Does diversity matter?

Soil microbial functioning and greenhouse gas fluxes in cover crop mixtures

Sytske M. Drost

Propositions

1. Cover crop mixtures stimulate microbial functional diversity.
   (this thesis)
2. Residue composition rather than diversity determines greenhouse gas emissions.
   (this thesis)
3. Validating results by repeating experiments in different settings is as important as novel research.
4. Meta-analyses increase scientific consensus and help in finding current knowledge gaps.
5. Allowing people to vote from the age of 12 will increase political awareness.
6. Current limited climate mitigation practices will lead to extinction of mankind.

Propositions belonging to the thesis, entitled:

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Sytske Maria Drost

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Thesis

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CHAPTER

General introduction

Problem description

Sustainable agricultural management is needed to benefit from soil-based life support functions for plant production while minimizing negative impacts on the environment. Already in the nineties, the importance of sustainable agriculture was discussed (Holmberg, Bass and Timberlake, 1991). Ecological processes, such as decomposition and carbon sequestration, are important life supporting functions and are strongly dependent on the composition, interactions and activity of soil biota. Intensive agriculture has a negative influence on soil biota as food web complexity decreases over time (Tsiafouli *et al.*, 2015). It is hypothesized that this will lead to a reduction of soil functioning. For instance, less efficient nutrient cycling by soil biota can result in increased leaching losses (Wagg *et al.*, 2014). Furthermore, intensification of agricultural land use management has led to reduced soil organic matter (SOM) (Poeplau *et al.*, 2011). SOM is important to stabilize the soil structure, increase microbial activity, act as reservoir of nutrients and increase water holding capacity (Johnston, Poulton and Coleman, 2009). Apart from adding organic residues like plant inputs to increase soil organic matter, it is becoming clear that an active microbial community can contribute significantly to build up stable SOM via microbial necromass (Kallenbach, Frey and Grandy, 2016; Angst *et al.*, 2021). In agriculture, there is a need for stabilizing or even increasing SOM and reduce nutrient losses by changing agricultural practices towards a more sustainable use of soil and land (Paustian *et al.*, 2016).

Why using cover crops?

The inclusion of cover crops in agricultural rotation is one of the options to enhance sustainability in agricultural systems (Bai *et al.*, 2019). Cover crops are beneficial by enhancing multiple soil health indicators like soil carbon content, aggregate stability and microbial diversity, which can be favorable for crop yield (Garland *et al.*, 2021; Wood and Bowman, 2021). Next to this, cover crops can (Blanco-Canqui *et al.*, 2015) suppress weeds (Smith, Atwood and Warren, 2014) and pathogens (Wang, McSorley and Kokalis-Burelle, 2006), increase SOM and labile C and N in the soil (Olson, Ebelhar and Lang, 2010; Zhou *et al.*, 2012; Strickland *et al.*, 2019), protect against erosion (Alonso-Ayuso, Gabriel and Quemada, 2014), stimulate nitrogen cycling and - fixation (Brozyna *et al.*, 2013; Bowen *et al.*, 2018) and stimulate soil biota including bacterivorous nematodes and mycorrhizal fungi (Boswell *et al.*, 1998; DuPont, Ferris and Van Horn, 2009; Nakamoto *et al.*, 2012). Planting of cover crop species with specific traits is a tool to achieve specific goals in agricultural management (Ramírez-García *et al.*, 2015). Cover crop traits include nutrient capture, associations with symbiotic microbes like nitrogen fixers, winter hardiness, rooting depth, maximum biomass and stoichiometric ratio (e.g. C:N ratio, lignin content). Grasses are for example more suitable as a catch crop as they are efficient in nutrient capture while legumes provide nitrogen to the soil via biological N-fixation (green manure).

In temperate climate zones, cover crops are sown after harvest of the main crop to reduce nutrient losses during the autumn and winter period in which the soil would otherwise be fallow (Kanders *et al.*, 2017). Nutrient capture and water retention by cover crops reduce leaching of nutrients to ground water. Before the main crop is sown, cover crops are incorporated in the soil, where they are decomposed by the soil microbial community. This will make the captured nutrients available for the following main crop (Figure 1).

Incorporating cover crop residues in the field can be used to increase C sequestration (Zhou *et al.*, 2012; Poeplau and Don, 2015; Bai *et al.*, 2019). Building up SOM is determined by the speed of decomposition of the incorporated residues which is influenced by the composition (amount of carbon and mineral nutrients) and abiotic conditions in the soil. In the Netherlands, the main motivation of farmers to use cover crops is to control plant-parasitic nematodes and reduce N-leaching after the main crop. So far, farmers are used to sow cover crop monocultures. The adoption of cover crop mixtures instead of monocultures can be interesting by increasing plant trait diversity.

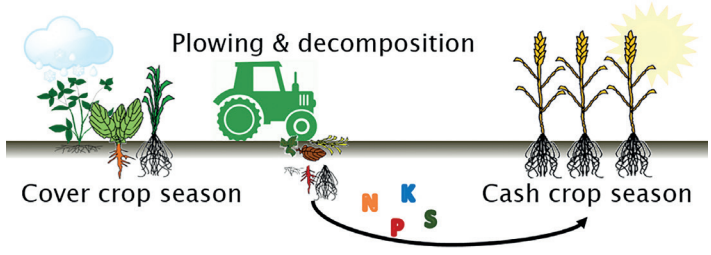

Figure 1. Cover crop management implemented in agricultural practice including cover crop growth, cover crop incorporation and decomposition, which is followed by a main crop.

Advantages of mixtures

It is hypothesized that combining plant species with different traits will have a positive effect on the soil. Complementary plant traits can have an effect on nutrient cycling, soil biota and plant productivity. Increased biomass production, yield stability and weed suppression in mixed plant communities compared to monocultures has been found in long term grassland experiments like the ones in Jena (Scherber *et al.*, 2010) and Cedar Creek (Tilman David *et al.*, 2001) as reviewed by Isbell *et al.* (2017). Plant species diversity effects have not only been studied in grasslands, but also in agriculture for intercropping or increased crop diversity in rotations (Bennett *et al.*, 2012; Cong *et al.*, 2014). Apart from increase in biomass yield, increased plant species diversity can enhance soil fertility (van Ruijven and Berendse, 2005; Fornara and Tilman, 2008) and soil organic matter (Steinbeiss *et al.*, 2008; Cong *et al.*, 2014). These positive plant-soil feedbacks by increasing plant diversity are attributed to increased exploration by roots of the soil profile, lower chances of pathogens to spread and increased amount of root exudates stimulating microbial nutrient cycling (Lange *et al.*, 2019; Thakur *et al.*, 2021).

Interestingly, also intercropping studies showed that organic matter content and aboveground biomass increases (Wang *et al.*, 2015) even in combinations without legumes (Cong *et al.*, 2014). Soil organic N increased twice as much as soil organic C independent of the two crop species used (Cong *et al.*, 2015a). Legumes in plant mixtures decrease the C:N ratio in the soil during crop growth. This leads to higher decomposition rates of plant residues (Cong *et al.*, 2015b, 2015c). Plant diversity on the one hand can increase SOM while on the other hand decomposition is increased as well. Increasing SOM is thought to be positive, however increased plant inputs by higher plant diversity stimulates microbial activity and decomposition (including priming) which will increase nutrient cycling and respiration (Janzen, 2006; Isbell *et al.*, 2017). The rate of decomposition of plant inputs (root exudates and residues) will determine accumulation of soil organic matter over the long term. Moreover, increase of microbial biomass can also contribute to increase of SOM as a large part of the stabile SOM is derived from microbial necromass (Angst *et al.*, 2021).

Plant traits are important for carbon inputs and carbon sequestration in the soil (De Deyn, Cornelissen and Bardgett, 2008). The functional characteristics of each plant species are different (Damour *et al.*, 2014; Tribouillois *et al.*, 2015). Plants differ in C:N:P ratio, lignin content, secondary metabolites, rooting architecture, ability to make symbiotic interactions with *Rhizobia* (legumes) or mycorrhizal fungi, resistance against pathogenic nematodes, etc. Furthermore, different plant species have different associations with the microbial community belowground (Berg *et al.*, 2016). These microbial associations can stimulate germination and plant growth, promote resistance and increase plant fitness (Mendes, Garbeva and Raaijmakers, 2013).

Cover crop mixtures can have comparable effects as seen for intercropping studies. This can lead to an increase in the belowground functional diversity, storage of C, N and P in the soil and higher primary production. Several studies showed that the use of a legume and a grass species mixture increased cover crop biomass production and SOM content due to biological N fixation by plant-symbiotic associations, increased incorporation of N in the plant material and reduced leaching (Nair and Ngouajio, 2012; Haque *et al.*, 2013; Tosti *et al.*, 2014). Furthermore, growing of cover crops can increase the abundance of arbuscular mycorrhizal fungi (AMF) in the soil (Lehman *et al.*, 2012). After incorporation of cover crops in the soil, part of the mineral nutrients will be released by soil microorganisms and become available for the main crop. This may reduce the need to use chemical fertilizers. In addition, increased abundance of arbuscular mycorrhizal fungi (AMF) can lead to increased main crop yields with possibilities to reduce artificial inputs even further (Lehman *et al.*, 2012). These examples illustrate that cover crops have a positive influence on the soil by affecting the activity and composition of the microbial community not only during cover crop growth, but also by having positive effects on the main crop (Lehman *et al.*, 2012; Nakamoto *et al.*, 2012; Mbuthia *et al.*, 2015; Alahmad *et al.*, 2019). Yet, more

information is needed to be able to understand the relationship between cover crop composition (mixtures *versus* monocultures) and soil functioning (Garland *et al.*, 2021).

Soil microbial community

Plants influence the soil microbial community. Each plant does select its own microbiome (Berg *et al.*, 2016) as plant traits like growth rate, N content and root exudates have an impact on the assembly of the community belowground (Sharma and Sharma, 2021; De Vries *et al.*, 2012b). Furthermore, increasing plant diversity will increase microbial biomass and respiration in the soil regardless of the ecosystem, however this effect is more pronounced with increasing plant stand age (perennial systems) (Chen *et al.*, 2019). This increased microbial biomass and diversity correlates with a higher diversity of root exudates in mixed plant communities (Steinauer, Chatzinotas and Eisenhauer, 2016b). High microbial biodiversity stimulates soil processes like decomposition and mineralization (Philippot *et al.*, 2013; Handa *et al.*, 2014).

Due to agricultural practices, the food web in the soil becomes less complex and less stable (Thiele-Bruhn *et al.*, 2012; Morriën *et al.*, 2017). This leads to a decrease of functional groups in the soil, for example mycorrhizal fungi (Wagg *et al.*, 2014) and can result in reduced carbon and nutrient use efficiency (Morriën *et al.*, 2017). Simplification of the soil community corresponded with a shift in ecosystem functioning (Wagg *et al.*, 2014; Delgado-Baquerizo *et al.*, 2016; Trivedi *et al.*, 2019; Bastida *et al.*, 2021). However, diversity loss does not per se lead to decline in functioning (Wertz *et al.*, 2006) as there is a high functional redundancy in soils (Wagg *et al.*, 2019; Delgado-Baquerizo *et al.*, 2020). It is hypothesized that communities with higher levels of soil biodiversity are more robust to changes (Wagg *et al.*, 2014). Furthermore, a reduction of the functional diversity and a shift from fungal dominated to bacterial dominated communities coincide with changes in nutrient cycling in the soil (Wardle *et al.*, 2004; De Vries and Bardgett, 2012). Stimulation of fungi in agricultural soils is beneficial as the presence of microbial communities with high fungal abundance often coincides with lower N losses via leaching and N_2O emissions (De Vries *et al.*, 2012b). Fungal biomass can be stimulated by the addition of carbon-rich organic residues in agricultural soils (Clocchiatti *et al.*, 2020). Including cover crops in agricultural fields can increase microbial biomass and modify microbial community structure (Muhammad *et al.*, 2021). This can lead to an increase in functional diversity. For example, cover crops can increase the amount of mycorrhizal fungi (Boswell *et al.*, 1998; Lehman *et al.*, 2012). This is correlated with increased growth and yield of the cash crop (Boswell *et al.*, 1998). Furthermore, individual cover crops may have different impacts on the microbial community. Mbuthia *et al.* (2015) found, for example, a relative high abundance of *Actinomycetes* under vetch cover crop. Vetch cover crops reduced the fungi:bacteria ratio in the soil. Leguminous cover crops increases soil N content by N fixation (Liu *et al.*, 2021). Increasing plant diversity can reduce soil available N during plant

growth via niche complementarity and increased root exploration of the soil profile (De Vries and Bardgett, 2012). After cover crop growth, residues of cover crops mixtures can enhance the functional diversity of the substrates added to the soil. This may stimulate a functionally more diverse microbial community.

Decomposition and nutrient cycling in the soil

Decomposition and mineralization are important processes for crop production to deliver nutrients to plants. The soil microbial community composition and the quality of the material affect decomposition and mineralization rates. Saprotrophic fungi are abundantly present in soils of (semi-)-natural ecosystems and play a major role in decomposition and nutrient cycling (van der Wal *et al.*, 2013). Fungal species are able to degrade complex organic substrates like lignin. Lignin can only be degraded by a few organisms including *Streptomyces*, brown-rot and white-rot fungi (Mester, Varela and Tien, 2004; Thevenot, Dignac and Rumpel, 2010). Arable soils are less favorable for saprotrophic fungi than forest soils due to low organic matter content and soil disturbance (Clocchiatti *et al.*, 2020). The addition of crop residues and organic material can stimulate the fungal community. The composition of the residues influence decomposition processes (Moorhead and Sinsabaugh, 2006; Hall *et al.*, 2020), as decomposition by microbes is dependent on the quality of the of organic matter such as the C:N:P ratio (Cornwell *et al.*, 2008). Easily available carbon and nutrients in residues determine the initial decomposition rate in the soil. Added mineral fertilizers have less effect on the microbial community than easily available C sources (Fanin *et al.*, 2016). Globally, the microbial biomass is strongly associated with the C content in the soil (Fierer *et al.*, 2009), but shifts in N and P availability over time have an impact on the composition of the microbial decomposer community (Fanin *et al.*, 2016). Growing plant mixtures or amending the soil with multiple litter qualities can increase litter decomposition (Handa *et al.*, 2014; Cong *et al.*, 2015c). This may be caused by an increase in microbial niches (Toljander *et al.*, 2006) and exchange of nutrients between litter types (Handa *et al.*, 2014). Microbial community interactions can influence degradation processes positively by mutualism, but also negatively by antagonism. Microbes can produce antagonistic compounds to suppress competitors and to protect occupied space (Hiscox and Boddy, 2016).

Microbes have specific niches and capabilities to degrade residues. Fungi have lower nutrient requirements compared to bacteria as fungi have higher nutrient use efficiencies (Strickland and Rousk 2010). Within the fungal phylum *Ascomycota,* the class *Chaetothyriomycetes* are related to resource-poor environments while *Leotiomycetes* and *Sordariomycetes* are related to resource-rich environments (Zechmeister-Boltenstern *et al.*, 2015). Within the bacteria, *Acidobacteria* and *Actinobacteria* are associated with lower quality resources while beta- and gamma-proteobacteria are more favored in soils with higher nutrient availability (Zechmeister-Boltenstern *et al.*, 2015). Fierer, Bradford

and Jackson (2007) showed that *Acidobacteria* are indeed dominant at sites with low C mineralization rates, while *Bacteroidetes* increased in abundance with increasing C mineralization rates. This shows that *Acidobacteria* are oligotrophs while *Bacteroidetes* show copiotrophic behavior. These studies indicate that microorganism groups can be divided in different life strategies, but within phyla, microorganisms are diverse (Ho, Di Lonardo and Bodelier, 2017a). Understanding decomposition, nutrient turnover and microbial composition and diversity in agricultural systems is important to estimate effects of addition of plant residues and organic amendments to make agriculture more sustainable.

Greenhouse gas fluxes

Management practices, physical and chemical soil properties, climate and crop species affect greenhouse gas (GHG) fluxes in agricultural fields (Ceschia *et al.*, 2010). During growth, cover crops withdraw nitrogen from the soil (less leaching) (Porre, 2020, chapter 2) but this will be returned when cover crops are incorporated in the soil during the preparation for cash crop cultivation. This can lead to increased CO_2 and N_2O emissions in arable fields compared to a fallow treatment (Abdalla *et al.*, 2012, 2014; Brozyna *et al.*, 2013; Sanz-Cobena *et al.*, 2014; Li *et al.*, 2016; Muhammad *et al.*, 2019). Abdalla *et al.*, (2014) found increased N_2O emissions after cover crop incorporation compared to no cover crop. N_2O can be produced during nitrification and denitrification. Some studies showed that denitrification is a main driver of N_2O emissions in agricultural fields after cover crop incorporation (Li *et al.*, 2016; Duan *et al.*, 2018), however, different soil texture or moisture conditions may lead to different conclusions (Wang *et al.*, 2021). Nitrogen retention and loss are correlated with the fungal:bacteria ratio and plant traits. Bacterial communities have higher N cycling rates compared to fungal dominated communities leading to increased N losses (Wardle *et al.*, 2004). Furthermore, cover crop residues can change the nutrient availability in the soil during decomposition. Legumes stimulate N mineralization as these residues have high nitrogen content (low C:N ratio) while grasses (high C:N ratio) immobilize N (Li *et al.*, 2016). Reduction of N availability by growing and incorporating cover crops with high C:N ratio has a positive effect of reducing N_2O emissions, which has a trade-off with yield (Perez-Alvarez *et al.*, 2013). Cover crop mixtures can reduce this trade-off by selecting combinations that increase the N content in the soil without reducing the N availability.

During crop growth, plants use CO_2 for photosynthesis, but root-microbial interactions via the production of root exudates can stimulate priming of SOM leading to increased CO_2 emissions (Bird, Herman and Firestone, 2011). After incorporation of the plant material in the soil, CO_2 emissions will be increased due to decomposition. Fresh litter inputs like cover crop residues increase soil microbial biomass (Barel *et al.*, 2019; Muhammad *et al.*, 2021). This can lead to increased decomposition of the SOM that was already present in

the soil leading to increased CO_2 emissions (Xiao *et al.*, 2015). The extent of this so-called priming of the resident SOM will determine if the SOM in the soil increases or decreases.

Methane consumption in soils can take up 10% of the global CH_4 sink (Saunois *et al.*, 2016). However, agricultural fields have lower methane consumption compared to abandoned fields or forests (Levine *et al.*, 2011). Still these soils are a possible sink for methane (Abdalla *et al.*, 2014). Microbial functional diversity can influence CH_4 cycling. Increased diversity of the methanotrophic community stimulates CH_4 consumption (Levine *et al.*, 2011; Bodelier *et al.*, 2013; Schnyder *et al.*, 2018). Furthermore, the application of organic residues like compost and cover crops in agricultural soils can stimulate CH_4 uptake (Sanz-Cobena *et al.*, 2014; Ho *et al.*, 2015a) which is linked to activity and growth of the methanotrophic community (Ho *et al.*, 2019, 2015a; Brenzinger *et al.*, 2021). Cover crops species slightly increased methane uptake in a long-term field experiment (Sanz-Cobena *et al.*, 2014), but knowledge on the effect of mixtures to enhance CH_4 uptake is lacking.

As shown by several studies, plant traits and management strategies influence the GHG fluxes in the soil. Accordingly, cover cropping can influence CO_2 and N_2O emissions and CH_4 uptake. It needs to be investigated if using cover crop mixtures can have a positive effect on the reduction of GHG emissions.

Aim of the thesis

The aim of this thesis is to compare the effects of cover crop mixtures and cover crop monocultures on composition and activity of the soil microbial community. To determine the possible effect of cover crop mixtures, the microbial community structure and functioning, nutrient cycling and GHG fluxes were measured over time both in pot and field experiments.

The hypothesis is that mixtures of cover crops species with different functional traits will increase the belowground microbial biomass and diversity by creating more niches in the soil. This will lead to increased microbial functional diversity resulting in more balanced element cycling processes. More specifically, I hypothesize that increased microbial biomass and – diversity will lead to an increased use of C and nutrients in the soil, thereby reducing GHG emissions and providing a more gradual delivery of nutrients to the cash crop (Figure 2).

This research is part of the Clever Cover Cropping project with two other PhD projects to capture a wide range of research questions related to cover crop mixtures. Ali Elhakeem focused in his PhD study on the productivity, resource capture, resource use efficiency and competitive relationships in cover crop mixtures (Elhakeem, 2021) while Rima Porre studied nitrogen and carbon cycling and decomposition of cover crop mixtures (Porre,

2020). Together, we setup a four-year field experiment to address these questions. This experiment is described in **Chapter 5**.

In **Chapter 2**, I evaluated in a controlled study if a mixture of cover crop residues increases microbial functional diversity and reduces GHG emissions compared to residues of cover crop monocultures. Cover crop residues, harvested right before incorporation in the soil, were mixed with soil in small pots and incubated for 8 weeks. I measured greenhouse gas fluxes, soil mineral N, microbial biomass and metabolic diversity with Biolog ECO plates. In **Chapter 3**, we compared various organic amendments (compost, digestate and sewage sludge) with cover crop residues. We used an incubation experiment to determine GHG dynamics, soil chemistry and microbial community structure. This was used to estimate the global warming potential (GWP) of the organic amendments and cover crop residues and to determine if microbial functional groups related to nutrient cycling and GHG consumption changed in abundance.

As microbial diversity is hypothesized to play an important role in decomposition and organic matter turnover, I evaluated in a meta-analysis (**Chapter 4**) if higher fungal diversity will increase decomposition rate. The focus on fungal species was made because saprotrophic fungi play a major role in decomposition and nutrient cycling. I collected papers studying diversity effect of litter decomposition (wood and leaf litter) in aquatic and terrestrial environments and both in lab incubations and field experiments.

In **Chapter 5**, I tested the effect of adding cover crop monocultures and mixtures to an agricultural rotation in a four-year field experiment. The effect of mixtures were compared to monocultures. I analyzed if mixtures can reduce GHG emissions and increase microbial biomass and diversity compared to monocultures. This was studied during cover crop growth, cover crop decomposition and main crop growth.

In the last chapter (**Chapter 6**), I synthesize the main findings of this thesis. I integrate the results, compare it with other research and suggest further research possibilities and the potential to use these results in current agricultural practice.

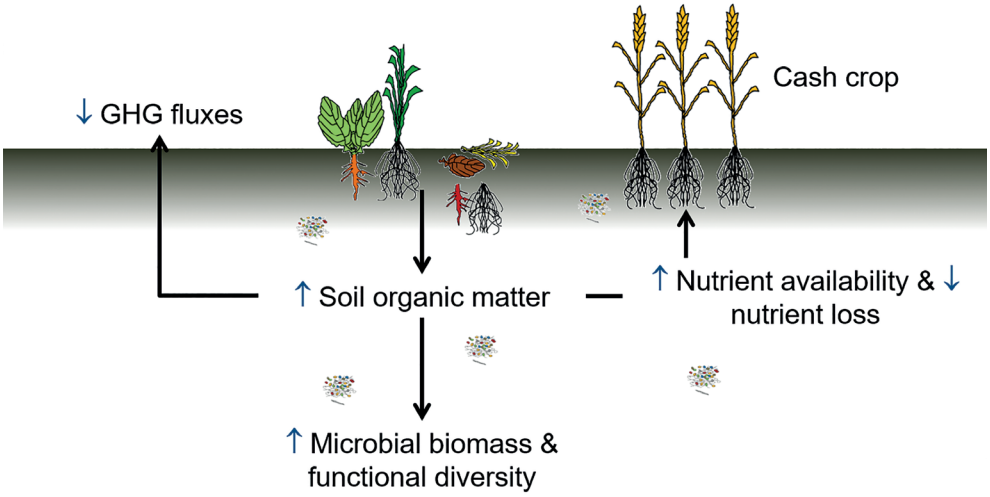

Figure 2. Hypotheses of the effect of cover crop mixtures compared to cover crop monocultures.

CHAPTER

2

Decomposition of mixtures of cover crop residues increases microbial functional diversity

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Abstract

To improve sustainability in agricultural systems, winter cover crops are increasingly replacing fallow to stimulate soil functions that reduce nutrient losses and greenhouse gas production, reduce pests for the next cash crops, increase soil organic matter pools and reduce erosion. Several of these functions are highly dependent on soil microbes decomposing cover crop residues. Since cover crop species differ in their traits it is hypothesized that plant species residue mixtures with complementary characteristics perform better by stimulating soil microbial functional diversity. To test this, residues of cover crop monocultures and mixtures were mixed with agricultural soil in a microcosm experiment, and fungal and bacterial biomass, microbial metabolic potential, greenhouse gas emissions and soil nutrients were measured during 50 days. Fungal biomass increased for all treatments compared to the control (no additions). However, there were no significant differences between cover crop mixtures and monocultures. Biolog ECO plates were used as a proxy for the metabolic potential of the microbial community. The number of substrates used was significantly higher in soil amended with residues of cover crop mixtures indicating an increased number of substrate niches for microbes. C:N ratio of cover crop residues was shown to be an important variable in explaining dynamics of CO_2 and N_2O emissions. Mixtures of cover crops showed reduced N_2O and CO_2 emissions compared to monocultures at the start of the experiment, but did not reduce greenhouse gas emissions over the whole incubation period. Adding nitrogen to the cover crop treatment with the highest C:N ratio (oat) did increase N_2O emissions, but not CO_2 emissions suggesting that decomposition rate of oat residues is not limited by nitrogen availability. Overall, mixtures of cover crops stimulated microbial functional diversity in soil incubations. Although this may have positive implications for soil quality and functioning in agricultural fields, further studies are needed to verify if these results hold under field conditions.

Keywords: microbial functional diversity, decomposition, cover crops, greenhouse gas emissions, sustainable agriculture

Introduction

Sustainable management of soils is essential to ensure balanced soil functioning with maintenance or increase of soil organic matter and minimal losses of inorganic nutrients, an idea already raised a few decades ago (Holmberg, Bass and Timberlake, 1991). Ecological processes such as decomposition are important for maintaining soil quality. These processes are modulated by soil biota, which are negatively affected by management in intensive agriculture (Tsiafouli *et al.*, 2015) such as predominant use of mineral fertilizers and monoculture cropping systems. This reduces the functional diversity in the soil (Tsiafouli *et al.*, 2015), which may affect plant performance. Creating more diverse cropping systems potentially leads to more sustainable agriculture as studies in (semi-)natural ecosystems have already shown that plant diversity improves soil microbial diversity leading to enhanced ecosystem functioning (Wardle *et al.*, 2004; Fierer *et al.*, 2009; Wagg *et al.*, 2014; Lange *et al.*, 2015). Additionally, it is known that high microbial diversity is important for maintaining multi-functionality in ecosystems (Eisenhauer, Reich and Isbell, 2012; Wagg *et al.*, 2014; Delgado-Baquerizo *et al.*, 2016). By stimulating the microbial diversity, the aim is to increase functional diversity belowground such as the ability to metabolize a wide spectrum of organic compounds (metabolic potential). Diversification of organic matter inputs into agricultural systems, e.g. use of cover crop mixtures, is expected to increase functional diversity belowground (Hattenschwiler, Tiunov and Scheu, 2005; Baumann *et al.*, 2009). This can have positive effects on nutrient availability (Olson, Ebelhar and Lang, 2010; Zhou *et al.*, 2012) and suppression of pathogens (Wang, McSorley and Kokalis-Burelle, 2006).

Cover crops grow during autumn and winter when the fields are usually fallow. During fallow, fields are more vulnerable to leaching of nutrients to groundwater. Cover crops can be harvested or are incorporated into the soil as green manure before the cash crops are introduced. This coincides with changes in the abundance and composition of microbes (Baumann *et al.*, 2009; Shi and Marschner, 2014; Tao *et al.*, 2017), being the biological catalysers of degradation. The compositional changes are related to the different substrates added to the soil (Baumann *et al.*, 2009). However, the effects on functioning of the increased microbes are less clear.

Soil microbial functioning depends strongly on substrate quality and quantity (Zechmeister-Boltenstern *et al.*, 2015; Mooshammer *et al.*, 2014a). Cover crops are usually fast growing species with a C:N ratio varying from 8 to 30, delivering residues with an initial stoichiometry that deviates from that of the microbial biomass as the C:N ratio of the soil microbial biomass is on average 7 (Xu, Thornton and Post, 2013). Mixtures with a wide range of plant species with deviating C:N ratios provide a range of substrates to the soil system, which may increase the niche breadth of the microbial community leading

to an increased metabolic potential. For example, soil amended with residues composed of mixtures of plants with a wide range of chemical qualities resulted in additive effects for C and N mineralization (Redin *et al.*, 2014). Besides C and N, other nutrients like P, S and K can be provided by cover crop residues and these nutrients are important for microbial growth and activity (Tao *et al.*, 2017). Furthermore, potassium concentrations are positively correlated with soil microbial biomass (Nair and Ngouajio, 2012). Hence, well-selected plant species mixtures may produce residues with a broader nutrient and stoichiometric spectrum thereby creating conditions facilitating the establishment of a diverse community with increased metabolic potential and higher microbial biomass compared to the use of monocultures (Nicolardot *et al.*, 2007; Baumann *et al.*, 2009, 2011).

Besides nutrients released from plant residues, turnover of microbial biomass formed during degradation of cover crop residues, may contribute to nutrient supply for the main crop following cover crop residue decomposition. However, sparse information is available considering nutrient release from microbial biomass in soils. It has been demonstrated that microbial products released after decomposition are the main precursors of SOM formation (Cotrufo *et al.*, 2013, 2015; Kallenbach, Frey and Grandy, 2016). Increases of SOM may be beneficial for plant growth by increasing the soil absorption complex and, therefore, nutrient retention (Lal, 2004; Lehmann and Kleber, 2015). SOM will only increase if the amount of carbon incorporation is higher than the carbon loss via respiration.

Both litter quality (e.g. C:N ratio) and agricultural practices (tillage) influence greenhouse gas emissions after cover crop incorporation (Petersen *et al.*, 2011; Abdalla *et al.*, 2014; McCourty, Gyawali and Stewart, 2018). Cover crop monocultures can have positive as well as negative effects on greenhouse gas emissions depending on the quality of the litter (Basche *et al.*, 2014). It is expected that the microbial community will be more efficient in decomposing residue mixtures compared to monocultures (Bardgett and Shine, 1999; Loreau, 2001). The effects of mixtures of cover crops, on GHG emissions have still to be investigated.

In this study, we executed a pot experiment in which residues of cover crop monocultures and - mixtures were added to a sandy, intensively managed agricultural soil with the aim to test the following hypotheses:

- Microbial biomass is positively affected by mixtures of cover crop residues due to increased substrate diversity.
- Mixtures of residues lead to a higher microbial functional diversity by increasing the niche breadth of the microbial community.
- Residue mixtures reduce greenhouse gas emissions due to increased nutrient use efficiency.

Materials and methods

Plant material and soil samples

Cover crop material was harvested in February 2017 from plots created and managed by Joordens Zaden company in an arable field in Neer, the Netherlands (51°18'24.7"N 6°05'08.8"E). Harvest was one week before the cover crops were ploughed in. Above- and belowground plant material of fifteen species was harvested from five different plant families (*Avena strigosa*, *Vicia sativa*, *Raphanus sativus*, *Guizotia abyssinica*, *Phacelia tanacetifolia*, *Sinapsis alba*, *Camelina sativa*, *Brassica carinata*, *Brassica napus*, *Lens culinaris*, *Medicago sativa*, *Pisum sativum*, *Trifolium alexantrinum*, *Secale cereale* and *Fagopyrum esculentum*). These fifteen species represent commonly used cover crops in the Netherlands. All cover crops were harvested from monoculture fields. Roots were collected simultaneously with the shoots by first making the soil loose with a shovel before pulling the complete plants out of the soil. The cover crops were dried at 40°C, cut in pieces of maximally 1 cm and stored at room temperature. Both above- and belowground plant material was used in the experiment (in ratio present at harvest and mixed during processing of the samples). Elemental composition (carbon (C), nitrogen (N), phosphorus (P), potassium (K) and sulfur (S)) was analyzed. C and N was analyzed by grinding 1 mg of plant material, transferred to tin cups and analyzed with an element analyzer (Flash EA 1112, Thermo Scientific). K, P and S were measured from 20 mg of grinded plant material with microwave-assisted digestion with nitric acid and hydrogen peroxide (Hansen *et al.*, 2009, 2013) and measured on an ICP-OES (iCAP 6500 DUO, Thermo Scientific).

Soil was collected in April 2017 from a conventionally managed agricultural field in Wageningen, the Netherlands (51°59'44.6"N 5°39'34.9"E). The soil type is loamy sand (83% sand, 12% silt and 2% clay). This is a highly disturbed arenic gleysol. Soil pH was 5.3 and soil organic matter content 3.1%. Prior to soil collection, the fields were from late summer after harvest of the cash crop (wheat). Fallow fields were used to ensure that the soil had no legacy of cover crops at the start of the experiment. The top 20cm of the soil was collected, as this is the plowing depth of the field. Prior to the experiment, the soil was sieved through a 4 mm sieve to remove stones. The sieved soil was stored at 4°C before the start of the experiment for maximally 1 month.

Experimental setup

250 gram of moist soil was mixed with 0.5 gram of plant material of different combinations, cut in pieces of 1 cm in length, and added in small plastic pots made from HDPE (6.5 cm in diameter and 14.5 cm height). The pots were loosely packed with a bulk density of 0.92 g/cm^3. The amount of added plant material is comparable with 5 ton per ha on an agricultural field scale, which is the common yield of cover crops in agriculture in the Netherlands. The treatments consisted of residues of monocultures of *Avena strigosa* (oat,

A), *Vicia sativa* (vetch, V) and *Raphanus sativus* (radish, R), a three species mixture of vetch, radish and oat (VRA) and a 15 species mixture (15sp) containing all harvested cover crops. The plant material in the mixtures was equally divided among the species in the mixture (0.17 gram plant material per species in the 3 species mixture and 0.03 gram plant material per species in the 15 species mixture). The three monocultures and three species mixture were chosen as these three species are commonly used as cover crops, representing three different plant families. The 15 species mixture was used to create a highly diverse mixture of plant traits. The nutrient content of plant material in the different treatments is shown in Table 1. To identify if the microbial decomposing activity is nitrogen limited, a treatment with added mineral nitrogen was used in the monoculture with the highest C:N ratio (oat) (A+N). In this treatment, 37 mg NH_4NO_3 was added to reduce the C:N ratio to approximately 10. As control (C), pots without added plant material were used.

The pots were incubated in the dark at 20°C for 50 days. The pots were watered twice a week to keep the water content of the soil at 60% of the water holding capacity (16.6 g water per 100 g of dry soil).

Soil samples were taken at 1, 3, 7, 12 and 50 days after the start of the experiment. At the start (T0), a soil sample was taken before mixing plant residues with the soil. The subsamples were taken destructively to prevent disturbance of the pots. Soil samples were taken after mixing the soil in pots to obtain a homogeneous sample.

Table 1: Nutrient content of the added plant material.

	N ($g*kg^{-1}$)	**C** ($g*kg^{-1}$)	**C:N**	**K** ($g*kg^{-1}$)	**P** ($g*kg^{-1}$)	**S** ($g*kg^{-1}$)
***V. sativa* (V)**	39.7	327.6	8.26	21.23	5.87	3.21
***R. sativus* (R)**	24.5	382.7	15.64	12.17	5.49	3.14
***A. strigosa* (A)**	12.0	373.0	31.16	2.79	1.91	1.16
3 species (VRA)	25.4	361.1	14.23	12.06	4.42	2.51
15 species (15sp)	18.1	364.6	20.16	9.56	3.31	2.32

Microbial biomass

Fungal biomass was measured in one gram of soil for all sampling time points using ergosterol as a proxy following the Bååth alkaline ergosterol extraction method (Bååth, 2001; De Ridder-Duine *et al.*, 2006). The ergosterol concentration was measured using LC-MSMS (6460 Triple Quad LC-MS, Agilent). Ergosterol was used to measure fungal biomass as this is a good method to estimate the saprotrophic fungi in the soil (Joergensen and Wichern, 2008). Bacterial biomass was measured using qPCR. DNA was extracted using DNeasy Powersoil Pro Kit (Qiagen, Venlo, The Netherlands) with 0.25 gram of soil according to the instructions provided by the supplier. The quantity and quality of the extracted

DNA was analyzed with a spectrophotometer (Nanodrop 2000, Thermo Scientific). qPCR assays were used to target the 16S rRNA gene for bacteria. Each assay was performed with 2 ng*µL^{-1} of DNA in duplicates for each DNA extract with the primer pair EUB338 and EUB518 (Fierer *et al.*, 2005). Each qPCR (total volume of 15 µL) consisted of 7.5 µL 29 SensiFAST SYBR (BIOLINE), 0.75 µL of forward and reverse primes each, 1.5 µL of bovine serum albumin (5 mg*mL^{-1}; Invitrogen), 1.5 µL DNase- and RNase-free water and 3 µL of the template DNA. Standard curves were obtained using 10-fold dilution series of a known amount of plasmid DNA from isolates of *Collimonas* (10^8 to 10^1 gene copies) containing the 16S rRNA fragment. The qPCR was performed with a C1000 Touch Thermal Cycler (CFX-real-Time system, Bio-Rad Laboratories, Inc.). As negative control, water was used instead of template DNA. Efficiencies for all assays were between 80.3 and 112.1 % with r^2 values between 0.951 and 0.989.

Bacterial biomass C was calculated by using the conversion factor 4.1 for 16s rRNA gene copy numbers for bacteria (Santelli *et al.*, 2008) and bacterial biomass estimates as described by (Bloem, Veninga and Shepherd, 1995). The ergosterol data were converted to fungal biomass C using the conversion factor 5.4 mg ergosterol*g biomass C^{-1} (Klamer and Bååth, 2004). Both calculations were used to estimate the total microbial biomass C (MBC) in the soil and the fungi:bacteria ratio.

Microbial functional diversity with Biolog ECO plates

Biolog ECO plates (Biolog Inc., Hayward, CA) were used to determine the ability of the microbial community to grow on different substrates (metabolic potential). This is used as a proxy for microbial functional diversity and niche breadth of the community (Mallon *et al.*, 2018). Biolog ECO plates contain 31 different C-substrates. To prepare Biolog ECO plates, microbial communities were extracted from 1 gram of soil with 10 ml TrisBis buffer as described by Rutgers *et al.* (2016). The soil samples were taken at 12 and 50 days after the start of the experiment. Three dilutions (factor 8) from the microbial suspension were prepared based on a test sample to identify the transition point between low and high well color development to be able to distinguish the differences between the treatments. The ECO plates were incubated at 20°C in the dark under humid conditions. The absorbance at 592 nm (purple) and at 750 nm (turbidity) was measured at 2h, 4h and 3, 5 and 7 days after plate preparation in a plate reader (Spectramax 250, Molecular Devices Corp., Memlo Park, CA). If the microbial community was able to grow on the substrate, absorbance 592 nm – 750 nm of the substrate was higher than the blank (water) with a threshold of 0.1, this was scored as "1". If there was no purple color development (no growth), this was scored with "0".

Nutrient analyses

At two time points (12 and 50 days), plant available soil nutrient concentrations (N, P, K and S) were measured. 50 mL 1 M KCl was added to 10 g of soil to extract NO_3 and NH_4. Clear supernatant was stored in the freezer before analysis on an AutoAnalyzer (SEAL QuAAtro SFA system, Beun- de Ronde B.V. Abcoude, The Netherlands).

P, K and S were determined after extraction of 3 g soil added to 30.0 mL 0.01M calcium chloride solution. This extraction procedure is used as it is considered as a proxy for plant available nutrients in the soil solution (Menzies, Donn and Kopittke, 2007). After 2 hours of shaking, the sample was centrifuged at 3000 rpm and the supernatant was filtered through a Whatman Puradisc Aqua 30 filter with CA membrane. 130 µL HNO_3 was added to 12.87 ml filtrate and stored for maximal one day at 4°C before analysis with an ICP-OES (iCAP 6500 DUO, Thermo Scientific).

Greenhouse gas measurements

Greenhouse gas (GHG) fluxes were measured daily up to 9 days. From day 9 on, measurement frequency was decreased to once a week until the end of the experiment. Thus, measurements were done at days: 1-9, 11, 12, 14, 16, 18, 21, 24, 28, 35, 42 and 50. CO_2 and N_2O fluxes were measured over an hour by closing the pots and taking an air sample at 0 and 60 min after closing. 20 mL of air was taken from the pots of which 14 ml was flushed in a 6 mL exetainer (Labco, UK) and the last 6 mL was used to create an overpressure of 1 bar. The exetainers were analyzed using an autosampler (TriPlus RSH, Thermo Fisher Scientific, Bleiswijk, The Netherlands) connected to a gas chromatograph (GC1300, Thermo Fisher Scientific) equipped with a Methanizer and a Flame Ionization Detector (FID) to detect CO_2 and CH_4 an electron capture detector (ECD) for detection of N_2O. The gas chromatograph contained two sets of a pair Rt-Q-Bond capillary columns (L; 15m and 30m, ID; 0.53mm, Restek, Interscience, Breda, The Netherlands). Chromeleon™ Chromatography Data System 7.1 (CDS, Thermo Fisher Scientific) software was used to analyze the obtained gas chromatograms from the GC. Fluxes of CO_2 and N_2O were calculated on basis of the accumulation during 1 hour. Gas concentrations were calculated in ppm values by comparing with calibration curves, which were generated by dilution of a certified gas mixture (1 ppm N_2O, 2 ppm CH_4 and 2000 ppm CO_2; Linde Gas, The Netherlands). The concentrations (ppm) were converted into absolute amounts (mmol) with the gas law: $pV = nRT$ in which p is the pressure in the pot, V is the volume, n is the amount gas in mol, R is the gas constant (8.31 $J{\cdot}K^{-1}mol^{-1}$) and T is the temperature. Cumulative fluxes of the GHG emissions were calculated by assuming that the time between the samples was similar to the average of both samples:

$$\text{Cumulative flux } (\Phi) = \Phi_{T_1} + T_{j-i} * \left(0.5 * \Phi_{T_i} + 0.5 * \Phi_{T_j}\right) + \cdots$$

where Φ is the flux at time point i and T is the time between the different time points (i and j).

Data analysis

The data were analyzed with R (version 3.5.1) with attached packages: agricolae, car and plyr for statistical analysis; ggplot2, grid and gridExtra for making figures; dplyr, survival, Formula and Hmisc for making correlation tables; and klaR and MASS for RDA analysis. First, the data were tested for normality and for homogeneity of variances. If this was not the case, Tukey's ladder of powers transformation in the package Rcompanion was used to meet the criteria for parametric statistical testing as log transformation was not sufficient to make the data normally distributed. The data were analyzed for significant treatment effects using a linear model and Anova. A post hoc Tukey test was used to identify the significant differences between the treatments. The greenhouse gas emissions data over time were not normally distributed and were analyzed using Kruskal-Wallis rank sum test combined with Scheffe test to determine treatment and time effects. Correlations were calculated between the GHG and nutrient content in the plant residues and in the soil.

The Biolog data were analyzed in R and Canoco (version 5). RDA analysis in Canoco was used to find the dissimilarity between the treatments. To determine if microbial biomass was driving differences in well color development, these parameters were added in the RDA analysis as environmental variables. All environmental variables were included in the analysis for visualization of the effecs. 500 permutations were used. Correlations were tested between the environmental variables.

Comparison of the effect of mixing of cover crops was done for the three species mixture. First, the expected values for contribution by the 3 species were determined by calculating the sum of all individual monocultures for a certain trait divided by the amount of species in the three species mixture. Next, it was tested if the values for the traits in the three species mixture were significantly different from the expected values. Results were statistical significant when the p-value was smaller than 0.05.

Results

Microbial biomass

Addition of plant material stimulated growth of fungi as indicated by increased soil ergosterol contents (Figure 1). The fungal biomass was not significantly different between the different residue treatments, but was higher than the control treatment (C) without added plant residues ($p < 0.001$). There was no significant difference between the different time points (Table S1, $p = 0.42$). Residue mixtures (VRA and 15sp) did not have an additive effect over monocultures, but mixtures showed a more gradual increase of ergosterol in the soil compared to the monocultures. Monocultures led to a fungal biomass peak at different time points, for example: vetch (V) at 3 days and oat (A) at 12 days.

Bacterial biomass (based on 16S rRNA gene copy numbers) was significantly different between cover crops additions three days after cover crop incorporation (Figure 2, $p < 0.001$). At this time point, radish (R), vetch alone and the three species mixture (VRA) led to an increase of the bacterial biomass compared to the control. Overall vetch, radish and both mixtures led to an increase of bacteria during the experiment compared to the other treatments, which disappeared after 50 days of incubation.

Dynamics of bacterial and fungal biomass development differed between treatments. Vetch led to an early increase of both fungal and bacterial biomass (at three days) while the oat residue led to an increase at a later time point (12 days). Mixtures had a more gradual increase and decrease and not a clear peak at one of the measured time points.

Microbial biomass C (MBC) was calculated to estimate the total amount of microbial biomass in the soil. The MBC ranged between 0.45 to 28 mg C per kg soil (Table S3). The highest values for radish monoculture and the three species mixture were mainly driven by high bacterial abundance in the samples compared to the other treatments. The F:B ratio increased during decomposition, after 50 days, this increase disappeared (Table S3).

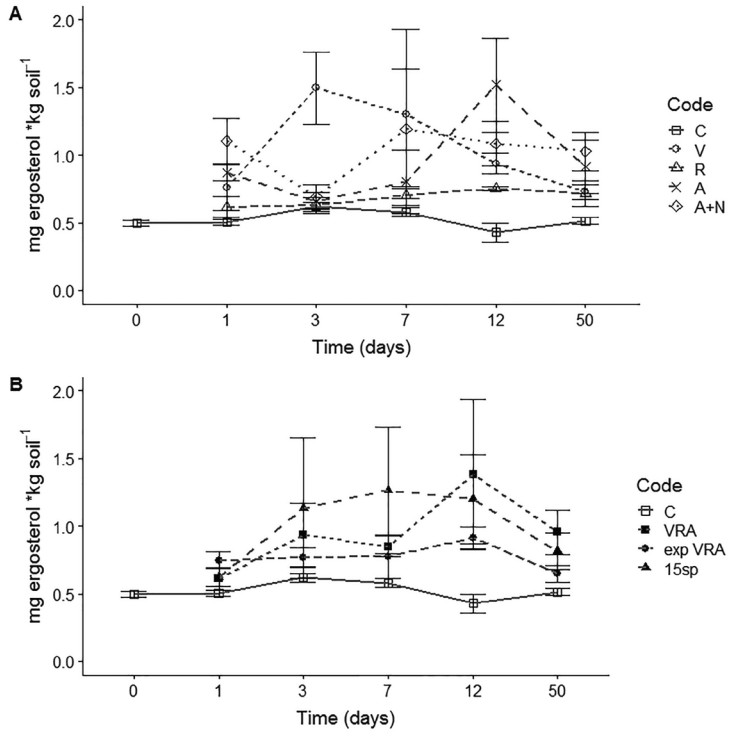

Figure 1: Fungal biomass (mg ergosterol *kg soil^{-1}) over time (mean ± SE; n = 4). **A:** control, monocultures V, R, A and A+N; **B:** control, mixtures VRA and 15sp. expVRA is the average of the three monocultures (V, R and A). The results of the statistical test are shown in Table S1.

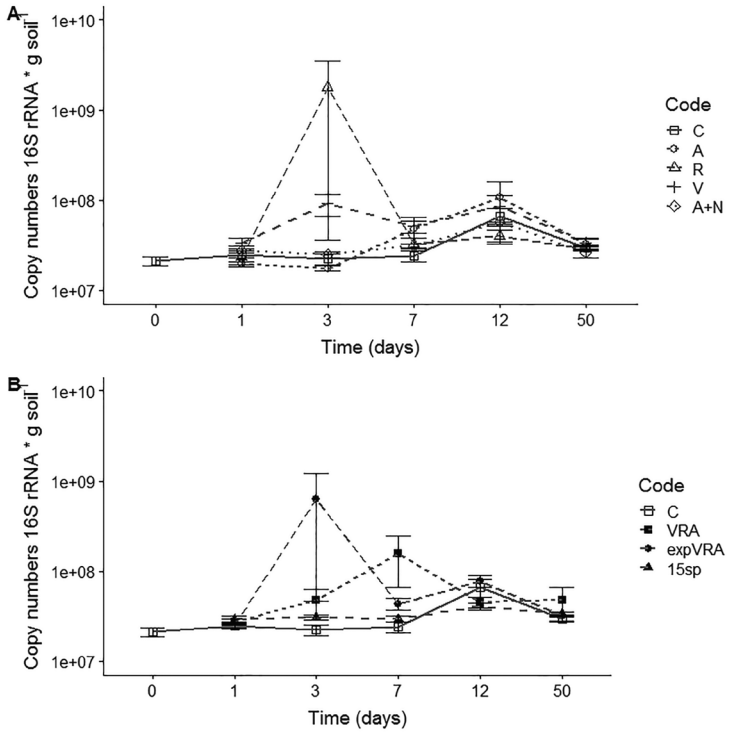

Figure 2: Bacterial biomass (based on copy numbers of the 16S rRNA gene with qPCR) over time (mean ± SE; n = 4). **A:** control, monocultures V, R, A and A+N; **B:** control, mixtures VRA and 15sp. expVRA is the average of the three monocultures (V, R and A). The results of the statistical test are shown in Table S2.

Microbial functional diversity

Microbial metabolic potential, as proxy for functional diversity, was measured with Biolog-ECO plates. Microbes in soil suspensions obtained from mixed residue additions showed a significant increase in substrate use compared to those obtained from monocultures at both time points (Figure 3 and Figure S1, $p = 0.0016$), except for vetch residue. For radish and oat (with and without added N), there was no significant difference compared to the control. Recalcitrant substrates like D-cellobiose (G1) and glycogen (F1) could be used at low concentrations by microbes derived from the mixtures of residues, while microbes from monocultures only degraded these substrates at the highest inoculum density. These differences between substrate use are shown in a RDA analysis (Figure 4). Radish, oat and oat with N grouped together, while 15sp and VRA are significantly different from vetch (V). Both mixtures could use several substrates including polymers and carboxylic acids while microbes from vetch-amended soil used more amino acids. Microbial biomass had no significant effect on substrate use in the Biolog plate ($p = 0.44$). Only C, 15sp and V explained a significant part of the variation observed in the redundancy analysis using forward selection (Table S8, adjusted P-values are lower than 0.05).

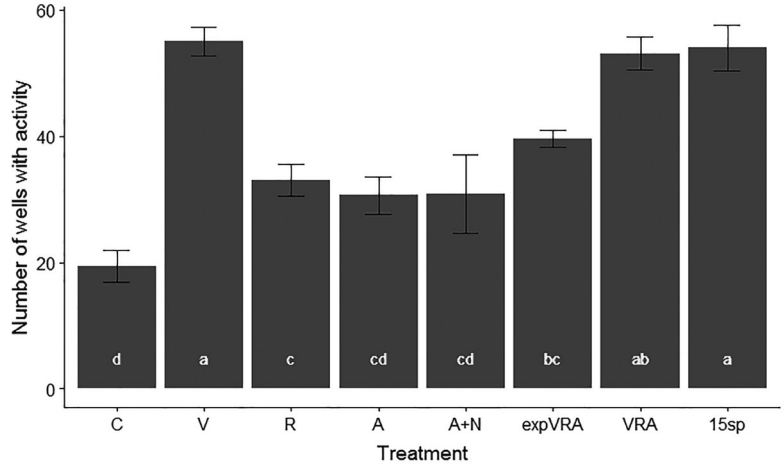

Figure 3: Microbial functional diversity: number of wells that showed a positive response in Biolog ECO plates after incubation of 7 days (mean ± SE; n = 4). As the treatments are not significantly different over time (p = 0.59), the average of both time points (T12 and T50) is shown here (complete graph is Figure S1). The different letters indicate significant differences between treatments. The results of the statistical test are shown in Table S6.

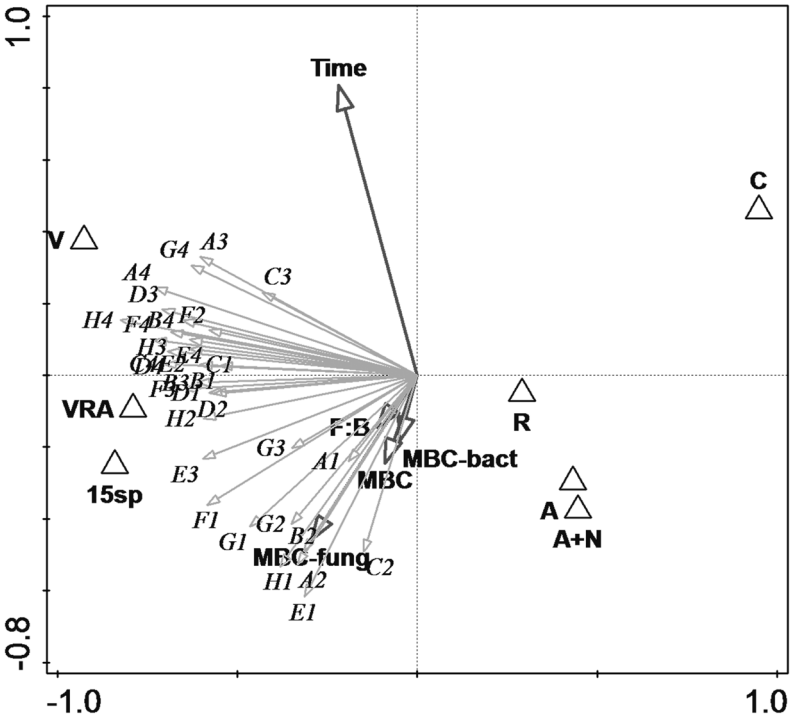

Figure 4: RDA (Redundancy Analyses) ordination plot based on positive/negative (1/0) results of the data from the Biolog ECO 96 well plates after 7 days of incubation. The letters indicate the different substrates and are listed in Table S9. Time was significantly related to the results of the Biolog ECO plates (P = 0.004), microbial biomass was not significantly correlated with the results. The results of the RDA are shown in Table S7 and Table S8. Correlations between the environmental variables are shown in Table S10.

Greenhouse gas fluxes

Cover crop addition to the soil showed an immediate response of greenhouse gas emissions. For all treatments, the first measurement at day 1 showed the highest GHG emissions. Vetch-amended soil showed the highest peak for both CO_2 and N_2O at the start followed by reduction that was faster than for the other residue treatments (Figure 5).

Cumulative fluxes showed that after 50 days, vetch decomposition resulted in the highest N_2O emissions while oat (with the highest C:N ratio) had the lowest (Figure S2B). Based on the average greenhouse gas emissions of the three monoculture treatments, expected values for the three species mixture were calculated. The N_2O emissions of the three species mixture was not significantly different from the expected N_2O emissions. Cumulative N_2O fluxes were negatively correlated with the C:N ratio of the initial plant material (Figure S4, $r = -0.57$, $p = 0.0014$), plant and soil N content correlated positively with N_2O emissions ($r = -0.67$, $p < 0.001$ and $r = -0.61$, $p < 0.001$ respectively).

CO_2 emissions showed a similar trend as N_2O (Figure 5A). For vetch- and radish amendments there was a decrease during prolonged incubation, while CO_2 fluxes for oat and oat with N were nearly constant over the experimental period. Cumulative CO_2 emissions did not correlate with C:N ratio of the residues (r -0.16, $p = 0.40$), but did correlate negatively with C content ($r = -0.37$, $p = 0.049$), C:P ($r = -0.66$, $p < 0.001$) and N:P ratio ($r = -0.57$, $p = 0.0016$). Interestingly, CO_2 emissions did correlate positively with initial plant P ($r = 0.73$, $p < 0.001$), K ($r = 0.71$, $p < 0.001$) and S ($r = 0.70$, $p < 0.001$) content and soil K ($r = 0.71$, $p < 0.001$) and S ($r = 0.65$, $p < 0.001$) content after 50 days (Figure S4).

The total global warming potential (GWP) was calculated based on the global warming potential for each separate gas from the cumulative fluxes over 50 days of incubation (CO_2 = 1 and N_2O = 265). Overall, cumulative greenhouse gas fluxes showed that amendment with vetch and radish only resulted in the highest greenhouse gas emissions after 50 days (Figure 6).

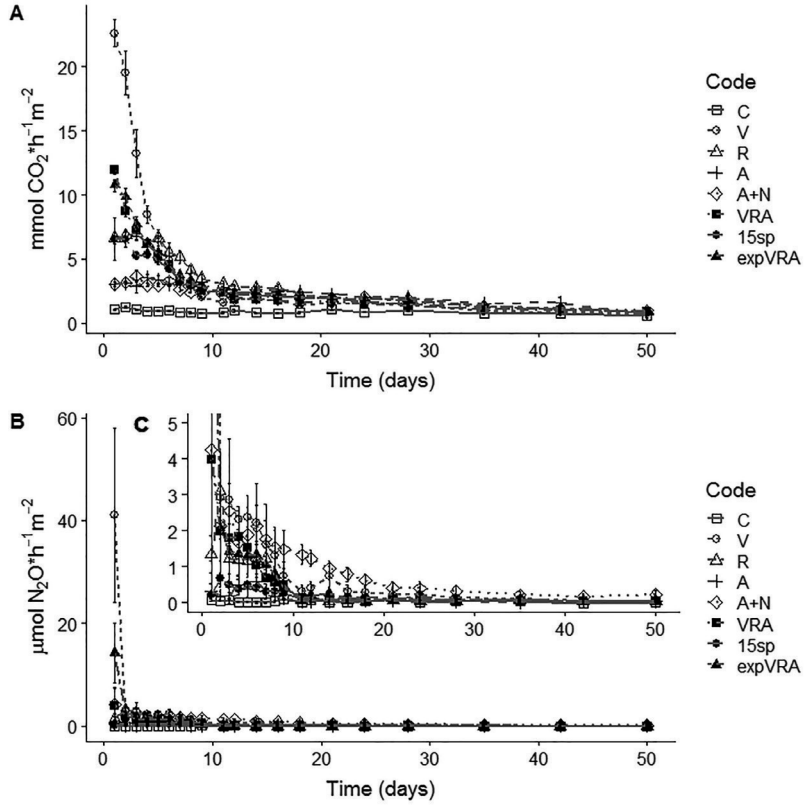

Figure 5: Greenhouse gas fluxes over time (days) (mean ± SE; n = 4). **A:** mmol CO_2 per hour per m^2; **B:** µmol N_2O per hour per m^2; **C:** close-up of N_2O graph. The results of the statistical test are shown in Table S11 and Table S12.

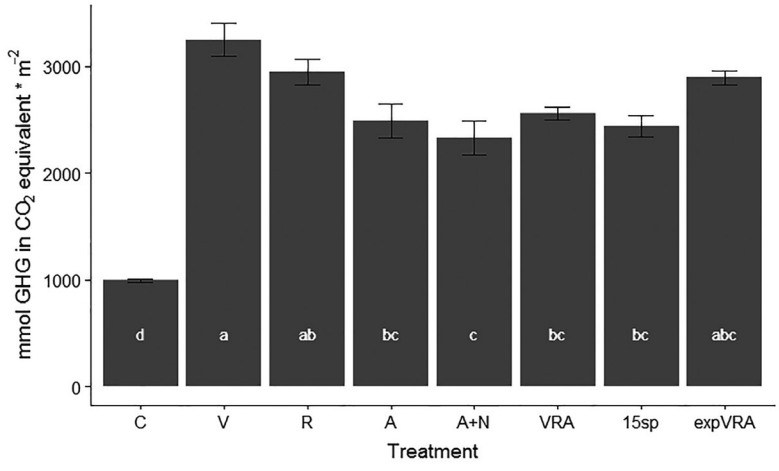

Figure 6: Cumulative greenhouse gas fluxes of both CO_2 and N_2O calculated as CO_2 equivalent values (mean ± SE; n = 4). The letters indicate significant differences between the treatments. The results of the statistical test are shown in Table S13. The separate cumulative greenhouse gas fluxes of both CO_2 and N_2O are shown in Figure S4.

Soil nutrients

Plant-available soil nutrient content was differently affected by cover crop residue additions. For N, only NO_3^- content significantly accumulated in the soil during decomposition (Figure 7A). The materials with the lowest C:N ratio (vetch) showed the largest mineral N accumulation at both time points. Oat alone immobilized N from the soil. Both mixtures show intermediate accumulation of mineral nitrogen in the soil. Mineral N accumulation for the three species mixture was similar to the expected value based on the three monocultures.

Both vetch and radish have high S contents (Table 1). This is reflected by the extractable S content in the soil after incubation at both 12 and 51 days after incubation (Figure S3C). Similar to S, vetch led to the highest extractable K and P content in soil as well (Figure S3A and B). Oat with added N had a significantly higher P increase over time than oat alone. The plant species mixtures had significantly higher concentrations of all three nutrients compared to oat, but not compared to the other two monocultures. Most of the K in the plant material was already available in the soil after 12 days. Approximately 90% of the K in the plant material was released for vetch, radish and the three species mixtures (Figure 8A), while only 5% of the P present in the total amount of residue (Figure 8B) and 50% of the added residue S was released in vetch and radish (Figure 8C), as measured with $CaCl_2$ extraction. Decomposing oat residues showed the lowest increase of mineral nutrients in the soil.

The amount of nutrients in the plant material added to the soil correlates positively with the nutrient content in the soil at the end of the experiment (Figure S4). The amount of P, K and S in the plant strongly correlates with each other not only in the plant residue ($r > 0.9$, $p < 0.001$), but also in the soil after 50 days ($r > 0.48$, $p < 0.01$). Furthermore, the C:N, C:P and N:P ratio of the added cover crop residues correlates negatively with the amount of K, P and S in the plant residue and in the soil.

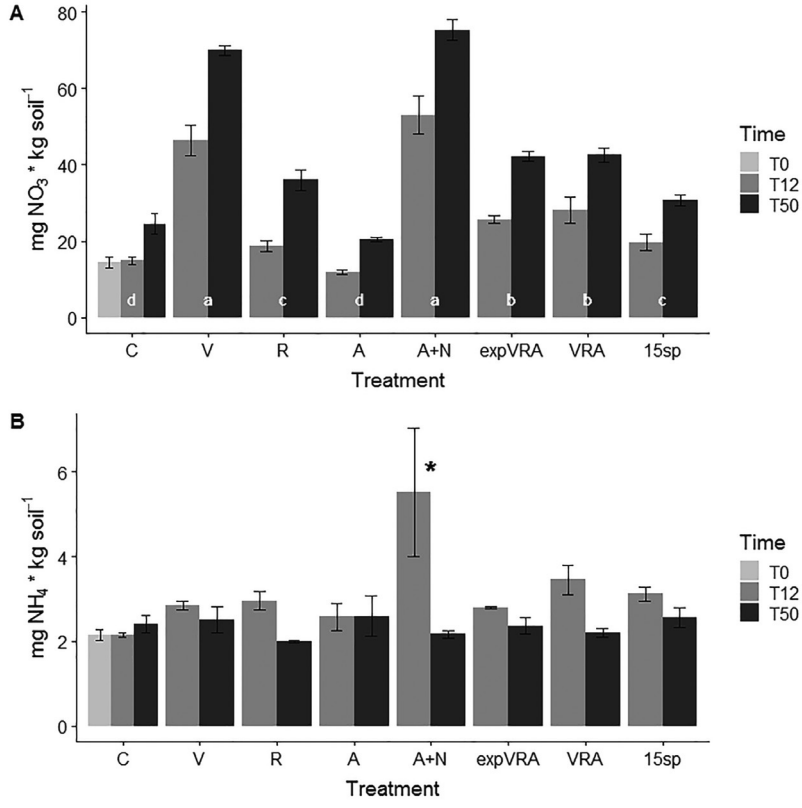

Figure 7: N content (mg per kg of dry soil) in the soil at the different time points (in days) (mean ± SE; n = 4). **A:** NO_3; Letters indicate significant differences between the treatments. The treatments show similar increase over time. **B:** NH_4. There are no significant differences between the treatments in B (only A+N was significantly different at T12), the asterisk indicate the significant difference between T12 and T50 of A+N. The results of the statistical test are shown in Table S14 and Table S15.

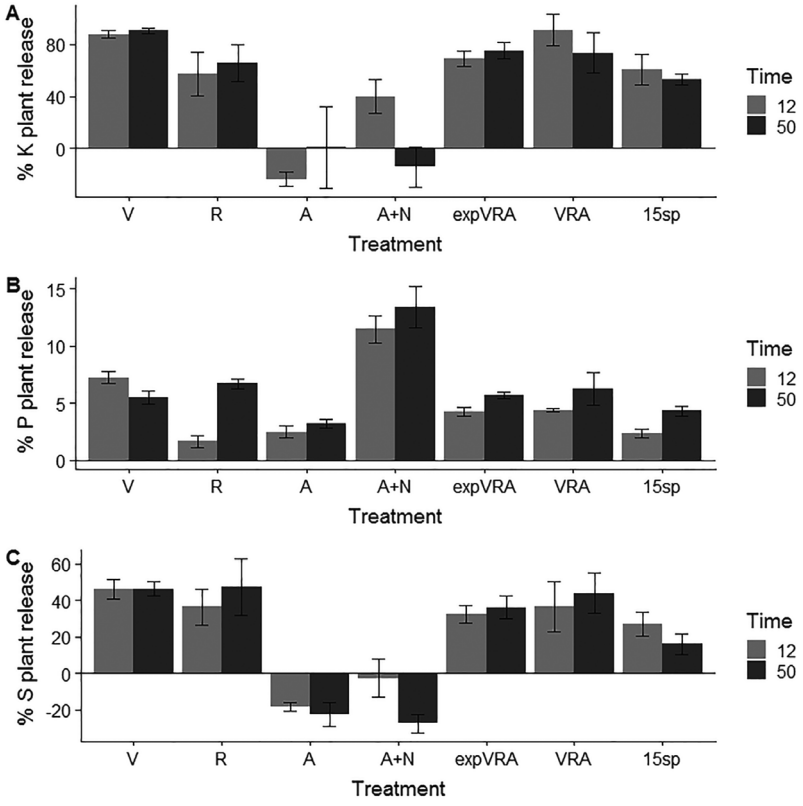

Figure 8: Percentage of nutrients that is released from the added plant material in the soil at the different time points (in days) (mean ± SE; n = 4). **A:** K; **B:** P; **C:** S.

Discussion

We investigated the effect of soil amendment with cover crop residues of mixtures and of monocultures on respiration, mineralization, greenhouse gas emission and microbial functional diversity. As previously shown, above- and belowground biodiversity is important to maintain ecosystem functioning (Wagg *et al.*, 2014). The current study aimed to determine if application of mixtures of cover crop residues has a positive effect on the functioning of soil microbes, compared to the residue of a monoculture cover crop. In this study, soil without a cover crop legacy was used to prevent effects of cover crops growing in the field (e.g. presence of root fragments). The cover crop residue amount used in this experiment is comparable to biomass production of winter cover crops. Although different cover crops in mixed cultures in the field do not produce the same amount of biomass, we used equal amounts of each species in the added mixtures to be able to identify if diversity effects could occur.

Microbial biomass dynamics

Fungal and bacterial biomass did increase when cover crop residues were added to the soil (Figure 1 & 2). The dynamics of fungal and bacterial biomass differed between residues added, which appeared to be mainly caused by C:N ratio of the plant residues. These results partially support the first hypothesis: different cover crop species can indeed lead to different microbial biomass dynamics. Mixtures of cover crop residues, however, did not result in increased biomass as compared to those of single species. Similar results have been published by (Shi and Marschner, 2014) who found only increased microbial activity, but no increased microbial growth in residue mixtures of root and shoot of a diverse set of plants as compared to either root or shoot residues alone. Furthermore, litter mixture effects in forests are mainly caused by the plant species identity (Hattenschwiler and Gasser, 2005) and do not lead to increased biomass of the decomposer community (Wardle *et al.*, 2006). These studies show that increase in microbial biomass also depends on the residue mixture, amount of residue and the environment.

Microbial functional diversity

Mixing plant material might lead to an increased amount of substrate niches that can lead to increased microbial functional diversity in the soil. In this experiment, the metabolic response of the microbial community as assessed by Biolog Eco plates was influenced by cover crop residue additions. As hypothesized, mixtures led to increased functional diversity as compared to monocultures leading to increased metabolic potential of the community (Figure 4). It should be considered that Biolog ECO plates, in potential, do reflect the substrate usage of the aerobic carbon utilizing microbial community. Dormant and microbes with another physiology are not captured. However, the substrate range of the ECO plates is so broad that a wide range of microbes will be detected, wide enough to assess effects of changes in environmental conditions in microbial functioning in comparative experimental designs. Hence, Biolog can be used as indicator of microbial potential for substrate usage and potential changes therein as the results of the changes in physical chemistry (i.e. niches) of the soil.

For natural systems, it was already shown that litter mixtures increase soil microbial diversity (Wardle *et al.*, 2006; Chapman and Newman, 2010; Chapman *et al.*, 2013; Byrnes *et al.*, 2014; Delgado-Baquerizo *et al.*, 2016). Several studies indicate that microbial functional diversity is enhancing soil ecosystem functions such as disease suppression (Van Elsas, Garbeva and Salles, 2002; Garbeva *et al.*, 2006; Postma *et al.*, 2008; Mallon, Elsas and Salles, 2015b), decomposition and nutrient cycling (Wagg *et al.*, 2014; Delgado-Baquerizo *et al.*, 2016, 2017). Van Elsas *et al.* (2012) showed that a higher microbial diversity could lead to reduced ability for pathogens to invade soil environments. This may be a result of the higher competition for resources in diverse microbial communities with a more diverse metabolic potential leading to reduced niche space for the invader (Mallon *et al.*,

2018, 2015a). As with disease suppression, decomposition may need a functional diverse community to be able to decompose residues faster and use the nutrients more efficiently (Loreau, 2001; Hattenschwiler, Tiunov and Scheu, 2005). This does not necessary mean that the microbial biomass is higher as well, as this experiment showed increased functional diversity, but not increased microbial biomass in mixtures compared to monocultures.

Greenhouse gas emissions

We hypothesized that residue mixtures lead to reduced GHG emissions compared to monocultures as we expected that the microbial community would be more efficient in using nutrients in residue mixtures compared to monocultures as a result of increased carbon use efficiency (Hattenschwiler, Tiunov and Scheu, 2005). In this experiment, mixtures did not reduce cumulative GHG emissions compared to residues from monocultures (Figure 6). Vetch had the highest cumulative emissions. The nitrogen concentration in the vetch residue was nearly double compared to the other residues (Table 1). This may have increased the decomposability of the vetch compared to the other residue treatments. Management practices, including cover crops and tillage, play an important role in mitigating GHG emissions (Kallenbach, Rolston and Horwath, 2010; Abdalla *et al.*, 2012, 2014). Furthermore, as shown by Basche *et al.* (2014) in a meta-analysis, decomposition of cover crops led to increased N_2O emissions, mainly caused by the amount of N added with cover crops. This shows that nutrient content of the plant material is a main driver of soil GHG fluxes and microbial activity in the soil (Basche *et al.*, 2014; Marschner, Hatam and Cavagnaro, 2015; Nguyen *et al.*, 2016b).

In the current study, the total amount of plant material was the same for all treatments, leading to different amounts of C and other nutrients added to the soil (Table 1) leading to correlations between the amount of C and N and greenhouse gas emissions. The correlation between the amount of C and CO_2 emissions was weaker compared to the amount of N with N_2O emissions, probably because the amount of C is not only important but also the recalcitrance of the material. Furthermore, adding N in the treatment with oat residue did increase mainly N_2O emissions but not CO_2 emissions. This lack of response in respiration may be explained by the recalcitrance of the material to degradation or shortage of other nutrients.

Nutrient release

The impact of decomposing cover crop residues on nutrient availability depended on the plant species, as shown in the results (Figure 7 & 8). K availability in the soil increased up to 90% of the amount added with the plant material while P availability only increased up to 10%. The available P released from the cover crops residues is low compared to a field study by (Cobo, Barrios and Delve, 2008) where only 30% of N and P remained in the plant material after 50 days of decomposition of two different leguminous plants. Rapid

release of K from decomposing plant material has been shown earlier (Cobo *et al.*, 2002). Decomposition of oat was not stimulated by the addition of NH_4NO_3, as the CO_2 emissions (respiration rate) are similar to oat without added N. The question arises: by what factor are these microbes limited other than N? Are other nutrients, e.g. P, K or S, missing? As shown before, adding nitrogen to decomposing plant residues can increase, decrease or have no effect on decomposition rates (Liu *et al.*, 2006; Hobbie, 2008; Norris *et al.*, 2013). In this experiment, the treatment with highest N content, vetch, led to an increase of the fungal biomass quickly after start of the experiment and higher CO_2 emissions, but vetch also had higher P, K and S amounts in the plant residues. Microbes may lack these nutrients in the treatment where only N was added to oat. Interestingly, P availability in the soil is increased when NH_4NO_3 is added, indicating that other nutrients are limiting. N addition to decomposing litter in other systems led to increased P availability as well (Liu *et al.*, 2006; Zhong *et al.*, 2017). These results show that nutrient content in residues at the start of the experiment is a major driver for decomposition and nutrient release (Sakala, Cadisch and Giller, 2000; Partey, Preziosi and Robson, 2014; Marschner, Hatam and Cavagnaro, 2015).

Microbial activity might be important for the incorporation of plant derived carbon and nutrients in soil organic matter (SOM). In this experiment, the microbial biomass ranged between 0.45 to 28 mg C per kg soil. Microbes can mobilize nutrients during decomposition of plant residues, but also as a result of turnover of the microbial biomass. In this experiment, the nutrient concentration in the soil mainly increased during the first two weeks. As shown by Achat *et al.* (2010), microbial P is important in the P cycling and availability of P for plants. The available P is mainly present in the microbial community and this P has a short turnover time of only a few days (Achat *et al.*, 2010). Thus, even though the microbial biomass represents a small portion of the soil, it can have large influence on nutrient availability.

Conclusion

In this study, decomposition of mixtures of cover crops and monocultures resulted in differences in dynamics of microbial biomass and functional diversity. The microbial biomass development in mixtures was more gradual as compared to monocultures. Furthermore, the microbial community became more functionally diverse in mixtures as shown by a higher ability to degrade substrates in Biolog ECO plates. Mixtures of residues did not increase GHG emissions and nutrient availability compared to the average of the monocultures. Overall, these results show that adding residue mixtures can lead to more balanced soil functioning. Further studies should test whether the microbial community will be more diverse in the field when using cover crop mixtures instead of monocultures to test if these microcosm results can be extrapolated to field conditions. This will help to

determine how long this microbial community will be positively changed due to residues of cover crop mixtures.

Acknowledgements

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Supplementary

Supplementary Tables

Table S1: Result statistical test of ergosterol (with Anova). Df is degrees of freedom, Sum Sq is sum of squares, Mean Sq is mean sum of squares.

	Df	Sum Sq	Mean Sq	F value	P-value
Treatment	7	2.1826	0.311802	8.0318	**5.353e-08**
Time	5	0.1946	0.038924	1.0026	0.4192
Time*Treatment	7	1.4066	0.050236	1.2940	0.1710
Residuals	146	4.6973	0.038821		

Table S2: Result statistical test of the copy numbers of the 16S rRNA gene (with Anova). Df is degrees of freedom, Sum Sq is sum of squares, Mean Sq is mean sum of squares.

	Df	Sum Sq	Mean Sq	F value	P-value
Treatment	7	1.3584e-13	1.9405e-14	7.0563	**4.473e-07**
Time	5	1.8413e-13	3.6826e-14	13.3909	**2.017e-10**
Time*Treatment	28	1.6517e-13	5.8990e-15	2.1450	**0.002356**
Residuals	123	3.3826e-13	2.7500e-15		

Table S3: Calculated microbial biomass C (MBC) in g C per kg soil and fungi:bacteria ratio (F:B) based on 16S and ergosterol content in the soil over time. The results of the statistical test are shown in Table S4 and S5.

Time		C	V	R	A	A+N	VRA	exp VRA	15sp
0	MBC	0.45±0.07							
	F:B	0.34±0.09							
1	MBC	0.51±0.04	0.49±0.07	0.51±0.11	0.73±0.13	0.69±0.15	0.60±0.04	0.58±0.06	0.61±0.09
	F:B	0.29±0.07	0.56±0.29	0.43±0.28	0.39±0.13	0.58±0.19	0.30±0.09	0.42±0.09	0.30±0.06
3	MBC	0.47±0.09	0.56±0.11	28.8±56.3	1.59±0.83	0.53±0.08	0.94±0.59	10.3±18.8	0.70±0.22
	F:B	0.34±0.09	1.00±0.34	0.16±0.13	0.10±0.05	0.32±0.06	0.26±0.13	0.18±0.13	0.41±0.35
7	MBC	0.50±0.11	1.00±0.74	0.66±0.19	0.97±0.28	0.72±0.13	2.67±2.89	0.88±0.28	0.71±0.16
	F:B	0.29±0.08	0.38±0.20	0.26±0.07	0.18±0.10	0.47±0.37	0.19±0.17	0.24±0.05	0.51±0.39
12	MBC	1.15±0.44	1.90±1.68	0.80±0.28	1.65±0.87	1.12±0.80	0.99±0.26	1.45±0.41	0.88±0.11
	F:B	0.10±0.07	0.17±0.09	0.23±0.09	0.32±0.35	0.27±0.11	0.38±0.30	0.17±0.03	0.36±0.24
50	MBC	0.57±0.44	0.67±1.68	0.60±0.28	0.71±0.87	0.61±0.80	0.94±0.26	0.66±0.41	0.70±0.11
	F:B	0.20±0.07	0.26±0.09	0.28±0.09	0.34±0.35	0.48±0.11	0.32±0.30	0.28±0.03	0.28±0.24

Table S4: Result statistical test of the soil microbial biomass C (MBC) (with Anova). Df is degrees of freedom, Sum Sq is sum of squares, Mean Sq is mean sum of squares.

	Df	Sum Sq	Mean Sq	F value	P-value
Treatment	7	1.9754	0.28220	5.7023	1.031e-05
Time	5	2.4063	0.48127	9.7249	7.923e-08
Time*Treatment	28	1.9986	0.07138	1.4423	0.09049
Residuals	121	5.9881	0.04949		

Table S5: Result statistical test of the fungi:bacteria ratio (with Anova). Df is degrees of freedom, Sum Sq is sum of squares, Mean Sq is mean sum of squares.

	Df	Sum Sq	Mean Sq	F value	P-value
Treatment	7	1.0004	0.142911	4.0883	0.0004628
Time	5	0.4695	0.093897	2.6861	0.0244125
Time*Treatment	28	2.4501	0.087502	2.5032	0.0003126
Residuals	121	4.2297	0.034956		

Table S6: Result statistical test of the biolog ECO plates (with Anova). Df is degrees of freedom, Sum Sq is sum of squares, Mean Sq is mean sum of squares.

	Df	Sum Sq	Mean Sq	F value	P-value
Treatment	7	11389.5	1627.08	20.9820	**5.525e-13**
Time	2	83.8	41.92	0.5406	0.58571
Time*Treatment	7	1321.4	188.77	2.4343	**0.03126**
Residuals	51	3954.9	77.55		

Table S7: Summary table RDA biolog ECO plates.

Statistic	Axis 1	Axis 2	Axis 3	Axis 4
Eigenvalues	0.3413	0.0727	0.0145	0.0099
Explained variation (cumulative)	34.13	41.4	42.85	43.84
Pseudo-canonical correlation	0.8497	0.8269	0.7102	0.6944
Explained fitted variation (cumulative)	75.17	91.18	94.37	96.55

Table S8: Analysis table of the RDA biolog ECO plates to identify significant contributors to the RDA plot. P-adjusted is P value corrected with Bonferroni correction.

Name	Explains %	pseudo-F	P-value	P-adjusted
Treatment: C	15.5	10.5	**0.002**	**0.024**
Treatment: V	9.6	6	**0.002**	**0.024**
Treatment: 15sp	8.2	5.1	**0.004**	**0.048**
Treatment: VRA	6.9	4.2	**0.014**	0.168
Time	6.6	4.1	**0.006**	0.072
MBC-fungus	5.2	3.1	**0.022**	0.264
Treatment: A+N	2.7	1.6	0.148	1
Treatment: A	2.5	1.5	0.162	1
MBC	1.5	0.8	0.436	1
Treatment: R	1.4	0.8	0.476	1
F:B	1.2	0.7	0.64	1
MBC-bacteria	1.1	0.6	0.702	1

Table S9: Substrates in Biolog ECO plates as carbon sources for the microbial community.

Code	Substrate	Substrate group
A1	Water	Blank
A2	β-Methyl-D-glucoside	Carbohydrate
A3	D-Galactonic acid γ-lactone	Carbohydrate
A4	L-Arginine	Amino acid
B1	Methylpyruvate (Pyruvic Acid Methyl Ester)	Ester
B2	D-Xylose	Carbohydrate
B3	D-Galacturonic acid	Carboxylic acid/sugar acid
B4	L-Asparagine	Amino acid
C1	Tween 40	Polymer
C2	i-Erythritol	Carbohydrate
C3	2-Hydroxybenzoate	phenolic compound
C4	L-Phenylalanine	Amino acid
D1	Tween 80	Polymer
D2	D-Mannitol	Carbohydrate
D3	4-Hydroxybenzoate	phenolic compound
D4	L-Serine	Amino acid
E1	α-Cyclodextrin	Polymer
E2	N-Acetyl-D-glucosamine	Carbohydrate
E3	γ-Hydroxy-butyric acid	Carboxylic acid
E4	L-Threonine	Amino acid
F1	Glycogen	Polymer
F2	D-Glucosaminic acid	Carboxylic acid

Table S9: Continued.

Code	Substrate	Substrate group
F3	Itaconic acid	Carboxylic acid
F4	Glycyl-L-glutamic acid	Amino acid
G1	D-Cellobiose	Carbohydrate
G2	Glucose-1-phosphate	Phosphorylated compound
G3	α-Ketobutyric acid	Carboxylic acid
G4	Phenyl-ethylamine	Amide/amine
H1	α-D-Lactose	Carbohydrate
H2	D,L-α-Glycerol phosphate	Phosphorylated compound
H3	D-Malic acid	Carboxylic acid
H4	Putrescine	Amide/amine

Table S10: Correlation table between the environmental variables used for the RDA analysis. Lower left part of the table are the correlation coefficients. Upper right panel are the P-values of the corresponding correlation.

	Time	Fungal biomass	Bacterial biomass	MBC	F:B ratio
Time		0.30	0.031	0.024	0.37
Fungal biomass	-0.14		0.49	0.082	<0.001
Bacterial biomass	-0.28	0.09		<0.001	<0.001
MBC	-0.29	0.23	0.99		0.014
F:B ratio	0.12	0.72	-0.43	-0.32	

Table S11: Result statistical test of the CO_2 emisisons over time (with Kruskal-Wallis rank sum test). Df is degrees of freedom.

	Chi-squared	Df	P-value
Treatment	185.76	7	**< 2.2e-16**
Time	356.99	19	**< 2.2e-16**

Table S12: Result statistical test of the N_2O emisisons over time (with Kruskal-Wallis rank sum test). Df is degrees of freedom.

	Chi-squared	Df	P-value
Treatment	186.29	7	**< 2.2e-16**
Time	246.08	19	**< 2.2e-16**

Table S13: Result statistical test of the cumulative GHG fluxes (with Anova). Df is degrees of freedom, Sum Sq is sum of squares, Mean Sq is mean sum of squares.

	Df	Sum Sq	Mean Sq	F value	P-value
Treatment	7	12855295	1836471	35.216	**4.041e-11**
Residuals	24	1251563	52148		

Table S14: Result statistical test of the NO_3 content in the soil (with Anova). Df is degrees of freedom, Sum Sq is sum of squares, Mean Sq is mean sum of squares.

	Df	Sum Sq	Mean Sq	F value	P-value
Treatment	7	15.3757	2.19653	102.2832	**<2e-16**
Time	2	3.9127	1.95637	91.0997	**<2e-16**
Time*Treatment	7	0.1087	0.01552	0.7229	0.6532
Residuals	51	1.0952	0.02148		

Table S15: Result statistical test of the NH_4 content in the soil (with Anova). Df is degrees of freedom, Sum Sq is sum of squares, Mean Sq is mean sum of squares.

	Df	Sum Sq	Mean Sq	F value	P-value
Treatment	7	0.045870	0.006553	2.2178	**0.04784**
Time	2	0.085545	0.042772	14.4764	**1.049e-05**
Time*Treatment	7	0.057577	0.008225	2.7838	**0.01568**
Residuals	51	0.150686	0.002955		

Table S16: Result statistical test of the K content in the soil (with Anova). Df is degrees of freedom, Sum Sq is sum of squares, Mean Sq is mean sum of squares.

	Df	Sum Sq	Mean Sq	F value	P-value
Treatment	7	3.0723e-05	4.389e-06	74.3937	**< 2.2e-16**
Time	2	1.3819e-06	6.910e-07	11.7119	**6.522e-05**
Time*Treatment	7	3.0570e-07	4.370e-08	0.7402	0.6391
Residuals	51	3.0089e-06	5.900e-08		

Table S17: Result statistical test of the P content in the soil (with Anova). Df is degrees of freedom, Sum Sq is sum of squares, Mean Sq is mean sum of squares.

	Df	Sum Sq	Mean Sq	F value	P-value
Treatment	7	3.7357	0.53366	50.7854	**< 2.2e-16**
Time	2	0.0354	0.01769	1.6831	0.196
Time*Treatment	7	0.6375	0.09108	8.6673	**5.745e-07**
Residuals	51	0.5359	0.01051		

Table S18: Result statistical test of the S content in the soil (with Anova). Df is degrees of freedom, Sum Sq is sum of squares, Mean Sq is mean sum of squares.

	Df	Sum Sq	Mean Sq	F value	P-value
Treatment	7	121.870	17.4100	26.7582	**5.524e-15**
Time	2	26.083	13.0415	20.0440	**3.774e-07**
Time*Treatment	7	2.493	0.3561	0.5473	0.7945
Residuals	7	121.870	17.4100	26.7582	**5.524e-15**

Supplementary Figures

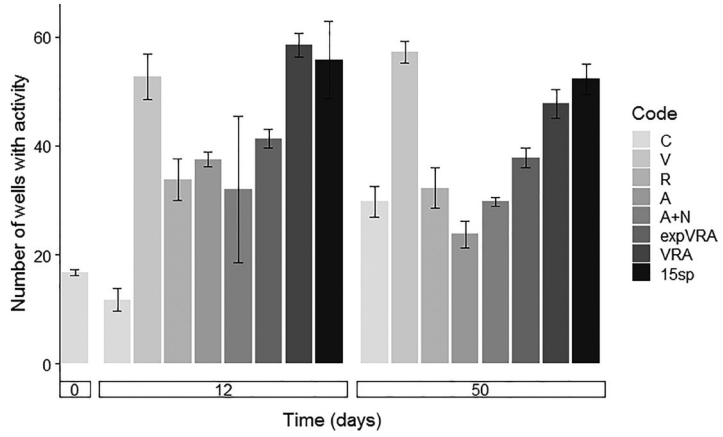

Figure S1: Microbial functional diversity: number of wells that showed a positive response in Biolog ECO plates after incubation of 7 days (mean ± SE; n = 4).

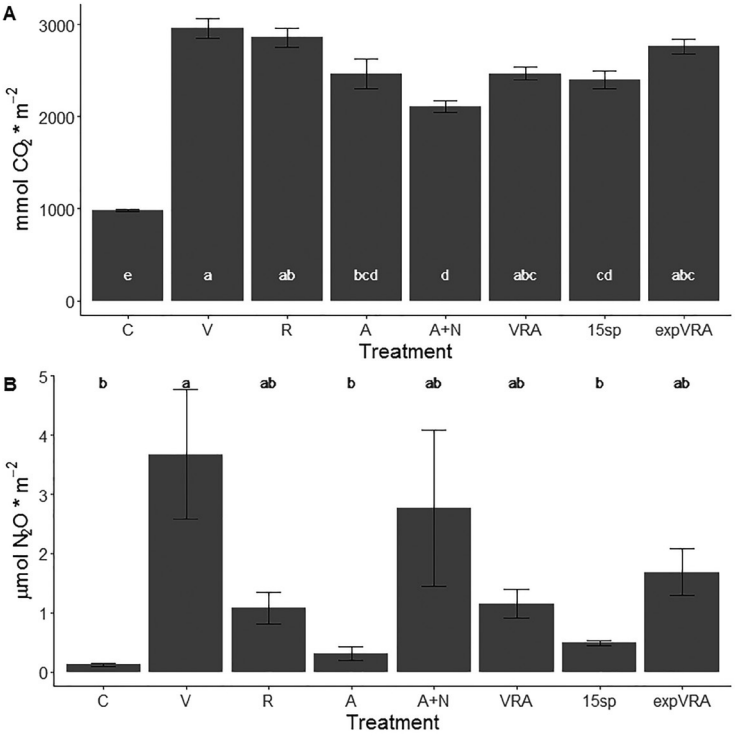

Figure S2: Cumulative greenhouse gas fluxes of CO_2 (A) and N_2O (B) (mean ± SE; n = 4). The letters indicate significant differences between the treatments.

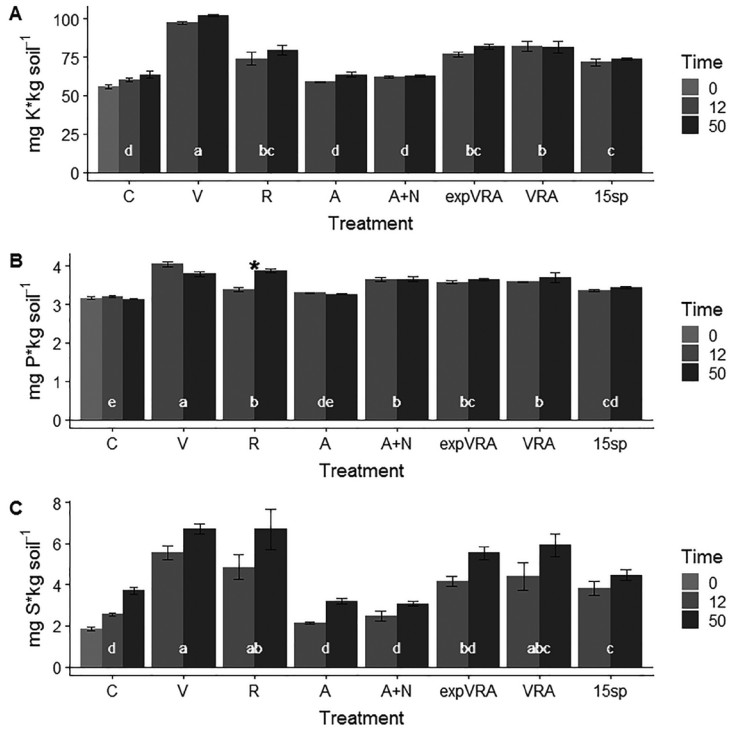

Figure S3: Nutrient content (mg per kg of dry soil) in the soil at the different time points (in days) (mean ± SE; n = 4). A: K; B: P; C: S. Letters indicate significant differences between the treatments. The asterisk in B indicate significant difference between T12 and T50 of R. In both A and C, all treatments show similar increase over time. The results of the statistical test are shown in Table S16, Table S17 and Table S18.

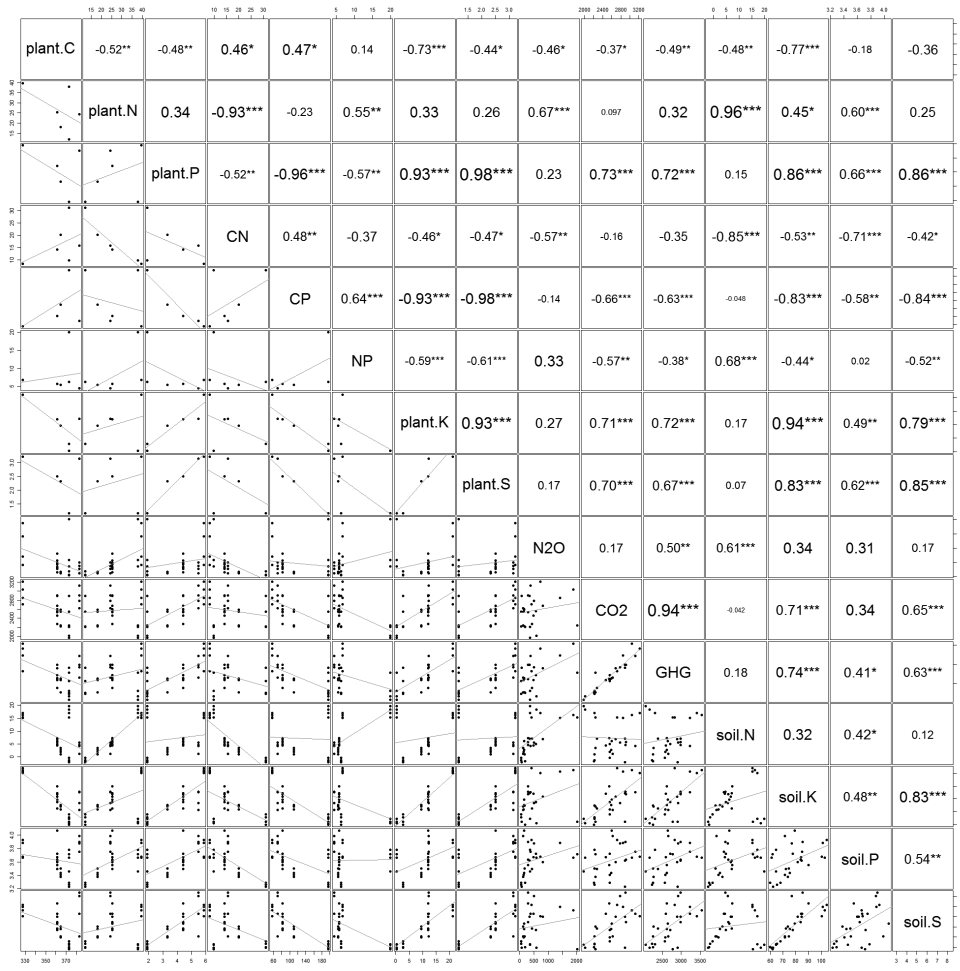

Figure S4: Correlation table between plant residue nutrient content (C, N, P, C:N, C:P, N:P, K and S), cumulative greenhouse gas emissions (N_2O, CO_2 and GHG based on GWP) and soil nutrient content (N, K, P and S) after 50 days of incubation. The numbers in the right panel are the correlation values between the parameters (Pearson correlation). If this correlation is significant, this is shown with asterisks: * if 0.01<p<0.05, ** if 0.001<p<0.01 and *** if p<0.001. The size of the values indicate stronger correlation.

CHAPTER

3

Organic residue amendments to modulate greenhouse gas emissions from agricultural soils

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Abstract

Organic fertilizers have been shown to stimulate CH_4 uptake from agricultural soils. Managing fertilizer application to maximize this effect and to minimize emission of other greenhouse gasses offers possibilities to increase sustainability of agriculture. To tackle this challenge, we incubated an agricultural soil with different organic amendments (compost, sewage sludge, digestate, cover crop residues mixture), either as single application or in a mixture and subjected it to different soil moisture concentrations using different amounts of organic amendments. GHG fluxes and *in vitro* CH_4 oxidation rates were measured repeatedly, while changes in organic matter and abundance of GHG relevant microbial groups (nitrifiers, denitrifiers, methanotrophs, methanogens) were measured at the end of the incubation. Overall the dynamics of the analysed GHGs differed significantly. While CO_2 and N_2O differed considerably between the treatments, CH_4 fluxes remained stable. In contrast, *in vitro* CH_4 oxidation showed a clear increase for all amendments over time. CO_2 fluxes were mostly dependent on the amount of organic residue that was used, while N_2O fluxes were affected more by soil moisture. Several combinations of amendments led to reductions of CO_2, CH_4 and/or N_2O emissions compared to un-amended soil. Most optimal GHG balance was obtained by compost amendments, which resulted in a similar overall GHG balance as compared to the un-amended soil. However, compost is not very nutrient rich potentially leading to lower crop yield when applied as single fertilizer. Hence, the combination of compost with one of the more nutrient rich organic amendments (sewage sludge, digestate) provides a trade-off between maintaining crop yield and minimizing GHG emissions. Additionally, we could observe a strong increase in microbial communities involved in GHG consumption in all amendments, with the strongest increase associated with cover crop residue mixtures. Future research should focus on the interrelation of plants, soil and microbes and their impact on the global warming potential in relation to applied organic amendments.

Keywords: Nitrous oxide, Carbon dioxide, Methane oxidation, agricultural soil, organic amendment, flux measurements, qPCR

Introduction

Recent novel insights led to the postulation that representatives of the newly discovered N_2O-reducing clade II can possibly turn soils into sinks of N_2O (Jones *et al.*, 2014; Domeignoz-Horta *et al.*, 2015). However, attempts to stimulate soil N_2O uptake by inoculation with a non-denitrifying *nosZ* clade II strain lowered the net potential emission but did not turn the soil into a sink of N_2O (Domeignoz-Horta *et al.*, 2016b). While the soil sink function of N_2O still has to be verified, CH_4 uptake can be found in several soils thereby contributing to cooling side of the GHG balance, representing 6% of the total global methane sink (Kirschke *et al.*, 2013; Tian *et al.*, 2016). However, fertilizer effects on the CH_4 sink function in agricultural soils have received far less attention as compared to wetlands and well-aerated non-agricultural soils. This is due the very low or negligible methane uptake capacity in these soils as compared to grassland and upland forest soils (Mosier and Delgado, 1997; Veldkamp, Koehler and Corre, 2013; Ciais *et al.*, 2014). By converting natural soils into agricultural soils, up to a seven fold reduction of CH_4 consumption was detected (Levine *et al.*, 2011), taking up to 80 years to recover to pre-land use change levels. It has been demonstrated that the decrease in methane uptake in agricultural soils is due to the destruction of the soil physical structure (e.g. plowing, soil compaction), disrupting the methane gradients in the soil, which are proposed to be crucial for high affinity atmospheric methane oxidation. Next to this, other agricultural practices (e.g. fertilization) have been demonstrated to have detrimental effects on atmospheric methane uptake (Bender and Conrad, 1992; Boeckx, Van Cleemput and Villaralvo, 1997; Hiltbrunner *et al.*, 2012). However, a recently published study (Ho *et al.*, 2015a) demonstrated strongly enhanced methane uptake rates after the addition of different organic amendments (e.g. compost, sewage sludge), to different agricultural soils. The observed rates of uptake were even comparable to the ones from well-aerated forest soils. Shackley and colleagues observed a similar effect upon addition of biochar which improved the GHG balance by reducing N_2O and CH_4 emissions from soil (Shackley *et al.*, 2016). These findings are further supported by another study which showed that the use of organic fertilizers (in this case biochar and compost) influence microbial processes which resulted in alterations of soil nutrient cycles thereby affecting agricultural properties (Ye *et al.*, 2016). Furthermore, the addition of plant-derived C compounds from external sources such as biochar or composts can increase soil C availability and may result in higher net CO_2 removals from the atmosphere (Paustian *et al.*, 2016) thereby lowering the global warming potential (GWP) (Järveoja *et al.*, 2016). Compared to fresh organic residues, mineralization of compost is slower after addition to soil, leading to a several fold greater mean residence time (Ryals *et al.*, 2015). Ho and colleagues (2015a) postulated that a well-balanced mix of different fertilizers could have a positive effect on GHG balance considering the creation of conditions for methanotrophs to take up atmospheric methane while at the same time keeping carbon dioxide and nitrous oxide emissions to a minimum by providing a greater variety of C- and

N-compounds to the microorganisms. However, not all organic fertilizers are suitable to serve this purpose, since in single application only a few organic residues showed the capability to increase soil CH_4 uptake and keep CO_2 and N_2O emissions to a minimum (Ho *et al.*, 2017b). However, to develop a strategy to reduce GHG emission from agricultural soils without decreasing crop yield requires understanding of the underlying mechanisms of how organic fertilizers influence GHG. This study aims to answer the following research questions: What is the influence of a combination of organic amendments (compost, digestate, sewage sludge and cover crop residues) on the GWP of agricultural soils? We hypothesize that methane uptake is stimulated while CO_2 and N_2O emissions are kept to a minimum compared to un-amended soil by application of mixes of organic amendment and mineral fertilizers. We test these hypotheses by performing soil incubations with various combinations of organic and mineral fertilizers and following GHG dynamics as well as soil chemistry and microbial functional gene abundance.

Material & Methods

Site description, soil sampling, and residues

The soil was collected in May 2017 at the research station of Wageningen University in Lelystad, the Netherlands (52˚32'26.4"N, 05˚33'34.7"E) representing a clay soil. The field was planted with onions and left fallow after harvest before sampling. Previously, soil physical-chemical properties have been determined (Ho *et al.*, 2015a). The upper 10 cm of the soils was collected in May 2017 from 1m x 1m using a shovel. The soil was air-dried at room temperature before being sieved (2 mm). The residues included in this study comprised materials with a broad C:N ratio ranging from 4.85 – 22.39 (Table 1) and were selected based on their CH_4 uptake performance (compost and sewage sludge) (Ho *et al.*, 2015a) or their common usage as bio-based additives in agricultural soil. The residues were air-dried at 30°C, the sewage sludge (S), digestate (D) and the cover crop residues (in the following referred to as CC residues) powder mixture were crushed and ground (< 2 mm) (Jaw Crusher Type BB-1/2, Aartselaar, Belgium). Both composts (C1 and C2) were broken down and sieved (< 6 mm), while the CC residues were cut with a scissor to smaller pieces (< 3 - 5 cm). Both the dried soils and residues were thoroughly mixed and sieved as per treatment prior to setup of the experiment to ensure standardized initial incubation conditions.

Table 1. Amendment description, total C and N contents of amendment and soil.

Soil/residues	Total C (µg C mg dw sample^{-1})	Total N (µg C mg dw sample^{-1})	C:N	Description (Source/location)
Soil	16.44 ± 0.34	1.12 ± 0.07	14.76	Clay soil from an agricultural field with onions as the last crop. (Lelystad, The Netherlands)
Sewage Sludge	202.74 ± 12.82 41.81 ± 1.80		4.85	Sampled from an anaerobic digester after sludge thickening (Vallei Veluwe, The Netherlands)
Digestate	290.07 ± 14.14	24.59 ± 1.64	11.82	Residue product of biogas formation from manure. (ACRRES, The Netherlands)
Compost1	145.68 ± 39.07	11.08 ± 2.19	13.04	Mature compost derived from organic materials e.g. plant clippings and grass. (Attero, The Netherlands)
Compost2	118.40 ± 13.77	6.25 ± 0.65	18.96	Van Iersel fungal dominant humic compost. Basic ingredient is wood shreds. (Soiltech, The Netherlands)
CC residue mixture	347.02 ± 15.78	15.50 ± 1.78	22.39	Consist of *Brassica carinata*, *Trifolium incarnatum*, *Secale cereal* collected from a field in November 2016. (Joordens, The Netherlands)

Experimental setup for in situ GHG flux measurements

The soil (200 g dry weight) and residues were mixed with a spoon in a pot and put in an incubation bottle (500 mL volume); deionized water was added to 65% or 40% of soil water holding capacity, respectively. The residue addition to the soil corresponded to a rate of either 20-ton ha^{-1}, which is typically used in agricultural practice (Diacono and Montemurro, 2010), or 5-ton ha^{-1}, which is the maximum amount of cover crop biomass incorporated in agricultural fields in spring. Incubation was performed using three replicates for each treatment in a climate chamber at 15 °C (mean annual temperature in the Netherlands is 10 °C) in the dark for approximately one month (for 28 days). Water loss, measured by weight, was compensated weekly. Periodically (0, 1, 3, 7, 14, 21, 28d) methane, nitrous oxide and carbon dioxide fluxes were measured under ambient air by closing the bottles tightly with a lid for three hours and measuring directly after closing, after 1.5h and after 3h. At every time point 20 mL of the headspace was withdrawn and stored in exetainers (5.9 mL) vials (Labco Limited, Lampeter, UK). The first 8ml of sample was used to flush the exetainer, followed by 12ml sample introduced into the exetainers creating a 2 bar overpressure. Introduction of the sample (1ml) into the GC was by an autosampler (TriPlus RSH, Thermo Fisher Scientific, Bleiswijk, The Netherlands) connected

to a gas chromatograph (GC1300, Thermo Fisher Scientific) equipped with a Methanizer and a Flame Ionization Detector (FID) to detect CH_4 and CO_2, an electron capture detector (ECD) for detection of N_2O and two sets of a pair Rt-Q-Bond capillary columns (L; 15m and 30 m, ID; 0.53 mm, Restek, Interscience, Breda, The Netherlands). Helium was used as a carrier gas, and oven temperature was set at 80 °C. Five different concentrations of CH_4 (0.1, 0.2, 0.6, 1.2, 2 ppm), CO_2 (100, 200, 600, 1200, 2000 ppm) and N_2O (0.05, 0.1, 0.3, 0.6, 1.0 ppm) from a gas mixture (2 ppm CH_4, 2000 ppm CO_2, 1 ppm N_2O) (Linde AG, Velsen-Noord, The Netherlands) were used as a standard. If higher concentrations of CO_2 and N_2O were measured, additional single gas calibration gases (Linde AG) of the respective gases (CO_2: 4000 and 10000 ppm; N_2O: 10 and 100 ppm) were used. Chromeleon™ Chromatography Data System 7.1 (CDS, Thermo Fisher Scientific) Software was used to analyse the obtained gas chromatograms from the GC and was used to calculate the standard curves. The gas flux rates were determined by linear regression from the three time points. All fluxes with a R_2<0.70 were discarded.

Measuring methane oxidation and organic matter

To determine near atmospheric soil methane emission or uptake under influence of the different amendments after 7, 14, 21 and 28d, the bottles were closed for 6 days and ~10 ppm CH_4 was added to the headspace. CH_4 decrease was measured every day in duplicates from each bottle using an Ultra GC gas chromatograph (Interscience, Breda, The Netherlands) equipped with a Flame Ionization Detector (FID) and a Rt-Q-Bond (L; 30 m, ID; 0.32 mm, Restek, Interscience) capillary column. Helium was used as a carrier gas, and oven temperature was set at 80 °C. Chromeleon™ Chromatography Data System 7.1 (CDS, Thermo Fisher Scientific) Software was used to analyse the obtained gas chromatograms from the GC.

Sample storage and soil organic matter measurements

After finishing the incubation ~10 g of soil samples were stored at -20 °C for later DNA extractions. Another ~50 g of soil was dried at 30 °C and stored for soil nutrient determination. To measure the soil organic matter content after incubation, 10-15 g of soil was dried in a porcelain cup at 105 °C for one day. Afterwards, the dried sample was burned in an oven at 430 °C for another day, both times the sample was weighed. To calculate the organic matter content per g 100g-1 dry soil the following formula was used: 100 * (g dry soil – g ashed soil) / g dry soil.

DNA extraction and qPCR assays

DNA was extracted using the DNeasy PowerSoil Kit (Qiagen, Venlo, The Netherlands) according to manufacturer's instruction. We performed qPCR assays targeting *amoA* for ammonium oxidizing Achaea (AOA) and bacteria (AOB), *nifH* (N-fixers), *nosZ* clade I/II (denitrifiers), *mcrA* (methanogens), *pmoA* (methane oxidizers), 16S rRNA gene for Archaea

and Bacteria as well as the 18S rRNA gene of fungi. Each assay was performed in duplicate for each DNA extract with primers, primer concentration, and PCR profiles as shown in Supplementary Table 1. Briefly, each qPCR (total volume 20 µl) for all assays consisted of 10 µl 2× SensiFAST SYBR (BIOLINE, Alphen aan den Rijn, The Netherlands), 1 µl of forward and reverse primers each (10 pmol μl^{-1}; Sigma-Alderich, Zwijndrecht, The Netherlands), 1 µl bovine serum albumin (5 µg μl^{-1}; Invitrogen, Breda, The Netherlands), 4.5 µl DNase- and RNase-free water and 2.5 µl diluted template DNA. The qPCR for the EUBAC (bacterial 16S rRNA gene) assay (total volume 15 µl) consisted of 7.5 µl 2× SensiFAST SYBR (BIOLINE), 0.75 µl of forward and reverse primers each (5 pmol μl^{-1}; Sigma-Alderich), 1.5 µl bovine serum albumin (5 µg μl^{-1}; Invitrogen), 1.5 µl DNase- and RNase-free water, and 3 µl diluted template DNA. Standard curves were obtained using serial 10-fold dilutions of a known amount of plasmid DNA from different pure cultures representing the target gene fragment (10^8 to 10^1 gene copies) containing the respective gene fragment. The qPCR was performed with an iCycler IQ5 (Applied Biosystem, Carlsbad, CA, USA). Negative controls were always run with water instead of template DNA. PCR reactions were done with 1:20 and 1:60 diluted DNA extracts. Amplification efficiencies for all assays were between 79-98 % with R_2 values between 0.969 and 0.995. Amplicon specificity was inferred from the melt curve.

Statistical analyses of collected data

All statistical analyses were done using R version 3.0.1 (R Development Core Team, 2013). The mean total GHG fluxes, the GWP, the organic matter loss and abundance of the different functional marker genes were tested for normality by Kolmogorov-Smirnov test and for homogeneity of variance by Levene's test. If necessary, normal distribution was achieved by log-transformation of the data. Treatment effects and differences between means were assessed using one-way ANOVA followed by Tukey's post hoc test. All levels of significance were defined at $p<0.05$

Results

GHG flux measurements

The fluxes of the three major GHG (CH_4, CO_2, N_2O) from the soils amended with the organic amendments were measured continuously through the experiment at different level of SM and different applied concentrations of organic amendments. An overview about values of the different GHG as well as the calculated GWP_{100yr} for the different samples is shown in Table 2.

Table 2. Overview of mean total CH_4. CO_2, N_2O and calculated GWP_{100yr} values of the different organic amendments, amounts and soil moisture concentration that were used. GWP_{100yr} calculations derived from the cumulative CH_4 (Supplementary Figure 1), CO_2 (Supplementary Figure 2) and N_2O (Supplementary Figure 3) fluxes. OA=organic amendments; un-amended=soil without organic amendment; C1=compost1; C2=compost2; cut CC=cut cover crop residue mixture; powder CC=powder cover crop residue mixture mix; D+C1=digestate + compost1; D+C2=digestate + compost2; S+C1=sewage sludge + compost1; S+C2=sewage sludge + compost2

OA	Amount [t ha^{-1}]	Mean total CH_4-C [µg kg $soil^{-1}$]		Mean total CO_2-C [mg kg $soil^{-1}$]		Mean total N_2O-N [mg kg $soil^{-1}$]		GWP_{100yr} [mg CO_2-C kg $soil^{-1}$]	
		40%SM	*65%SM*	*40%SM*	*65%SM*	*40%SM*	*65%SM*	*40%SM*	*65%SM*
Un-amended	*none*	75 ± 50.2	83 ± 18.0	958 ± 71.3	1068 ± 132.6	0.015 ± 0.008	0.063 ± 0.034	959 ± 71.2	1072 ± 134.8
C1	*20*	100 ± 28.6	70 ± 53.7	1830 ± 117.5	2357 ± 256.4	0.057 ± 0.008	0.290 ± 0.052	1834.4 ± 117.1	2375.9 ± 257.7
	5	142 ± 123.4	125 ± 23.8	818 ± 464.6	1717 ± 73.3	0.002 ± 0.015	0.0414 ± 0.014	819 ± 464.8	1744 ± 79.9
C2	*20*	91 ± 27.1	88 ± 42.7	1058 ± 27.3	1586 ± 14.3	0.013 ± 0.001	0.042 ± 0.014	1060 ± 27.1	1589 ± 13.7
	5	91 ± 24.1	88 ± 32.5	777 ± 35.2	1426 ± 75.7	0.009 ± 0.005	0.425 ± 0.457	778 ± 34.9	1453 ± 13.6
cut CC	*20*	134 ± 52.2	70 ± 37.4	32372 ± 2762.6	46157 ± 1289.2	13.651 ± 1.879	16.877 ± 2.182	33218 ± 2670.6	47201 ± 1381.7
	5	70 ± 46.5	84 ± 22.8	6303 ± 1057.9	11689 ± 1220.0	0.107 ± 0.033	5.482 ± 2.244	6310 ± 1059.5	12028 ± 1358.8
powder CC	*20*	118 ± 27.9	70 ± 30.5	20098 ± 1538.7	26177 ± 1006.6	19.345 ± 3.967	6.397 ± 2.67	21295 ± 1422.1	26688 ± 1031.1
	5	82 ± 19.1	109 ± 47.2	5286 ± 1205.6	7996 ± 1429.6	0.031 ± 0.013	4.666 ± 5.425	5289 ± 1205.9	8236 ± 1143.8
Digestate	*20*	80 ± 40.3	-77 ± 21.3	4554 ± 780.5	6583 ± 316.5	0.280 ± 0.335	6.204 ± 2.207	4572 ± 800.9	6966 ± 183.7
	5	104 ± 53.8	107 ± 94.0	2322 ± 277.4	2750 ± 490.8	0.032 ± 0.049	2.029 ± 0.457	2325 ± 279.4	2877 ± 129.9
D+C1	*20*	48 ± 32.1	30 ± 25.7	2734 ± 177.8	3807 ± 348.0	0.105 ± 0.060	2.259 ± 0.189	2741 ± 180.8	3947 ± 359.0
	5	46 ± 28.8	18 ± 55.1	1560 ± 383.7	2070 ± 95.6	-0.033 ± 0.093	1.059 ± 0.30	1558 ± 389.5	2135 ± 84.4
D+C2	*20*	75 ± 74.9	-70 ± 61.1	2135 ± 34.1	3848 ± 1239.4	0.056 ± 0.049	8.183 ± 10.67	2139 ± 31.9	4354 ± 1895.3
	5	57 ± 15.4	12 ± 29.6	1118 ± 208.5	1711 ± 103.1	0.028 ± 0.011	0.586 ± 1.211	1120 ± 208.1	1747 ± 70.0
S+C1	*20*	53 ± 23.9	-40 ± 44.5	4884 ± 362.0	6057 ± 2144.8	1.485 ± 0.271	28.589 ± 15.345	4976 ± 345.2	7825 ± 3042.5
	5	85 ± 40.8	9 ± 38.9	1853 ± 137.3	2527 ± 63.0	0.263 ± 0.190	9.306 ± 4.354	1870 ± 130.0	3102 ± 289.9
S+C2	*20*	107 ± 71.2	16 ± 23.8	4561 ± 336.3	6170 ± 209.3	0.707 ± 0.047	32.501 ± 3.094	4605 ± 336.1	8178 ± 399.2
	5	88 ± 24.1	62 ± 64.5	1648 ± 203.7	2266 ± 186.1	-0.016 ± 0.091	8.756 ± 2.053	1647 ± 208.7	2808 ± 313.5

CH_4

The CH_4 flux measurements under 65% SM (Supplementary Figure 1A-B) showed variation over time considering uptake or emission of CH_4. Both amounts of organic amendments applied (5 and 20 t/ha) led to similar fluxes during the incubation without fluctuation. However, total CH_4 fluxes (Figure 1A-B) varied between treatments, mostly releasing CH_4 over time irrespective of the amount of organic amendment used. Only three amendments (digestate, D + C2, S + C1 at 20 t/ha) led to increased methane uptake. Under 40% SM, minor fluctuations in CH_4 fluxes over time were detected with both organic amendment amounts (Supplementary Figure 1C-D). Calculated mean cumulative CH_4 fluxes (Figure 1C-D) demonstrated that all samples emitted CH_4 during the incubation.

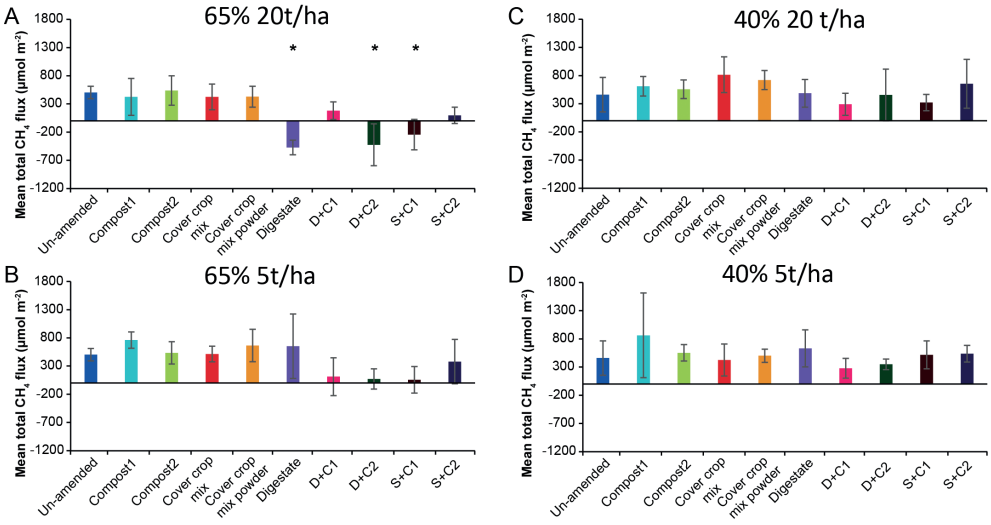

Figure 1. Mean total CH_4 emitted or consumed over the period of 28d in un-amended clay soil and after amendments with compost1, compost2, CC residues mixtures, digestate, digestate+compost1, digestate+compost2, sewage sludge+compost1 and sewage sludge+compost2 (mean ± SD; n = 3) at (A) high amount (20 t/ha) and high water content (65%), (B) low amount (5 t/ha) and high water content, (C) high amount and low water content (40%) and (D) low amount and low water content, derived from the cumulative CH_4 (Supplementary Figure 1) fluxes. Asterisk (*) indicate significant differences in the mean total CH_4 fluxes between the soils with organic amendments and the un-amended soil within the four separate superordinate treatments (ANOVA: P value < 0.05).

CO_2

Measured CO_2 fluxes under 65% SM (Supplementary Figure 2A-B) showed the same trends, irrespective of the amounts of organic amendment applied. Highest CO_2 fluxes were observed for cut and powdered cover crop residues, respectively, followed by digestate and the sewage sludge + compost 2 combination. Independent of the amount applied, cut as well as powdered CC residues continuously released CO_2 over the complete incubation. Both types of compost led to the lowest CO_2 fluxes among the organic amendments used and were comparable or lower than the CO_2 fluxes of the un-amended soil. The mean

cumulative CO_2 fluxes (Figure 2A-B) reflect the dynamics of the CO_2 fluxes over time and treatments (Supplementary Figure 2A-B). Highest CO_2 emissions were observed for cut CC residue material, followed by powdered CC residue, digestate and the sewage sludge amendments. This was true for both tested amounts. Highest CO_2 fluxes under 40% SM were always observed for cut CC residue material followed by powdered CC residues, digestate and the two sewage sludge treatments (Supplementary Figure 2C-D). While high amounts of CC residues showed emission of CO_2 over the whole incubation period, no emissions were detected after 21d with low amounts. Similarly, cumulative CO_2 fluxes (Figure 2C-D) were always lower with lower amounts of organic amendments, the extent of which differed between the type of organic amendment. While both cover crop residue treatments were 4-5 fold higher, all other organic amendments were only 1.4-2.7 fold higher when 20 t ha^{-1} was applied.

Lower SM always lead to lower CO_2 fluxes when same amounts organic amendments were applied.

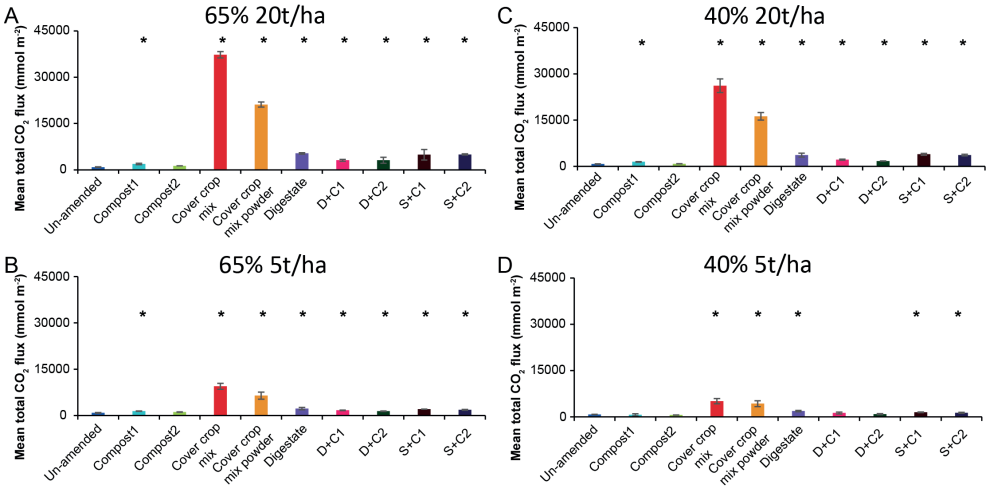

Figure 2. Mean total CO_2 emitted over the period of 28d in un-amended clay soil and after amendments with compost1, compost2, CC residues mixtures, digestate, digestate+compost1, digestate+compost2, sewage sludge+compost1 and sewage sludge+compost2 (mean ± SD; n = 3) at (A) high amount (20 t/ha) and high water content (65%), (B) low amount (5 t/ha) and high water content, (C) high amount and low water content (40%) and (D) low amount and low water content, derived from the cumulative CO_2 (Supplementary Figure 2) fluxes. Asterisk (*) indicate significant differences in the mean total CO_2 fluxes between the soils with organic amendments and the un-amended soil within the four separate superordinate treatments (ANOVA: P value < 0.05).

N_2O

Both sewage sludge combinations showed the highest N_2O flux rates at 65% SM, regardless of the applied amounts of organic amendments, followed by digestate and cut CC residue material (Supplementary Figure 3A-B). Both composts, as well as the un-amended soil,

showed almost no N_2O fluxes. In general, 20 t ha^{-1} led to higher overall measurable N_2O fluxes. These findings are also underlined by the cumulative N_2O fluxes (Figure 3). The N_2O fluxes of both sewage sludge combination, digestate, digestate + compost 1 and both CC residue mixtures were 2-4-fold lower with 5 t ha^{-1}. The digestate + compost 2 amendment showed a 13-fold reduction, while the un-amended and both single compost applications did not lead to any N_2O emission at all. After 14d of incubation both combinations of digestate with compost at an application rate of 5 t ha^{-1} resulted in lower N_2O emissions.

Only low N_2O emissions were detected at 40% SM (Supplementary Figure 3C-D). All organic amendments applied at a rate of 5 t ha^{-1} showed no N_2O emissions during the complete incubation period while at 20 t ha^{-1} only small amounts of N_2O were released in the first 14d of incubation. After 14d both CC residue amendments (cut and powdered) showed a rapid increase in N_2O emissions, which peaked at day 21. After 28d the cut CC residues still released N_2O from the soil, while the powdered CC residue enabled soil N_2O uptake from this point onward.

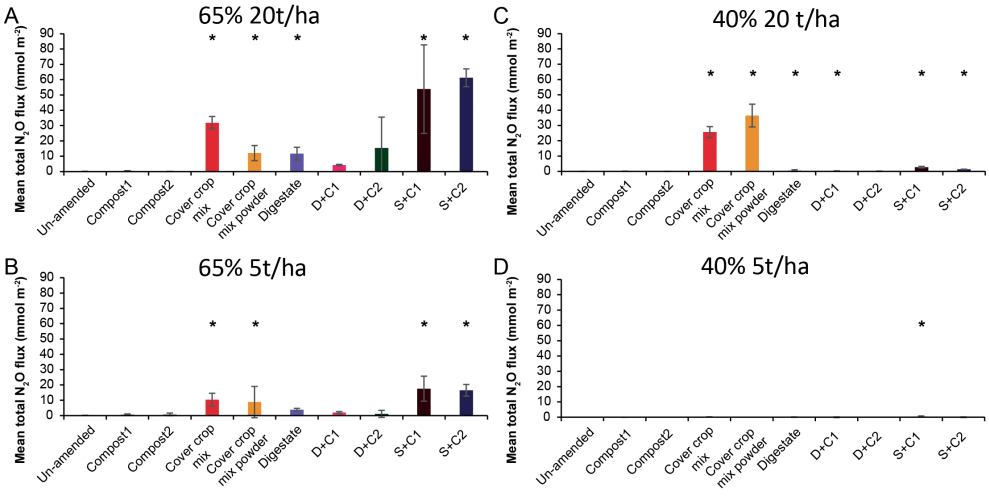

Figure 3. Mean total N_2O emitted over the period of 28d in un-amended clay soil and after amendments with compost1, compost2, CC residues mixtures, digestate, digestate+compost1, digestate+compost2, sewage sludge+compost1 and sewage sludge+compost2 (mean ± SD; n = 3) at (A) high amount (20 t/ha) and high water content (65%), (B) low amount (5 t/ha) and high water content, (C) high amount and low water content (40%) and (D) low amount and low water content, derived from the cumulative N_2O (Supplementary Figure 3) fluxes. Asterisk (*) indicate significant differences in the mean total N_2O fluxes between the soils with organic amendments and the un-amended soil within the four separate superordinate treatments (ANOVA: P value < 0.05).

GWP analyses

We derived the GWP in mg CO_2 equivalent per kg soil (IPCC, 2007) by combining the cumulative CH_4, CO_2 and N_2O flux (Supplementary Figs. 1-3). In these calculations, the

GWP value for CH_4 and N_2O are considered to be 28 and 265, respectively over a hundred-year time frame, while the GWP value for CO_2 is considered to be 1 (IPCC, 2013).

The GWP values showed similar trends as the cumulative CO_2 fluxes, irrespective of the SM and amount of organic amendment (Figure 4). Notably, compost1 and 2 treatments led to lower GWP as compared to un-amended soil with low amounts applied under 40% SM. (Fig 4).

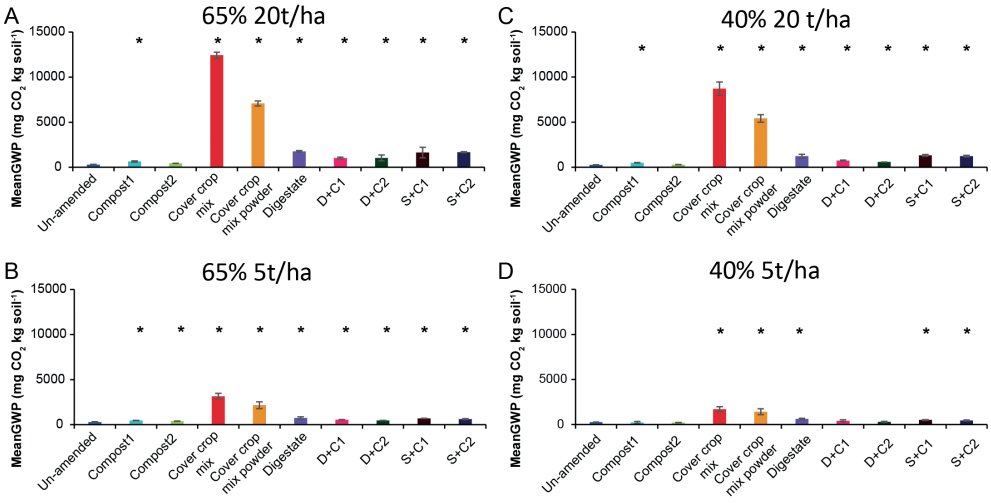

Figure 4. Mean global warming potential (GWP) over the period of 28d in un-amended clay soil and after amendments with compost1, compost2, CC residues mixtures, digestate, digestate+compost1, digestate+compost2, sewage sludge+compost1 and sewage sludge+compost2 (mean ± SD; n = 3) at (A) high amount (20 t/ha) and high water content (65%), (B) low amount (5 t/ha) and high water content, (C) high amount and low water content (40%) and (D) low amount and low water content, derived from the cumulative CH_4 (Supplementary Figure 1), CO_2 (Supplementary Figure 2) and N_2O (Supplementary Figure 3) fluxes. Asterisk (*) indicate significant differences in the GWP between the soils with organic amendments and the un-amended soil within the four separate superordinate treatments (ANOVA: P value < 0.05).

CH_4 fluxes after addition of 10 ppm CH_4

CH_4 fluxes after the addition of 10 ppm CH_4 at multiple times, did not differ significantly between the four major treatments irrespective of SM and organic amendment rate applied. (Supplementary Figure 4). The fluxes in most cases vary between 0 and -0.003 µmol m^{-2} min^{-1}, which can be referred to as CH_4 uptake. At the last sampling point the amendment with compost2 at 40% SM and 5 t ha^{-1} increased to an uptake of -0.008 µmol m^{-2} min^{-1}, which was the highest uptake measured. However, most organic amendments improve their CH_4 uptake over time.

Organic matter

When low amounts of organic amendment are applied at 65% SM, the organic matter loss is constant through all treatments ranging from -0.4 to -0.6% loss of the original

OM content which was around 2.5 to 3% (Figure 5). At high concentration of organic amendments the loss of OM is lower being around -0.4% with exception of the cut CC residue amendment, resulting in 1.4% loss in organic matter. In general incubations at 40% SM lost more organic matter than their counterpart at 65% SM (Figure 5). The lowest losses were observed for digestate, compost1 and D+C1 with a loss of ~ -0.55%. These organic amendments are followed by compost2, D+C2, S+C1 and S+C2 with a loss of -0.8 to -1.0% organic matter content. The highest loss could be observed for cut and powdered CC residue mixture with -1.2% and -1.4%, respectively.

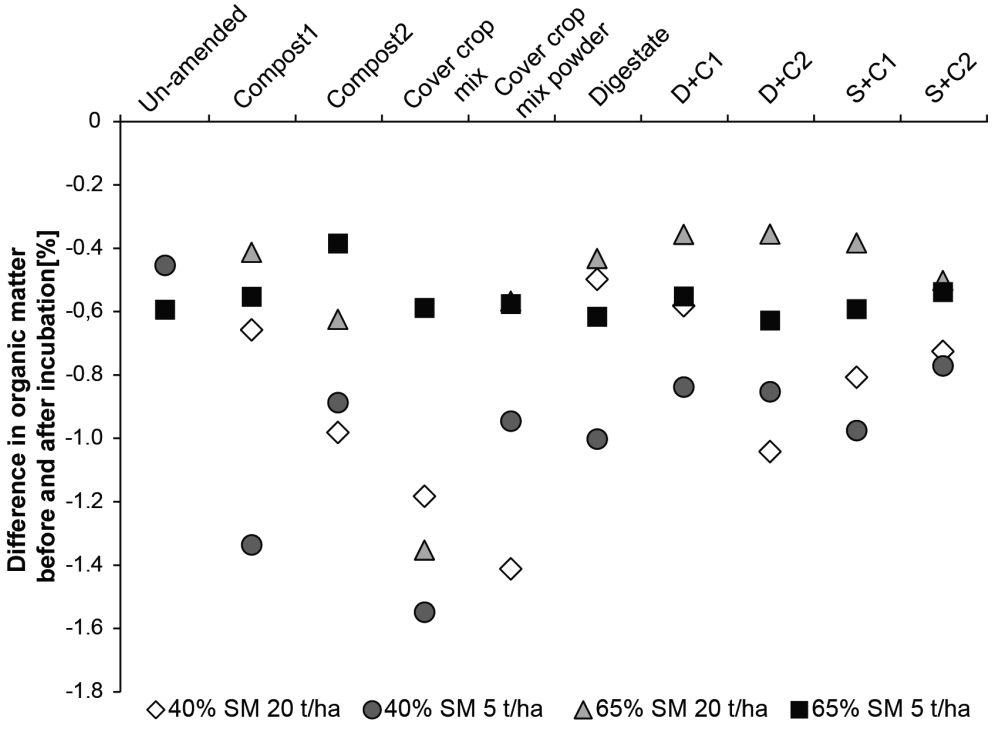

Figure 5. Loss in organic matter content during the incubation period of 28d in un-amended clay soil and during amendments with compost1, compost2, CC residues mixtures, digestate, digestate+compost1, digestate+compost2, sewage sludge+compost1 and sewage sludge+compost2 (mean ± SD; n = 3) at (▲) high amount (20 t/ha) and high water content (65%), (■) low amount (5 t/ha) and high water content, (◊) high amount and low water content (40%) and (●) low amount and low water content.

Abundance analyses of microbial groups

To assess changes in the abundance of the microbial communities, the ratio was calculated between gene copy numbers of the analysed genes in the initial soil and at the end of the incubation. The individual gene copy numbers of all samples analysed can be found in Sup Table 2 and 3.

The overall bacterial abundance stayed either stable or increased over time (Figure 6A), with high amounts of CC residues leading to the highest stimulation in abundance (4-7 fold). All other organic amendments at high application rate led to at least to a doubling of bacterial numbers, while numbers in the un-amended remained constant. When applying low amounts of organic amendments, microbial abundances did not change in any of the treatments.

In contrast to the bacterial abundance, archaea communities either remained stable or decreased over the time (Figure 6B). Typically, all digestate combinations, both composts and sewage sludge combinations at high application rate did not lead to change in archaeal abundance, while it decreased in all other treatments.

Overall, fungal abundance was rather constant during the incubation (Figure 6C). However, the cut CC residue mixture led to a 15- and 5-fold increase in fungal abundance at high and low organic amendment application rate, respectively while the 20 t ha^{-1} powdered CC residue treatment increased around 3-fold. Compost 2 at low application led a 10-fold in increase. All other treatments at high application rate did not lead to change in fungal abundance.

For most of the functional marker genes there was no change in the un-amended soil, except for a decrease of AOAs and a doubling of *nosZ* clade II (Figure 7).

Both *nosZ* clades showed an increase in abundance, in all organic amendment-treatments, irrespective of the application rate (Figure 7A-B). While the two clades with low amendments increased mainly between 1.2-2.5-fold, a 2-7-fold increase was observed with 20 t ha^{-1}. The highest increase occurred in the incubation with cut CC residue material with 28-fold in *nosZ* clade I. In general, the *nosZ* clade II was 10- to 100-fold more abundant than *nosZ* clade I (Supplementary Table 2).

At low application rates organic amendments had no effect on the bacterial *amoA* abundance (Figure 7C). At high concentrations, the cut CC residue, both sewage sludge combinations and all treatments with digestate lead to an increase in bacterial *amoA* of 2-8-fold (Figure 7C).

In contrast to the abundance of the bacterial *amoA*, archaeal *amoA* abundance decreased in all organic amendment-treatments (Figure 7D). The strongest decrease was observed for the digestate and sewage sludge combinations with both composts, which decreased 3-4-fold in both applied concentrations. In all compost, CC residue and digestate amendments AOA gene copy numbers were 2- to 10-fold higher than for AOBs. This is contrast with the sewage sludge treatments, which at low amendment led to higher

numbers of AOA, whereas AOBs showed a 2- to 4-fold higher abundance at high organic amendment (Supplementary Table 2).

The abundance of N-fixers in the cut and powdered CC residue mixture increased in the application with 20 t ha^{-1} by 3- and 6-fold, respectively (Figure 7E). The only other treatment with a positive effect on the abundance of *nifH* was the sewage sludge + compost 2 amendment, which showed an increase of approximately 3-fold.

The methanogenic abundance did not changed for both cover crop treatments, but increased 3-fold for compost1, 5-fold for sewage sludge+compost1 and between 10 to 14-fold for the remaining organic amendments at high rates of application while at low rates *mcrA* gene abundance stayed stable (Figure 7F).

Gene copy number of methanotrophs (*pmoA*) increased for all samples with 20 t ha^{-1}, except in the digestate amendment, in which no differences to un-amended soil were reported (Figure 7G). The compost2 amendment and the combination with compost2 showed the strongest effect on the copy numbers with a 4- to 6-fold increase. Low organic amendment application rates only showed minor positive effects on the abundance of methanotrophs.

The abundance of the two CC residue amendments at low SM and high organic amendment application behaved very similar for all analyzed genes (Supplementary Table 3). The abundance of the archaeal 16S rRNA gene and archaeal *amoA* dropped by 2-fold, while it stayed stable for *nifH*, *mcrA* and *pmoA*. A 5-fold increase was observed for the fungal 18S rRNA gene and *nosZ* clade I for the cut CC residues, while the powder led to a 3- and 2-fold increase, respectively. *nosZ* clade II numbers increase for both CC residue materials around 3-fold. While the cut CC residue material resulted in a 2-fold increase for the bacterial 16S rRNA gene and the bacterial *amoA*, the powdered CC residue material did not show a change for these two genes.

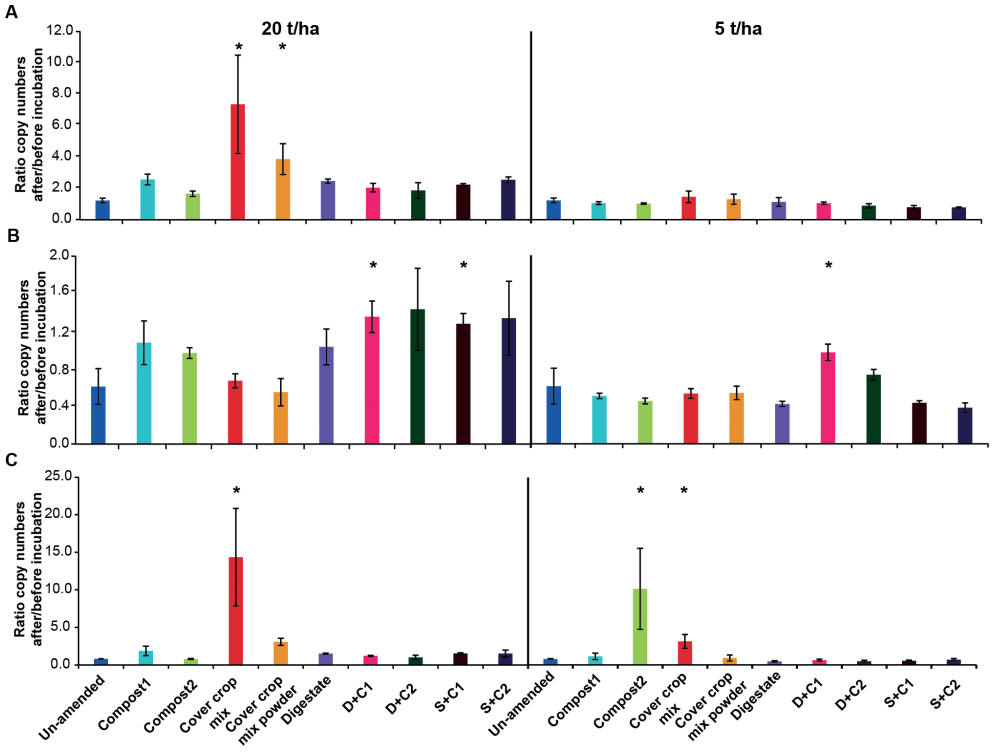

Figure 6. Ratio of the copy numbers of (A) bacterial 16S rRNA gene, (B) archaeal 16S rRNA gene and (C) fungal 18S rRNA gene after and before an incubation of un-amended clay soil and during amendments with compost1, compost2, CC residues mixtures, digestate, digestate+compost1, digestate+compost2, sewage sludge+compost1 and sewage sludge+compost2 (mean ± SD; n = 3) for 28d. Asterisk (*) indicate significant differences in the ratio of the individual genes in the soils with organic amendments and the un-amended soil within the four separate superordinate treatments (ANOVA: P value < 0.05).

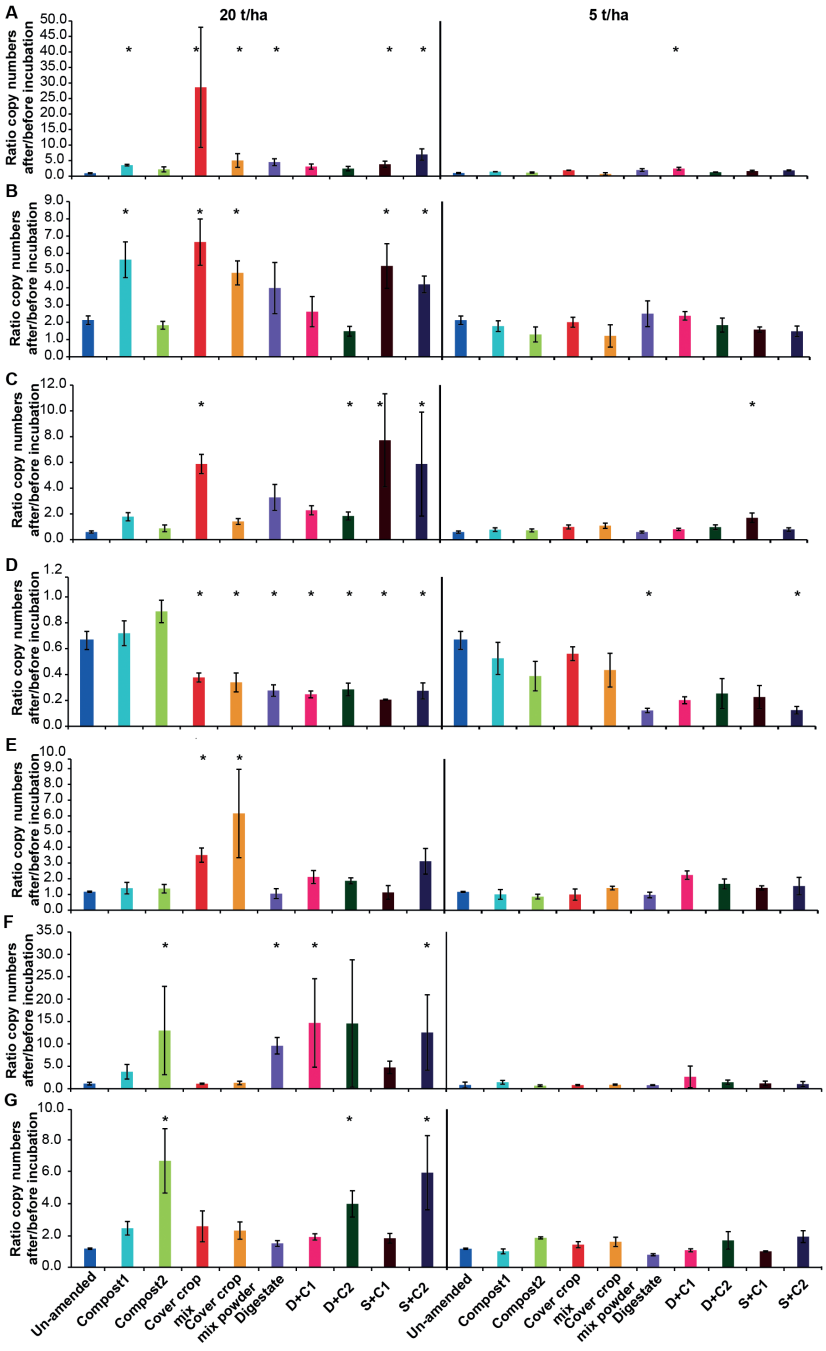

Figure 7. Ratio of the copy numbers of the functional marker genes (A) *nosZ* clade I, (B) *nosZ* clade II, (C) bacterial *amoA*, (D) archaeal *amoA*, (E) *nifH*, (F) *mcrA* and (G) *pmoA* after and before an incubation of un-amended clay soil and during amendments with compost1, compost2, CC residues mixtures, digestate, digestate+compost1, digestate+compost2, sewage sludge+compost1 and sewage sludge+compost2 (mean ± SD; n = 3) for 28d. Asterisk (*) indicate significant differences in the ratio of the individual genes in the soils with organic amendments and the un-amended soil within the four separate superordinate treatments (ANOVA: P value < 0.05).

Discussion

In this study, we investigated the influence of combinations of organic amendments on the GHG balance and the CH_4 uptake as well as on dynamics of different soil microbial groups that are involved in producing or reducing GHGs in agricultural soil. Several combinations of amendments led to reductions of CO_2, CH_4 and/or N_2O emissions compared to un-amended soil. Most optimal GHG balance was obtained by compost amendments, which resulted in similar overall GHG balance as compared to the un-amended soil. Additionally, we could observe a strong increase in microbial communities involved in GHG consumption in all amendments, with the strongest increase associated with cover crop residue mixtures.

GHG dynamics and GWP in relation to different organic amendments and manipulation of soil moisture

CH_4

We did not observe significant uptake of CH_4 in any of our samples except for digestate (D), D+C2, S+C1 at high SM and high application rate, which led to CH_4 uptake over the complete incubation period (Figure 1). However, the *in vitro* methane uptake capacity at near atmospheric (i.e. 10ppm) methane concentrations increased in all samples over time. As proposed by Ho *et al.* (2015a), it seems that the methanotrophic community needs elevated methane to gear up the enzyme machinery. A similar result was found in rice soils where high methane concentration spikes were necessary to induce atmospheric methane uptake (Cai *et al.*, 2016). Especially the 5 t ha^{-1} compost2 treatment under 40% SM showed a very strong improvement in CH_4 uptake at the end of the incubation. Potentially, the release of rare earth metals (e.g. La, Ce, Nd), which are stored in the compost (La ~ 2.2 µg g^{-1}; Ce ~ 3.8 µg g^{-1}; Nd ~ 2.2 µg g^{-1} (El-Ramady, 2011)) stimulated the CH_4 uptake (Keltjens *et al.*, 2014). Recent studies found that for some methanotrophs rare earth metals are essential as cofactors in the active center of an alternative methanol dehydrogenase (Keltjens *et al.*, 2014; Pol *et al.*, 2014; Shiller *et al.*, 2017). Furthermore, it was shown that the La-dependent methanol dehydrogenase can also be more efficient hydrolytic catalysts because they are stronger Lewis acids (electrophilic electron acceptor) than the Ca dependent one (Lim and Franklin, 2004). This La-dependent methanol dehydrogenase which can also be found in the newly isolated atmospheric CH_4 oxidizer belonging to the USCα cluster (Pratscher *et al.*, 2018). However, all studies with rare earth metals and their effect on CH_4 oxidation were performed in liquid cultures. Another possible explanation for the increase in CH_4 oxidation rates at the end of the incubation in compost2 incubations, could be its relatively low C- and N-content in comparison to the other organic amendments. This could lead to higher amounts of essential substrates (O_2) or lower amounts of inhibiting compounds (e.g. NH_4^+) for methane oxidation (Conrad

and Rothfuss, 1991; Bender and Conrad, 1992; Malyan *et al.*, 2016). In contrast, the higher amount of C- and N-compounds in the other organic amendments could result in a reduced or delayed start of CH_4 oxidation. Furthermore, it is known that compost could lead to an increase in the soil's cation binding capacity (Epstein, Taylor and Chaney, 1976), leading to lowering of the availability of ammonium ions, potentially inhibiting the particulate methane monooxygenase (Singh and Seneviratne, 2017).

CO_2

The first addition of water induced a direct emission of CO_2 from the soil in samples with organic amendments. The extend of these CO_2 emissions is strongly dependent on the amendment used. The lowest CO_2 emissions were obtained with both compost amendments, showing similar values as the un-amended soil, where the fungal based compost emitted less CO_2 than the compost from green cut materials (Table 2). The reason for this could be a low total C concentration together with not easily degradable C-compounds (Ryals *et al.*, 2015). Based on this it seems that the different material of the compost can contribute better or worse to reducing GHG, which would need further analyses.

The decrease respiration in organic matter added through the experiment did not correlate with most of the CO_2 fluxes. Only the CO_2 fluxes under moist conditions (R_2=0.633) and high amount of organic amendment (R_2=0,783) correlated with the decrease of organic matter. This is in accordance with our previous study (Ho *et al.*, 2017b), demonstrating that C:N alone is not a good predictor of amendment effects on GHG fluxes. In this study, the organic amendment with the highest C:N ratio was the fungal based compost which showed the lowest measurable CO_2 fluxes of all organic amendments. However, the highest measured CO_2 fluxes were emitted by both CC residue mixtures which indeed have the second highest C:N ratio. We observed a correlation between the total C concentration measured in the organic amendments and the CO_2 fluxes. The quality and composition of the amendments, seem to be more important for influencing the CO_2 fluxes. For example the sewage sludge+compost2 amendment has the same total C-content as compost1, but emitted 4-fold higher CO_2 fluxes. In accordance with this, digestate has a lower total C concentration compared to CC residue material, but emitted 15-fold less CO_2. One explanation is that the digestate is not as easily degradable as the plant material for the microorganisms, since its origin is already anaerobically digested manure. It was already shown that CO_2 respiration from digestate is highly dependent on the initial source from which the digestate is produced, which led to a broad range of CO_2 respiration rates (Alburquerque, de la Fuente and Bernal, 2012). According to our results, this statement can be extended to a variety of organic amendments.

Surprisingly, we saw a second peak of increased CO_2 emission after 21d in almost all treatments. This may be explained by the fact after 14d substrates which are more difficult to degrade are reduced to a more accessible form of shorter chain molecules. Succession in microbial community composition may be involved which can take place in just a short period of time (13-15d) following amendment with organic residues as shown by Ho *et al.* (2017b). Additionally, changes in soil parameters (e.g. O_2 availability, N availability) may cause a second phase of CO_2 respiration due to alleviation of initial limitations.

N_2O

Surprisingly, the highest N_2O fluxes were not observed from the N richest organic amendment (digestate), but from the combinations of sewage sludge with compost, followed by the CC residue mixtures (Table 2). Hence, the N_2O emission is not only depending on the N-content of the organic amendments, but also in which form the N-source is provided to the microorganisms. These observations are similar to our findings of the relatively weak correlation between C-content and CH_4 / CO_2 fluxes. Additionally, we could not find any correlation of C:N or C-content to N_2O fluxes (data not shown). Contrary to a recent study we also did not observe a linear relation between N fertilization and N_2O emission (Shcherbak, Millar and Robertson, 2014) in a study where all soil and environmental parameters were kept stable.

Only in case of the high organic amendment application, we observed a second N_2O flux peak after 21d of incubation. In these incubations, the existing input of fresh N through the organic amendments was probably already processed and either turned into gaseous N, microbial N or remains in refractory form. The microbial biomass or refractory N may release ammonium by mineralization, but this may take more time explaining the temporal pattern observed. Another explanation may be that the soil parameters changed and stimulated the production of N_2O again (e.g. through more anoxic zones). The results of the abundance analyses from these samples (Supplementary Table 3) revealed a strong increase of fungi in these samples, which could be causing the observed N_2O production in our incubations. Fungi are known for possessing denitrification genes to produce N_2O, but as yet have not been demonstrated to harbor N_2O-reductase gene (Takaya, 2002; Shoun *et al.*, 2012). It was also shown that denitrifying fungi already prefer drier conditions than denitrifying bacteria (Chen, Mothapo and Shi, 2015a). Additionally, since a SM of 40% normally does not favor denitrification processes (Dijk, Ball and Skiba, 2002; Bateman and Baggs, 2005), changes in soil structure or chemistry (e.g. pH, O_2 availability, aggregate composition) could have occurred leading to 'hotspots' of N_2O production as proposed to be responsible for local, temporary high denitrification activity (Groffman *et al.*, 2009).

The water content has a more pronounced influence on the N_2O emission than on the CH_4 and CO_2 fluxes. At low SM almost no N_2O emission was detected. Since high SM reduces

O_2 availability and gas diffusivity and therefore will favor denitrification (Dijk, Ball and Skiba, 2002), it can be assumed that in our incubation denitrification processes are the main source of N_2O production. It was already observed in other studies that an increasing SM led to an increase of N_2O production by denitrification (peak above 65% water-filled pore space), since the optimal SM concentration for nitrification peaks at around 55-65% water-filled pore space (Bateman and Baggs, 2005; Vargas *et al.*, 2014; Sanz-Cobena *et al.*, 2016, p.). Contrary to this, the high amount of CC residue mixtures showed a strong increase in N_2O emission at a low SM (Figure 4) just after 15d. Even more surprising was the uptake of N_2O after 28d for the powdered CC residue mixture. This can either be caused by the high concentrations of N_2O stimulating N_2O reducers, or by a change in the soil characteristics (e.g. pH, O_2 availability). Growth of fungi, which occurred in the CC residue bottles after some days of incubation, could also increase production of N_2O activating the N_2O-reducing community in the soil. It was shown recently that through application of plant residues, hotspots of N_2O emission can occur, by enhanced water absorption from the plant residues which will lead to reduced O_2 concentrations in the surrounding (Kravchenko *et al.*, 2017). Combined with mineralized N and fungal growth this could explain the N_2O peak caused by CC residues. To our knowledge this is the first time that such a behavior of N_2O emission/consumption was observed after applying crop residues to the soil. More studies that confirm these results need to be conducted in the future.

Abundance of microorganisms in relation to GHG fluxes and organic amendment application

Microbial dynamics following application of organic amendments clearly offers scope for modulating functional groups involved in consumption of GHGs. In this light, the CC residues materials showed the best results, by increasing the abundance of the denitrifiers (*nosZ)*, methanotrophs (*pmoA)* and nitrogen fixers (*nifH)* genes, while only moderately increasing the nitrifiers (AOB) and methanogens (*mcrA)*. This could be either through the introduction of microbes already present in the organic amendments or stimulation of growth from indigenous microorganisms harboring these genes. Here, the effect is highly related to the amount of organic amendment applied to the soil. Small amounts of organic amendments have only a minor effect on the different microbial groups, which is also in accordance with the distinct lower GHG flux measurements from these incubations. On the opposite site, organic amendments cannot only increase the gene copy numbers, but can also lead to a decrease of microbial groups (AOA) in comparison to an un-amended soil.

The overall bacteria and fungi abundance correlate quite well with the CO_2 respiration rates (R_2=0.942/ R_2=0.858, respectively). The strong increase, especially in the CC residue application in bacterial and fungal abundance, could mainly occur due to the high application rate of the CC residue in our experiment. Normally, around 4-6-fold lower amounts of CC residues are plowed under in the field after the winter (Marinari *et al.*, 2015;

Coombs *et al.*, 2017). However, we observe also an increase in the fungal abundance at the low amount of applied CC residues, which is comparable to recent studies (Maul *et al.*, 2014).

The differences in abundance of the different groups are highly influenced by the different organic amendments that are used. For example, the application with the fungi based compost has a great effect (7-fold increase) on the abundance of the methanotrophs, compared to the green cut compost material which (like the other organic amendments) had only a doubling effect on the abundance of methanotrophs. Like mentioned before, a stimulation of rare-earth metal-dependent methanotrophs, which harbor the *XOXF* dependent methanol dehydrogenase gene, in these samples could be a possible explanation (Gu and Semrau, 2017; Krause *et al.*, 2017). However, in a previous study (Ho *et al.*, 2015a) USCα *pmoA* sequences, which are known to poses the XOXF enzyme and is capable of atmospheric CH_4 oxidation, was not detected in soil samples from the same location. This would rather support the hypotheses that the increase in *pmoA* copies is due to the introduction of methanotrophs by the organic amendment.

In contrast to the methanotrophic community, we observe more distinct differences of the effect of organic amendments on the methanogenic abundance. Especially organic amendments (compost and digestate) that undergo a treatment in which anoxic habitats are formed to provide a perfect environment for methanogens (Hellmann *et al.*, 1997; Alburquerque, de la Fuente and Bernal, 2012). Especially, CC residue amendment increased the ratio of methanotrophs to methanogens, which can harbor a positive effect on the ratio of CH_4 consumption to CH_4 production (Conrad, 2007).

In our soil the newly found *nosZ* clade II (Jones *et al.*, 2013) is 10- to 100-fold more abundant than *nosZ* clade I. While clade I is mainly associated with soil type (clay), nutrient status, total organic carbon, organic matter or C:N ratio, it is unclear what the drivers for the abundance of clade II in soils are (Highton *et al.*, 2016; Hallin *et al.*, 2018). Our soil is a clay soil, which would be expected to show a higher correlation to *nosZ* clade I bacteria, but instead we see a clear preference of N_2O-reducers with a *nosZ* clade II gene. We think that the differentiation between the two clades cannot be broken down to just one or two single soil characteristics. More knowledge about the ecology of *nosZ* clade II bacteria, which seem to be the major drivers for soil N_2O sink capacity (Jones *et al.*, 2014; Domeignoz-Horta *et al.*, 2016a), is necessary. This knowledge may be used to design strategies to enrich agricultural soils either directly with *nosZ* clade II microorganisms or using amendments that are rich in these denitrifiers. In our study almost all organic amendments had a stimulating effect on the two *nosZ* clades. The rise in N_2O production may have stimulated the N_2O-reducers during the incubation (Hallin *et al.*, 2018).

The archaeal 16s rRNA gene and archaeal *amoA* are the only two genes that are decreasing during the incubation. For archaea and especially the AOA inside the archaea kingdom it was already shown that they are more affected by rewetting stress compared to bacteria and AOB (Conrad *et al.*, 2014; Thion and Prosser, 2014). The decrease in the archaeal *amoA* seem to be higher with the addition of either CC residues, digestate or sewage sludge to the soil (Figure 7). Potentially, the high N-content in these organic amendments, along with the high water level is known to favor denitrification processes (Dijk, Ball and Skiba, 2002). Furthermore, it is believed that the addition of fertilizer normally lead to an increase in the AOB/AOA ratio (Wertz, Leigh and Grayston, 2012; Hartmann *et al.*, 2013; Kastl *et al.*, 2014), since it was shown that AOB grow faster after the addition of fertilizer, this may also be true for our study. Even though a recent study showed that this effect is not occurring in every occasion by showing that AOA and AOB had changed in the same way during an incubation (Orellana *et al.*, 2018).

It is not surprising that the treatments with CC residues harbored the highest abundance of N-fixing bacteria, since 1/3 of the CC residues mixtures we added were legumes (Sprent, Ardley and James, 2017). N-fixers cannot directly be linked to a GHG production or consumption, but can have an indirect effect on N_2O production by converting N_2 to NH_4 which then can be consumed by nitrifiers in the soil (Galloway *et al.*, 1995).

Conclusion

In our study we analyzed different organic amendments and their influence on the GWP as well as functional microbial groups which are involved in GHG transformations in an agricultural soil. Our results indicate that compost amendments perform best with respect to the soil GWP calculated from the three major GHGs (CH_4, CO_2, N_2O) and have a similar GWP as the un-amended soil (Table 2). Combinations of sewage sludge and digestate with both composts have also moderate effects on the soil GWP and will provide higher nutrients supply for plants. Although CC residues had the least favorable GWP, it still harbors a great long-term benefit to reduce GHG emissions from agricultural soils in manipulating the microbial communities. The CC residue amendment increased microbial groups that are involved in the reduction of GHGs (N_2O-reducers, methanotrophs) or keeping the producing microbial community stable (methanogens, nitrifiers) compared to other organic amendments and the un-amended soil. This could provide a better GWP in the long-term. The next step would be to study the effect of plants on the GWP and have a deeper investigation of the associated microbial communities that are involved in GHG consumption and perform a longer running long-term incubation experiment to verify the short-term results. Further well-aerated agricultural soils need to be investigated in their potential as a sink for CH_4, especially in combination with organic fertilizers and the potential of rare earth metals in these organic amendments. Understanding the

underlying mechanisms of how organic fertilizers influence and possibly decrease GHG would allow us to develop a strategy to reduce GHG emission from agricultural soils without affecting the plant yield.

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Supplementary

Supplementary Tables

Supplementary Table 1. Primers and PCR conditions used to amplify fragments of functional marker genes nosZI/II, archaeal amoA, bacterial amoA, nifH, pmoA, archaeal and bacterial 16S rRNA genes as well as fungal 18S rRNA gene by qPCR.

Gene	Primer sets	Forward primer	Reverse primer	PCR conditions	PCR product length (bp)	References
nosZI	nosZ2F/ nosZ2R	CGCRACGGCAAS AAGGTSMSSGT	CAKRTGCAKSG CRTGGCAGAA	95 °C/5min, 45 cycles (95°C/10sec, 64°C/10sec, 72°C/20sec), 65 to 95°C (+0.5°C/ sec) for denaturation curve.	267	(Henry *et al.*, 2006)
nosZII	nosZ-II-F/ nosZ-II-R	CTIGGICCIYTKCAYAC	GCIGARCARAA ITCBGTRC	95 °C/15min, 40 cycles (95°C/15sec, 54°C/30sec, 72°C/30sec, 80°C/15sec), 65 to 95°C (+0.5°C/sec) for denaturation curve.	745	(Jones *et al.*, 2013)
Archaeal *amoA*	Arch-amoAF/ Arch-amoAR	STAATGGTCTGG CTTAGACG	GCGGCCATCCA TCTGTATGT	95 °C/15min, 40 cycles (95°C/10sec, 64°C/10sec, 72°C/20sec), 65 to 95°C (+0.5°C/ sec) for denaturation curve.	635	(Francis *et al.*, 2005)
Bacterial *amoA*	amoA1F/ amoA2R	GGGGTTTCTACT GGTGGT	CCCCTCKGSAAA GCCTTCTTC	95 °C/10min, 40 cycles (95°C/10sec, 65°C/25sec, 72°C/30sec), 65 to 95°C (+0.5°C/ sec) for denaturation curve.	491	(Rotthauwe, Witzel and Liesack, 1997)
nifH	PolF/ PolR	TGCGAYCCSAAR GCBGACTC	ATSGCCATCATY TCRCCGGA	95 °C/2min, 40 cycles (95°C/5sec, 54°C/10sec, 72°C/20sec), 60 to 95°C (+1°C/ sec) for denaturation curve.	360	(Poly, Monrozier and Bally, 2001)
mcrA	mlas/ mcrA-rev	GGTGGTGTMGGD TTCACMCARTA	CGTTCATBGCGTA GTTVGGRTAGT	95 °C/3min, 40 cycles (95°C/10sec, 60°C/10sec, 72°C/25sec), 65 to 95°C (+1°C/ sec) for denaturation curve.	645	(Steinberg and Regan, 2008)
pmoA	A189/ Mb661R	GGNGACTGGGA CTTCTGG	CCGGMGCAAC GTCYTTACC	95 °C/3min, 45 cycles (95°C/10sec, 58°C/15sec, 72°C/25sec, 82°C/10sec), 70 to 99°C (+1°C/sec) for denaturation curve.	472	(Costello and Lidstrom, 1999)
Archaeal 16S rRNA gene	915/ 1017r	AGGAATTGGCGG GGGAGCAC	GGCCATGCACC WCCTCTC	95 °C/10min, 40 cycles (95°C/10sec, 60°C/10sec, 72°C/20sec), 65 to 95°C (+0.5°C/ sec) for denaturation curve.	112	(Klindworth *et al.*, 2013)
Bacterial 16S rRNA gene	Eub338/ Eub518	ACTCCTACGGGA GGCAGCAG	ATTACCGCGG CTGCTGG	95 °C/3min, 40 cycles (95°C/10sec, 53°C/10sec, 72°C/25sec), 60 to 95°C (+1°C/ sec) for denaturation curve.	180	(Fierer *et al.*, 2005)
Fungal 18S rRNA gene	FF390/ FR1	CGATAACGAAC GAGACCT	AICCATTCAAT CGGTAIT	95 °C/2min, 40 cycles (95°C/5sec, 52°C/10sec, 72°C/20sec), 65 to 95°C (+0.5°C/ sec) for denaturation curve.	390	(Vainio and Hantula, 2000)

Supplementary Table 2. Abundance analyses measured with qPCR of the overall bacterial, archaeal and fungal community as well as of several functional marker genes (amoA, nosZ, nifH, pmoA) that are involved in producing or reducing GHG in soil, under a soil moisture content of 65%. (n = 3 ± STD)

Treatment	Archaeal l6S rRNA	Bacterial 16S rRNA	Fungal 18S rRNA	Archaeal *amoA*	Bacterial *amoA*	*nifH*	*nosZ clade I*	*nosZ clade II*	*mcrA*	*pmoA*
Initial soil	2.5E+08 ± 5.1E+07	1.8E+09 ± 2.2E+08	5.5E+06 ± 9.0E+05	1.4E+08 ± 2.6E+07	1.4E+07 ± 3.4E+06	6.8E+06 ± 2.9E+06	1.0E+06 ± 2.2E+05	2.4E+07 ± 5.0E+06	1.2E+05 ± 1.9E+04	1.3E+06 ± 3.1E+05
Un-amended	1.5E+08 ± 4.7E+07	2.2E+09 ± 2.7E+08	4.3E+06 ± 1.3E+05	9.5E+07 ± 1.0E+07	8.1E+06 ± 1.2E+06	7.9E+06 ± 2.5E+05	9.4E+05 ± 2.1E+05	5.1E+07 ± 6.0E+06	1.3E+05 ± 1.9E+04	1.6E+06 ± 4.0E+05
High concentrations (20 t/ha)										
Compost1	2.6E+08 ± 5.7E+07	4.6E+09 ± 6.1E+08	1.0E+07 ± 3.5E+06	1.0E+08 ± 1.4E+07	2.4E+07 ± 4.3E+06	9.5E+06 ± 2.5E+06	3.7E+06 ± 2.7E+05	1.4E+08 ± 2.5E+07	4.4E+05 ± 7.6E+04	2.8E+06 ± 4.1E+05
Compost2	2.4E+08 ± 1.4E+07	3.0E+09 ± 3.1E+08	4.4E+06 ± 3.6E+05	1.3E+08 ± 1.2E+07	1.2E+07 ± 3.5E+06	9.2E+06 ± 1.9E+06	2.3E+06 ± 8.3E+05	4.4E+07 ± 5.5E+06	1.5E+06 ± 4.6E+05	6.5E+06 ± 2.0E+06
Cut cover crop	1.6E+08 ± 1.8E+07	1.3E+10 ± 5.7E+09	7.9E+07 ± 3.6E+07	5.4E+07 ± 5.0E+06	8.0E+07 ± 1.0E+07	2.4E+07 ± 3.0E+06	3.0E+07 ± 2.0E+07	1.6E+08 ± 3.2E+07	1.3E+05 ± 1.8E+04	3.3E+06 ± 9.4E+05
Powdered cover crop	1.4E+08 ± 3.6E+07	7.0E+09 ± 1.8E+09	1.7E+07 ± 2.6E+06	4.9E+07 ± 1.0E+07	1.9E+07 ± 3.1E+06	4.2E+07 ± 1.9E+07	5.3E+06 ± 2.3E+06	1.2E+08 ± 1.7E+07	1.5E+05 ± 2.7E+04	2.9E+06 ± 5.3E+05
Digestate	2.5E+08 ± 4.7E+07	4.4E+09 ± 2.4E+08	8.3E+06 ± 3.5E+05	4.0E+07 ± 6.4E+06	4.5E+07 ± 1.4E+07	7.1E+06 ± 2.1E+06	4.7E+06 ± 1.1E+06	9.6E+07 ± 3.6E+07	1.1E+06 ± 9.9E+04	2.0E+06 ± 5.4E+05
Digestate + compost1	3.3E+08 ± 4.1E+07	3.7E+09 ± 4.8E+08	6.7E+06 ± 4.1E+05	3.5E+07 ± 3.7E+06	3.1E+07 ± 4.8E+06	1.4E+07 ± 2.8E+06	3.2E+06 ± 8.4E+05	6.3E+07 ± 2.1E+07	1.9E+06 ± 1.1E+06	2.2E+06 ± 3.6E+05
Digestate + compost2	3.5E+08 ± 1.1E+08	3.4E+09 ± 4.8E+08	5.6E+06 ± 1.6E+06	4.1E+07 ± 7.0E+06	2.5E+07 ± 4.3E+06	1.3E+07 ± 1.3E+06	2.6E+06 ± 7.4E+05	3.6E+07 ± 6.9E+06	1.7E+06 ± 6.7E+05	3.9E+06 ± 6.0E+05
Sewage sludge + compost1	3.1E+08 ± 2.7E+07	4.0E+09 ± 1.2E+08	8.5E+06 ± 4.6E+05	2.9E+07 ± 5.6E+05	1.1E+08 ± 4.9E+07	7.6E+06 ± 2.9E+06	4.0E+06 ± 1.0E+06	1.3E+08 ± 3.1E+07	5.3E+05 ± 6.9E+04	2.1E+06 ± 2.9E+05
Sewage sludge + compost2	3.3E+08 ± 9.6E+07	4.6E+09 ± 5.0E+08	8.4E+06 ± 2.5E+06	3.9E+07 ± 8.9E+06	8.0E+07 ± 5.5E+07	2.1E+07 ± 5.5E+06	7.3E+06 ± 1.9E+06	1.0E+08 ± 1.2E+07	1.5E+06 ± 4.8E+05	5.8E+06 ± 2.3E+05

Supplementary Table 2. Continued.

Treatment	Archaeal l6S rRNA	Bacterial 16S rRNA	Fungal 18S rRNA	Archaeal *amoA*	Bacterial *amoA*	*nifH*	*nosZ clade I*	*nosZ clade II*	*mcrA*	*pmoA*
Low concentrations (5 t/ha)										
Compost1	1.2E+08 ± 7.0E+07	1.9E+09 ± 1.6E+08	6.2E+06 ± 2.4E+06	7.5E+07 ± 1.8E+07	1.1E+07 ± 1.9E+06	6.8E+06 ± 2.1E+06	1.4E+06 ± 2.9E+04	4.3E+07 ± 7.5E+06	1.5E+05 ± 3.7E+04	1.4E+06 ± 3.8E+05
Compost2	1.1E+08 ± 7.4E+07	1.8E+09 ± 7.1E+07	5.6E+07 ± 3.0E+07	5.6E+07 ± 1.6E+07	9.9E+06 ± 1.5E+06	5.8E+06 ± 1.0E+06	1.1E+06 ± 2.0E+05	3.1E+07 ± 1.0E+07	2.4E+05 ± 5.0E+04	1.7E+06 ± 1.0E+05
Cut cover crop	1.3E+08 ± 1.3E+07	2.6E+09 ± 6.6E+08	1.7E+07 ± 5.2E+06	8.0E+07 ± 7.6E+06	1.4E+07 ± 2.2E+06	6.8E+06 ± 2.4E+06	1.9E+06 ± 3.2E+04	4.8E+07 ± 6.9E+06	1.2E+05 ± 2.2E+04	1.7E+06 ± 4.0E+05
Powdered cover crop	1.3E+08 ± 1.7E+07	2.4E+09 ± 5.9E+08	5.1E+06 ± 2.2E+06	6.2E+07 ± 1.9E+07	1.5E+07 ± 2.8E+06	9.5E+06 ± 7.8E+05	6.8E+05 ± 4.0E+05	2.9E+07 ± 1.6E+07	1.3E+05 ± 2.3E+04	1.8E+06 ± 5.4E+05
Digestate	1.0E+08 ± 6.9E+07	2.0E+09 ± 4.5E+08	2.6E+06 ± 4.0E+05	1.8E+07 ± 2.4E+06	8.2E+06 ± 1.0E+06	6.6E+06 ± 1.2E+06	2.0E+06 ± 4.7E+05	6.0E+07 ± 1.8E+07	4.4E+05 ± 1.5E+05	1.1E+06 ± 6.7E+04
Digestate + compost1	2.4E+08 ± 2.1E+07	1.9E+09 ± 1.4E+08	3.5E+06 ± 6.9E+05	2.9E+07 ± 3.9E+06	1.1E+07 ± 1.2E+06	1.5E+07 ± 1.8E+06	2.4E+06 ± 4.7E+05	5.7E+07 ± 6.0E+06	2.3E+05 ± 2.7E+04	1.4E+06 ± 9.4E+04
Digestate + compost2	1.8E+08 ± 1.3E+07	1.6E+09 ± 2.5E+08	2.7E+06 ± 6.9E+06	3.6E+07 ± 1.7E+06	1.3E+07 ± 2.5E+06	1.1E+07 ± 2.1E+06	1.2E+06 ± 1.0E+05	4.4E+07 ± 9.8E+06	2.1E+05 ± 5.5E+04	1.7E+06 ± 5.3E+05
Sewage sludge + compost1	1.1E+08 ± 5.4E+07	1.4E+09 ± 2.0E+08	3.0E+06 ± 5.3E+05	3.2E+07 ± 1.3E+06	2.3E+07 ± 5.1E+06	9.6E+06 ± 9.0E+05	1.6E+06 ± 2.9E+05	3.8E+07 ± 3.7E+06	1.7E+05 ± 3.5E+04	1.3E+06 ± 2.9E+04
Sewage sludge + compost2	9.4E+07 ± 1.2E+07	1.4E+09 ± 6.2E+07	3.9E+06 ± 8.3E+05	1.8E+07 ± 4.3E+06	1.1E+07 ± 1.8E+07	1.0E+07 ± 3.7E+06	1.8E+06 ± 1.7E+05	3.6E+07 ± 7.2E+06	2.8E+05 ± 5.5E+04	1.7E+06 ± 3.6E+05

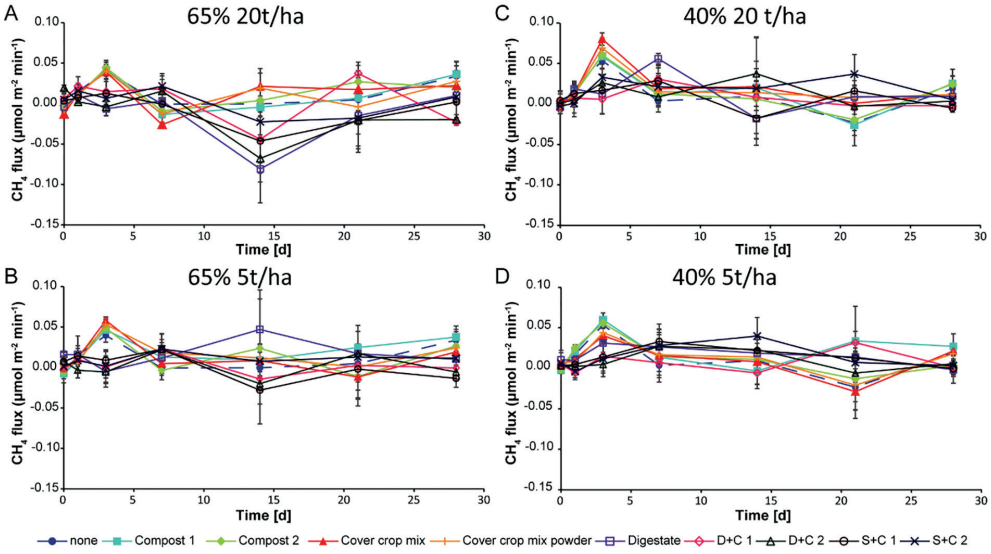
Supplementary Table 3. Abundance analyses measured with qPCR of the overall bacterial, archaeal and fungal community as well as of several functional marker genes (amoA, nosZ, nifH, mcrA, pmoA) that are involved in producing or reducing GHG in soil, under a soil moisture content of 40% and high concentration (20 t/ha) of the OA cover crop. (n = 3 ± STD)

Treatment	Archaeal l6S rRNA	Bacterial 16S rRNA	Fungal 18S rRNA	Archaeal *amoA*	Bacterial *amoA*	*nifH*	*nosZ clade I*	*nosZ clade II*	*mcrA*	*pmoA*
Cut cover crop	2.2E+08 ± 3.1E+07	4.9E+09 ± 2.0E+08	2.8E+07 ± 2.6E+06	8.8E+07 ± 1.7E+07	2.9E+07 ± 4.7E+06	8.4E+06 ± 3.7E+06	5.3E+06 ± 1.4E+06	7.5E+07 ± 1.7E+07	1.5E+05 ± 3.5E+04	2.5E+06 ± 7.2E+05
Powdered cover crop	1.3E+08 ± 2.8E+07	3.2E+09 ± 5.6E+08	2.0E+07 ± 3.2E+06	9.3E+07 ± 2.2E+07	1.3E+07 ± 2.2E+06	1.0E+07 ± 3.7E+06	2.1E+06 ± 6.4E+05	6.2E+07 ± 1.7E+07	1.3E+05 ± 3.1E+04	1.6E+06 ± 6.0E+05

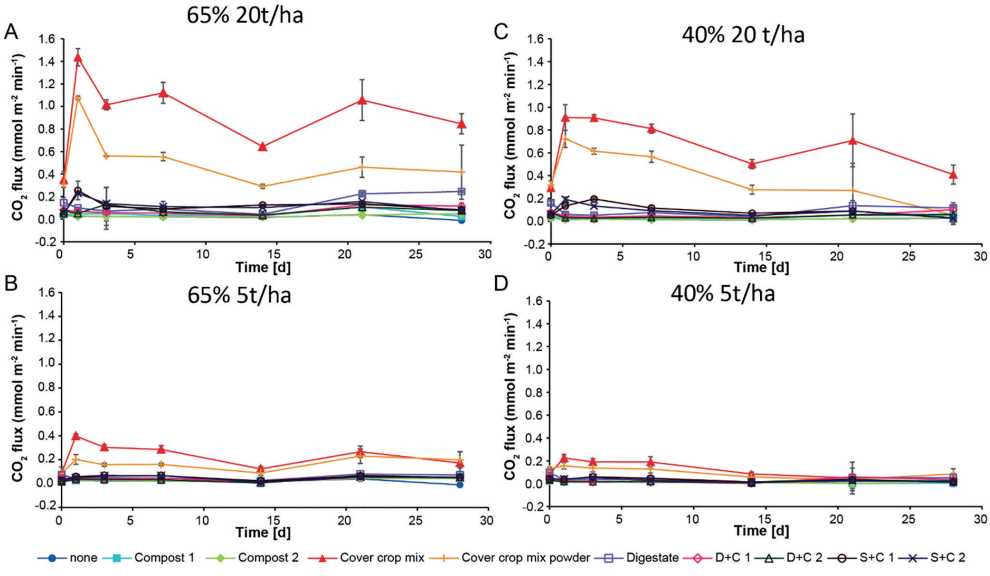
Supplementary Table 4. Ratio of the abundance, measured with qPCR, of the initial microbial soil community and the microbial community after a 28d incubation based on the overall bacterial, archaeal and fungal community as well as of several functional marker genes (amoA, nosZ, nifH, mcrA, pmoA) that are involved in producing or reducing GHG in soil, under a soil moisture content of 40% and high concentration (20 t/ha) of the OA cover crop. Different letters indicate significant differences in the ratio of the individual genes (ANOVA: P value < 0.05). (n = 3 ± STD)

Treatment	Archaeal l6S rRNA	Bacterial 16S rRNA	Fungal 18S rRNA	Archaeal *amoA*	Bacterial *amoA*	*nifH*	*nosZ clade I*	*nosZ clade II*	*mcrA*	*pmoA*
Cut cover crop	0.892 a ± 0.122	2.744 a ± 0.334	4.995 a ± 0.574	0.615 a ± 0.153	2.092 a ± 0.473	1.249 a ± 0.261	5.102 a ± 0.908	3.131 a ± 0.462	1.268 a ± 0.114	1.826 a ± 0.263
Powdered cover crop	0.537 a ± 0.112	1.764 b ± 0.075	3.544 a ± 0.666	0.647 a ± 0.178	0.965 a ± 0.217	1.565 a ± 0.240	2.048 b ± 0.473	2.582 a ± 0.650	1.102 a ± 0.084	1.058 a ± 0.267

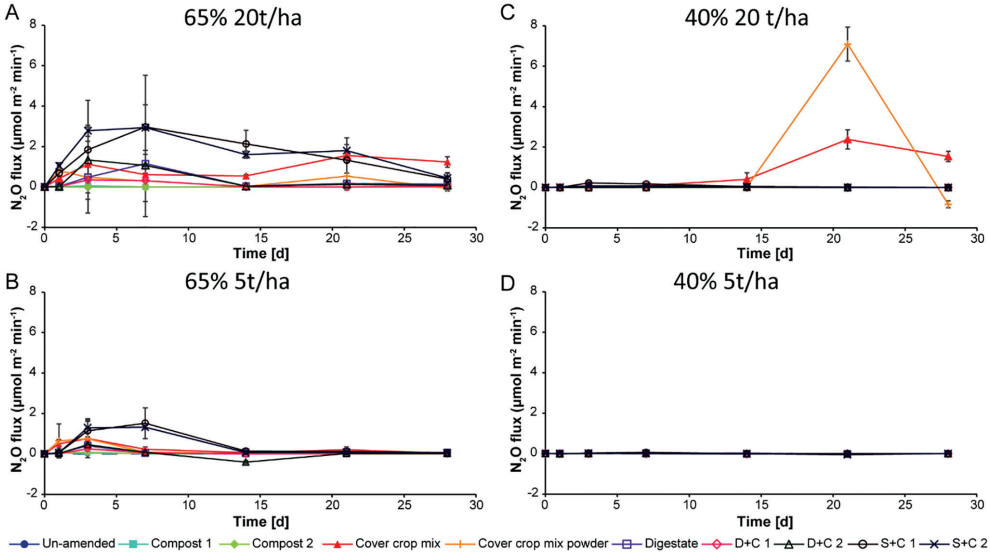
Supplementary Figures



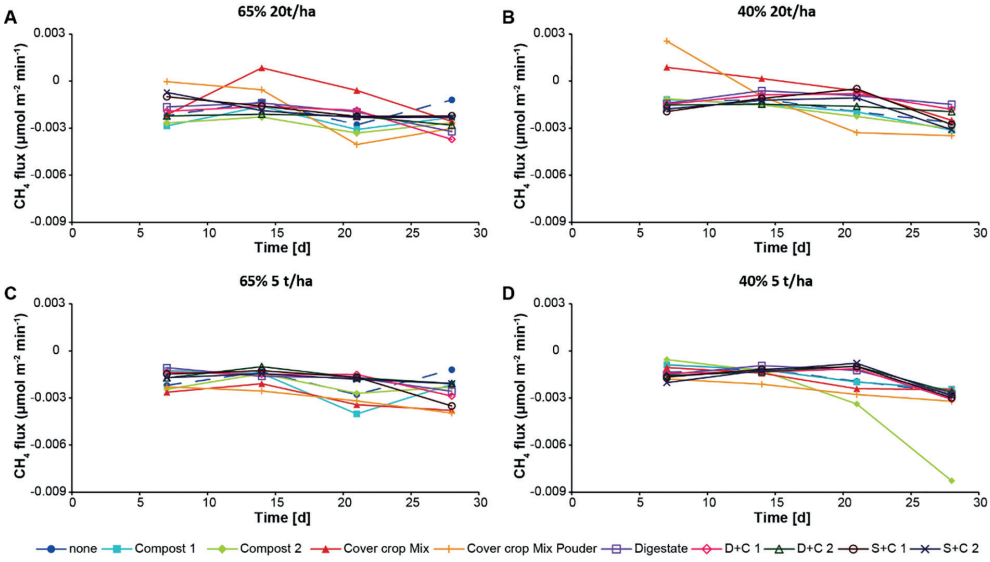
Supplementary Figure 1. Methane flux over the period of 28d in un-amended clay soil and after amendments with compost1, compost2, cover crop mixtures, digestate, digestate+compost1, digestate+compost2, sewage sludge+compost1 and sewage sludge+compost2 (mean ± SD; n = 3) at (A) high amount (20 t/ha) and high water content (65%), (B) low amount (5 t/ha) and high water content, (C) high amount and low water content (40%) and (D) low amount and low water content. Methane flux measurements were performed under ambient gas concentrations.



Supplementary Figure 2. Carbon dioxide flux over the period of 28d in un-amended clay soil and after amendments with compost1, compost2, cover crop mixtures, digestate, digestate+compost1, digestate+compost2, sewage sludge+compost1 and sewage sludge+compost2 (mean ± SD; n = 3) at (A) high amount (20 t/ha) and high water content (65%), (B) low amount (5 t/ha) and high water content, (C) high amount and low water content (40%) and (D) low amount and low water content. Carbon dioxide flux measurements were performed under ambient gas concentrations.



Supplementary Figure 3. Nitrous oxide flux over the period of 28d in un-amended clay soil and after amendments with compost1, compost2, cover crop mixtures, digestate, digestate+compost1, digestate+compost2, sewage sludge+compost1 and sewage sludge+compost2 (mean ± SD; n = 3) at (A) high amount (20 t/ha) and high water content (65%), (B) low amount (5 t/ha) and high water content, (C) high amount and low water content (40%) and (D) low amount and low water content. Nitrous oxide flux measurements were performed under ambient gas concentrations.



Supplementary Figure 4. Methane uptake over the period of 28d in un-amended clay soil and after amendments with compost1, compost2, CC residues mixtures, digestate, digestate+compost1, digestate+compost2, sewage sludge+compost1 and sewage sludge+compost2 (mean ± SD; n = 3) at (A) high amount (20 t/ha) and high water content (65%), (B) low amount (5 t/ha) and high water content, (C) high amount and low water content (40%) and (D) low amount and low water content. Methane uptake were measured after the addition of 10 ppm CH_4 at each sampling point.

CHAPTER

Assessing the role of fungal diversity in decomposition: A meta-analysis

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Abstract

Fungi play an important role in carbon - and nutrient cycling. It is, however, unclear if diversity of fungi is essential to fulfill this role. With this meta-analysis, we aim to understand the relationship between fungal diversity and decomposition of plant materials (leaf litter and wood) in terrestrial and aquatic environments. The selection criteria for papers were the presence of a fungal diversity gradient and quantification of decomposition as mass loss. In total 40 papers met the selection criteria. We hypothesized that increase of fungal species will result in stronger decomposition, especially in species poor communities. Both artificial inoculated and naturally assembled fungal communities were included in the analysis in order to assess whether manipulated experiments are representative for field situations. We found a significant positive effect of increased fungal diversity on decomposition. However, in manipulated experiments this relationship was only positive when a control treatment of one fungus was compared with multispecies communities. This relationship became negative when comparisons of higher initial richness (at least two fungal species as "control") were included. In contrast, under natural field conditions increased fungal diversity coincided with increased decomposition. This suggests that manipulated experiments are not representative for field situations. Possible reasons for this are discussed. Yet, both in manipulated and field experiments, environmental factors can influence diversity – decomposition relationships as indicated by a negative relationship of increasing C:N ratio on the effect of fungal diversity on decomposition. Overall, our results show that fungal diversity can have an important role in decomposition, but that design of experiments (manipulated or field) and quality of the plant material should be taken into account for interpretation of this diversity-functioning relationship.

Introduction

Understanding the consequences of decreasing biodiversity on the functioning of natural ecosystems is one of the highest research priorities in ecological research (Coleman and Whitman, 2005; Lecerf and Richardson, 2010; Delgado-Baquerizo *et al.*, 2017). An important aspect of the biodiversity-functioning relationship is the role of belowground biodiversity on soil functioning such as carbon- and nutrient cycling (Wagg *et al.*, 2014; Delgado-Baquerizo *et al.*, 2016). Saprotrophic fungi are an important group of soil microorganisms involved in decomposition of organic materials and mineral nutrient cycling (van der Wal *et al.*, 2013). It is estimated that 1.5 million fungal species occur worldwide (Hyde *et al.*, 2007). They are abundantly present in (undisturbed) ecosystems like grasslands and forest floors, but also in aquatic systems like streams that receive input of terrestrial organic matter (Grossart *et al.*, 2019). Due to their hyphal growth form and ability to produce a wide range of polymer hydrolyzing and -oxidizing enzymes, saprotrophic fungi have a key role in the degradation of solid, lignocellulose-rich organic materials (van der Wal *et al.*, 2013). The number of fungal species with a predominant saprotrophic lifestyle is tremendous and there are strong differences in their abilities to degrade organic compounds (van der Wal *et al.*, 2013). Yet, the importance of this high taxonomical and functional diversity of saprotrophic fungi for decomposition processes is not well understood.

Diversity-functioning relationships are mostly studied in experimental settings after the inoculation of a limited number of fungal isolates on sterile plant residues. Many of these studies have been executed in aquatic experimental settings with terrestrial leaf litter that under field conditions naturally falls into streams (Duarte *et al.*, 2006; Pascoal *et al.*, 2010; Andrade, Pascoal and Cássio, 2016). Manipulated diversity studies in terrestrial ecosystems are less common and do often involve woody materials (Toljander *et al.*, 2006; Wagg *et al.*, 2014; Hiscox *et al.*, 2016). The diversity gradients range from 1 to a maximum of 16 species, which is a common amount of cultivated fungal species retrieved from aquatic ecosystems (Duarte *et al.*, 2010). However, in terrestrial ecosystems much higher diversity levels are found (Deacon *et al.*, 2006) and with sequencing techniques even more species are detected, but their function and activity are not yet known (Grossart *et al.*, 2019).

Interactions between fungi are an important aspect to be included in the prediction of the effect of increasing diversity on ecosystem functioning. Fungi can compete for the same resources leading to competitive exclusion. This can lead to a reduction of the initial inoculated diversity levels at the end of experiments (Toljander *et al.*, 2006). In wood logs, competitive interactions are visible between wood-rot fungi (zones between fungal species as described by van der Wal *et al.* (2013)). When interactions are neutral, fungal species co-occur in the same environment without exhibiting harmful or beneficial effects. Decomposition rates are not expected to be different from the average decomposition of

each fungus in monoculture (additive effect). Complementarity and facilitation can lead to increased decomposition. For example, Tiunov and Scheu (2005) showed a positive effect by combining cellulolytic fungi and sugar fungi. The production of cellulase supported the consumption of the released sugars by sugar fungi.

Another aspect that can influence the diversity-decomposition relationship is the chemical composition of the material that is decomposed. Fungal species are adapted to the specific composition of the organic materials they decompose. For example, to overcome lignin barriers in complex organic substrates, degradation (white rot fungi) or modification (brown rot fungi) strategies have been evolved in wood decomposition (Mester, Varela and Tien, 2004). Nutrient availability, lignin content and toxic elements can influence wood and litter decomposition as well as the success and outcome of interactions of fungal species during decomposition.

In general, even though interaction effects can have different directions, it is assumed that diversity is important for decomposition (Baerlocher, 2005; Gessner *et al.*, 2010; Hättenschwiler, Fromin and Barantal, 2011). Yet, there is still a debate on the extent of diversity importance as redundancy effects within the community may occur, since many species are able to break down organic matter. We performed a meta-analysis to better understand the relationship between fungal diversity and decomposition. We screened manipulated diversity studies from both aquatic and terrestrial ecosystems including leaf and woody materials. The selection criteria for papers were 1) the presence of an initial fungal diversity gradient and 2) quantification of decomposition as mass loss. Decomposition measured as CO_2 emissions were not included as fungal interactions can affect carbon substrate use efficiencies of individual fungal species leading to difficulties with interpretation of the relationship between respiration and decomposition (Hiscox *et al.*, 2015). The following hypotheses were tested:

1. Diversity increases decomposition as increasing species diversity will lead to increased potential of the community to degrade diverse material (niche differentiation/ complementarity).
2. The diversity effect will be flattened off with increasing species richness (redundancy effect with increasing species richness).
3. The diversity effect is expected to be higher in litter or woody materials with lower C:N ratio as substrates with a higher C:N ratio are more difficult to degrade and will require specialized fungi. These fungi have to invest a lot of energy in their specialization (e.g. lignolytic enzymes) and prevent competition from other microbes by creating unfavorable growth conditions (Boddy and Hiscox, 2016), resulting in decreased fungal diversity in substrates with a high C:N ratio.

4. The diversity effect will be reduced when measuring mass loss at later time points as compared to earlier time points within the same substrate; facilitation/ complementarity is expected to have larger influence during early stages of decomposition as nutrient content is more diverse leading to a possible increased importance of niche differentiation.

Environmental studies that tested decomposition under field conditions were selected as well to understand if the diversity-functioning relationship was different under field conditions with spontaneous developed fungal diversity compared to manipulated fungal diversity in laboratory settings. In these studies, differences in diversity of fungal species are based on differences in natural assembly processes on similar organic starting materials. We therefore additionally hypothesize that:

5. Fungal diversity effects will be larger in field settings compared to experimental settings especially in fungal rich communities. In field settings, natural colonization processes (no artificial inoculation) and changing environmental conditions will result in an increase in possible niches.

Material and methods

Literature search

The two main criteria for selection of papers on fungal diversity – decomposition relationships were: (1) decomposition is based on mass loss as % loss of plant material and (2) fungal diversity (richness) differences have been compared (at least 2 levels of fungal diversity per study). Web of Science was used as database for literature search using the following search words: "fungal diversity" AND ("litter decomposition" OR "wood decomposition"). This yielded 142 papers in Web of Science. Papers were included until the 1st of March 2021. Reference lists of the papers were also checked to include articles that were missed by the literature search in Web of Science. We included laboratory experiments with manipulated fungal diversity levels and environmental experiments where the environment created differences in diversity due to differences in colonization processes in replicates or treatments. Studies that were excluded from the analysis compared/examined: (1) contrasting environments or plant residues, (2) decomposition compared between successional stages of the experiment (no diversity differences between treatments but over time), (3) toxicity effects of metals or other harmful compounds. Based on these criteria, we kept 16 manipulated studies and 24 environmental studies. The list of included studies is shown in Table 1.

Data on decomposition and diversity were extracted from the articles using the online tool WebPlotDigitizer. Data related to plant quality and environment were extracted

as well. As most studies did not measure the quality of the plant material used in the experiments, the TRY-database (Kattge *et al.*, 2020) was used to get an estimation of the C:N ratio of the plant material.

Treatments with similar diversity levels, but different species composition, were not analyzed separately. Such treatments were pooled before the analysis using the formulas: $\%\, mean\ mass\ loss\ = average(\%\ mass\ loss_1 + \%\ mass\ loss_2 + \cdots + \%\ mass\ loss_n)$ and $\text{standard deviation } (\sigma) = sum(\sigma_{\%\ mass\ loss_1} + \sigma_{\%\ mass\ loss_2} + \cdots + \sigma_{\%\ mass\ loss_n})^{\frac{1}{n}}$ where n is the amount of different treatments with the same diversity level. Some environmental papers measured k decomposition rates and not % mass loss. These values were calculated into % mass loss with the formula: $\%\, mass\ loss = 100\% - (100\% * e^{-k*t})$ where k is the decomposition rate and t is the duration of the incubation (in days or years).

Statistical analyses

Data were analyzed with R (version 4.0.3) with attached packages for analyses and visualization: car, carData, plyr, dplyr, grid, gridExtra, cowplot. The packages metafor and forestplot were used to perform the meta-analysis (Viechtbauer, 2010). Individual effect sizes within each study were estimated by calculating the difference between 2 treatments: $D = mass\ loss\ \%_{diverse} - mass\ loss\ \%_{control}$ and $Variance_D = \frac{\sigma^2{}_{diverse}}{n_{diverse}} + \frac{\sigma^2{}_{control}}{n_{control}}$ where n is replicates per treatment (diverse or control) (Makowski, Piraux and Brun, 2019). Within each combination, the lowest diversity level was used as "control". All possible combinations in each study were analyzed leading to multiple effect sizes per study when more than two diversity levels were measured. In total 458 combinations obtained from the 40 selected studies were tested. With a random effect model and the REML method overall effect size was estimated. As most studies had more than one individual effect size (D), we corrected for this within the analysis to prevent overestimation of a single study with more combinations. To determine differences between experimental design (manipulated or field), environment (aquatic or terrestrial) and plant material (litter or wood), these factors were analyzed separately within the analysis. To analyze redundancy effects, an extra factor was added within the manipulated experiments to compare the individual effect size in measured combinations from real control treatments (1 fungal species) and other treatments with a diverse community already (at least 2 fungal species) as lowest diversity level. A forest plot was made to visualize the individual and overall effect sizes and a funnel plot to estimate publication bias (Sterne and Egger, 2001). Regression analysis (with packages ggplot2, ggpubr) was used to assess whether environmental factors and quality of the plant material could explain the magnitude and direction (positive/negative) of individual effect sizes (ES).

Within the environmental studies, seven studies did not analyze treatments, but individual replicates. To estimate the diversity effect on decomposition, linear regression models

were used to estimate r and the variance of the relationship between fungal diversity and % mass loss. To be able to compare this approach with the group design of all other studies, the regression results were transformed into d statistics with $d = \frac{2r}{\sqrt{1-r^2}}$ and $d = \frac{D}{\sqrt{Variance_D}} = \frac{\%\ mass\ loss_{diverse} - \%\ mass\ loss_{control}}{\sqrt{Variance_D}}$ (Nakagawa and Cuthill 2007) where r is the regression coefficient and D is the estimated difference. These estimates of D were added to the other 33 studies to measure the overall effect as described before.

Table 1: List of papers used in meta-analysis

	Paper	Environment	Plant type	Plant species	Study
1	Andrade, Pascoal and Cássio (2016)	Aquatic	Litter	Alnus glutinosa	Manipulated
2	Bärlocher and Corkum (2003)	Aquatic	Litter	Quercus robur	Manipulated
3	Barros *et al.* (2016)	Aquatic	Litter	Alnus glutinosa	Manipulated
4	Costantini and Rossi (2010)	Aquatic	Litter	Alnus glutinosa, Phragmites australis, Quercus cerris	Manipulated
5	Dang, Chauvet and Gessner (2005)	Aquatic	Litter	Alnus glutinosa, Quercus robur	Manipulated
6	Duarte *et al.* (2006)	Aquatic	Litter	Alnus glutinosa	Manipulated
7	Fernandes, Pascoal and Cássio (2011)	Aquatic	Litter	Alnus glutinosa	Manipulated
8	Geraldes, Pascoal and Cássio (2012)	Aquatic	Litter	Alnus glutinosa	Manipulated
9	Gonçalves, Graça and Canhoto (2015)	Aquatic	Litter	Quercus robur	Manipulated
10	Jabiol *et al.* (2013)	Aquatic	Litter	Quercus robur	Manipulated
11	Kivlin and Treseder (2015)	Terrestrial	Litter	Festuca altaica	Manipulated
12	Pascoal *et al.* (2010)	Aquatic	Litter	Tilia cordata	Manipulated
13	Raviraja, Nikolcheva and Baerlocher (2006)	Aquatic	Litter	Tilia cordata	Manipulated
14	Wagg *et al.* (2014)	Terrestrial	Litter	Lolium multiflorum	Manipulated
15	Venugopal *et al.* (2017)	Terrestrial	Wood	Pinus sylvestris	Manipulated
16	Toljander *et al.* (2006)	Terrestrial	Wood	Picea abies	Manipulated
17	Encalada *et al.* (2010)	Aquatic	Litter	Alnus acuminate, Inga spectabilis	Field
18	Ferreira *et al.* (2006)	Aquatic	Litter	Alnus glutinosa, Quercus robur	Field
19	Miura *et al.* (2013)	Terrestrial	Litter	Saccharum officinarum	Field
20	Pascoal, Cassio and Marvanová (2005)	Aquatic	Litter	Alnus glutinosa	Field
21	Pérez, Descals and Pozo (2012)	Aquatic	Litter	Alnus glutinosa	Field
22	Maamri *et al.* (2001)	Aquatic	Litter	Salix pedicellata	Field
23	Lecerf and Chauvet (2008)	Aquatic	Litter	Alnus glutinosa	Field
24	Simon, Simon and Benfield (2009)	Aquatic	Litter	Quercus prinus	Field
25	Tolkkinen *et al.* (2013)	Aquatic	Litter	Alnus incana	Field

Table 1: Continued.

	Paper	Environment	Plant type	Plant species	Study
26	Tolkkinen *et al.* (2015a)	Aquatic	Litter	Alnus incana	Field
27	Mesquita, Pascoal and Cassio (2007)	Aquatic	Litter	Eucalyptus globulus	Field
28	Rincon and Santelloco (2009)	Aquatic	Litter	Ficus	Field
29	Sudheep and Sridhar (2013)	Aquatic	Litter	Ficus benghalensis, Anacardium occidentale	Field
30	Krevs *et al.* (2017)	Aquatic	Litter	Alnus glutinosa, Alnus negundo	Field
31	Miura *et al.* (2015)	Terrestrial	Litter	Saccharum officinarum	Field
32	Hoppe *et al.* (2016)	Terrestrial	Wood	Fagus sylvatica, Picea abies	Field
33	Kubartová, Ottosson and Stenlid (2015)	Terrestrial	Wood	Picea abies	Field
34	Harrop, Marks and Watwood (2009)	Aquatic	Litter	Alnus oblongifolia, Platanus wrightii	Field
35	Tolkkinen *et al.* (2015b)	Aquatic	Litter	Alnus incana	Field
36	Bärlocher and Graca (2002)	Aquatic	Litter	Eucalyptus globulus, Castanea sativa	Field
37	Xiao *et al.* (2019)	Terrestrial	Wood	Mixture tree species	Field
38	Purahong *et al.* (2017)	Terrestrial	Wood	Schima superba, Pinus massoniana	Field
39	van der Wal, Ottoson and De Boer (2015)	Terrestrial	Wood	Larix kaempferi	Field
40	van der Wal *et al.* (2016)	Terrestrial	Wood	Larix kaempferi, Quercus rubra	Field

Results

Diversity effect

The overall statistical analysis, including all 40 studies, revealed a significant positive effect ($D = 1.57 \pm 0.41$, $p < 0.001$) of fungal diversity on decomposition (Figure 1), supporting hypothesis 1. The overall effect size was based on individual effect sizes as revealed by a mixed effect model using REML as method (AIC = 3254.2, QE = 14515.9, $p<0.001$, Table S1). Most of the included studies were performed in aquatic ecosystems and used leaf litter (28 studies). Wood decomposition studies were only done in terrestrial ecosystems (8 studies). Correlation based diversity effects in field studies (7 studies; see M&M) did not change the outcome of the analysis, thus it was not needed to distinguish these field experiments from the other experiments. In the analysis, ecosystem (aquatic or

terrestrial), resource (leaf litter or wood), experiment (manipulated or natural assembly) and comparison (control or diverse) were analyzed separately to identify the influence of these experimental factors. The control/diverse grouping was only used in manipulated experiments as in field experiments a fixed control treatment with only 1 fungus was not present. All examined comparisons were statistically significant in explaining differences in the diversity effects on decomposition (QM = 34.72 and $p<0.001$).

In the manipulated experiments, the effect size of increasing diversity was negative ($D = -1.76\pm0.56$, $p<0.001$) when a low diverse community (at least two fungal species) was compared with a higher diversity level, while increasing fungal diversity from one fungus (no diversity) to more fungal species had a positive effect size ($D = 2.96\pm0.46$, $p<0.001$). This indicates a reduced diversity effect with increasing species richness, which is in line with the role of redundancy proposed in hypothesis 2. In field experiments, increasing diversity was positively related to decomposition ($D = 2.82\pm0.78$, $p<0.001$) supporting hypothesis 5. When considering the individual effect sizes (D) of all studies, no significant correlation was seen with the size of the lowest fungal diversity level (control) within each comparison (Figure 2, $p = 0.27$). This indicates that for field experiments no evidence for a redundancy effect was found.

Resource type had a significant effect as litter decomposition was significantly increased with increasing fungal diversity ($D = 1.65\pm0.36$, $p<0.001$), whereas this was not the case for decomposition of wood ($D = 0.32\pm1.61$, $p=0.84$). However, the amount of studies with data on wood decomposition was low (8 studies).

To estimate if the selected studies had a publication bias towards studies publishing a significant diversity effect, funnel plot analysis was used to estimate missing studies (Figure 3, Table S2). This resulted in an estimation of missing studies only at the right side of the plot (estimated 110 ± 13.9 studies missing), indicating that in the included studies there was a publication bias for no or negative effects of fungal diversity on decomposition.

	Ecosystem	Resource	Experiment	Comparison	Average
11	Aquatic	Litter	Manipulated	Control	2.90
2	Terrestrial	Litter	Manipulated	Control	6.62
2	Terrestrial	Wood	Manipulated	Control	1.23
15			**Manipulated**	**Control**	**2.96**
11	Aquatic	Litter	Manipulated	Diverse	-1.82
2	Terrestrial	Litter	Manipulated	Diverse	1.89
2	Terrestrial	Wood	Manipulated	Diverse	-3.49
15			**Manipulated**	**Diverse**	**-1.76**
16	Aquatic	Litter	Field	Diverse	3.04
2	Terrestrial	Litter	Field	Diverse	6.76
6	Terrestrial	Wood	Field	Diverse	1.37
24			**Field**	**Diverse**	**2.82**
40	**All**				**1.57**

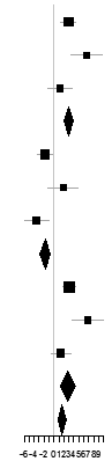


Figure 1. Overall effect size (D) for fungal diversity-decomposition interactions (forest plot), for all studies (All) and different categories within manipulated and field experiments. All data with individual effect sizes of each study calculated as D in forest plot is shown in Figure S1. Numbers in the left column represent the amount of individual studies used in the analysis with a total of 40 papers selected.

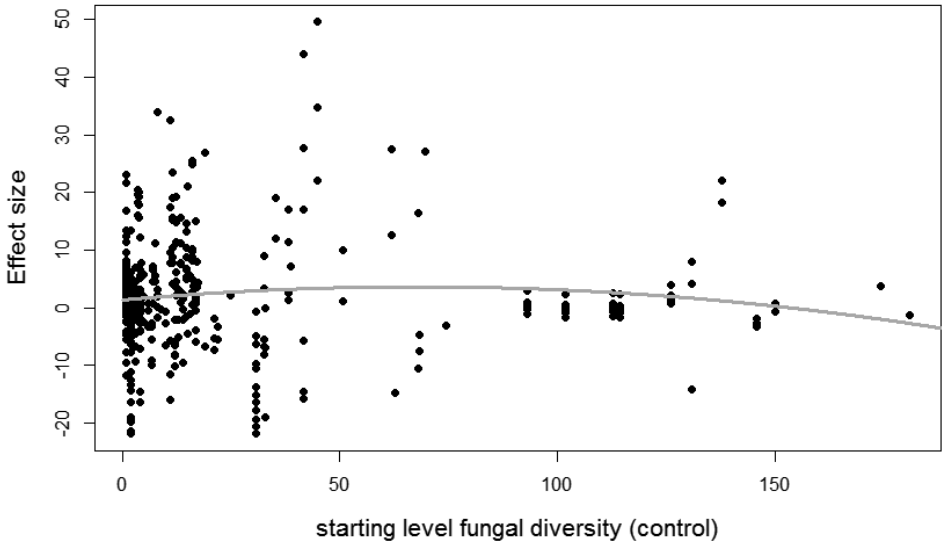


Figure 2. Relationship between effect size (D) and the starting level of fungal diversity within each comparison. Grey line is predicted model based on quadratic relationship (p = 0.27).

Plant quality

Correlation analysis revealed a significant negative relationship between the C:N ratio of plant residues and diversity effect size (r = -0.033±0.016, R = -0.11, p = 0.034, Figure 4), supporting hypothesis 3. The C:N ratio of the plant material was based on an estimate from the TRY-database. For some plant species, however, this data was not available in the database.

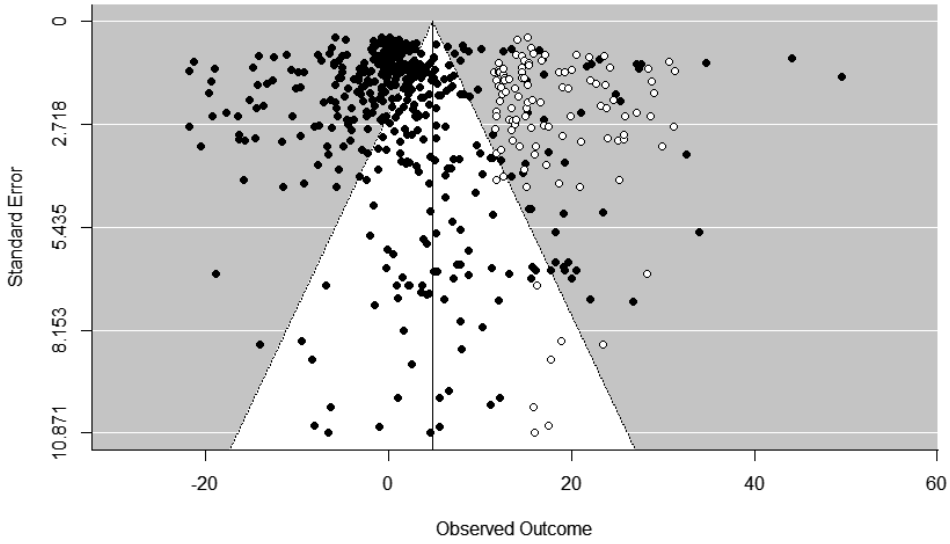


Figure 3. Estimate of publication bias (funnelplot): Individual diversity effect sizes are presented on the x-axis together with the average (black line) and the standard error of the individual effects is presented on the y-axis. Black dots represent individual measurements and white dots indicate possible missing measurements as the distribution of these measurements is expected to be symmetric around the average of all studies.

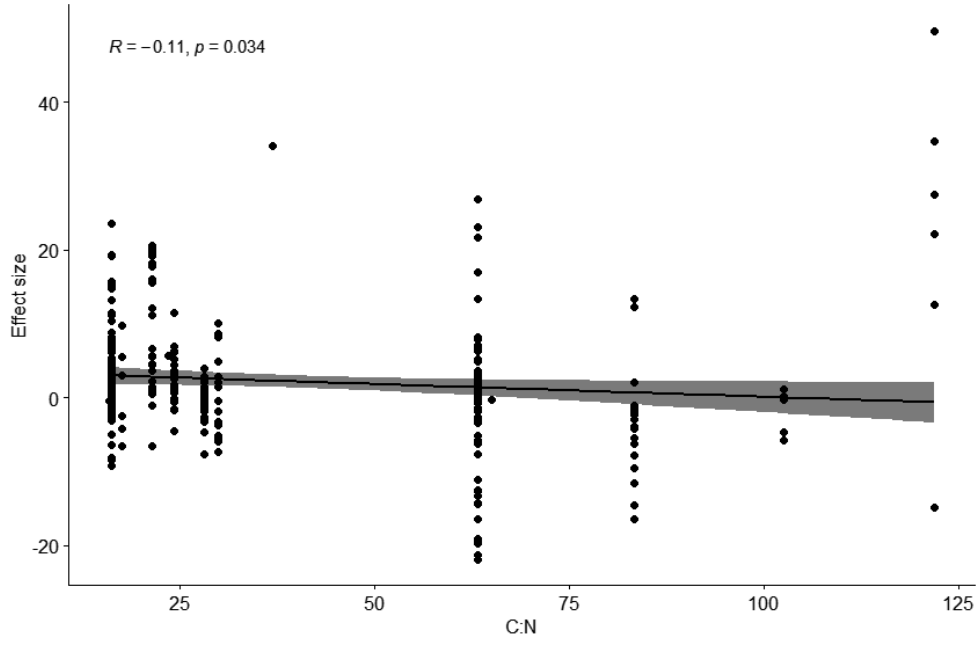


Figure 4. Correlation of estimated C:N ratio of plant material with individual diversity effect size D.

Time effect

To study if time of harvest had an influence on the diversity effect size, the most used plant species was selected to compare different experiments. *Alnus glutinosa* was used in 7 manipulated experiments and 6 field experiments. These experiments were analyzed separately as laboratory conditions are not comparable with field conditions. For both types of experiments the time after which % mass loss was measured did not have a significant negative effect on the individual diversity effect sizes (p = 0.65 and p = 0.98 for manipulated and field experiments respectively, Figure 5), rejecting hypothesis 4.

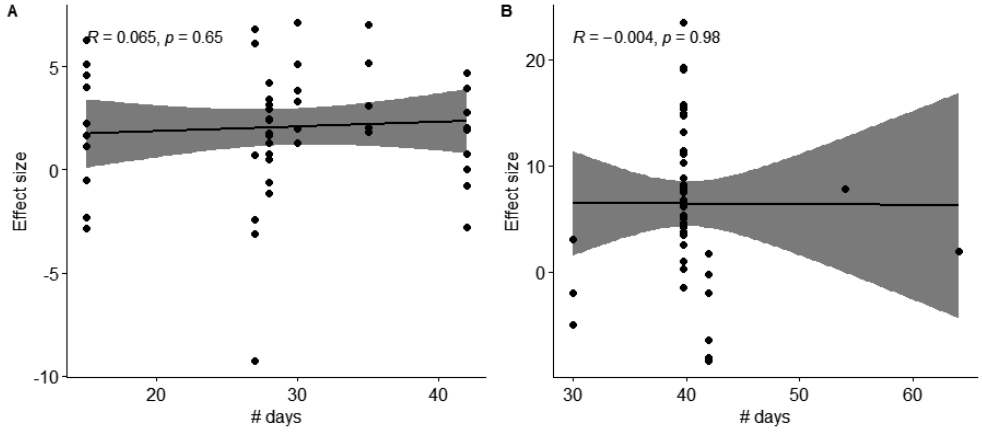


Figure 5. Correlation between time of mass loss measurements and diversity effect size D for 13 studies with leaf litter of *Alnus glutinosa* D. A: manipulated experiments (7) ad B: field experiments (6).

Discussion

Soil biodiversity plays an important role in ecosystem functioning and nutrient cycling (Thiele-Bruhn *et al.*, 2012; Wagg *et al.*, 2014; Delgado-Baquerizo *et al.*, 2016). As fungi play a prominent role in decomposition and carbon cycling (Creamer *et al.*, 2016), the relationship with fungal diversity warrants special attention. Surprisingly, fungal diversity and decomposition are both well studied, but an overall analysis of their relationship has not been addressed. The current meta-analysis examined this both for controlled differences of diversity in experimental settings and for spontaneous assembly differences of diversity in field studies.

In this meta-analysis, we did find an overall positive effect of fungal diversity on decomposition (D = 1.57±0.41, p < 0.001, Figure 1), as stated in hypothesis 1. A positive effect of diversity in manipulated experiments was found when incubations with one fungal species were compared with incubations of two or more fungal species. Comparisons of higher initial richness (at least two fungal species as "control") with multispecies communities did not show such an effect, supporting the expected redundancy effect (hypothesis 2). However, studies of decomposition of naturally colonized substrates in field

sites indicate an overall positive relationship between fungal diversity and decomposition regardless of the lowest diversity levels, thereby rejecting hypothesis 2. These differences between manipulated experiments and natural ecosystems show a possible discrepancy in the representativeness of manipulated experiments for field situations as stated in hypothesis 5. This can be explained in four ways as already discussed by Fukasawa and Matsukura (2021).

First, the fungal diversity levels used to determine the effect of increasing diversity levels on decomposition in experimental settings are the initial levels constructed by the researcher. Competitive exclusion may have leveled off the initial diversity differences in manipulated experiments. For example, Toljander *et al.* (2006) showed that only one or two species existed on decaying wood blocks after 6 months of incubation even though the highest inoculation diversity started with 16 species. Unfortunately, most studies did not take this species loss into account and used the initially inoculated numbers of species to calculate diversity effects on the decomposition as determined at the end of the incubation. This can result in a bias of studies reporting no significant effect of diversity on decomposition as shown in Figure 3. Both competitive interactions leading to a reduction in species and interactions in the remaining species-poor decomposer communities will greatly determine the effect on decomposition (Hiscox *et al.*, 2015; Hiscox, O'Leary and Boddy, 2018). In field studies, fungal community composition and decomposition (weight loss) are determined at the same time. Hence, different from artificially manipulated diversity experiments, the relationship between decomposition and fungal diversity in field experiments is not based on the initial, but rather on the final fungal diversity. Therefore, the lack of effect of differences in initial species richness on decomposition in artificially assembled fungal communities cannot be considered as an appropriate test for diversity effects when final richness levels have not been included. Furthermore, in manipulated experiments, a random assembly of fungal species is used as inoculation while in field experiments succession and colonization are not random and can be selected by forces based on functioning, stimulating co-occurrence (Palmer, Stanton and Young, 2003; Dickie *et al.*, 2012; van der Wal *et al.*, 2013).

Second, the amount of detected species in field experiments is larger than the amount of used species in laboratory conditions (Grossart *et al.*, 2019; Fukasawa and Matsukura, 2021). Only one experiment in this meta-analysis used dilution-to-extinction (Wagg *et al.*, 2014) as a method to reduce diversity in laboratory conditions. Yet, this method might be more representative for differences in diversity levels in field situations as dilutions are made from naturally assembled fungal communities.

Third, differences in homogeneity and quality of the plant material within manipulated and field experiments can lead to differences in decomposition. In manipulated experiments, the plant material needs to be sterile before fungal communities can be inoculated. These

pre-treatments do change the quality of the plant material. Comparisons between partly and fully sterilized material as a method to dilute fungal diversity is therefore not suitable to estimate diversity effects as reported previously (Valentín *et al.*, 2014; Muszynski *et al.*, 2021). Furthermore, homogenization of the material in laboratory conditions may reduce substrate complexity compared to field conditions. Increasing complexity of the environment and substrate can lead to reduced exclusion during competition and, therefore, lead to co-existence (Lee *et al.*, 2019; Chan *et al.*, 2021). Even in field situations, reduced resource complexity can lead to reduced diversity of fungal communities (Baessler *et al.*, 2014).

Fourth, higher environmental heterogeneity in field experiments as compared to controlled incubation conditions may lead to differences in the relationship between fungal diversity and decomposition. Environmental heterogeneity can lead to increased possibilities for co-existence of fungal species (Bradford *et al.*, 2014). Fungal species have specific ranges of abiotic stress tolerance (niche width) (Maynard *et al.*, 2019). Therefore, spatial and temporal fluctuations in environmental conditions can facilitate species coexistence which can coincide with increased decomposition (Toljander *et al.*, 2006). In this meta-analysis we included both aquatic and terrestrial ecosystems. Even though aquatic and terrestrial ecosystems are different, fungi fulfill an important role as decomposers of terrestrial plant material in both environments. In aquatic ecosystems, an important source of terrestrial plant material consists of fallen leaves and more than 50% of the organic matter in lakes has a terrestrial origin (Wilkinson, Pace and Cole, 2013). Fungal diversity in streams and lakes is simpler compared to terrestrial systems as a possible consequence of reduced environmental fluctuations in temperature and water availability in aquatic ecosystems (Bärlocher and Boddy, 2016; Grossart *et al.*, 2019). Even though the presence of these differences, fungal functioning and interactions play similar roles in relationship to the degradation of plant material. However, a limited amount of studies was found that studied decomposition of plant litter in terrestrial ecosystems (4 studies) compared to aquatic ecosystems (28 studies). This bias hampers comparison of both ecosystems. A similar problem accounts for wood decomposition as well. Wood decomposition in aquatic ecosystems is rarely studied even though not only leaf litter but also twigs, branches and even trunks fall into streams as organic matter input (Allan and Castillo, 2007). This limited attention for aquatic wood decomposition is probably due to the assumed very slow decomposition of submerged wood, which has recently been questioned (Ferrer *et al.*, 2020). More experimental studies analyzing fungal diversity within decomposing plant material in a diverse set of ecosystems is needed to be able to make better predictions. In addition, by using knowledge from decomposition models that include microbial parameters, it will be possible to better estimate decomposition rates under a changing climate and in different land use types. Until now, only a few models have been developed including microbial traits (Treseder *et al.*, 2011; Allison, 2012; Sainte-Marie *et al.*, 2021). Our results show that fungal biomass on its own is not enough and fungal diversity

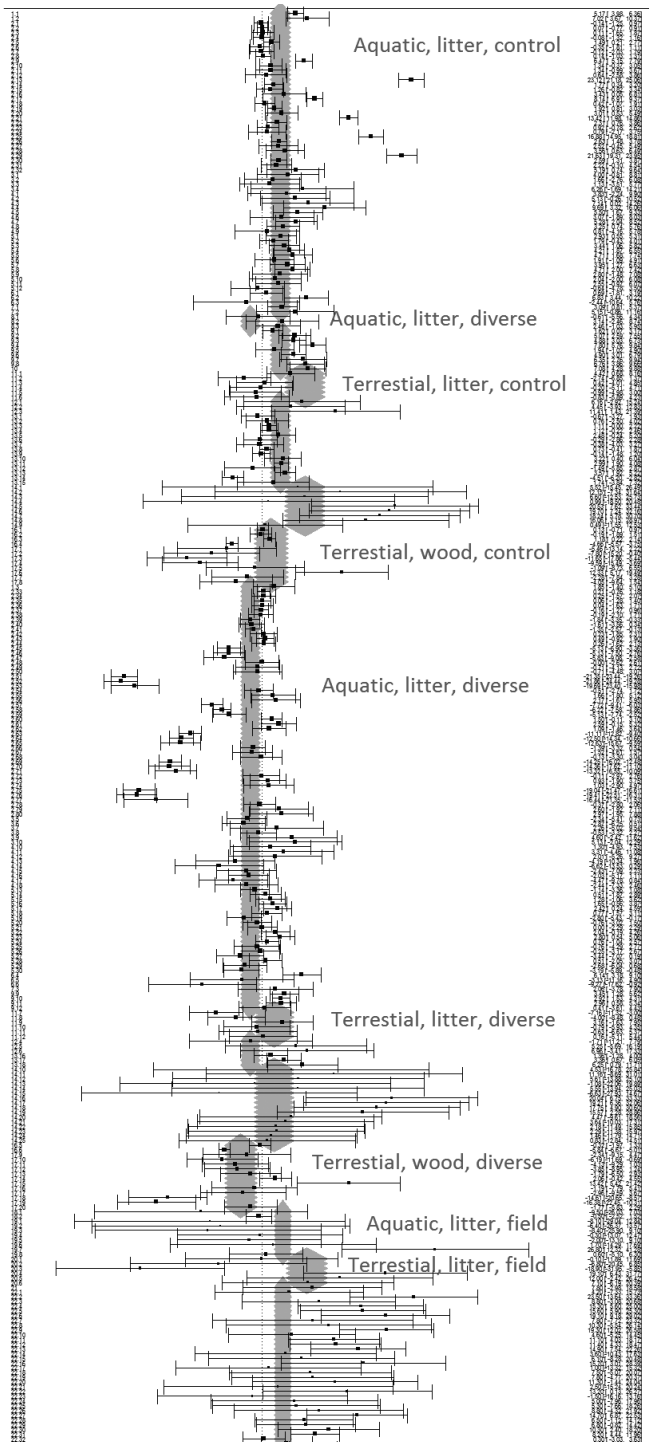
should be included in models to better estimate and understand ecosystem functioning. For example, in a modeling approach by Bastida *et al.* (2021) an estimate was made of both microbial diversity and biomass worldwide.

Apart from tested differences in the fungal diversity-decomposition relationship between manipulated and field experiments, we evaluated if plant quality influenced the fungal diversity effect on decomposition. We found a negative relationship between C:N ratio and the effect size supporting hypothesis 3. In the current analysis, it was not possible to quantify the effect of the quality of the plant material in more detail as this information was lacking. Only one out of 40 papers measured C:N ratio. To overcome this problem, we extracted C:N data from the TRY-database (Kattge *et al.*, 2020). However, this is an estimation and an average of known measurements, but may not accurately reflect the wood and litter quality used in the experiments. Yet, we did find a negative effect of C:N ratio on the calculated diversity effect sizes ($r = -0.033 \pm 0.016$, $R = -0.11$, $p = 0.034$, Figure 4). This suggests that lower plant quality can lead to a reduction of positive effects of fungal diversity on decomposition. Antagonistic forces, such as the production of secondary metabolites and modification of the environment (e.g. reducing pH) to protect occupied space and nutrient sources by the fungal species (Hiscox and Boddy, 2016; Baldrian, 2017), can result in reduced diversity. For example, Baldrian *et al.* (2016) showed that individual wood logs were dominated by one or a few fungal species. Other information about the quality of the plant material, like lignin concentration, can give more insight in the relationship between the plant quality and decomposition (Hall *et al.*, 2020). In the selected studies, only a limited amount of data was available for such plant traits. Therefore, other traits related to quality of litter and wood used for the decomposition studies were not included in the analyses.

To test if incubation time had an effect on diversity-decomposition relationships (hypothesis 4), we selected only studies that used *Alnus glutinosa* leaf litter. Within these studies, the differences between harvesting time periods were minimal (ranging from 15 days to 42 days in laboratory conditions and 30 to 64 days in field conditions). This might be the reason for not finding a significant effect and rejecting hypothesis 4. To be able to understand the effect of time on fungal diversity-decomposition relationships, it is better to analyze decomposition at different time points within the same study to account for environmental differences. For example, Fernandes *et al.* (2009) measured decomposition over time, but this study could not be used since the temperature treatments created a diversity effect (exclusion reason 1: contrasting environment or plant material).

In conclusion, fungal diversity is an important parameter to take into account to estimate and understand decomposition. Experimental studies examining fungal diversity effects on ecosystem functioning do not represent natural environments, leading to the need of doing more field experiments to determine fungal diversity-functioning relationships.

Supplementary



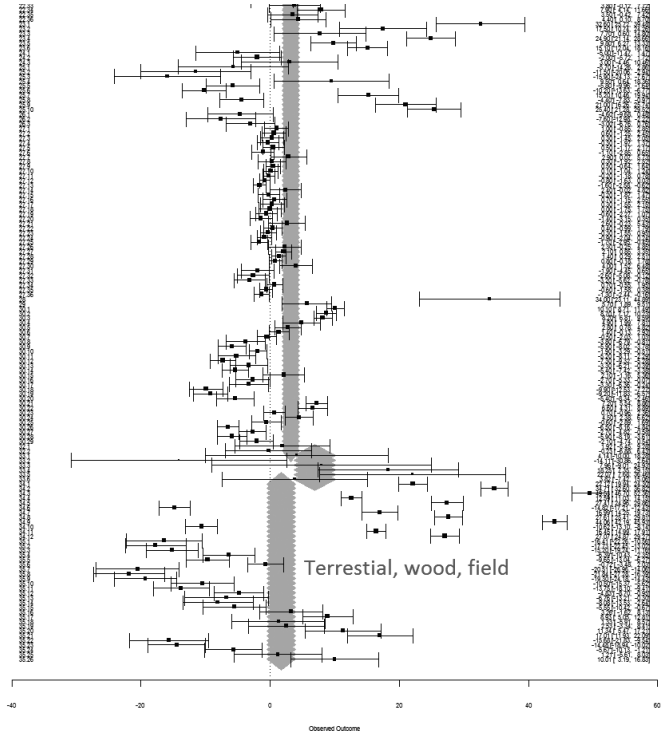


Figure S1. All individual effect sizes for each study indicated by the numbers on the left side: first number is publication number, second number is the number of the combination/interaction within each study. Numbers on right side indicate average ± confidence interval. Grey labels indicate average effect size of the individual groups based on the 4 factors (see Figure 1 for average of these groups).

Table S1: Mixed model results (tau^2 = 65.47±4.84, I^2 = 99.85%, H^2 = 670.80, R^2 = 6.59%)

	estimate	SE	z-score	p-value	Lower confidence interval	Upper confidence interval
Intercept	19.0288	3.3442	5.6902	<0.0001	12.4744	25.5832
Experiment (manipulated)	-4.8658	0.9771	-4.9799	<0.0001	-6.7808	-2.9507
Comparison (control)	-4.7240	1.0748	-4.3953	<0.0001	-6.8306	2.6175
Ecosystem (aquatic)	3.7136	1.5400	2.4114	0.0159	0.6952	6.7319
Resource (litter)	-5.3852	1.7876	-3.0124	0.0026	-8.8889	-1.8814

Table S2: Random effect model, funnel plot analysis with estimate number of missing studies on the right side of 110±13.9 (tau^2 = 99.59±6.40, I^2 = 99.90%, H^2 = 980.61, Q (df = 567) = 30406.67, p<0.001)

	estimate	SE	z-score	p-value	Lower confidence interval	Upper confidence interval
Intercept	4.6466	0.4376	10.6193	<0.0001	3.7890	5.5042

CHAPTER

5

Assessing the effects of cover crop mixtures *versus* monocultures on microbial community composition and greenhouse gas fluxes in a multiannual field experiment

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Abstract

To reduce negative impacts from intensive agricultural practices, cover crops are used to minimize nutrient losses and increase organic matter inputs. Growing mixtures of cover crops and incorporation of their residues is expected to have even more beneficial effects compared to monocultures as a result of the increase of plant functional dissimilarity. We hypothesized that cover crop mixtures will lead to reduced greenhouse gas (GHG) fluxes and functionally more diverse soil microbial communities compared to cover crop monocultures. We established a field experiment for four years with three cover crop species (*Avena strigosa*, *Vicia sativa* and *Raphanus sativus*) in all possible combinations (monoculture, 2-species mixture and 3-species mixture). We tested if cover crop mixtures reduce GHG fluxes and stimulate the soil microbial biomass (qPCR), diversity (16S and ITS partial gene amplicon sequencing) and activity (functional genes related to N and C cycling). GHG (CO_2, N_2O and CH_4) fluxes were measured regularly over the years during cover crop growth, after cover crop incorporation and during main crop growth. Microbial parameters were studied both in the first and last year of the experiment. Contrasting to our expectations, mixtures did overall not perform better than monocultures. GHG fluxes increased by adding cover crops compared to the fallow treatment both during cover crop growth and after incorporation. Mixtures with *V. sativa* and *R. sativus* resulted in highest emissions mainly during decomposition after incorporation of the residues. Increased N_2O fluxes correlated with higher denitrification and nitrification rates. Furthermore, contrasting to other studies we did not find a difference in microbial biomass or diversity between plots with cover crops (mixtures or monocultures) and fallow plots. Collectively, our results indicate that growing cover crop mixtures does not decrease GHG emissions or increase functioning and diversity of the microbial community differently than cover crop monocultures.

Introduction

Climate change and intensive usage of soil with associated soil erosion and environmental pollution affect multiple ecosystem services in agricultural ecosystems (Geisen, Wall and van der Putten, 2019), potentially leading to irreversible changes (Steffen *et al.*, 2015, 2018; IPCC 2021). More sustainable agriculture management, geared towards increased carbon sequestration and reduced usage of synthetic fertilizers is essential in order to mitigate the negative effects of anthropogenic activity (Paustian *et al.*, 2016). Sustainable management could increase soil organic matter (SOM) which is important to stabilize the soil structure, increase storage of nutrients and increase water holding capacity (Johnston, Poulton and Coleman, 2009; Wagg *et al.*, 2014; Tsiafouli *et al.*, 2015). One of the options to increase sustainability in agriculture is the inclusion of cover crops (Paustian *et al.*, 2016; Abdalla *et al.*, 2019). In temperate climates, cover crops are mostly grown in fall and winter and are used as green manure and have the potential to reduce nutrient leaching and to increase SOM (Olson, Ebelhar and Lang, 2010; Zhou *et al.*, 2012; Blanco-Canqui *et al.*, 2015; Poeplau and Don, 2015). Earlier studies have shown the importance of including cover crops in crop rotation to provide a wide range of ecosystem services (Mbuthia *et al.*, 2015; Alahmad *et al.*, 2019; Garland *et al.*, 2021). To increase diversity in agriculture, European regulations stimulate the use of cover crop mixtures in agricultural practice instead of growing cover crops as monocultures (European-Commission, 2019). The underlying assumption for these regulations is that a diverse set of cover crops will provide greater ecological services compared to single species (Blanco-Canqui *et al.*, 2015). Since each plant species has a certain set of specific traits (e.g. rooting depth, composition of exudates), adding more plant species will lead to increased differentiation of traits in mixtures compared to monocultures. This will increase functional complementarity in the field. It has been shown that complementarity in plant traits in grasslands and arable fields with intercropping result in increased biomass production (De Deyn *et al.*, 2009; Cong *et al.*, 2014). However, it needs to be investigated if this also holds under sub-optimal conditions (reduced light intensity and temperature) in autumn and winter when cover crops are grown. It has been shown that, compared to monocultures, mixtures of cover crops can increase overall cover crop yield when grown in autumn (Wendling *et al.*, 2017). Furthermore, mixtures of legumes and grasses increase biomass production due to biological nitrogen fixation (Nair and Ngouajio, 2012; Haque *et al.*, 2013; Tosti *et al.*, 2014). This can lead to increased SOM content and higher input of N from the plant material in spring. Residues from cover crop mixtures represent a more diverse set of substrates for the soil microbial community (Damour *et al.*, 2014; Tribouillois *et al.*, 2015) which is hypothesized to increase decomposition and nutrient turnover.

Microbial communities are key players in nutrient cycling and will, therefore, have a critical role in the effects of cover crops on soil functioning. It has been shown that cover

crops mainly increase C and N pools leading to a stimulation of microbial activity and biomass (Nair and Ngouajio, 2012; Zhou *et al.*, 2012; Strickland *et al.*, 2019). As each cover crop has certain traits, microbial community composition might be changed depending on the cover crop type. For example, by adding oat as cover crop, mycorrhizal fungi are stimulated (Boswell *et al.*, 1998; Lehman *et al.*, 2012). Adding several cover crop species instead of single species is expected to increase complementarity and the number of niches in the soil leading to increased microbial diversity and metabolic potential (Drost *et al.*, 2020). This is expected to have a positive effect on the functioning of the ecosystem (Delgado-Baquerizo *et al.*, 2016).

As nutrient pools in the soil change by cover crop addition, changes in microbial activity will affect nutrient cycling. During cover crop growth both C and N are captured in plant biomass. After incorporation, decomposition of cover crop residues can lead to increased greenhouse gas (GHG) emissions (Abdalla *et al.*, 2012, 2014; Brozyna *et al.*, 2013; Basche *et al.*, 2014; Sanz-Cobena *et al.*, 2014; Li *et al.*, 2016). Microbial decomposition of cover crops can increase N_2O emissions by consuming C leading to reduced oxygen and enhanced denitrification in the soil (Duan *et al.*, 2018) or by increased nitrifier N_2O production. However, growing of cover crops can still lead to a positive GHG balance as compared to bare soils as a result of increased carbon storage and reduced nutrient losses during cover crop growth (Petersen *et al.*, 2011; Abdalla *et al.*, 2014, 2019; Tribouillois, Constantin and Justes, 2018). We expect that cover crop mixtures will be more beneficial than monocultures due to increased organic matter input in the soil and increased dissimilarity of residues leading to reduced GHG emissions. It is expected that the microbial community will use nutrients more efficient with increasing diversity of plant material; e.g. diversity of C:N ratio of residues increase diversity and decomposition of microbial community (Hattenschwiler, Tiunov and Scheu, 2005; Chen *et al.*, 2019). Furthermore, organic matter input from organic residues like compost, sewage sludge and cover crop residues has been shown to increase CH_4 uptake from the atmosphere (Brenzinger *et al.*, 2018, 2021; Ho *et al.*, 2015b). This increases the GHG sink function of agricultural soils as well as the abundance of microbes involved in GHG consumption (Brenzinger *et al.*, 2018, 2021). We expect that cover crop mixtures will be beneficial in enhancing methane uptake compared to cover crop monocultures.

A four-year field experiment was established to study the effects of growth and their residue incorporation of cover crop monocultures and mixtures on microbial community and greenhouse gas fluxes within a common agricultural rotation. The aim of the field experiment was to test the following hypotheses:

1. Cover crop mixtures increase microbial diversity and activity in the soil compared to cover crop monocultures by creating higher substrate diversity and availability for

the microbial community (increased presence of functional genes and increased nutrient cycling);
2. Cover crop mixtures reduce GHG emissions from soil by increased substrate use efficiency of the microbial community compared to cover crop monocultures;
3. The differences between cover crop mixtures and monocultures are most pronounced during cover crop residue decomposition in spring. After cover crop incorporation, high amounts of carbon are added to the soil. This is expected to be favorable for microbial activity leading to larger differences between treatments as compared to other moments during the year (e.g. cover crop growth and main crop growth).

Materials and methods

Experimental design

A field experiment was established in August 2016 at the experimental farm of Wageningen University Research (WUR), Wageningen, the Netherlands (51°59'41.9"N, 5°39'17.5"E) (as described by Porre (2020) thesis chapter 2). The agricultural history of the field was conventional arable rotation (e.g. potato, maize, wheat, and beetroot) with winter wheat grown in 2016 before the start of the experiment. The soil type is sandy (83% sand, 12% silt and 2% clay). Soil pH measured in water (1:2.5 (w/v) suspension of soil in water) was 5.3 and soil organic matter content, measured as weight loss on ignition, was 3.1%. Temperature and rainfall over the experimental period are shown in Figure 1. The field experiment was set up as a randomized block-design with 5 blocks of 8 plots of 6*10m (Figure 2). Each block contained 1 replicate of 8 treatments consisting of 8 cover crop treatments sown in August: three single species (*Raphanus sativus* (R), *Avena strigosa* (A) and *Vicia sativa* (V)), all possible 2- and 3- species combinations of these single cover crops and a fallow treatment. The cover crops were sown in rows with row spacing of 12.5 cm and at a depth of 2 cm. Seeding rates of *R. sativus*, *V. sativa* and *A. strigosa* were 30 kg*ha^{-1}, 110 kg*ha^{-1} and 70 kg*ha^{-1} respectively, according to farmers' practice. Species were mixed according to a replacement design, using 50% and 33% of each monoculture seeding rate (in the 2- and 3-species mixtures, respectively). To ensure good establishment, cover crops were fertilized with 30 kg*ha^{-1} in a form of calcium ammonium nitrate (CAN: 27% N - 6% CaO - 4% MgO). The fallow treatment was not fertilized. In 2016, weeds were removed by hand in September due to massive growth of *Chenopodium album*. The growing season of the cover crop ended in spring, latest in March (Figure 3). Cover crops were chopped and incorporated in the soil up to a depth of 5 cm by a cultivation machine. After at least 2 weeks of decomposition, the soil was prepared for the main crop and in all plots (including the fallow) a main crop was sown. The main crop changed every year according to a common Dutch agricultural rotation practice. The crop rotation is shown in Figure 3. Main crops were maize, potato and barley for 2017, 2018 and 2019 respectively. Maize (LG 31.211, supplied by Agrifirm, Apeldoorn, The Netherlands) was fertilized with 300 kg

K*ha^{-1} (Kali60), 59 kg P*ha^{-1} (Triple superphosphate) and 95 kg N*ha^{-1} (CAN). Herbicide was applied as roundup to avoid weed growth between the rows. Due to a drought period the maize was irrigated with water on the 20-6-2017 with twice 15 mm. Potato (variety "Eersteling") was fertilized with 110 kg N*ha^{-1} (CAN), 46 kg P_2O_5*ha^{-1} and 60 kg K_2O*ha^{-1}. Barley (variety "IRINA") was fertilized with 60 kg N*ha^{-1} (CAN).

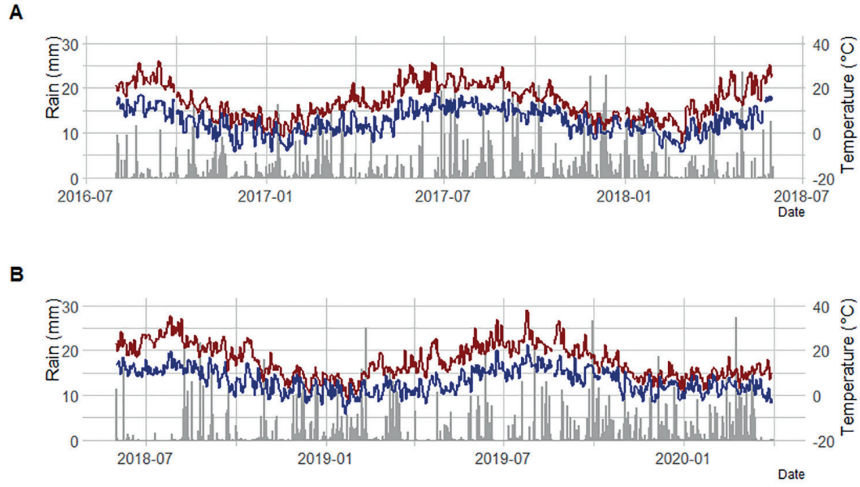

Figure 1: Daily data from Veenkampen weather station of Wageningen University Research (WUR): grey bars represent rainfall in mm; red line is maximum day temperature and blue line is minimum day temperature. A: 1st of August 2016 until 31st of May 2018; B: 1st of June 2018 until 31st of March 2020

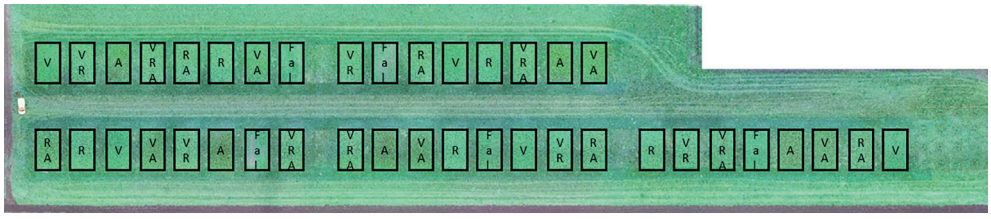

Figure 2: Drone image of the field including plot borders and treatment description. Letters inside plots indicate all different cover crop treatments with Fal: fallow treatment, V: *V. sativa* (vetch), R: *R. sativus* (radish), A: *A. strigosa* (oat) and combinations of letters are two or three species mixtures. The experimental plots are 6*10 m, space between the plots is 3 m within a block and 9 m between blocks.

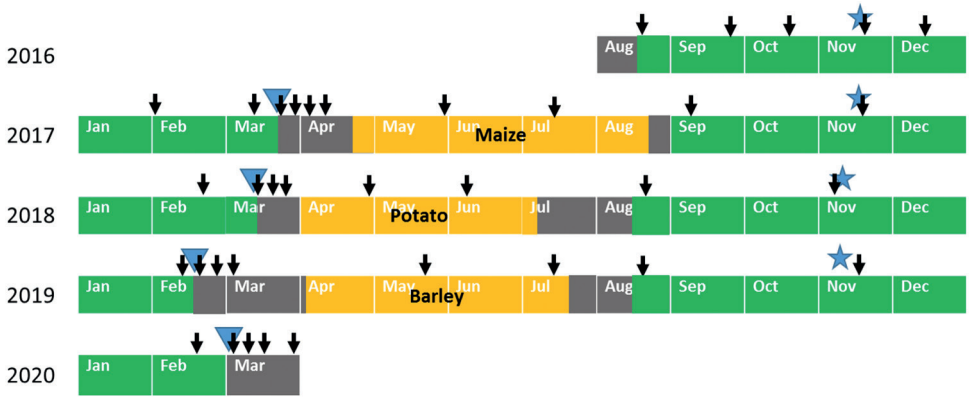

Figure 3: Timeline of the experiment. Stars indicate sampling time of cover crops in November, 12 weeks after sowing; triangles indicate incorporation time of cover residues into the soil; and arrows indicate time points when greenhouse gasses were measured. Colors indicate time approximately when cover crops (green) or main crops (yellow) were present and when the field was bare (brown).

Biomass of cover crop and main crop

Cover crop biomass was determined in November, 12 weeks after cover crop sowing, to estimate the maximum standing biomass present on the field. In an area of 2 m^2, aboveground plant material was harvested. Weeds were removed and plant material was washed to remove adhering soil and weeds. During the last three years of the experiment, biomass of the individual cover crop species was separated. The aboveground plant material was dried at 70°C for 48 hours to estimate dry weight. In 2016, cover crop root biomass was estimated by sampling with a 12 cm × 30 cm × 30 cm (length*width*depth) frame, which was placed perpendicular on the rows so multiple rows were included in the sample. In 2017-2019, four root cores (8 cm Ø, 30 cm depth) were extracted. Two cores were sampled in the rows and two cores between the rows. Each core was divided into four depth layers, 0-5 cm, 5-10 cm, 10-20 cm and 20-30 cm. Samples from each plot were pooled together by depth. Root biomass was rinsed with water, in order to remove adhering soil and organic matter particles, and dried at 70 °C for 48 h.

In spring just before cover crop incorporation, aboveground biomass was harvested in 2018, 2019 and 2020. One m^2 was harvested and handled similarly as aboveground plant material harvested in November.

Main crop biomass was determined in July or August by harvesting a net plot (excluding border rows) of 8 by 4 m (2017 maize, 2018 potato and 2019 barley) (Figure 3). Total aboveground biomass was measured for maize and barley. For potatoes, only tuber yield was estimated. A subsample of the biomass was dried at 70°C for 48 hours to determine the main crop dry weight.

Soil samples

Soil samples were taken four times a year: 1 day after sowing cover crops in August, in November 12 weeks after cover crop sowing, before cover crop residue incorporation and two weeks after cover crop residue incorporation. Per plot, 7 random samples (0-20 cm) were pooled. These soil samples were used for nutrient and microbial analyses. The soil was sieved through a 4 mm sieve and frozen at -26°C for microbial analyses. Soil samples were dried at 40°C for 48 hours for nutrient analyses.

Nutrient analyses

Nutrient content of both cover crops and main crop were measured. C and N was analyzed by grinding 1 mg of plant material (for cover crops harvested in November separated for above and belowground material), transferred to tin cups and analyzed with an Element Analyzer (Flash EA 1112, Thermo Scientific, Breda, The Netherlands).

Mineral nitrogen (NH_4^+ and NO_3^-) content of the soil was analyzed each sampling time by extraction with 0.01M $CaCl_2$ (soil:liquid ratio of 1:10 (w/v)). After 2 hours of shaking at 250 rpm, the suspension was centrifuged for 10 min at 3000 rpm and filtered through a Whatman Puradisc filter with CA membrane. Clear supernatant was stored for maximal 1 day in the fridge at 4°C until analysis on the AutoAnalyzer (SEAL QuAAtro SFA system, Beun- de Ronde B.V. Abcoude, The Netherlands).

Soil organic matter (SOM) was determined by loss on ignition. Soil samples were dried at 105°C for 24 h and 20 g of dried soil was heated up to 550°C in a Nabertherm oven (Nabertherm GmbH, Lilienthal, Germany) for 24 hours and weighed. SOM was measured at the start of the experiment and 2 weeks after cover crop incorporation in the 3rd and 4th year of the experiment.

Greenhouse gas fluxes

Greenhouse gas fluxes were measured regularly during cover crop growth, cover crop decomposition and main crop growth (Figure 3). GHG fluxes were measured just after sowing the cover crops in August, during cover crop growth in November, before cover crop incorporation in February and 1 day, 1 week and 2 weeks after cover crop incorporation and before sowing of the main crop. During main crop growth, GHGs were measured twice. In the first year of the experiment, we took monthly samples during cover crop growth. Greenhouse gasses were measured in static opaque PVC chambers, equipped with a battery driven internal ventilator for 1.5h. Chambers with a diameter of 30 cm and 40 cm high were used (volume 28 L). PVC rings were inserted 5-10 cm into the soil and chambers were mounted on the rings, closed off by an internal rubber seal ensuring airtightness of the chamber system (Figure 4). The chambers were covered with isolation foil to prevent temperature increases inside the chamber during measurements. After

closing the chamber, headspace samples (60 ml) were collected after 0, 20, 40, 60, 75 and 90 minutes using a disposable syringe equipped with a needle to penetrate the septum in the chamber sampling port. Roughly, 54 ml of sample was used to flush a 6 ml exetainer vial (Labco, UK). The last 6 ml of sample was introduced in the vial after removing the outlet needle, thereby creating an overpressure of 1 bar in the vial. The vials were stored at room temperature until analysis. CO_2, N_2O and CH_4 were measured simultaneously in the same sample. Samples from the exetainers were introduced into a GC using an autosampler (TriPlus RSH, Thermo Fisher Scientific, Bleiswijk, The Netherlands) connected to a gas chromatograph (GC1300, Thermo Fisher Scientific) equipped with a Methanizer and a Flame Ionization Detector (FID) to detect CO_2 and CH_4 and an electron capture detector (ECD) for detection of N_2O. The gas chromatograph contained two sets of a pair Rt-Q-Bond capillary columns (L; 15m and 30m, ID; 0.53mm, Restek, Interscience, Breda, The Netherlands). Nitrogen was used as a carrier gas, and oven temperature was set at 80 °C. Chromeleon™ Chromatography Data System 7.1 (CDS, Thermo Fisher Scientific) software was used to analyze the obtained gas chromatograms from the GC. Fluxes were calculated based on the accumulation or reduction over the 1.5h measured. Gas concentrations were calculated in ppm values by comparing with calibration curves, which were generated by dilution of a certified gas mixture (1 ppm N_2O, 2 ppm CH_4 and 2000 ppm CO_2; Linde Gas, The Netherlands). The concentrations (ppm) were converted into absolute amounts (mmol) using the ideal gas law: $pV = nRT$ in which p is the pressure in the flux chamber (assuming equal to outside pressure), V is the volume, n is the amount of gas in mol, R is the gas constant (8.31 $J*K^{-1}mol^{-1}$) and T is the temperature. Afterwards we used the measured GHG fluxes to determine fluxes per m^2 (surface area of the chamber was 0.071 m^2).

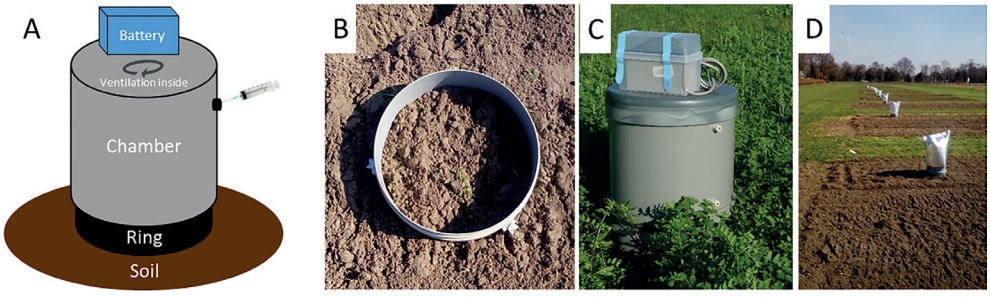

Figure 4: Chambers for measurements of GHG fluxes. A: schematic drawing of the chamber; B: picture of ring inserted in the soil; C and D: picture of chamber installed in the field during cover crop growth (C) and after cover crop incorporation covered by isolation foil (D).

Microbial activity assays

Four activity assays (nitrification (Coci, Bodelier and Laanbroek, 2008), denitrification (Philippot *et al.*, 2013; Kim *et al.*, 2015), N_2-fixation (Hardy, Burns and Holsten, 1973) and methane oxidation (Bodelier *et al.*, 2013; Ho *et al.*, 2015a)) were performed both in the first

year and in the last year 2 weeks after cover crop incorporation. The activity assays were conducted after sieving wet soil through 4 mm sieves. Gravimetric water content of the soil was determined to be able to calculate a normalized activity per gram of dry soil.

Nitrification was measured as described by Coci, Bodelier and Laanbroek (2008). 5 g of wet soil was weighed into 50 ml bottles and 25 ml medium consisting of 5 mM $(NH_4)_2SO_4$, 0.8 mM K_2HPO_4 and 0.2 mM KH_2PO_4 in MilliQ water with a pH of 7.5 was added to the bottles. Bottles were incubated at room temperature. Slurry samples were collected over a 5 day period at approximately 0, 3, 6, 18, 24, 48, 72 and 96 hours after the start of the incubation. The soil slurry was centrifuged at 16100 g for 10 minutes. The supernatant was transferred to a new tube and stored at -20°C before analysis of NO_3^--N with an AutoAnalyzer (SEAL QuAAtro SFA system, Beun- de Ronde B.V. Abcoude, The Netherlands). Short-term potential nitrification rates were calculated based on the first 5 sampling times (until 24h from the start, with no or limited growth of nitrifying microbes) and long-term potential nitrification rates were measured based on the results on the last three days (potential increase of nitrifying microbes).

Denitrification was analyzed using the acetylene inhibition method (Philippot *et al.*, 2013; Kim *et al.*, 2015). 10 g of fresh soil was supplemented with 15 ml of 1 mM KNO_3 in MilliQ water in 120 ml bottles. An additional carbon source was not added to be able to measure denitrification fed by the carbon of the cover crop residues present in the soil. Bottles were closed with rubber stoppers and aluminum caps (SUPELCO open center seals, 20 mm). 10% of acetylene was added in the headspace to prevent complete denitrification from N_2O to N_2. Bottles were incubated in the dark at room temperature on a gyratory shaker at 120 rpm. Oxygen was not removed from the bottles. N_2O concentration was measured at 0, 3, 18 and 24h on the GC (GC1300, Thermo Fisher Scientific). Denitrification activity was calculated based on the increase of N_2O over these time points.

N_2-fixation of free living bacteria in the soil was measured using 10 g of fresh soil in 120 ml bottles amended with 10% acetylene in the headspace (Hardy, Burns and Holsten, 1973). The bottles were closed with rubber stoppers and aluminum caps (SUPELCO open center seals, 20 mm). The conversion of acetylene to ethylene is a proxy of the N_2-fixation rate. Ethylene concentration was measured 0, 24 and 48 hours after the start of the incubation on the GC (Trace Ultra GC, Thermo Fisher Scientific) equipped with a Thermal Conductivity Detector (TCD) to detect C_2H_4. The gas chromatograph contained Rt-Q-Bond capillary columns (Restek, Interscience, Breda, The Netherlands). Chromeleon™ Chromatography Data System 7.1 software (CDS, Thermo Fisher Scientific) was used to analyze the obtained chromatograms from the GC. Helium and H_2 were used as carrier and burning gas, respectively. Gas concentrations were calculated in ppm values by comparing with a calibration curve, which was generated by dilution of a pure ethylene gas (concentrations

from 1 to 100ppm). The increase of ethylene over time, was used to calculate the potential N_2 fixation by free living diazotrophs.

Near atmospheric methane oxidation rates were measured using 10 g of soil in 120 ml bottles closed with rubber stoppers and aluminum caps (SUPELCO open center seals, 20 mm) (Bodelier *et al.*, 2013; Ho *et al.*, 2015a). Methane (quality 4.5; Linde Gas, The Netherlands) was added to the headspace to reach 10 ppmv of CH_4. During a period of 1 month, CH_4 concentration was measured in the incubations by using a GC (Trace Ultra GC, Thermo Fisher Scientific) equipped with Rt-Q-Bond (L; 30 m, ID; 0.32 mm) capillary column and FID. The temperature of the column, injector and detector was adjusted to 80, 150, and 250 °C, respectively. Helium and H_2 were used as the carrier and burning gases, respectively. Linear regression of the methane concentration over time was used to calculate the methane oxidation rate in the soil.

Microbial community composition

The composition of the microbial community as well as abundance of bacteria/fungi and specific functional microbial guilds was analyzed using MiSeq Illumina sequencing and qPCR. DNA was extracted using DNeasy Powersoil Pro Kit (Qiagen, Venlo, The Netherlands) using 0.25 g of soil according to the instructions provided by the supplier. The quantity and quality of the extracted DNA was analyzed with a spectrophotometer (Nanodrop 2000, Thermo Scientific). Bacterial and fungal copy numbers were measured using qPCR. qPCR assays were used to target the 16S rRNA gene for bacteria and ITS region for fungi. Each assay was performed with 2 ng*µL^{-1} of DNA in duplicates for each DNA extract with the primer pair EUB338 and EUB518 for bacteria (Fierer *et al.*, 2005) and ITS1 and 5.8S for fungi (Fierer *et al.*, 2005). Each qPCR (total volume of 15 µL) consisted of 7.5 µL 29 SensiFAST SYBR (BIOLINE) for ITS and 7.5 µL I taq SYBRGreen (Biorad) for 16S rRNA gene, 0.75 µL of forward and reverse primers each, 1.5 µL of bovine serum albumin (5 mg*mL-1; Invitrogen), 1.5 µL DNase- and RNase-free water and 3 µL of the template DNA. Standard curves were obtained using 10-fold dilution series of a known amount of plasmid DNA from isolates of *Collimonas* sp. (10^8 to 10^1 gene copies) containing the 16S rRNA gene fragment for bacteria and *Trichoderma koningii* (EF4/TIS4) containing the ITS region for fungi. The qPCR was performed with a C1000 Touch Thermal Cycler (CFX-real-Time system, Bio-Rad Laboratories, Inc.). As negative control, water was used instead of template DNA. Efficiencies for bacterial assays were between 105.9 and 136.5 % with r^2 values between 0.905 and 0.929. For the fungal assays, the efficiencies were between 63.4 and 69.4% with r^2 values between 0.967 and 0.999.

qPCR of functional genes were performed to determine possible differences between treatments in genes related to nitrogen cycling (*nosZI*, *nosZII*, *nifH*, *amoA* bacteria and *amoA* archaea) and methane oxidation (*pmoA*) as described by Brenzinger *et al.* (2018). A

short description is given here. Each assay was performed with 2.5 µL of DNA. For *pmoA* we used 10 µL of 29 SensiFAST SYBR (BIOLINE), 2 µL of each primer pair, 1 µL of bovine serum albumin and 2.5 µL DNase- and RNase-free water. For all other functional genes, each qPCR consisted of 7.5 µL of 29 SensiFAST SYBR (BIOLINE), 0.75 µL of each primer pair, 0.75 µL of bovine serum albumin and 2.75 µL DNase- and RNase-free water. The specific primers and program are shown in Table 1. Standard curves were obtained using serial 10-fold dilutions of a known amount of plasmid DNA from different pure cultures representing the target gene fragment (108–101 gene copies) containing the respective gene fragment (plasmid *Methylosinus trichosporium* OB3b for *pmoA*; vector pCR2.1 *Pseudomonas stutzzeri* for *nosZI* and *nosZII*; plasmid PCR4-topo *Nitrososphaera viennensis* EN76 for *amoA* archaea; plasmid PCR4-topo *N. multiformis* ATCC25196 for *amoA* bacteria; and plasmid pol 1 for *nifH*). The qPCRs were performed with a C1000 Touch Thermal Cycler (CFX-real-Time system, Bio-Rad Laboratories, Inc.). As negative control, water was used instead of template DNA. The efficiencies of the qPCR were between 73.9 and 88.2 (*pmoA*), 87.7 and 89.1 (*nosZI*), 59.4 and 61.4 (*nosZII*), 80.3 and 84.3 (*nifH*), 97.8 and 99.8 (*amoA* bacteria) and 92.2 and 135.3 (*amoA* archaea) with r^2 values between 0.975 and 0.998.

Microbial diversity was assessed using Illumina Miseq amplicon sequencing in the last year. DNA samples were send for bacterial and fungal sequencing using Illumina MiSeq PE250, which was performed by McGill University and Genome Quebec Innovation Centre, Montreal, Canada. The following primers were used for sequencing: EUB515f/806r (Caporaso *et al.*, 2011) and ITS4r/9f (Ihrmark *et al.*, 2012) for bacteria and fungi, respectively. The resulting sequences can be accessed at the European Nucleotide Archive (accession number PRJEB49947).

Table 1: qPCR protocol for functional genes

Gene	Primers (10 pmol μL^{-1})	Primer sequence	qPCR program	Reference
pmoA	A189F Mb661R	Forward (5' to 3'): GGNGACTGGGACTTCTGG Reverse(3' to 5'): CCGGMGCAACGTCYTTACC	Initial denaturation for 3 min at 95°C; 45 cycles of 10 sec denaturation at 95°C, 10 sec annealing at 60°C and amplification at 72°C for 25 sec (data acquisition) and 82°C for 8 sec (data acq.); and 65-99°C (+0.5°C) for denaturation curve	(Costello and Lidstrom, 1999)
nosZI	nosZ2F nosZ2R	Forward: CGCRACGGCAASAAGGTSMSSGT Reverse: CAKRTGCAKSGCRTGGCAGAA	Initial denaturation for 3 min at 95°C; 45 cycles of 10 sec denaturation at 95°C, 20 sec annealing at 64°C and amplification at 72°C for 20 sec (data acq.); and 65-99°C (+0.5°C) for denaturation curve	(Henry *et al.*, 2006)
nosZII	nosZIIF nosZIIR	Forward: CTIGGICCIYTKCAYAC Reverse: GCIGARCARAAITCBGTRC	Initial denaturation for 5 min at 95°C; 45 cycles of 30 sec denaturation at 95°C, 45 sec annealing at 54°C and amplification at 72°C for 60 sec (data acq.) and 80°C for 15 sec (data acq.); and 65-95°C (+0.5°C) for denaturation curve	(Jones *et al.*, 2013)
nifH	polF polR	Forward: TGCGAYCCSAARGCBGACTC Reverse: ATSGCCATCATYTCRCCGGA	Initial denaturation for 3 min at 95°C; 40 cycles of 10 sec denaturation at 95°C, 20 sec annealing at 64°C and amplification at 72°C for 20 sec (data acq.); and 65-95°C (+0.5°C) for denaturation curve	(Poly, Monrozier and Bally, 2001)
amoA archea	Arch amoAF Arch amoAR	Forward: STAATGGTCTGGCTTAGACG Reverse: GCGGCCATCCATCTGTATGT	Initial denaturation for 3 min at 95°C; 45 cycles of 10 sec denaturation at 95°C, 20 sec annealing at 64°C and amplification at 72°C for 20 sec (data acq.); and 65-95°C (+0.5°C) for denaturation curve	(Francis *et al.*, 2005)
amoA bacteria	Amo1F AmoA2R	Forward: GGGGTTTCTACTGGTGGT Reverse: CCCCTCKGSAAAGCCTTCTTC	Initial denaturation for 10 min at 95°C; 45 cycles of 10 sec denaturation at 95°C, 25 sec annealing at 65°C and amplification at 72°C for 30 sec (data acq.); and 65-95°C (+0.5°C) for denaturation curve	(Rotthauwe, Witzel and Liesack, 1997)

Bioinformatic analyses

For bacterial and fungal communities, bioinformatics pipeline and subsequent analyses were conducted using R v4.02 (Team, 2019). Forward and reverse PCR primers were removed from the reads by using the cutadapt plugin v2.10 (Martin, 2011) with the DADA2 v1.8 pipeline, and then demultiplexed paired-end fastq files processing (Callahan *et al.*, 2016). Forward and reverse reads were trimmed to 200 base pairs and 190 base pairs, respectively, and at the location of the first occurrence of a base calls or containing greater than or equal to 18 estimated errors, and merged with a minimum overlap of 12 bases. Chimeric sequences were removed and merged reads dereplicated. Taxonomy was assigned to amplicon sequence variants (ASVs) using the SILVA v138 database (McLaren, 2020).

The read counts in ASV table were filtered based on taxonomy to remove unknown taxa at Phylum level and chloroplasts reads. The ASVs abundance were summarized at Genus taxonomic level. Low-occurring microbes were defined as being presence in less than 5 samples and aggregated into a single variable named 'rare'. Then, data were transformed to the center log-ratio (CLR) using the Bayesian-multiplicative replacement of count zeros (CZM method). The filtered ASV table was used for alpha-diversity and beta-diversity determination of bacterial and fungal communities using R with the package phyloseq version 1.34.0.

Statistical analyses

The data were analyzed with R (version 4.0.3) with attached packages: agricolae, car and plyr for statistical analysis; ggplot2, grid and gridExtra for making figures; and dplyr, survival, Formula and Hmisc for making correlation tables. First, the data were tested for the assumption of normality and for homogeneity of variances. In the case of violation of the assumption, log transformation was used to meet the criteria for parametric statistical testing. The data were analyzed for significant treatment effects using a mixed linear model and Anova. A post hoc Tukey test was used to identify the significant differences between the treatments. The greenhouse gas fluxes were analyzed using repeated measures Anova as samples were taken repeatedly from the same location in the field. We tested if the treatments were significantly different from each other ($p < 0.05$), but also if the cover crop treatments were different from the fallow treatment and if cover crop mixtures performed differently compared to cover crop monocultures.

Correlations were calculated between the GHG fluxes, soil measurements (microbial activity assays, mineral N) and plant data (biomass and carbon and nitrogen content). To determine correlations between the different measurements, data were grouped together in three different groups according to the season in the agricultural field: (1) cover crop growth, (2) cover crop decomposition and (3) main crop growth. If a measurement

was taken several times within a season, averages were calculated before estimating correlation between the different measurements. For example, soil samples were taken at four time points: in August (3), November (1), February (1) and March (2) two weeks after cover crop incorporation.

Results

Cover crop and main crop biomass

Cover crop biomass was harvested in November each year, 12 weeks after sowing (Figure 5). Total cover crop biomass in mixtures was not significantly different from the most productive monoculture each year. Monoculture of *V. sativa* (V) was each year the least productive, while *R. sativus* (R) was most productive in 2017 and *A. strigosa* (A) in 2016, 2018 and 2019. Root biomass was significantly higher in treatments with taproot of *R. sativus* (R) present ($p = 4.64*10^{-10}$). The last three years of the experiment, one of the mixtures had the highest measured total biomass (VRA in 2017, VR in 2018 and RA in 2019). In the last 3 years of the experiment aboveground cover crop biomass was also measured just before cover crop incorporation to have an estimation of the biomass incorporated in the soil (Figure 6). In 2018 and 2019 cover crop (CC) biomass in spring was not significantly different between monocultures and mixtures excluding low *V. sativa* (V) biomass. In 2020, A and VA had significantly more standing biomass compared to V, R and VR. In all years, aboveground cover crop biomass significantly decreased from November to February ($p = 0.00099$).

Main crop biomass did not significantly differ between the treatments. Only in 2018, potato yield was reduced in the fallow treatment compared to VR and RA treatments (Figure 7).

Carbon and nitrogen content were measured for total cover crop biomass in November and main crop biomass (Tables 2-4). Carbon content showed similar results compared to cover crop and main crop biomass. Treatments with leguminous *V. sativa* (V) had higher N content in the plant material. This resulted in highest N content present in the VR treatment in most years. In the main crops, nitrogen content was only significantly different between the treatments in 2019 (barley). VR and VRA treatments resulted in significantly higher N compared to the fallow treatment.

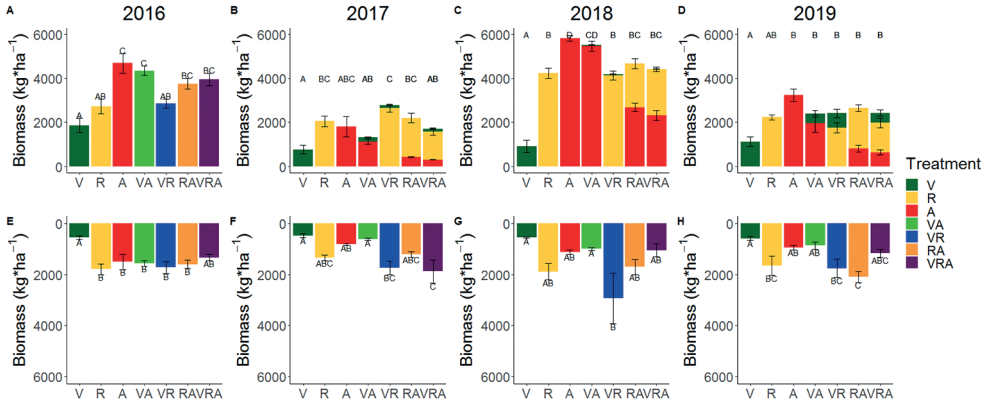

Figure 5: Cover crop biomass harvested in November each year (12 weeks after cover crop sowing). A-D aboveground biomass, E-H belowground biomass; A and E 2016, B and F 2017, C and G 2018, and D and H 2019. Letters on the x-axis indicate all different cover crop treatments with V: *V. sativa* (vetch), R: *R. sativus* (radish), A: *A. strigosa* (oat) and combinations of letters are two or three species mixtures. Aboveground biomass was split in species in 2017, 2018 and 2019. Bars are averages of 5 replicates ± SE. Letters indicate significant differences between the treatments (analyzed by Anova with post-hoc Tukey test with significance level $p < 0.05$).

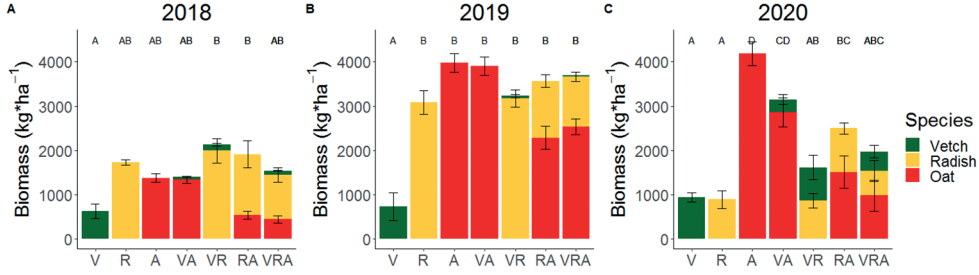

Figure 6: Aboveground cover crop biomass harvested in spring (February or March) just before cover crop incorporation: A: 2018, B: 2019, C: 2020. Letters on the x-axis indicate all different cover crop treatments with V: *V. sativa* (vetch), R: *R. sativus* (radish), A: *A. strigosa* (oat) and combinations of letters are two or three species mixtures. Bars are averages of 5 replicates +/- SE. Letters indicate significant differences between the treatments (analyzed by Anova with post-hoc Tukey test with significance level $p < 0.05$).

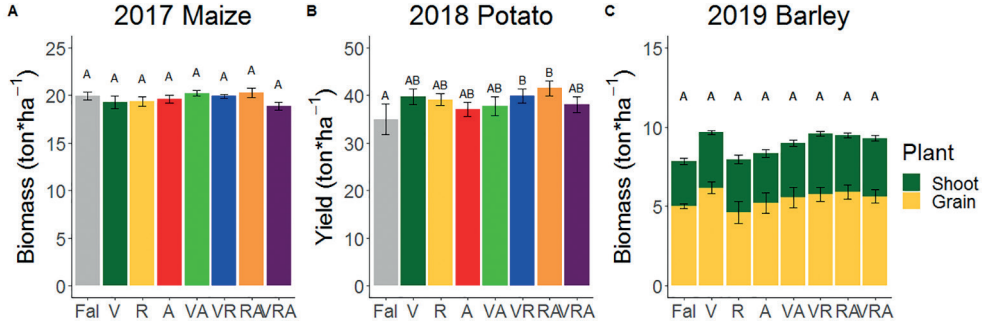

Figure 7: Main crop biomass: A 2017 aboveground biomass maize, B 2018 potato tuber yield, C 2019 aboveground biomass barley split in shoot and grain. Letters on the x-axis indicate all different cover crop treatments with Fal: fallow treatment, V: *V. sativa* (vetch), R: *R. sativus* (radish), A: *A. strigosa* (oat) and combinations of letters are two or three species mixtures. Bars are averages of 5 replicates +/- SE. Letters indicate significant differences between the treatments (analyzed by Anova with post-hoc Tukey test with significance level $p < 0.05$).

Table 2: Carbon and nitrogen content of both aboveground and belowground cover crops harvested in November (mean ± SE). Letters of the cover crop treatments indicate V: V. sativa (vetch), R: R. sativus (radish), A: A. strigosa (oat) and combinations of letters are two or three species mixtures. Letters indicate significant differences between the cover crop treatments in each year (analyzed by Anova with post-hoc Tukey test with significance level $p < 0.05$).

	2016		2017		2018		2019	
	C (kg*ha^{-1})	N (kg*ha^{-1})	C (kg*ha^{-1})	N (kg*ha^{-1})	C (kg*ha^{-1})	N (kg*ha^{-1})	C (kg*ha^{-1})	N (kg*ha^{-1})
V	903.16 ± 75.41 ^{A}	79.60 ± 7.50 ^{A}	533.96 ± 62.61 ^{A}	48.12 ± 8.81 ^{A}	592.07 ± 111.68 ^{A}	47.93 ± 11.42 ^{A}	679.62 ± 112.66 ^{A}	56.73 ± 10.28 ^{A}
R	1742.04 ± 230.43 ^{B}	71.59 ± 9.53 ^{A}	1368.37 ± 130.89 ^{BCD}	88.85 ± 11.86 ^{AB}	3047.68 ± 691.38 ^{AB}	191.03 ± 55.30 ^{B}	1472.69 ± 148.79 ^{A}	81.94 ± 8.78 ^{AB}
A	2698.62 ± 203.75 ^{D}	95.22 ± 9.08 ^{A}	1115.26 ± 166.54 ^{ABC}	66.77 ± 15.30 ^{A}	4765.57 ± 1811.80 ^{B}	199.92 ± 66.64 ^{B}	1643.46 ± 79.90 ^{A}	74.59 ± 4.88 ^{A}
VA	2499.09 ± 89.18 ^{CD}	106.96 ± 6.19 ^{A}	821.08 ± 71.55 ^{AB}	54.80 ± 5.19 ^{A}	2696.66 ± 83.37 ^{AB}	129.56 ± 9.80 ^{B}	1294.60 ± 195.41 ^{A}	75.20 ± 13.84 ^{A}
VR	1820.03 ± 73.16 ^{BC}	94.64 ± 6.71 ^{A}	1805.03 ± 79.94 ^{D}	129.57 ± 12.49 ^{B}	2778.16 ± 383.12 ^{AB}	163.73 ± 13.32 ^{B}	1546.99 ± 116.31 ^{A}	117.20 ± 3.28 ^{B}
RA	2274.36 ± 141.57 ^{BCD}	83.16 ± 9.13 ^{A}	1398.99 ± 92.14 ^{BCD}	91.03 ± 11.29 ^{AB}	2801.09 ± 430.54 ^{AB}	157.09 ± 25.55 ^{B}	1814.63 ± 126.79 ^{A}	90.87 ± 8.87 ^{AB}
VRA	2206.95 ± 172.12 ^{BCD}	98.82 ± 8.78 ^{A}	1463.51 ± 244.97 ^{CD}	93.41 ± 18.67 ^{AB}	2412.90 ± 413.64 ^{AB}	132.39 ± 22.35 ^{B}	1354.60 ± 21.73 ^{A}	94.55 ± 6.57 ^{AB}

Table 3: Carbon and nitrogen content of main crops (mean ± SE): 2017 maize aboveground plant material, 2018 potato tubers, and 2019 barley aboveground plant material. Letters of the cover crop treatments indicate Fal: fallow treatment, V: V. sativa (vetch), R: R. sativus (radish), A: A. strigosa (oat) and combinations of letters are two or three species mixtures. Letters indicate significant differences between the cover crop treatments in each year (analyzed by Anova with post-hoc Tukey test with significance level $p < 0.05$).

	2017 Maize		2018 Potato		2019 Barley	
	C (Mg*ha^{-1})	N (kg*ha^{-1})	C (Mg*ha^{-1})	N (kg*ha^{-1})	C (Mg*ha^{-1})	N (kg*ha^{-1})
Fal	8.54 ± 0.19 ^{A}	227.72 ± 5.88 ^{A}	13.80 ± 1.24 ^{A}	464.83 ± 38.18 ^{A}	3.33 ± 0.10 ^{A}	91.65 ± 3.47 ^{A}
V	8.29 ± 0.31 ^{A}	218.54 ± 14.33 ^{A}	15.64 ± 0.67 ^{A}	513.88 ± 23.63 ^{A}	4.09 ± 0.18 ^{A}	110.79 ± 4.36 ^{AB}
R	8.32 ± 0.24 ^{A}	226.33 ± 7.89 ^{A}	15.40 ± 0.45 ^{A}	545.72 ± 25.70 ^{A}	3.40 ± 0.40 ^{A}	108.02 ± 8.98 ^{AB}
A	8.44 ± 0.16 ^{A}	209.32 ± 5.79 ^{A}	14.59 ± 0.61 ^{A}	492.85 ± 21.91 ^{A}	3.55 ± 0.36 ^{A}	101.59 ± 10.15 ^{AB}
VA	8.69 ± 0.12 ^{A}	220.65 ± 12.63 ^{A}	14.96 ± 0.82 ^{A}	524.70 ± 36.44 ^{A}	3.80 ± 0.35 ^{A}	119.61 ± 7.27 ^{AB}
VR	8.59 ± 0.08 ^{A}	238.36 ± 10.54 ^{A}	15.74 ± 0.64 ^{A}	516.49 ± 22.46 ^{A}	4.11 ± 0.23 ^{A}	130.70 ± 5.47 ^{B}
RA	8.76 ± 0.24 ^{A}	234.99 ± 3.64 ^{A}	16.04 ± 0.76 ^{A}	548.19 ± 30.95 ^{A}	4.03 ± 0.21 ^{A}	120.16 ± 3.80 ^{AB}
VRA	8.11 ± 0.17 ^{A}	230.92 ± 11.20 ^{A}	15.04 ± 0.66 ^{A}	510.52 ± 25.04 ^{A}	3.97 ± 0.24 ^{A}	128.62 ± 4.32 ^{B}

Table 4: Carbon and nitrogen content of cover crops harvested in spring 2020 (mean ± SE). Letters of the cover crop treatments indicate V: V. sativa (vetch), R: R. sativus (radish), A: A. strigosa (oat) and combinations of letters are two or three species mixtures. Letters indicate significant differences between the cover crop treatments in each year (analyzed by Anova with post-hoc Tukey test with significance level $p < 0.05$).

	2020	
	C (kg*ha^{-1})	**N (kg*ha^{-1})**
V	376.14 ± 39.05 ^{A}	33.07 ± 3.21 ^{A}
R	329.52 ± 75.67 ^{A}	38.52 ± 8.49 ^{AB}
A	1716.20 ± 108.43 ^{D}	81.55 ± 5.00 ^{C}
VA	1272.21 ± 178.20 ^{CD}	77.09 ± 10.28 ^{BC}
VR	630.73 ± 133.34 ^{AB}	69.99 ± 15.66 ^{ABC}
RA	983.69 ± 125.96 ^{BC}	74.51 ± 6.33 ^{BC}
VRA	783.24 ± 120.48 ^{ABC}	67.87 ± 7.32 ^{ABC}

Soil nutrients

Soil mineral N was measured three or four times each year (Figure 8). Only in the second year of the experiment, the mineral N content differed between mixtures and monocultures in August ($p = 0.014$), where the VR combination showed significantly higher mineral N content as compared to oat (A) alone (Figure 8E). In all years, soil mineral N content was lowest in the samples taken in November (Figure 8B, 8F, 8J and 8N). In the first and last year of the experiment, soil mineral N content under *V. sativa* (V) was significantly higher compared to the other treatments ($p < 0.001$ and $p = 0.0056$ respectively for year 1 and 4) (Figure 8B and 8N). In the first and last year, mineral N was also measured just before cover crop incorporation. We found no significant differences between the treatments (Figure 8C and 8O). Two weeks after cover crop incorporation (Figure 8D, 8H, 8L and 8P), soil mineral N was higher in cover crop treatments compared to the fallow treatment in the first and third year of the experiment with either VR or RA as treatment with highest mineral N (analyzed by Anova with posthoc Tukey test with significance level $p < 0.05$ for each individual time point).

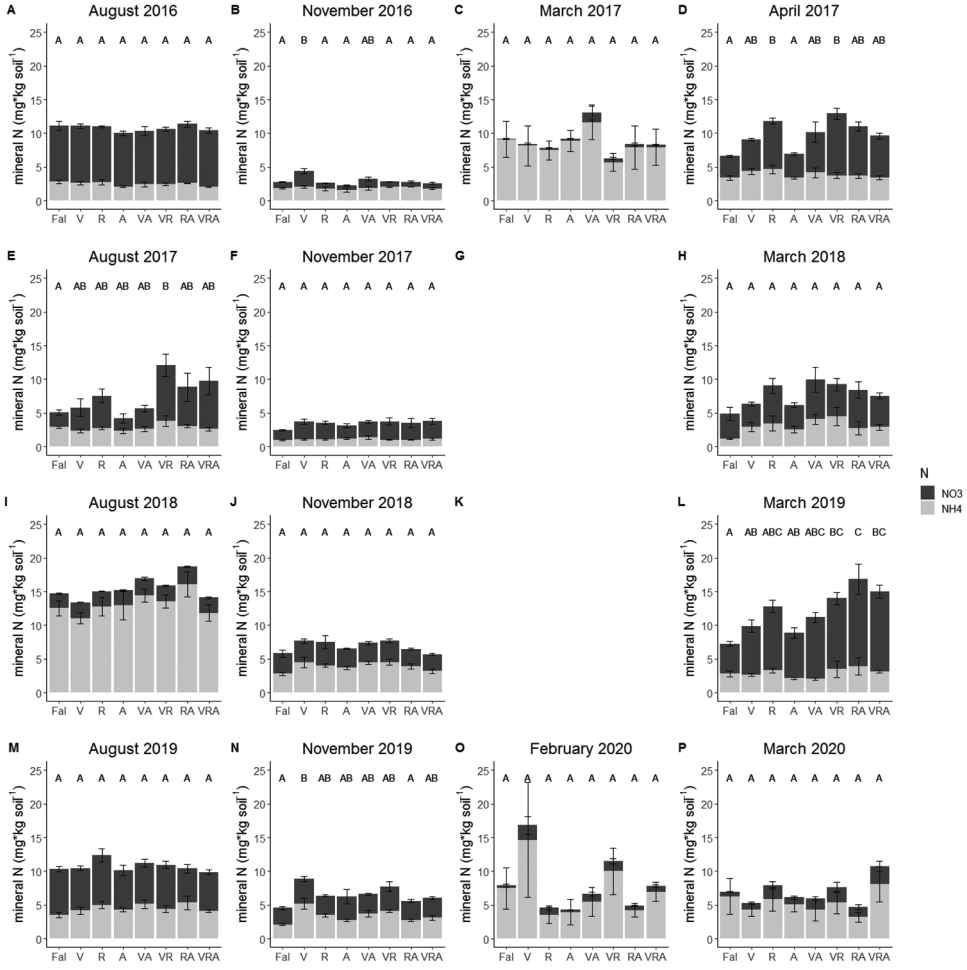

Figure 8: Soil mineral N (NO_3^- and NH_4^+) of each measured time point. A, E, I and M: measurement at sowing time cover crops before fertilization; B, F, J and N: measurement in November 12 weeks after cover crop sowing; C and O: measurement before cover crop incorporation; and D, H, L and P: measurements 2 weeks after cover crop incorporation. In 2018 and 2019, soil samples were not analyzed before cover crop incorporation (G and K). Letters on the x-axis indicate all different cover crop treatments with Fal: fallow treatment, V: *V. sativa* (vetch), R: *R. sativus* (radish), A: *A. strigosa* (oat) and combinations of letters are two or three species mixtures. Bars are averages of 5 replicates +/- SE. Letters indicate significant differences between the treatments at each individual time point of total mineral N measured (NO_3^- and NH_4^+) (analyzed by Anova with post-hoc Tukey test with significance level $p < 0.05$).

Microbial diversity

Bacterial and fungal DNA were analyzed with Illumina sequencing to estimate microbial diversity of the last year. The diversity indices richness, Chao1 and Shannon-index were used to determine differences between the treatments at each measured time point. Both richness measurements (Observed and Chao1) and evenness (Shannon) were not significantly different between the treatments ($p > 0.05$) for both bacteria and fungi

(Figure 9 and 10). Cover crop treatments were not significantly different from the fallow treatment. Furthermore, neither the treatments nor the time points did group differently in NMDS plot for both bacteria and fungi (Figure 11).

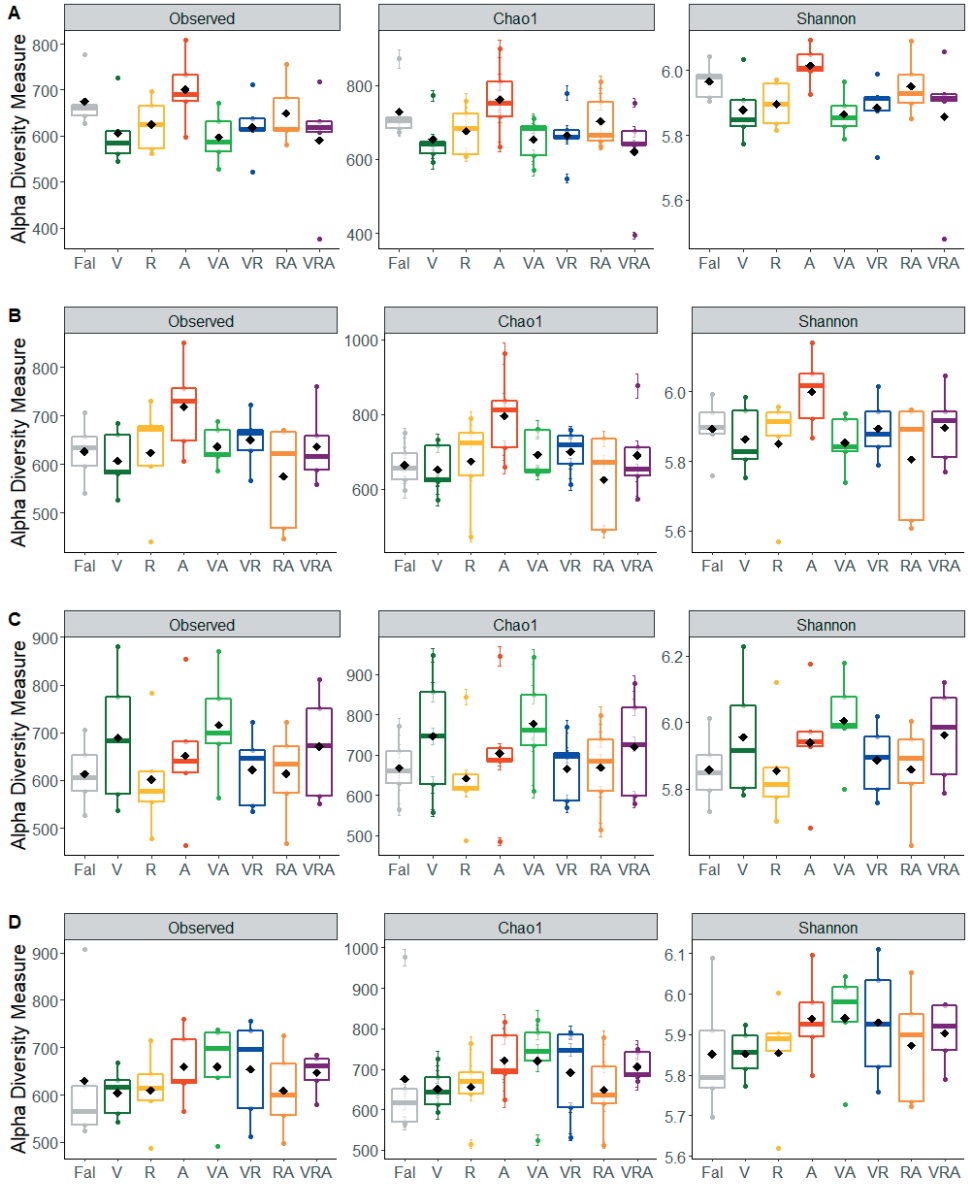

Figure 9: Bacterial diversity indices (richness, Chao1 and Shannon index) of year 4 at all different time points. A: August; B: November 12 weeks after cover crop sowing; C: February before cover crop incorporation; D: 2 weeks after cover crop incorporation. Letters on the x-axis indicate all different cover crop treatments with Fal: fallow treatment, V: *V. sativa* (vetch), R: *R. sativus* (radish), A: *A. strigosa* (oat) and combinations of letters are two or three species mixtures. Boxplots represent "minimum", first quartile (Q1), median, third quartile (Q3), and "maximum". Each individual sample is represented by the colored dots in the figure. Black diamonds represent the mean.

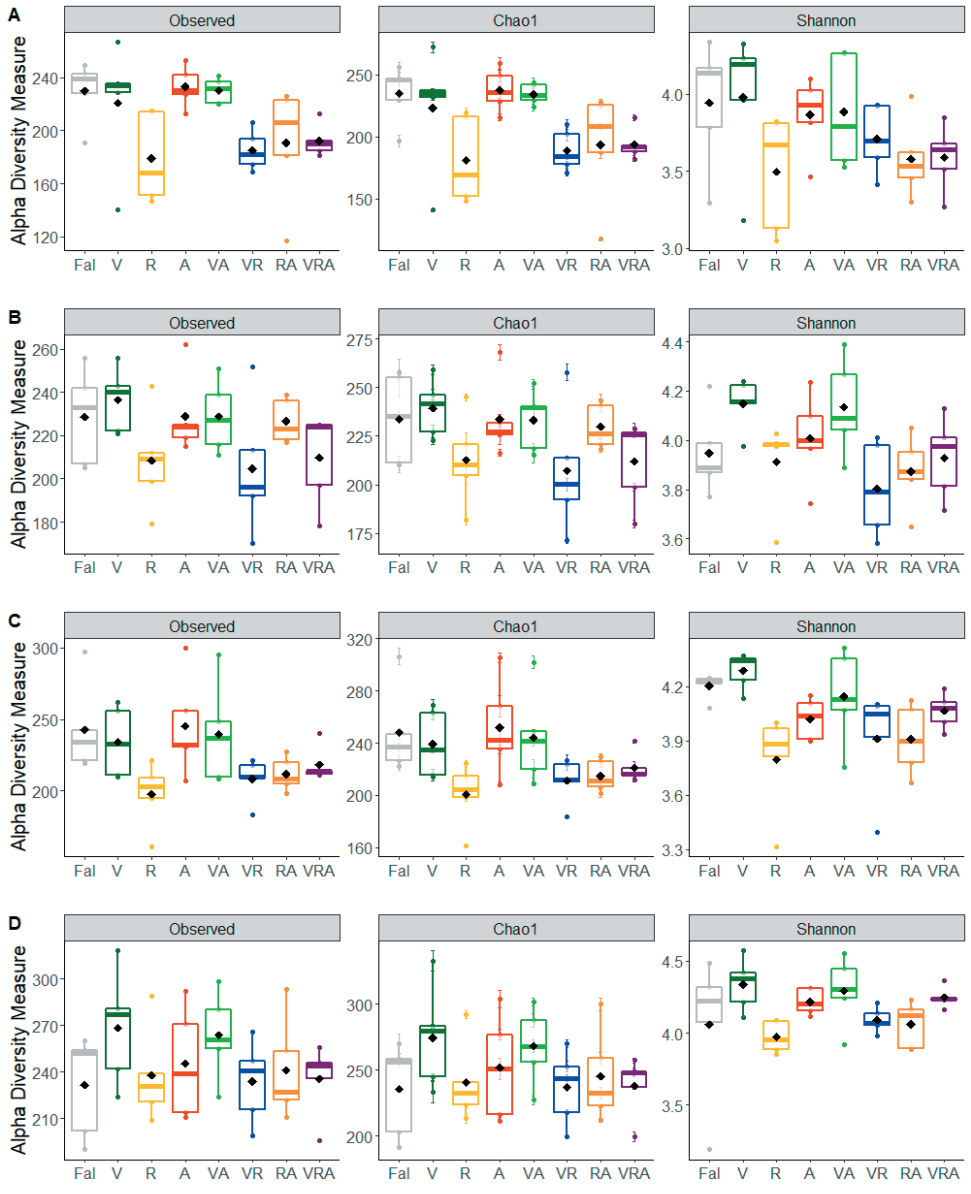

Figure 10: Fungal diversity indices (richness, Chao1 and Shannon index) of year 4 at all different time points. A: August; B: November 12 weeks after cover crop sowing; C: February before cover crop incorporation; D: 2 weeks after cover crop incorporation. Letters on the x-axis indicate all different cover crop treatments with Fal: fallow treatment, V: *V. sativa* (vetch), R: *R. sativus* (radish), A: *A. strigosa* (oat) and combinations of letters are two or three species mixtures. Boxplots represent "minimum", first quartile (Q1), median, third quartile (Q3), and "maximum". Each individual sample is represented by the colored dots in the figure. Black diamonds represent the mean.

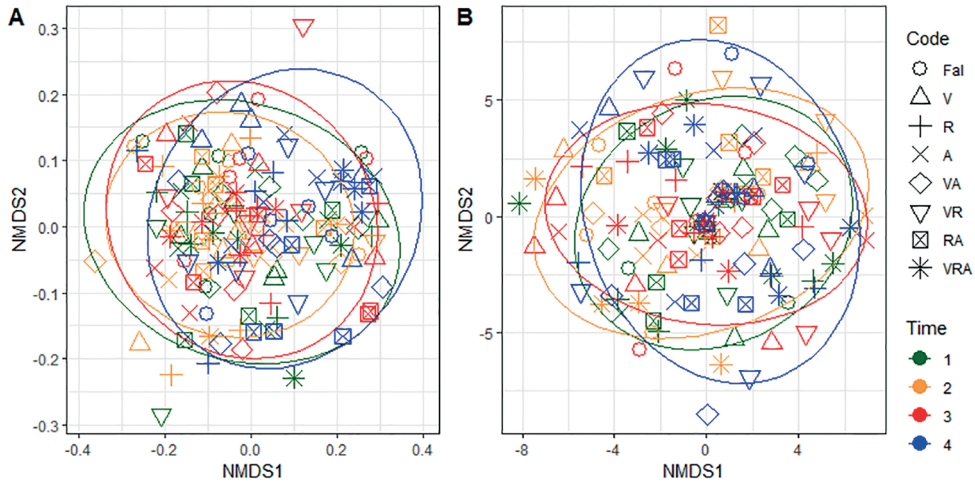

Figure 11: NMDS plot of microbial diversity of year 4. A: 16S, B: ITS. The colors indicate the 4 different time points: 1: August; 2: November 12 weeks after cover crop sowing; 3: February before cover crop incorporation; 4: 2 weeks after cover crop incorporation. Letters of the codes indicate all different cover crop treatments with Fal: fallow treatment, V: *V. sativa* (vetch), R: *R. sativus* (radish), A: *A. strigosa* (oat) and combinations of letters are two or three species mixtures.

qPCR results

Abundance of bacteria and fungi were measured for each soil sampling event in the first and last year of the experiment. For the functional genes, qPCR analyses were only done at the start and at the end of the experiment, concomitant with microbial activity assays.

Both bacterial and fungal gene copy numbers did not significantly differ between the treatments ($p = 0.65$ and $p = 0.20$, for 16S and ITS respectively) at each individual time point tested using mixed linear model and Anova (Figure 12 and 13). Cover crops (mixtures) did not increase bacterial and fungal biomass, even not after cover crop incorporation in spring (Figure 12D, 12H, 13D and 13H). However, significant differences were present between the measured time points regardless of the treatments ($p < 2.2*10^{-16}$ and $p = 7.91*10^{-14}$, for 16S and ITS respectively) (Figure 12 and 13). Bacterial biomass was high at the start and at the end of the experiment with reduced amount at the other measured time points (Figure 12). Fungal biomass was significantly higher in the last year compared to the first year (Figure 13).

The functional genes (*pmoA*, *amoA bacteria*, *amoA archea*, *nosZI*, *nosZII* and *nifH*) were not significantly different at the start of the experiment between the treatments. In the last year, 2 weeks after cover crop incorporation (Figure 14), only *amoA* bacteria was significantly different ($p = 0.0038$) with R and RA having higher copy numbers of *amoA* bacteria compared to the fallow treatment (Figure 14B).

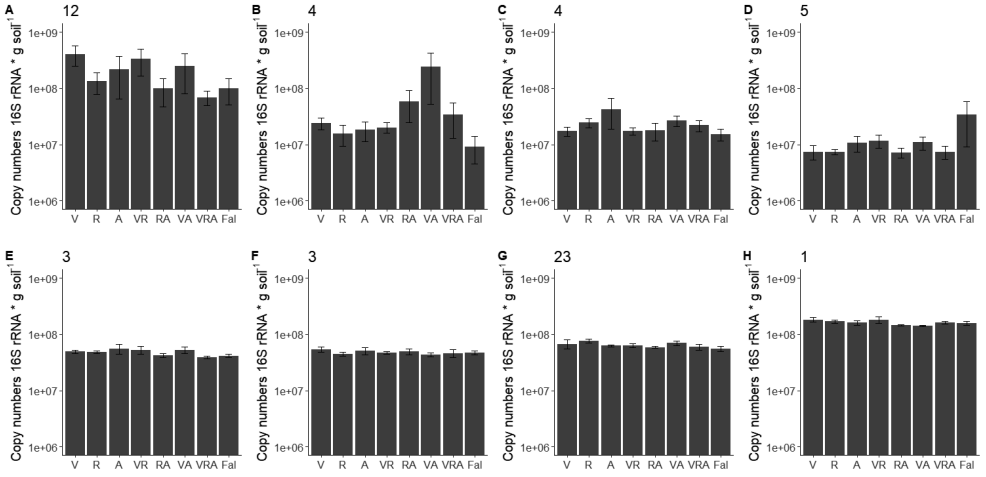

Figure 12: 16S copy numbers per gram soil measured year 1 (A-D) and year 4 (E-H). A and E: August; B and F: November 12 weeks after cover crop sowing; C and G: March/February before cover crop incorporation; D and H: 2 weeks after cover crop incorporation. Letters on the x-axis indicate all different cover crop treatments with Fal: fallow treatment, V: *V. sativa* (vetch), R: *R. sativus* (radish), A: *A. strigosa* (oat) and combinations of letters are two or three species mixtures. Bars are averages of 5 replicates +/- SE. Numbers on top of y-axis indicate significant differences between the time points, treatments were not significantly different at all individual time points (analyzed by Anova with post-hoc Tukey test with significance level $p < 0.05$).

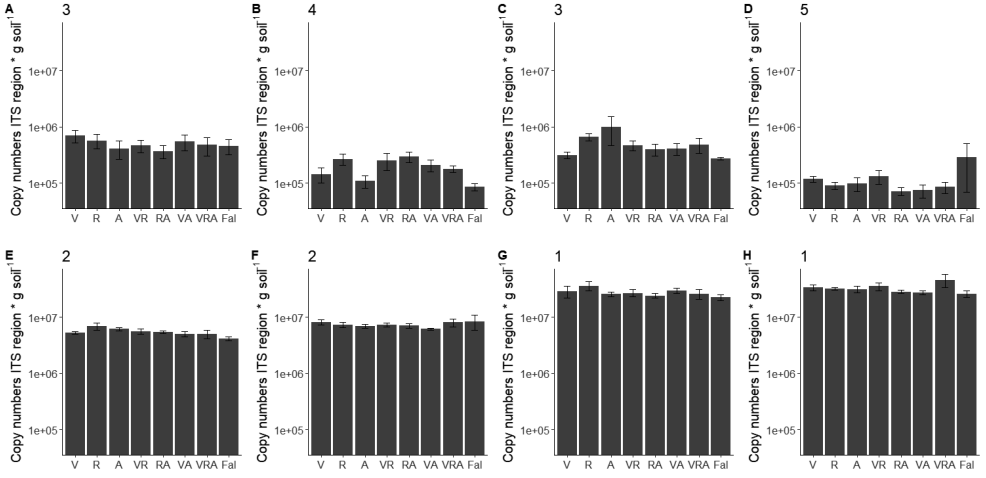

Figure 13: ITS copy numbers per gram soil measured year 1 (A-D) and year 4 (E-H). A and E: August; B and F: November 12 weeks after cover crop sowing; C and G: March/February before cover crop incorporation; D and H: 2 weeks after cover crop incorporation. Letters on the x-axis indicate all different cover crop treatments with Fal: fallow treatment, V: *V. sativa* (vetch), R: *R. sativus* (radish), A: *A. strigosa* (oat) and combinations of letters are two or three species mixtures. Bars are averages of 5 replicates +/- SE. Numbers on top of y-axis indicate significant differences between the time points, treatments were not significantly different at all individual time points (analyzed by Anova with post-hoc Tukey test with significance level $p < 0.05$).

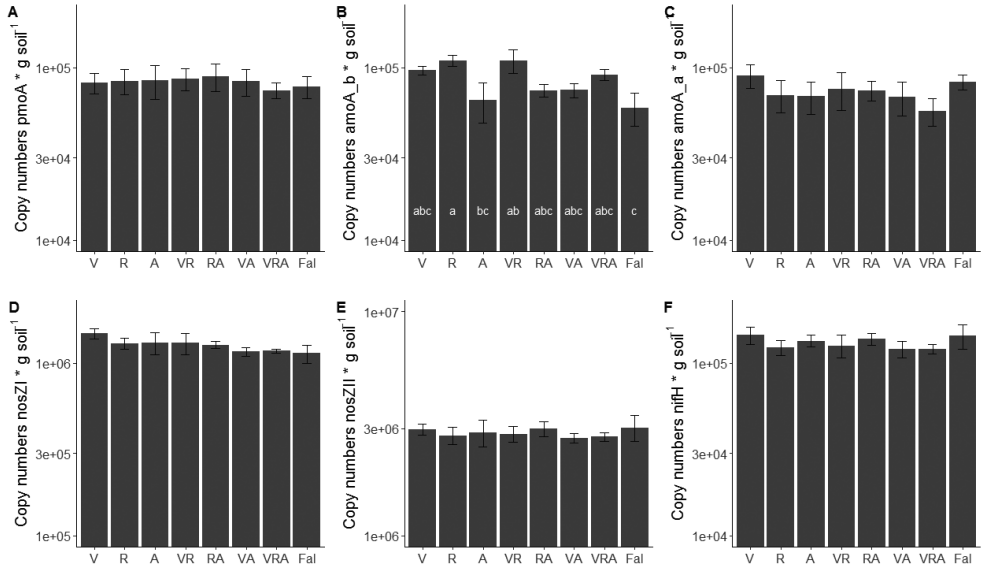

Figure 14: Copy numbers of functional genes at the last measured time point (two weeks after cover crop incorporation after 4 years). A: *pmoA*, B: *amoA bacteria*, C: *amoA archea*, D: *nosZI*, E: *nosZII* and F: *nifH*. Letters on the x-axis indicate all different cover crop treatments with Fal: fallow treatment, V: *V. sativa* (vetch), R: *R. sativus* (radish), A: *A. strigosa* (oat) and combinations of letters are two or three species mixtures. Bars are averages of 5 replicates +/- SE. Letters in bars of *amoA* bacteria (B) indicate significant differences between the treatments. Treatments were not significantly different for all other genes (analyzed by Anova with post-hoc Tukey test with significance level $p < 0.05$).

Microbial activity assays

Activity assays were measured in the first year and at the end of the experiment, 2 weeks after cover crop incorporation. In the first year, we did not find significant differences between the treatments at all measured time points. At the end of the experiment after 4 years of cover crops and 2 weeks after cover crop incorporation, both denitrification and nitrification were lower in the fallow treatment compared to the three species mixture (Figure 15A and B). Initial nitrification rate was highest in treatments including *R. sativa* (R, VR and VRA significantly different from fallow and *A. strigosa* monoculture). Nitrification rate correlated significantly positive ($r = 0.315$, $p = 0.047$) with measured gene copy numbers of bacterial *amoA*. Methane oxidation rate was not significantly different between treatments (Figure 15C). For N_2-fixation, measured as ethylene production, we did not find significant activity in any of the treatments. For all analyzed activity assays, we found only significant differences between single species treatments and mixtures for initial nitrification with mixtures having higher nitrification compared to monocultures, ($p = 0.25$, $p = 0.036$, $p = 0.62$ for denitrification, initial nitrification and methane oxidation, respectively).

Soil mineral N and gravimetric water content of the soil were measured at the same time point as these activity assays (Table S1). Denitrification rates correlated negatively with

soil mineral N (r = -0.27, $p = 0.014$) and positively with nitrification rate (r = 0.46, $p < 0.001$) while water percentage correlated positively with denitrification rate (r = 0.68, $p < 0.001$) and negatively with nitrification rate (r = -0.87, $p < 0.001$).

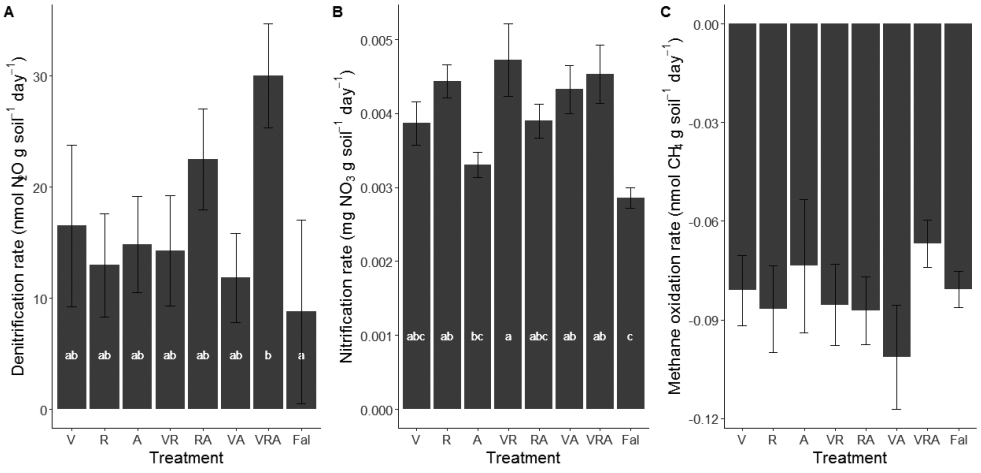

Figure 15: activity assays in year 4, 2 weeks after cover crop incorporation. A: denitrification, B: nitrification and C: methane oxidation. Letters on the x-axis indicate all different cover crop treatments with Fal: fallow treatment, V: *V. sativa* (vetch), R: *R. sativus* (radish), A: *A. strigosa* (oat) and combinations of letters are two or three species mixtures. Bars are averages of 5 replicates +/- SE. Letters indicate significant differences between treatments. As treatments were not significantly different for methane oxidation, letters are not shown here (analyzed by Anova with post-hoc Tukey test with significance level $p < 0.05$).

Greenhouse gas fluxes

Greenhouse gas (GHG) fluxes were measured regularly during 4 years of the experiment. CO_2 fluxes were fluctuating over the years (Figure 16). N_2O fluxes were negligible during cover crop growth, but peaked during cover crop decomposition (Figure 17). CH_4 fluxes were most of the time negative indicating uptake of CH_4 in the soil (Figure 18).

Analysis at individual measured time points did not result in significant differences between the treatments. However, when analyzing average GHG fluxes between the different seasons with repeated measures Anova (cover crop growth, cover crop decomposition or main crop growth) significant differences were observed for CO_2 ($p = 6.77*10^{-8}$) and N_2O ($p = 1.25*10^{-6}$) but not for CH_4 ($p = 0.128$) (Table S2). During the main crop season, GHG fluxes were not significantly different ($p = 0.138$, $p = 0.513$ and $p = 0.328$ for CO_2, N_2O and CH_4 respectively). CO_2 emissions (plant and soil respiration) were lower in the fallow treatment than during cover crop growth ($p = 0.00786$) and decomposition ($p = 5.81*10^{-5}$). However, the three species mixture was not significantly different from the fallow treatment (Figure 19). N_2O emissions were not significantly different between the treatments during cover crop growth ($p = 0.841$). Only during cover crop decomposition, the VR mix showed

significantly increased N_2O emissions compared to the fallow treatment ($p = 1.19*10^{-04}$, Figure 20). Differences occurred mainly between cover crop treatments and the fallow treatment and not between single species and mixtures. Furthermore, differences between treatments were most pronounced during cover crop decomposition.

Weather data during the experimental period were used to analyze correlation with GHG fluxes. Rainfall was calculated cumulative for 14 days before GHG measurements to take into account the weather history of the field. CO_2 fluxes did correlate positively with temperature ($r = 0.34$, $p < 0.0001$) and negatively with rainfall ($r = -0.13$, $p < 0.0001$). N_2O fluxes correlated positively with both temperature and rainfall, but the correlations were weak ($r = 0.054$, $p = 0.040$ and $r = 0.055$, $p = 0.038$ respectively).

To compare data that was measured less frequently with GHG fluxes, GHG data was merged in 3 groups following seasonal differences (cover crop growth, cover crop decomposition and main crop growth). Averages of the individual measurements within these seasonal groups per year were used to analyze correlation with other data (plant biomass, nutrient content in plant and soil, qPCR results, activity assays) (Table S1). Only significant relationships are described here. CO_2 fluxes did correlate significantly with soil water content (% water determined by drying of fresh soil) measured in the soil ($r = -0.34$, $p = 0.0017$), soil organic matter (SOM) content ($r = 0.36$, $p = 0.00094$) and soil mineral N ($r = 0.17$, $p = 0.034$) during cover crop decomposition. N_2O fluxes did correlate significantly with soil water percentage ($r = 0.36$, $p = 0.00087$) and SOM percentage ($r = 0.22$, $p = 0.049$). Aboveground cover crop biomass harvested before cover crop incorporation did not correlate with GHG fluxes. Standing biomass measured in November did correlate positively with both CO_2 and N_2O emissions ($r = 0.241$, $p = 0.0041$ and $r = 0.239$, $p = 0.0044$ respectively). Higher biomass resulted in increased respiration and GHG emissions measured during cover crop growth (Table S1).

CO_2 and N_2O fluxes correlated significantly with bacterial and fungal biomass (qPCR 16S rRNA gene and ITS region), whereas CO_2 fluxes were negatively correlated with microbial biomass ($r = -0.48$, $p < 0.0001$ and $r = -0.43$, $p < 0.0001$ for 16S rRNA gene and ITS region, respectively), N_2O fluxes were positively correlated ($r = 0.26$, $p = 0.019$ and $r = 0.19$, $p < 0.087$ for 16S rRNA gene and ITS region, respectively). Measured nitrification and denitrification activity at the end of the experiment during cover crop decomposition correlated significantly with CO_2 as well as with N_2O fluxes. Nitrification correlated positively with CO_2 ($r = 0.563$, $p < 0.001$) and negatively with N_2O ($r = -0.249$, $p = 0.026$), while denitrification showed the opposite result ($r = -0.306$, $p = 0.0058$ and $r = 0.238$, $p = 0.033$ for CO_2 and N_2O, respectively) (Table S1).

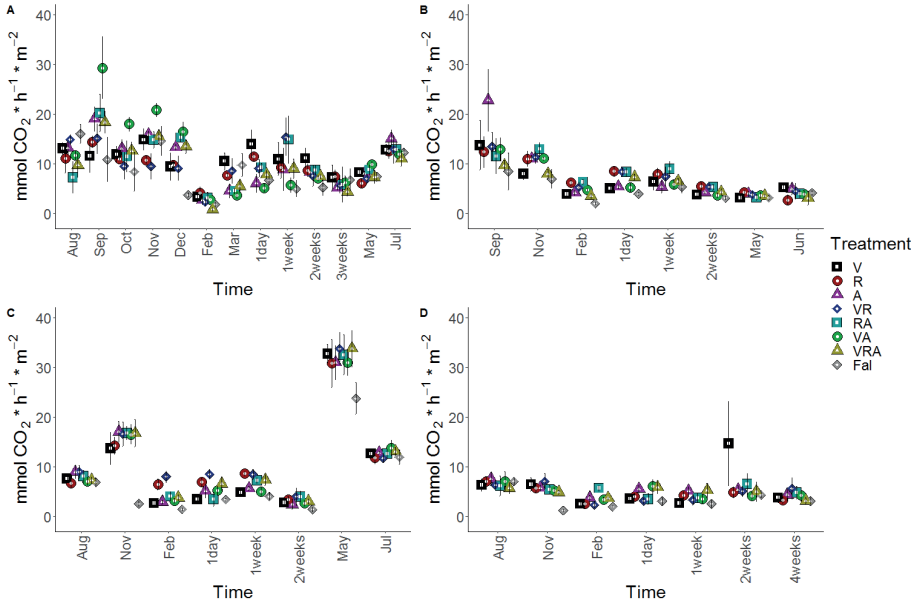

Figure 16: CO_2 fluxes of all 4 years starting from August 2016 until March 2020. A: first year -, B: second year -, C: third year -, D: last year of the experiment. Treatment letters indicate Fal: fallow treatment, V: *V. sativa* (vetch), R: *R. sativus* (radish), A: *A. strigosa* (oat) and combinations of letters are two or three species mixtures. Points are averages of 5 replicates +/- SE. Time on x-axis indicate measured time point, 1day/1week/etc. are measurements after cover crop incorporation before main crop was present.

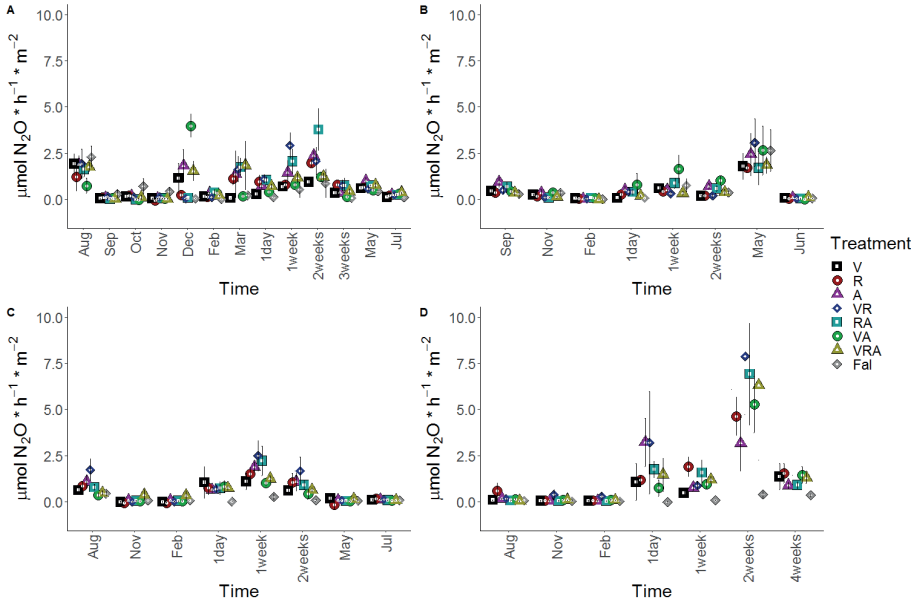

Figure 17: N_2O fluxes of all 4 years starting from August 2016 until March 2020. A: first year -, B: second year -, C: third year -, D: last year of the experiment. Treatment letters indicate Fal: fallow treatment, V: *V. sativa* (vetch), R: *R. sativus* (radish), A: *A. strigosa* (oat) and combinations of letters are two or three species mixtures. Points are averages of 5 replicates +/- SE. Time on x-axis indicate measured time point, 1day/1week/etc. are measurements after cover crop incorporation before main crop was present.

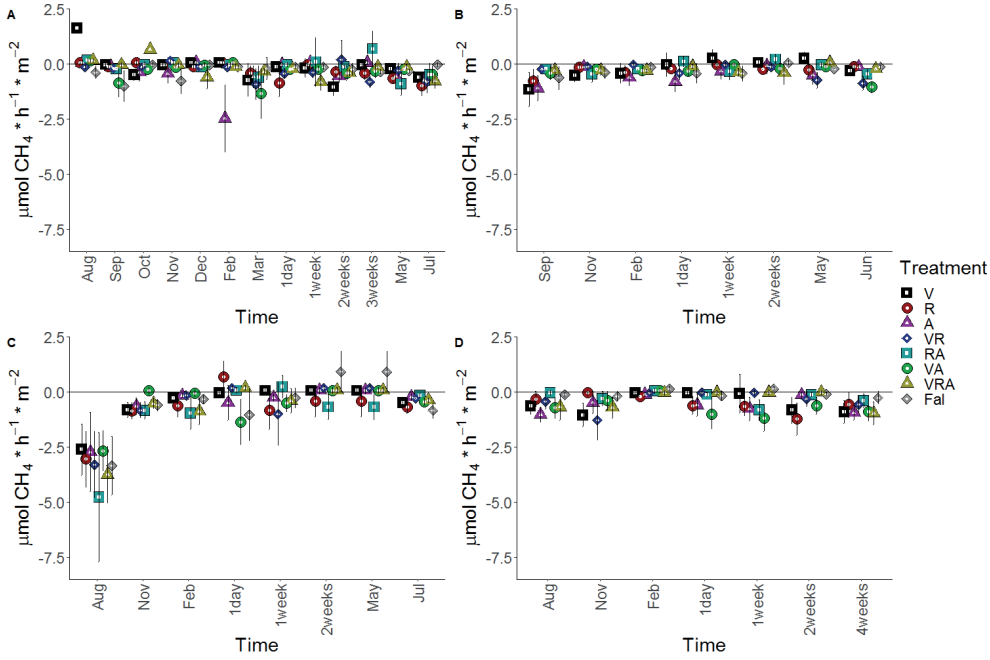

Figure 18: CH_4 fluxes of all 4 years starting from August 2016 until March 2020. A: first year -, B: second year -, C: third year -, D: last year of the experiment. Treatment letters indicate Fal: fallow treatment, V: *V. sativa* (vetch), R: *R. sativus* (radish), A: *A. strigosa* (oat) and combinations of letters are two or three species mixtures. Points are averages of 5 replicates +/- SE. Negative emissions are methane uptake into the soil. Time on x-axis indicate measured time point, 1day/1week/etc. are measurements after cover crop incorporation before main crop was present.

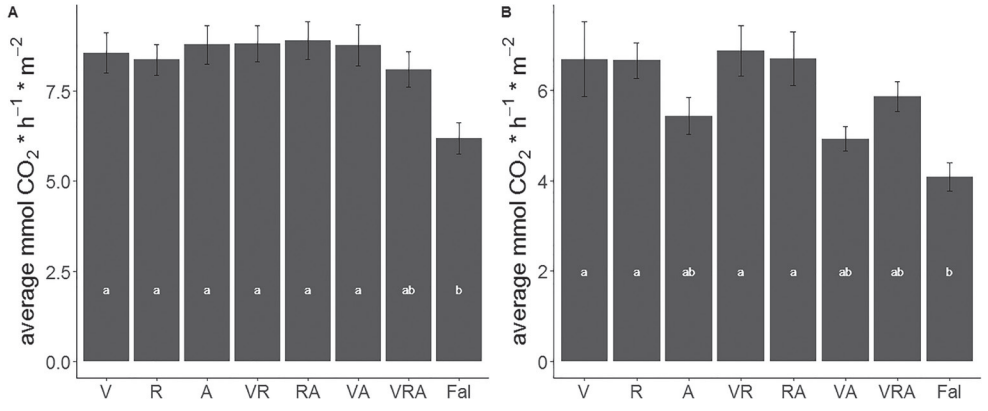

Figure 19: Average CO_2 fluxes measured over the whole experiment (A) and in spring during cover crop decomposition (B). Letters on the x-axis indicate all different cover crop treatments with Fal: fallow treatment, V: *V. sativa* (vetch), R: *R. sativus* (radish), A: *A. strigosa* (oat) and combinations of letters are two or three species mixtures. Points are averages of 5 replicates +/- SE. Letters inside bars indicate significant differences between treatments (analyzed with repeated measures Anova and post-hoc test with $p < 0.05$).

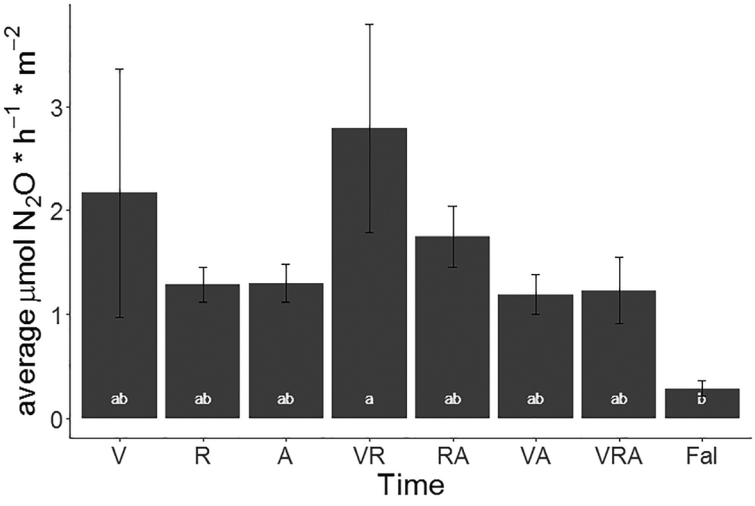

Figure 20: Average N_2O fluxes measured in spring during cover crop decomposition. Letters on the x-axis indicate all different cover crop treatments with Fal: fallow treatment, V: *V. sativa* (vetch), R: *R. sativus* (radish), A: *A. strigosa* (oat) and combinations of letters are two or three species mixtures. Points are averages of 5 replicates +/- SE. Letters indicate significant differences between treatments (analyzed with repeated measures Anova and post-hoc test with $p < 0.05$).

Discussion

Sustainable agriculture is needed to reduce losses of soil organic matter (SOM) and to ensure crop production with minimal negative impact on the environment. Growing of cover crops is an interesting option in this respect as it provides organic carbon to the system. Cover crops are commonly used to improve soil functions like nutrient uptake and carbon sequestration and therefore are a potential strategy in sustainable agriculture (Tsiafouli *et al.*, 2015). Cover crop mixtures are expected to increase the benefits of monoculture cover crops as it is hypothesized that combining plant species with different traits and functions have a positive effect on nutrient cycling and plant performance (Blanco-Canqui *et al.*, 2015). Several studies evaluated the use of cover crop monocultures (e.g. Zhou *et al.*, 2012; Strickland *et al.*, 2019) or mixtures (e.g. Nair and Ngouajio, 2012) to increase soil carbon, nitrogen retention and microbial biomass and activity, but a full factorial comparison between monocultures and mixtures in a field experiment is lacking. In this study we evaluated the effect on C- and N-cycling of three cover crop monocultures and all possible mixed combinations in a four-year field experiment. We tested if cover crop mixtures improve soil microbial biomass and diversity. Overall, we found no differences between cover crop mixtures and monocultures. The only differences we observed were between the fallow treatment and cover crop treatments for GHG fluxes and microbial activity (nitrification and denitrification). Below we discuss the results in more detail.

To determine if GHG fluxes can be reduced when using cover crop mixtures, we estimated GHG fluxes for four years during cover crop growth, cover crop decomposition and main crop growth. Contrary to our hypothesis, we did not find significant differences between the cover crop monocultures and mixtures. Only the fallow treatment had lower CO_2 and N_2O emissions compared to the cover crop treatments (Figures 19 and 20). These results are similar with those of previous studies where GHG emissions in cover crop plots where higher than in the fallow plots (Abdalla *et al.*, 2014; Basche *et al.*, 2014; Muhammad *et al.*, 2019). In our study, VR treatment had the highest N_2O emissions. Nitrification was also high in the treatment with *V. sativa* and *R. sativus* (VR) (Figure 15B). In our experimental fields, cover crop species reduced nitrogen leaching during cover crop growth (Porre, 2020 thesis chapter 2). This may compensate nitrogen losses via N_2O from the field as mineral nitrogen is taken up during cover crop growth. Furthermore, SOM significantly increased after 4 years with both VR and the three species mixture (VRA) compared to the start of the experiment (Porre, 2020 chapter 6). All other treatments were not significantly different in the 4 years of this field trial. Olson, Ebelhar and Lang (2010) did not find C sequestration after 8 years of cover crops either. In our agricultural field, prolonged growing of cover crops might be needed to realize increased C sequestration. Both CO_2 and N_2O fluxes fluctuated over the years and seasons. Environmental factors (temperature and moisture content) influence GHG fluxes, although only weak correlations were observed in our data (Table S1). Duan *et al.* (2018) found also higher year-to-year variation of GHG fluxes in a cover crop experiment than treatment differences. This shows that it is necessary to measure GHG fluxes regularly to be able to distinguish environmental fluctuations from treatment effects. Apart from CO_2 and N_2O, CH_4 fluxes were measured as well, but none of the treatments differed from each other (Figure 18). Potential methane oxidation was not different either (Figure 15C). We measured net uptake of CH_4 in the field in all treatments. This shows that agricultural fields are a possible sink for atmospheric methane, albeit with low uptake rates (Abdalla *et al.*, 2014). Other studies did find increased methane uptake by the addition of organic residues (Brenzinger *et al.*, 2018, 2021; Ho *et al.*, 2015b). However, 5 ton*ha^{-1} of cover crop residues might not be enough to stimulate the methanotroph community as these studies added at least 10 ton*ha^{-1}.

After 4 years we found increased nitrification and denitrification of the cover crop treatments compared to the fallow treatment (Figure 15). Two and three species mixtures containing *R. sativa* cover crop had highest denitrification and nitrification activities. The absence of differences between cover crop monocultures or mixtures may be explained by the lack of differences in soil mineral N after cover crop incorporation in the fourth year (Figure 8P). In our study, cover crops did not increase soil mineral N while others found increase of N by adding cover crops compared to a fallow with mineral fertilization (Tosti *et al.*, 2014; Alahmad *et al.*, 2019). Nitrification activity correlated with the abundance of bacterial *amoA* gene copies (Figure 14). Previous research showed that functional groups

related to nitrogen cycling and GHG consumption increased by the addition of cover crop mixtures (Brenzinger *et al.*, 2018, 2021). They found that mixed cover crop residues increased the abundance of *amoA*, *nirK*, *nirS*, *nifH* and *nosZ* when at least 10 ton*ha^{-1} of cover crop residue was added, however this disappeared when 5 ton*ha^{-1} was added (Brenzinger *et al.*, 2018). In our field experiment, the maximum aboveground biomass in four years was around 5 ton*ha^{-1} (Figure 5) which might not be enough to stimulate microbial community in the soil due to limited availability of carbon sources and unequal distribution in the field.

We did not find an increase of microbial biomass in the cover crop treatments at all measured time points in the first and last year (Figure 12 and 13). This is contrasting with results of other researchers who found an increase of microbial biomass in field plots with cover crops compared to fallow plots (Barel *et al.*, 2019; Muhammad *et al.*, 2021). Barel *et al.* (2019) did find increased microbial biomass using PLFA quantification in cover crop treatments compared to fallow treatment in a similar experimental field (sandy soil) and sampling depth (30 cm compared to 20 cm in our study). It is unclear if these differences can be attributed to the different quantification methods used. In our field experiment microbial biomass carbon (MBC) determined by using the fumigation extraction method did not show increased biomass in cover crop treatments either (Porre, 2020 thesis chapter 6). Even though microbial biomass was not different between the treatments, modification of the microbial community is possible (Zhang *et al.*, 2005; Alahmad *et al.*, 2019). We did find increased CO_2 emissions suggesting increased microbial activity. This could be due to enhanced activity in the existing community or to changes in community structure rather than increase in size (Zhang *et al.*, 2005; Sadet-Bourgeteau *et al.*, 2019). However, as we did not find differences between the diversity indices after four years of the experiment (Figure 9 and 10), it is expected that only minor changes of the microbial community occurred at the measured time points. Others found increased bacterial evenness (Alahmad *et al.*, 2019) which can indicate increase of functional complementarity which has been reported to enhance stability and functioning of the ecosystem (Wagg *et al.*, 2019). However, Alahmad *et al.* (2019) did not find increased richness or metabolic potential (measured using Biolog ECO plates) showing that 4 years of field experiment may not be enough to change microbial functional diversity in the soil by the addition of cover crops even though in a short term pot experiment cover crops significantly increased metabolic potential (Drost *et al.*, 2020).

As described above, cover crop treatments were only different compared to the fallow treatment and not between monocultures or mixtures rejecting hypothesis 1 and 2. The differences between cover crops and the fallow treatments were seen mainly after cover crop incorporation during decomposition, confirming hypothesis 3. During cover crop decomposition, N_2O fluxes and nitrification were significantly higher when comparing

all mixtures with all monocultures. All other measured microbial data (qPCR, microbial diversity, microbial activity and CO_2 and CH_4 fluxes) were not significantly different between cover crop monocultures and mixtures. Many studies analyzed differences between cover crop (mixture) and fallow treatments (Tosti *et al.*, 2014; Mbuthia *et al.*, 2015; Alahmad *et al.*, 2019; Garland *et al.*, 2021; Wood and Bowman, 2021). Yet, only a limited number of studies compared effects of monocultures and mixed cultures (Zhou *et al.*, 2012; Smith, Atwood and Warren, 2014). Zhou *et al.* (2012) did not find differences in soil C and N pools and microbial biomass and activity (MicroResp) between cover crop monocultures and mixtures. Furthermore, in a meta-analysis, cover crop diversity did not result in increased bacterial diversity. However, duration of cover crop cultivation positively influenced soil microbial diversity and nutrient cycling (Garland *et al.*, 2021). In our study, we analyzed both GHG fluxes and microbial activity and diversity during four years of cover crop cultivation, but we did not find advantages of cover crop mixtures compared to monocultures. Furthermore, soil microbial biomass and diversity in cover crop treatments were not significantly different from the fallow treatment. Several factors may have contributed to this lack of responses. First, sampling depth of 20 cm of soil was taken for DNA analyses while cover crops were only incorporated in the top 5 cm. This might have diluted effects of decomposing cover crop residues. Second, weeds were not removed from the fields. Hence, although there were no cover crops, biomass of weeds was incorporated in the fallow treatment. However, the biomass of the weeds was low with maximally 1000 $kg*ha^{-1}$ in November, except in 2018 (3000 $kg*ha^{-1}$). Weed biomass was not measured in spring, but total biomass in November was significantly lower in the fallow treatment compared to the cover crop treatments. Third, effects of cover crops may be short term. GHG fluxes were significantly different after cover crop incorporation indicating increased microbial activity. It is possible that the microbial turnover is high or that the carbon - and nitrogen use efficiency (CUE and NUE) are reduced leading to no increase in microbial biomass (Blagodatskaya and Kuzyakov, 2013; Dijkstra *et al.*, 2015; Mooshammer *et al.*, 2014b).

From this study we can conclude that during a four-years period of cover crop cultivation, mixtures did not develop benefits compared to monocultures with respect to GHG fluxes, microbial biomass, - diversity and - activity. Cover crop treatments increase microbial activity and GHG fluxes compared to the fallow treatment mainly during cover crop decomposition in spring. Measurements of the full carbon and nitrogen cycle are needed to determine possible positive effects of cover crop mixtures in longer term field experiments.

Supplementary

Table S1: Correlation table of data GHG fluxes (CH_4, CO_2, N_2O), weather (rain (cumulative over 14 days) and temperature), qPCR (copy numbers 16S, ITS, pmoa, amoA bacteria, amoA archea, nosZI, nosZII and nifH), microbial activity (nitrification, denitrification, N-fixation and methane oxidation), water % soil, soil mineral nitrogen, soil organic matter (SOM), aggregates (macro and micro), cover crop plant biomass (CC B) and cover crop nutrient content (CC C:N, CC N and CC C) all measured in spring 2 weeks after cover crop incorporation[1]. Stars indicate significance level with *** $p < 0.001$, ** $0.001 < p < 0.01$ and * $0.01 < p < 0.05$, if no star is present $p > 0.05$. Color indicate correlation coefficient with white indicating -1 and dark grey indicating 1.

	CH_4	CO_2	N_2O	Rain	Temp	16S	ITS	pmoA	amoA_b	amoA_a	nosZI	nosZII	nifH	nitr	denitr	N-fix	CH_4 ox
CH_4	1	-0.08	-0.03	-0.19*	0.00	-0.18	-0.18	0.38*	0.06	0.37*	0.11	0.40*	0.47**	0.15	-0.09	0.12	-0.07
CO_2	-0.08	1	0.22**	-0.26***	0.42***	-0.47***	-0.42***	0.22	0.27	0.29	0.36*	0.18	0.01	0.56***	-0.30**	-0.11	-0.30**
N_2O	-0.03	0.22**	1	0.35***	0.03	0.26*	0.19	0.04	0.21	0.22	0.30	-0.05	0.03	-0.24*	0.23*	0.17	0.09
Rain	-0.19*	-0.26***	0.35***	1	-0.17*	0.94***	0.87***	7.07	6.77	6.81	1.18	1.41	9.86	-0.94***	0.67***	0.56***	0.32**
Temp	0.00	0.42***	0.03	-0.17*	1	-0.65***	-0.59***	0.37*	0.08	0.21	0.14	0.48**	0.37*	0.69***	-0.44***	-0.08	-0.17
16S	-0.18	-0.47***	0.26*	0.94***	-0.65***	1	0.90***	0.52***	0.64***	0.49**	0.76***	0.41**	0.39*	-0.89***	0.67***	0.48***	0.30**
ITS	-0.18	-0.42***	0.19	0.87***	-0.59***	0.90***	1	0.25	0.50***	0.05	0.35*	0.27	0.01	-0.83***	0.67***	0.57***	0.28*
pmoA	0.38*	0.22	0.04	7.07	0.37*	0.52***	0.25	1	0.55***	0.79***	0.67***	0.67***	0.57***	-0.17	0.18	0.85	-0.56***
amoA_b	0.06	0.27	0.21	6.77	0.08	0.64***	0.50***	0.55***	1	0.46**	0.67***	0.40**	0.19	0.31*	0.07	0.53	-0.27
amoA_a	0.37*	0.29	0.22	6.81	0.21	0.49**	0.05	0.79***	0.46**	1	0.70***	0.40**	0.44**	-0.19	0.01	0.65	-0.46**
nosZI	0.11	0.36*	0.30	1.18	0.14	0.76***	0.35*	0.67***	0.67***	0.70***	1	0.53***	0.50***	-0.13	0.05	0.32	-0.23
nosZII	0.40*	0.18	-0.05	1.41	0.48**	0.41**	0.27	0.67***	0.40**	0.40**	0.53***	1	0.74***	-0.06	-0.07	0.84	-0.44**
nifH	0.47**	0.01	0.03	9.86	0.37*	0.39*	0.01	0.57***	0.19	0.44**	0.50***	0.74***	1	-0.17	0.03	0.76	-0.29
nitr	0.15	0.56***	-0.24*	-0.94***	0.69***	-0.89***	-0.83***	-0.17	0.31*	-0.19	-0.13	-0.06	-0.17	1	-0.64***	-0.44**	-0.31**
denitr	-0.09	-0.30**	0.23*	0.67***	-0.44***	0.67***	0.67***	0.18	0.07	0.01	0.05	-0.07	0.03	-0.64***	1	0.34*	0.22*
N-fix	0.12	-0.11	0.17	0.56***	-0.08	0.48***	0.57***	0.85	0.53	0.65	0.32	0.84	0.76	-0.44**	0.34*	1	0.07
CH_4 ox	-0.07	-0.30**	0.09	0.32**	-0.17	0.30**	0.28*	-0.56***	-0.27	-0.46**	-0.23	-0.44**	-0.29	-0.31**	0.22*	0.07	1
Water %	-0.21	-0.34**	0.36***	0.93***	-0.51***	0.88***	0.80***	0.43**	0.01	0.31*	0.32*	0.33*	0.35*	-0.86***	0.68***	0.56***	0.28*
Soil N	0.11	0.16*	0.03	-0.26***	0.12	-0.34**	-0.17	-0.02	0.28	-0.01	0.17	0.10	0.13	0.46***	-0.27*	-0.15	-0.07
SOM	0.07	0.36***	0.22*	0.04	0.69***	0.04	0.00	0.60***	0.30	0.45**	0.34*	0.64***	0.42**	0.13	-0.06	0.77	-0.62***
DOC	0.09	0.04	-0.27*	-0.74***	-0.01	0.23	0.21	0.18	0.26	0.27	0.25	0.09	-0.12	-0.04	-0.07	0.52	-0.12
MBC	0.42***	0.18	-0.02	-0.52***	0.20	0.02	0.10	0.41**	0.20	0.19	0.17	0.49**	0.22	0.19	0.06	0.99***	-0.31
Macro-aggr	-0.02	0.07	0.30	3.37	-0.35*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Micro-aggr	0.32	-0.11	0.23	2.54	-0.81***	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Silt+clay	-0.33	-0.24	-0.19	2.50	0.50*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
CC B	-0.05	0.04	-0.02	-0.00	0.19*	-0.14	-0.07	0.15	-0.36*	0.04	-0.00	0.04	-0.01	-0.31	0.00	0.91	0.05
CC C:N	-0.10	0.22	-0.08	4.19	0.10	-0.29	-0.24	0.04	-0.52**	-0.03	-0.10	0.03	0.07	-0.33*	-0.03	0.72	-0.01
CC N	0.10	0.01	0.01	4.83	0.00	0.09	0.14	0.24	-0.04	0.16	0.14	0.02	-0.08	-0.24	0.14	0.93	0.11
CC C	-0.01	0.09	-0.09	2.25	0.08	-0.15	-0.08	0.13	-0.37*	0.02	-0.00	0.04	-0.01	-0.30	-0.00	0.91	0.06

Table S1: Continued.

	Water %	Soil N	SOM	DOC	MBC	Macro-aggr	Micro-aggr	Silt+clay	CC B	CC C:N	CC N	CC C
CH_4	-0.21	0.11	0.07	0.09	0.42***	-0.02	0.32	-0.33	-0.05	-0.10	0.10	-0.01
CO_2	-0.34**	0.16*	0.36***	0.04	0.18	0.07	-0.11	-0.24	0.04	0.22	0.01	0.09
N_2O	0.36***	0.03	0.22*	-0.27*	-0.02	0.30	0.23	-0.19	-0.02	-0.08	0.01	-0.09
Rain	0.93***	-0.26***	0.04	-0.74***	-0.52***	3.37	2.54	2.50	-0.00	4.19	4.83	2.25
Temp	-0.51***	0.12	0.69***	-0.01	0.20	-0.35*	-0.81***	0.50*	0.19*	0.10	0.00	0.08
16S	0.88***	-0.34**	0.04	0.23	0.02	NA	NA	NA	-0.14	-0.29	0.09	-0.15
ITS	0.80***	-0.17	0.00	0.21	0.10	NA	NA	NA	-0.07	-0.24	0.14	-0.08
pmoA	0.43**	-0.02	0.60***	0.18	0.41**	NA	NA	NA	0.15	0.04	0.24	0.13
amoA_b	0.01	0.28	0.30	0.26	0.20	NA	NA	NA	-0.36*	-0.52**	-0.04	-0.37*
amoA_a	0.31*	-0.01	0.45**	0.27	0.19	NA	NA	NA	0.04	-0.03	0.16	0.02
nosZI	0.32*	0.17	0.34*	0.25	0.17	NA	NA	NA	-0.00	-0.10	0.14	-0.00
nosZII	0.33*	0.10	0.64***	0.09	0.49**	NA	NA	NA	0.04	0.03	0.02	0.04
nifH	0.35*	0.13	0.42**	-0.12	0.22	NA	NA	NA	-0.01	0.07	-0.08	-0.01
nitr	-0.86***	0.46***	0.13	-0.04	0.19	NA	NA	NA	-0.31	-0.33*	-0.24	-0.30
denitr	0.68***	-0.27*	-0.06	-0.07	0.06	NA	NA	NA	0.00	-0.03	0.14	-0.00
N-fix	0.56***	-0.15	0.77	0.52	0.99***	NA	NA	NA	0.91	0.72	0.93	0.91
CH_4 ox	0.28*	-0.07	-0.62***	-0.12	-0.31	NA	NA	NA	0.05	-0.01	0.11	0.06
Water %	1	-0.40***	0.52***	0.03	0.50***	NA	NA	NA	0.24	0.20	0.23	0.23
Soil N	-0.40***	1	-0.02	0.42***	0.33**	0.09	0.04	-0.17	0.26**	-0.21	-0.00	-0.14
SOM	0.52***	-0.02	1	-0.07	0.18	0.33	-0.45	0.20	-0.03	0.12	0.01	0.08
DOC	0.03	0.42***	-0.07	1	0.39***	0.13	-0.27	-0.22	0.26*	-0.17	0.11	-0.06
MBC	0.50***	0.33**	0.18	0.39***	1	-0.16	-0.05	-0.08	0.16	0.12	0.05	0.12
Macro-agr	NA	0.09	0.33	0.13	-0.16	1	0.16	-0.31	-0.20	NA	NA	NA
Micro-aggr	NA	0.04	-0.45	-0.27	-0.05	0.16	1	-0.32	0.04	NA	NA	NA
Silt+clay	NA	-0.17	0.20	-0.22	-0.08	-0.31	-0.32	1	0.02	NA	NA	NA
CC B	0.24	0.26**	-0.03	0.26*	0.16	-0.20	0.04	0.02	1	0.84***	0.82***	0.99***
CC C:N	0.20	-0.21	0.12	-0.17	0.12	NA	NA	NA	0.84***	1	0.42*	0.85***
CC N	0.23	-0.00	0.01	0.11	0.05	NA	NA	NA	0.82***	0.42*	1	0.81***
CC C	0.23	-0.14	0.08	-0.06	0.12	NA	NA	NA	0.99***	0.85***	0.81***	1

Table S2: Result of measures Anova including treatment effect, block effect and season. Only p-values are shown in table for corresponding analyses.

	CO2	**N2O**	**CH4**
ALL DATA			
Treatment effect	$6.77*10^{-8}$	$1.25*10^{-6}$	0.12795
Block effect	0.379	0.932	0.00394
ONLY COVER CROP GROWTH			
Treatment effect	0.000289	0.841	0.1567
Fallow vs. cover crop	0.00786		
Monoculture vs mixture (excluding fallow)	0.615		
AFTER COVER CROP INCORPORATION			
Treatment effect	$5.46*10^{-5}$	$2.28*10^{-10}$	0.168
Fallow vs. cover crop	$5.81*10^{-5}$	0.000119	
Monoculture vs mixture (exluding fallow)	0.92	0.00498	
DURING MAIN CROP GROWTH			
Treatment effect	0.136	0.513	0.328

CHAPTER

6

Synthesis and outlook

Cover crops are increasingly used by farmers as sustainable management strategy and different aspects of their possible contribution to agricultural sustainability are studied (e.g. Wood and Bowman 2021; Garland *et al.* 2021). It is known that growing of plant mixtures instead of monocultures in grasslands stimulates plant biomass, microbial biomass and - functioning and reduces nutrient losses (Isbell *et al.*, 2017; Thakur *et al.*, 2021). Sowing of cover crop mixtures instead of monocultures after the harvest of the main crop may give similar beneficial effects. In this thesis, I compared several effects of cover crop mixtures with those of cover crop monocultures. In pot experiments, I studied the effect of cover crops residue assembly on decomposition and microbial metabolic functioning, in field experiments the focus was on microbial biomass, microbial diversity, microbial activity and greenhouse gas (GHG) emissions. In field experiments, the effects of both the standing cover crops as well as of incorporated residues were included. I hypothesized that mixtures of cover crop species with different functional traits will increase microbial biomass and – diversity. As consequence thereof, I expect an increased efficiency of use of carbon and nutrients by soil microbes, thereby reducing GHG emissions and providing a more gradual delivery of nutrients to the cash crop. In this chapter, I will summarize the main results of the thesis and discuss these in relation to current knowledge.

Cover crop mixtures

The effects of cover crops mixtures were studied both in pot experiments (Chapter 2 and 3) and in a field experiment (Chapter 5). If farmers use cover crops in their field to avoid the fallow period in autumn and winter, cover crops are grown under sub-optimal environmental circumstances, such as low temperatures and short periods of daylight. In spring, when preparing the soils for sowing or planting of cash crops, cover crops are incorporated in the soil. In this synthesis, I split the effects of standing cover crops and incorporated cover crop residues as the effects of cover crop mixtures may be different during residue decomposition than during cover crop growth.

Cover crop growth

In a four-year field experiment, cover crops were included in a full factorial design with three monocultures (*Vicia sativa*, *Raphanus sativus* and *Avena strigosa*) and all possible combinations of 2-species - and 3-species mixtures. Cover crop biomass in the mixtures was as high as the best performing monoculture 12 weeks after sowing (Chapter 5). The best performing monoculture differed between the years. This indicates that cover crop mixtures reduce the risk of sub-optimal growth compared to monocultures and that yield variability in cover crop mixtures is reduced (Elhakeem *et al.*, 2021). This is thought to be the result of plant complementarity and facilitation effects between the different cover crop species in a mixture (Wendling *et al.*, 2017). Furthermore, during cover crop growth, nitrogen is captured in plant biomass. This reduced leaching compared to fallow plots

(Porre 2020, chapter 2). Only in the monoculture of *V. sativa* nitrogen was lost via leaching probably due to low plant biomass compared to the other cover crop treatments. In mixtures, this disadvantage of the leguminous species disappeared.

We observed increased CO_2 emissions during cover crop growth compared to the fallow treatment, but this was not the case for N_2O emissions (Chapter 5). CO_2 emissions are derived from both plant - and microbial respiration in the soil. Plant root exudates are known to stimulate microbial activity, respiration and microbial biomass in the soil (Steinauer, Chatzinotas and Eisenhauer, 2016b). Furthermore, part of the plant material dies off and is decomposed already during the growing season, which can stimulate the microbial community. Yet, we did not find increased microbial biomass in the first and last year of the field experiment during cover crop growth (Chapter 5). This might be a result of low temperatures or the soil sampling procedure, which included both rhizosphere and bulk soil. Hence, zones of high microbial activity (rhizosphere, dead plant parts) are mixed with bulk soil zones of low microbial activity. Yet, it is clear that a possible positive effect of growing cover crops on microbial biomass does not spread throughout the whole upper (20 cm) soil layer.

Overriding effects of single dominant species may also contribute to reported lack of differences between addition of cover crop mixtures or monocultures regarding nutrient cycling and weed suppression despite reduced fluctuations in cover crop mixtures (Zhou *et al.*, 2012; Smith, Atwood and Warren, 2014). It may be necessary to include more monocultures in a full factorial design to determine which cover crops are performing best for different soils (e.g. clay or sandy) and environmental conditions or to achieve certain purposes (increase SOM and microbial activity and/or reduce leaching, pathogens and weeds) in agricultural rotations. This can result in tailor-made solutions for finding the best performing mixtures for specific soil conditions and planned cash crops.

After cover crop incorporation

When cover crops are incorporated in the soil, this will lead to increased carbon- and mineral nutrient sources and, consequently, to increases in microbial biomass and -activity (Nakamoto *et al.*, 2012; Muhammad *et al.*, 2021). We found higher denitrification and nitrification rates in the fourth year of the field experiment in cover crops plots than in fallow plots (Chapter 5). Furthermore, it is expected that addition of cover crops will increase microbial diversity in the field (Mbuthia *et al.*, 2015; Garland *et al.*, 2021). However, we did not find increased microbial biomass or diversity in the field (Chapter 5). Comparable with our results, Alahmad *et al*. (2019) did not find increased richness of the microbial community. Only microbial evenness increased and not functional diversity (measured with Biolog ECO plates) (Alahmad *et al.*, 2019). Furthermore, a meta-analysis indicated that bacterial diversity was more affected by management intensity and duration of cover

crop cultivation than diversity of the cover crops (Garland *et al.*, 2021). However, in a pot experiment, microbial biomass and functional diversity measured with Biolog ECO plates were increased after addition of cover crop residues (Chapter 2). Residues of cover crop mixtures increased metabolic potential compared to monoculture residues, while these differences were not seen for microbial biomass.

Stimulation of microbial activity after incorporation of cover crop mixtures can occur (Shi and Marschner, 2014). Yet, environmental fluctuation can be larger than treatment effects in field experiments resulting in the absence of significant differences between cover crop mixtures and monocultures (Duan *et al.*, 2018). Furthermore, plant species identity might be more important in bringing about changes of the microbial biomass and activity (Hattenschwiler and Gasser, 2005; Shi and Marschner, 2014). In our pot experiment, monoculture residue of *V. sativa* did result in high microbial functional diversity, demonstrating the possibility of a major contribution of one plant species to microbial functioning (Chapter 2).

After cover crop incorporation, microbial activity increases as can be seen from increased greenhouse gas emissions (Abdalla *et al.*, 2012, 2014; Sanz-Cobena *et al.*, 2014; Muhammad *et al.*, 2019). Indeed, we found increased CO_2 and N_2O emissions after cover crop incorporation both in the pot and field experiments compared to the control treatments without added cover crop residues (Chapter 2 and 5). N_2O emissions were highest with *V. sativa* and *R. sativus* mixtures in the field (Chapter 5) and *V. sativa* monoculture in the lab (Chapter 2), probably as a result of highest plant N content (low C:N ratio) in these treatments. Cover crop mixtures did neither increase nor decrease GHG emissions compared to cover crop monocultures (Chapter 2 and 5). Plant nutrient content and C:N ratio can be major drivers of GHG fluxes as we found highest emissions in treatment with highest plant N content (Chapter 2). This shows that residue composition rather than diversity is a predictive parameter determining GHG fluxes (Basche *et al.*, 2014; Muhammad *et al.*, 2019; Nguyen *et al.*, 2016a).

Long-term effects

To determine if cover crop mixtures are beneficial over the long-term, we ran a four-year field experiment. Within these years, weather conditions varied considerably. Year variation does influence the results of the experiment and it is important to take this into account as this affects cover crop performance and plant quality (Duan *et al.*, 2018). In our field experiment, we found no differences between monoculture and mixed cover crop treatments on GHG emissions and microbial biomass and diversity.

Addition of cover crops can increase SOM content in the soil, which is beneficial for microbial activity, nutrient cycling and soil structure (Johnston, Poulton and Coleman,

2009; Strickland *et al.*, 2019). SOM content was only significantly increased in two treatments after 4 years, namely in VR (*V. sativa* and *R. sativus*) and VRA (*V. sativa*, *R. sativus* and *A. strigosa*) (Porre 2020, chapter 6). Other studies indicate that a longer time may be necessary to find significant increases in SOM leading to increased soil quality (Olson, Ebelhar and Lang, 2010; Bai *et al.*, 2019).

Cash crop performance

In the four-year field experiment, three main crops were analyzed (biomass/yield and nutrient content). Overall, biomass and nutrient content of the main crop was not affected by growing and incorporating cover crops (Chapter 5). This is in contrast to other studies which showed that cover crops can increase main crop biomass and quality (Nakamoto *et al.*, 2012; Barel *et al.*, 2018; Brenzinger *et al.*, 2021). Differences between our field experiment and other studies may be a result of reduced fertilizer applications for the main crops after cover crops residue incorporation in other studies. In the last year, when barley was the main crop, we split all plots in a fertilized and an unfertilized part. Within the unfertilized plots, the barley biomass was significantly lower in the fallow treatment (Fal) as well as in the treatments with *A. strigosa* (A) and *V. sativa* (V) monocultures compared to the other treatments. Monoculture of *R. sativus* and cover crop mixtures in which *R. sativus* was present, did not result in significantly lower biomass compared to fertilized fields (Figure 1). This gives a possibility to consider reduced fertilizer use for main crops when residues of cover crops are incorporated.

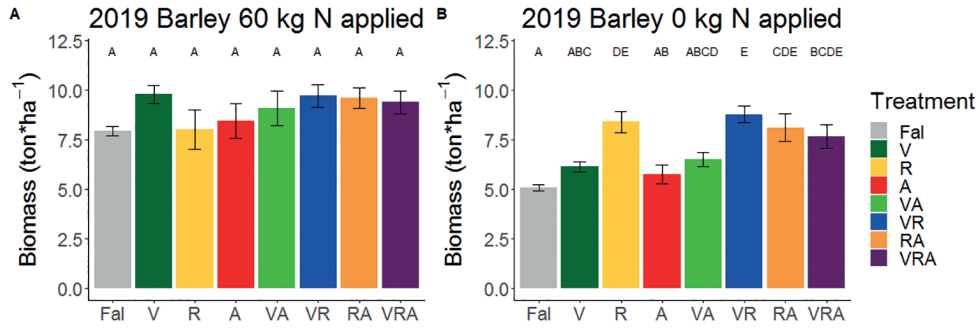

Figure 1: Total aboveground biomass of barley in 2019. A: biomass with applied fertilizer (60 kg N ha^{-1}); B: biomass without applied fertilizer. Letters on the x-axis indicate all different cover crop treatments with Fal: fallow treatment, V: *V. sativa* (vetch), R: *R. sativus* (radish), A: *A. strigosa* (oat) and combinations of letters are two or three species mixtures. Bars are averages of 5 replicates +/- SE. Letters indicate significant differences between the treatments of both panel A and B (analyzed by Anova with post-hoc Tukey test with significance level $p < 0.05$).

Microbial community

Microbial biomass

It is expected that soil microbial biomass increases after addition of organic residues. This increase can be persistent after addition of woody residues (Clocchiatti *et al.*, 2020). However, in our pot experiments, the microbial biomass was not higher after 8 weeks of cover crop incorporation compared to the non-amended treatments (Chapter 2). Fungal biomass was increased in the first weeks of the experiment (Chapter 2 and 3) and bacterial biomass did increase in the first days until 2 weeks depending on the cover crop treatment (Chapter 2). This short-term effect may be due to limited amount of cover crop residues added and easy decomposability of the material leading to high microbial turnover rates (Harden and Joergensen, 2000; Rousk and Baath, 2011). Bacterial biomass was significantly increased only up to 4 weeks when 20 ton ha^{-1} of cover crop residues was applied (Chapter 3). High microbial biomass turnover rates may be a result of increased predation by protists and nematodes (Gao *et al.*, 2019). In the field, such short-term effects are difficult to capture with the used sampling procedure with only one sampling shortly after residue incorporation, leading to no significant differences between the fallow treatment and cover crop treatments (Chapter 5). Contrasting to our results, others found increased microbial biomass in the soil after cover crop incorporation in field experiments (Barel *et al.*, 2019; Muhammad *et al.*, 2021). Sampling time is important to capture differences in the field and 2 weeks might be too short as Barel *et al*. (2019) found microbial biomass increase after 4 weeks. More frequent soil sampling might be advantageous to capture differences between treatments that might be lost when sampling only once. Furthermore, differences between lab and field experiments may be explained by increased heterogeneity of residues in the field as residues are bigger in size leading to less homogeneous distribution in the soil. Barel *et al.* (2018) mixed the residues in the soil twice with a 1-week interval while, in our field experiment, the residues were only mixed once. Mixing cover crop residues in the soil twice may have increased homogeneity which does make it easier to capture microbial biomass increase.

Microbial diversity

Available nutrient and carbon sources have a strong impact on the structure of the soil microbial community. Individual plant traits change the microbial community during growth via composition of root exudates, but also after incorporation of residues in the soil (Moorhead and Sinsabaugh, 2006; Berg *et al.*, 2016; Hall *et al.*, 2020; Sharma and Sharma, 2021; De Vries *et al.*, 2012a). Increasing plant diversity can increase microbial functional diversity in the soil via a more diverse input of organic resources. In Chapter 2, we showed that adding cover crop residue mixtures increases microbial metabolic potential (measured with Biolog ECO plates), pointing at the possibility for soil microbes to occupy a broader spectrum of metabolic niches. In cover crop monoculture residues,

the diversity in chemical composition of the plant material is lower, leading to reduced metabolic potential. However, this is species dependent as decomposing residues of *V. sativa* monocultures triggered a high microbial metabolic potential (Chapter 2). Increased microbial functional diversity can enhance ecosystem processes like decomposition and nutrient cycling (Chapter 4; Wagg *et al.*, 2014; Delgado-Baquerizo *et al.*, 2016, 2017). This may increase nutrient use efficiency of the microbial community by increased functional niche complementarity leading to reduced nutrient losses (Hattenschwiler, Tiunov and Scheu, 2005). However, Alahmad *et al.* (2019) did not find increased metabolic potential in a field experiment as addition of cover crops did not lead to increased functional diversity compared to a fallow treatment. In our field experiment, we could not find increased microbial diversity either (Chapter 5). However, this was analyzed with Illumina sequencing. This gives an indication of the diversity of microbes, but not of the functional capabilities of the community. It is necessary to understand species functioning and not only species identity in diverse microbial communities (Reiss *et al.*, 2009; Lustenhouwer *et al.*, 2020) as it is proposed that traits might reflect community composition more closely than taxa per se (Crowther *et al.*, 2019; Maynard *et al.*, 2019; Lustenhouwer *et al.*, 2020). This is still problematic in microbial research as many species are not yet fully sequenced. Metagenomics is a possibility to overcome this limitation. However, knowledge about the function of genes in the environment is not yet complete. Fungal species have been classified into functional groups with FUNGuild based on phylogenetic identity after sequencing analyses (Nguyen *et al.*, 2016a), but many species stay unclassified and their contribution to ecosystem processes like decomposition is unknown. Knowledge about functioning and diversity of traits in microbial communities is necessary to get a full understanding of diversity effects of microbial communities regarding ecosystem processes (Hättenschwiler, Fromin and Barantal, 2011; Krause *et al.*, 2021).

Decomposition

The resident microbial community plays an important role in decomposition of organic residues (Creamer *et al.*, 2016a). Increasing microbial diversity can lead to increased nutrient cycling and increased decomposition (Chapter 4; Hattenschwiler, Tiunov, and Scheu 2005; Loreau 2001). However, this is mostly studied in manipulated laboratory experiments. In our meta-analysis on the relationship between fungal diversity and decomposition we found a significant difference between manipulated experiments and natural ecosystems (Chapter 4). The fungal diversity effect on decomposition was saturated at two species in artificial assembled communities while in natural ecosystems a positive effect of increasing diversity was still present at high diversity levels (Chapter 4). This indicates that there is a need to study manipulated diversity-functioning relationships with other approaches, for example by dilution-to-extinction of environmental samples

(e.g. Wagg *et al.* 2014) instead of the inoculation of selected, cultivable microbial strains on sterile organic residues.

Furthermore, quality of plant material is an important factor determining decomposition (Cornwell *et al.*, 2008). For example, residues with a high C:N ratio or lignin content are mainly decomposed by fungal species due to their capability of producing a wide range of enzymes (van der Wal *et al.*, 2013). When organic residues are difficult to degrade, microbes invest energy in specialization and try to prevent competition by creating unfavorable conditions for other microbes (Hiscox and Boddy, 2016). Within these low-quality residues, effects of microbial diversity may be less important (Chapter 4). Furthermore, residues with low quality can result in immobilization of nutrients from the environment during decomposition. We found immobilization of nutrients (N, K and S) by the addition of *A. strigosa* residues (Chapter 2). In agricultural fields, application of residues that immobilize nutrients are not favorable when no other sources of mineral nutrients are applied before main crop growth, as this will reduce the main crop yield. Addition of cover crop mixture residues can reduce this negative effect by adding high quality litter such as *V. sativa* and *R. sativus*. Increasing plant residue diversity can increase microbial diversity in the soil by increasing metabolic niches in the soil as a result of the presence of a range of plant nutrient contents and decomposabilities (Handa *et al.*, 2014; Delgado-Baquerizo *et al.*, 2016). This is why we hypothesized in our field and pot experiments an increase in microbial diversity and nutrient cycling in the soil after addition of mixed cover crop residues compared to single species (Chapter 2 and 5). However, we only found a diversity effect during decomposition in our pot experiment (Chapter 2) and not in the field (Chapter 5). This may be a result of different methodology as described in the previous section. Furthermore, diversity of residue mixtures does not necessarily increase decomposition rates as compared to residues of monocultures (Wardle *et al.*, 2006; Porre *et al.*, 2020). Within two to eight species mixtures, decomposition rates are mainly predictable from the decomposition rates of the individual species. However, when plant diversity does have a positive effect on the microbial diversity, this can lead to a cascading effect of increased decomposition and nutrient cycling. In agricultural fields, this is favorable, as nutrients from decomposed residues will become more quickly available for the following main crop after cover crop residue incorporation.

GHG fluxes

As previously described, greenhouse gas fluxes were significantly higher during decomposition of incorporated cover crop residues in the soil (Chapter 2, 3 and 5; Abdalla *et al.*, 2012, 2014; Muhammad *et al.*, 2019). Mixed cover crops residues did not reduce the GHG emissions compared to monoculture cover crops both in the field and in the lab (Chapter 2 and 5). To be able to compare laboratory results (Chapter 2 and 3) with the fluxes

found in the field (Chapter 5), global warming potential (GWP) per day was calculated. All GHG fluxes were calculated based on the GWP of each separate gas (CO_2 = 1, CH_4 = 28 and N_2O = 265) (IPCC 2014). For both pot experiments, the GWP was calculated for cover crop residues cumulative over the full time of the experiment resulting in an average of approximately 60 mmol CO_2 equivalent day^{-1} m^{-2} over 50 days (Chapter 2) and 107 mg CO_2 equivalent kg $soil^{-1}$ day^{-1} over 28 days (Chapter 3), respectively, which is approximately 36 mmol CO_2 equivalent day^{-1} m^{-2} (assuming 10 cm soil depth and 1.3 kg m^{-3} bulk density). In the field experiment, GHG fluxes were approximately -1 µmol CH_4 h^{-1} m^{-2}, 2 µmol N_2O h^{-1} m^{-2} and 6 mmol CO_2 h^{-1} m^{-2} during the first 2 weeks after cover crop incorporation (Chapter 5). The GWP in the field was on average 156 mmol CO_2 equivalent day^{-1} m^{-2}. This is two to five times higher than the emission rates estimated for the laboratory experiments. The differences between the field and laboratory experiments can be explained in two ways. First, measurement times were different for the three experiments. Sampling for a short time after cover crop incorporation may have captured only the decomposition peak while measuring for 8 weeks did include basal respiration after initial microbial response. Second, the measurements in the field were conducted during day-time under fluctuating environmental conditions (temperature and soil moisture) while the pot experiments were incubated continuously in the dark at a constant temperature of 15°C or 20°C in Chapter 3 and Chapter 2, respectively. Single-day flux measurements can induce biases due to diurnal variation in the field with a peak mostly during daytime (Hall, Winters and Rogers, 2014; Wu *et al.*, 2021). Changes in soil temperature during the day are thought to cause a day-time peak of GHG fluxes. It is estimated that mid-morning sampling yielded emission rates with the smallest bias for N_2O emissions as mid-morning closely corresponds to daily average temperature (Wu *et al.*, 2021). However this can be different between N_2O, CO_2 and CH_4 (Hall, Winters and Rogers, 2014). We did measure GHG fluxes in the field between 9h and 15h with dark chambers to prevent influence from radiation. However, as we sampled per block, differences over time in the field will not change treatment effects as a block effect was added in the statistical analyses. However, even though there are difficulties with calculating GWP over a longer time in the field based on single-day flux measurements, it is still possible to compare differences between the treatments of each individual measurement both in the lab and in the field. Furthermore, in field experiments, fluxes change over time by changing environmental conditions (soil moisture and temperature). This indicates a need to develop methods capturing GHG fluxes over time and estimate gains and losses in the field. Mathematical models that include environmental conditions, microbial activity and nutrient content may be able to predict GHG fluxes in the field throughout the season (e.g. Lehuger *et al.* 2011). For example, the DAYCENT model estimates GHG emissions, C sequestration and N losses (Del Grosso *et al.*, 2009) and can be used to determine the potential of organic amendments to reduce GHG emissions and increase C storage (Ryals *et al.*, 2015).

Even though cover crops increase GHG emissions especially after incorporation in the soil, cover crops have a positive effect by changing the microbial community in the soil. Cover crops increase the biomass of microbial functional groups related to GHG consumption and nutrient cycling (Chapter 3; Brenzinger *et al.*, 2021). This can lead to reduced GWP in the long-term by increased activity of methanotroph bacteria, for example. High amounts of organic residues (corresponding to 20 ton ha^{-1}) stimulated the microbial community while small amounts (corresponding to 5 ton ha^{-1}) only had a limited effect (Chapter 3). In agricultural fields cover crop biomass is commonly not higher than 5 ton ha^{-1} aboveground and 2 ton ha^{-1} belowground (Chapter 5; Elhakeem 2021, chapter 2). This appeals towards addition of combinations of organic residues including cover crops to increase residue biomass and stimulate the microbial community related to nutrient cycling and GHG consumption and increase SOM and nutrient availability for the main crop and reduce GHG emissions.

Outlook and further research

The main starting hypothesis of my thesis research was that mixtures of cover crops species with different functional traits will increase microbial biomass and - diversity. This will lead to an increased use of carbon and nutrients in the soil, thereby reducing GHG emissions and providing a more gradual delivery of nutrients to the cash crop. We found that cover crops can increase microbial biomass and diversity in pot experiments, but in the field experiment we did not measure changes in the microbial community structure. Furthermore, we did not find differences in the field between cover crop monocultures and mixtures. GHG emissions were not significantly different compared to monocultures and adding cover crop mixtures did not lead to a higher main crop yield. These conclusions lead to new questions and further research possibilities. In the following sections I discuss remaining questions and possibilities for further research.

Carbon and nutrient cycling

As mentioned before, it is difficult to estimate losses of GHGs based on measurements in the field as these measurements are influenced by diurnal variation (Wu *et al.*, 2021) and variation in environmental conditions (Duan *et al.*, 2018). To be able to understand the benefits of cover crop species compared to a fallow soil, we need to understand how much nutrients are lost via leaching or GHG emissions. A high resolution of field sampling throughout the year can support the development of models to estimate losses in the field. For example, Ryals *et al.* (2015) measured weekly GHG fluxes over three years to estimate the climate change mitigation potential of compost with the DAYCENT model. Increased sampling frequency to determine GHG emissions, leaching losses and soil C dynamics will improve our understanding of nutrient cycling in the soil and the effect of adding organic residues and sowing cover crops in the field.

Soil sampling

In our field experiment we choose to take a mixed sample of the top 20 cm of the soil. This is the plowing depth in the field and most of the root biomass of the cover crops is present in this layer. However, subsampling the profile within this layer would add more information. When cover crops are incorporated in the field, the residues are only mixed in the top 5 cm of the soil while in our pot experiments the residues were completely mixed with the soil. Furthermore, cover crop residues in the field were not cut in small pieces leading to heterogeneity compared to pot experiments. This can lead to different effects on microbial biomass and - composition. In the field, we diluted the possible effect within the top 5 cm by sampling until 20 cm depth. When analyzing several layers it is possible to understand changes occurring within the soil profile in more detail. Alahmad *et al.* (2019), for example, found differences in the microbial community composition between shallow and deep layers in the soil. Microbial taxonomic richness was only increased after cover crop addition in the layer from 10-30 cm, while Shannon evenness increased in all layers from 0 to 50 cm. Furthermore, in many studies, samples are only taken at the end of field experiments (e.g. Zhou *et al.*, 2012; Mbuthia *et al.*, 2015; Barel *et al.*, 2018). This can result in biases leading to positive or negative effects that might not be consistent throughout the year. It is expected, however, that long term effects should be present in a bulk soil sample after several years of cover crop addition due to common soil homogenizing agricultural practices like plowing.

Long term effects

We determined cover crop mixture effects in the field for 4 years. However, we did not find differences between cover crop mixtures and monocultures. This indicates that, in agricultural fields, mixture effects are either not present or more years are needed to determine the possible effects of mixtures compared to monocultures. In grassland studies, it is common to study plant diversity effects on soil properties and ecosystem functioning for more than ten years (Fornara and Tilman, 2008; Isbell *et al.*, 2017). However, even after 4 years high plant diversity in grasslands increased soil organic matter and fertility (van Ruijven and Berendse, 2005; Steinbeiss *et al.*, 2008). In our experiment, SOM only increased in two treatments over four years (Porre, 2020) while including cover crops over the long term in agricultural fields (up to 50 years) can result in significant increases of SOC as reviewed by (Poeplau and Don, 2015). This appeals towards continuation of experiments over a long term in agricultural settings.

Cover crops in combination with organic residues

In Chapter 3, we found that cover crop amendments stimulated microbial communities involved in nutrient cycling and CH_4 consumption. However, this effect was most pronounced when high amounts of residues were applied in the pots (corresponding to 20 ton ha^{-1}). This amount of cover crop biomass is impossible to attain in agricultural

fields as cover crops are grown under suboptimal circumstances in autumn and winter over a short time frame (Elhakeem, 2021). Combining cover crops with organic residues like compost and manure can reduce the use of mineral fertilizers and can enhance the positive effects of cover crops on microbial activity. Many studies have shown the added value of organic residue application in agricultural fields by increasing microbial functional diversity and aggregate stability (Wortmann and Shapiro, 2008; Nair and Ngouajio, 2012; Brenzinger *et al.*, 2021). Furthermore, compost can increase stable SOC in agricultural systems while cover crops are beneficial to increase labile carbon in the soil (White *et al.*, 2020). A combination of compost and cover crops might be beneficial to increase SOM and to stimulate microbial activity and nutrient cycling. This can result in increased microbial turnover and create microbial necromass which is a substantial amount of stable SOM (Angst *et al.*, 2021). The combination of organic residues in agricultural fields with cover crops is a possibility to increase residue biomass and stimulate the microbial activity in the soil. This can reduce the use of chemical fertilizers for optimal main crop yield.

Soil type

In experimental settings, soil type can have a large influence on the effects of organic amendments. In this thesis, the focus was mainly on effects of cover crops in sandy soils, while effects in clay soils may be different. Meta-analyses are used to determine differences between experiments and soil types in relationship to the addition of cover crops (e.g. Muhammad *et al.*, 2019, 2021; Garland *et al.*, 2021; Wood and Bowman, 2021). These studies showed that soil clay content is a strong driver of microbial community structure, GHG fluxes, aggregate stability and SOM. Clay soils had, for example, lower bacterial biomass and higher N_2O emissions compared to sandy soils regardless of the cover crop treatment. It would be interesting to develop similar experiments at different locations and soil types to reduce the bias of different treatments that are used in individual experiments and to understand the effect of addition of cover crop species under several soil types and environmental conditions. Studies on European transects are already used to estimate microbial community composition and soil chemistry to develop knowledge on soil biodiversity leading to the possibility to protect soil by policy makers (Stone *et al.*, 2016). Collaborative projects using similar techniques and similar treatments within experimental settings will gain knowledge on differences between soil types and treatment effects as this reduces the variability in experiments. This can help improving the choice of the most suitable cover crop (mixture) for certain soil conditions and environments.

Determining best performing cover crop (mixture)

In our experiments, we used three cover crop species from three plant families, representing differences in plant functionality and residue stoichiometry. However, differences with other cover crop species might be larger. Elhakeem (2021, chapter 2)

analyzed the possibilities of 25 individual cover crops to attain high biomass. Such a set-up could also be used to determine the effects of plant stoichiometry on the microbial community in the field after residue incorporation. Decomposition and nutrient turnover of the added residue is determined by the stoichiometry of the material and carbon and nutrients present in the soil (Fierer *et al.*, 2009; Fanin *et al.*, 2016). Microbial community changes are affected by the quantity and composition of degradable resources (Zechmeister-Boltenstern *et al.*, 2015). By adding a stoichiometric range of plant residues within experimental settings, it is possible to test which plant species combinations are favorable to attain certain goals in agricultural fields such as increasing SOM, reducing GHG emissions and increasing microbial biomass and functional diversity.

Farmers' advice

Taking together our results regarding the use of cover crop mixtures versus monocultures, mixtures can have advantages compared to monocultures. Mixtures ensure the possibility of obtaining high biomass compared to monocultures (Elhakeem 2021). Furthermore, mixtures can lead to increased SOM (Porre 2020 chapter 6). Cover crop mixtures reduce leaching during cover crop growth (Porre 2020 chapter 2) and do not have a negative effect regarding GHG emissions or microbial community structure and functioning (Chapter 2, 3 and 5). However, it is unknown if cover crop mixtures are beneficial to reduce plant pathogens compared to a monoculture cover crop (e.g. use of *R. sativus* to reduce plant pathogenic nematodes). When farmers want to increase soil quality and sustainability, it might be advantageous to sow cover crop mixtures during fallow period in agricultural rotation in combination with other organic residues to enhance SOM.

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Summary
Samenvatting

Summary

Sustainable agricultural management is needed to reduce negative effects of current intensive agricultural practices. Intensification of agricultural land use management can lead to reduced soil organic matter (SOM) and microbial functional diversity in the soil. One of the options to increase sustainability in agricultural practices is the diversification of the cropping system via the use of wider crop rotations, intercropping and growing of cover crops. A major reason to grow cover crops is to reduce nutrient losses during the fallow period of the soil. Furthermore, cover crops are used to suppress weeds, plant pathogens, as well as to protect against erosion and stimulate nutrient cycling in the soil. There are indications that cover crops are beneficial by enhancing SOM content, aggregate stability and microbial functioning. Reducing the fallow period of the soil can be beneficial to enhance microbial diversity and main crop yields. It is hypothesized that using cover crop mixtures instead of cover crop monocultures will have an extra positive effect as mixtures will increase complementarity, possibly leading to increased biomass production and chemically more diverse root exudates and plant residues. In this PhD project, I studied if cover crop mixtures are beneficial compared to cover crop monocultures both during cover crop growth and after cover crop incorporation in the soil. Field and pot experiments were executed to determine if cover crop mixtures enhance nutrient cycling in the soil, reduce GHG emissions, stimulate a diverse microbial community and enhance yield of the main crop. To determine long term effects, the field experiment was conducted for four years.

First, we studied in a pot experiment if diversity of cover crop residues will increase microbial functional diversity during decomposition (**Chapter 2**). Cover crop residues of single species and diverse mixtures of 3 or 15 species were mixed with arable soil. Addition of cover crop residues rapidly increased microbial biomass in the soil during the first two weeks of the experiment. However, microbial biomass increase did not differ between monocultures and mixtures. With the use of Biolog ECO plates, we tested if residue diversity increased microbial metabolic potential as a proxy of functional diversity. Microbial communities extracted from pots amended with residue mixtures were able to use significantly more substrates than the ones extracted from pots amended with residues derived from a single cover crop species. Microbial communities from pots with residues of *Vicia sativa* showed high functional diversity as well. However, metabolic activity of the microbes from the *V. sativa* amended pots was mainly associated with easily degradable substrates while microbial communities from mixed residue amended pots were able to consume a wider set of substrates. This indicates that residues from cover crop mixtures increase the substrate utilization range and therefore the number of niches for microbes compared to residues derived from a single species. Furthermore, we found that the C:N ratio of cover crop residues rather than residue diversity influences

greenhouse gas (CO_2 and N_2O) fluxes. This indicates that composition of the residues is important for nutrient cycling while diversity of cover crop residues influences the soil microbial functional diversity.

To be able to evaluate the potential of organic residues, including cover crops, to mitigate greenhouse gasses (GHGs), pot experiments in controlled climate chambers were set up to determine if residues can minimize emissions. The aim of **Chapter 3** was to test differences between organic amendments (compost, sewage sludge, digestate and cover crop mixture) and understand how (combinations of) organic amendments influence the global warming potential (GWP) of an agricultural soil. Organic residues were added to the soil in two different amounts (5 and 20 ton*ha^{-1}) at different soil moisture levels (40% and 65% of soil water holding capacity). GHG fluxes (CO_2, CH_4 and N_2O) were measured over time while abundance of microbial groups involved in nutrient cycling (*nosZ*, bacterial and archaeal *amoA*, *nifH*, *mcrA* and *pmoA*) was measured at the beginning and at the end of the incubation. Compost resulted in the lowest GHG balance while the mixture of cover crop residues showed the highest GHG emissions. However, the used compost is poor in mineral nutrients and can reduce yield of the main crop. To overcome this problem, combining compost with nutrient rich organic amendment (e.g. sewage sludge or digestate) can minimize the trade-off between obtaining high yields and minimizing GHG emissions. Additionally, all amendments increased microbial communities involved in nutrient cycling and GHG consumption. Mixed cover crop residues led to the highest increase. However, this is dependent on the amount of the added residues. Applying 20 ton per hectare of residues strongly increased microbial groups, while 5 ton per hectare did not significantly increase these microbial groups compared to un-amended soil.

Apart from studying the effect of plant diversity of cover crop residues, microbial diversity itself can influence processes like decomposition and nutrient cycling. As fungi play a major role in decomposition, the aim of **Chapter 4** was to determine if fungal diversity enhance decomposition, which was assessed by executing a meta-analysis. An extensive literature search was performed to find papers that studied decomposition with a fungal diversity gradient both in manipulated and field experiments and in aquatic and terrestrial environments. Increased fungal diversity coincided with increased decomposition rate of plant residues (leaf litter and wood). However, in artificially manipulated experiments, fungal diversity rapidly reached a saturation level at two fungal species and further increase of diversity did not enhance decomposition. In field experiments, however, fungal diversity was positively correlated with decomposition regardless of the diversity level. This suggests that manipulated experiments are not representative for field situations and that it is necessary to study microbial diversity in the field. Furthermore, plant residue quality influences the fungal diversity – decomposition relationship. Increasing the C:N ratio of the residue reduced the positive effect of diversity on decomposition. These

results indicate that both microbial diversity and residue quality are important to estimate decomposition rates of plant residues.

In **Chapter 5,** results of cover crop diversity effects of a multiannual field experiment are described both during cover crop growth and after cover crop incorporation in the soil. We hypothesized that cover crop mixtures reduce greenhouse gas (GHG) fluxes and increase soil microbial diversity compared to cover crop monocultures. Three cover crop species (*Avena strigosa*, *Vicia sativa* and *Raphanus sativus*) were grown in randomized block design with all possible combinations of the three species. GHG fluxes were measured regularly throughout the year. We found increased emissions in the plots with cover crops compared to fallow plots, both during cover crop growth and after cover crop incorporation. Cover crop mixtures did not reduce GHG fluxes compared to cover crop monocultures. N_2O emission peaked after cover crop incorporation and was correlated with increased denitrification and nitrification rates. Furthermore, contrasting to our hypothesis, we did not find effects of cover crops on microbial biomass or - diversity in the field. This indicates that, in this field experiment, cultivating mixtures of cover crops for four years did not develop benefits compared to monocultures with respect to GHG fluxes and microbial biomass, - diversity and - activity.

Overall, my thesis research compared performance of mixtures and monocultures of cover crops both in pot experiments during decomposition and in a four-year field experiment. The results show that residues of cover crop mixtures have the potential to increase microbial functional diversity and stimulate microbial groups involved in nutrient cycling. This indicates a promising perspective to use cover crop mixtures in the field. However, in our field experiment, mixtures did not result in reduced GHG emissions or increased microbial biomass or diversity. Continuation of field experiments for a longer period is needed to determine if mixtures are advantageous compared to monocultures to increase sustainability in agricultural systems.

Samenvatting

Duurzaam landbouwbeheer is nodig om de negatieve effecten van de huidige intensieve landbouw te verminderen. Intensief landgebruik kan tot een vermindering van organisch materiaal en verlies van microbiële functionele diversiteit in de bodem leiden. Eén van de mogelijkheden om de landbouw te verduurzamen is het gebruik van een divers gewassysteem met gewasrotaties, mengteelt en wintergewassen. Wintergewassen kunnen nutriëntenverliezen verminderen tijdens de periode dat de bodem braak ligt. Daarnaast kunnen wintergewassen gebruikt worden om onkruiden en pathogenen te onderdrukken, maar ook om de bodem te beschermen tegen erosie en de nutriëntencyclus te stimuleren. Er zijn aanwijzingen dat wintergewassen gunstig zijn voor het laten toenemen van bodem organisch materiaal, aggregaat stabiliteit en microbieel functioneren. Het verkorten van de periode dat de bodem braak ligt kan de microbiële diversiteit en gewasopbrengsten verhogen. De hypothese is dat het gebruik van mengsels van wintergewassen ten opzichte van monoculturen een extra positief effect heeft, omdat in mengsels de functionele complementariteit van de gewassen groter is. Dit kan leiden tot verhoogde biomassaproductie, chemisch diverse wortel-exudaten en plantenresten. In dit PhD-project heb ik bestudeerd of mengsels van wintergewassen gunstiger zijn dan monoculturen tijdens de groei en na het inwerken van plantmateriaal in de bodem. Veld- en potexperimenten zijn uitgevoerd om te bepalen of mengsels van wintergewassen de nutriëntencyclus in de bodem stimuleren, broeikasgasemissies verminderen, een diverse microbiële gemeenschap stimuleren en leiden tot een verhoogde opbrengst van het hoofdgewas. Om langetermijneffecten te bepalen, is het veldexperiment gedurende vier jaar uitgevoerd.

Ten eerste hebben we in een potexperiment bestudeerd of diversiteit van wintergewasresiduen de microbiële functionaliteit tijdens afbraak verhoogt (**Hoofdstuk 2**). Wintergewasresiduen van monoculturen en mengsels van 3 of 15 soorten werden met landbouwgrond gemengd. Het toevoegen van wintergewasresiduen leidde snel tot een verhoogde microbiële biomassa tijdens de eerste twee weken van het experiment. Deze verhoogde microbiële biomassa was niet verschillend tussen de mengsels en de monoculturen. Met Biolog ECO-platen hebben we bepaald of residu-diversiteit het metabool potentieel, als indicator voor de functionele diversiteit van de microbiële gemeenschap, kan verhogen. De geëxtraheerde microbiële gemeenschap uit potten met residu-mengsels kon significant meer soorten substraten afbreken dan de geëxtraheerde microbiële gemeenschap uit residu-monoculturen. De microbiële gemeenschap uit de potten met *Vicia sativa* had ook een hoge functionele diversiteit, maar de metabolische activiteit van deze micro-organismen was vooral geassocieerd met makkelijk afbreekbare substraten, terwijl micro-organismen uit de potten met de mengsels een grotere variatie aan substraten konden afbreken. Dit toont aan dat residuen van mengsels

van wintergewassen de reikwijdte van substraatgebruik verhogen, resulterend in een verhoging van het aantal niches voor micro-organismen ten opzichte van residu-monoculturen. Daarnaast vonden we dat de C:N ratio van wintergewassen, en niet de diversiteit van het plantmateriaal, invloed heeft op broeikasgasemissies (CO_2 en N_2O). Dit toont aan dat de samenstelling van het residu belangrijk is voor de nutriëntencyclus terwijl diversiteit van de wintergewasresiduen invloed heeft op de microbiële functionele diversiteit.

Om te onderzoeken of organische residuen, inclusief wintergewassen, het potentieel hebben om broeikasgasemissies te verminderen, is een potexperiment opgezet in gecontroleerde klimaatkamers. Het doel van **Hoofdstuk 3** was om verschillende organische materialen (compost, rioolslib, digestaat en een mengsel van wintergewassen) te onderzoeken en te begrijpen hoe (combinaties van) deze residuen invloed hebben op de emissies en het opwarmingsvermogen in een landbouwbodem kunnen verminderen. De residuen zijn aan de bodem toegevoegd in twee verschillende hoeveelheden (5 en 20 ton*ha^{-1}) en met verschillende vochtgehaltes van de bodem (40% en 65% waterhoudend vermogen). Broeikasgasfluxen (CO_2, CH_4 en N_2O) werden gedurende het experiment regelmatig gemeten, terwijl de biomassa van microbiële groepen die de nutriëntencyclus beïnvloeden (*nosZ*, bacterieel en archaea *amoA*, *nifH*, *mcrA* en *pmoA*) alleen aan het begin en eind van het experiment gemeten werden. Compost resulteerde in de laagste broeikasgasbalans terwijl een mengsel van wintergewassen de hoogste emissies liet zien. Compost bevat echter weinig nutriënten en kan de opbrengst van een hoofdgewas verlagen. Om dit probleem op te lossen, kan compost worden gecombineerd met een residu rijk aan nutriënten, bijvoorbeeld rioolslib en digestaat. Dit kan voor een compromis tussen gewasopbrengst en broeikasgasemissies zorgen. Daarnaast verhoogden alle residuen de microbiële biomassa van micro-organismen die betrokken zijn bij de nutriëntencyclus en broeikasgasopname. Residuen van mengsels van wintergewassen zorgden voor de hoogste toename, maar dit is afhankelijk van de hoeveelheid toegevoegd residu. Het toevoegen van 20 ton per hectare verhoogde sterk de biomassa van deze microbiële groepen, terwijl 5 ton per hectare geen significant effect had ten opzichte van een bodem zonder organische residuen.

Naast het bestuderen van de effecten van de diversiteit van residuen van wintergewassen kan de microbiële diversiteit op zichzelf ook invloed op processen zoals afbraak en nutriëntencyclus uitoefenen. Omdat schimmels een belangrijke rol spelen in de afbraak, was het doel van **Hoofdstuk 4** om te bepalen of schimmeldiversiteit de afbraak versnelt. Dit is onderzocht met een meta-analyse. Door middel van uitgebreid literatuuronderzoek zijn studies gevonden die de afbraak van plantmateriaal onderzochten in een diversiteitgradiënt van schimmels, zowel in gecontroleerde experimenten met gemanipuleerde schimmeldiversiteit als in veldexperimenten met een natuurlijke schimmeldiversiteit en in aquatische en terrestrische milieus. Verhoogde

schimmeldiversiteit viel samen met versnelde afbraak van plantresiduen (strooisel en hout). In de gecontroleerde experimenten bereikte de diversiteit echter snel een plafond bij twee soorten schimmels en leidde een verdere toename van diversiteit niet tot versnelde afbraak. In veldexperimenten met natuurlijke schimmeldiversiteit, daarentegen, leidde een verhoogde diversiteit tot een versnelde afbraak ongeacht het diversiteitsniveau. Dit suggereert dat gecontroleerde experimenten niet representatief zijn voor veldsituaties en dat het noodzakelijk is om microbiële diversiteit in het veld te bestuderen. Daarnaast had de kwaliteit van het plantresidu invloed op de relatie tussen schimmeldiversiteit en afbraak. Een hogere C:N ratio van het residu resulteerde in een verminderd positief effect van diversiteit op de afbraak. Deze resultaten laten zien dat zowel microbiële diversiteit als residukwaliteit een belangrijke rol spelen bij de afbraak van plantresiduen.

In **Hoofdstuk 5** zijn de resultaten van het effect van de diversiteit van wintergewassen in een meerjarig veldexperiment tijdens de groei en na inwerking van de wintergewassen in de bodem beschreven. De hypothese was dat mengsels van wintergewassen broeikasgasemissies verminderen en microbiële diversiteit in de bodem verhogen ten opzichte van monoculturen. Drie wintergewassen (*Avena strigosa* (Japanse haver), *Vicia sativa* (voederwikke) en *Raphanus sativus* (bladrammenas)) groeiden in alle mogelijke combinaties van deze drie soorten in het veld in een gerandomiseerde blokopzet. Broeikasgasemissies werden regelmatig gedurende het jaar gemeten. Emissies gemeten in plots met wintergewassen waren hoger dan braakliggende grond, zowel tijdens de groei als na het inwerken van de wintergewassen. Mengsels van wintergewassen reduceerden de broeikasgasemissies niet ten opzichte van monoculturen. N_2O-emissies piekten na inwerken van de wintergewassen en waren gecorreleerd met verhoogde nitrificatie en denitrificatie. Verder vonden we, in tegenstelling tot onze hypothese, geen effect van wintergewassen op de microbiële biomassa of diversiteit in het veld. Dit toont aan dat in dit veldexperiment het telen van mengsels van wintergewassen geen voordelen opleverde ten opzichte van monoculturen voor broeikasgasfluxen en microbiële diversiteit, biomassa en activiteit.

Alles overziend bestudeerde ik in mijn onderzoek de effecten van mengsels en monoculturen van wintergewassen in potexperimenten tijdens de afbraak en in een vierjarig veldexperiment. De resultaten laten zien dat residuen van mengsels van wintergewassen het vermogen hebben om de microbiële functionele diversiteit te verhogen en microbiële groepen die betrokken zijn bij de nutriëntencyclus te stimuleren. Dit is een veelbelovend perspectief om mengsels van wintergewassen te gebruiken in het veld. In ons veldexperiment resulteerden mengsels van wintergewassen echter niet in verlaagde broeikasgasemissies of een verhoogde microbiële biomassa en diversiteit. Het voortzetten van veldexperimenten voor een langere periode is noodzakelijk om te bepalen of mengsels van wintergewassen gunstig zijn ten opzichte van monoculturen om de duurzaamheid in landbouwsystemen te verhogen.

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Of course, I did not enjoy these years without all board games and outdoor activities with my friends and family. Thank you for supporting me, your willingness to listen if I wanted to share something (especially Sophie and Sharell), but most of all giving me the feeling of being at home and having fun.

About the author

Sytske Maria Drost was born 29th of December in 1991 in Tilburg, The Netherlands. In 2013, she finished her BSc degree in Biology at the Wageningen University (WUR). After that, she continued with an MSc in Biology at the same university with specializations in Bio-interactions and Molecular Ecology. During her study, she got interested in microbial interactions in the soil. She did an MSc thesis at the Netherlands Institute of Ecology (NIOO-KNAW) where she studied the impact of bacterial interactions on antibiotic production. In 2014, she started an internship at the National Institute for Public Health and the Environment (RIVM) and analyzed community level physiological profiles (CLPPs) from Dutch and European soils. Afterwards, she did a thesis at the Swedish University of Agricultural Sciences (SLU) in Uppsala and studied the *Plasmodiophora brassicae-Arabidopsis* responses. In 2015, she finished her MSc at Wageningen University and started her PhD research under the supervision of Dr. Paul Bodelier and Prof. Dr. Wietse de Boer. She joined the NWO project Clever Cover Cropping and researched how cover crop mixtures can stimulate microbial functional diversity and alter greenhouse gas emissions. The results of her PhD research are described in this thesis.

List of publications

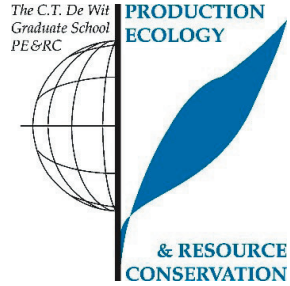
Rutgers, M., Wouterse, M., **Drost, S.M.**, Breure, A.M., Mulder, C., Stone, D., Creamer, R.E., Winding, A., Bloem, J., 2016. Monitoring soil bacteria with community-level physiological profiles using Biolog™ ECO-plates in the Netherlands and Europe. Applied Soil Ecology 97, 23–35. http://dx.doi.org/10.1016/j.apsoil.2015.06.007

Brenzinger, K., **Drost, S.M.**, Korthals, G., Bodelier, P.L.E., 2018. Organic Residue Amendments to Modulate Greenhouse Gas Emissions From Agricultural Soils. Frontiers in Microbiology 9, 3035. https://doi.org/10.3389/fmicb.2018.03035

Drost, S.M., Rutgers, M., Wouterse, M., de Boer, W., Bodelier, P.L.E., 2020. Decomposition of mixtures of cover crop residues increases microbial functional diversity. Geoderma 361, 114060. https://doi.org/10.1016/j.geoderma.2019.114060

Drost, S.M., van der Wal, A., de Boer, W., Bodelier, P.L.E., 2021. Assessing the role of fungal diversity in decomposition: A meta-analysis. bioRxiv 2021.09.29.462096. https://doi.org/10.1101/2021.09.29.462096

PE&RC Training and 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 (4.5 ECTS)
- The effect of plant traits on soil microbial functioning (2016)

Writing of project proposal (2 ECTS)
- Clever cover cropping: optimizing resource stoichiometry for increased microbial diversity and functioning (2016)

Post-graduate courses (4.7 ECTS)
- Soil ecology and the planetary boundaries; PE&RC (2016)
- Frontiers in microbial ecology; RSEE / PE&RC / SENSE (2018)
- Mixed linear models; PE&RC (2019)
- Meta-analysis; PE&RC (2020)

Competence strengthening / skills courses (4 ECTS)
- Competence assessment; WGS (2016)
- Efficient writing strategies; WGS (2017)
- Time management and self-management PhD; Leiden University (2018)
- Ethics in plant and environmental sciences; WGS (2019)
- Career orientation; WGS (2020)

PE&RC Annual meetings, seminars and the PE&RC weekend (2.85 ECTS)
- PE&RC First year weekend (2015)
- NERN Symposium current themes in ecology (2015)
- PE&RC Day (2015, 2016)
- Plant-soil-microbe workshop (2016)
- PE&RC Midterm weekend (2018)
- PE&RC Last years afternoon; online (2020)

Discussion groups / local seminars or scientific meetings (6 ECTS)
- Sustainable Intensification of Agricultural Systems (SIAS) discussion group (2016-2017)
- Plant-Soil Interactions (PSI) discussion group (2016-2020)
- NIOO Monday seminars (2016-2020)

International symposia, workshops and conferences (10.7 ECTS)
- NAEM; poster presentation; Lunteren, the Netherlands (2016)
- SOMmic; poster presentation; Leipzig, Germany (2016)
- SOM; poster presentation; Harpenden, United Kingdom (2017)
- Ecology of soil microorganisms (ESM); oral and poster presentation; Helsinki, Finland (2018)
- EGU; poster presentation; Vienna, Austria (2019)

Lecturing / supervision of practicals / tutorials (3.2 ECTS)
- Soil-plant interactions (2016-2019)
- Supervision 2 MBO students (2016-2018)
- Ecological aspects of bio-interactions (2017)

Colophon

The research presented in this thesis was conducted at the Department of Microbial Ecology of the Netherlands Institute of Ecology (NIOO-KNAW) in Wageningen. The research described in this thesis was financially supported by the Netherland Organization for Scientific Research (NWO green, grant number 870.15.073) with co-financing from seed producers (Agrifirm, Vandinter Semo, P.H. Petersen Saatzucht, and Joordens Zaden).

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