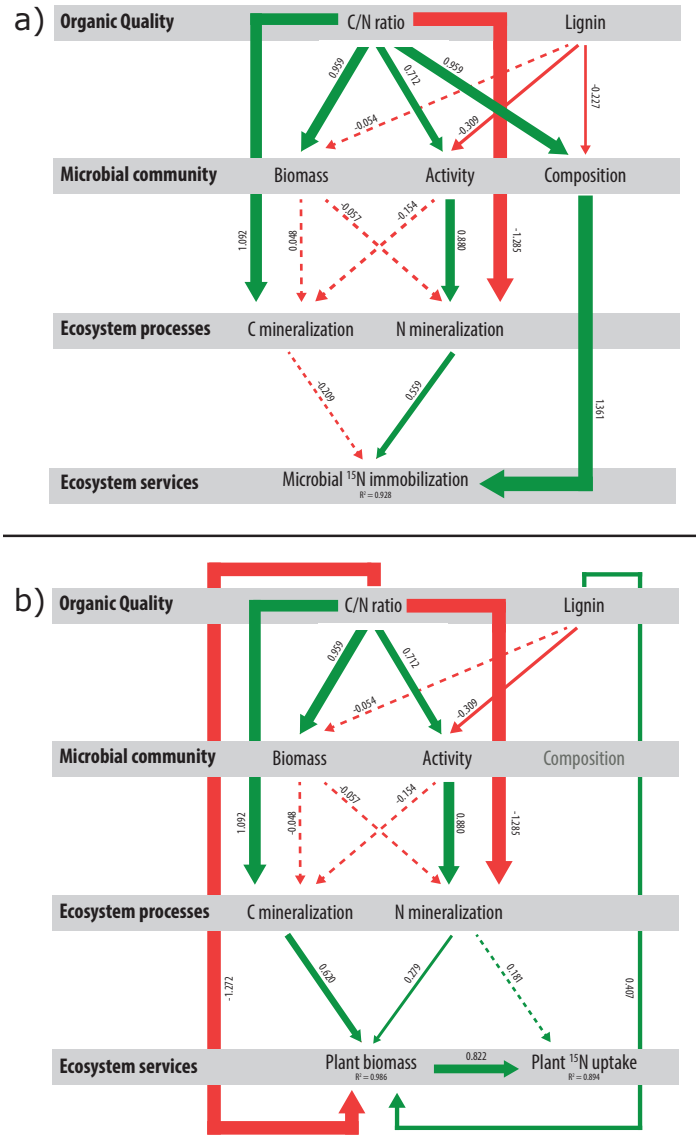

Figure 5.4 Results of structural equation model linking organic amendment treatments to soil microbial characteristics and ecosystem processes and services of interest 132 days after application of organic amendments. Green arrows indicate a positive and red arrows a negative effect on the predicted variable. Solid lines visualize a significant relationship, whereby arrow widths correspond to the relative effect size of each variable. (a) Visualization of direct and indirect effects on microbial ^{15}N immobilization. Results of model fitting: χ^2=18.457, p=0.141, df=13, n=18. (b) The direct and indirect effects of organic amendment quality on plant biomass and plant ^{15}N uptake. Results of model fitting: χ^2=15.442, p=0.218, df=12, n=18.

5.4 Discussion

5.4.1 Effects of contrasting organic amendments on soil microbial biomass, activity and community composition

The aim of this experiment was to study the effects of contrasting organic amendments, in combination with mineral fertilizer, on the soil microbial community structure and functioning, plant growth and associated mineral fertilizer utilization. In agreement with our hypothesis, we saw that application of all four types of organic amendments increased microbial biomass and activity. The higher microbial biomass and activity with organic amendments is in correspondence with studies that determined microbial biomass (e.g. Hu *et al.*, 1999; Peacock *et al.*, 2001; Malik *et al.*, 2013) or microbial growth (e.g. Lazcano *et al.*, 2013) after application of organic matter treatments. Soil microbes are generally C limited (Demoling *et al.*, 2007), which explains the increase in microbial biomass and activity with addition of decomposable C in the form of organic amendments. The amount of organic amendments was standardized for equal N input, which was most likely the cause for a generally small and non-significant increase of microbial biomass and activity with application of low C:N ratio organic materials. The use of PLFA analyses in combination with multivariate statistical techniques proved to be a powerful tool (Frostegård *et al.*, 2011) that enabled us to detect effects of organic amendments on the soil microbial community composition, showing in particular a shift towards a fungal dominated community when high C:N ratio amendments were applied. The higher fungal/bacterial ratio with organic amendments was caused by a strong increase (up to 5-fold) in fungal biomass, compared to a small increase (50% or less) in bacterial biomass. This result is in accordance with previous studies, which showed that soil fungi have an advantage over bacteria in N-limited soils because of their ability to utilize a greater fraction of applied organic materials (de Vries *et al.*, 2006; de Vries & Bardgett, 2012).

5.4.2 Effects of organic amendments on mineral fertilizer recovery

In this study, the stable isotope ^{15}N was used as a label to trace the fate of applied mineral N. Although this method did not close the complete N budget, it gives important insights in processes as mineral N retention, recovery and immobilization, as being important mechanisms in combating agricultural mineral N losses. Mineral ^{15}N uptake by the plant was fairly high in most treatments of this pot experiment, with recovery values varying between 45 and 80% in all treatments after 69 days, except for addition of wheat straw which had a plant ^{15}N recovery of only 8% (Figure 5.3). Most of these N recovery values are high as compared to recoveries in field studies (Cassman *et al.*, 2002), which we attribute to the limited size of pots relative to plant canopy. Organic amendments with a high C:N ratio had a significant lower plant ^{15}N recovery compared to the control treatment, in contrast to application of low C:N ratio organic material. High values of microbial N immobilization,

following application of high C:N ratio amendments, are likely to have negatively affected available plant N, and thereby plant biomass and plant N retention. Higher values of microbial ^{15}N immobilization were also associated with higher values of ^{15}N retention in the soil (both labile and remaining), which also indicates that organic amendments with a high C:N ratio promote nutrient retention through the decomposition and immobilization by the soil microbes. For all treatments, the actual plant ^{15}N uptake and microbial ^{15}N immobilization was likely underestimated, because ^{15}N measurements were only performed for the above ground plant biomass and for microbial biomass and soil N pools in bulk soil, while a considerable part of fertilizer-derived N can be found in plant roots and associated rhizosphere microbial communities (e.g. Ladd *et al.*, 1981; Mader *et al.*, 2000). Besides the underestimation of microbial and plant N pools, it was presumably loss by denitrification of ^{15}N that caused total system ^{15}N recovery to remain well below 100% (max. 90%). Future work should investigate how N immobilization and mineralization in response to organic amendments are linked to N loss pathways such as denitrification and nitrate leaching, in order to optimize N management strategies.

5.4.3 Effects of soil microbial biomass, activity and community composition on ecosystem processes & services

It was shown that ecosystem process rates of C and N mineralization were mainly affected by microbial activity, while microbial N immobilization was more affected by microbial community composition (Figure 5.4). Microbial community composition values in the SEM represented the PC1 axis as shown in Figure 5.1c, which shows that this axis is mainly driven by the relative amount of fungal PLFAs. These results indicate that a high relative amount of fungal biomass increases the microbial N immobilization capacity in the soil. Mineralization has been viewed as a 'broad' ecosystem process with high functional redundancy since they are carried out by a wide array of soil microbial organisms (Schimel *et al.*, 2005). Mineralization is then not very sensitive to *who* is present but rather to the *amount* of total microbial activity. Schimel *et al.* (2005) argue that mineralization could be depending on microbial community composition if soil microbes have difficulties with physical access to the substrate or when exoenzymes are taken into account. However, in this study sufficient organic material was well mixed through the soil, which caused that the soil microbial community was not constrained by physical access to the substrate. Microbial N immobilization can be seen as a more 'narrow' process as it is mainly impacted by microbial community composition. The dependency of N immobilization on community composition rather than on microbial activity is likely related to the presence of microbial groups that have the ability to decompose and process particular compounds of organic matter and incorporate N in their biomass (de Vries & Bardgett, 2012). The use of different organic amendments in this study created microbial communities with different rates of N immobilization. The higher the C:N ratio of the organic amendment, the higher the N immobilization. This

is a plausible result, as when microbes decompose soil organic matter with a low N content they may extract mineral N from the soil solution for the production of microbial cells with relatively high N contents. Part of this microbial biomass may have been turned over into organic residues found in the remaining soil N.

5.4.4 Implications for agricultural fertilization management

Given current high fertilizer-N losses in agriculture, Drinkwater & Snapp (2007) suggested that a new paradigm needs to be adopted to emphasize a more ecosystem-based approach to nutrient management. In their view, NUE can be increased by improving the N retention capacity of organic and mineral reservoirs that can be accessed by both microbial and plant-controlled processes (Drinkwater & Snapp, 2007). Combined addition of organic amendments with mineral N fertilizers could help to increase temporal N retention in the soil ecosystem with the aim to ensure sufficient N supply at all crop growth stages (Grandy *et al.*, 2013). The quality of organic amendments is likely to have a large effect on the delicate balance between N mineralization, microbial N immobilization, soil N retention and N uptake by the plant. This study shows that mainly the C:N ratio of the organic amendments had a large effect, i.e. high C:N ratio enhanced soil microbial biomass, activity and ^{15}N immobilization and decreased plant growth and ^{15}N uptake. The enhancing effect of organic amendment with a high C:N ratio on soil microbial biomass and activity was probably due to the fact that organic application levels were standardized for N content, causing that high C:N ratio treatments received more C compared to low C:N ratio treatments, while C availability is known as a limiting factor for microbial growth. For future research it would therefore be interesting to perform a similar experiment combining mineral N and varying organic amendments, but then with a standardized C content and variable N application levels. Such an additional study will provide the possibility to test which amendment effects are caused by differing C:N ratios rather than differences in the amount of applied C.

When comparing the contrasting treatments, it seems that addition of lucerne silage had overall the best results as plant biomass as well as plant ^{15}N uptake was increased, but also microbial ^{15}N immobilization was doubled at the expense of total ^{15}N loss, compared to the control treatment which received no organic amendment. However, we cannot rule out that the relatively high amount of plant-available N contributed to the high plant ^{15}N recovery and lower ^{15}N loss fraction in the lucerne treatment, via promotion of early root growth and hence N interception capacity of the plant. Although the cattle manure amendment was comparable to the lucerne silage amendment in terms of C:N ratio and plant biomass, the effects of manure amendments on the soil microbial community and coherent processes were barely distinguishable from the control treatment, while total ^{15}N loss with manure was about twice as large as with lucerne silage. This is attributed to the higher recalcitrance of manure as compared to lucerne (Table 5.1). Jenkinson (1977) viewed the soil microbes are the 'eye of the needle', through which all organic matter passes towards

nutrition and retention. In that perspective the present results indicated that organic matter amendments with relatively low N contents lead to high rates of microbial N immobilization and soil N retention, sometimes but not always at the expense of plant growth.

Finally, what can our findings contribute to the optimization of N management in agroecosystems? The results from this study are in line with previous studies and demonstrate that the chemical composition of the amended organic matter is important for finding a balance between plant nutrition and soil nutrient retention. In the present study, the addition of lucerne silage appeared to be the best in terms of combining plant nutrition with reducing N losses through increased soil N retention. A next step might be to analyse which particular combinations of organic amendments can exploit the soil ecosystem functions to promote overall N recovery by crops while reducing N losses to the environment for a more sustainable agriculture.

Table S5.1 Recovery of stable isotope ^{15}N originating from applied ^{15}N labelled mineral fertilizer after 69 and 132 days. Values correspond to the means of 4 pots per treatment and those treatments that do not share the same letter are significantly different ($p<0.05$; Tukey post-hoc test or Mann-Whitney test with Bonferroni correction in case of non-normal distributed data). P-values are the results from ANOVAs or the non-parametric Kruskal-Wallis test in case of non-normal distributed data.

			Treatment					
^{15}N recovery	**Unit**	**Time (days)**	**Control**	**Manure**	**Lucerne**	**Maize**	**Straw**	**p-value**
^{15}N plants	%	69	58.41 abc	77.82 a	70.60 a	44.87 b	7.55 c	0.003**
		132	41.37 a	55.70 b	54.98 ab	39.59 ab	12.36 ab	0.007**
^{15}N microbes	%	69	1.60 abc	1.66 a	3.04 abc	7.95 b	13.87 c	0.005**
		132	0.87 a	0.43 a	3.56 ab	7.19 b	6.25 ab	0.005**
^{15}N labile	%	69	0.09 a	0.09 a	0.58 ab	3.30 ab	10.92 b	0.005**
		132	0.05 a	0.22 a	0.16 a	0.81 ab	6.06 b	0.001**
^{15}N soil	%	69	10.14 abc	3.70 b	15.15 a	8.53 a	24.90 c	0.004**
		132	20.94 ab	14.48 a	22.50 ab	31.13 ab	37.28 b	0.013*

Table S5.2 Composition of the labile N pool for each organic amendment treatment after both 69 and 132 days. Values correspond to the means of 4 pots per treatment and those treatments that do not share the same letter are significantly different ($p<0.05$; Tukey post-hoc test or Mann-Whitney test with Bonferroni correction in case of non-normal distributed data). P-values are the results from ANOVAs or the non-parametric Kruskal-Wallis test in case of non-normal distributed data.

			Treatment					
	Unit	Time (days)	Control	Manure	Lucerne	Maize	Straw	p-value
N-organic	(mg/pot)	69	9.49 a	12.86 a	19.42 a	36.64 b	186.76 b	<0.001***
		132	20.59	50.01	33.04	30.71	104.94	0.06235
$NO_2^- + NO_3^-$	(mg/pot)	69	8.34 ab	6.76 a	8.47 ab	16.54 c	12.26 bc	<0.001***
		132	11.18 a	15.72 ab	19.33 bc	19.32 bc	24.80 c	<0.001***
NH_4^+	(mg/pot)	69	-6.78 abc	-4.16 ab	5.19 a	-2.64 b	325.67 c	<0.01**
		132	8.22 a	55.08 b	12.54 ab	11.39 a	164.06 c	<0.001***

Chapter 6

Local functioning, landscape structuring: drivers of soil microbial community structure and function in peatlands

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Abstract

Agricultural peatlands are essential for a myriad of ecosystem functions, and play an important role in the global carbon (C) cycle through C sequestration. Peatlands are managed regularly, which influences environmental drivers controlling soil microbiota. Changes in the functioning of soil microbes is directly driven by environmental conditions, but the extent to which these have an effect is constrained by the microbial community structure. Management of agricultural peatlands takes place at different spatial scales, ranging from local to landscape management, and drivers of soil microbial community structure and function may be scale-dependent. Effective management for an optimal biogeochemical functioning thus requires knowledge of the drivers on soil microbial community structure as well as functioning and the spatial scales upon which they are influenced. During two field campaigns, we examined the importance of different drivers (i,e. soil characteristics, nutrient management, vegetation composition) at two spatial scales (local vs landscape) for respectively the soil microbial community structure (determined by PLFA) and soil microbial community functional capacity (as assessed by CLPP**)** in agricultural peatlands. We show that soil communities are controlled by a distinct set of drivers at local versus landscape scale. Moreover, our results suggest that microbial structure is more controlled at a landscape scale by phosphorus related variables, whereas microbial functional capacity is driven locally through plant community feedbacks. Optimal peatland management strategies should consider the scale-dependent action of soil microbial community drivers, for example by first optimizing microbial structure at the landscape scale by targeted areal management, and then optimizing soil microbial function by local vegetation management.

6.1 Introduction

Peatlands play an important role in Earth's biogeochemical cycles by storing about a third of all terrestrial carbon (C) (Turetsky *et al.*, 2002; Turunen *et al.*, 2002). In Europe, the majority of peatlands is in use as agricultural land (Joosten & Clarke, 2002). Despite their potential to sequester C, agricultural peatlands typically act as significant C sources. Worldwide drainage of such peatlands has increased the rates of peat oxidation and hence microbial decomposition, causing high rates of C losses and greenhouse gas emissions (Drösler *et al.*, 2008). However, due to the large C sequestration potential of agricultural peatlands, they could play an important role in efforts to increase soil C storage, such as the recently launched '4 per 1000 initiative', which seeks to increase C storage in agricultural soils with 4‰ per year (Le Foll, 2015).

Current peatland management influences microbe-mediated biogeochemical functions, for example by maintaining waterlogged conditions to prevent microbial peat oxidation and thereby reduce peat subsidence and CO_2 emissions (Kløve *et al.*, 2017). Restoration of peat ditches often seeks to optimize nutrient removal and reduce eutrophication, both of which have links to the microbial processes of nitrogen (N) and phosphorus (P) conversion. Microbial activities are clearly critical to the success of peatland management strategies, for instance for C storage, yet management practises rarely consider potential impacts on soil-borne microbial communities. With future climate change pressures in mind, the management of ecosystems for minimal microbial mediated CH_4 and N_2O emission is expected to become ever more important (Taft *et al.*, 2017).

Traditionally regarded as random noise, spatial variability in soil microbial communities is now widely acknowledged to exist (Ettema & Wardle, 2002), and it displays consistent and informative patterns at different spatial scales (O'Brien *et al.*, 2016). With mounting evidence for scale-dependent ecological processes acting on microbial communities, the need for examining multiple spatial scales to understand the patterns in soil microbial communities has become apparent (Yergeau *et al.*, 2010; Martiny *et al.*, 2011). This has led to the study and discovery of clear examples of small scale (cm to m) patterns (e.g. Franklin & Mills 2003), as well as large continental (Waldrop *et al.*, 2017) and global biogeographic patterns in soil microbial communities (Nemergut *et al.*, 2011). These scales of study are not always in line with the scales on which the management of ecosystems takes place. For agricultural peatlands the most obvious scale of management is that of the field level, with local farmers carrying out customary management practices such as fertilization, grazing and mowing. Another relevant scale is that of the landscape level at which spatial planning and water level management occurs. To adequately steer management towards optimization of microbial communities, there is a need to match the spatial scale of land management and the study of spatial microbial community patterns.

While identification of patterns in soil microbial composition is in itself relevant,

there is a clear need to go beyond pattern description and towards identification of the underlying drivers of community structure and functioning (Martiny *et al.*, 2011; Hanson *et al.*, 2012). Soil microbial community structure and functioning are often assumed to be driven by the same factors (O'Brien *et al.*, 2016). This would imply that management aimed at an optimal microbial structure will also result in the desired functioning of the ecosystem. Alternatively, soil microbes are often considered to have high functional redundancy (Strickland *et al.*, 2009); and therefore drivers of soil microbial community structure may have minimal effect on soil microbial functioning. This perspective would imply that management practices designed to control only the drivers of community function would be sufficient to achieve the desired ecosystem functions.

Soil microbial community structure has been shown to be controlled by a wide spectrum of drivers: soil pH and C:N ratio (Lauber *et al.*, 2008; Fierer *et al.*, 2009, 2012; Kuramae *et al.*, 2012; Zhang *et al.*, 2013; Ramirez *et al.*, 2014),vegetation (O'Donnell *et al.*, 2001; de Vries *et al.*, 2012),external nutrient load (O'Donnell *et al.*, 2001; O'Brien *et al.*, 2016), and soil moisture (Brockett *et al.*, 2012). Drivers of soil microbial community function have not been examined in as much detail, but it has been shown that soil microbial community functions are controlled by soil moisture (Brockett *et al.*, 2012), C:N ratio (Kuramae *et al.*, 2014), and external nutrient load and pH (Wakelin *et al.*, 2013). These different drivers of community structure and functioning are themselves dependent on the spatial scale of examination. As land management is carried out at scales orders of magnitude larger than those experienced by microbes directly, it remains to be tested if and how microbial communities respond to changes in these drivers. If soil microbial community structure and functioning are influenced by scale-dependent drivers, information on the scale-dependency of these microbial community drivers can be useful for informing management designed to improve peatland functioning.

In this study, we assessed the impacts of several drivers of soil microbial structuring and functional capacity at local and landscape scales in agricultural peatlands. We combine data from two sampling campaigns across agricultural peatlands in The Netherlands (Figure 6.1a,b), one that examined drivers of soil microbial community structure, as determined by phospholipid fatty acid (PLFA) analysis, and one that examined drivers of soil microbial community functional capacity, as estimated by community level physiological profiling (CLPP). These patchwork agricultural landscapes are highly heterogeneous, making them effective model systems for examining the effects of multiple environmental drivers on soil microbial communities (Vasseur *et al.*, 2013). Comparison of samples within and between different polder areas made it possible to analyse drivers at two different spatial scales: local scale (sites within a sampled area) and landscape scale (differences between sampled polder areas).

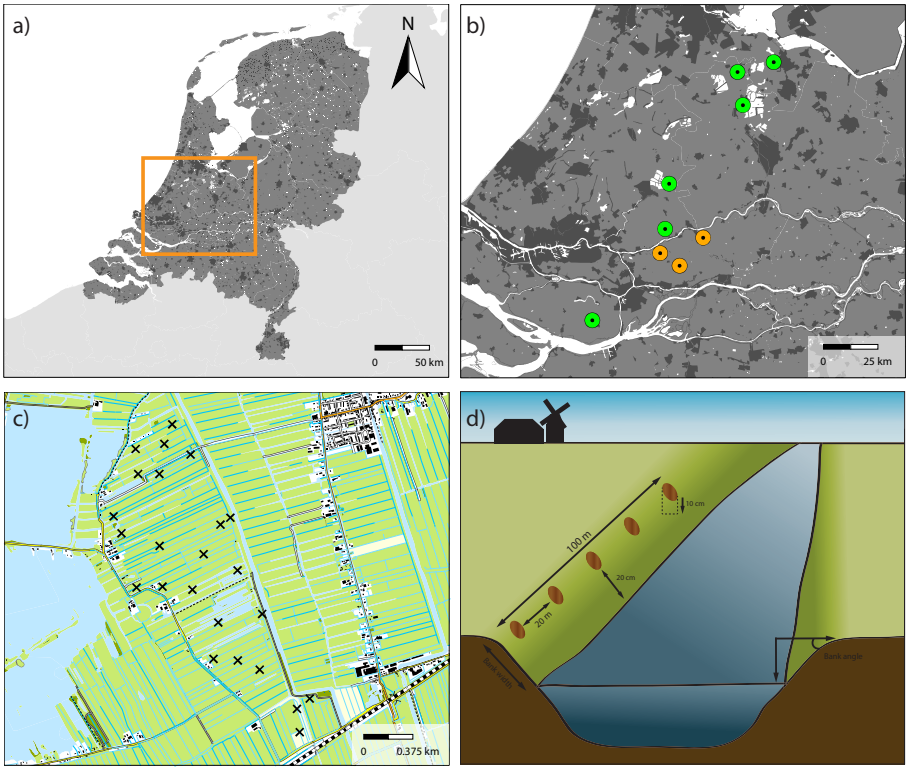

Figure 6.1 Overview of sample sites and sample locations. a) The Netherlands with the studied region indicated in an orange rectangle. b) Map with the areas sampled in 2013 for PLFA analyses (orange) and in 2014 for CLPP analyses. c) Detailed map of one of the areas indicating the location of 24 sampling locations. d) Schematic representation of how samples were collected along the waterside of ditches.

6.2 Methods

6.2.1 Study site and design

The field sites used in this study are situated in a peat area in the West of The Netherlands (Figure 6.1a) and comprise nine ±200 ha peatlands (Figure 6.1b). All areas are characterized by a mixture of intensive and extensively managed peatlands intersected by ditches, resulting in a mosaic of land uses. In the summer of 2013, a total of three agricultural peatland areas were sampled, and another six areas were sampled in the subsequent summer of 2014. In each area, 24 transects were laid out on field margins (Figure 6.1c), as such edges account for 96% of the total vegetation species richness of a field (Kleijn *et al.*, 2001). Each transect had a total length of 100 meter, where the vegetation was surveyed for the sloping part of each transect up to the waterside (Figure 6.1d). Vegetation abundance was assessed using Tansley abundance classes (Tansley, 1946), which were subsequently converted into abundance

percentages (Supplementary Table S6.1). To analyse soil physical-chemical properties and microbial community structure and functional capacity, five soil samples (10 cm deep) were taken in each transect, 20 meters apart from each other and 20 cm from the waterside (Figure 6.1d). Soil samples were mixed per transect after removal of the vegetation layer, sieved (2 mm mesh) and stored at -80°C as one composite sample.

6.2.2 Phospholipid fatty acid (PLFA) analyses

Soil microbial community structure was determined by analysing Phospholipid Fatty Acids (PLFA) extracted from soil samples taken in the areas that were sampled in summer 2013 (three areas, 72 samples). PLFAs were extracted from 4 grams of soil per composite sample using an adapted protocol, following White *et al.* (1979) and Frostegård & Bååth (1996). Lipid fractionation took place over prepacked Bond Elut SI solid phase extraction columns, after which lipid extracts were identified by gas chromatography (GC-FID, 7890A, Agilent technologies, Delaware, USA). The following PLFA markers were taken into account when determining soil microbial community structure: i14:0, 14:0, i15:0, a15:0, 15:0, i16:0, 16:1ω5, 16:1ω9, 16:0, i17:0, a17:0, cy17:0, 17:0, 18:2ω6, 18:1ω9, 18:1ω7 and 18:0. Abundance of each PLFA biomarker was expressed as nmol PLFA g^{-1} dry weight of soil.

6.2.3 Community level physiological profiling (CLPP)

Functional diversity of the soil microbial community was determined in soil samples originating from the areas that were sampled in summer 2014 (six areas, 144 samples) by the use of Biolog EcoPlates (Biolog, Hayward, CA, USA). These 96-well plates contain three replicate sets of 31 ecologically relevant C substrates, along with a tetrazolium redox dye (Insam, 1997). Microbial use of these substrates is reflected by colour change in each of the wells, as the redox dye is reduced to tetrazolium violet (Pohland & Owen, 2009). Eco-plate wells were inoculated with diluted soil slurries (150 µl), obtained by mixing 0.5 gram of soil with 49.5 mL of milli-Q water, shaken (200 rpm) for 30 minutes on an orbital shaker, and 10^{-4} diluted by serial dilution. Three technical replicates were included for each of the 144 soil samples. Eco-plates were incubated in the dark at 25°C, and colour development was recorded as optical density (OD_{590}, OD_{750}) at the start and after 24, 48, 72, 96, 168 and 192 hours, on a Biotek Synergy HT plate reader (Biotek Instruments, Winooski, United States).

Conceptually, the function of Ecoplate-substrate utilisation through time consists of four distinct phases (Supplementary Figure S6.1a). The substrate utilization function captures the signals of community respiration, but also that of the substrate consumed for community growth. For our purposes, we were interested only in the respiration of the originally sampled community. To remove the signal of reproduction from the data, we used a modified method of Brouns *et al.* (2016). The rationale behind this method is that by removing the exponential-growth signal from the exponential phase of the substrate-use function, only the substrate use of the initial community

remains. The exponential phase is characterized by plentiful substrate where growth of organisms is not limited by its availability. By fitting a log-linear regression to the extracted exponential phase (Supplementary Figure S6.1b), we determined the initial community substrate use (y-intercept). In contrast to the existing methodology, we determined the phase of true exponential growth from the second derivative of a polygonal curve fit. By finding the inflection point, the point where the second derivative changes from positive to negative, the convex, true exponential, part of the curve is determined. We also accounted for the possible existence of a lag phase by removing non-positive values (i.e. zeroes).

6.2.4 Soil chemical analyses

Soil pH was measured after shaking a soil-water (1:2.5 w/v) suspension on an orbital shaker at 200 rpm for 2 hours. Total C, N and P analyses were performed on oven-dried (60°C, 96 h) and ground (1.0 mm, Retsch SM 100, Haan, Germany) soil samples. Total C and N were determined using an Elemental Analyser (Thermo Electron, Milan, Italy). Total P was determined according to the method of Murphy and Riley (1962). Soil samples were ashed at 550 °C for 30 minutes, after which P was re-suspended by digestion with 2.5% (w/v) acid persulphate in an autoclave (30 minutes at 121°C). Total P was measured colorimetrically, on a continuous flow analyser (SEAL analytical, Abcoude, The Netherlands). Soil moisture was determined as the percentage weight loss upon oven drying.

6.2.5 Cartographic information

Soil typological information, yearly fertilizer use and land management were extracted from geographical maps (Alterra, PAWN; Natuur op Kaart, Kadaster 2013/14, SNL, IPO 2013/14) using ArcGIS 10.1. With this information we determined fertilizer use and N and P loadings per hectare. In determining artificial and organic fertilizer (manure), we assumed that farmers used the maximum amount of admissible fertilizer based on national legislation. Fields with specific nutrient management schemes, such as areas with natural grassland management generally use less artificial fertilizer due to a resting period where no fertilizer can be applied or due to legal restrictions on artificial fertilizer use. Also, manure application may be constrained due to the resting period or further limited to solid manure application for certain types of nature management. In designated natural grassland sites, neither artificial nor organic fertilizer application is allowed. We estimated inorganic and organic N and P loadings per hectare (ha) per year for fields in each polder (Supplementary Table S6.2).

6.2.6 Data analysis

All analyses were performed in R version 3.2.1 using the vegan, KernSmooth, PCNM, packfor and VennDiagram packages. In this study, we use two separate

datasets on soil microbial communities. One dataset contained data on soil microbial community structure (PLFA data), and another dataset contained data on soil microbial community functional capabilities (CLPP data). We first examined general patterns and clustering in polder areas using a principal component analysis (PCA). For each dataset, we tested the importance of general drivers and the existence of polder level differences further using distance-based redundancy analysis models (dbRDA; Legendre & Anderson, 1999), where microbial community variation (in composition or functional capacity) between sites was expressed as an Odum's percentage difference distance. Thus, large distances indicate very different sites and small distances indicate comparable sites in terms of community structure or functioning. Next, we defined two spatial scale levels of analysis, the local field level within polders and between polder areas at the landscape level. At both scale levels we carried out a variation partitioning analysis (Peres-Neto *et al.*, 2006) using dbRDA. Prior to variation partitioning, a dbRDA analysis on the full data set was carried out for different models. Next, all models were subjected to a forward selection procedure prior to variation partitioning (Blanchet *et al.*, 2008). We subsequently assessed the importance of underlying variables in shaping the microbial community structure and functional capacity at the two scale levels by examining the explained variation (R^2_{adj}) of the selected variables in isolation.

6.2.7 Local scale: model definition

Differences in community composition or functional capacity at the local scale may result from differences in environmental quality between field edges. To analyse general patterns at the local level we applied the approach described by Declerck *et al.* (2011). Briefly, dummy-coded polder identifiers delineate the different study areas. These polder identifiers were used as covariates in the analysis to control for large-scale patterns in the data. By controlling for the polder identity we could effectively study local scale patterns in community structure and function independently of landscape scale patterns. We distinguished four explanatory models at the level of the polder: a soil characteristics model (SOIL), a nutrient management model (NUT), a vegetation composition model (VEG) and a spatial model (SPACE).

The SPACE model was composed of Moran Eigenvector Map (MEM) variables that explain the spatial autocorrelation between sites in the landscape based on geographical distances (PCNM: Dray *et al.* 2006). By using staggered matrices of MEM eigenvectors (Declerck *et al.*, 2011; Legendre *et al.*, 2013), we described spatial autocorrelation within polders without the confounding effects of between polder autocorrelation. We only selected the eigenvectors with positive spatial correlations for analyses. The latter was already accounted for through the inclusion of polder identity as a covariate. With this approach, we could study small scale spatial patterning in community variation. Our SOIL model consisted of variables describing the quality of the soil (pH, C, N and P content, C:N, C:N and N:P ratio, moisture), morphometric characteristics (bank angle, bank width) and soil typology. Our NUT

model consisted of loadings of organic and inorganic N and P applied to the field along with dummy coded variables of the occurring nutrient management schemes (Supplementary Table S6.4). Our VEG model consisted of a staggered matrix of the principal axes resulting from a principal coordinate analysis (PCoA) per polder. Because many axes of a PCoA explained little to no variation, we selected for relevant axes based on a broken stick model of explained variation, with all axes before the break point being selected. The resulting axes were arranged into a staggered matrix (Legendre *et al.*, 2010) with the goal of only representing within polder differences. All models were subjected to forward selection based on a double stopping criterion (R^2_{adj} and a>0.05) and tested for significance using 99,999 permutations constrained within polder identity levels.

6.2.8 Regional scale: model definition

Differences in community composition or functioning at the landscape scale may result from differences in environmental quality between polders. Environmental gradients existing at the spatial grain of the landscape may be markedly different from those at the field level. Hence, an examination of these gradients irrespective of the variation explained within polders is appropriate. To this end, we used an approach where we first constructed a statistical model explaining community variation by dummy-coded polder identity variables. By taking the predicted values of this polder model, we obtained a matrix of community variation present between polder landscapes only. We used this matrix as our response matrix in subsequent analyses of drivers of community variation between polders, allowing us to make models that only explain community variation encompassed by the polder model. Here, we distinguished three explanatory models at the level of the landscape: a soil characteristics model (SOIL), a nutrient loading model (NUT) and a vegetation composition model (VEG). For the sake of interpretation, the explained variation of the models was rescaled to the total community variation captured by the polder model.

Our SOIL and NUT models consisted of the same variables as those used at the local scale. Our VEG model was created by transformation of an Odum's percentage dissimilarity matrix of the vegetation composition of all field edges within the scope of the respective data set by means of a principal coordinate analysis (PCoA). For the model explaining community variation, we made use of the resulting PCoA axes. This approach differs from the one used at the local scale in that we did not use PCoA axes per polder, but rather examined variation over all polders. As for the local model, we selected for the relevant PCoA axes based on a broken stick model of explained variation, with all axes before the break point being selected. The uniquely explaining part of the variation of the polder model, the part not explained by environmental drivers, may be interpreted as spatial patterning at the landscape level that is not directly related to the measured environment. A formal permutation test is not viable with the limited number of different polders. Hence, forward selection was carried

out using the increase in R^2_{adj} as the only criterion. Additionally, when models were found to be non-significant in explaining patterns in the full data, irrespective of spatial scale, we disregarded the model in this analysis.

6.3 Results

6.3.1 Drivers of soil microbial community structure at different spatial scales

Soil microbial community structure was examined by PLFA fingerprinting. In a first examination of PCA results (Supplementary Figure S6.2), we found clear differences between the different polder landscapes examined. A dbRDA of the PLFA data revealed that 19.8% of the community variation could be explained between polders (Figure 6.2a; Supplementary Table S6.3c) and a mere 4.0% could be explained within polders (Figure 6.2b; Supplementary Table S6.3a). This leaves a majority of the variation unexplained. Nonetheless, we were able to identify consistent, significant gradients explaining community structure (Supplementary Tables S6.3 and S6.4). At the local scale (Figure 6.2b), only the NUT model proved explain a significant portion of community variation (R^2_{adj}=4.0%, $p<0.01$). This leaves large parts of the total variation explained at the level of the full dataset unaccounted for (Supplementary Table S6.4). This variation was found to be explained at the landscape scale (Figure 6.2a) by means of the SOIL, NUT and VEG models. Only 2.9% (ns) of the variation was unique to the polder model, and not captured by one of the other models (Figure 6.4a, Supplementary Table S6.3a). The SOIL model was the most important explaining environmental component (R^2_{adj}=16.1%), encompassing large parts of the explained variation of the NUT (2.7%+1.1%=3.8%) and VEG model (3.0%+1.1%=4.1%).

We ranked variables underlying the main drivers identified in the variation partitioning in terms of their importance (Table 6.1). At the landscape scale, PLFA structuring responded most strongly to nutrient-related variables, the soil P content (10.3%), inorganic N fertilization (3.1%), soil N content (1.7%), organic P (1.0%) and N fertilization (0.6%). In addition to nutrient-related parameters, soil type (7.8%), the presence of nature management schemes (2.6%), agri-environmental schemes (0.2%), and the resident vegetation composition (4.6%) were shown to be important variables in explaining landscape level community structure. At the local scale variation in PLFA data was less well explained, with organic P fertilization being the most pronounced driver (5.7%) of microbial community structure.

6.3.2 Drivers of microbial community functional capacity at different spatial scales

Community level physiological profiling (CLPP) was used as a proxy for the functional capacity of the microbial community. In a first examination of PCA

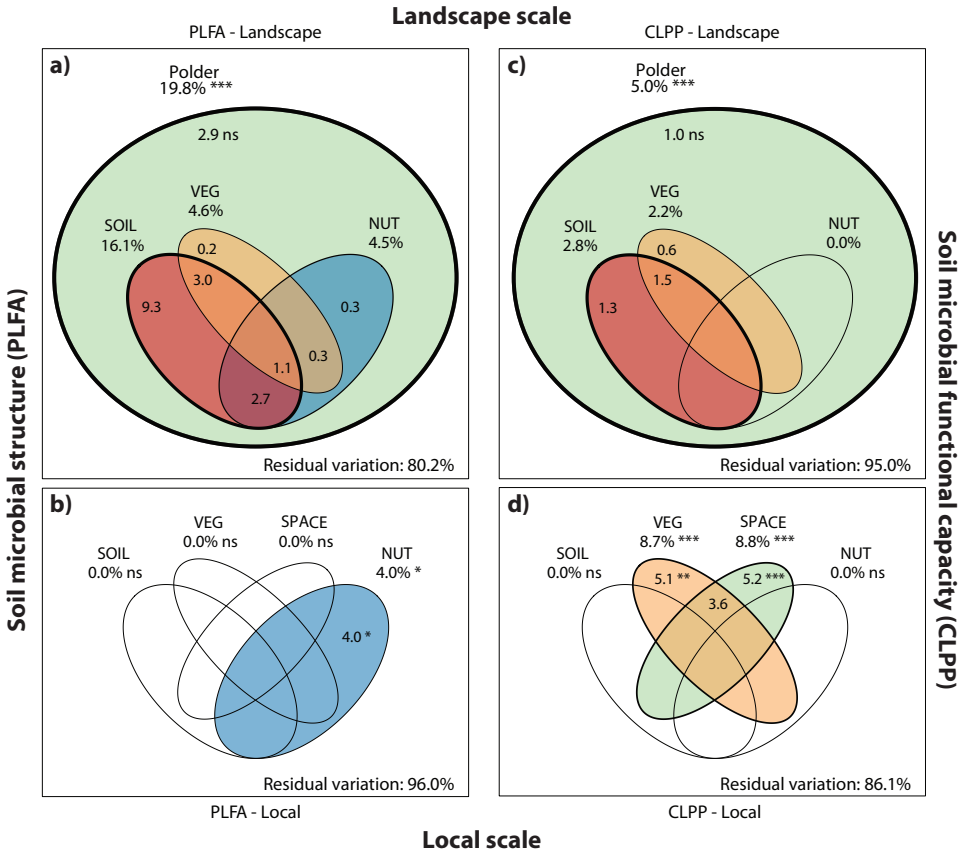

Figure 6.2 Drivers of microbial community structure and functioning on local and landscape scale. Venn diagrams showing the variation partitioning of different statistical dbRDA models: a soil characteristics model (SOIL), a nutrient management model (NUT) and a vegetation composition model (VEG) and a spatial model (SPACE). These models explain soil microbial structure (PLFA) and functioning (CLPP) by different drivers at the landscape and local scale. Stars indicate significance and numbers express the adjusted R^2 (%) of the model partitions.

results (Supplementary Figure S6.3), we found a strong overlap between sites of the different polder areas under examination. This was also reflected in RDA analyses of the data, with only 5.0% of the total variation in community functional capacity being explained by the polder model (Figure 6.2c). Despite this small part of the variation being explained, we did find that part of the CLPP variation between landscapes was attributable to soil characteristics (R^2_{adj}=2.8%) and vegetation (R^2_{adj}=2.2%) (Figure 6.2c). Nutrient management was found to be non-significant in explaining patterns in the full dataset (Table 6.1), and it therefore did not explain any of the variation encompassed by differences between polders. At a local scale (Figure 6.2d), we could explain a larger part of the variation (13.9%), which was attributed to the vegetation composition model (R^2_{adj}=8.7%, p<0.001) and spatial patterns in community functional capacity (R^2_{adj}=8.8%, p<0.001).

Table 6.1 Importance of variables underlying soil microbial community structure (PLFA) at both scale levels (local and landscape).

		Explained variation*	
Model	**Variable**	**Local**	**Landscape**
Soil characteristics (SOIL)	Soil P content	-	10.3
	Soil type: Organic top soil on deep peat layer	-	7.8
	Soil N content	-	1.7
Nutrient management (NUT)	Organic P fertilization	5.7	1.0
	Management: Nature - Moist meadow-bird grassland	0.8	2.6
	Inorganic N fertilization	-	3.1
	Organic N fertilization	-	0.6
	Management: AES - Meadow-bird nest protection	-	0.2
Spatial patterns (SPACE)	ns	-	-
Vegetation composition (VEG)	Vegetation composition	-	4.6

* Explained variation of each variable is given as R^2 (%) of the variable

\- Variable was not selected in the forward selection of the specific model

We identified the primary driving variables related to soil microbial community functional capacity (Table 6.2). At a the landscape scale, soil pH (1.1%) and soil type (0.9%) and soil P ratios (soil N:P: 0.7% and soil C:P: 0.6%) were found to be most explaining for the variation in functional capacity. The local scale was explained by the vegetation community and a spatial MEM model based on geographical distance between field edges. The latter showed that most patterns were described by the highest order MEM variable (7.7%), indicative of a relatively coarse spatial patterning of community functioning.

6.3.3 Comparing community structure and functional capacity

Comparing the two datasets, the two analyses of community variation yielded highly disparate results with respect to the scale at which different environmental factors could explain variation in the data (Figure 6.2). Community structure data (PLFA) was most influenced by environmental factors between different polders, *i.e.* at a large landscape scale (Figure 6.2, Supplementary Table S6.3). In contrast, functional data was poorly explained at this scale; rather environmental variation within polders offered the greatest level of explanatory power (Figure 6.2). Despite the difference in total explained variation, at the landscape scale the general partitioning and relative weight of the drivers was comparable for both PLFA structure and CLPP (Figure 6.2, Supplementary Table S6.3). Both microbial community properties were most explained by the SOIL model with a small contribution of the variance being

Table 6.2 Importance of variables underlying soil microbial functional capacity (CLPP) at both scale levels (local and landscape).

		Explained variation*	
Model	**Variable**	**Local**	**Landscape**
Soil characteristics (SOIL)	Soil pH	-	1.1
	Soil type: Sand	-	0.9
	Soil N:P	-	0.7
	Bank angle	-	0.6
	Soil C:P	-	0.6
	Soil type: Clay on peat	-	0.6
	Soil N content	-	0.6
	Soil C:N	-	0.3
Nutrient management (NUT)	ns	-	-
Spatial patterns (SPACE)†	MEM1	7.7	-
	MEM2	3.1	-
	MEM3	1.4	-
Vegetation composition (VEG)	Vegetation composition	8.7	2.2

* Explained variation of each variable is given as R^2 (%) of the variable

\- Variable was not selected in the forward selection of the specific model

† Spatial patterns model is composed of Moran Eigenvector Map (MEM) variables based on geographical distance as per Dray *et al.*, 2006. Variables of increasing order indicated decreasing scale of spatial patterning.

explained by VEG. Moreover, variation was highly collinear between the different models. On a local scale, patterns were markedly different between community structure and functional capacity.

6.4 Discussion

Understanding the drivers of soil microbial processes at relevant scales can help to improve management of agricultural peatlands to protect and improve desired ecosystem functioning. Through our analyses, we have examined the driving forces of microbial community structure and functioning in field margins along agricultural banks at two different scale levels; within polders (local) and between polders (landscape). We found local and landscape scale drivers to be distinct at different scale levels. The underlying variables were found to be largely different as well. This implies that the spatial scale of soil microbial studies is important when talking about driving forces of soil microbial community structure and functioning, enforcing the idea that the scale of soil management and the scale of study of soil microbial structure and functioning need to be well aligned.

6.4.1 Local functioning, landscape structuring

While somewhat anecdotal due to the separate collection of the datasets, we showed that soil microbial community structure (PLFA) was more strongly regulated at the landscape scale, while functional capacity (CLPP) was more strongly driven at the local scale. Explained variation, while not being exceptionally high (15-20%), was comparable to other studies using similar multivariate community analysis approaches (Van der Gucht *et al.*, 2007; Sayer *et al.*, 2017). Across polder regions, i.e. at the landscape scale, the results indicate a driving role for soil characteristics, with vegetation being largely collinear with soil characteristics, which is similar to earlier findings of Kuramae *et al.* (2010). We conclude from this that, with respect to soil microbial structure, differences in vegetation and nutrient management between polders are well reflected in the soil characteristics. Local microbial structure could only be led back to the applied nutrient management of the field and explained little variation. In contrast, variation in community functional capacity could be explained better by vegetation composition and spatial patterns at the local scale, with both explaining distinctly different parts of the community variation. The overlap in drivers at the landscape scale is likely due to the fact that the studied areas vary in land-use, land-history and management, which leads to landscape-scale vegetation and nutrient availability patterns that leave clear imprints in the soil. Locally, the small-scale heterogeneity of fields becomes more important in driving the specific microbial function. This mismatch in scale between structure and function has been described previously for specific microbes and their functions (Veraart *et al.*, 2016).

6.4.2 Drivers of soil microbial community structuring and functional capacity

Drivers of community variation may differ strongly with scale (Yergeau *et al.*, 2010; Prober *et al.*, 2015), and our analyses support this premise. At both scale levels community structure was driven by nutrient management. The latter result is in agreement with previous research (O'Donnell *et al.*, 2001; O'Brien *et al.*, 2016) that has shown the importance of fertilization regimes for soil microbial communities. In turn, the addition and manner in which nutrients are added can have direct consequences for ecosystem functions such as nutrient retention and plant uptake (Heijboer *et al.*, 2016). We, however, did find clear differences in underlying drivers of nutrient management of the within and between polder scales, with organic P loading and inorganic N loading being most important. This highlights the importance of identifying underlying drivers (Martiny *et al.*, 2011). By focussing on a single scale level important drivers may be overlooked and incorrect conclusions may arise, potentially leading to mismanagement of the agricultural landscape.

Our conclusions regarding landscape scale patterns are complicated by the lack of extensive replication at the landscape level, making formal testing of the drivers encapsulated within the polder model problematic. While we acknowledge these

limitations within our study, our results are strengthened by the strong significant patterns found in tests of the entire data set (Table S6.4). As large parts of the total variation that can be explained by our models remain unexplained at the local scale (e.g. Figure 6.2b), it is reasonable to assume that this variation may be explained at the landscape scale.

A surprising similarity in soil characteristic drivers of soil microbial community structure and functioning can be found for nutrient-related drivers (soil N:P ratio, soil P and N content). Specifically, soil N content was found as the only variable that was important in determining landscape scale community structure, as well as the community functional capacity. Additionally, for community structure, specifically the P-related processes were important drivers at a local (organic P fertilization) and at a landscape scale (soil P content, organic P fertilization). In existing literature, little attention has been paid to the effects of P on peatland microbial communities and functioning (Lin *et al.*, 2014; Veraart *et al.*, 2015). Our results suggest that these effects of P enrichment on peatland microbial communities deserve additional consideration.

The relevance of the resident vegetation community for local microbial functional capacity, but not local microbial structure, is a noteworthy result. This could be caused by the study design in which we compare different polder areas with slightly different plant communities. An ecological explanation for this may be found in the stimulating role of plant presence and diversity on the function of soil microbes by (e.g. Zak *et al.*, 2003). Furthermore, a well-developed, species-rich riparian zone will influence water and nutrient retention (Hefting *et al.*, 2005) and thereby microbial functioning (Korol *et al.*, 2016). This development of a riparian zone depends strongly on local disturbance by mowing and cattle grazing. We did not directly quantify these factors, although they should in part be represented in the nutrient and land management schemes. However, within these schemes, there is room for variation in grazing and mowing regimes at the digression of the land manager. As land managers tend to own different nearby fields within a landscape, this variation in mowing a grazing is likely to be spatially structured. Our results, where vegetation and spatial structure explain local functional capacity, may thus be (partially) explained by these unmeasured differences in management regimes.

We found evidence for spatial patterns that could not be explained by any of the measured environmental drivers at the level of the local functional capacity (uniquely explained variation of the SPACE model), which may represent a possible signal of dispersal limitation (Dray *et al.*, 2006). While dispersal-limitation has been shown to be plausible within microbial communities (Evans *et al.*, 2017; Langenheder *et al.*, 2017), it is rarely a significant driver of microbial community structure (Martiny *et al.*, 2011; O'Brien *et al.*, 2016). Hence, our observed spatial patterns are likely to be caused by spatially-structured environmental variables (e.g. light climate, soil redox conditions) that were not taken into account in this study (Martiny *et al.*, 2006).

6.4.3 Management of soil microbial communities in peatlands: an integrative approach

We provide some indications that microbial function is regulated by multiple distinct drivers that are distinct from those driving soil microbial structure, and that these drivers act at different spatial scales. This complicates the task of managing agricultural peatlands for desired ecological functioning. The traditional view maintains that environmental drivers influence community structure and that this structure in turn influences community functioning (Allison & Martiny, 2008). However, this paradigm has been proven to be insufficient to explain microbial functional patterns in nature (Strickland *et al.*, 2009; Weedon *et al.*, 2017). Microbial functions have been shown to change independently of microbial community structure (Tian *et al.*, 2016; Weedon *et al.*, 2017) and respond to different variables than structure (Boeddinghaus *et al.*, 2015). However, disregarding community structure entirely and solely focussing on functioning is also clearly inappropriate, as microbial community structure serves as a constraint on the realized functioning of the community and the ecosystem as a whole (Pérez-Valera *et al.*, 2015; Heijboer *et al.*, 2016).

We argue that for effective management of desired functioning to optimize the different societal benefits obtained from the landscape, both soil microbial structure and functioning need to be considered. Based on our study, environmental quality changes relevant for soil microbial functional capacity were most pronounced at the local scale. As local environmental quality shifts, this will lead to a direct shift in realized functioning away from the desired function (Figure 6.3, horizontal axis). However, the magnitude of this shift may be limited by the community structure, which constraints the extent of the shift in function (e.g. compare Figure 6.3, central-right and bottom-right, respectively unconstrained vs constrained situation). Changes in environmental drivers governing structure (Figure 6.3, vertical axis) were primarily found to manifest themselves at the level of the landscape within the context of this study. A change in environment at the landscape level may hamper realization of the desired function by constraining the realised function negatively as well (e.g Figure 6.3, top-left). Hence, a thorough understanding of the community structure and its potential to facilitate the desired function is an imperative first step in soil microbial management followed by optimization of the conditions directly driving required soil microbial functioning. Throughout this process the spatial scale at which microbial structure and functioning responds to these changes needs to be taken into account. Landscape measures, such as water level fluctuations and spatial planning set the constraints for the potential functioning (i.e. structure), and once this stage has been set, local management options such as mowing and fertilization regimes are decisive in determining if the desired functioning can be achieved.

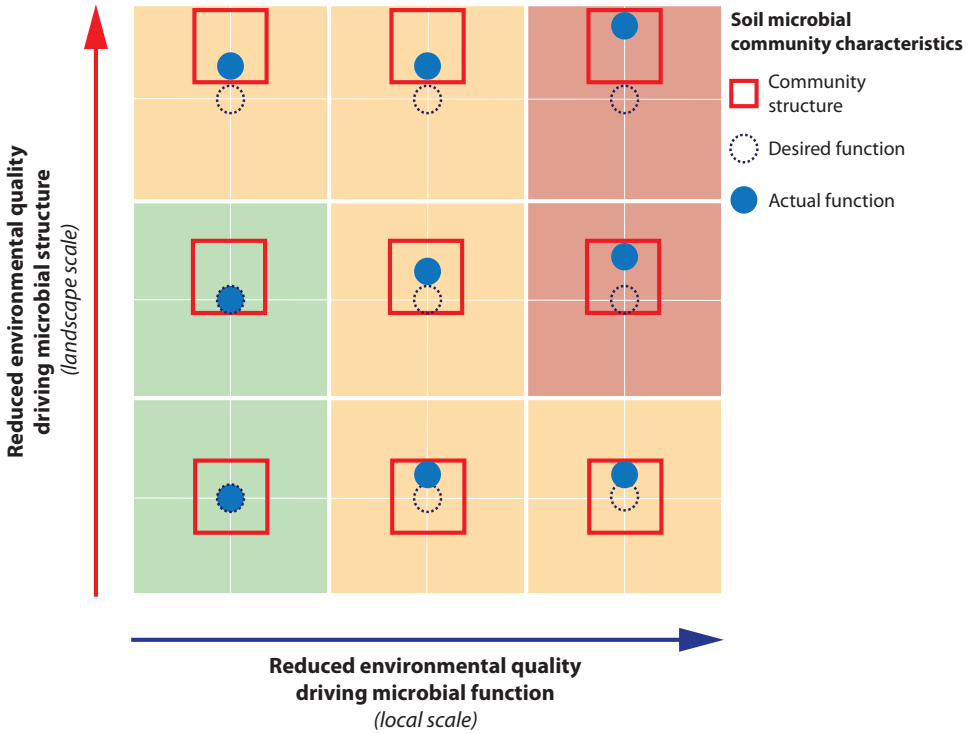

Figure 6.3 Schematic representation of the effects of reduced environmental quality on soil microbial community structure and functioning. This conceptual figure illustrates how reduced environmental quality of drivers relevant for functional capacity will directly lead to shifts of soil microbial functioning away from its desired function. Reduced environmental quality relevant for microbial structural composition will cause shifts in the soil microbial community structure box. This can ultimately also shift desired soil microbial community function through its constraint on microbial function. Within the context of study the environmental drivers of microbial functioning were found to manifest at the local scale whereas the drivers shaping structure manifest at the landscape scale.

6.5 Concluding remarks

Our study showed that soil microbial communities of agricultural peatlands are being driven by different factors at distinct, management relevant spatial scales. Furthermore, our study provides a first indication that soil community structure and function does not respond to the same factors, or at the same spatial scales. We argue that it is important to take both these soil microbial community characteristics (structure and function) into account for management of these important ecosystems. Based on this study, we suggest optimizing management of microbial ecosystem functioning in peatlands by first focussing on landscape restoration, followed by suitable local scale management optimization. This has direct relation to recent initiatives such as the 4‰ initiative for increasing soil C storage in agricultural areas (Le Foll, 2015) and efforts to optimize long-term biogeochemical functioning of agricultural peatlands.

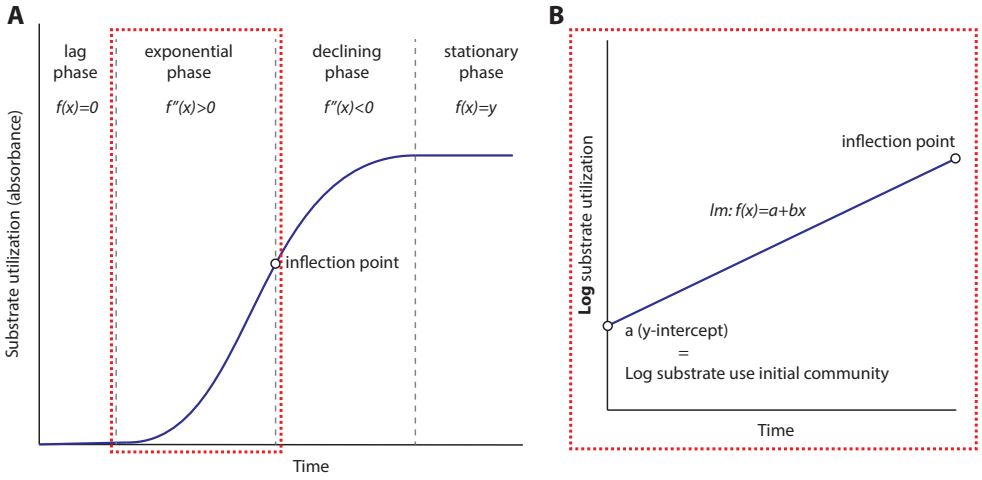

Figure S6.1 Schematization of the steps to calculate substrate use by the initial microbial community (CLPP). A) Conceptually, the function of substrate utilisation through time consists of four distinct phases. The lag phase in which no measurable amount of substrate is utilized, the exponential phase in which the function is convex (f"(x)>0), the declining phase in which the function is concave and a stationary phase in which the substrate is depleted. B) By extracting the exponential phase of the function (delimited by positive y values and the inflection point) and fitting a log-linear model, the y-intercept is found. This yields the substrate use without growth, the substrate utilization of the initial community.

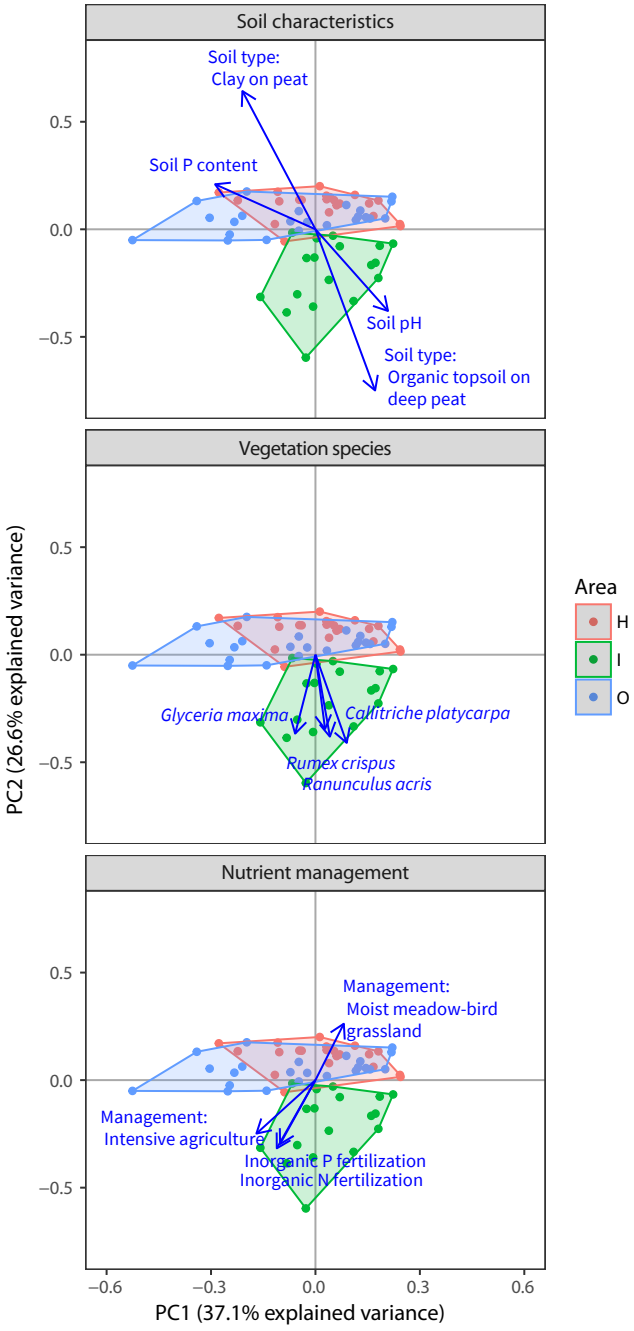

Figure S6.2 Multidimensional scaling of microbial community structure and its driving variables. PCA plots of soil microbial structure data (PLFA) for the three different groups of drivers (Soil characteristics, Nutrient management and Vegetation). Arrows are projected variables showing the four variables with the highest squared correlation coefficients. Different colours indicated the different sampled areas.

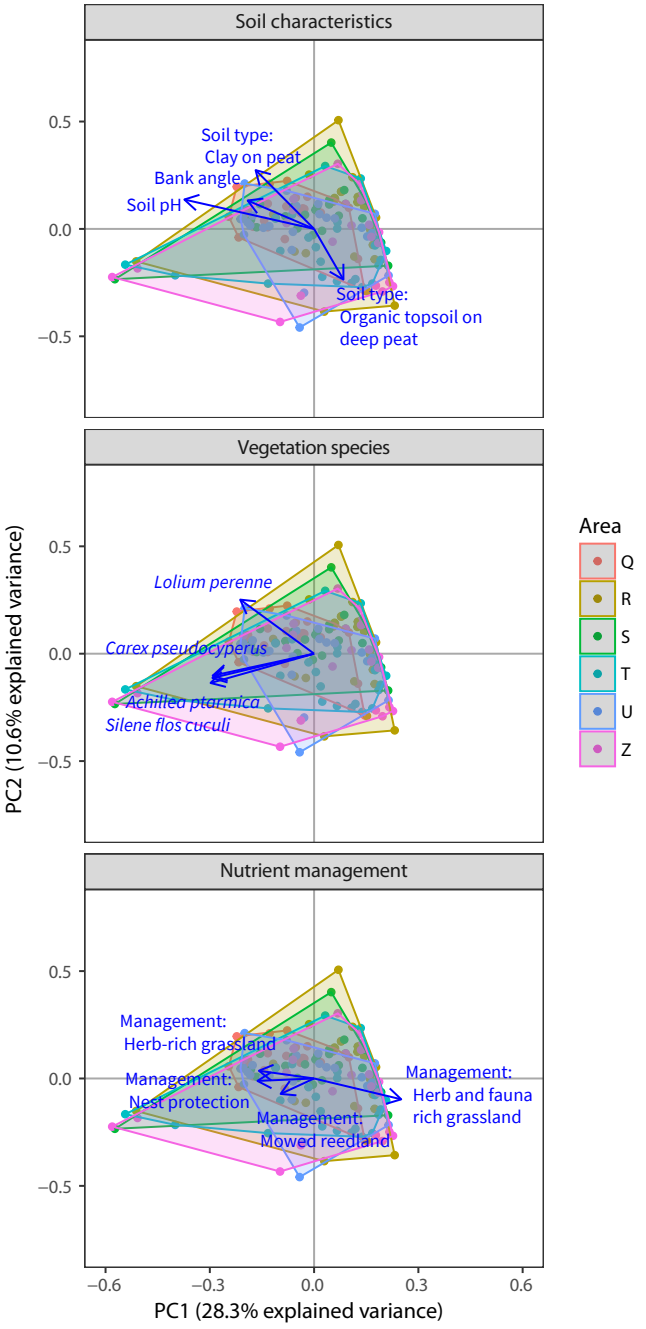

Figure S6.3 Multidimensional scaling of microbial community functional capacity and its driving variables. PCA plots of soil microbial functional capacity data (CLPP) for the three different groups of drivers (Soil characteristics, Nutrient management and Vegetation). Arrows are projected variables showing the four variables with the highest squared correlation coefficients. Different colours indicated the different sampled areas.

Table S6.1 Conversion of Tansley abundance classes to numeric classes and percentage cover.

Tansley score	Tansley numeric score	Cover (%)
d	11	60
cd	10	40
ld	9	22
a	8	15
la	7	9
f	6	8
lf	5	3
o	4	2
lo	3	1
r	2	0.5
s	1	0.1

Table S6.2 Nutrient management schemes. Description of the different nutrient management schemes and their respective N and P fertilization limits based on resting periods and legislation.

Nutrient management practice		$N_{organic}$ limit	$N_{inorganic}$ limit	P limit	Times mown (per year)	First mowing day
Agri-environmental schemes	*Meadow-bird grassland to June 1*	170.0	18.8	67.7	4.0	153
	Meadow-bird grassland to June 15	109.2	0.0	39.1	3.0	167
	Meadow-bird grassland, pre-grazed	170.0	51.0	79.2	3.0	167
	Meadow-bird nest protection	170.0	58.6	82.0	4.0	134
	Herb-rich meadow-bird grassland	161.3	0.0	57.8	2.0	174
Nature	*Swamp*	0.0	0.0	0.0	0.0	-
	Mowed reed land	0.0	0.0	0.0	1.0	106
	Moist hay meadow	170.0	0.0	70.0	1.0	153
	Herb and fauna rich grassland	170.0	0.0	70.0	1.0	153
	Moist meadow-bird grassland	170.0	0.0	70.0	2.0	153
Intensive agriculture	*Intensive agriculture*	170.0	95.0	95.0	5.0	134

Table S6.3 Variation partitioning of soil microbial community drivers.

A) Variation partitioning of PLFA data at the landscape scale

Model[#]		R^{2*}	F	p	DF model	DF res
Soil characteristics	full	16.07	-	-	3	59
	partial	9.30	-	-	3	50
Vegetation community	full	4.62	-	-	4	58
	partial	0.16	-	-	4	50
Polder	full	19.83	8.67	0.0002 ***	3	59
	partial	2.93	1.01	0.3658 ns	3	59
Nutrient management	full	4.48	-	-	5	57
	partial	0.33	-	-	5	50

B) Variation partitioning of CLPP data at the landscape scale

Model[#]		R^{2*}	F	p	DF model	DF res
Soil characteristics	full	2.85	-	-	8	135
	partial	1.27	-	-	8	130
Vegetation community	full	2.17	-	-	5	138
	partial	0.59	-	-	5	130
Polder	full	4.99	29.10	0.0020 **	5	138
	partial	1.04	10.21	0.4434 ns	5	119
Nutrient management	full	0.00	-	-	NA	NA
	partial	0.00	-	-	NA	NA

C) Variation partitioning of PLFA data at the local scale

Model[#]		R^{2*}	F	p	DF model	DF res
Soil characteristics	full	0.00	NA	NA ns	NA	NA
	partial	0.00	NA	1.0000 ns	NA	NA
Vegetation community	full	8.75	4.50	0.0010 ***	4	134
	partial	5.14	3.09	0.0051 **	4	128
Spatial patterns	full	8.76	3.34	0.0010 ***	6	132
	partial	5.16	2.42	0.0007 ***	6	128
Nutrient management	full	8.76	NA	NA ns	NA	NA
	partial	0.00	NA	1.0000 ns	NA	NA

D) Variation partitioning of CLPP data at the local scale

Model#		R^{2*}	F	p	DF model	DF res
Soil characteristics	full	0.00	NA	NA ns	NA	NA
	partial	0.00	NA	1.0000 ns	NA	NA
Vegetation community	full	0.00	NA	NA ns	NA	NA
	partial	0.00	NA	1.0000 ns	NA	NA
Spatial patterns	full	0.00	NA	NA ns	NA	NA
	partial	0.00	NA	1.0000 ns	NA	NA
Nutrient management	full	4.02	2.59	0.0241 *	2	58
	partial	4.02	2.59	0.0241 *	2	58

* Explained variation is expressed as the adjusted R^2 in %

Partial models are constrained for all variables contained in other models, giving the unique variation explained by the model under consideration, excluding the polder model where applicable.

- Indicates a model which was not tested due to limited replication at the landscape level.

Table S6.4 Importance of different drivers in explaining the full variation in soil microbial community structure (PLFA) and functional capacity (CLPP) irrespective of spatial scale.

	Model	R^{2*}	F	p	DF res
PLFA	*Soil characteristics*	19.76	2.27	0.0034 **	50
	Vegetation composition	15.11	2.58	0.0069 **	55
	Polder	19.83	8.67	0.0002 ***	60
	Nutrient management	10.77	2.07	0.0276 *	55
CLPP	*Soil characteristics*	9.50	2.07	0.0131 *	129
	Vegetation composition	9.15	2.80	0.0001 ***	135
	Polder	4.99	2.50	0.0022 **	138
	Nutrient management	3.38	1.42	0.1309 ns	131

* Explained variation is expressed as the adjusted R^2 in %

Chapter 7

General discussion

Amber Heijboer

The soil food web is critical to the Earth's biogeochemical cycles through the decomposition of soil organic matter, thereby playing an important role in a wide range of ecosystem services like food production, carbon (C) storage and nutrient retention. To anticipate the high speed of environmental change that our planet is currently facing, a detailed understanding of soil life and related functioning is crucial to ensure a sustainable management of our soils. Even though the microbial part of the soil food web makes up 90% of the belowground biomass, and is responsible for the majority of soil organic matter decomposition, detailed information on the microbial processing of different types of organic substrates is missing. This information is however crucial for targeted management of soil life, a strategy which is increasingly being referred to as the prospective tool to anticipate environmental change, and to reach optimal soil functioning (Sanders *et al.*, 2014; Tardy *et al.*, 2015). The main research aim of this thesis was therefore to disentangle the soil microbial food web in relation to an important type of environmental change: land use change. In this thesis, I explored the use of empirical techniques to gain more insight into the detritus-based soil microbial community and to examine the fate of different qualities of organic substrates through the microbial part of the soil food web in response to land use change.

7.1 Disentangling soil microbial food webs

One of the central questions in this thesis is: how can we increase the level of detailed understanding in the diverse microbial part of the detrital based soil food web (*research objective 1*)? **Chapter 2** of this thesis starts by exploring what type of empirical information is needed to create and extend empirically based food web models, with a main focus on energy flow webs describing flows of matter and energy. Of the empirical methods addressed in this chapter, Stable Isotope Probing (SIP) is most applicable to disentangle soil microbial C flows. This upcoming empirical technique in food web research is used to trace flows of stable isotope-labelled matter in food webs at a fine taxonomic scale, by combining molecular techniques with the tracing of stable isotopes in molecular biomarkers. In this thesis, SIP was applied in the studies described in **chapters 3 and 4** to trace the fate of respectively stable isotope labelled litter and different quality organic C substrates through the soil microbial community. The biomarkers assessed in this research were phospholipid fatty acids (PLFA, chapter 3 and 4) and RNA (chapter 4). PLFA-SIP served as a first analysis to quantify general C flows through distinctive microbial groups. Although the taxonomic assignment of PLFAs is rather difficult, it is a very sensitive method that enabled us to study C flow patterns through initial phases of soil microbial decomposition. Based on PLFA-SIP results, a selection of soil samples was subjected to the more labour-intensive, yet more detailed, RNA-SIP approach to assess soil microbial C flows in greater taxonomic detail. While this extension provides far greater taxonomic resolution, one should keep in mind that RNA-SIP also reduces statistical power and the ability to yield quantitative information. By applying a combination of these two approaches, the

research presented in **chapter 4** gives a first quantitative and comprehensive picture of soil microbial key players in the decomposition of different qualities of soil organic matter substrates in response to changing land use (*research objective 2*). What were the main findings of these studies and what do they contribute to our understanding of detritus based soil microbial food webs?

I first focus on the new insights that can be gleaned from these studies with regard to detritus-based soil microbial food webs, irrespective of land use change. When studying the decomposition of litter as well as different qualities of organic substrates, it became apparent that there is a very strong successional pattern of soil microbial groups during the initial phases of litter decomposition. Especially in the study described in **chapter 3**, which examines a high number of samples over time, it is clearly shown that each soil microbial group as determined by PLFA-SIP, has its own temporal niche when it comes to the maximal amount of litter-derived C incorporation. In addition, it was clear that distinctive soil microbial groups showed large differences in the absolute amounts of litter-derived C, with a remarkable similarity between the order of soil microbial succession (early to late) and their quantitative contribution (high to low) of soil microbial groups after litter addition: fungi > G^- bacteria > G^+ bacteria > actinomycetes > micro-fauna. These results support the idea of having trophic levels within the soil microbial community, a concept that is not yet defined as such in classical soil food web models. **Chapter 4** extends this work by tracing different qualities of organic C substrates, that were assessed by both PLFA-SIP as well as RNA-SIP to get a deeper insight into soil microbial resource partitioning. These approaches enabled me to show that intra-kingdom microbial resource partitioning takes place in both the bacterial as well as the fungal community. For this specific model system, different bacterial species form a continuum of active decomposers along the easy-soluble/labile to recalcitrant organic matter gradient. For the fungal community, there is a clear case of soil microbial resource partitioning, with specific active fungal decomposers for different qualities of organic C substrates.

Since I would like to use these new insights in the soil microbial food web to understand the effects of environmental change on soil microbial functioning, I assessed soil microbial C flows in a land-use change model system: a chronosequence of ex-arable soils. In both **chapters 3 and 4,** I compared recent and long-term abandoned soils in order to assess the effects of land use change on soil microbial C flows. The successional pattern of soil microbial decomposers was shown to be unaffected by time since land abandonment, which means that the order of microbial groups/classes in the soil microbial food web remains similar in the different soils. Besides soil microbial decomposer succession, there was also intra-kingdom soil microbial resource partitioning, a phenomenon that was affected by time since land abandonment. This was most apparent in the emergence of a specialized fungal and bacterial communities involved in the decomposition of recalcitrant material in long-term abandoned soils, but also by a relative shift in decomposing efficiency from G^- towards G^+ bacteria.

What do these results teach us about the topological information of soil microbial food webs? The main insights in the soil microbial food web advanced by this research are:

1. There is intra-kingdom soil microbial resource partitioning.
2. There is a clear succession of soil microbial decomposers, indicating the existence of microbial trophic levels.
3. The topological structure of the microbial soil food web is unaffected by land use.
4. The relative importance of specific soil microbial energy and matter flows, as defined in energy flow webs, is affected by land use change.

Although somewhat obvious, I would like to emphasize that more research on soil microbial food webs is clearly needed to standardize above standing findings. Even though there is already existing literature confirming the existence of intra-kingdom soil microbial resource partitioning (Paterson *et al.*, 2008; Drigo *et al.*, 2010; Kramer *et al.*, 2016; Pausch *et al.*, 2016) and soil microbial decomposer succession (Kramer *et al.*, 2016), we are only at the onset of exploring soil microbial food webs and more insights are needed in order to create reliable soil microbial food web models that can equip us with important information regarding the long-term consequences of environmental change for soil functioning. This research only addresses one specific type of environmental change, land use change as a result land abandonment, and more scenarios should be examined to define the stability/flexibility of soil microbial food webs in response to changing environmental conditions.

7.2 Linking soil microbial communities to ecosystem processes

The last research objective of this thesis focused on linking soil microbial community structure to functioning in response to land use (*research objective 3*). Two different types of case studies were used to study this complicated link, with the aim of assessing the importance of soil microbial community structure for ecosystem functioning. In **chapter 5**, a pot experiment was performed in which soils were amended by four contrasting types of organic amendments and subsequently used as potting soils for the growth of Brussels Sprouts. This experiment allowed us to 1) test the effect of organic amendments on soil microbial community biomass, activity and structure, and 2) assess the effects of potential changes in the soil microbial community structure on ecosystem services in terms of plant biomass, C and N cycling, and N retention. This study showed that a number of soil microbial functions were not affected by the composition of the soil microbial community, but rather by soil microbial activity: N mineralization rates, plant N uptake and plant biomass. Microbial N immobilization was affected by the composition of the soil microbial community. These results indicate the existence of both 'broad' and 'narrow' microbe-mediated ecosystem processes, whereby broad processes are ecosystem processes that are characterized by a high level of functional redundancy, and therefore relatively insensitive to *who* is

present bur rather sensitive the *amount* of microbial activity (Schimel, 1995). Narrow microbial mediated processes on the other hand, depend more on specific types of microbes present, and therefore rely more strongly on the composition of the soil microbial community.

These results suggest that manipulating soil microbial community composition, might be a viable strategy to optimize specific soil microbial functions. These findings are in line with the growing understanding among soil scientists, that targeted management of soil microbes, e.g. via specific organic inputs, might help to optimize sustainable soil functioning (Sanders *et al.*, 2014; Tardy *et al.*, 2015). In the light of these advancements, there is an increasing demand for information on the drivers of both soil microbial community composition and functioning, and the possible overlaps and differences between these drivers. To this end, **chapter 6** describes a study in which a set of potential soil microbial community drivers are assessed at two spatial scales that are relevant to soil management: local vs landscape. This research demonstrates that soil microbial community structure and functioning are driven by distinct sets of drivers operating on different spatial scales, with phosphorous-related microbial community structure drivers acting at the landscape scale and vegetation-related microbial function drivers predominating at the local scale. Since soil microbial community structure and functioning are also directly related (e.g. **chapter 5**), this means that both soil microbial structure and functioning should be taken into account for the design of effective soil management strategies.

7.3 New frontiers in soil microbial food web research

This thesis discusses both disentangling the soil microbial food web, as well as the link between the structure of these soil microbial communities and their functioning. What type of research lines do I think should be addressed in the future to advance our understanding of soil microbial food webs and their role in ecosystem functioning?

7.3.1. Linking empirical and modelling approaches in soil food web ecology

Important technological advances in empirically characterizing trophic networks are increasing possibilities to study soil food web dynamics in greater detail. However, in order to link these new insights to the advancements that are being in made in soil food web modelling (Moore *et al.*, 2018), it is crucial that soil empiricists and soil modellers work very closely. As **chapter 3** points out, bringing modellers and empiricists together should be the start of any type of research in this area, to assure that empirical studies take into account all model-relevant parameters and variables. From the other perspective, it is important that modellers be aware of the types of information that can be derived from empirical studies. Combining modellers and empiricists into large-scale research projects will significantly advance the field of soil food web research and will provide new insights that could dramatically improve our view on the dynamics and functioning of soil food webs.

7.3.2 Assess the effects of emerging soil microbial food web views on food web stability

In this thesis I show evidence of the existence of intra-kingdom microbial succession and intra-kingdom microbial resource partitioning, findings that go against the prevailing idea of separate fungal and bacterial energy channels in classical soil food web models. However, the existence of these energy channels has been mentioned as an important factor in the stability of soil food webs (e.g. Moore & Hunt, 1988; Moore *et al.*, 2005; Moore & de Ruiter, 2012). To what extent does the existence of intra-kingdom soil microbial resource partitioning destabilize the commonly held view regarding the importance of fungal and bacterial energy channels for soil food web stability? The concept of intra-kingdom soil microbial resource partitioning still leaves room for the concept of resource compartmentation (Moore & Hunt, 1988), however it would mean that we have to let go of the classical bacterial and fungal energy channels within the soil microbial community. Having energy channels that are based on decomposability and accessibility of organic matter, rather than taxonomic segregation of microbial kingdoms, creates new possibilities to study the role of resource compartmentation in soil food web stability.

7.3.3 Integrating green and brown C flows into a soil microbial food web model

Over the last few years, there have been a number of soil microbial SIP studies that have increased our insight into the cycling of carbon and nutrients through the microbial part of the soil food web (e.g. Hannula *et al.*, 2017, 2012; Kramer *et al.*, 2016; Malik *et al.*, 2015; Morriën *et al.*, 2017). However, all of these studies have focussed on the root-based (green) microbial food web (e.g. Hannula *et al.*, 2017, 2012; Malik *et al.*, 2015; Morriën *et al.*, 2017) or the detritus-based (brown) soil food web (e.g.Kramer *et al.*, 2016). In **chapters 3 and 4** of thesis, it is suggested that the interplay between these different types of C inputs in the soil might be key in determining the overall ecosystem functioning, especially in response to environmental change. Where our SIP studies show a decreased microbial efficiency in processing detritus-derived matter flows with time since land abandonment, Morriën *et al.* (2017) show an increased microbial efficiency of plant-derived microbial C flows in the same ex-arable soils. So combining this current work with other studies examining soil microbial food webs in the same model soil system (Hannula *et al.*, 2017; Morriën *et al.*, 2017), might therefore lead to new insights that could significantly affect our understanding of soil food web dynamics. This is also in line with the suggestions of previous studies that propose a new conceptual model in which plant-derived C flows directly to the detritus pool, but also indirectly via mycorrhizal fungi (Drigo *et al.*, 2010; de Vries & Caruso, 2016).

To study the interactions between the green and brown soil food web, one would ideally wish to study plant-derived and detritus-derived microbial flows simultaneously.

When using SIP techniques, this approach faces technical complications, since green food web interactions are usually assessed in intact soils, while brown food web interactions are mainly studied by mixing bulk soil with labelled organic substrates. Upcoming techniques like RAMAN-SIP, able to trace the fate of stable isotope-labelled substrates at the single-cell level in a non-destructive manner, and CHIP-SIP, able to trace multiple SIP-labelled elements simultaneously, could play a major role in these type of advancements (Li *et al.*, 2013; Wang *et al.*, 2016; Mayali *et al.*, 2012). With such an approach, the level of disruption is minimal, so that it is also possible to take organic matter accessibility to microbes into account, a concept that is increasingly seen as a major determinant of soil microbial community structure and functioning (Lehmann & Kleber, 2015).

Figure 7.1 presents a conceptual model, integrating new insights from this thesis and recent studies on the soil microbial food web. The detritus pool of this model incorporates both the range of decomposability, as well as the accessibility of soil organic matter as proposed in the soil continuum model of Lehmann & Kleber (2015). The detritus pool receives input of organic material via (1) dead plant material, (2) dead soil life, (3) plant root exudates, or (4) via mycorrhizal fungi (4). In the classical detrital food web model only the solid brown arrows (detritus flows) were included, as well as the arrow for root herbivory (5). This thesis focusses on the connection between the detritus pool and the soil microbial community to further disentangle the processing of detrital inputs by the soil microbial community. Where classical soil food web models only consider a flow from the detritus pool towards the microbial community, I think it is also important to consider the role that soil microbial decomposition has on both the quality and accessibility of the detritus pool (brown dashed arrows). The results in this thesis demonstrate that soil microbial community structure is important to consider when unravelling the relationship between organic inputs, the soil microbial community and overall ecosystem functioning. This is depicted in Figure 7.1 by including both inter-kingdom resource partitioning (horizontal) as well as decomposer succession (vertical) into the soil microbial community. Implementation of high-resolution microbial data in these types of models will further increase our understanding of soil microbial food webs and their functioning and improve models to better strengthen predictions on soil ecosystem processes. As an example one could think of nutrient and C retention in the soils. Although mostly assumed as a 'broad' ecosystem service that is independent of soil microbial community structure, the results of **chapter 4** indicate that microbial community structure is an important determinant of soil ecosystem functioning. Therefore, the role of soil microbial community structure should clearly not be underestimated (Graham *et al.*, 2016).

7.4 Reflection and final conclusions

'It always seems impossible until it's done,' is a famous quote of the late Nelson Mandela, which I think could also be easily applied to many of the things we are

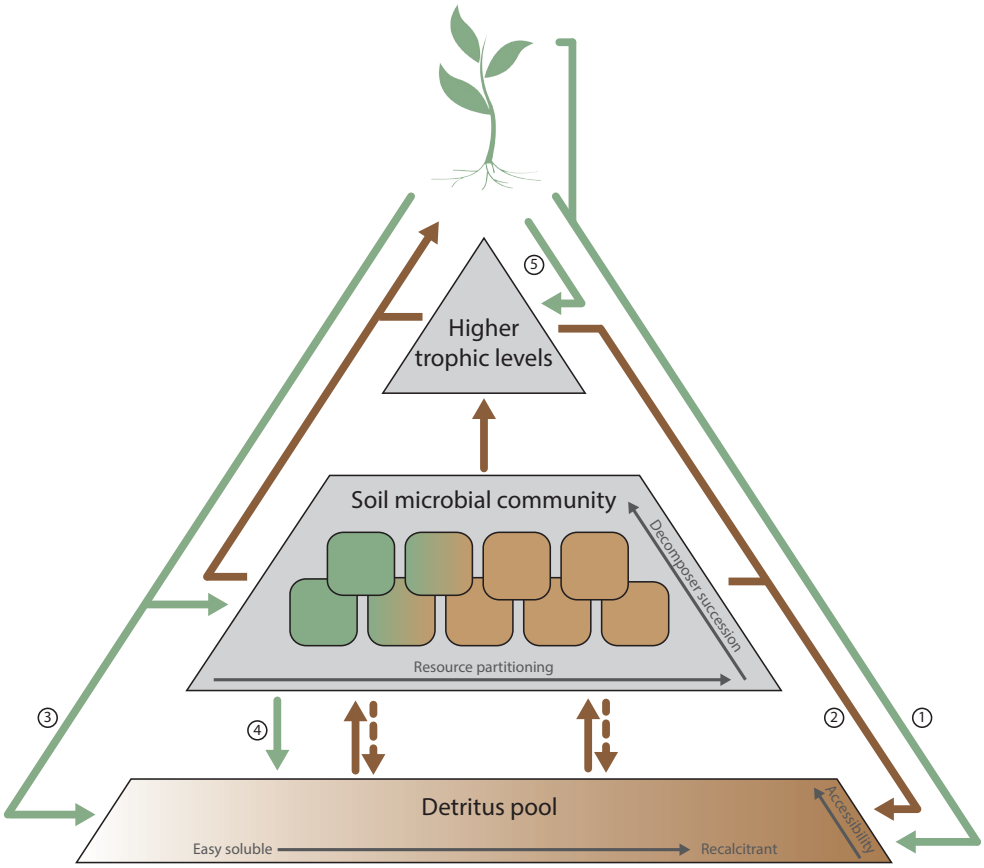

Figure 7.1 Conceptual model to integrate plant-derived (green) and detritus-derived (brown) matter flows in soil food webs, whereby the arrows depict matter flows from between the different components: plant, detritus pool, soil microbial community and the higher trophic levels. The soil microbial community consists of a number of microbial groups (green and brown boxes) that share the same resource. Specific matter flows are: 1) flow of dead plant material to the detritus pool, 2) flow of dead soil organisms to the detritus pool, 3) flow of plant root exudates to the detritus pool, 4) flow of root-derived matter to the detritus pool via the soil microbial community, i.e. AM fungi, 5) plant herbivory by soil organisms as part of the higher trophic levels of the soil food web. Dashed brown arrows indicate the effect that soil microbial decomposition has on the quality and accessibility of the detritus pool.

doing in science. The reason that detailed information on the flow of contrasting types of organic compounds is still missing in soil food web models, is because until recently it was simply impossible to disentangle the soil microbial food web. With emerging empirical methods, this is now possible, as demonstrated in this thesis in which these approaches are reviewed and applied to study detritus-based soil microbial food webs in more detail, and in relation to ecosystem functioning.

This thesis gives the first high-resolution and quantitative image of detritus-based microbial food webs as affected by land use change. The use of Stable Isotope Probing

(SIP) enabled us to trace the fate of (contrasting types of) organic matter through the microbial part of the soil food web, demonstrating that microbial C flows are not restricted to the fungal and bacterial energy channels. Instead, microbial C flows are mediated by the soil microbial community properties: intra-kingdom resource partitioning and soil microbial decomposer succession. Studying soil microbial food webs in a chronosequence of ex-arable fields, revealed that these microbial community properties are important to understand the effect of land-abandonment on the functioning on soil microbial food webs. Where the overall microbial community structure, as well as the topology of litter-derived C flows, were rather unaffected by time since abandonment, there was still an effect on soil microbial functioning, with the soil microbial community becoming less efficient in decomposing SOM as a function of time since abandonment. Detailed analyses of microbial C flows revealed that the soil microbial community (both fungal and bacterial) partly develops into a specialized microbial community dealing with the decomposition of recalcitrant organic matter. The importance of subtle changes in soil microbial community structure was also shown in **chapter 5**, where it was revealed that the soil microbial community structure was important for the N retention capacity of the soil microbial community. **Chapter 6** provides important information on how detailed analysis of soil microbial community structure can be used to target optimal soil management strategies, as it was shown that soil microbial structure and functioning are linked, but driven by different drivers that operate at different spatial scales.

This thesis has advanced the understanding of soil food webs, but the journey of disentangling and understanding these communities are far from over. Our ability to disentangle soil microbial food webs is increasing, and our understanding of the relationship between soil microbial communities and the functioning of soil food webs is progressing. In this thesis a first start has been made to disentangle soil microbial C flows of diverse organic substrates. Taken together, this brings me to the conclusion that a deeper understanding of soil microbial community structure and intra-kingdom variation in soil microbial C processing is of vital importance to enhance our appreciation of soil microbial community functioning in response to environmental change, which is the key to success for targeted management of soil life.

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Summary

Soils are crucial for a large number of ecosystem services and occupy an important position in driving the Earth's biogeochemical cycles. Soils are therefore essential for e.g. agricultural food production, carbon sequestration, water purification and nutrient cycling. These soil functions are to a large extent governed by the huge biodiversity of soil life, which can be depicted in the form of a soil food web: a model that describes the feeding relationships among groups of species that live in the soil. A number of soil ecosystem services, as governed by soil life, are currently under considerable threat due to e.g. soil degradation, atmospheric nitrogen deposition and land use change. A proper understanding of the mechanisms underlying soil ecosystem functioning, in relation to global change, is important to anticipate these threats and to help ensure optimal functioning of our soils.

Soil food web models have proven to be highly useful in the study of the long-term consequences of environmental change on soil communities and associated ecosystem functioning. Perhaps the most important ecosystem process driven by the soil food web is the decomposition of detritus: plant residues and soil organic matter. Via the decomposition of detritus, soil organisms determine the critical balance between sequestration and mineralization of carbon (C) and nutrients, affecting soil CO_2 emissions to the atmosphere and nutrient availability for plants. Soil microbes (bacteria, fungi and protozoa) play a very important role in the decomposition of detritus by being the first consuming trophic level and by making up more than 90% of the total belowground biomass. In this way, soil microbes are the main influencers of C and nitrogen (N) dynamics in soil. However, detailed information on the microbial processing of different types of organic substrates in soil food webs is still missing. Due to the important role of soil microbial communities in C and N cycling, this information is crucial to incorporate in soil food web models in order to study the long-term consequences of global change on ecosystem functioning. This is especially important if one wants to use this information for targeted management of soil life, which is seen as a promising management tool to target optimal soil functioning in anticipation of a changing world. The main research aim of this thesis was therefore to disentangle the soil microbial food web in relation to an important type of environmental change: land use change.

In **chapter 2**, I start with discussing how state-of-the-art empirical techniques can be used to collect trophic information that is needed to construct different types of empirically-based food webs: connectedness webs, semi-quantitative webs, energy flow webs and functional webs. I explain what types of information is needed from

molecular and biogeochemical studies to create such soil food web models. I thereby give a comprehensive overview of the available empirical techniques with respect to the type of information they can provide for soil food web models.

In **chapter 3**, I study litter-derived C flows through the soil microbial food web in six different ex-arable soils. In a 56-day incubation experiment, I compared the fate of litter-derived C flows through the soil microbial communities of recent and long-term abandoned soils. Soils were amended with ^{13}C-labelled plant litter and microbial C flows were studied by tracing the labelling of biomarkers in the form of Phospholipid Fatty Acids Stable Isotope Probing (PLFA-SIP). PLFA-SIP revealed that soil microbial communities are less efficient in decomposing litter-derived C in long-term compared to recently abandoned soils. The reduced efficiency of litter-derived C decomposition is most likely due to a net shift of organic matter-derived C to root-derived C input in relation to time since abandonment of agricultural practices. The study further revealed a clear succession of microbial decomposers, both in time and quantity that was similar across all examined fields: fungi > G^- bacteria > G^+ bacteria ≥ actinomycetes > micro-fauna. This information gives a first quantitative insight in how litter-derived C flows through the detritus-based soil microbial food web.

In **chapter 4**, I continue assessing C flows through the soil microbial community in more detail, by tracing the fate of three contrasting types of organic substrates. The same set of ex-arable soils as examined in chapter 3 were incubated for 28 days after the addition of a mixture of glycine, cellulose and vanillin. In each of the treatments one or none of these compounds was ^{13}C-labelled, to trace the fate of a specific organic compound. Application of both PLFA-SIP and RNA-SIP analyses allowed me to 1) quantify substrate-derived C flows through the soil microbial food web and 2) assess soil microbial resource partitioning beyond the concepts of the bacterial and fungal energy channels. The analyses revealed the emergence of a specific microbial community that deals with the decomposition of recalcitrant material in long-term abandoned soils. Furthermore, the existence of soil microbial decomposer succession was further confirmed by revealing both intra-kingdom microbial decomposer successional patterns and intra-kingdom microbial resource partitioning on the taxonomic level of fungal and bacterial classes. These results further enhance the view that the understanding of soil microbial decomposition goes beyond the concepts of bacterial and fungal energy channels.

In **chapter 5**, I assess the effects of contrasting types of organic matter inputs on microbial biomass, activity and community structure, as well as related ecosystem processes like N mineralization, microbial N immobilization, plant growth and nutrient uptake. In a pot experiment, Brussels sprouts were grown on arable soils that were mixed with ^{15}N-labelled mineral fertilizer and a contrasting type of organic amendments. The experiment revealed that a number of ecosystem processes were

directly related to soil microbial activity, while microbial N immobilization was mostly dependent on the soil microbial community structure. These outcomes support the idea that soil microbial community structure is important to take into account when assessing the effects of the soil organic inputs on soil ecosystem functioning and can be used to design nutrient management strategies for more sustainable agriculture.

In **chapter 6,** I study the drivers of both soil microbial community structure and function on two spatial scales (landscape and local scale). It is shown that these two soil microbial community characteristics are controlled by a distinct set of drivers at local versus landscape scale. I show that soil microbial community structure is driven on the landscape level by phosphorous related variables, whereas soil microbial functioning is driven locally through vegetation patterns. It is therefore important that management strategies consider the scale-dependent action of soil microbial community drivers and take both soil microbial community function and structure into account to target the desired biogeochemical functioning of soils.

Overall, this thesis gives the first high-resolution and quantitative image of detritus-based microbial food webs as affected by land use change and advances our understanding of soil food webs. Studying soil microbial food webs in a chronosequence of ex-arable fields revealed that a good understanding of soil microbial C flows, beyond the level of bacterial and fungal energy channels, is crucial to understand the effect of land-abandonment on the functioning of soil food webs. A thorough understanding of intra-kingdom variation in soil microbial C processing is therefore of vital importance to enhance our understanding of soil microbial functioning in response to global change, which is the key to success for targeted management of soil life in a changing world.

Samenvatting

Bodems zijn van cruciaal belang voor een groot aantal ecosysteemdiensten en spelen een belangrijke rol in biogeochemische kringlopen. Bodems zijn daarmee essentieel voor onder andere landbouw, koolstofopslag, waterzuivering en nutriëntenkringlopen. Deze bodemfuncties worden voor een groot deel gereguleerd door het bodemleven. Het bodemleven kan je visualiseren als een bodemvoedselweb: een model dat de voedselrelaties beschrijft tussen verschillende groepen organismen in de bodem. Een aantal van de door het bodemleven gereguleerde ecosysteemdiensten worden bedreigd door onder andere bodemdegradatie, atmosferische stikstofdepositie en veranderingen in landgebruik. Om te kunnen anticiperen op deze bedreigingen, en om ervoor te zorgen dat bodems zo optimaal mogelijk functioneren, is het van groot belang dat we goed begrijpen welke processen ten grondslag liggen aan deze bodemfuncties en hoe deze processen onderhevig zijn aan veranderingen in omgeving en klimaat.

Bodemvoedselwebmodellen hebben bewezen zeer bruikbaar te zijn bij het bepalen van de lange termijn gevolgen van klimaat- en omgevingsveranderingen op het bodemleven en de daaraan gerelateerde ecosysteemprocessen en -functies. Misschien wel het meest belangrijke ecosysteemproces dat wordt gereguleerd door het bodemleven is de afbraak van detritus: plantenresten en organische stof in de bodem. Met de afbraak van detritus reguleert het bodemleven de balans tussen het vastleggen en de mineralisatie van koolstof (C) en nutriënten. Dit heeft grote invloed op de koolstofdioxide (CO_2) emissies van de bodem naar de atmosfeer, maar ook de nutriëntenbeschikbaarheid voor planten. Bodemmicroben (bacteriën, schimmels en protozoa) spelen een belangrijke rol in de afbraak van detritus doordat ze het eerste consumerende trofische niveau zijn en omdat meer dan 90% van de totale ondergrondse biomassa uit microben bestaat. Op deze manier hebben microben van al het bodemleven de meeste invloed op de koolstof- en stikstofdynamiek. Gedetailleerde informatie over de microbiële afbraak van verschillende typen organisch materiaal is echter nog niet aanwezig in de huidige bodemvoedselwebmodellen. Door deze informatie op te nemen in bestaande modellen kan met grotere precisie bepaald worden wat de lange termijn gevolgen zijn van klimaat- en omgevingsveranderingen op bodemfuncties. Deze informatie is met name van belang om bodemleven doelgericht te kunnen beheren, iets wat wordt gezien als een veelbelovende beheerstrategie om optimaal bodemgebruik te bewerkstelligen. Het belangrijkste onderzoeksdoel van dit proefschrift is daarom het ontrafelen van het microbiële deel van het bodemvoedselweb in relatie tot veranderingen in landgebruik.

In **hoofdstuk 2** bediscussieer ik welk type empirische informatie uit moleculaire en biogeochemische onderzoeken kan worden gebruikt om bodemvoedselwebmodellen

te construeren op basis van empirische gegevens. Daarnaast geef ik een uitgebreid overzicht van de beschikbare empirische technieken in relatie tot het type informatie dat ze kunnen toevoegen aan voedselwebmodellen.

In **hoofdstuk 3** bestudeer ik de microbiële afbraak van strooisel in zes voormalige landbouwgronden, die onderdeel zijn van een chronosequentie. Gedurende een incubatie experiment van 56 dagen vergeleek ik de microbiële koolstofstromen in verschillende bodems. Strooisel (gelabeld met het stabiele isotoop ^{13}C) werd toegevoegd aan de bodems, waarna microbiële koolstofstromen in kaart werden gebracht door het traceren van de stabiele isotopen in microbiële fosfolipiden (PLFA-SIP). De resultaten lieten zien dat microbiële gemeenschappen in de bodems die al langere periode uit productie waren genomen, minder effectief waren in de afbraak van strooisel vergeleken met bodems die recent uit productie waren genomen. De afnemende efficiëntie van strooiselafbraak komt waarschijnlijk doordat velden die al langere tijd uit productie zijn, relatief meer koolstof krijgen via wortels dan via organische stof. Verder liet het experiment zien dat er een duidelijke successie is van microbiële groepen gedurende het decompositieproces, zowel in tijd als kwantiteit. Deze volgorde was gelijk over alle bestudeerde bodems: schimmels > G^- bacteriën > G^+ bacteriën ≥ actinomyceten > micro-fauna. Dit experiment geeft daarmee voor het eerst een kwantitatief inzicht in de microbiële koolstofstromen van het bodemvoedselweb.

In **hoofdstuk 4** bestudeer ik wederom de microbiële koolstofstromen, maar nu in nog meer detail door te kijken naar de afbraak van drie verschillende typen organische substraten. Dezelfde zes voormalige landbouwgronden als in hoofdstuk 3 kregen een mix toegediend van glycine, cellulose en vanilline en werden vervolgens 28 dagen geïncubeerd. In ieder type behandeling was slechts één van deze substraten gelabeld met ^{13}C, zodat de afbraak van één specifiek substraat kon worden bestudeerd. Door zowel PLFA-SIP als RNA-SIP toe te passen kon ik 1) microbiële koolstofstromen afkomstig van specifieke substraten kwantificeren en 2) kijken of er sprake was van microbiële specialisatie met betrekking tot de afbraak van specifieke substraten. De analyses lieten zien dat bodems die al langere tijd uit productie waren genomen een microbiële gemeenschap hadden die gespecialiseerd was is in de afbraak van recalcitrant materiaal. Daarnaast was er zowel binnen het schimmelrijk als binnen het bacteriële rijk sprake van successie van microbiële groepen gedurende het afbraakproces. Tevens was er sprake van microbiële afbraakspecialisatie, zowel binnen het schimmelrijk als het bacteriële rijk Deze resultaten versterken het idee dat microbiële afbraak van organisch materiaal op een gedetailleerder niveau plaats vind dan momenteel in bodemvoedselwebmodellen wordt gesuggereerd.

In **hoofdstuk 5** test ik het effect van vier contrasterende typen organische meststoffen op zowel de microbiële biomassa, activiteit en gemeenschapsstructuur, als ook de ecosysteemprocessen N mineralisatie, microbiële N immobilisatie, plantproductiviteit en nutriëntenopname door planten. In een potexperiment werd akkergrond gemixt met ^{15}N-gelabelde kunstmest en één van de vier typen organische

meststof. Daarop werd vervolgens spruitkool geplant. Het experiment liet zien dat een aantal ecosysteemprocessen direct afhankelijk waren van de microbiële activiteit in de bodem, terwijl N immobilisatie vooral afhankelijk was van de microbiële gemeenschapsstructuur. Deze resultaten bevestigen dat het belangrijk is rekening te houden met de structuur van de microbiële bodemgemeenschap om te kunnen bepalen wat het effect is van organische bemesting op ecosysteemfuncties. Dit type informatie kan belangrijke inzichten opleveren voor de ontwikkeling van duurzame bemestingsstrategieën.

In **hoofdstuk 6** bestudeer ik welke omgevingsvariabelen de structuur en het functioneren van de microbiële gemeenschap in de bodem beïnvloeden op zowel lokaal als landschapsniveau. Het onderzoek laat zien dat deze twee microbiële eigenschappen op lokaal en landschapsniveau worden gereguleerd door verschillende omgevingsvariabelen. De resultaten laten zien dat microbiële gemeenschapsstructuur vooral op landschapsniveau wordt bepaald door fosfor gerelateerde variabelen, terwijl functie met name lokaal wordt bepaald door vegetatiepatronen. Om bodems biogeochemisch gezien zo optimaal mogelijk te laten functioneren is het daarom belangrijk rekening te houden met de schaal waarop beheersmaatregelen plaats vinden, en de potentiele effecten van deze beheersmaatregelen op zowel microbiële gemeenschapsstructuur als functie.

Deze thesis brengt voor het eerst het microbiële bodemvoedselweb gedetailleerd en kwantitatief in beeld, waarbij ook is gekeken naar de gevolgen van veranderingen in landgebruik. Het bestuderen van microbiële bodemvoedselwebben in een chronosequentie van voormalige landbouwgronden laat zien dat een goed begrip van microbiële koolstofstromen essentieel is om de effecten van landgebruik op bodemvoedselwebben te doorgronden. Resultaten laten daarnaast zien dat het cruciaal is om meer inzicht te krijgen in de variatie aan koolstofstromen binnen het bacterie- en schimmelrijk, zodat we nog beter kunnen begrijpen hoe microbiële bodemgemeenschappen en bijbehorende functies zullen reageren op klimaat- en omgevingsveranderingen. Deze kennis zal uiteindelijk de sleutel zijn tot het succesvol en doelgericht beheren van bodemleven, zodat we maximaal kunnen anticiperen op de veranderende wereld om ons heen.

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About the author

Amber Heijboer was born on 4 September 1989 in Westdorpe in Zeeland, the Netherlands. She completed secondary school at Scholengroep Cambium in Zaltbommel in 2007 and afterwards she started a BSc study Biology at the Utrecht University. During her BSc studies she focussed on ecology and natural resource management and she obtained her BSc cum laude in 2010. Directly after her BSc study, she continued her education at the same university with the research master Environmental Biology. During her major research project she studied the effects of changed snow cover on the soil biotic processes in a mountain bog in the Swiss Jura, under the supervision of Dr Bjorn Robroek and Dr Luca Bragazza. During this research project she stayed for four months at the École Polytechnique Fédérale de Lausanne in Switzerland. This research project resulted in a publication in a peer-reviewed scientific journal. Her minor research project took place at the Free University in Amsterdam under the supervision of Prof. Rien Aerts and focussed on the feedback of warming-induced increased Sphagnum growth on soil respiration of given peat strata. She obtained her MSc diploma cum laude in 2012. In October 2012 Amber started her PhD research at the Wageningen University in the Mathematical en Statistical Methods group Biometris. The project was supervised by the promotors Prof. Peter de Ruiter and Prof. George Kowalchuk from Utrecht University. The supervision team was completed by Dr Jaap Bloem (Wageningen Environmental Research) and Dr Paul Bodelier (Netherlands Institute for Ecology). A considerable part of her PhD project was carried out within the Ecology and Biodiversity group at Utrecht University. The results of her PhD research are presented in this thesis. Since 2017 Amber works as science officer at the Institute for Biodiversity and Ecosystem Dynamics (IBED) at the University of Amsterdam and as PhD Programme Coordinator in the PE&RC graduate school.

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PE&RC Training & Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)

- Empirical methods of identifying and quantifying trophic interactions for constructing soil food web models (2016)

Post-graduate courses (8 ECTS)

- Soil ecology; PE&RC (2012)
- New frontiers in microbial ecology; RSEE PE&RC (2013)
- Molecular analysis of trophic interactions; Universität Innsbruck (2014)
- Structural equation modelling; PE&RC/WIMEK (2015)

Laboratory training and working visits (3.5 ECTS)

- Laboratory training PLFA analysis; Lund University, Sweden (2013)
- Laboratory training RNA-SIP; Soil Science Institute, Nanjing, China (2016)

Invited review of (unpublished) journal manuscript (2 ECTS)

- Applied Soil Ecology: soil microbial ecology (2016)
- Scientific Reports: soil microbial ecology (2016)

Competence strengthening / skills courses (2.6 ECTS)

- PhD competence assessment; WGS (2013)
- Communication with the media and the general public; WGS (2013)
- Entrepreneurship in and outside science; WGS (2014, 2015)
- Analytic storytelling; FNWI, UvA (2017)

PE&RC Annual meetings, seminars and the PE&RC weekend (3.15 ECTS)

- PE&RC Introduction weekend (2012)
- PE&RC Biodiversity symposium (2012)
- PE&RC Day: extreme life (2012)
- PE&RC Day: biomimicry, unlocking nature's secrets (2013)
- PE&RC Last year weekend (2016)

- PE&RC Day: one's waste.. another's treasure? (2015)
- PE&RC Day: PE&RC's got talent! (2016)
- PE&RC Day: preventing the end of the world (2017)

Discussion groups / local seminars / other scientific meetings (4.5 ECTS)

- Food web discussion group (2012-2014)
- Ecological Theory & Application discussion group (2012-2013)
- Institute for Environmental Biology PhD meetings; UU (2014-2016)
- Centre for Soil Ecology meeting (2012)
- Microbial communication (Micom) conference (2012)
- Current Themes in Ecology: soil, biodiversity and life (2012)
- Meeting of the KNPV working group Soilborne Pathogens and Soil Microbiology (2013)
- Netherlands Annual Ecology meeting (2014)
- Symposium Biodiversiteit Werkt (NWO) (2014, 2015)
- Wageningen PhD Symposium (2015)
- Institute for Environmental Biology (IEB) symposium (2016)
- Current Themes in Ecology: microbial power (2015)
- Current Themes in Ecology: ecological networks (2016)
- Final symposium Biodiversiteit Werkt (NWO) (2016)

International symposia, workshops and conferences (4.7 ECTS)

- Netherlands Annual Ecology Meeting (2013)
- Food webs: science for impacts; Rauischolzhausen, Germany (2013)
- Netherlands Annual Ecology Meeting (2015)
- Wageningen Soil Conference (2015)
- Netherlands Annual Ecology Meeting (2016)
- International workshop: soil food webs: linking structure, energy flux and function; Berlin, Germany (2016)
- Ecological Society of America (ESA); Fort Lauderdale, USA (2016)

Lecturing / Supervision of practicals / tutorials (14.7 ECTS)

- Living soil; WUR (2014, 2015, 2017)
- Ecology & natural resource management; UU (2014, 2016)
- Experiment & statistiek; UU (2015)
- Wiskunde M; WUR (2013, 2016)
- Wiskunde; WUR (2016)

Supervision of MSc students (6 ECTS)

- The effects of land use, spatial patterns and plant communities on soil microbial community structure
- Substrate quality-matrix quality interaction in a secondary succession chronosequence: only in early stage decomposition

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