Relevance of functional organic matter fractions for soil denitrification

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List of abbreviations

ANOVA	Analysis of Variance
a.s.l.	Above sea level
C	Carbon
C_2H_2	Acetylene
$CaCl_2$	Calcium chloride
CO_2	Carbon dioxide
DNRA	Dissimilatory nitrate reduction to ammonium
DOC	Dissolved organic matter
Fe_d	Iron (Fe) extractable by dithionite-citrate-bicarbonate
fPOM	Free particulate organic matter
Fu	Fuhrberg soil
FYM	Farmyard manure
GC	Gas chromatograph
Gi	Giessen soil
He	Helium
ICP-OES	Inductively coupled plasma optical emission spectrometry
KCl	Potassium chloride
KNO_3	Potassium nitrate
MOM	Mineral-associated organic matter
N	Nitrogen
N_2	Molecular Nitrogen
N_2O	Nitrous oxide
$NaHCO_3$	Sodium hydrogen carbonate
Nar	Nitrate reductase
NH_3	Ammonia
NH_4^+	Ammonium
Nir	Nitrite reductase
NirK	Genes encoding copper-containing NO_2^- reductase
NirS	Genes encoding cytochrome cd1 NO_2^- reductase
N_{min}	Mineral nitrogen
NO	Nitric oxide

NO_2^-	Nitrite
NO_3^-	Nitrate
Nor	Nitric oxide reductase
Nos	Nitrous oxide reductase
NosZ I	Genes encoding nitrous oxide reductase (clade I)
NosZ II	Genes encoding nitrous oxide reductase (clade II)
O_2	Molecular oxygen
OC	Organic carbon
OM	Organic matter
ON	Organic nitrogen
oPOM	Occluded particulate organic matter
P	Phosphorus
POM	Particulate organic matter
Ro	Rotthalmünster soil
$SUVA_{280}$	Specific UV absorbance at 280 nm
TN	Total nitrogen
WEOC	Water-extractable organic carbon
WEOM	Water-extractable organic matter
WEON	Water-extractable organic nitrogen
WETN	Water-extractable total nitrogen
WFPS	Water-filled pore space

1 General introduction

1.1 Factors controlling soil denitrification and its products

An increasing input of nitrogen (N) fertilizers to agricultural fields since the beginning of the industrial age resulted in increasing concentrations of reactive N in agricultural soils and surrounding ecosystems (Mosier et al., 1998; Erisman et al., 2008; IPCC, 2013). This has several serious environmental consequences, such as eutrophication, loss of biodiversity, health problems, and effects on the climate system (Sutton et al., 2011). Particularly, emissions of climate-relevant nitrous oxide (N_2O) and pollution of ground water by nitrate (NO_3^-) leaching from fields are increasing. Denitrification is the key process returning reactive nitrogen as dinitrogen (N_2) to the atmosphere through a series of enzymatic steps and intermediate compounds: $NO_3^- \rightarrow_{(Nar)} NO_2^- \rightarrow_{(Nir)} NO \rightarrow_{(Nor)} N_2O \rightarrow_{(Nos)} N_2$ (Hayatsu et al., 2008). Thus, it plays an important role in the closure of the global N cycle (Figure 1.1; Philippot et al., 2007). However, depending on the fertilization, a significant portion of the applied N can be released as nitrogenous gas through denitrification, and thus, limit crop production (Aulakh et al., 1992). Furthermore, incomplete denitrification results in emissions of N_2O, which exhibits the largest warming potential of all biogenic greenhouse gases (298 times that of CO_2) and accounts for about 6% of the current global greenhouse effect (Cicerone, 1989; Bouwman et al., 1995; IPCC, 2013). In addition, it contributes to the stratospheric ozone depletion (Ravishankara et al., 2009). Denitrification reactions are considered as the predominant source of N_2O in natural ecosystems and especially temperate agricultural soils (e.g., Opdyke et al., 2009). Consequently, understanding the factors controlling denitrification and its $N_2O/(N_2O+N_2)$ product ratio is crucial for evaluating climatic effects by denitrification and for developing strategies to minimize N losses, especially in the form of N_2O. Compared to N_2O, the N_2 production is rarely studied. The large background concentrations of N_2 in air and water render it difficult to detect N_2 release by denitrification (Groffman et al., 2006).

Denitrification is a microbial respiratory process by bacteria, archaea, and fungi when oxygen (O_2) is not available for aerobic respiration (Zumft, 1997). The availability of readily decomposable carbon (C) as electron donor and a basic substance for the formation of their own body substance is essential for any denitrification process (Beauchamp et al., 1989). In contrast to aquatic systems, where autotrophic denitrification with inorganic electron donors often occurs (Rivett et al., 2008),

heterotrophic denitrification, where organic compounds are oxidized, generally predominates in soils (Davidson and Seitzinger, 2006). Denitrifiers can represent up to 5% of the total soil microbial community (Tiedje, 1988; Henry et al., 2004, 2006), but some of them produce only N_2O or N_2 as end product of the denitrification cascade (Philippot et al., 2007). However, the share of N_2O depends not only on the composition of the denitrifier community but also, like the denitrification rate itself, on numerous biotic and abiotic environmental factors. Nitrous oxide reductase (*Nos*) was found to be more sensitive to O_2, C/N ratios, and soil pH (Betlach and Tiedje, 1981; Tiedje, 1988). Due to declining O_2 availability, denitrification mainly contributes to N_2O formation when soil moisture increases over 60% water-filled pore space (WFPS), with a shift to N_2 in soils with WFPS above 80% (Linn and Doran, 1984; Davidson, 1991). Lower temperatures and pH values (<6) tend to cause decreased denitrification rates but larger molar $N_2O/(N_2O+N_2)$ ratios (Smith, 1997; Čuhel et al., 2010). Another important factor influencing the share of N_2O is the ratio of bioavailable OC to NO_3^--N, as demonstrated by (Senbayram et al., 2012).

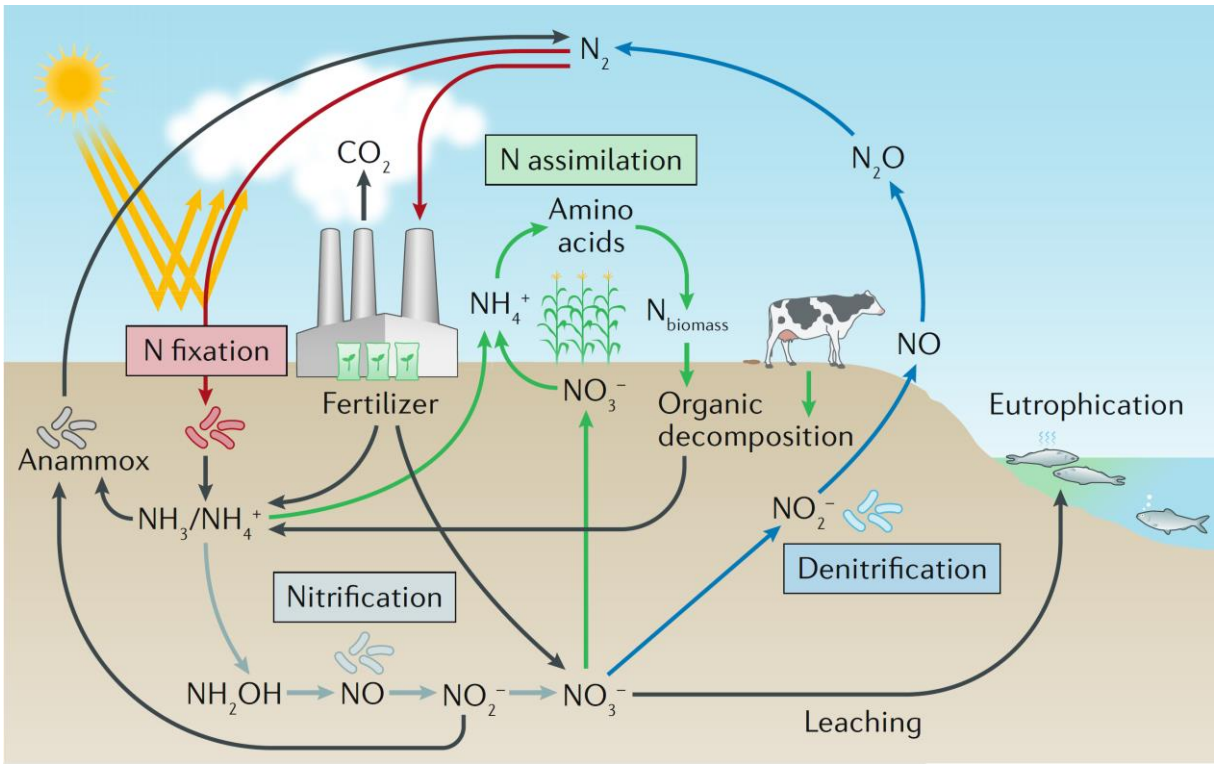

Figure 1.1 The main species and interconversions in the nitrogen cycle (Lehnert et al., 2018). N_2 is reduced to NH_3 through biological or industrial N_2 fixation (red arrows), providing N containing fertilizers for plants (green arrows). However, excess NH_3 is processed by microorganisms in the soil by nitrification (pale-blue arrows) and denitrification (blue arrows), which transform the N containing fertilizer into environmental pollutants.

In soil, denitrification mainly occurs in anoxic microhabitats ('hot spots') where sufficient nitrogenous oxides (NO_3^-, NO_2^-, NO, or N_2O) as electron acceptors and suitable electron donors such as organic carbon compounds are bioavailable (e.g., Groffman et al., 2009). Suboxic or anoxic conditions in soil can be caused and promoted by moisture, respiration, aggregation, compaction, and waterlogging (Granli and Bøckman, 1994; Ruser et al., 2006). Not only biochemical soil properties, such as pH and cation exchange capacity (Senesi and Loffredo, 1998; Baldock and Broos, 2011), but also these physical conditions are directly or indirectly influenced by soil OM. Extremely high contents of readily bioavailable carbon can enhance aerobic respiration, and thus, cause O_2 depletion in microsites. Here, the O_2 consumption is higher than its supply through diffusion (Linn and Doran, 1984). Soil macroaggregates with anoxic centers are often 'hot spots' for denitrification, despite oxic conditions in the surrounding soil (Schlüter et al., 2018). Thus, denitrification can also occur in soils with a WFPS of less than 60%. Agricultural topsoils are a favorable habitat for denitrifying microorganisms, since oxic and anoxic conditions frequently alternate or coexist, and nitrogenous electron acceptors are usually abundant.

In summary, denitrification in agricultural soils is controlled by various regulators acting on various scales. Soil organic matter may directly and indirectly affect denitrification**.** Its quantity, quality, and distribution within the soil matrix are decisive in driving denitrification.

1.2 Bioavailability of functional soil organic matter fractions for denitrifying organisms

Soil OM is the most important source of energy and nutrients for soil flora and fauna. It comprises a heterogeneous mixture of structurally diverse plant-, animal-, and microbial-derived components in different stages of transformation and degradation, and with different types of stabilization. Besides selective preservation (relative accumulation of recalcitrant C compounds) and spatial inaccessibility (e.g., by occlusion in aggregates), especially interactions with mineral surfaces can slow down decomposition of OM and prevent mineralization (von Lützow et al., 2006). Thus, soil OM fractions differ strongly in bioavailability, and thus, in their potential to fuel soil denitrification. While most abiotic factors on denitrification reactions, such as temperature und pH, are well studied, the relevance of different soil OM fractions and C compounds is largely unexplained, probably

due to their high complexity (Beauchamp et al., 1989). Previous studies have shown that addition of fresh plant biomass or well-defined low-molecular-weight compounds affects denitrification rates, product ratios, and denitrifier populations (e.g., Beauchamp et al., 1989; Miller et al., 2008; Palmer et al., 2012). Despite evidence of large differences in release and biodegradability of water-extractable OM (WEOM) (e.g., Don and Kalbitz, 2005; Kalbitz et al., 2005; Mastný et al., 2018), much less information is available on the effects of more complex soil OM fractions, such as particulate and mineral-associated OM (POM, MOM) (Lavallee et al., 2020).

Water-extractable organic matter

Water-soluble compounds of soil OM are readily bioavailable and disperse within the liquid phase through the soil profile (Boyer and Groffman, 1996). Thus, WEOM represents the most labile and mobile fraction of soil OM and is considered to be most effective in promoting denitrification (e.g., Bremner and Shaw, 1958; Burford and Bremner, 1975). Accordingly, the addition of plant-derived WEOM (water extracts of maize stalk) to repacked soil results in increased CO_2 and N_2O emissions (Qiu et al., 2015). However, its chemical composition can vary greatly. It may comprise low-molecular-weight C compounds, such as sugars, amino acids, and proteins, but also high-molecular-weight components, such as microbial-derived extracellular polymeric or humic substances (Kalbitz et al., 2003a; Marschner and Kalbitz, 2003). While WEOM leached from fresh plant residues and bacterial extracellular polymeric substances is rich in hydrophilic components, such as polysaccharides, WEOM derived from well humified forest floor layers typically is enriched in phenolic compounds, such as lignin or lignocellulose, and, thus, much less biodegradable (Kalbitz et al., 2003a, 2003b; Marschner and Kalbitz, 2003). When comparing different C substances, soluble low-molecular-weight compounds are much more effective in promoting denitrification and related bacterial populations (e.g., organic acids, glycerol ≫ glucose, methanol) than insoluble polymers, such as cellulose and especially lignin (e.g., Valera and Alexander, 1961; deCatanzaro and Beauchamp, 1985; Rashid and Schaefer, 1988; Akunna et al., 1993). In addition, Henry et al. (2008) showed that artificial root exudates with higher proportion of sugars (80%) caused a much lower $N_2O/(N_2O+N_2)$ product ratio than a solution rich in organic acids and amino acids (40% sugars). Thus, the extent of denitrification as well as the gas products depend on the type of low-molecular-weight organic compounds (Morley and Baggs, 2010;

Morley et al., 2014). However, very limited information exists about the effect of more complex WEOM substrates and their chemical composition on denitrification.

Particulate organic matter

Particulate OM mainly consists of fresh and slightly degraded plant residues originating from litter, crop residues, or organic fertilizers (Yamashita et al., 2006). Therefore, POM is generally more abundant in the topsoil than in the subsoil. The decomposability of POM varies depending on its chemical composition and microbial accessibility. Above- and below-ground plant residues are mainly composed of polysaccharides, such as starch, cellulose, hemicellulose, pectin (50–60%), and lignin (15–20%) (von Lützow et al., 2006). The proportion of compounds which are less biodegradable due to their structural composition varies widely between plant species (Kögel-Knabner, 2002). In addition, the relatively fast turnover time of POM in well aerated soils (Gregorich et al., 2006) can be significantly slowed down by its occlusion in aggregates (Besnard et al., 1996). However, POM is commonly considered as a labile pool which is very sensitive to changes in soil management und temperature (Gosling et al., 2013; Benbi et al., 2014).

Previous studies emphasize that microheterogeneities caused by fresh plant residues might explain the patchy distribution of denitrification 'hot spots' in soil (Aulakh et al., 1984; Christensen et al., 1990; Murray et al., 1995). Those microsites with increased denitrification activity are often associated with decomposing plant residues (Parry et al., 2000; Kravchenko et al., 2017). Parkin (1987) found that POM was responsible for up to 85% of the total denitrification activity in intact soil cores. Further, Gaillard et al. (2003) showed that soluble C from POM enters the adjacent soil (several mm) and fuels microbial processes. Although cover crops and intercrops are increasingly used to reduce soil erosion and provide efficient N utilization, they may also stimulate N_2O emissions (Li et al., 2015, 2016). While it is well known that exudates from living plant roots can cause higher denitrification activities in the rhizosphere than in bulk soil (Smith and Tiedje, 1979; Højberg et al., 1996), the relevance of dead root debris for total soil denitrification is largely unresolved. The addition of root residues results in significantly lower N_2O and CO_2 emissions compared to aboveground plant biomass, such as shoot litter (Rummel et al., 2020). Nevertheless, young root detritus can stimulate extracellular enzyme activities stronger than living roots (Spohn and Kuzyakov, 2014) and might be important for the formation of denitrification 'hot spots-hot moments' in soil. Consequently, POM-derived

WEOC could be of crucial importance for the stimulation of denitrification processes. Although leaching and subsequent degradation of soluble compounds have been recognized as initial steps of litter decomposition (Berg and McClaugherty, 2014), possible effects of WEOM from different plant residues on potential denitrification have not been evaluated so far. At present, the contribution of POM to soil denitrification is still unclear.

Mineral-associated organic matter

Up to 90% of soil OC is associated with minerals (Kögel-Knabner et al., 2008). Thus, MOM represents by far the most abundant OM fraction in soil, particularly in POM-poor subsoil horizons. Since association of OM with minerals increases its persistence in soil, MOM generally has a higher mean age than non-mineral-associated OM (Kleber et al., 2015). Not only the bioavailability but also the C/N ratios of OM in mineral-organic associations are usually lower compared to those of POM (Swanston et al., 2002; Sollins et al., 2006; Gentsch et al., 2015). Mineral-organic associations are basically formed by adsorption and precipitation reactions where organic compounds react with mineral surfaces or metals (Kaiser and Guggenberger, 2000; Kleber et al., 2015). Consequently, the amount and composition of MOM in a given soil is strongly affected by the composition of dissolved OM as well as the presence and reactivity of pedogenic minerals (Kaiser and Guggenberger, 2000; Mikutta et al., 2009, 2010). While MOM in loamy soils is generally richer in polysaccharides, MOM in sandy soils is often more aliphatic (Kögel-Knabner et al., 2008). Interactions between OM and mineral surfaces include ligand exchange, polyvalent cation bridges, and weak interactions such as van der Waals forces and H-bonding (von Lützow et al., 2006). Thus, MOM represents a continuum of mineral-bound C substances and their biodegradation is linked to its ability to become desorbed, hence, to produce dissolved OM (e.g., Mikutta et al., 2007). Incubation experiments under oxic conditions indicate that the bioaccessible OC portion of sorbed OM is in the range of ~2–10% during a period of several months (Swanston et al., 2002; Crow et al., 2007; Saidy et al., 2012; Jagadamma et al., 2014) but can also exceed 60% (Mikutta et al., 2011). Especially in topsoils with high OC inputs and mineral C loadings, OC can repeatedly be displaced and desorbed from mineral surfaces (Leinemann et al., 2016; Liebmann et al., 2020a). Consequently, MOM is a potential source of OC fueling denitrification, with its contribution probably depending on the amount and quality of WEOM.

1.3 Objectives

Although the availability of organic matter (OM) is well known to be one of the main factors controlling soil denitrification, the role of OM quality in shaping the spatial and temporal patterns of denitrification is still rather unknown. The overarching goal of this thesis was to contribute to an improved understanding of the relevance of different functional fractions of OM in driving soil denitrification. Despite many studies suggest an important contribution of WEOM to denitrification, there is no study available that systematically addressed the role of the molecular WEOM composition and concentration in soil denitrification. Furthermore, it is currently unknown to what extent the POM and MOM fractions provide readily available OC, i.e., WEOC, for denitrification reactions. The three specific aims of the thesis were

(i) to test the effect of soil OM composition as caused by different fertilization regimes on potential denitrification, the $N_2O/(N_2O+N_2)$ product ratio, and abundances of functional genes of denitrification (Chapter 2),

(ii) to examine the potential effect of agriculturally important plant residues on denitrification with special emphasis on WEOM (Chapter 3), and

(iii) to test short-term effects of POM and MOM on potential denitrification and to estimate the contribution of POM- and MOM-derived WEOC to denitrification and CO_2 production of three agricultural topsoils (Chapter 4).

2 Differences in labile soil organic matter explain potential denitrification and denitrifying communities in a long-term fertilization experiment

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Author contributions:
RS, JB, and RM conceived the experiment. RS, EL, S Heilek, and S Henjes conducted the experiments. RS, LS, S Henjes, and CWM analyzed the data. RS prepared the first manuscript draft and all authors contributed to its refinement.

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2.1 Abstract

Content and quality of organic matter (OM) may strongly affect the denitrification potential of soils. In particular, the impact of soil OM fractions of differing bioavailability (soluble, particulate, and mineral-associated OM) on denitrification remains unresolved. We determined the potential N_2O and N_2 as well as CO_2 production for samples of a Haplic Chernozem from six treatment plots (control, mineral N and NP, farmyard manure - FYM, and FYM + mineral N or NP) of the Static Fertilization Experiment Bad Lauchstädt (Germany) as related to OM properties and denitrifier gene abundances. Soil OM was analyzed for bulk chemical composition (^{13}C-CPMAS NMR spectroscopy) as well as water-extractable, particulate, and mineral-associated fractions. Soils receiving FYM had more total OM and larger portions of labile fractions such as particulate and water-extractable OM. Incubations were run under anoxic conditions without nitrate limitation for seven days at 25 °C in the dark to determine the denitrification potential (N_2O and N_2) using the acetylene inhibition technique. Abundances of *nirS*, *nirK*, and *nosZ (I+II)* genes were analyzed before and after incubation. The denitrification potential, defined as the combined amount of N released as N_2O+N_2 over the experimental period, was larger for plots receiving FYM (25.9–27.2 mg N kg^{-1}) than pure mineral fertilization (17.1–19.2 mg N kg^{-1}) or no fertilization (12.6 mg N kg^{-1}). The CO_2 and N_2O production were well related and up to three-fold larger for FYM-receiving soils than under pure mineral fertilization. The N_2 production differed significantly only between all manured and non-manured soils. Nitrogenous gas emissions related most closely to water-extractable organic carbon (WEOC), which again related well to free particulate OM. The larger contribution of N_2 production in soils without FYM application, and thus, with less readily decomposable OM, coincided with decreasing abundances of *nirS* genes (NO_2^- reductase) and increasing abundances of genes indicating complete denitrifying organisms (*nosZ I*) during anoxic conditions. Limited OM sources, thus, favored a microbial community more efficient in resource use. This study suggests that WEOC, representing readily bioavailable OM, is a straightforward indicator of the denitrification potential of soils.

2.2 Introduction

When oxygen (O_2) is absent and organic carbon (OC) sources are available denitrifying microorganisms reduce nitrate (NO_3^-) via a series of enzymatic steps to NO_2^-, NO, N_2O, and finally N_2 (Philippot et al., 2007). Therefore, denitrification can cause considerable N losses in form of nitrogenous gases from agricultural soils, resulting in limited crop production (Aulakh et al., 1992). In addition, N_2O exhibits the largest warming potential of all biogenic greenhouse gases (298 times that of CO_2) and accounts for about 6% of the current global greenhouse effect (Bouwman et al., 1995; IPCC, 2013). Its atmospheric concentration increased since pre-industrial times by approximately 20%, mainly in the wake of the increasing use of N fertilizers (WMO, 2017). Compared to N_2O, the N_2 production is rarely studied, due to the large background concentrations of N_2 in air and water, rendering it difficult to detect N_2 release by denitrification (Groffman et al., 2006). Complete denitrification to N_2 still results in a net loss of N but has no such effect on climate change as N_2O. Consequently, better understanding of factors controlling the N_2O/N_2 product ratio is of crucial importance for evaluating climatic effects by denitrification.

Denitrification in soil mainly occurs in anoxic microhabitats ('hot spots') where enough NO_3^- and carbon (C) are available (e.g., Groffman et al., 2009). Previous studies have shown that addition of plant biomass or well-defined low-molecular weight compounds, such as glucose or sucrose, affects denitrification rates, product ratios and denitrifier populations (e.g., Beauchamp et al., 1989; Miller et al., 2008; Palmer et al., 2012). Much less information is available on effects of ecologically more relevant OM fractions, such as particulate and mineral-associated OM, on potential denitrification rates and resulting product ratios. Water-extractable organic C (WEOC) is considered to be readily decomposable and most effective in promoting denitrification (e.g., Bremner and Shaw, 1958; Burford and Bremner, 1975). Accordingly, the addition of plant-derived dissolved OM (water extracts of maize stalk) to repacked soil results in increased CO_2 and N_2O emissions (Qiu et al., 2015). Effects of sources, availability, and composition of WEOC on denitrification, however, have not been addressed so far. In agricultural soils, additional factors need to be taken into account when evaluating the relevance of different OM fractions, including rates and type of fertilizer application (organic versus mineral) and crop sequences (Janzen et al., 1992; Edmeades, 2003; Diacono and Montemurro, 2010). Mineral fertilization has controversial and at most indirect effects on the content

and quality of OM (e.g., He et al., 2015; Dou et al., 2016). For example, sole addition of mineral N significantly accelerates the decomposition of OM with the decomposition products then becoming stabilized in mineral-organic associations (Neff et al., 2002). Manure application, by contrast, results in larger contents of plant-derived sugars (Xie et al., 2014), a generally higher proportion of labile OC, and an overall higher microbial activity (e.g., Aoyama et al., 1999; Hai et al., 2010; Wang et al., 2015). Randall et al. (1995) also observed that OM in a manured silty clay loam of the Broadbalk Experiment at Rothamsted (UK; monoculture of winter wheat since 1843) was slightly enriched in O/N-alkyl C and alkyl C components compared to soils under mineral NPK fertilization. Consequently, also the abundances of denitrifying organisms and the N_2O production can be higher in manured than in mineral fertilized soils (e.g., Sun et al., 2015; Cui et al., 2016). So far, the relationship between fertilization-induced changes in functional OM fractions and the respective potential denitrification as well as gene abundances have only been rarely addressed; with most denitrification studies neglecting the emission of N_2 relative to N_2O.

The objective of this study was, therefore, to test the effect of soil OM composition as caused by different fertilization regimes on (i) potential denitrification, (ii) the $N_2O/(N_2O+N_2)$ product ratio, and (iii) respective gene abundances. We used soil samples from six plots of the long-term Static Fertilization Experiment Bad Lauchstädt (Germany) to obtain a wide range of organic matter composition under similar textural properties. Based on the assumption that the long-term application of different fertilizers changed the amount and composition of soil OM, we hypothesize that there are specific and measurable OM fractions that allow for explaining and predicting denitrification rates and product ratios. We assume that treatments causing stronger accumulation of readily decomposable OM, indicated by larger portions of water-extractable and particulate OM, and (O/N-)alkyl C components, result in increased denitrification with increased proportions of N_2O. In addition, we surmise that the denitrifier community (abundances of NO_2^- and N_2O reductase genes) is directly linked to the amount and composition of OM fractions or their bioavailability.

2.3 Materials and methods

2.3.1 Soil sampling

Soil samples (four field replicates) were randomly collected at 0–30 cm depth from six treatment plots of the Static Fertilization Experiment Bad Lauchstädt, Germany (51°23' N, 11°52' E), in October 2016. The site is characterized by an exceptional homogenous soil, with very little variation in basic properties, such as soil texture, but providing a wide range of different compositions of soil organic matter (e.g., Ludwig et al., 2007). The loamy soil is classified as Haplic Chernozem with the topsoil (Ap horizon) having about 70% silt, 20% clay, and 10% sand on all experimental plots (Altermann et al., 2005; Ludwig et al., 2007). The mean annual precipitation and temperature at the site is 486 mm and 8.8 °C, respectively. The Static Fertilization Experiment was established in 1902 and consists of eight strips, each divided into 18 treatment plots (except for strip number 4 and 5). In this study, only the following six treatments of strip number 2 were used: control, i.e., without any fertilization; mineral N (N) and N+P fertilization (NP); application of farmyard manure (FYM), also combined with mineral N and P (FYM+N and FYM+NP). The study is focused on the relevance of organic matter fractions for potential denitrification and less on the effect of fertilization. Nevertheless, in accordance with previous work, the sampled soils are designated according to the respective fertilization treatment. Calcium ammonium nitrate (27% N) and superphosphate were used as mineral N and P sources. The amount of mineral fertilizers depended on the crop and amount of applied FYM (Appendix 1). Farmyard manure (30 t ha^{-1}) has been applied every second year with root crops (sugar beets, potatoes). In 2015, the original crop rotation (sugar beet – spring barley – potatoes – winter wheat) has been modified to: silage maize – spring barley – silage maize – winter wheat. FYM is now applied in years with maize cropping. Additional information is given by Körschens et al. (1994), Merbach and Körschens (2002), and Merbach and Schulz (2013).

2.3.2 Basic characterization of bulk soils

Soil samples were stored at 4 °C in the dark for a maximum of four days after collection. Large plant particles and stones were removed by sieving to <2 mm. To estimate available P (Olsen, 1954), 2.5 g of field-fresh soil suspended in 50 ml 0.5 M $NaHCO_3$ (pH 8.5) solution were shaken for 30 minutes. After centrifugation (3000 × g) for 10 minutes (Heraeus™

Cryofuge 8500i, Thermo Fisher Scientific, Waltham, MA, USA), the supernatant was passed through a 0.45-µm membrane filter (Supor®-450, Pall Cooperation, New York, NY, USA). Concentrations of P in the extracts were analyzed using ICP-OES (Ultima 2, Horiba Jobin-Yvon, Longjumeau, France). N_{min} (NO_3^- and NH_4^+) was extracted from field-fresh soils into 1 M KCl solution at a soil-to-solution ratio of 1:5 (wt./v), with centrifugation and filtration as described above, and determined using a Continuous-Flow Analyzer (ScanPlus, Skalar Analytical B.V., Breda, The Netherlands). Soil reaction was estimated by potentiometric measurement of pH in the supernatant of a soil suspension in 0.01 M $CaCl_2$ (1:5 wt./v). Air-dried and sieved bulk soils were analyzed for total C und N (TN) with a Vario Max Cube (Elementar Analysensysteme GmbH, Langenselbold, Germany). Inorganic C was not detectable (Vario Max Cube, Elementar), therefore, total C was assumed to represent OC. The difference TN − N_{min} is an estimate of organic N (ON). All values were normalized to the respective total dry matter, determined gravimetrically after oven-drying at 105 °C.

2.3.3 Solid-state ^{13}C-NMR spectroscopy

Air-dried and sieved bulk soils (n = 24, including field replicates) were ground in an agate mortar, combined into a composite sample for each fertilization treatment (n = 6), and analyzed by solid-state ^{13}C cross-polarization magic angle spinning NMR spectroscopy (^{13}C-CPMAS NMR spectroscopy) with an Avance III 200 spectrometer (Bruker BioSpin GmbH, Karlsruhe, Germany). Samples were placed into a 7-mm zirconia rotor that was spun at 6.8 kHz around a 'magic angle' of 54.74°. Contact time was 1 ms and the recycle delay time was set to 0.4 s. The spectra were processed with 100 Hz line broadening, phase adjusted, and baseline corrected; no spinning side bands appeared in the spectra. Peaks were assigned to four integration areas: –10–45 ppm (alkyl C), 45–110 ppm (O/N-alkyl C), 110–160 ppm (aromatic C), and 160–220 ppm (carboxylic/carbonyl C). All spectra were well resolved, indicating no interfering effects of paramagnetic materials, such as iron oxides, on the measurements (Appendix 2).

2.3.4 Characterization of functional OM fractions

Soil samples were fractionated according to density using a modified version of the procedure described by Christensen (1992). The procedure provides three fractions:

particulate OM (POM) not or only weakly associated with mineral particles (i.e., free POM = fPOM), POM occluded within water-stable aggregates (oPOM), and OM strongly bound to mineral phases (mineral-bound OM = MOM). In brief, 25 g of air-dried soil (<2 mm) were gently suspended in 125 ml of sodium polytungstate (SPT; 1.6 g cm^{-3}) in a 500-ml centrifuge beaker. After one hour, the suspension was centrifuged (6800 × *g*) for 30 minutes at 20 °C (Cryofuge 8500i). The supernatant with the floating fPOM material was aspirated and passed through a 0.45-µm membrane filter (Supor®-450, Pall). Soils were re-suspended in SPT solution and the centrifugation–filtration procedure was repeated once again. The fPOM on the filter was washed with distilled water until the electrical conductivity in washing solution was <50 µS cm^{-1}, and then air-dried at 40 °C, and weighed. The soil was then re-suspended in SPT solution and sonicated at 60 J ml^{-1} (Sonoplus UW 2200, Bandelin electronic GmbH, Berlin, Germany) to release oPOM from aggregates. The selected sonication energy was shown to be sufficient to disrupt all aggregates in a preparatory test according to Cerli et al. (2012). Centrifugation, filtration, and washing were carried out as for the fPOM. The remaining soil material, representing the MOM fraction, was subjected to several washing–centrifugation cycles until the conductivity of the washing solution was <50 µS cm^{-1}. Subsequently, the MOM fraction was freeze-dried and weighed. The MOM fraction was analyzed for OC and TN using a Vario Max Cube; analyses of fPOM and oPOM were carried out with a Vario EL analyzer (Elementar Analysensysteme GmbH). The total contents of OC and TN with the fractions in soil were calculated by multiplying the respective bulk soil OC and TN contents with the proportional contribution of the individual fractions to the sum of OC and TN in all fractions.

Water-extractable organic C (WEOC) and N (WEON) were determined on fresh soil samples. Briefly, 20 g of soil suspended in 100 ml deionized water were shaken for one hour. After centrifugation (3000 × *g*) for 10 minutes (Cryofuge 8500i), the supernatant was passed through a 0.45-µm membrane filter (Supor®-450) and analyzed for OC and total N, using a DIMATOC® 100 (Dimatec Analysetechnik GmbH, Essen, Germany).

2.3.5 Incubation and gas measurements

Bulk soils were anoxically incubated at 25 °C in the dark for seven days, as most N_2O production in soil occurs at timescales of less than two weeks (Kuzyakov and Blagodatskaya, 2015). To reactivate the microbial community, subsamples (110 g dry

mass) of each soil were wetted to 40% water holding capacity and aerobically pre-incubated for seven days at 25 °C in the dark. Then, 100 g (dry mass) of each soil were packed into 500-ml glass infusion bottles to 1.3 g cm^{-3} bulk density, using a plunger. A KNO_3 solution (50 mg NO_3^--N kg^{-1} dry soil) was added to achieve 80% water-filled pore space and avoid nitrate limitation during the incubation period. The latter ensured that differences in denitrification related primarily to differences in OM content and quality. All bottles were sealed with a bromine-butyl-rubber stopper and crimped with an aluminum cap (32 mm; Chroma Globe GbR, Kreuznau, Germany). An O_2-free atmosphere for anoxic incubations was obtained by evacuating (below 250 hPa), and then rinsing the bottles with He gas (99.999%, Air Liquide, Düsseldorf, Germany) for three times, reaching a final pressure of about 1025 hPa. After one hour, the first (t_0) gas sample (18 ml) was taken with a gastight syringe (25 ml, 25MDR-LL-GT; SGE Analytical Science Pty. Ltd., Ringwood, VIC, Australia), equipped with a push button valve (Luer Lock; SGE Analytical Science) and a 0.7-mm ID cannula (Sterican G26, 25 mm; B. Braun AG, Melsungen, Germany), and transferred into pre-evacuated (10 hPa residual pressure, rinsed with He) 12-ml Exetainer® vials (IVA Analysentechnik e.k., Meerbusch, Germany). This resulted in an overpressure of >200 hPa in the Exetainer® vials, which was necessary to avoid contamination with air during storage and for measuring gas concentrations with the gas chromatography system described below. An additional septum (12 mm, silicone–PTFE, 1.5 mm; IVA) was placed in the screw caps above the chlorobutyl rubber septum to achieve gas tightness. To avoid low pressure in the incubation bottles, 18 ml He at ~1000 hPa were injected after daily gas sampling, resulting in constant absolute pressure of ~1025 hPa during incubation. The absolute pressure in the bottles was measured before and after gas sampling as well as after He injection, using a GMSD 2 BA-K31-L01 pressure sensor coupled with a Greisinger GMH 3151 reader (GSG Geologie-Service GmbH, Würzburg, Germany).

All gas samples were analyzed on a custom-tailored gas chromatography system by Chromtech (Bad Camberg, Germany), using an Agilent HP 7890B GC as basis. The samples were introduced into the injector by an autosampler (PAL GC-xt; CTC Analytics AG, Zwingen, Switzerland), using an open needle syringe. Calibration was done online, using standard gas cups connected to bottles with certified calibration gases with known concentrations of CO_2 and N_2O in He (Linde Gas AG, Pullach, Germany). After injecting the sample at a liner temperature of 150 °C, it was transferred to a Shin Carbon chromatographic column (2 m, 0.53 mm ID, Restek GmbH, Bad Homburg, Germany) using

He as carrier gas (purity 99.9999%) and directly transferred to a He ionization detector (Vici AG International, Schenkon, Switzerland) run at 180 °C. The GC oven was set to a starting temperature of 60 °C, kept constant for three minutes, increased to 110 °C at a rate of 10 °C min^{-1}, kept for one minute, and finally increased to 220 °C at a rate of 50 °C min^{-1}, kept for three minutes. The limit of detection for both gases was calculated using 10 blank samples that were drawn and measured in the same way as all other samples. The limit of detection was calculated as 0.1 ppb and 11.76 ppm for N_2O and CO_2, respectively. Precision and accuracy were analyzed injecting a reference standard every ten samples. On average, precision was 4.8 and 0.5% relative standard error for N_2O and CO_2, respectively, while accuracy was 3.3 and 1.5% offset from the specified concentration for N_2O and CO_2, respectively.

Cumulative emissions of gases represent the sum of daily produced amounts, i.e., the detected gas mass in the headspace at time point t_x minus the gas mass at time point t_{x-1}. For setting up the acetylene inhibition technique (Yoshinari and Knowles, 1976), the entire incubation procedure described above was repeated with injection of 60 ml of C_2H_2 (99.6%; Air Liquide) in exchange for 60 ml He, resulting in an initial C_2H_2 concentration of ~10% (v/v) directly after flushing with He and one hour before the first gas sampling. Considering the dilution by the He addition after gas sampling and assuming a microbial metabolism of <2.5% of added C_2H_2 over seven days (Terry and Duxbury, 1985), the C_2H_2 concentration was high enough (>5% v/v) to prevent the reduction of N_2O to N_2 over the entire incubation period (Yeomans and Beauchamp, 1978). To estimate the N_2 production, the produced amount of N_2O was subtracted from the respective amount of N_2O in presence of C_2H_2 (representing N_2O+N_2). The ratio of the two N_2O amounts was used to determine the molar N_2O-N/(N_2O+N_2)-N ratio for each incubation day. Proportional NO_3^--N losses as N_2O-N and N_2-N were calculated based on cumulative gas emissions within seven days and the initial (natural + added) NO_3^--N content. The portion of mineralized OC was derived by relating the cumulative CO_2-C produced within seven days to the initial bulk soil OC as well as the WEOC content. Changes in soil pH in response to the incubations were little (±0.3 pH units) and revealed no consistent patterns between differently fertilized soils.

2.3.6 DNA extraction and qPCR assay

Pre-incubated and incubated soil samples were frozen and stored at –20 °C prior to analyses of abundances of NO_2^- and N_2O reductase genes. While gene abundances in pre-incubated soils were mainly determined by the different long-term fertilization, changes over the incubation time represent short-time effects. The core reaction of denitrification is the conversion of soluble NO_2^- into gaseous NO, catalyzed by NO_2^- reductases. Canonical denitrifiers have either a copper-containing NO_2^- reductase or a cytochrome cd1 NO_2^- reductase, encoded by *nirK* or *nirS* genes, respectively (Gao et al., 2016). The last step, the reduction of N_2O to N_2, is catalyzed by the N_2O reductase encoded by *nosZ* genes, which can be divided into clade I and II (Hallin et al., 2017). Most organisms with *nosZ I* genes are complete denitrifiers, i.e., they also have *nirS* or *nirK* genes, whereas many organisms with *nosZ II* are non-denitrifying N_2O reducers (Graf et al., 2014). DNA was extracted from 0.3 g of soil, using the DNeasy Soil Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA concentration was measured spectrophotometrically (DS-11 FX, DeNovix, Wilmington, NC, USA) and *nirK*, *nirS*, as well as *nosZ* (clade I and II) were amplified by quantitative polymerase chain reaction (PCR) in a CFX Connect thermocycler (Bio-Rad Laboratories, Hercules, CA, USA), using the primer pairs F1aCu (ATCATGGTSCTGCCGCG) / R3Cu (GCCTCGATCAGRTTGTGGTT; Hallin and Lindgren, 1999), cd3aF (GTSAACGTSAAGGARACSGG) / R3cd (GASTTCGGRTGSGTCTTG; Throbäck et al., 2004) , nosZ1840F (CGCRACGGCAASAAGGTSMSSGT) / nosZ2090R (CAKRTGCAKSGCRTGGCAGAA; Henry et al., 2006), and nosZ-II-F (CTIGGICCIYTKCAYAC) / nosZ-II-R (GCIGARCARAAITCBGTRC; Jones et al., 2013), respectively. Standard curves for each qPCR assay were derived from plasmids containing the respective cloned gene from either a soil sample (*nirK*, *nirS*) or pure culture (*Bradyrhizobium japonicum*, *nosZ I*; *Bacillus azotoformans*, *nosZ II*), where the inserts were amplified with primers specific for the multiple cloning site (Zaprasis et al., 2010; Palmer and Horn, 2015). The gene copy numbers were related to dry mass of soil. Details on the composition of the PCR mastermixes and thermocycling protocols are given in the Appendix 3. The amplification of standards and samples was done in triplicate and the specificity of the reaction was tested via gel electrophoresis. The absence of PCR-inhibiting substances, such as humic acids, was shown by spiking a number of the soil DNA extracts with a known amount of standard DNA before subjecting them to qPCR and comparing the resulting Ct-values to those of the same standard DNA in pure water.

2.3.7 Statistical evaluation

Basic statistical analyses were performed using Sigma Plot 11.0 (Systat Software Inc., Erkrath, Germany). One-way ANOVA (fertilization treatment as independent variable, n = 6) followed by the Tukey HSD test was used for testing for differences in biochemical soil and OM properties, abundances of denitrification genes, as well as initial, maximum, and cumulative emissions of N_2O, N_2, and CO_2. Data not normally distributed were log-transformed to achieve normality, or the non-parametric Kruskal-Wallis test followed by the Tukey HSD test was used. Linear regression analyses were used to test for relationships between gas emissions and soil properties of different fertilization treatments after confirming the normal distribution of data by the Shapiro-Wilk test (n = 24). One-way ANOVA followed by the Tukey HSD test was also used for testing for differences between cumulative N_2 emissions from all manured and non-manured soils (for each n = 12). For ^{13}C-NMR spectroscopy data, linear regression analyses with mean values of gas emissions and gene abundances were performed using values of the six composite samples of each fertilization treatment (n = 6). Correlation coefficients were determined and considered statistically significant at $p < 0.05$.

2.4 Results

2.4.1 Chemical soil properties under different long-term fertilization

Different fertilization over 114 years altered the chemical soil properties (Table 2.1). Except for one replicate of the NP treatment with a pH of only 5.5, the pH values of soils with FYM application were lower (6.1–6.9) than those of soils without (6.9–7.4). The mean OC content of the FYM-treatments was almost 44% larger than under the other three fertilization regimes (control, N, NP). Within each of the two groups, the OC contents varied only slightly (Table 2.1). The average OC/ON ratio was slightly less in soils with FYM application (12.6–12.8) than under mineral fertilization (13.6) and in the control (14.3). FYM application increased the contents of available P (Olsen P); sole calcium ammonium nitrate (N treatment) application resulted in decreased contents of available P (Table 2.1). The largest contents of KCl-extractable NO_3^- occurred in the manured (FYM, FYM+N, FYM+NP) and N-fertilized soils (13.8–18.9 mg N kg^{-1} dry soil); the control and NP treatments had 8.0 and 9.8 mg NO_3^--N kg^{-1} dry soil, respectively. Extractable ammonium (NH_4^+) was not detectable in any treatment.

Table 2.1 Soil pH, total organic C (OC), OC/ON ratio, KCl-extractable NO_3^--N, and $NaHCO_3$-extractable P of soils from the Static Fertilization Experiment (control, mineral N and NP, farmyard manure – FYM, and FYM + mineral N or NP). Since no ammonium was detected in any sample, NO_3^--N represented total mineral N. Values represent means (n = 4) ± standard deviation. Different letters indicate significant differences between treatments ($p < 0.05$).

Fertilization treatment	pH[a] ($CaCl_2$)	Total OC [g kg^{-1}]	OC/ON ratio	NO_3^--N [mg kg^{-1}]	Olsen P [mg kg^{-1}]
Control	6.9–7.4	15.5 ± 0.4b	14.3 ± 0.4a	8.0 ± 1.2b	11.0 ± 5.2d
N	7.1–7.4	16.0 ± 0.3b	13.6 ± 0.6ab	14.7 ± 3.3ab	4.4 ± 0.9d
NP	5.5–7.2	16.5 ± 0.5b	13.6 ± 0.5ab	9.8 ± 2.6b	35.4 ± 3.6c
FYM	6.4–6.9	22.3 ± 0.5a	12.8 ± 0.3bc	13.9 ± 4.6ab	48.1 ± 3.4b
FYM+N	6.2–6.7	23.3 ± 1.6a	12.6 ± 0.3c	17.8 ± 4.6a	39.3 ± 4.1c
FYM+NP	6.1–6.7	22.3 ± 0.5a	12.7 ± 0.1c	18.9 ± 3.6a	57.5 ± 4.8a

[a] Range of replicated samples (n = 4).

2.4.2 Organic matter composition

The overall contribution of fPOM-C and oPOM-C to total soil OC was 1–3% and 2–7%, respectively (Table 2.2). Soils receiving FYM had about twice the content of fPOM (1.8–2.4 g kg^{-1} soil) as compared to mineral fertilized soils and the control (0.8–1.0 g kg^{-1}). N-fertilized soils and manured soils receiving additional mineral fertilizers (N and especially NP) had the largest oPOM contents (2.7–4.0 g kg^{-1} soil). In all soils, most OC resided within the MOM fraction (91–97%). The OC/TN ratios of fPOM and oPOM in soils with FYM application were about 30% smaller than those of the other treatments (Table 2.2). The OC/TN ratio of MOM was 5 and 9% less for manured soils than for soils under mineral fertilization and for the control, respectively.

^{13}C-CPMAS NMR spectroscopy revealed that the contribution of O/N-alkyl C to total soil OC ranged from 34 to 38% in the order control < N, NP < FYM < FYM+N, FYM+NP; smaller proportions of aryl C and carboxyl/carbonyl C were measured for soils under mineral fertilization, with or without FYM (Figure 2.1). The application of mineral N and P, especially without FYM, resulted in slightly more aliphatic C. Soils with FYM application showed about 13% smaller alkyl C-to-O/N-alkyl C ratios (0.5–0.6) than in those of the other three treatments. For all treatments, the proportion of WEOC and WEON varied

between 0.1–0.2% of total OC and between 0.3–0.7% of total ON. In accordance with their larger OC and ON contents, the manured soils had larger amounts of WEOC and WEON of than the mineral fertilized soils and the control soil (Table 2.2). The WEOC/WEON ratios were almost similar for all fertilization treatments (4.3–5.5). WEOC contents were positively correlated with the fPOM-C/soil OC ratio ($r = 0.63$, $p < 0.01$, $n = 24$) and, in turn, negatively to the MOM-C/soil OC ratio ($r = -0.63$, $p < 0.01$, $n = 24$) and OC/TN ratio of fPOM ($r = -0.85$, $p < 0.001$, $n = 24$).

Table 2.2 Contents of water-extractable organic C (WEOC) and N (WEON), contribution of free and occluded particulate OC (fPOM, oPOM) and mineral-associated OC (MOM) to total soil OC as well as OC/TN ratios of the three fractions in soils from the Static Fertilization Experiment (control, mineral N and NP, farmyard manure – FYM, and FYM + mineral N or NP). Values represent means (n = 4) ± standard deviation. Different letters indicate significant differences between treatments ($p < 0.05$).

Fertilization treatment	WEOC [mg kg^{-1}]	WEON [mg kg^{-1}]	Proportion in total OC [%]			OC/TN ratio		
			fPOM-OC	oPOM-OC	MOM-OC	fPOM	oPOM	MOM
Control	23.0 ±4.7c	4.4 ±0.7b	2.1 ±0.1ab	3.4 ±1.0bc	94.5 ±1.1ac	30.8 ±5.1a	21.7 ±2.0ab	13.7 ±0.4a
N	28.5 ±1.9bc	6.9 ±1.5ab	1.8 ±0.7ab	6.6 ±1.4a	91.6 ±2.1b	29.5 ±4.7a	20.1 ±1.9ab	13.0 ±0.6ab
NP	28.8 ±7.1bc	5.6 ±0.9ab	1.3 ±0.3b	2.1 ±0.5c	96.6 ±0.3a	29.6 ±5.1a	27.1 ±8.4a	13.1 ±0.3ab
FYM	37.9 ±2.4ab	8.6 ±1.7a	3.3 ±1.1a	4.2 ±1.1abc	92.5 ±2.1bc	20.8 ±5.2ab	15.7 ±1.5b	12.5 ±0.3b
FYM+N	38.6 ±4.1ab	7.6 ±2.3ab	3.3 ±1.3a	5.5 ±1.1ab	91.3 ±2.4b	18.3 ±3.0b	16.3 ±1.3b	12.4 ±0.2b
FYM+NP	39.8 ±5.4a	8.7 ±2.1a	3.2 ±0.6a	6.0 ±2.1ab	90.8 ±1.6b	20.4 ±3.3ab	15.2 ±2.0b	12.4 ±0.3b

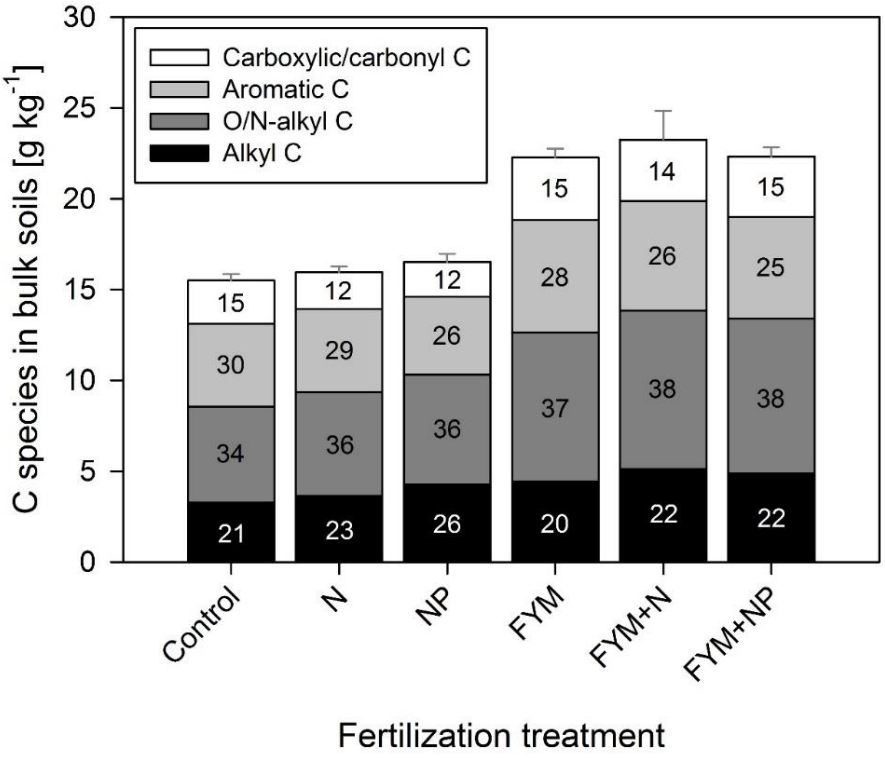

Figure 2.1 Distributions of C species in bulk soils from the Static Fertilization Experiment (control, mineral N and NP, farmyard manure – FYM, and FYM + mineral N or NP). Bars represent the soil's bulk mean OC contents (n = 4); error bars indicate standard deviation in one direction only. Stacks within bars represent the percentage contribution of the different integrated chemical shift regions.

2.4.3 Initial and cumulative production of CO_2, N_2O, and N_2

Initial (after one day) as well as cumulative N_2O and CO_2 production within seven days were significantly higher for soils receiving FYM than for soils under pure mineral fertilization and fertilizer deprivation (Figures 2.2, 2.3). Additional N and P input (FYM+N, FYM+NP) resulted in the highest average cumulative N_2O and CO_2 production (7.3–7.4 mg N kg^{-1} and 30.6–31.7 mg C kg^{-1}, respectively). The cumulative N_2O production of the control and the pure mineral N fertilization treatment reached its maximum within the first two days and dropped close to zero after seven days (Figure 2.2). The maximum cumulative N_2O production for soils with FYM application was reached after five days and subsequently stagnated (FYM+N, FYM+NP) or decreased slightly (FYM). The NP treatment showed a similar N_2O production trend as the FYM-treatments but on a lower level (Figure 2.2). The initial N_2 production was about 25% higher for mineral fertilization than for FYM application but the manured soils released almost 18% more N_2 over the entire seven days than soils receiving mineral fertilization (Figure 2.4). There was no

significant difference in the initial and cumulative N_2 production among all fertilization treatments, but the average cumulative N_2 production was significantly larger for all manured soils (n = 12) than for soils receiving no FYM application (n = 12). The cumulative amount of N_2 emitted from the control (unfertilized) was substantially less than from the fertilized soils. After seven incubation days, about 40% of the initial (natural + added) NO_3^--N was lost as N_2O and N_2 in soils with FYM application, while mineral fertilized soils (N, NP) and the unfertilized control soil emitted only 26, 32, and 22% of the initial NO_3^--N, respectively. The cumulative CO_2-C production accounted for 23–49% (non-manured soils) and 64–82% (manured soils) of the initial WEOC content.

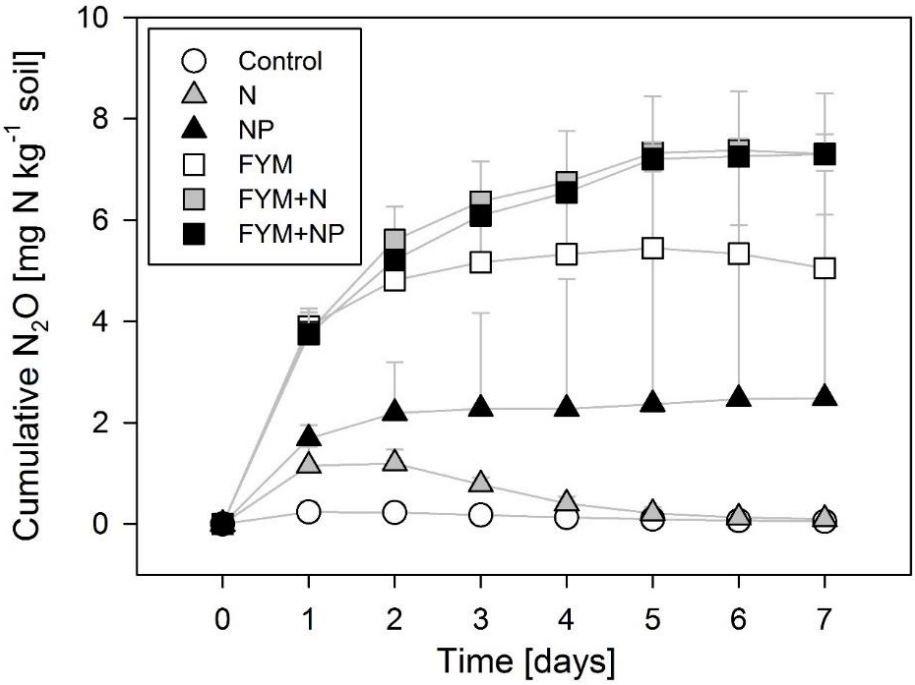

Figure 2.2 Cumulative N_2O emission during anoxic incubation under excess nitrate (50 mg NO_3^--N kg^{-1}) at 25 °C for soils from the Static Fertilization Experiment (control, mineral N and NP, farmyard manure – FYM, and FYM + mineral N or NP). Error bars show standard deviation of mean values (n = 4) per day in one direction only.

When pooling all fertilization treatments, we found a number of statistically significant relations between gas emissions with soil and OM properties: The initial N_2O and cumulative N_2 emissions over seven days correlated positively with the content of WEOC (r = 0.82 and 0.69, respectively, p <0.001, n = 24). In accordance with the narrow range of WEOC/WEON ratios this held also true for WEON (r = 0.70 and 0.77, respectively, p <0.001, n = 24). In addition, the cumulative N_2O emissions were highly correlated to the cumulative release of CO_2 over the seven incubation days (r = 0.96, p <0.001, n = 24). The

maximum N_2O production was significantly correlated with the proportion of O/N-alkyl C of bulk soils (r = 0.92, p <0.01, n = 6) and the CO_2-C/WOEC ratio (r = 0.94, p <0.001, n = 24; Figure 2.5a). No statistical relations were found between the absolute contents of particulate organic material (fPOM, oPOM) or their proportions in bulk soil OC (POM-C/soil OC) as well as of contents of MOM-C and respective CO_2, N_2O, and N_2 emissions. Likewise, pH (before and after incubation), soil OC/ON ratio, and content of available P were not significantly related to measured gas emissions.

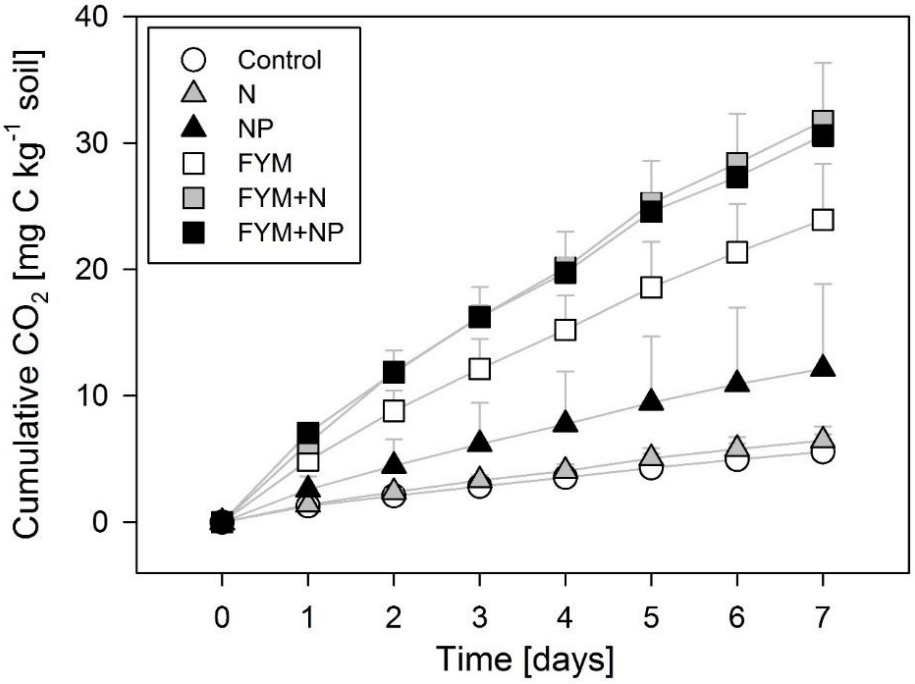

Figure 2.3 Cumulative CO_2 emission during anoxic incubation under excess nitrate (50 mg NO_3^--N kg^{-1}) at 25 °C for soils from the Static Fertilization Experiment (control, mineral N and NP, farmyard manure – FYM, and FYM + mineral N or NP). Error bars show standard deviation of mean values (n = 4) per day in one direction only.

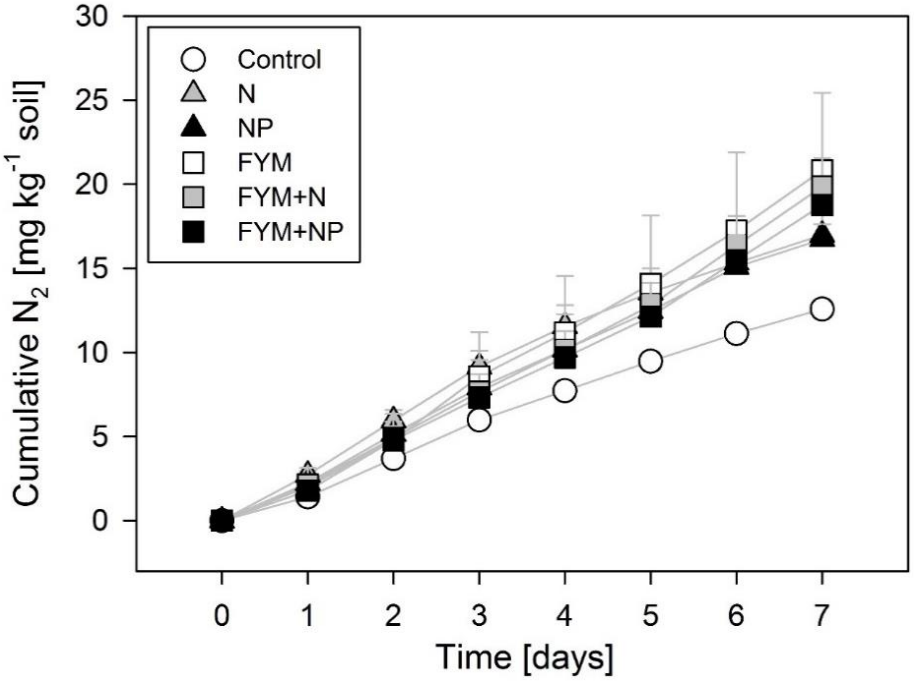

Figure 2.4 Cumulative N_2 emission during anoxic incubation under excess nitrate (50 mg NO_3^--N kg^{-1}) at 25 °C for soils from the Static Fertilization Experiment (control, mineral N and NP, farmyard manure – FYM, and FYM + mineral N or NP). Error bars show standard deviation of mean values (n = 4) per day in one direction only.

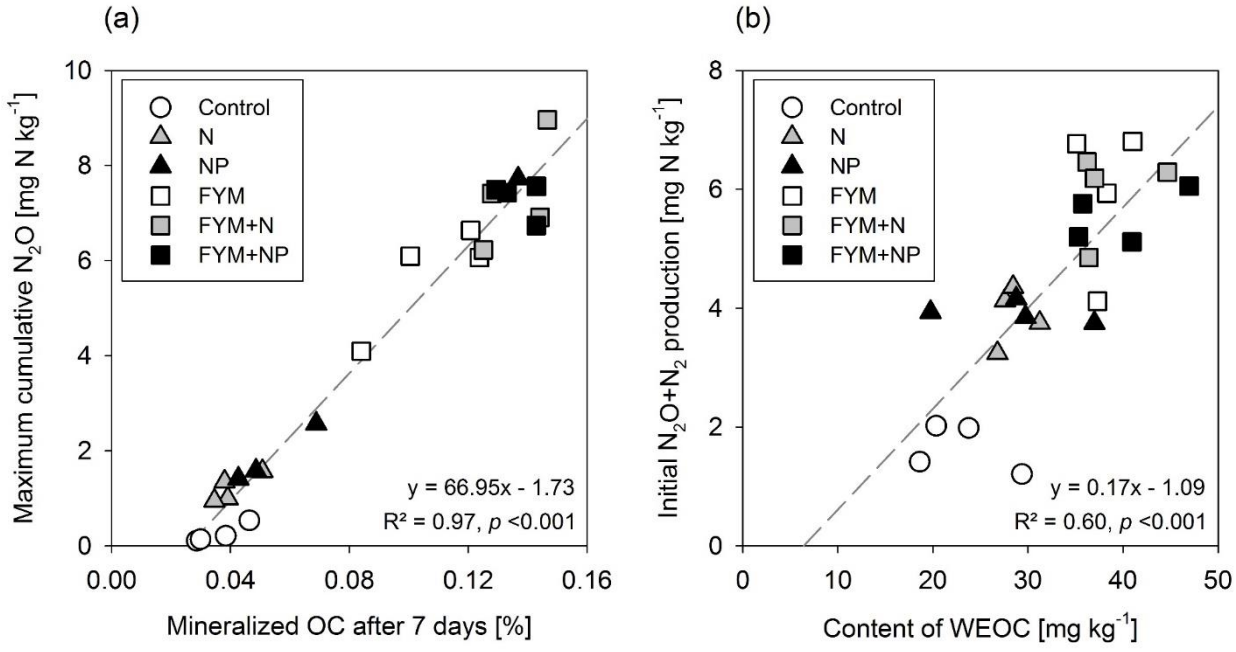

Figure 2.5 Relationship between (a) mineralized OC (CO_2-C/OC) after seven days of anoxic incubation and maximum cumulative N_2O production as well as (b) between the content of water-extractable OC (WEOC) and initial N_2O+N_2 production for soils from the Static Fertilization Experiment (control, mineral N and NP, farmyard manure – FYM, and FYM + mineral N or NP).

2.4.4 Potential denitrification and product ratios

After one day of incubation, N_2O was the dominant denitrification product of all manured soils (64–68%); for soils receiving mineral or no fertilization, N_2O contributed merely to 14–43% (Table 2.3). The initial molar $N_2O/(N_2O+N_2)$ product ratio was negatively correlated to the soil OC/ON ratio and positively to the WEOC content (r = –0.87, r = 0.75, respectively, p <0.001, n = 24). The initial total denitrified N (N_2O+N_2 after one day of incubation) of manured soils was significantly higher (by 49%) than for soils under mineral fertilization (Table 2.3). Similar to N_2O production, the initial total denitrified N was well correlated to WEOC (Figure 2.5b) and WEON (r = 0.78 and 0.76, respectively, p <0.001, n = 24). During seven days of anoxic incubation, the molar $N_2O/(N_2O+N_2)$ ratio decreased for all treatments to 0.2–0.3 (FYM-treatments), 0.01–0.1 (mineral fertilization), and 0.00 (control) (Table 2.3). At the end of incubation, the total release of nitrogenous gases by denitrification from the soils receiving only mineral fertilizers were still significantly lower (31%) than of those with FYM application but still 44% larger than of the control soil (Table 2.3). There was no significant difference in denitrified N (25.9–27.2 mg kg^{-1}) within the seven incubation days between manured soils receiving no or additional mineral fertilizers. The cumulative N_2O+N_2 production over the seven days was again positively correlated with the WEOC content (r = 0.75, p <0.001, n = 24).

2.4.5 Abundance of denitrifier genes

For pre-incubated soils, the different fertilization regimes had no distinct effect on the abundance of NO_2^- reductase genes (*nirS* and *nirK*) and the N_2O reductase gene *nosZ II* (Figures 2.6a-d). However, *nosZ I* (N_2O reductase) genes were significantly more abundant in soils receiving FYM than in soils under other fertilization regimes, even before anoxic incubation (Figure 2.6c). In non-manured soils, abundances of *nirS* and *nosZ II* genes decreased during anoxic incubation by 35–45% and about 50%, respectively, while the abundances of *nosZ I* genes increased by 130% (Figures 2.6a, c, d). In soils under FYM application, *nosZ I* genes increased as well but the increases were by 58–92% lower than in non-manured soils. Overall, soils receiving FYM showed significantly higher absolute abundances of NO_2^- reductase genes (*nirS* + *nirK*) and N_2O reductase genes (*nosZ I* + *nosZ II*) after anoxic incubation than soils receiving either no or only mineral fertilizers (Figures 2.6a-d). The abundances of *nirS* genes were substantially higher than those of

nirK for all treatments. While *nosZ I* gene copy numbers were mostly smaller than *nosZ II* before anoxic incubation, except for the FYM+N treatment, *nosZ I* was the dominant N_2O reductase gene at the end of incubation.

Table 2.3 Total release of nitrogenous gases (N_2O+N_2) and molar $N_2O/(N_2O+N_2)$ ratios of cumulative gas emissions after one and seven days of incubation of soils from the Static Fertilization Experiment (control, mineral N and NP, farmyard manure – FYM, and FYM + mineral N or NP). Values represent means (n = 4) ± standard deviation. Different letters indicate significant differences between treatments ($p < 0.05$).

Fertilization treatment	Cumulative N_2O+N_2 [mg N kg^{-1}]		$N_2O/(N_2O+N_2)$ ratio	
	After 1 day	After 7 days	After 1 day	After 7 days
Control	1.7 ± 0.4c	12.6 ± 0.3c	0.14 ± 0.09c	0.00 ± 0.00b
N	3.9 ± 0.5b	17.1 ± 1.7bc	0.29 ± 0.06bc	0.01 ± 0.00ab
NP	3.9 ± 0.2b	19.2 ± 3.3b	0.43 ± 0.05b	0.11 ± 0.15ab
FYM	5.9 ± 1.3a	25.9 ± 3.5a	0.68 ± 0.14a	0.20 ± 0.09ab
FYM+N	6.0 ± 0.7a	27.2 ± 2.4a	0.64 ± 0.03a	0.27 ± 0.03ab
FYM+NP	5.5 ± 0.5a	26.1 ± 1.5a	0.68 ± 0.05a	0.28 ± 0.01a

No significant relation between pH (before and after incubation) and abundance of denitrifier genes were observed. However, the initial *nosZ I* gene abundances (after aerobic pre-incubation) were positively correlated to Olsen P ($r = 0.80$, $p < 0.001$, $n = 24$). The initial abundances of *nirK* and *nosZ I* as well as the gene copy numbers of *nirS* and *nosZ I* after anoxic incubation correlated positively with the WEOC content ($r = 0.76$, 0.86, 0.81, and 0.79, respectively, $p < 0.001$, $n = 24$; Figures 2.7a, b). The abundance of NO_2^- reductase genes showed no relationship to the N_2O production. Also, the abundances of N_2O reduction genes were not significantly correlated to the production of N_2. In contrast, the initial abundances of *nosZ I* were highly correlated with the initial N_2O production ($r = 0.90$, $p < 0.001$, $n = 24$).

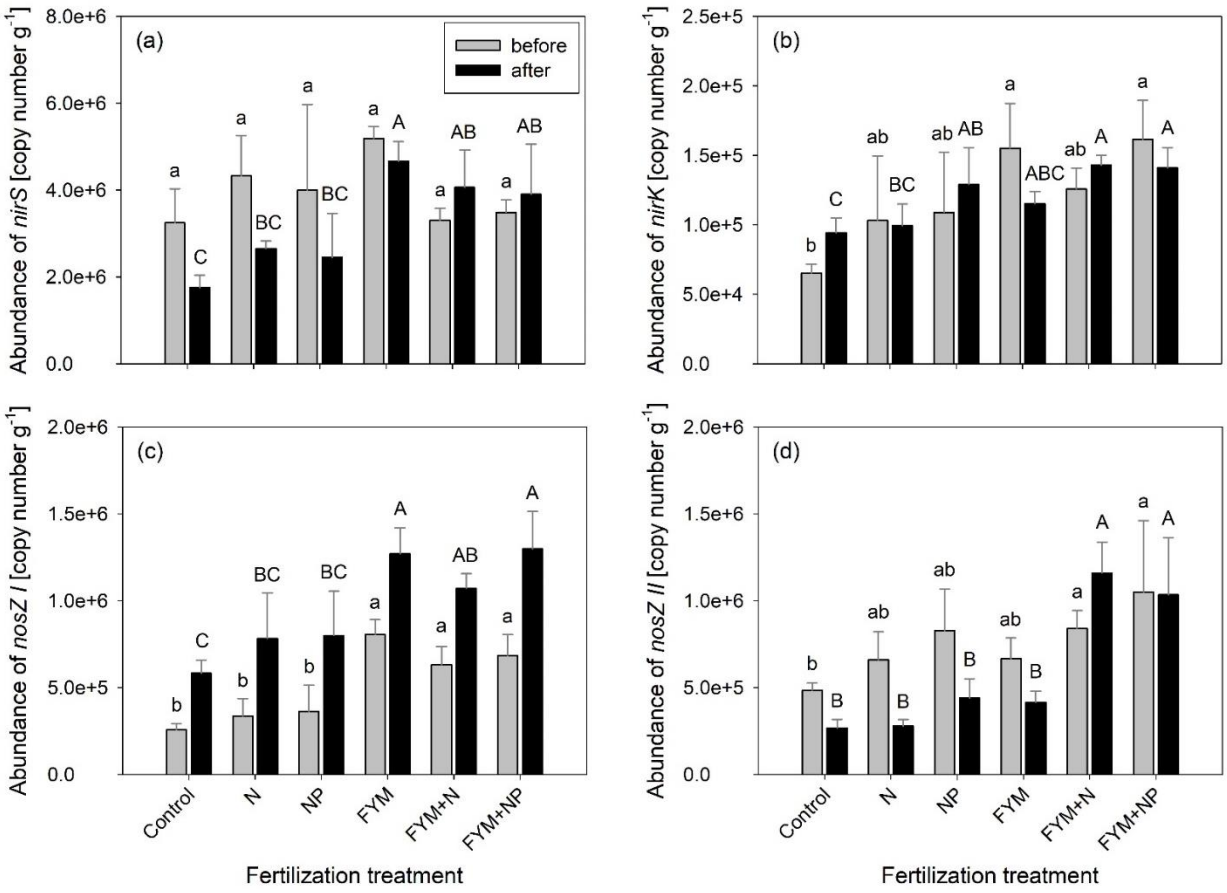

Figure 2.6 Abundance of (a) *nirS*, (b) *nirK*, (c) *nosZ I*, and (d) *nosZ II* genes before (aerobically pre-incubated) and after anoxic incubation under nitrate excess for seven days at 25 °C. Different letters indicate significant differences (p <0.05) between different fertilization treatments (control, mineral N and NP, farmyard manure – FYM, and FYM + mineral N or NP) before (small letters) and after (capital letters) anoxic incubation. Error bars show standard deviation of mean values (n = 4) in one direction only.

2.5 Discussion

In accordance with our initial assumption, we found that different fertilization regimes affected not only chemical soil properties and total OC contents but also amounts of readily decomposable OM, as indicated by higher proportions of water-extractable and particulate OM as well as O/N-alkyl C components. In the following we relate these changes to the denitrification potential, gas product ratios, and gene abundances.

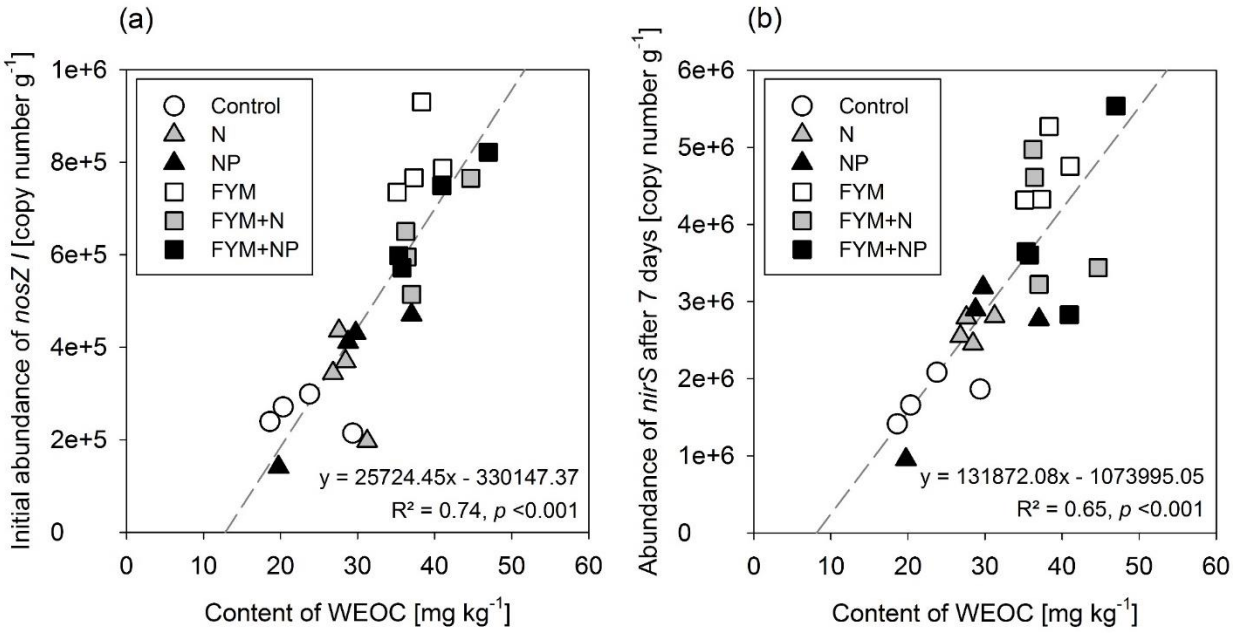

Figure 2.7 Relationship between (a) the water-extractable OC (WEOC) content and the initial abundance of *nosZ I* genes and (b) abundance of *nirS* genes after seven days of anoxic incubation for soils from the Static Fertilization Experiment (control, mineral N and NP, farmyard manure – FYM, and FYM + mineral N or NP).

2.5.1 Potential denitrification and product ratios as related to OM functional fractions

Denitrification potential and product ratio are both controlled by soil reaction (e.g., Bremner and Shaw, 1958; Saggar et al., 2013). Lower pH values tend to cause decreased denitrification rates but larger molar $N_2O/(N_2O+N_2)$ product ratios (Čuhel et al., 2010). However, except for NP, the soil acidity (pH) varied only slightly between fertilization treatments (Table 2.1). Accordingly, we observed no distinct relations between soil reaction and CO_2, N_2O, and N_2 emissions. In contrast to soil reaction, the fertilizer-induced variation in OM appeared as major control on denitrification. Despite POM with adhering microorganisms being considered to have substantial effects on soil denitrification (Parkin, 1987; Parry et al., 2000), we observed no direct relations between POM-C and the N_2O and N_2 production. Gaillard et al. (2003) showed that soluble OC from POM enters the adjacent soil (several mm) and fuels microbial processes. Consequently, POM-derived WEOC could be a major factor in denitrification. In accordance, we found a positive relationship between the content of WEOC and the share and quality (OC/TN ratio) of fPOM as well as between the WEOC content and the production of N_2O and N_2 (Figure 2.5b). These findings suggest that POM facilitates denitrification rather due to its large fraction of leachable C than because of being an easily accessible C source.

The positive correlation between WEOC contents and the contribution of the ratio of fPOM-C to bulk soil OC suggests that the main part of soluble OC derived from fPOM, despite MOM-C the main portion of bulk soil OC (91–97%). The observed negative correlation between WEOC and the OC/TN ratio of fPOM is consistent with the fact that plant residues with low C/N ratio decompose more rapidly than residues with higher C/N ratio (Aulakh et al., 1991; Lynch et al., 2016), and thus, releasing more soluble OC and causing higher CO_2 and N_2O emissions (Huang et al. 2004). Gaillard et al. (2003) showed that water-soluble OC in residues (young rye leaves) can comprise up to 23% of the total residue-C. Thus, plant residues are a major source of leachable OC in surface soils (McCarty and Bremner, 1993). The low and strikingly invariable WEOC/WEON ratios (4.3–5.5) suggest that those leachable components contained a large proportion of proteinaceous material, possibly originating from POM-associated microbial biomass. The C/N ratio of microbial biomass usually varies in the range of 6 to 9 (e.g., Cleveland and Liptzin, 2007).

Our results suggest, therefore, that the amount of fPOM and the related production of water-soluble OM determine the denitrification potential. This is in line with the notion that dissolved OM is the most important substrate and electron donor in denitrification reactions (Bremner and Shaw, 1958; Ottow, 2011). When pooling all fertilization treatments, the anoxic OC mineralization (CO_2 production) was well related to the N_2O production (Figure 2.5a). This again supports the idea that denitrification is fueled by soluble OM and that a high bioavailability of C sources promotes incomplete denitrification (high N_2O/N_2 product ratio) in situations where oxygen is absent and nitrate not limited ('hot spots').

We also assume that the declining bioavailability of WEOC over the incubation time caused the decrease or leveling off in cumulative N_2O over the course of the incubation (Figure 2.2). Since manured soils contained more labile OM than mineral fertilized and unfertilized soils, readily available C was longer available to denitrifying organisms, resulting in larger overall gas emissions and continued gross N_2O production over the entire incubation period. In contrast, N_2O was no longer produced in N-fertilized soils and the control after only a few days, along with the complete depletion of previously accumulated N_2O at the end of incubation. The decreasing molar $N_2O/(N_2O+N_2)$ ratio over time was mainly due to continuous production of N_2, while the gross N_2O formation remained at low level and previously accumulated N_2O was gradually reduced to N_2. Although the average N_2 production after seven days was significantly lower for all non-

manured samples than for the FYM-treatments, the differences in N_2 emission between fertilization regimes were small (Figure 2.4). This indicates that the N_2O production was more strongly affected by the OM bioavailability than the N_2 emission or the total denitrification rate. The loss of 22–40% of the initial (natural + added) NO_3^--N as N_2O or N_2 during incubation and the CO_2-C production representing 23–82% of the initial WEOC may indicate that the microbial use efficiency of C and nitrate differed between fertilization regimes. Under exclusion of plant effects and related N limitation, the total emission of nitrogenous gases (N_2O+N_2) was, therefore, significantly higher in soils with FYM application than in soils with mineral and no fertilization (Table 2.3). As hypothesized, the accumulation of readily decomposable OM in manured soils, reflected by higher portions of components rich in O/N-alkyl C, fPOM, and WEOC, resulted in increased denitrification potential with increased proportions of N_2O. Our results show that not only the potential denitrification rate in total but also the product ratio was strongly affected by the content of WEOC that mostly derived from easily degradable POM sources.

2.5.2 Responses of denitrifier gene abundances to fertilization-induced changes in soil organic matter

Manured soils had significantly higher abundances of *nosZ I* genes than other soils, even before anoxic incubation (Figure 2.6c). This observation may be ascribed to the fact that the higher C availability in manured soils results in faster C mineralization and O_2 consumption, and thus, – over long periods of time – supports larger abundances of complete denitrifiers, especially within anoxic microsites ('hot spots') where nitrate becomes limited. This could also explain why the initial gene abundances of *nosZ I* were related to initial WEOC contents (Figure 2.7a). During anoxic incubation, *nosZ I* gene abundances increased for all treatments, especially in those under mineral fertilization, whereas abundances of *nosZ II* genes either decreased or remained roughly constant (Figures 2.6c, d). One explanation for this observation could be that N_2O reduction kinetics of organisms having *nosZ II* genes differ from those with *nosZ I* genes. For example, Conthe et al. (2018) reported that non-denitrifying N_2O reducers with *nosZ II* genes have a lower affinity for N_2O than canonical denitrifiers (bacteria with *nosZ I*) under N_2O- and C-limiting conditions. In addition, most organisms with *nosZ I* genes also possess *nirS* or *nirK* genes, thus, are able to reduce NO_2^- (Graf et al., 2014), while organisms with

nosZ II often respire N_2O alone. This might explain why the initial N_2O emissions were only related to *nosZ I* and not to *nosZ II* gene abundances.

Changes in *nirK* gene abundances during incubation were only small and irregular across treatments (Figure 2.6b). Considering the low gene copy numbers, *nirK* might play a minor role in N_2O production compared to *nirS*. The abundances of *nirS* genes decreased within seven incubation days only in soils without FYM application (Figure 2.6a). Since not only the abundances of *nosZ I* but also of *nirS* genes after incubation were positively related to initial WEOC contents, this was probably due to limitation of suitable C substrates. This could also explain why the linear relationship between *nirS* gene abundance after incubation and WEOC contents was closer for non-manured soils than for manured soils (Figure 2.7b). Henderson et al. (2010) found higher *nosZ I* gene abundances in soils (coarse loamy till; pH 6) amended with different POM materials than in soil amended with glucose, while the abundance of $nirS_p$ gene-bearing denitrifiers (*P. mandelii* and related species) was only increased by glucose addition. This is in line with our assumption that NO_2^--reducing organisms (having *nirS* genes) were more dependent on readily available C substrates than N_2O-reducing organisms with *nosZ I* genes. Accordingly, large abundances of *nirS* genes occurred in manured soils even at the end of the incubation (Figure 2.6a). This suggests that the availability of WEOC not only determined the amount of denitrified N but also the $N_2O/(N_2O+N_2)$ product ratio. Correspondingly, gross N_2O production still continued after seven days, resulting in higher molar $N_2O/(N_2O+N_2)$ ratios for manured soils (0.2–0.3) than for soils under mineral fertilization (0.01–0.1; Table 2.3). Based on our results, we assume that small amounts of bioavailable OM favored complete denitrifiers (i.e., N_2O-reducing organisms) with *nosZ I* genes when NO_3^- was not limiting. Those complete denitrifiers are generally in advantageous position, since the maximum energy production of the complete reduction to N_2 is 10% higher than for the incomplete denitrification (release of N_2O) (Ottow, 2011). Consequently, N_2 was the dominant product in mineral or unfertilized soils, even at the beginning of the incubation (Table 2.3). In turn, larger amounts of WEOC favored increased denitrification with larger shares of N_2O. The fact that abundances of *nosZ I* and *nirS* genes after incubation as well as the production of N_2O+N_2 increased upon FYM application and both, gene abundance and denitrification potential, were well related to the content of WEOC underpins the relevance of WEOC for denitrification in agricultural soils.

2.6 Conclusions

As hypothesized, fertilization treatments causing stronger accumulation of labile OM resulted in increased denitrification with larger proportions of N_2O, while treatments causing smaller portions of readily decomposable OM favored complete denitrifying organisms. Therefore, this study highlights the close link between soil OM and denitrification potential, with larger portions of labile C substrates promoting denitrification reactions. In particular, we found that water-soluble OC readily available to denitrifiers shapes their community composition on a short-term, and thus, determines the overall denitrification and the $N_2O/(N_2O+N_2)$ product ratio in situations where oxygen is absent and nitrate not limited ('hot spots'). Despite soil OC was mainly present in the MOM fraction, water-soluble OC itself appears to largely derive from fPOM (i.e., undecomposed organic debris, especially enriched in manured plots); its source strength for water-soluble OC seemingly increases with decreasing C/N ratio of the fPOM. Consistent with our hypotheses, readily decomposable OM, especially water-soluble OC, seems to be a general and easily measurable indicator of a soil's immediate denitrification potential. The additional determination and characterization of fPOM might offer a possible estimate for the production potential of water-soluble OC, and consequently, for the denitrification potential along longer time scales. The observed control of water-soluble OC on the potential denitrification also prompts investigating the possible effects of other, similar easily decomposable organic substrates, such as root exudates.

2.7 Acknowledgments

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3 Potential denitrification stimulated by water-soluble organic carbon from plant residues during initial decomposition

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Author contributions:
RS, JB, and RM conceived the experiment. RS and CMS conducted the experiments. RS, LS, and CWM analyzed the data. RS prepared the first manuscript draft and all authors contributed to its refinement.

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3.1 Abstract

Denitrification usually takes place under anoxic conditions and over short periods of time, and depends on readily available nitrate and carbon sources. Variations in CO_2 and N_2O emissions associated with plant residues have mainly been explained by differences in their decomposability. A factor rarely considered so far is water-extractable organic matter (WEOM) released to the soil during residue decomposition. Here, we examined the potential effect of plant residues on denitrification with special emphasis on WEOM. A range of fresh and leached plant residues was characterized by elemental analyses, ^{13}C-NMR spectroscopy, and extraction with ultrapure water. The obtained solutions were analyzed for the concentrations of organic carbon (OC) and organic nitrogen (ON), and by UV-VIS spectroscopy. To test the potential denitrification induced by plant residues or three different OM solutions, these carbon sources were added to soil suspensions and incubated for 24 hours at 20 °C in the dark under anoxic conditions; KNO_3 was added to ensure unlimited nitrate supply. Evolving N_2O and CO_2 were analyzed by gas chromatography, and acetylene inhibition was used to determine denitrification and its product ratio. The production of all gases, as well as the molar (N_2O+N_2)-N/CO_2-C ratio, was directly related to the water-extractable OC (WEOC) content of the plant residues, and the WEOC increased with carboxylic/carbonyl C and decreasing OC/ON ratio of the plant residues. Incubation of OM solutions revealed that the molar (N_2O+N_2)-N/CO_2-C ratio and share of N_2O are influenced by the WEOM's chemical composition. In conclusion, our results emphasize the potential of WEOM in largely undecomposed plant residues to support short-term denitrification activity in a typical 'hot spot–hot moment' situation.

3.2 Introduction

Denitrification, the microbial reduction of nitrate (NO_3^-) via a series of enzymatic steps to NO_2^-, NO, N_2O, and finally N_2 (Philippot et al., 2007), results in net ecosystem losses of N and the release of climate-relevant N_2O and CO_2. Consequently, understanding the factors controlling denitrification and its N_2O/N_2 product ratio is crucial for developing strategies to minimize emissions of greenhouse gases.

In soil, denitrification mainly occurs in anoxic microhabitats ('hot spots') where sufficient nitrogenous oxides (NO_3^-, NO_2^-) as alternative electron acceptors and organic carbon (OC) as electron donor are bioavailable (e.g., Groffman et al., 2009). Those microsites are mostly associated with particulate organic matter derived from farmyard manure or plant residues (e.g., Parkin, 1987; Parry et al., 2000). When comparing different plant residues, variations in mineralization rates and gas emissions were mostly explained by their chemical properties, such as C/N and lignin/N ratios (Aulakh et al., 1991; Vanlauwe et al., 1996; Chantigny et al., 2002; Lynch et al., 2016). A few studies, however, hinted at the possible role of soluble C compounds in the denitrification of residue-amended soils (e.g., deCatanzaro and Beauchamp, 1985; deCatanzaro et al., 1987). Soluble C is considered most effective in fueling denitrification over a period of a few days (e.g., Bremner and Shaw, 1958; Burford and Bremner, 1975). Despite evidence of large differences in release, chemical composition, and biodegradability of water-extractable organic matter (WEOM) from different litter materials (Kalbitz et al., 2003a; Don and Kalbitz, 2005; Mastný et al., 2018), most previous studies only compared well-defined C compounds such as glucose, glycerol, and organic acids, for their potential to promote emissions of CO_2 and nitrogenous gases (e.g., Valera and Alexander, 1961; Jacobson and Alexander, 1980; Akunna et al., 1993). While WEOM leached from fresh aboveground plant residues is rich in easily degradable, low molecular-weight C compounds, such as simple sugars, amino acids, and proteins, WEOM derived from partly decomposed plant material and roots is enriched in less biodegradable high-molecular-weight and phenolic compounds (e.g., Marschner and Kalbitz, 2003; Clemente et al., 2013). Although leaching and subsequent degradation of soluble compounds have been recognized as initial steps of litter decomposition (Berg and McClaugherty, 2014) that may cause formation of anoxic microsites in soil (Kravchenko et al., 2017), possible effects of WEOM from different plant residues on potential denitrification have not been evaluated so far.

We hypothesized that plant residues of different composition and particle size provide differing amounts of water-extractable OC (WEOC) that fuel denitrifying organisms during short-term anoxic conditions. To address this hypothesis, we examined the effects of residues of various agriculturally important plant species (ryegrass, maize, and alfalfa) on potential denitrification and CO_2 production; including plant parts (roots, stems, and leaves) with a wide range in C/N ratios and varying in molecular composition (estimated by ^{13}C nuclear magnetic resonance spectroscopy). In addition, we compared two different particle sizes and three leaching stages of ryegrass residues. Further, we expected that potential denitrification, its product ratio, and CO_2 production are not only controlled by the WEOC content but also by the quality, i.e. chemical composition, of WEOM. This was studied using WEOM solutions of equivalent C concentrations prepared from maize straw and ryegrass leaves, as well as maize root exudates.

3.3 Materials and methods

3.3.1 Test soil

In summer 2016, we collected topsoil (0–20 cm) material of a C-poor arable Haplic Luvisol with a silty loam texture (19% sand, 71% silt, and 10% clay) and a pH ($CaCl_2$) of 6.7 from a long-term trial site at Höhere Landbauschule Rotthalmünster, approximately 150 km east of Munich, Germany (latitude N48°21′, longitude E13°11′, elevation 360 m a.s.l.; Yamashita et al., 2006). The mean annual precipitation and temperature at the site is 890 mm and 8.2 °C, respectively (Ellerbrock and Kaiser, 2005). The soil was air-dried, and then sieved to <2 mm to remove coarse plant debris and stones. The test soil had 13.4 g OC kg^{-1}, 1.5 g organic nitrogen (ON) kg^{-1}, an OC/ON ratio of 9.0, 219.4 mg WEOC kg^{-1}, and 26.8 mg water-extractable ON (WEON) kg^{-1}, as well as 18.5 mg NO_3^--N kg^{-1} and 2.0 mg NH_4^+-N kg^{-1} (extracted with 0.1 M KCl). To reactivate the microbial community of the soil, subsamples were rewetted to 50% water holding capacity and aerobically pre-incubated for two weeks at 20 °C in the dark before anoxic incubation.

3.3.2 Plant residues

In 2016, ryegrass plants (*Lolium perenne* L.), i.e., stems+leaves and roots, as well as maize straw (*Zea mays* L.) were collected from experimental fields (Reinshof and Relliehausen,

respectively) of the University of Göttingen, Germany; alfalfa (*Medicago sativa* L.) stems and leaves were collected near the field trial Ewiger Roggenbau in Halle (Saale), Germany. Ryegrass roots were washed with ultrapure water to remove adherent soil before air-drying. Maize roots were obtained from plants grown for root exudate sampling (see below). Pre-leached, double, and triple-leached rye grass residues were produced by multiple extractions with water (see below), followed by air-drying. All plant residues were ground to <1 mm using an impact mill (Rekord A, Gebr. Jehmlich GmbH, Nossen, Germany). A separate portion of non-leached ryegrass (stems+leaves) was additionally ground to <3 mm (in the following referred to as coarse ryegrass), to determine the possible effect of particle size.

Solid plant residue samples were analyzed for total C (assuming total C = OC) and total nitrogen (TN) using a Vario Max Cube (Elementar Analysensysteme GmbH, Langenselbold, Germany). In addition, the residues' contents of WEOC and water-extractable total N (WETN) were determined. Briefly, 50 mg of ground plant residues were suspended in 25 ml of ultrapure water and shaken horizontally for one hour. The supernatants were passed through 0.45-µm membrane filters (Supor®-450, Pall Cooperation, New York, NY, USA). The extracts were analyzed for OC and TN, using a multi N/C® 3100 analyzer (Analytik Jena AG, Jena, Germany). Concentrations of N_{min} (NO_3^- and NH_4^+) were determined using a Continuous Flow Analyzer (ScanPlus, Skalar Analytical B.V., Breda, The Netherlands). WEON (or ON) was calculated by: (WE)ON = (WE)TN – N_{min}. All reported (WE)OC and WE(ON) contents refer to dry mass determined at 105 °C. The specific UV absorbance at 280 nm, as an estimate of the aromaticity of WEOM (Chin et al., 1994), was determined using a photometer (SPECORD® 210 PLUS, Analytik Jena AG).

3.3.3 Root exudates and water-extractable organic matter

Maize plants (*Zea mays* L. var. Ronaldinio) were grown in nutrient solution (Mengutay et al., 2013) for 5–6 weeks in a greenhouse. To sample root exudates, the dipping method described by Neumann and Römheld (2007) was used. Maize roots were soaked in deionized water for 3 × 5 minutes to remove nutrient solution from the root surfaces. Then, roots were submerged in the trap solution (aerated distilled water) for two hours. The exudate solution was filtered through a 4-µm-filter paper (MN615 1/4 Ø 90 mm, Macherey-Nagel GmbH, Düren, Germany), flash frozen in liquid nitrogen, freeze-dried,

and stored at −20 °C prior to the experiments. For analysis and incubation, freeze-dried root exudates were re-dissolved in ultrapure water in a sonication bath for 15 minutes.

To obtain sufficiently large and reproducible amounts of plant-derived WEOM for incubation experiments, either 50 g ryegrass (stems+leaves; cut to <10 cm) or 25 g maize straw (cut to <10 cm) were soaked in 500 ml ultrapure water for 18 hours at ~20 °C in the dark. The parent residue materials were removed by passing the suspensions first through 0.7-µm-glass microfiber filters (13440-130------K, Sartorius Stedim Biotech GmbH, Göttingen, Germany) using a pressure filter holder with barrel (16274, Sartorius Stedim Biotech GmbH), and then, through 0.45-µm membrane filters (Supor®-450). The resulting filtrates, containing the WEOM, were used in the incubation experiments.

Re-dissolved root exudates and WEOM from ryegrass and maize straw were analyzed for pH (SenTix® 41, WTW, Xylem Analytics Germany Sales GmbH & Co. KG, Weilheim, Germany), OC, ON, and specific UV absorbance at 280 nm as detailed above.

3.3.4 Solid-state ^{13}C-NMR spectroscopy

Plant residues were ground with a vibratory disc mill (RS 100, Retsch, Haan, Germany) before analysis by solid-state ^{13}C cross-polarization magic angle spinning NMR spectroscopy (^{13}C-CPMAS NMR spectroscopy) with an Avance III 200 spectrometer (Bruker BioSpin GmbH, Karlsruhe, Germany). Samples were placed into a 7-mm zirconia rotor that was spun at 6.8 kHz around a 'magic angle' of 54.74°. Contact time was 1 ms and the recycle delay time was set to 0.4 s. The spectra were processed with 100 Hz line broadening, phase adjusted, and baseline corrected; no spinning side bands appeared in the spectra. Peaks were assigned to four integration areas: −10–45 ppm (alkyl C), 45–110 ppm (O/N-alkyl C), 110–160 ppm (aromatic C), and 160–220 ppm (carboxylic/carbonyl C); spectra are shown in the Appendix 4.

3.3.5 Incubation and gas measurements

Anoxic incubations were carried out at 20 °C in the dark for 24 hours, to assess potential short-term effects in a typical 'hot spot–hot moment' situation (McClain et al., 2003). Homogeneous soil suspensions were prepared by filling 10 g (dry mass) of pre-incubated soil material into 250-ml glass infusion bottles and mixing with amounts of different residues equivalent to 2 g C kg^{-1} dry soil, and then adding 50 ml of KNO_3 solution (50 mg

NO_3^--N kg^{-1} dry soil; soil/water ratio = 1/5 w/v), to avoid NO_3^- limitation during at least the first eight hours of incubation. As indicated by the WEON/total N ratios of plant residues (average 34%), a significant portion of ON is structurally bound (e.g., as proteins) and would require depolymerization within the experimental period to yield NH_4^+. Since the oxidation of NH_4^+ to NO_3^- is unlikely under anoxic conditions and the input amount of residue-NH_4^+ is negligible (only for alfalfa residues; <1.0 g kg^{-1} dry matter), no significant effect of the initially present NH_4^+ on determined gas productions was expected. In soil incubations with WEOM and root exudates, KNO_3 was added with the 50 ml of respective solutions. Again, the C addition amounted to 2 g C kg^{-1} dry soil.

All incubations were carried out in triplicate, with the bottles sealed with a bromine-butyl-rubber stopper and crimped with an aluminum cap (32 mm; Chroma Globe GbR, Kreuznau, Germany). An O_2-free atmosphere was obtained by evacuating (<250 mbar), and then flushing the bottles including the suspensions with He gas (99.999%, Air Liquide, Düsseldorf, Germany) three times; the final pressure was about 1025 mbar. Soil suspensions were homogenized by horizontal shaking during incubation. After one hour of incubation, the first (t_0) gas sample (18 ml) was taken with a gastight syringe (25 ml, 25MDR-LL-GT; SGE Analytical Science Pty. Ltd., Ringwood, VIC, Australia), equipped with a push button valve (Luer Lock; SGE Analytical Science) and a 0.7-mm ID cannula (Sterican G26, 25 mm; B. Braun AG, Melsungen, Germany), and transferred to pre-evacuated (90 mbar residual pressure, rinsed with He) 12-ml Exetainer® vials, which were sealed with a double septum cap (IVAVC329; IVA Analysentechnik e.k., Meerbusch, Germany). This resulted in an overpressure of >200 mbar in the Exetainer® vials, which was necessary to avoid contamination with air during storage and for measuring gas concentrations with the gas chromatography system described below. To avoid low pressure in the incubation bottles, 18 ml He at ~1 bar were injected after gas sampling, resulting in constant absolute pressure of ~1025 mbar during incubation. The absolute pressure in the bottles was measured before and after gas sampling as well as after He injection, using a GMSD 2 BA-K31-L01 pressure sensor coupled with a Greisinger GMH 3151 reader (GSG Geologie-Service GmbH, Würzburg, Germany). Gas samples were taken after 0, 2, 4, 6, 8, and 24 hours.

All gas samples were analyzed on a custom-tailored gas chromatography system by Chromtech (Bad Camberg, Germany), using an Agilent HP 7890B GC as basis. The samples were introduced into the injector by an autosampler (PAL GC-xt; CTC Analytics AG, Zwingen, Switzerland), using an open needle syringe. Calibration was done online, using

standard gas cups connected to bottles with certified calibration gases with known concentrations of CO_2 and N_2O in He (Linde Gas AG, Pullach, Germany). After injecting the sample at a liner temperature of 150 °C, it was transferred to a Shin Carbon chromatographic column (2 m, 0.53 mm ID, Restek GmbH, Bad Homburg, Germany) using He as carrier gas (purity 99.9999%) and directly transferred to a He ionization detector (Vici AG International, Schenkon, Switzerland) run at 180 °C. The GC oven was set to a starting temperature of 60 °C, kept constant for three minutes, increased to 110 °C at a rate of 10 °C min^{-1}, kept for one minute, and finally increased to 220 °C at a rate of 50 °C min^{-1}, kept for three minutes. The limit of detection for both gases was calculated using 10 blank samples that were drawn and measured in the same way as all other samples. The limit of detection was calculated as 0.1 ppb and 11.76 ppm for N_2O and CO_2, respectively. Precision and accuracy were analyzed injecting a reference standard every ten samples. On average, precision was 4.8 and 0.5% relative standard error for N_2O and CO_2, respectively, while accuracy was 3.3 and 1.5% offset from the specified concentration for N_2O and CO_2, respectively.

Cumulative emissions of gases represent the sum of produced amounts, i.e., the detected gas mass in the headspace plus estimated gas mass in the suspension at time point t_x minus the total gas mass at time point t_{x-1}, considering the removed gas amount during each gas sampling. Gas masses in the suspension were calculated by using the respective Henry's law constant, volume of solution, headspace pressure and gas concentration; for different CO_2 species the pH was also taken into account. For the acetylene inhibition technique (Yoshinari and Knowles, 1976), a second line of incubations as described above was carried out with additional injection of 30 ml of C_2H_2 (99.6%; Air Liquide) in exchange for 30 ml He, resulting in an initial C_2H_2 concentration of ~10% (v/v). An C_2H_2 concentration of >5% (v/v) was expected to be maintained during the 24 hours of incubation (Yeomans and Beauchamp, 1978; Terry and Duxbury, 1985). Therefore, the amount of N_2O in presence of C_2H_2 was assumed to represent total denitrification (N_2O+N_2). Consequently, the difference between the N_2O amounts of incubations with and without C_2H_2 addition was an estimate of the N_2 production, while the ratio of the two N_2O species was used to determine the molar N_2O-N/(N_2O+N_2)-N ratio. Cumulative amounts of N_2O in presence of C_2H_2 and CO_2 in incubations without C_2H_2 were used to determine the molar (N_2O+N_2)-N/CO_2-C ratio, which indicates the contribution of denitrification to total CO_2 production. Based on the stoichiometry of complete and incomplete denitrification (Ottow, 2011), ratios between 0.8 and 1.0

indicate that the CO_2 production is exclusively linked to denitrification reactions. Main potential problems in application of the acetylene inhibition technique, i.e., underestimated N_2 production due to acetylene inhibition of nitrification and incomplete inhibition of N_2O reduction caused by low NO_3^- concentrations or acetylene diffusion effects (Knowles, 1990; Almaraz et al., 2020), can be excluded for our incubation approach (anoxic, excess NO_3^-, soil suspension). Alleviation of acetylene inhibition by sulfide compounds potentially released from plant materials is unlikely, given that the reported effect typically shows a delay of 2–3 days (Yeomans and Beauchamp, 1982; Knowles, 1990), which beyond the time frame of our 24-hour experiments.

3.3.6 Statistical evaluation

Basic statistical analyses were performed using Sigma Plot 11.0 (Systat Software Inc., Erkrath, Germany). One-way ANOVA with type of OM addition as independent variable followed by the Tukey HSD test was used for testing for differences in cumulative emissions of CO_2, N_2O, and N_2O+N_2, and respective ratios. Linear regression analyses were used to test for relationships between chemical properties of residues or their water extracts and resulting gas emissions, or respective ratios after confirming the normal distribution of data by the Shapiro-Wilk test. In some cases, regression analyses were additionally carried out under exclusion of ryegrass roots because, unlike the other residues, they were washed to remove adherent soil, while alfalfa residues were partly excluded for being depleted in NO_3^- at the end of incubation.

3.4 Results

3.4.1 Characterization of plant residues, root exudates, and water-extractable organic matter

The OC/ON ratios of plant-derived residue types ranged from 9.0 (alfalfa leaves) to 83.7 (ryegrass roots). Multiple leaching of ryegrass with ultrapure water resulted in an increasing OC/ON ratio (Table 3.1). Triple-leached ryegrass was enriched in O/N-alkyl C and aryl C but proportions of alkyl C and carboxylic/carbonyl C were reduced (Table 3.2). Root residues contained relatively less (O/N-)alkyl C than respective aboveground residues while proportions of aryl C and carboxylic C were higher. The WEOC contents of not pre-leached residues varied between 21.3 g kg^{-1} (maize straw) and 126.9 g kg^{-1}

(alfalfa leaves) (Table 3.1). Triple-leaching of ryegrass caused a decrease in WEOC and WEOC/WEON ratio by 90% and 64%, respectively, while the specific UV absorbance (at 280 nm) of the respective WEOM increased by 77%. The WEOC content of the residues was significantly related to the residues' OC/ON ratio ($r = -0.67$, $p < 0.05$, $n = 10$; and $r = -0.89$, $p < 0.001$, $n = 9$, when ryegrass roots were excluded; Figure 3.1a), the residues' proportion of carboxylic/carbonyl C determined by ^{13}C-NMR spectroscopy ($r = 0.94$, $p < 0.01$, $n = 7$; Figure 3.1b), and specific UV absorbance (at 280 nm) of respective WEOM solutions ($r = -0.56$, $p < 0.001$, $n = 10$; and $r = -0.97$, $p < 0.001$, $n = 8$, when ryegrass roots and alfalfa leaves were excluded).

Root exudates and WEOM solutions used for incubation experiments differed in their chemical composition (Table 3.3). Maize straw-derived WEOM and root exudates had lower OC/ON ratios but higher specific UV absorbances (at 280 nm) than ryegrass WEOM.

Table 3.1 Contents of total (OC and ON) and water-extractable organic C (WEOC) and N (WEON) as well as the OC/ON and WEOC/WEON ratios of residue types. The specific UV absorbance at 280 nm ($SUVA_{280}$) was measured on WEOM solutions.

Residue type	OC [g kg^{-1}]	ON [g kg^{-1}]	OC/ON ratio	WEOC [g kg^{-1}]	WEON [g kg^{-1}]	WEOC/ WEON	$SUVA_{280}$ [l mg^{-1} C cm^{-1}]
Ryegrass	400.8 d[a]	11.7 cd	34.3 e	52.6 c	4.7 d	11.3 b	0.013 e
Coarse ryegrass	400.8 d	11.7 cd	34.3 e	46.3 d	4.3 d	10.7 b	0.012 e
Pre-leached ryegrass	405.5 cd	10.9 d	37.2 de	17.7 f	1.8 ef	9.9 bc	0.017 c
Double-leached ryegrass	413.3 ab	10.7 d	38.6 d	10.4 g	1.6 f	6.5 d	0.020 b
Triple-leached ryegrass	418.9 a	9.3 de	45.0 c	5.2 h	1.3 f	4.1 e	0.023 a
Ryegrass roots	411.3 bc	4.9 f	83.7 a	28.6 e	0.9 f	30.5 a	0.006 g
Maize straw	400.7 d	7.3 ef	54.9 b	21.3 f	2.5 e	8.6 c	0.016 d
Maize roots	409.9 bc	14.1 c	29.1 f	56.1 c	6.4 c	8.8 c	0.009 f
Alfalfa stems	390.8 e	19.8 b	19.7 g	72.2 b	13.9 b	5.2 de	0.005 g
Alfalfa leaves	409.2 bc	45.6 a	9.0 h	126.9 a	21.0 a	6.0 d	0.011 e

[a] Values followed by the same letters within a column are not significantly different ($p < 0.05$) based on a one-way ANOVA test followed by the Tukey HSD test. Values represent means ($n = 3$).

Table 3.2 Distribution of C species in different plant residue types. Values represent the percentage contribution of the different integrated chemical shift regions determined by ^{13}C-CPMAS NMR spectroscopy.

Residue type	Alkyl C [%]	O/N-alkyl C [%]	Aryl C [%]	Carboxylic/ carbonyl C [%]
Ryegrass	12	79	6	3
Triple-leached Ryegrass	7	85	7	1
Ryegrass roots	3	78	14	5
Maize straw	6	80	11	3
Maize roots	4	78	12	6
Alfalfa stems	10	72	9	9
Alfalfa leaves	21	44	18	17

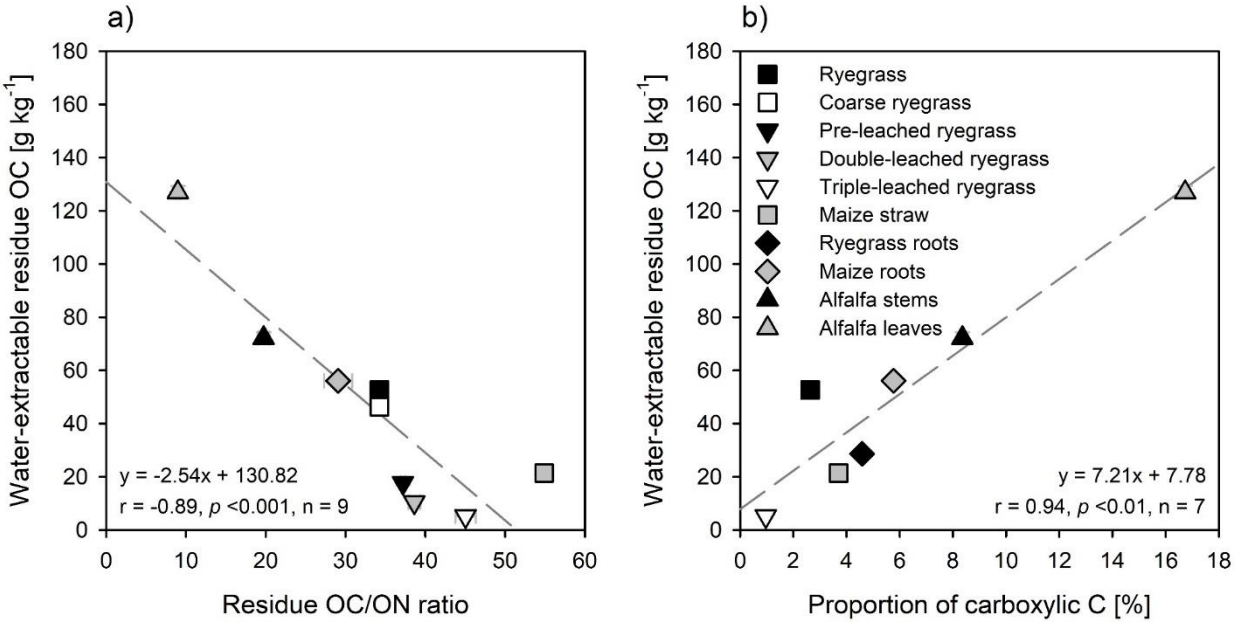

Figure 3.1 Relationship between the content of water-extractable OC and (a) the OC/ON ratio of different plant residue types (except for ryegrass roots) as well as (b) the proportion of carboxylic C in residues determined by ^{13}C-NMR spectroscopy. Error bars represent standard deviation of means (n = 3).

Table 3.3 Chemical properties and composition of plant residue-derived water-extractable organic matter (WEOM) solutions and the maize root exudate solution. Values represent the pH, organic nitrogen (ON) concentration, the ratio of organic C (OC) to ON of solutions with an OC concentration of 400 mg l^{-1} used for the incubation experiments. $SUVA_{280}$ refers to the specific UV absorbance at 280 nm.

WEOM type	pH	ON [mg l^{-1}]	OC/ON ratio	$SUVA_{280}$ [l mg^{-1} C cm^{-1}]
Ryegrass WEOM	6.1	30.5	13.1	0.007
Maize straw WEOM	7.3	36.5	10.9	0.013
Maize root exudates	9.4	39.9	10.0	0.014

3.1.1 Effect of residues on potential denitrification and CO_2 production

The addition of different residues to the soil resulted in a wide range of cumulative N_2O+N_2, N_2O, and CO_2 production rates (Figures 3.2a–c). Soil amended with alfalfa stems or leaves reached the highest cumulative amounts of N_2O+N_2 and CO_2 over the entire incubation period (Figures 3.2a, c). Differences between maize roots and alfalfa residues were small by the end of incubation (82.2–92.8 mg N kg^{-1} and 117.3–142.1 mg C kg^{-1}). Lowest cumulative production of N_2O+N_2 and CO_2 after 24 hours was determined for multiple leached ryegrass (about 5 mg N kg^{-1} and 20 mg C kg^{-1}, respectively). Pre-leaching caused substantial decreases in the potential denitrification (84%) and CO_2 production (58%) after 24 hours; double- and triple-leaching reduced the release of N_2O+N_2 by 86 and 95%, respectively, and the CO_2 production by about 73% compared to not-leached ryegrass. Cumulative amounts of N_2O+N_2 after 24 hours were closely related to WEOC contents of residues ($r = 0.98$, respectively, $p < 0.001$, $n = 9$, when excluding alfalfa leaves; Figure 3.3a) and cumulative amounts of CO_2 ($r = 0.99$, $p < 0.001$, $n = 10$; Figure 3.3b). Multiple leaching of ryegrass also resulted in decreasing molar (N_2O+N_2)-N/CO_2-C ratios, which were significantly lower than those of the control (Table 3.4). The molar (N_2O+N_2)-N/CO_2-C ratios after 24 hours were positively related to contents of WEOC for all residues ($r = 0.94$, $p < 0.001$, $n = 8$, when excluding alfalfa residues).

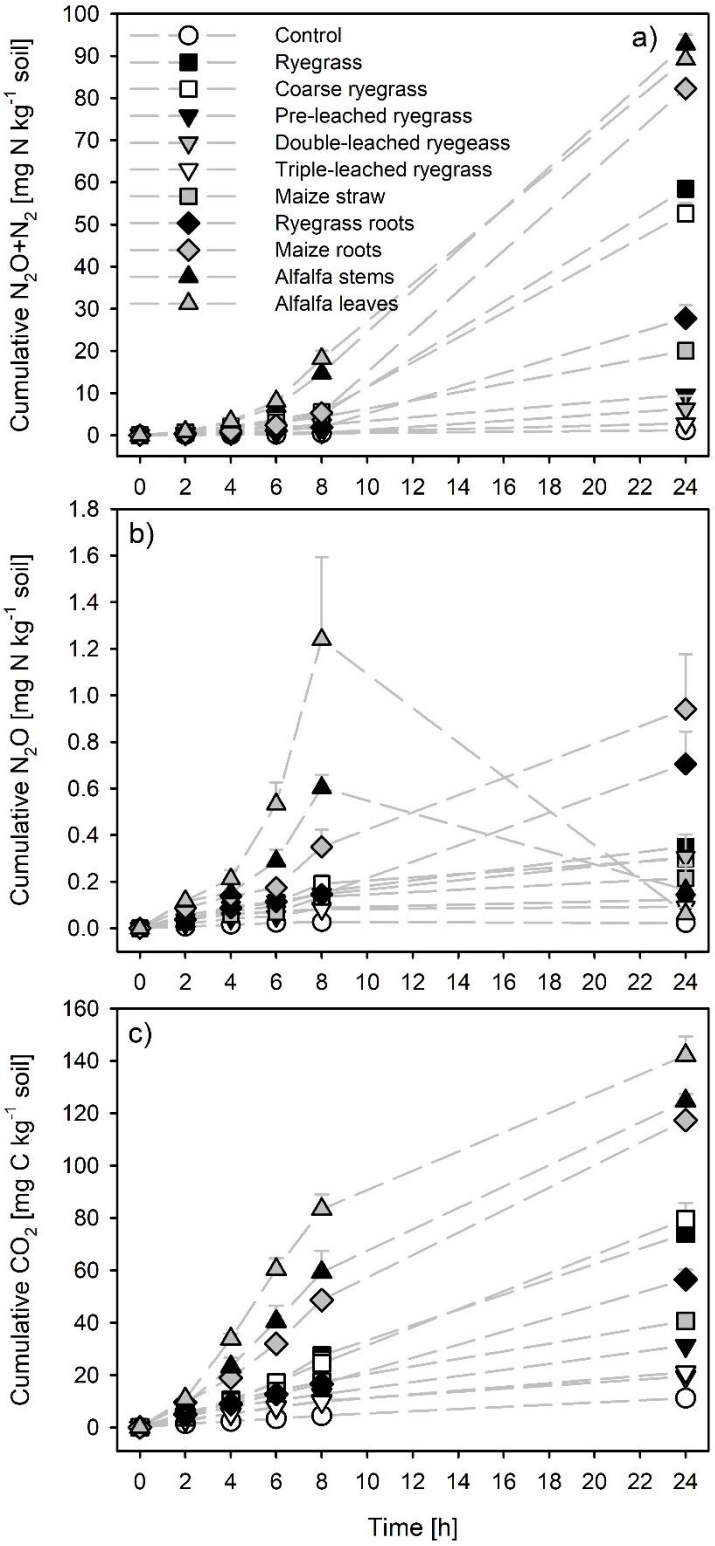

Figure 3.2 Cumulative (a) N_2O+N_2, (b) N_2O, and (c) CO_2 production during anoxic incubation at 20 °C of soil without (control) and with addition of different plant residues. Error bars represent standard deviation of means (n = 3). All incubations received initial KNO_3 additions of 50 mg NO_3^--N^{-1} kg dry soil. Total denitrification (N_2O+N_2) was determined by using the acetylene inhibition technique.

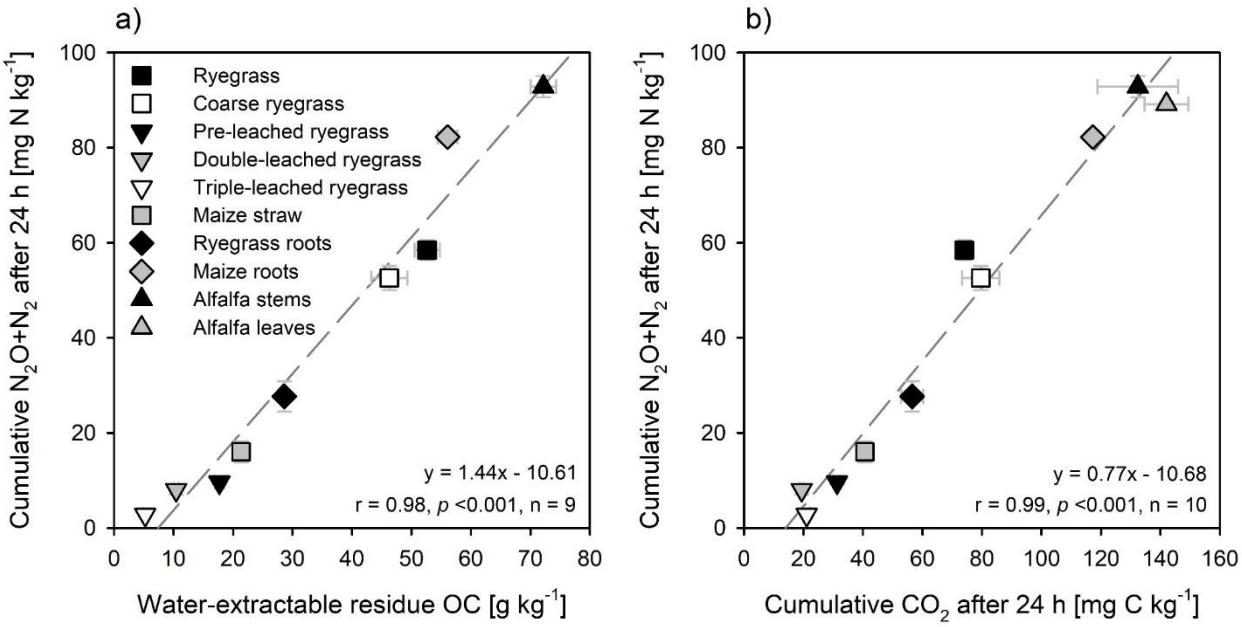

Figure 3.3 Relationship between (a) water-extractable residue OC and cumulative N_2O+N_2 production after 24 hours of anoxic incubation (except for alfalfa leaves because of NO_3^- depletion) as well as (b) between cumulative amounts of CO_2 and N_2O+N_2 at the end of incubation. Error bars represent standard deviation of means (n = 3).

The cumulative N_2O production during the first eight hours (no NO_3^- limitation) was highest for maize roots and especially alfalfa residues (0.4–1.2 mg N kg^{-1}) (Figure 3.2b). However, the soil suspensions with alfalfa were depleted in NO_3^- after 24 hours, and therefore, N_2O decreased significantly. Application of ryegrass roots and especially maize roots resulted in increased denitrification rates after about eight hours, and thus, relatively high cumulative N_2O production (0.7–0.9 mg N kg^{-1}) by the end of incubation. Cumulative amounts of N_2O after eight hours were also most closely related to residue WEOC (r = 0.93, p <0.001, n = 10). The molar $N_2O/(N_2O+N_2)$ ratio was generally low after two hours (0.16 on average) and decreased strongly over the entire incubation period for all residues (Table 3.4).

The particle size of ryegrass had only little effect on cumulative gas emissions (Figures 3.2a–c) and respective ratios (Table 3.4).

Table 3.4 Molar $N_2O/(N_2O+N_2)$ ratios and (N_2O+N_2)-N/CO_2-C ratios after 2, 8, and 24 hours of anoxic incubation at 20 °C of soil without (control) and with addition of different plant residues, WEOM solutions, and root exudates. All incubations received initial KNO_3 additions of 50 mg NO_3^--N^{-1} kg dry soil.

Organic matter type	$N_2O/(N_2O+N_2)$ ratio			(N_2O+N_2)-N/CO_2-C ratio		
	2 h	8 h	24 h	2 h	8 h	24 h
Control	0.03 e[a]	0.02 d	0.01 def	0.13 ab	0.22 a	0.26 de
Residue types						
Ryegrass	0.07 de	0.03 d	0.01 def	0.11 abc	0.15 bc	0.68 a
Coarse ryegrass	0.04 e	0.03 d	0.01 ef	0.15 a	0.19 ab	0.57 b
Pre-leached ryegrass	0.04 e	0.04 d	0.01 de	0.10 bcd	0.17 ab	0.26 de
Double-leached ryegrass	0.63 a	0.21 b	0.04 a	0.03 gh	0.06 d	0.35 cd
Triple-leached ryegrass	0.23 bc	0.12 c	0.03 ab	0.05 efgh	0.06 d	0.11 f
Ryegrass roots	0.11 cde	0.08 cd	0.03 c	0.06 efg	0.10 cd	0.42 c
Maize straw	0.06 de	0.03 d	0.01 d	0.10 bcd	0.21 ab	0.34 c
Maize roots	0.19 cd	0.07 cd	0.01 de	0.04 fgh	0.09 cd	0.60 ab
Alfalfa stems	0.12 cde	0.04 d	0.00 f	0.08 cde	0.22 ab	0.60 ab
Alfalfa leaves	0.14 cde	0.07 cd	0.00 f	0.07 def	0.19 ab	0.54 b
OM solutions						
Ryegrass WEOM	0.05 e	0.04 d	0.00 f	0.13 ab	0.19 ab	0.19 ef
Maize straw WEOM	0.07 de	0.04 d	0.00 f	0.04 fgh	0.09 d	0.22 e
Maize root exudates	0.35 b	0.30 a	0.00 f	0.02 h	0.07 d	0.55 b

[a] Values followed by the same letters within a column are not significantly different (p <0.05) based on a one-way ANOVA test followed by the Tukey HSD test. Values are means (n = 3).

3.1.2 Effect of different WEOM and root exudates on potential denitrification and CO_2 production

Differences between WEOM solutions and root exudates in promoting the production of nitrogenous gases and CO_2 were visible but showed no consistent patterns (Figures 3.4a–c). Cumulative amounts of N_2O+N_2 differed only slightly within the first eight hours (Figure 3.4a). After 24 hours, the cumulative N_2O+N_2 production was significantly higher for maize root exudates than for ryegrass and maize straw WEOM. While the CO_2 production for ryegrass WEOM was lowest after eight hours (about 45 mg C kg^{-1} dry soil), it was highest (about 275 mg C kg^{-1} dry soil) after 24 hours among all three WEOM types (Figure 3.4b). The addition of ryegrass WEOM resulted in the highest molar (N_2O+N_2)-N/CO_2-C ratio after eight hours (0.19). The maximum ratio after 24 hours was observed for maize root exudates (0.55), being about 2.7 times higher compared to ryegrass and maize straw WEOM (Table 3.4).

The addition of maize root exudates caused the highest cumulative N_2O emission (2.2 mg N kg^{-1} dry soil) and molar $N_2O/(N_2O+N_2)$ ratio (Table 3.4) during the first eight hours. Not only NO_3^- but also N_2O in the headspace was depleted by the end of incubation for all three OM solutions.

We found no distinct relationships between chemical properties of OM solutions (Table 3.3) and resulting gas emissions (Figures 3.4a–c) or their respective ratios (Table 3.4).

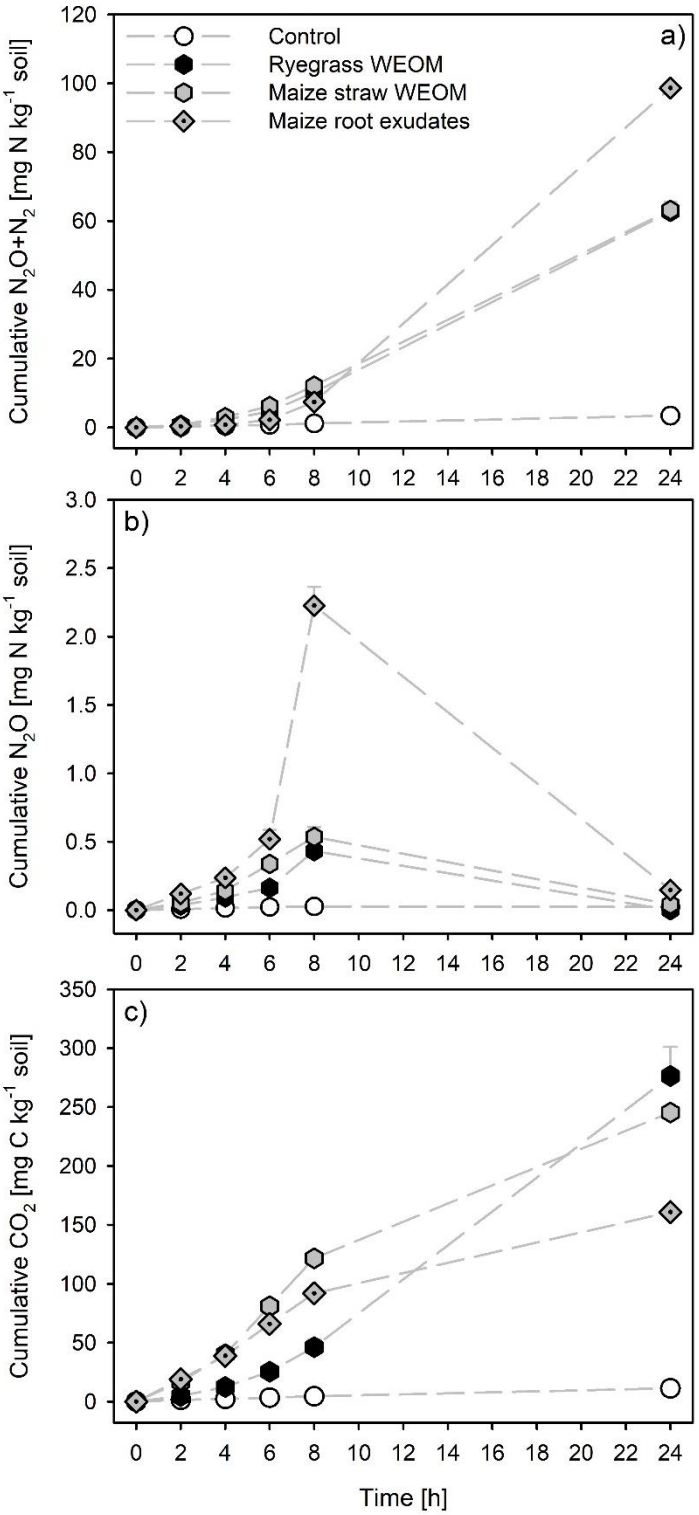

Figure 3.4 Cumulative (a) N_2O+N_2, (b) N_2O, and (c) CO_2 production during anoxic incubation at 20 °C of soil without (control) and with addition of ryegrass- and maize straw-derived water-extractable organic matter (WEOM) and maize root exudates. Error bars represent standard deviation of means (n = 3). All incubations received initial KNO_3 additions of 50 mg NO_3^--N^{-1} kg dry soil. Total denitrification (N_2O+N_2) was determined by using the acetylene inhibition technique.

3.2 Discussion

3.2.1 WEOC content of residues determines potential denitrification and CO_2 production

As hypothesized, and in agreement with previous studies (e.g., Bremner and Shaw, 1958), variations in cumulative gas emissions from soil amended with different plant residues were closely related to residue WEOC (Figure 3.3a). Also, the molar (N_2O+N_2)-N/CO_2-C ratios were related to residue WEOC contents. These findings suggest that during short-term anoxic conditions denitrifying organisms rely on soluble compounds from fresh residues as major C sources. Pre-leaching of ryegrass resulted in a strongly decreased production of CO_2, N_2O, and N_2 (Figures 3.2a–b), which additionally underlines that, at least during short anoxic periods, residue WEOC represents the most important C source for denitrifying organisms. This is consistent with Rummel et al. (2020), who reported close relations between WEOC and N_2O and CO_2 emissions from a repacked topsoil (gleyic Fluvisol) amended with maize root and shoot litter under aerobic conditions. Our findings support their suggestion that litter can stimulate denitrification by creating plant litter-associated anaerobic microsites. Those 'hot spots' have a high potential to accelerate N losses but are only activated under certain circumstances (Bernhardt et al., 2017). Especially in situations where soil moisture and NO_3^- concentrations are high, incorporated plant residues of varying age and freshness could substantially contribute to the emission of nitrogenous gases via denitrification. The WEOC content being a determining factor in plant residue-induced denitrification is well in line with studies showing that soluble low-weight compounds (e.g., organic acids and glycerol >> glucose, methanol) are much more effective in promoting denitrification than insoluble polymers such as cellulose and especially lignin (e.g., Valera and Alexander, 1961; Rashid and Schaefer, 1988; Akunna et al., 1993).

Consistent with Hadas et al. (2004), the WEOC release from plant residues increased with lower OC/ON ratios. Similarly, Reinertsen et al. (1984) found that the initial microbial biomass production and decomposition rate of wheat straw with different OC/ON ratios are linked to the size of the soluble C pool. When testing residue effects on denitrification, Huang et al. (2004) found accumulation of DOC for residues with low OC/ON ratios under conditions with limited N supply, i.e., when no denitrification took place. This underlines the strong control of WEOC on denitrification and the link between residue OC/ON ratios and their WEOC contents. In addition, WEOC contents were

positively related to proportions of carboxylic C (Figure 3.1b), suggesting that acidic compounds are a major source of potentially soluble carbon. Leaching of ryegrass resulted in decreasing proportions of carboxylic C (Table 3.2), which underlines the water solubility of organic acids. Hence, the proportions of carboxylic C as well as the OC/ON ratios of plant residues can be used as proxy for their WEOC contents. The insignificant effect of the residue particle size on WEOC (Table 3.1) and gas emissions (Figures 3.2a–c) apparently contrast some observations of varying decomposition and production rates of climate-relevant gases depending on the residue particle size (Angers and Recous, 1997; Shelp et al., 2000; Ambus et al., 2001). However, such effects may only become relevant for larger differences in the particle size and over time periods longer than those considered in our study.

Overall, the results suggest that plant residues have the potential to directly control denitrification rates and the share of N_2O by releasing soluble OM when environmental conditions promote the formation of 'hot spot–hot moment' situations.

3.2.2 Effect of soluble OM composition on potential denitrification and CO_2 production

We found that residues with low OC/ON ratios produced not only more WEOC, but also that specific UV absorbances at 280 nm were lower. According to Chin et al. (1994), this indicates lower aromaticity of WEOM. Since aromatic compounds are less decomposable than proteins and organic acids (Marschner and Kalbitz, 2003), the quality of WEOM derived from N-rich residues could be also higher. Hence, we assume that at similar concentrations, WEOC released from different residues might cause different productions of CO_2, N_2O, and N_2, depending on the chemical composition of WEOM.

Direct comparison revealed that the addition of maize straw WEOM resulted only in significantly higher CO_2 emissions than ryegrass-derived WEOM during the first eight hours, while differences in the N_2O and N_2 production were very small over the entire incubation period (Figures 3.4a–c). Consequently, the molar (N_2O+N_2)-N/CO_2-C ratio (Table 3.4) was more affected by the WEOM composition than total denitrification. The small molar (N_2O+N_2)-N/CO_2-C ratios, especially during the first hours, indicate that the contribution of denitrification to total CO_2 production was initially minor and that other processes such as fermentation likely dominated.

The addition of maize root exudates resulted in a much higher cumulative production of N_2O+N_2 and also in the highest molar (N_2O+N_2)-N/CO_2-C ratio after 24 hours (Figure 3.4a), with NO_3^- becoming depleted towards the end of incubation for all three OM solutions. Based on mass balance calculations using the acetylene inhibition technique, about 40% of the initial NO_3^- was likely reduced to NH_4^+ in the WEOM incubations. This is supported by Fazzolari et al. (1998), who concluded that the dissimilatory nitrate reduction to ammonium (DNRA) is favored over denitrification at OC/NO_3^--N ratios above 4 (initial ratio of WEOM treatments was ~20). Thus, our findings suggest that NO_3^- distribution between denitrification and DNRA can also be affected by the chemical composition of readily available C compounds and is not only regulated by the total WEOC concentration (Fazzolari et al., 1998). Akunna et al. (1993) demonstrated that the addition of glucose and glycerol to anaerobic sludges resulted in a high share of DNRA and production of volatile fatty acids, particularly acetic acid, while no DNRA was observed for the application of acetic acid and lactic acid. Consequently, differences in effects between WEOM solutions and root exudates can be probably explained by differences in their molecular composition. Correspondingly, higher emissions of N_2O in the incubations with maize root exudates than in those with maize straw and ryegrass WEOM (Figure 3.4b) could be due to smaller shares of sugars, such as glucose, and higher proportions of organic or amino acids (Henry et al., 2008).

In summary, the observed variations in molar (N_2O+N_2)-N/CO_2-C ratios and shares of N_2O with the soluble OM's molecular composition were likely due to anaerobic processes other than denitrification, such as fermentation and DNRA, becoming prominent at high WEOC availability.

3.3 Conclusions

As hypothesized, the potential denitrification of arable topsoils is – at least during the initial phase of residue decomposition – closely related to the release of WEOM from plant residues. The potential to release WEOM is closely linked to the plant residues' chemical composition, which, in turn, reflects the residues' source and degradation stage. Also as hypothesized, the chemical composition of soluble OM affects the extent and product ratio of denitrification, at least in situations where NO_3^- availability is not limited. In summary, the results of the study indicated the potential of WEOM in largely undecomposed plant

residues to support the formation of anoxic microhabitats. Thus, WEOM is a very important factor for the denitrification potential of agricultural soils, especially in situations where soil moisture and NO_3^- concentrations are relatively high. This study aimed to improve basic mechanistic understanding of the effect of soluble OM for potential denitrification using incubation experiments under controlled conditions and additional studies need to address the relevance of observed effects under field conditions. Likewise, the results call for more efforts in the molecular characterization of readily available OM and their subsequent transformation during denitrification.

3.4 Acknowledgments

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4 Contribution of particulate and mineral-associated organic matter to potential denitrification of agricultural soils

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Author contributions:
RS, KK, JB, and RM conceived the experiment. RS and CMS conducted the experiments. RS, CMS, and CWM analyzed the data. RS prepared the first manuscript draft and all authors contributed to its refinement.

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4.1 Abstract

Water-extractable organic carbon (WEOC) is considered as most important carbon (C) source for denitrifying organisms but the contribution of individual organic matter (OM) fractions (i.e., particulate − POM and mineral-associated − MOM) to its release, and thus, to denitrification remains unresolved. Here we tested short-time effects of POM and MOM on potential denitrification and estimated the contribution of POM- and MOM-derived WEOC to denitrification and CO_2 production of three agricultural topsoils. Suspensions of bulk soils with and without addition of soil-derived POM or MOM were incubated for 24 hours under anoxic conditions. Acetylene inhibition was used to determine the potential denitrification and respective product ratio at constant nitrate supply. Normalized to added OC, effects of POM on CO_2 production, total denitrification, and its product ratios were much stronger than those of MOM. While the addition of OM generally increased the (N_2O+N_2)-N/CO_2-C ratio, the $N_2O/(N_2O+N_2)$ ratio changed differently depending on the soil. Gas emissions and the respective shares of initial WEOC were then used to estimate the contribution of POM- and MOM-derived WEOC to total CO_2, N_2O, and N_2O+N_2 production. Water-extractable OC derived from POM accounted for 53 to 85% of total denitrification and WEOC released from MOM accounted for 15 to 47%. Total gas emissions from bulk soils were partly over- or underestimated, mainly due to non-proportional responses of denitrification to the addition individual OM fractions. Our findings show that MOM plays a role in providing organic substrates during denitrification but is generally less dominant than POM. We conclude that the denitrification potential of soils is not predictable based on the C distribution over POM and MOM alone. Instead, the source strength of POM and MOM for WEOC plus the WEOC's quality turned out as most decisive determinants of potential denitrification.

4.2 Introduction

Agricultural soils vary in terms of the content and composition of organic matter (OM), which may affect their denitrification potential. Denitrification, the microbial reduction of nitrate (NO_3^-) via a series of enzymatic steps to NO_2^-, NO, N_2O, and finally N_2 (Philippot et al., 2007), results in net ecosystem losses of nitrogen (N) and release of the greenhouse gases N_2O and CO_2. Considering the role of N_2O in atmospheric processes, understanding the factors controlling denitrification and its N_2O/N_2 product ratio is crucial for developing strategies to minimize emissions of greenhouse gases. In soil, denitrification mainly occurs in anoxic microhabitats ('hot spots') where electron acceptors, nitrogen oxides (e.g., NO_2^-, NO_3^-), and electron donors, organic carbon (OC), are sufficiently bioavailable (e.g., Groffman et al., 2009). Despite OM is known to serve as an electron donor, the role of OM quality in shaping the spatial and temporal patterns of denitrification is still rather unknown. Previous studies have shown that addition of fresh plant biomass or well-defined low-molecular-weight compounds affects denitrification rates, product ratios, and denitrifier populations (e.g., Beauchamp et al., 1989; Miller et al., 2008; Palmer et al., 2012). When comparing different C substances, soluble low-molecular-weight compounds are much more effective in promoting denitrification and related bacterial populations (e.g., organic acids, glycerol ≫ glucose, methanol) than insoluble polymers, such as cellulose and especially lignin (e.g., Valera and Alexander, 1961; deCatanzaro and Beauchamp, 1985; Rashid and Schaefer, 1988; Akunna et al., 1993). In addition, Henry et al. (2008) showed that artificial root exudates with higher proportion of sugars (80%) caused a much lower $N_2O/(N_2O+N_2)$ product ratio than a solution rich in organic acids and amino acids (40% sugars). Despite evidence of large differences in release and biodegradability of water-extractable OM (WEOM) (e.g., Don and Kalbitz, 2005; Kalbitz et al., 2005; Mastný et al., 2018), much less information is available on the effects of more complex soil OM fractions, such as particulate and mineral-associated OM (POM, MOM) (Lavallee et al., 2020).

Water-extractable OM is considered to be readily decomposable and most effective in promoting denitrification (e.g., Bremner and Shaw, 1958; Burford and Bremner, 1975) but its chemical composition can vary greatly. It may comprise low-molecular-weight C compounds, such as sugars, amino acids, and proteins, but also high-molecular-weight components, such as microbial-derived extracellular polymeric or humic substances (Kalbitz et al., 2003a; Marschner and Kalbitz, 2003). While WEOM leached from fresh

plant residues and bacterial extracellular polymeric substances is rich in hydrophilic components, such as polysaccharides, WEOM derived from well humified forest floor layers typically is enriched in phenolic compounds, such as lignin or lignocellulose, and, thus, much less biodegradable (Kalbitz et al., 2003a, 2003b; Marschner and Kalbitz, 2003). In previous studies, we have shown that water-extractable OC (WEOC) is a straightforward indicator of the denitrification potential of soils, well related to the chemical composition of the source materials, such as POM and fresh plant residues (Surey et al., 2020a, 2020b). A substantial role in denitrification of POM with adhering microorganisms has been also suggested by Parkin (1987) and Parry et al. (2000). Further, Gaillard et al. (2003) showed that soluble C from POM enters the adjacent soil (several mm) and fuels microbial processes. Consequently, POM-derived WEOC could be of crucial importance for the stimulation of denitrification processes. Nevertheless, MOM comprises up to 90% of total soil OC, particularly in subsoil horizons (Kögel-Knabner et al., 2008), but is usually much less bioavailable than POM (e.g., Swanston et al., 2002). Mineral-organic associations are basically formed by adsorption and precipitation reactions where dissolved OM reacts with mineral surfaces (e.g., Kaiser and Guggenberger, 2000; Kleber et al., 2015). The amount and composition of MOM in a given soil is strongly affected by the composition of dissolved OM as well as the presence and reactivity of pedogenic minerals (Kaiser and Guggenberger, 2000; Mikutta et al., 2009, 2010). Thus, MOM represents a continuum of mineral-bound C substances of varying availability and degradability (e.g., Mikutta et al., 2007). Especially in topsoils with high OC inputs and mineral C loadings, OC can repeatedly be displaced and desorbed from mineral surfaces (Leinemann et al., 2016; Liebmann et al., 2020a). Consequently, MOM is a potential source of OC fueling denitrification, with its contribution probably depending on the amount and quality of WEOC.

In summary, it is currently unknown to what extent the POM and MOM fractions provide readily available OC, i.e., WEOC, for denitrification reactions. We expect that not only the potential denitrification but also its product ratios depend on the release of WEOC from POM and MOM. We hypothesize that WEOC derived from MOM contributes to potential denitrification but is less effective in driving denitrification than POM-derived WEOC. Moreover, we hypothesize that the contribution of POM and MOM to potential denitrification of bulk soils depends less on their mass proportions but can instead be estimated from the respective WEOC contents. To address our research questions, we isolated POM and MOM fractions from three agricultural soils and determined their WEOC

contents. The bulk soils were subsequently amended with isolated POM and MOM fractions, and the potential denitrification and $N_2O/(N_2O+N_2)$ product ratios were measured over a period of 24 hours.

4.3 Materials and methods

4.3.1 Sampling sites and soil characteristics

In summer 2016, we collected topsoil (0–20 cm) material at three different sites in Germany (Table 4.1). The first soil, a Haplic Luvisol with silty loam texture and pH ($CaCl_2$) of 6.7 under arable management, was from a long-term trial at Höhere Landbauschule Rotthalmünster, approximately 150 km east of Munich (latitude N48°21′, longitude E13°11′, elevation 360 m a.s.l.; Yamashita et al., 2006). In the following, it is referred to as Rotthalmünster (Ro) soil. A clayey grassland soil (Fluvic Gleysol) with a pH ($CaCl_2$) of 5.7, referred to as Giessen (Gi) soil, was collected at the Environmental Monitoring and Climate Impact Research Station Linden near Giessen (latitude N50°32′, longitude E8°41.3′, elevation 172 m a.s.l.; Jäger et al., 2003). The third soil was taken approximately 1.5 km south of Fuhrberg (latitude N52°32.9′, longitude E9°50.6′, elevation 43 m a.s.l.; Böttcher et al., 1999). The sandy arable soil with a pH ($CaCl_2$) of 4.8 is classified as a Gleyic Podzol and referred to as Fuhrberg (Fu) soil. Soil samples were air-dried, and large plant particles and stones removed by sieving to <2 mm. The clay fraction of the three soils had a comparable clay mineral composition, dominated by illite and kaolinite, with a substantial contribution of vermiculite in the Ro soil (Table 4.1, Appendix 5). Contents of pedogenic Fe oxides, expressed as dithionite-citrate-bicarbonate-extractable Fe (Fe_d), varied strongly among soils, following the order: Ro > Gi > Fu (Table 4.1).

4.3.2 Isolation of organic matter fractions

Soils were fractionated according to density using sodium polytungstate solution adjusted to 1.6 g cm^{-3} as described in Surey et al. (2020a) to estimate the distribution of soil OC over POM and MOM fractions. The fraction <1.6 g cm^{-3} comprises free POM (i.e., not or only weakly associated with mineral particles; fPOM) and occluded POM present within water-stable aggregates (oPOM); the fraction >1.6 g cm^{-3} represents MOM. Since density fractionation might release soluble C from plant residues, we used electrostatic attraction (Kaiser et al., 2009) to recover unaltered POM material for the incubation experiments.

Before separation, soil aggregates of the three study soils were gently crushed with mortar and pestle. Subsequently, POM was collected using a plastic stick electrostatically charged by rubbing; washing was avoided to leave the amounts of WEOC unaltered.

Table 4.1 Basic characteristics of sampling sites and soils used for incubation experiments.

Sampling site/soil	Rotthalmünster	Giessen	Fuhrberg
Classification (soil type)	Haplic Luvisol	Fluvic Gleysol	Gleyic Podzol
Land management	Arable cropping	Grassland	Arable cropping
Mean annual air temperature [°C]	8.2[a]	9.3[b]	8.2[c]
Mean annual precipitation [mm]	890[a]	600[b]	680[c]
Parent material	Loess[a]	Fluviatile sediments[b]	Fluviatile sediments[c]
Texture	Silt loam	Clay loam	Sand
Sand [% w/w]	19	32	91
Silt [% w/w]	71	41	6
Clay [% w/w]	10	27	3
Dominant clay minerals[d]	Vermiculite, illite, kaolinite	Illite, kaolinite	Illite, kaolinite
Fe_d [g kg^{-1}][e]	11.8	7.1	2.1
pH ($CaCl_2$)	6.7	5.7	4.8
OC [g kg^{-1}]	13.4	28.8	24.8
ON [g kg^{-1}]	1.5	2.8	1.6
OC/ON ratio	9.0	10.2	16.0
NO_3^--N [mg kg^{-1}][f]	18.5	12.2	18.9
NH_4^+-N [mg kg^{-1}][f]	2.0	1.5	1.8

[a] Jahn and Guggenberger (2003)
[b] Jäger et al. (2003)
[c] Böttcher et al. (1999)
[d] determined by X-ray diffraction (see Appendix 5)
[e] Fe extractable by dithionite-citrate-bicarbonate
[f] extracted with 0.1 M KCl

4.3.3 Chemical characterization of organic matter fractions

Prior to analyses and incubation experiments, POM materials were ground to <1 mm using an impact mill (Rekord A, Gebr. Jehmlich GmbH, Nossen, Germany). The MOM fractions were analyzed for total C and N (TN) using a Vario Max Cube; analyzes of POM were carried out with a Vario EL cube (Elementar Analysensysteme GmbH, Langenselbold, Germany). Inorganic C was not detectable (Vario Max Cube, Elementar), therefore, total C was assumed to represent OC. Water-extractable OC and total N (WETN) were determined by suspending either 5 g of the MOM fractions or 500 mg of POM in 25 ml ultrapure water and shaking for one hour. After centrifugation (only necessary for MOM) at 3,000 × *g* for 10 min (Cryofuge 8500i, Thermo Fisher Scientific, Waltham, MA, USA), the supernatants were passed through 0.45-µm membrane filters (Supor-450, Pall Cooperation, New York, NY, USA). All WEOM solutions were analyzed for dissolved OC (DOC) and TN using a multi N/C 3100 (Analytik Jena AG, Jena, Germany). Concentrations of N_{min} (NO_3^- and NH_4^+) were determined using a Continuous-Flow Analyzer (ScanPlus, Skalar Analytical B.V., Breda, The Netherlands). Water-extractable organic N (WEON) or ON was calculated by: (WE)ON = (WE)TN – N_{min}. All reported (WE)OC and (WE)ON contents refer to dry mass determined at 105 °C. The specific UV absorbance at 280 nm (an estimate of aromatic compounds) of the WEOM was determined using a photometer (SPECORD 210 PLUS, Analytik Jena AG) by normalizing the absorbance to the OC concentration.

Before analysis of POM and MOM fractions by solid-state ^{13}C cross-polarization magic angle spinning NMR spectroscopy (^{13}C-CPMAS NMR spectroscopy) with an Avance III 200 spectrometer (Bruker BioSpin GmbH, Karlsruhe, Germany), both fractions were ground with a vibratory disc mill (RS 100, Retsch GmbH, Haan, Germany). Samples were placed into a 7-mm zirconia rotor that was spun at 6.8 kHz at a 'magic angle' of 54.74°. Contact time was 1 ms and the recycle delay time was set to 0.4 s. The spectra were processed with 100 Hz line broadening, phase adjusted, and baseline corrected; no spinning side bands appeared in the spectra. Peaks were assigned to four integration areas: –10–45 ppm (alkyl C), 45–110 ppm (O/N-alkyl C), 110–160 ppm (aromatic C), and 160–220 ppm (carboxylic/carbonyl C); spectra are shown in Appendix 6.

4.3.4 Incubation, gas measurements, and calculations

Soil samples were rewetted to 50% water holding capacity and aerobically pre-incubated for two weeks at 20 °C in the dark to stimulate microbial growth before anoxic incubation. Subsequently, 10 g (dry mass) of soil were placed into 250-ml glass infusion bottles, either without (control/bulk soil) or with addition of soil-derived OM fractions (POM or MOM). The OM additions amounted to 2 g C kg^{-1} dry soil and increased the total OC of Ro, Gi, and Fu soil by 15, 7, and 8%, respectively. Then, 50 ml of a KNO_3 solution (50 mg NO_3^--N kg^{-1} dry soil) were added and well mixed with the soil materials to achieve homogeneous soil suspensions (soil/water ratio = 1/5, w/v) and to avoid nitrate limitation during incubation. Using soil suspensions instead of repacked soil minimized accessibility limitations, and thus, allowed for comparing potential effects of OM fractions. The incubation bottles were sealed with a bromine-butyl-rubber stopper and crimped with an aluminum cap (32 mm; Chroma Globe GbR, Kreuznau, Germany). An O_2-free atmosphere for anoxic incubations was obtained by evacuating (<250 mbar), and then flushing the bottles three times with Helium (He) gas (99.999%, Air Liquide, Düsseldorf, Germany); the final pressure was about 1025 mbar. Soil suspensions were horizontally shaken during incubation at 20 °C in the dark for 24 hours, as this study focused on short-time effects in a typical 'hot spot–hot moment situation' (McClain et al., 2003). All incubations were carried out in triplicate.

As described in Surey et al. (2020b), gas samples (18 ml) were taken after 0, 2, 4, 6, 8, and 24 hours, using a gastight syringe (25 ml, 25MDR-LL-GT; SGE Analytical Science Pty. Ltd., Ringwood, VIC, Australia), equipped with a push button valve (Luer Lock; SGE Analytical Science) and a 0.7-mm ID cannula (Sterican G26, 25 mm; B. Braun AG, Melsungen, Germany), and then transferred into pre-evacuated (90 mbar residual pressure, flushed with He) 12-ml Exetainer vials, which were sealed with a double septum cap (IVAVC329; IVA Analysentechnik e.k., Meerbusch, Germany). This resulted in an overpressure of >200 mbar in the Exetainer vials, which was necessary to avoid contamination with air during storage and for measuring gas concentrations. To avoid low pressure in the incubation bottles, 18 ml He (~1 bar) were injected after gas sampling, resulting in constant absolute pressure of ~1025 mbar during incubation. The absolute pressure in the bottles was measured before and after gas sampling as well as after He injection, using a GMSD 2 BA-K31-L01 pressure sensor coupled with a Greisinger GMH 3151 reader (GSG Geologie-Service GmbH, Würzburg, Germany). Gas samples were

analyzed for CO_2 and N_2O concentrations on a custom-tailored gas chromatography system by Chromtech (Bad Camberg, Germany), using an Agilent HP 7890B GC as basis (Surey et al., 2020a).

Cumulative emissions of gases represent the sum of produced amounts, i.e., the detected gas mass in the headspace at time point t_x minus the mass at time point t_{x-1}, considering the removed gas amount during each gas sampling. Gas masses in suspension were calculated by using the respective Henry's law constant, volume of solution, headspace pressure, and gas concentration; pH was considered for estimating the different CO_2 species. For setting up the acetylene (C_2H_2) inhibition technique (Yoshinari and Knowles, 1976), the entire incubation procedure described above was also carried out with injection of 30 ml of C_2H_2 (99.6%; Air Liquide) in exchange for 30 ml He, resulting in an initial C_2H_2 concentration of ~10% (v/v). An C_2H_2 concentration of >5% (v/v) was expected to be maintained during the 24 hours of incubation (Yeomans and Beauchamp, 1978; Terry and Duxbury, 1985). The amount of N_2O in presence of C_2H_2 represented the total denitrification products (N_2O+N_2). Consequently, the ratio of N_2O measured without and with C_2H_2 addition is an estimate of the molar N_2O-N/(N_2O+N_2)-N product ratio. Cumulative amounts of N_2O in presence of C_2H_2 and of CO_2 in incubations without C_2H_2 were used to determine the molar (N_2O+N_2)-N/CO_2-C ratio, which reveals the contribution of denitrification to total CO_2 production. According to the stoichiometry of denitrification (Ottow, 2011), ratios between 0.8 and 1.0 indicate that the CO_2 production is exclusively linked to denitrification reactions. In order to test for the microbial use efficiency of the WEOC in each POM and MOM fraction, we calculated the percentage increase/decrease (Δ%) in cumulative gas emissions per Δ% initial WEOC induced by the addition of OM fractions to bulk soil. This allows for comparison of all OM fractions across the different soils. Potential problems of the acetylene inhibition technique, i.e., underestimated N_2 production due to acetylene inhibition of nitrification and incomplete inhibition of N_2O reduction caused by either low NO_3^- concentrations or acetylene diffusion effects (Knowles, 1990; Almaraz et al., 2020), can be excluded for the incubation conditions used (anoxic, excess NO_3^-, soil suspension).

4.3.5 Estimation of contributions of POM and MOM to total gas production of bulk soils

Contributions of POM and MOM to total gas emission of bulk soils were calculated using the determined gas emissions from soils with added soil-derived OM fractions (POM and MOM). Based on the idea that gas production rates were mainly controlled by the composition of WEOC, we estimated the contribution of the OM fractions as follows. We assumed that POM-induced gas emission from amended soil (E_{Pa}) was equal to that induced by the POM originally present in the bulk soil (E_{Pb}), considering the respective POM/MOM ratio (equation 1),

$$\frac{E_{Pb}}{P_b/M_b} = \frac{E_{Pa}}{(P_a + P_b)/M_b} \quad (1)$$

where P_b and P_a represent the amount of POM-derived WEOC in the bulk soil and the amount of added POM-derived WEOC, respectively, and M_b denotes the MOM-derived WEOC in the bulk soil. For estimating the gas emission induced by the POM originally present in the bulk soil, equation 1 can be rearranged into equation 2.

$$E_{Pb} = \frac{E_{Pa}\, P_b}{(P_a + P_b)} \quad (2)$$

The same applies to the MOM-induced emission from the bulk soil (E_{Mb}), which can be calculated by equation 3.

$$E_{Mb} = \frac{E_{Ma}\, M_b}{(M_a + M_b)} \quad (3)$$

As in equation 2, E_{Ma} represents the emission of MOM-amended bulk soil; amounts of MOM-derived WEOC in the bulk soil and added MOM-derived WEOC are denoted by M_b and M_a, respectively. The sum of E_{Pb} and E_{Mb} should be similar to the actually determined total emission from the bulk soil (E_b) as described in equation 4.

$$E_b = \frac{E_{Pa}\, P_b}{(P_a + P_b)} + \frac{E_{Ma}\, M_b}{(M_a + M_b)} \quad (4)$$

Since air-drying of soil samples increases the release of WEOC (Kaiser et al., 2001), WEOC contents of bulk soils were determined for pre-incubated soils (soil/water ratio = 1/2, w/v; only stirred for 1 min; Table 4.2). Also, the WEOC contents of dried MOM fractions were extremely high (Table 4.3). Using these figures resulted in estimates of gas emissions exceeding those actually measured for bulk soils without OM addition by far (eq. 4). Therefore, the contents of MOM-derived WEOC in bulk soil (M_b) were estimated

by total WEOC of the bulk soil minus the measured POM-derived WEOC in the bulk soil (P_b). Here, P_b was, in turn, determined by multiplying the proportion of WEOC in POM-OC (for POM obtained by electrostatic attraction) with the respective total POM-OC content of the soil.

Table 4.2 Content of water-extractable organic carbon (WEOC) and the WEOC/WEON ratio as well as the content of free and occluded particulate organic matter (fPOM, oPOM), and their proportions in total OC and their OC/ON ratios for soils used for incubation experiments. Values represent means (n = 3) ± standard deviation.

Soil	Rotthalmünster	Giessen	Fuhrberg
WEOC [mg kg^{-1}][a]	19.0 ± 0.8	64.3 ± 2.4	15.5 ± 0.6
WEOC/WEON ratio	8.2 ± 0.4	9.5 ± 0.4	9.2 ± 0.6
fPOM [g kg^{-1}]	0.8 ± 0.0	2.1 ± 0.2	3.1 ± 0.4
oPOM [g kg^{-1}]	1.6 ± 0.3	1.2 ± 0.3	1.2 ± 0.3
fPOM-OC/total OC [%]	1.2 ± 0.1	2.3 ± 0.2	4.0 ± 0.5
oPOM-OC/total OC [%]	4.7 ± 0.9	1.9 ± 0.4	2.1 ± 0.5
fPOM-OC/ON ratio	21.9 ± 2.4	27.3 ± 0.8	20.8 ± 0.8
oPOM-OC/ON ratio	18.6 ± 2.5	19.6 ± 0.7	41.5 ± 1.1

[a] Values represent initial amounts of pre-incubated soils (soil/water ratio = 1/2, w/v; only stirred for 1 min), which were used for the calculation of contributions of POM and MOM to total gas emissions of bulk soils.

4.3.6 Statistical evaluation

Basic statistical analyses were performed using Sigma Plot 11.0 (Systat Software Inc., Erkrath, Germany). One-way ANOVA with incubation treatment or time (after 2, 8, and 24 hours) as independent variable followed by the Tukey HSD test was used for testing for differences in molar $N_2O/(N_2O+N_2)$ and (N_2O+N_2)-N/CO_2-C ratios.

Table 4.3 Content of organic carbon (OC) and water-extractable OC (WEOC) as well as the OC/ON and WEOC/WEON ratios of particulate and mineral-associated organic matter (POM, MOM) fractions used for incubation experiments. POM was extracted by electrostatic attraction and MOM by density fractionation of the soils from Rotthalmünster – Ro, Giessen – Gi, and Fuhrberg – Fu. The specific UV absorbance at 280 nm ($SUVA_{280}$) was measured for extracted WEOM solutions from each POM and MOM fraction; values were related to the respective OC concentration. All values represent means (n = 3) ± standard deviation.

Organic matter type	OC [g kg^{-1}]	OC/ON ratio	WEOC [g kg^{-1}]	WEOC/ WEON	$SUVA_{280}$ [l mg^{-1} C cm^{-1}]
Ro POM	198.7 ± 0.3	25.1 ± 0.2	3.0 ± 0.6	9.0 ± 0.5	0.011 ± 0.003
Gi POM	271.6 ± 0.2	21.6 ± 0.1	4.7 ± 0.2	7.8 ± 0.3	0.013 ± 0.001
Fu POM	259.5 ± 0.3	19.4 ± 0.1	1.8 ± 0.1	5.3 ± 0.1	0.015 ± 0.002
Ro MOM	10.5 ± 0.4	8.6 ± 0.1	0.2 ± 0.0	8.1 ± 0.1	0.040 ± 0.002
Gi MOM	28.9 ± 1.6	9.3 ± 0.1	1.0 ± 0.1	9.2 ± 0.6	0.015 ± 0.002
Fu MOM	17.3 ± 0.6	15.1 ± 0.3	0.1 ± 0.0	9.1 ± 0.3	0.040 ± 0.001

4.4 Results

4.4.1 Distribution and chemical composition of soil organic matter fractions

Despite the Ro soil contained the lowest amounts of total POM (fPOM+oPOM) and total OC, the content of WEOC was about 23% higher than of the Fu soil (Tables 4.1, 4.2). While the Fu and Gi soils had more fPOM than oPOM, oPOM dominated in the Ro soil (Table 4.2). The Gi soil had the highest contents of total OC and WEOC but the lowest contribution of POM to total OC (4.2%). Soil WEOC contents were not related to total OC contents or proportions of POM fractions. The WEOC/WEON ratios of bulk soils differed little (8.2–9.5). Except for the higher OC/ON ratios of fPOM in the Gi soil (27), and especially of oPOM in the Fu soil (42), the OC/ON ratios of POM fractions were fairly similar (19–22). The OC/ON ratios of POM fractions showed no relations to the WEOC contents and WEOC/WEON ratios of bulk soils.

Likewise, WEOC contents of POM and MOM fractions used for incubation experiments were not linked to their OC/ON ratios and OC contents (Table 4.3). WEOC from the Fu POM and MOM fractions accounted for only 0.6–0.7% of their total OC

contents, while it was 1.5–1.7 and 2.3–3.4% for POM and MOM of the other two soils, respectively. The WEOC/WEON ratio of Fu POM was remarkably lower (5.3) than that of the other two POM fractions (7.8–9.0), while WEOC/WEON ratios of MOM fractions were fairly similar (8.1–9.2). Also, the specific UV absorbance of WEOM from the Gi MOM fraction was 63% less than that from Ro and Fu MOM (Table 4.3). The POM and MOM fractions isolated from the Fu soil contained significantly more alkyl C than the other OM fractions, while proportions of O/N-alkyl C and aryl C were, in turn, smaller (Table 4.4). The distribution of C species across OM fractions, especially POM, from the Ro and Gi soil, differed little. The more Fe oxide-rich Ro soil contained the highest proportion of carboxylic/carbonyl C in the MOM fraction, nevertheless, the OC contents of MOM fractions did not coincide with Fe_d contents (Tables 4.1, 4.3, 4.4).

Table 4.4 Distribution of C species in particulate and mineral-associated organic matter (POM, MOM) fractions obtained by density fractionation from soils collected at Rotthalmünster – Ro, Giessen – Gi, and Fuhrberg – Fu. For the three POM fractions, a mixture of free and occluded POM (according to mass contributions in bulk soils) was used. Values represent the percentage contribution of the different integrated chemical shift regions determined by ^{13}C-CPMAS NMR spectroscopy.

Organic matter type	Alkyl C [%]	O/N-alkyl C [%]	Aryl C [%]	Carboxylic/ carbonyl C [%]
Ro POM	17	50	26	7
Gi POM	13	52	28	7
Fu POM	43	35	15	7
Ro MOM	15	39	27	19
Gi MOM	20	43	20	17
Fu MOM	32	36	19	13

4.4.2 Effect of organic matter fractions on potential denitrification and CO_2 production

Addition of MOM resulted in increases in total denitrification (by about 55%) and cumulative CO_2 production (by 25–54%) for all three soils (Figures 4.1a, c). Addition of POM to the Gi soil caused about 93% more N_2O+N_2 and 33% more CO_2 emissions; for the

Ro und Fu soils, the emissions of N_2O+N_2 increased by 350% and those of CO_2 by 150%. Adding POM to the Ro soil caused larger N_2O+N_2 emissions after 24 hours than to the more OC-rich Gi soil (14.7±0.0 and 12.7±0.3 mg N kg^{-1} dry soil, respectively). Cumulative CO_2 emissions after 24 hours were 22–28% or 37–63% of initial WEOC in soils with either MOM or POM addition, respectively.

The cumulative N_2O release from the Ro soil was generally low and decreased slightly after eight hours for the control and MOM addition but not for POM addition (Figure 4.1b). For the Gi soil, POM and MOM additions caused drastic decreases in cumulative N_2O after eight hours as compared with the soil without amendment. By contrast, the cumulative N_2O emission from the Fu soil increased linearly during the entire incubation time, irrespective of OM addition. The Fu soil amended with POM generally released the highest amounts of N_2O.

The effect on cumulative N_2O+N_2 and CO_2 emissions (Δ%) was generally highest for the Fu POM when normalized to respective percentage increase in initial WEOC caused by OM additions (Figures 4.2a, c). Addition of POM to the Ro soil caused the maximum effect on N_2O emission after 24 hours (about 5% N_2O-N per 1% initial WEOC increase; Figure 4.2b). Addition of POM to the Gi soil caused higher effects on N_2O-N per Δ% initial WEOC than all MOM fractions after two hours but declined during incubation and was even negative after 24 hours, similar as for the MOM addition. As with POM, the effect of MOM on cumulative N_2O+N_2 and CO_2 emissions per Δ% initial WEOC was highest for the Fu soil; it was similar to that of Gi POM during the first eight hours (Figures 4.2a, c).

4.4.3 Effect of organic matter fractions on denitrification product ratios

Compared with the untreated control samples, additions of OM resulted in lower shares of N_2O for the Gi and Fu soils but not for the Ro soil. Irrespective of OM addition, the release of N_2O dominated in the Fu soil, resulting in the highest molar $N_2O/(N_2O+N_2)$ ratios (Table 4.5). In the Fu soil without OM addition, no N_2 production occurred within the first eight hours, as indicated by the molar $N_2O/(N_2O+N_2)$ ratio of 1. Across soils and treatments, the molar $N_2O/(N_2O+N_2)$ ratios decreased and the molar (N_2O+N_2)-N/CO_2-C ratio increased over incubation time (Table 4.5). Addition of POM caused high molar (N_2O+N_2)-N/CO_2-C ratios, being highest for the Ro and Fu soils (0.41 and 0.45, respectively); the effects of MOM additions were negligible.

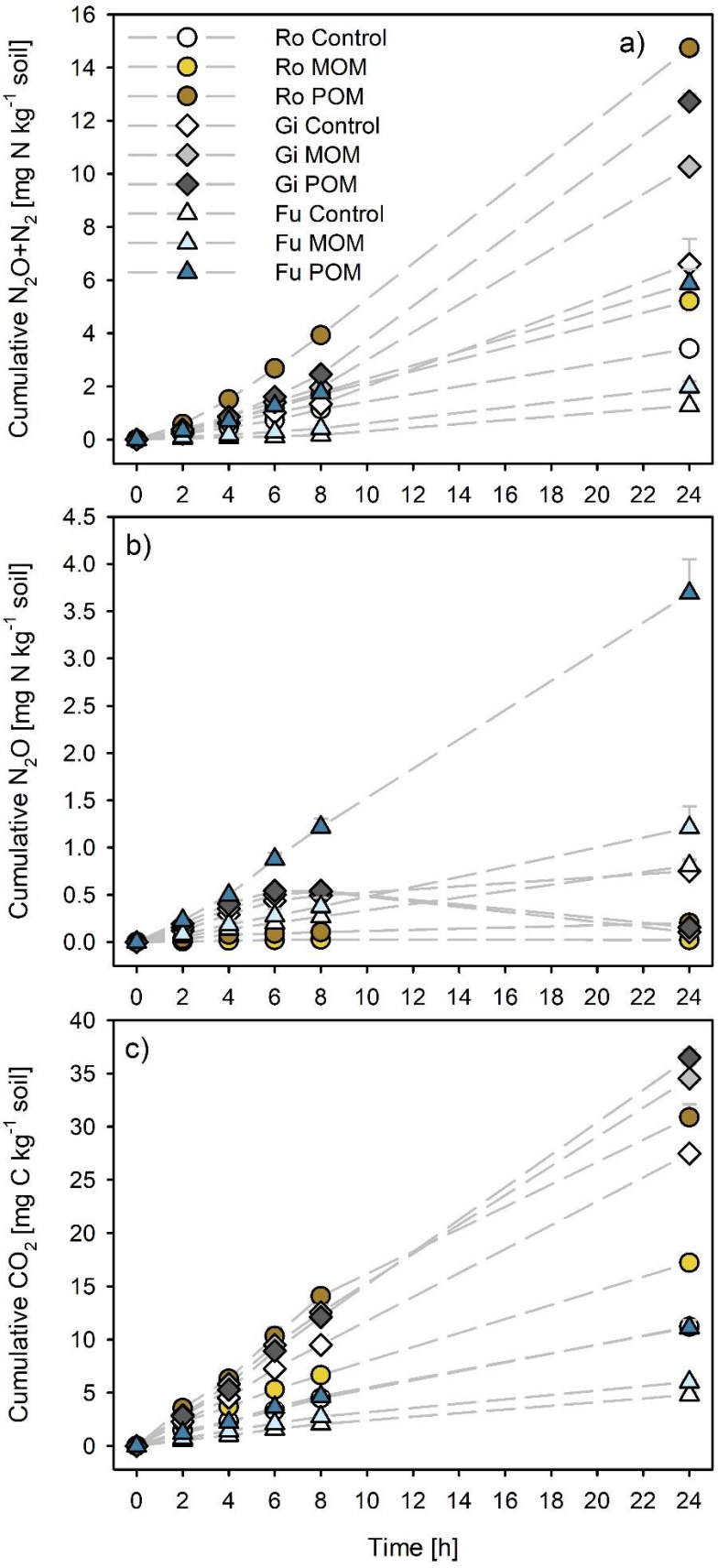

Figure 4.1 Cumulative (a) N_2O+N_2, (b) N_2O, and (c) CO_2 production during anoxic incubation at 20 °C of the soils from Rotthalmünster – Ro, Giessen – Gi, and Fuhrberg – Fu without (control) and with addition of soil-derived particulate and mineral-associated organic matter (POM, MOM). Error bars represent standard deviation of means (n = 3). All incubations received initial KNO_3 additions of 50 mg NO_3^--N kg^{-1} dry soil.

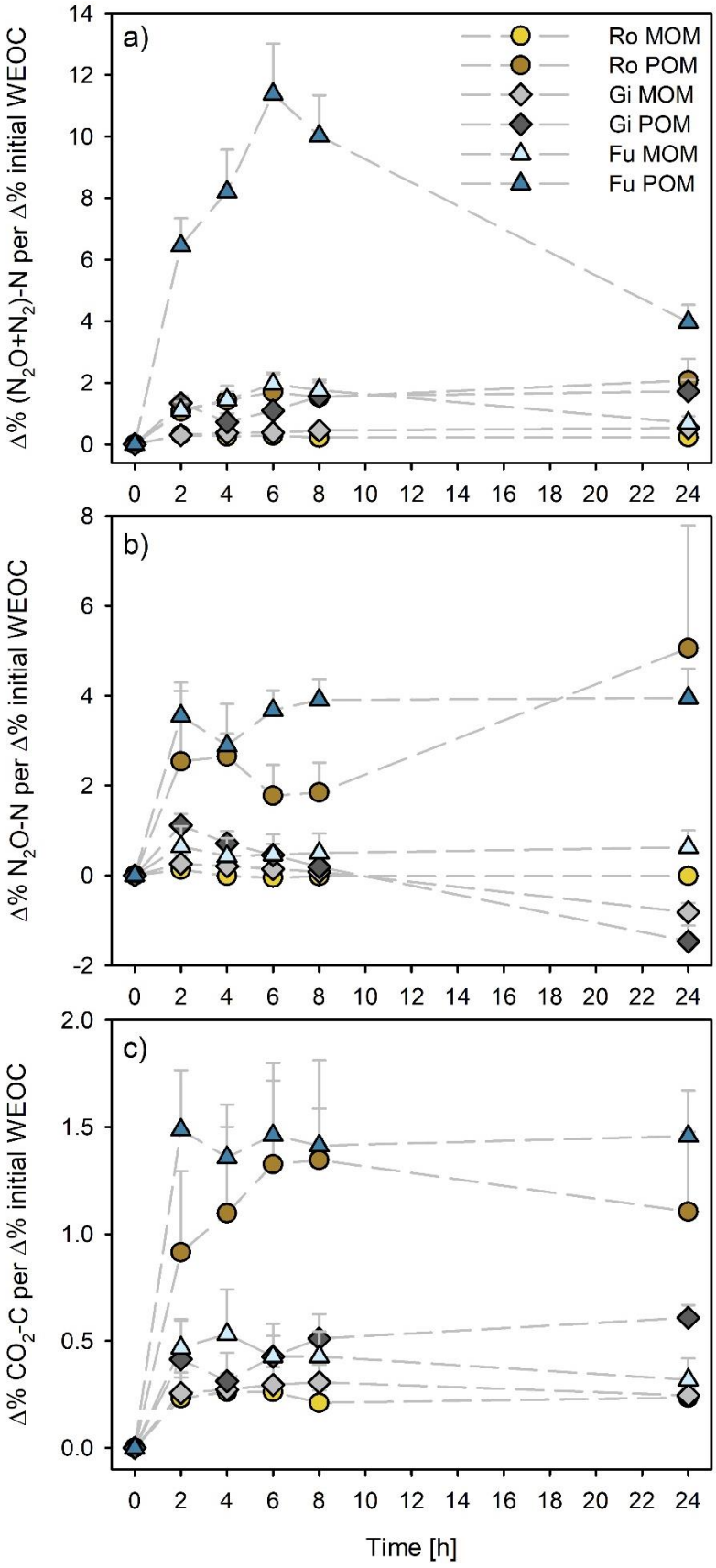

Figure 4.2 Percentage effect of additions of soil-derived particulate and mineral-associated organic matter (POM, MOM) on cumulative (a) N_2O+N_2, (b) N_2O, and (c) CO_2 production normalized to percentage initial WEOC increases compared to the control (without OM addition) during anoxic incubation at 20 °C of the soils from Rotthalmünster – Ro, Giessen – Gi, and Fuhrberg – Fu. Error bars represent standard deviation of means (n = 3).

Table 4.5 Molar $N_2O/(N_2O+N_2)$ and (N_2O+N_2)-N/CO_2-C ratios after 2, 8, and 24 hours of anoxic incubation at 20 °C of soils from Rotthalmünster – Ro, Giessen – Gi, and Fuhrberg – Fu without (control) and with addition of soil-derived particulate or mineral-associated organic matter (POM, MOM). All incubations received initial KNO_3 additions of 50 mg NO_3^--N^{-1} kg dry soil. All values represent means (n = 3) ± standard deviation.

Treatment	Molar $N_2O/(N_2O+N_2)$ ratio			Molar (N_2O+N_2)-N/CO_2-C ratio		
	2 h	8 h	24 h	2 h	8 h	24 h
Ro Control	0.03 ± 0.01 d[a]	0.02 ± 0.00 e	0.01 ± 0.00 b*[b]	0.13 ± 0.03 bc	0.22 ± 0.04 bc*	0.26 ± 0.02 bc*
Ro MOM	0.02 ± 0.00 d	0.02 ± 0.00 e	0.00 ± 0.00 b**	0.14 ± 0.01 b	0.22 ± 0.02 bc*	0.26 ± 0.02 bc*
Ro POM	0.06 ± 0.03 d	0.03 ± 0.00 de	0.01 ± 0.01 b*	0.14 ± 0.03 b	0.24 ± 0.02 b*	0.41 ± 0.02 a**
Gi Control	0.55 ± 0.06 c	0.37 ± 0.03 c*	0.11 ± 0.02 b**	0.08 ± 0.01 c	0.12 ± 0.01 de	0.21 ± 0.03 c**
Gi MOM	0.53 ± 0.09 c	0.27 ± 0.03 cd*	0.01 ± 0.00 b**	0.08 ± 0.01 c	0.13 ± 0.01 d*	0.26 ± 0.01 bc**
Gi POM	0.51 ± 0.04 c	0.22 ± 0.03 cde*	0.01 ± 0.00 b**	0.12 ± 0.01 bc	0.17 ± 0.01 cd*	0.30 ± 0.01 bc**
Fu Control	1.00 ± 0.09 a	1.00 ± 0.10 a	0.63 ± 0.06 a**	0.08 ± 0.01 c	0.07 ± 0.01 e	0.23 ± 0.02 bc**
Fu MOM	0.90 ± 0.23 ab	0.89 ± 0.22 ab	0.61 ± 0.13 a	0.11 ± 0.01 bc	0.13 ± 0.01 d	0.28 ± 0.03 bc**
Fu POM	0.69 ± 0.10 bc	0.69 ± 0.08 b	0.63 ± 0.08 a	0.24 ± 0.03 a	0.32 ± 0.03 a	0.45 ± 0.05 a**

[a] Values followed by the same letters within a column are not significantly different (p <0.05) based on a one-way ANOVA test followed by the Tukey HSD test.
[b] Significant differences between values at different incubation times are indicated by * (against "2 h" only) or ** (against both "2 h" and "8 h").

4.4.4 Contribution of POM and MOM to total gas emissions from bulk soils

Based on our calculations, about 63, 32, and 69% of WEOC of the Ro, Gi, and Fu soil derived from POM, respectively; the remaining share was from the MOM. Accordingly, the calculated contribution of POM to total cumulative gas emissions from bulk soils varied widely (Figures 4.3a–i). The contribution of POM-derived WEOC to total N_2O+N_2, N_2O, and

CO_2 emissions was dominant in the Ro (81, 91, and 79%, respectively) and Fu soil (85, 82, and 72%, respectively) but was almost equal to MOM-derived WEOC in the Gi soil (48–53%). The contribution of POM-derived WEOC to CO_2 emissions was smaller than to the production of nitrogenous gases in all three soils. The relative contribution of OM fractions differed only slightly with incubation time but percentage differences between calculated (sum of POM- and MOM-induced gas emissions) and measured total bulk soil emissions during incubation varied strongly for N_2O for all three soils (–87 to +169%), and especially for N_2O+N_2 for the Fu soil (+142 to 460%). Except for the Fu soil, the calculated cumulative N_2O+N_2, N_2O, and CO_2 emissions within the first eight hours were well in line with those actually measured for the bulk soils (Figures 4.3a–i). Lowest percentage differences with little variations over the entire incubation period occurred for CO_2 (–9 to +10%) for the Ro and Gi soils, and for N_2O+N_2 (+7 to 33%) for the Gi soil.

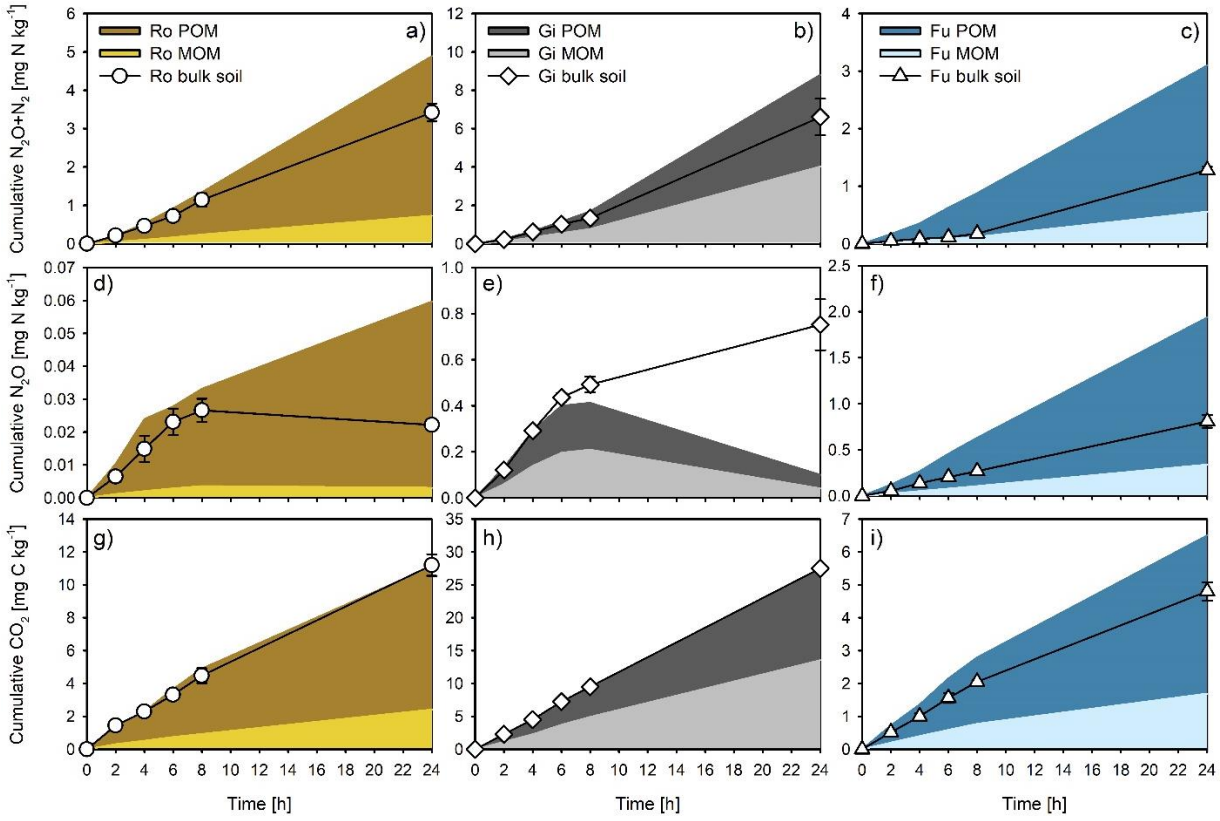

Figure 4.3 Estimated cumulative (a–c) N_2O+N_2, (d–f) N_2O, and (g–i) CO_2 production induced by WEOC derived from particulate and mineral-associated organic matter (POM, MOM), and measured cumulative production of the respective gases for the bulk soils, during anoxic incubation at 20 °C of the soils from Rotthalmünster – Ro, Giessen – Gi, and Fuhrberg – Fu. Error bars represent standard deviation of means (n = 3).

4.5 Discussion

4.5.1 Effect of organic matter fractions on potential denitrification and CO_2 production

The incubation experiment revealed that short-time effects of POM on potential denitrification and CO_2 production are much stronger than those of MOM when normalized to C equivalents (Figures 4.1a–c). Thus, POM clearly has a higher potential to fuel denitrification than MOM. This is well in line with previous studies showing that MOM comprises mainly less bioavailable C compounds (e.g., Kalbitz et al., 2005; Liebmann et al., 2020). Correspondingly, we found that only 22–28% of the initial WEOC in soils with MOM addition was emitted as CO_2 within 24 hours, while 37–63% of initial WEOC was mineralized in soils with POM addition. The results, thus, support the assumption that POM is a major driver of denitrification in soil (Parkin, 1987; Parry et al., 2000; Surey et al., 2020a). Nevertheless, all fractions contribute to CO_2 and N gas emissions, largely according to their different composition and the related WEOC source strengths (Table 4.3).

The effect of POM addition on cumulative gas emissions (Δ%) was highest for the Fu soil when normalized to the induced increases in WEOC (Figures 4.2a–c). This could be due to high quality of WEOM, as indicated by the narrow WEOC/WEON ratio of 5.3 (Table 4.3). Such a narrow C/N ratio suggests the presence of a large proportion of microbial-derived OM. The C/N ratio of microbial biomass usually varies in the range of 6 to 9 (e.g., Cleveland and Liptzin, 2007). Due to the small WEOC contents of the Fu POM, rapid depletion of such readily available C compounds could be the reason for the declining effect of added Fu POM on cumulative N_2O+N_2 already after six hours, when the maximum was reached (Figure 4.2b). Thus, as already shown in a previous study (Surey et al., 2020b), potential denitrification depends not only on the plant residues' content of WEOM but also on its chemical composition (e.g., C/N ratio, aromaticity, shares of sugars and organic acids). Consequently, when predicting denitrification potentials of different soils, the quantity of WEOC as well as its quality needs to be considered. Since the Ro POM was also more efficiently used than the other OM fractions (Figures 4.2b, c), we suggest that microorganisms in WEOC-poor soils are better adapted to POM-derived WEOM.

In summary, as hypothesized, the contribution of POM to potential denitrification is much higher than that of MOM (based on C equivalents). Also, the stronger percentage

effects of added POM and its larger contents of readily mineralizable WEOC than of MOM underline the importance of POM-derived WEOM for the potential denitrification in soils.

4.5.2 Effect of organic matter fractions on denitrification product ratios

Previous studies have shown that not only the rate of denitrification but also the $N_2O/(N_2O+N_2)$ ratio is strongly affected by environmental conditions and soil properties (e.g., Bremner and Shaw, 1958; Knowles, 1982). Lower pH values tend to cause decreased denitrification rates but larger molar $N_2O/(N_2O+N_2)$ ratios (Čuhel et al., 2010). Therefore, differences in soil pH (Table 4.1) could explain that the molar $N_2O/(N_2O+N_2)$ ratio was highest for the Fu soil. Another important factor influencing the share of N_2O is the ratio of bioavailable OC to NO_3^--N, as demonstrated by Senbayram et al. (2012). The effects of POM and MOM additions on N_2O production (Figures 4.1a, 4.2b) and molar $N_2O/(N_2O+N_2)$ ratio (Table 4.5) were well in line with this finding. Higher concentrations of WEOC at the beginning of the incubation of the OM-amended acidic Fu soil might have facilitated complete denitrification to N_2, resulting in lower molar $N_2O/(N_2O+N_2)$ ratios than for the Fu soil without OM addition, despite high initial NO_3^- concentrations. The addition of POM and MOM to the Gi soil resulted in the molar $N_2O/(N_2O+N_2)$ ratios declining to about zero by the end of incubation. Consequently, the OC-rich Gi bulk soil (high DOC/NO_3^--N ratio) without OM addition released more N_2O within 24 hours than with additions of POM and MOM (Figure 4.1b). By contrast, the addition of OM fractions to the OC-poor Ro soil (low DOC/NO_3^--N ratio) increased the share of N_2O, especially at the beginning of incubation. Therefore, in situations where NO_3^- is not limited and O_2 absent ('hot spot/hot moment'), OM fractions releasing WEOC control the total denitrification as well as its molar $N_2O/(N_2O+N_2)$ ratio. In accordance with effects discussed in the previous section, additions of soil-derived POM resulted also in higher molar (N_2O+N_2)-N/CO_2-C ratios than MOM additions; the ratio being maximum for the Fu soil with POM addition (Table 4.5). This supports the idea that the product ratios of denitrification responds to the chemical composition of WEOM (Surey et al., 2020b) and again indicates a higher quality and use efficiency of WEOM derived from plant residues than from MOM.

As hypothesized, not only the rate of denitrification but also the molar $N_2O/(N_2O+N_2)$ and (N_2O+N_2)-N/CO_2-C product ratios are affected by the release of WEOM from MOM and especially from POM. Thus, WEOM in combination with the soil pH and available NO_3^--N determines the relative contribution of the different gas products.

4.5.3 Estimation of potential denitrification based on functional organic matter fractions

Apart from overestimations discussed below, our calculations suggest that the gas emissions from the OC-poor and silty Ro soil, as well as from the sandy Fu soil, were largely fueled by POM-derived WEOC (Figures 4.3a, c, d, f, g, i). This accords with findings of N_2O+N_2 emissions from silty soils (Haplic Chernozem; 1.6–2.3% total OC) of a long-term fertilization experiment being closely related to total WEOC (23–40 mg kg^{-1}), which again related well to shares of POM (Surey et al., 2020a). This might also explain why denitrification 'hot spots' are often linked to POM (e.g., Parkin, 1987; Parry et al., 2000). Further, the lower contribution of POM-derived WEOC to CO_2 emissions than to cumulative N gas emissions for all three soils (Figures 4.3a–i) reflects higher molar (N_2O+N_2)-N/CO_2-C product ratios for POM than for MOM additions and emphasizes again the importance of plant residues as a C source for denitrifying organisms (e.g., Bremner and Shaw, 1958; Burford and Bremner, 1975; Surey et al., 2020b). However, the estimated contributions of Gi POM and MOM to total gas emissions were almost similar (Figures 4.3b, e, h), basically due to the relatively large WEOC contents of the MOM fraction (Table 4.3). In addition, Gi MOM-derived WEOM was characterized by a remarkably low (comparable to POM-derived WEOM) specific UV absorbance at 280 nm (Table 4.3), indicating smaller portions of aromatic C compounds (Chin et al., 1994), which are less easily decomposable than proteins and low-molecular-weight organic acids (Marschner and Kalbitz, 2003). Therefore, the activity of denitrifying organisms in the clayey grassland soil is likely less limited by readily decomposable OM but rather by the presence of NO_3^-. The much higher contributions of POM-derived WEOC to total gas emissions for the Ro than for the Gi soil, despite lower amounts of POM (Table 4.2), suggests that the contribution of OM fractions to total gas emissions of bulk soils depends less on their mass proportions but more on their WEOM content and quality. In addition, the Fu soil contained most POM (Table 4.2) but the total WEOC content was the lowest among all three soils. This might be due to its sandy texture and low aggregation level, facilitating strong leaching of POM (and MOM) during precipitation events, and thus, promoting large losses of WEOC (Bremner and Shaw, 1958; Surey et al., 2020b). Also, the high ratios of alkyl C to O/N-alkyl C (Table 4.4) indicate advanced decomposition of the Fu POM and MOM (Baldock et al., 1997). Consequently, the total amount of WEOC and the related denitrification potential of different soils cannot be estimated from the plant residue

content alone. We conclude, therefore, that the distribution of OM fractions is not a suitable direct predictor of the denitrification potential of soils.

Deviations between estimated (sum of POM- and MOM-induced gas amounts) and measured bulk soil emissions (Figures 4.3a–i) indicate that for part but not all soils the contribution of POM and MOM to potential denitrification can be well predicted based on respective WEOC contents. We suggest that the general overestimation of the sum of POM- and MOM-induced gas emissions from the Fu soil was mainly due to an inaccurate estimate of the contribution of WEOC from individual OM fractions. This generally remains a major experimental challenge, as not all OM fractions can be extracted from soil without alterations in physicochemical properties. Also a priming effect, i.e., accelerated decomposition of native soil OC due to OM additions, cannot be excluded, as it can vary widely among soils and with the chemical composition of added C substrates (Liu et al., 2020). However, the CO_2 production of the Ro and Gi soils could be estimated remarkably well, which basically supports the validity of our approach. Deviations from measured N_2O and N_2O+N_2 emissions can be mainly explained by changes in molar $N_2O/(N_2O+N_2)$ and (N_2O+N_2)-N/CO_2-C product ratios caused by OM additions (Table 4.5; see section 4.5.2). This indicates that also under field conditions the contribution of POM and MOM to the bulk soil WEOC can vary over time, thus influencing denitrification rates and product ratios.

Overall, our calculations imply that the contributions of POM and MOM to total gas emissions from arable and grassland soils can vary widely and depend less on the mass distribution of OM fractions but rather on the respective content and quality of their WEOC. Contrary to our second hypothesis, the contribution of POM and MOM to potential denitrification of bulk soils could be well estimated by use of respective WEOC contents for only two soils and not in general. Nevertheless, our results again imply that WEOC derived from POM is more relevant for promoting denitrification reactions but MOM-derived WEOC may significantly contribute in soils with relatively large shares of mineral-bound OC.

4.6 Conclusions

We showed that contributions of POM and MOM to denitrification can vary widely among soils, depending on physicochemical soil properties (texture, total OC content) and the

chemical composition of OM fractions. Generally, POM is far more effective in fueling denitrification than MOM. This could partly explain why denitrification 'hot spots' are often associated with plant residues. Additional studies focusing on the contribution of OM fractions to soil denitrification in intact soils and under aerobic conditions could add to better understanding of the relevance of individual OM fractions for soil denitrification. The extraction methods to obtain soil OM fractions and to determine respective WEOC contents should be chosen carefully. Since WEOC contents and denitrification potentials of soils were not directly related to their contents of POM, our study emphasizes that the distribution of OM fractions is not suitable to predict the denitrification potential of soils in general. It appears recommendable to rely on direct determination of WEOC and its chemical composition. In addition, improved estimates of amounts and composition of WEOM produced during decomposition of plant residues might support better prediction of the denitrification potential of agricultural soils.

4.7 Acknowledgments

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5 Synthesis

5.1 Discussion

5.1.1 Importance of plant residues for soil denitrification

The addition of plant residues to the soil can significantly modify soil microbial activity and net N mineralization, and thus, denitrification (Chen et al., 2013; Li et al., 2013). According to a meta-analysis including 21 laboratory studies and 7 field studies that covered 43 species of plant residues, residue effects on N_2O emissions were negatively associated to the residues' C/N ratio and strongly influenced by the texture and water content of soils (Chen et al., 2013). Consistent with this, the results of Chapter 4 showed that POM, largely composed of plant residues, is generally more effective in promoting denitrification compared to MOM, and thus, may be the primary source of OC for denitrifying organisms, especially in OC-poor or acidic and sandy arable soils. In addition, we found that WEOC, representing the most important driver for denitrification reactions, is related to the proportion of fPOM-OC and the OC/TN ratio of fPOM for soils of a long-term fertilization experiment (Chapter 2). However, in Chapter 4 we concluded that denitrification potentials of very different soils were not directly related to their contents of POM, which indicates that the distribution of OM fractions is not suitable to predict the denitrification potential of soils in general. This can be explained by the fact that the source strength of plant residues for WEOC and the potential to drive denitrification reactions largely depends on their chemical composition. As shown in Chapter 3, not only the denitrification rate but also the molar $N_2O/(N_2O+N_2)$ and $(N_2O+N_2)–N/CO_2–C$ ratios are directly related to the amount und composition of WEOM derived from differed plant residues. The content of WEOC increased with carboxylic/carbonyl C and decreasing OC/ON ratio of the plant residues. Thus, the negative relationship between the C/N ratio of plant residues and denitrification products found in many studies is probably not only a function of increased N additions, but also due to higher amounts of WEOC. Consequently, the composition of plant residues plays a crucial role in soil denitrification reactions. This points towards the relevance of crop species and the type of fertilization (organic vs. inorganic) for N_2O and N_2 emissions from agricultural soils.

However, in order to address the aspect of OM quality, the studies presented in this thesis focus on the denitrification potential, i.e., under conditions without NO_3^- limitation

and O_2 inhibition. Under field conditions, the importance of plant residues for soil denitrification and, especially, the emission of N_2O might be even higher than estimated in Chapter 4, since 'hot spots' of denitrification are often associated to increased moisture and respiration in and around plant residues (Parkin, 1987; Kravchenko et al., 2017). Consequently, the positive effect of plant residue additions on denitrification and N_2O emissions is often linked to a stimulation of microbial respiration, and thus, the formation of anaerobic microsites (Flessa and Beese, 1995; Miller et al., 2008; Chen et al., 2013). Li et al. (2016) showed that catch crop residues can stimulate N_2O emissions via denitrification over a wide range of soil moisture conditions. However, the application of plant residues to soils with a WFPS ≥90% often results in decreased N_2O emissions (Chen et al., 2013). Li et al. (2013) suggested that the bioavailability of NO_3^- is reduced by inhibited production of NO_3^- via nitrification and increased microbial N assimilation after plant material addition under O_2-limited conditions. Consequently, the reduction of N_2O to N_2 is enhanced, since denitrifying organisms are more efficient in resource use under NO_3^- limitation. Consistent with this, a meta-analysis including 112 scientific assessments of crop residue returning revealed that N_2O emissions were significantly enhanced (+24%) by the addition of crop residues for upland soils but significantly inhibited (-27%) for paddy soils (Shan and Yan, 2013). Also the effect of different plant particle sizes and types of residue placement/incorporation on denitrification and N_2O emissions is largely dependent on environmental conditions, resulting in contrasting findings (Aulakh et al., 1991; Shelp et al., 2000; Ambus et al., 2001).

The results described in Chapter 3 suggest that POM-associated denitrification depends not only on the source of plant residues but also on their degradation stage. We showed that pre-leaching of POM significantly reduced the content of WEOC but the effect of decomposition, bacterial colonization, and dry-wet or freeze-thaw alteration on POM-induced denitrification is rather unresolved. After harvest, plant residues often remain on or in the soil and are decomposed from autumn to spring, with unknown consequences for their potential to promote denitrification. As shown in Chapter 3, fresh harvest residues represent high-quality litter with strong impacts on soil denitrification and respective gas products, but already decomposed residues persisting over winter might have less effect on denitrification. Previous studies testing effects of aerobic decomposition of plant residues on denitrification under anaerobic conditions showed conflicting results. In contrast to Shelp et al. (2000), McKenney et al. (1993) found that a 5-day aerobic pre-incubation of the soil amended with plant residues resulted in

increased N_2O production during a subsequent anaerobic period, likely due to more readily available OC released from plant residue decomposition. However, Shelp et al. (2000) suggested that the remaining OC in plant residues is less available for denitrifiers after a period of aerobic incubation, due to the consumption of most available substrates. This is consistent with McCarty and Bremner (1992), who concluded that WEOC derived from plant residues is rapidly decomposed in surface soils, and thus, residue additions have a short-lived effect on denitrification, especially for subsoils. Cellulose and hemicellulose compounds are the major components of most plant residues, which are decomposed immediately after the water-soluble fraction (Beauchamp et al., 1989). Under anaerobic conditions, the decomposition of plant residues results in the production of organic acids, such as acetic acid and butyric acid, by fermentative decomposers. Greenwood and Lees (1960) reported that those volatile fatty acids did not accumulate in the presence of NO_3^-, suggesting that they provide a carbon source for denitrifying organisms (Beauchamp et al., 1989). Consistent with this, Paul and Beauchamp (1989) found a close relation between volatile fatty acids in manure and denitrification. Thus, the literature provides evidence of interaction between denitrifiers and fermentative bacteria during anaerobic decomposition of plant residues, and hints at the importance of organic acids for denitrification. However, in Chapter 2 we found that the share of N_2O decreased over the time of anoxic incubation even at constant NO_3^- excess, indicating limited availability of high-quality C compounds. Nevertheless, the total denitrification rate was not affected. Improved estimation of amounts and composition of WEOM produced during decomposition of plant residues is necessary for better prediction of the denitrification potential of agricultural soils.

As shown in Chapter 3, multiple pre-leaching of fresh ryegrass residues drastically reduced their effect on denitrification. Thus, not only decomposition rates but also leaching processes need to be considered when evaluating the relevance of degraded plant residues. On the other hand, processes promoting the water-soluble fraction of plant residues, such as dry-wet and freeze-thaw cycles, can result in significantly increased denitrification rates (McCarty and Bremner, 1993; Risk et al., 2013). In Chapter 2 and partly in Chapter 3 and 4, we found low WEOC/WEON ratios (<9) suggesting that leachable components often contain a large proportion of proteinaceous material, possibly originating from POM-associated microbial biomass (Cleveland and Liptzin, 2007). Therefore, not only fresh plant residues but also degraded and bacterially

colonized POM represents a major source of WEOM in topsoils, which can be capable of promoting denitrification in subsoils (McCarty and Bremner, 1993).

5.1.2 Contribution of MOM to denitrification depends on physico-chemical soil properties

The high bond strength between OM and the mineral surface results in an increased resistance against microbial decomposition and is generally enhanced by a high soil acidity (Kleber et al., 2015). Soil OC associated with minerals is related to the content of clay, due to the high specific surface area of clay-sized particles facilitating colonization by microorganisms and direct adsorption of organic matter (Kögel-Knabner et al., 2008). Thus, the presence of sorptive mineral phases, such as Fe and Al oxides or clay minerals, in finely textured soils might significantly reduce the OC availability for denitrification, which is less likely in coarse textured soils with a minor contribution of reactive minerals. Consistent with this, we found a negative correlation between the proportion of MOM-OC and WEOC content, which related most closely to the denitrification potential of differently fertilized soils (Chapter 2).

On the other hand, MOM could be used as a source of OC by denitrifying organisms through processes that cause the release and destabilization of previously mineral-bound OC (e.g., microbial degradation, transformation, displacement or leaching) (Gu et al., 1995; Sollins et al., 1996; Sanderman et al., 2008). Especially in topsoils with high OC inputs/contents and mineral C loadings, OC can repeatedly be displaced and desorbed from mineral surfaces (Leinemann et al., 2016; Liebmann et al., 2020b), and thus, promote denitrification. Consistent with this, the positive effect of MOM addition on CO_2, N_2O, and N_2O+N_2 emissions was much higher for the OC-rich and clayey grassland soil from Giessen compared to the two arable soils with less OC and clay contents from Rotthalmünster and Fuhrberg (Chapter 4). Based on these results and respective WEOC contents, we estimated that MOM contributed to about 50% of the total denitrification in the Giessen soil, while more than 80% of N_2O+N_2 was induced by WEOC derived from POM in the other two soils. Consequently, in soils with high OC and clay contents MOM may represent a potential OC source for denitrifying organisms through the release of WEOC.

By contrast, in sandy and acidic soils, such as the Fuhrberg soil, MOM probably plays only a minor role in promoting denitrification because of the low contribution to total soil WEOC. However, the effect on cumulative N_2O+N_2 emissions of MOM isolated from the

Fuhrberg soil was significantly higher compared to the other MOM fractions when related to percentage increases in WEOC caused by MOM addition, but only during the first eight hours of incubation (Figure 4.2a). This could indicate that WEOM initially released from MOM in sandy and acidic soils can have a high quality and use efficiency by denitrifying organisms. Consistent with previous findings (e.g., von Lützow et al., 2006; Kögel-Knabner et al., 2008), the MOM fraction from the sandy and acidic Fuhrberg soil had a higher proportion of alkyl C, indicating aliphatic C compounds, and higher C/N ratio than to the two MOM fractions isolated from soils with much higher proportions of clay and silt (Tables 4.3 and 4.4). However, we found no significant differences in the chemical composition (WEOC/WEON, $SUVA_{280}$) of WEOM derived from the three MOM fractions. The results indicate that readily decomposable OM compounds can also be released in sandy and acidic soils when environmental conditions, especially the water content and pH, change, but this has only a very short-term impact on total denitrification.

Nevertheless, we have shown that POM is far more effective in fueling denitrification than MOM (Chapter 4). We only tested short-term effects for 24 hours, but it is likely that limitations in the bioavailability of easily decomposable C compounds occur much more rapidly when denitrifiers are only supplied by the OC release from MOM fractions. This is consistent with previous studies showing lower turnover rates of MOM compared to POM (Alvarez et al., 1998; Swanston et al., 2002), and could partly explain why denitrification 'hot spots' are mostly associated with plant residues (Parkin, 1987). Mineral-associated OM might play a larger role in providing organic substrates during denitrification in subsoils where the proportion of other OC sources, such as POM, is often negligible. However, also the strength of bonding between OM and mineral surface usually increases with soil depth (Kögel-Knabner et al., 2008). Accordingly, McCarty and Bremner (1992) reported evidence that denitrification in subsoils is limited by a lack of OC that can be utilized by denitrifying microorganisms. Also Siemens et al. (2003) concluded that DOM leached from soils does not contribute significantly to the natural attenuation of NO_3^- leached to aquifers, since the bioavailability of leached DOM is generally low and spatially decoupled from reduced NO_3^- concentrations. However, the authors also pointed out that the short timescale and exclusion of sorption processes of laboratory incubations could underestimate the amount of degradable fractions and their bioavailability. McCarty and Bremner (1993) showed that WEOM derived from surface soils is much more effective in stimulating denitrification in subsoils when soils were frozen or air-dried compared to field-moist soil. Although the authors suggested that plant residues are the main source

of WEOM, dry-wet and freeze-thaw cycles may also be relevant for OC release from MOM. Mineral-bound OM can generally be displaced by freshly released WEOM from plant residues and dead microbial biomass and move vertically through the soil profile with water (Kaiser and Kalbitz, 2012). However, old mineral-bound C compounds are often less degradable than the fresh OM substances by which they were displaced, and thus, are likely less effective in promoting denitrification.

5.1.3 Water-extractable OC is a straightforward indicator of the denitrification potential of agricultural soils

As discussed in the previous sections, the water-soluble fraction of plant residues is most effective in promoting denitrification reactions, and the bioavailability of mineral-bound OC is favored mainly by desorption resulting from leaching or displacement processes. Consequently, WEOC might be a suitable quality marker of soil OM in terms of potential denitrification. Accordingly, we found a close relation between CO_2, N_2O, and N_2O+N_2 emissions, as well as the molar (N_2O+N_2)-N/CO_2-C ratio, and the WEOC content of fresh plant residues (Chapter 3). Furthermore, pre-leaching of ryegrass resulted in a strongly decreased production of CO_2, N_2O, and N_2 (Chapter 3), which again underlines that, at least during short anoxic periods, residue WEOC represents the most important C source for denitrifying organisms. Also in Chapter 4 we concluded that the denitrification potential of soils is not predictable based on the C distribution over POM and MOM alone, but is more dependent on the source strength of POM and MOM for WEOC plus the WEOC's quality. Our findings suggest, therefore, that during short-term anoxic conditions denitrifying organisms rely on the release of soluble compounds from POM and MOM fractions. This is well in line with previous studies showing that soluble low-weight compounds (e. g., organic acids and glycerol ≫ glucose, methanol) are much more effective in promoting denitrification than insoluble polymers such as cellulose and especially lignin (e.g., Valera and Alexander, 1961; Rashid and Schaefer, 1988; Akunna et al., 1993). In addition, variations in potential dentification and abundances of denitrifying organisms of soils from a long-term fertilization experiment could be well explained by different contents of WEOC (Chapter 2). Consequently, WEOC, representing readily bioavailable OM, can be a straightforward indicator of the denitrification potential of soils. This is consistent with previous studies also pointing at the relevance of WEOC for denitrification reactions under anaerobic conditions (Burford and Bremner, 1975;

McCarty and Bremner, 1993). Since denitrification occurs mainly in 'hot spots' and during 'hot moments', i.e., in situations where O_2 is absent and both NO_3^- and readily decomposable OC are bioavailable (Groffman et al., 2009), these results are also relevant for soils under field conditions. In accordance with this, Rummel et al. (2020) reported close relations between WEOC and N_2O and CO_2 emissions from a repacked topsoil (gleyic Fluvisol) amended with maize root and shoot litter even under aerobic conditions.

However, the contribution of POM and MOM to potential denitrification of bulk soils could be well estimated by use of respective WEOC contents for only two soils and not in general (Chapter 4). Over- or underestimations of total gas emissions from bulk soils can mainly be explained by nonproportional responses of denitrification to the addition of individual OM fractions but could also be partly due to effects of different WEOM qualities. As shown in Chapter 3, not only the WEOC concentration but also the chemical composition of WEOM has an impact on denitrification rates and product ratios. Consequently, soils with similar WEOC contents can have different denitrification potentials and slight differences in WEOC can be misinterpreted. In these cases, the chemical composition of the WEOM must also be considered. However, although it is known that additions of different C compounds result in large variations in denitrification rates, it is currently unclear how different molecular compositions of WEOM affect the denitrification potential of soils.

Despite the large importance of plant residues for denitrification (see section 5.1.1), WEOC contents and denitrification potentials of soils were not directly related to their contents of POM (Chapter 4). Again, this suggests that the amount and composition of water-soluble OC compounds in plant residues are much more important for potential soil denitrification than the total POM content (see Chapter 3). Accordingly, our study in Chapter 4 emphasizes that the distribution of OM fractions is not suitable to predict the denitrification potential of soils in general. It is recommendable to rely on direct determination of WEOC and its chemical composition.

5.2 Conclusions

This thesis highlights the close link between soil OM and denitrification potential, with larger portions of labile C substrates promoting denitrification reactions. The results of Chapter 2 showed that fertilization treatments causing stronger accumulation of labile OM resulted in increased denitrification with larger proportions of N_2O, while treatments causing smaller portions of readily decomposable OM favored complete denitrifying organisms. The potential denitrification of arable topsoils is closely related to the release of WEOM from POM, such as plant residues. The potential for WEOM release is highly dependent on the chemical composition of the plant residues, which in turn reflects their source and degradation stage (Chapter 3). Processes promoting the water-soluble fraction of POM and MOM, such as bacterial colonization and dry-wet or freeze-thaw cycles, might facilitate denitrification reactions. Generally, the WEOC in POM has a much higher potential to support short-term denitrification activity in a typical 'hot spot–hot moment' situation than MOM (Chapter 4). This could partly explain why denitrification 'hot spots' are often associated with plant residues. However, in OC-rich soils with high clay contents MOM can play an important role in providing organic substrates during denitrification.

Overall, WEOM is a very important factor for the denitrification potential of agricultural soils, especially in situations where soil moisture and NO_3^- concentrations are relatively high. This thesis suggests that the distribution of OM fractions is not suitable to predict the denitrification potential of soils in general. It is recommendable to rely on direct determination of WEOC and its chemical composition. However, the extraction method for determination of WEOC of different soils should be chosen carefully, since the storage condition, extraction time, and type of filtration can affect the concentration and chemical composition of WEOC.

5.3 Outlook

The observed control of WEOC on the potential denitrification calls for more efforts in the molecular characterization of readily available OM and their subsequent transformation during denitrification. In addition, improved estimates of amounts and composition of WEOM produced during decomposition of plant residues might support better prediction of the denitrification potential of agricultural soils. Since previous studies have shown conflicting results, the effects of plant residue particle size and placement or degree of incorporation on denitrification reactions need to be investigated more in detail. Also the effects of bacterial colonization as well as dry-wet and freeze-thaw alteration on POM- and MOM-induced denitrification remain rather unrevealed. Testing effects of different minerals or soil-derived MOM fractions on potential denitrification would give valuable information on the quantitative relevance of mineral-organic associations for soil denitrification. The studies presented in this thesis aimed to improve basic mechanistic understanding of the effect of functional soil OM fractions for potential denitrification using incubation experiments under controlled conditions, but additional studies need to address the relevance of observed effects under field conditions. In addition, it is still uncertain whether the content of WEOC, and thus, the denitrification potential can be generally estimated for a wide variety of soils based on easily determinable soil properties, such as total OC, C/N ratio, and texture. The development of pedotransfer functions by statistical analyses could be a valuable approach for the estimation of denitrification potentials of soils.

Summary

Although the availability of organic matter (OM) is well known to be one of the main factors controlling soil denitrification, the role of OM quality in shaping the spatial and temporal patterns of denitrification is still rather unknown. The overarching goal of this thesis was to contribute to an improved understanding of the relevance of different functional fractions of OM in driving soil denitrification. Despite many studies suggest an important contribution of water-extractable OM (WEOM) to denitrification, there is no study available that systematically addressed the role of the molecular WEOM composition and concentration in soil denitrification. Furthermore, it is currently unknown to what extent particulate and mineral-associated OM fractions (POM, MOM) provide readily available organic carbon, i.e., water-extractable organic carbon (WEOC), for denitrification reactions. The three specific aims of the thesis were

(i) to test the effect of soil OM composition as caused by different fertilization regimes on potential denitrification, the $N_2O/(N_2O+N_2)$ product ratio, and abundances of functional genes of denitrification (Chapter 2),
(ii) to examine the potential effect of agriculturally important plant residues on denitrification with special emphasis on WEOM (Chapter 3), and
(iii) to test short-term effects of POM and MOM on potential denitrification and to estimate the contribution of POM- and MOM-derived WEOC to denitrification and CO_2 production of three agricultural topsoils (Chapter 4).

i) The potential production of N_2O, N_2, and CO_2 under anoxic conditions was determined for samples of a Haplic Chernozem from six treatment plots of the Static Fertilization Experiment Bad Lauchstädt (Germany) and related to their OM properties and denitrifier gene abundances. Nitrogenous gas and CO_2 emissions related most closely to WEOC, which again related well to free POM. Limited OM sources favored a microbial community more efficient in resource use, i.e., complete denitrifying organisms (*nosZ I*).

ii) The effects of residues of various agriculturally important plant species (ryegrass, maize, and alfalfa) on potential denitrification and CO_2 production were determined; including plant parts (roots, stems, and leaves) with a wide range in C/N ratios and

varying in molecular composition. In addition, we compared two different particle sizes and three leaching stages of ryegrass residues, and WEOM solutions of equivalent C concentrations prepared from maize straw and ryegrass leaves, as well as maize root exudates. The production of all gases, as well as the molar (N_2O+N_2)–N/CO_2–C ratio, was directly related to the WEOC content of the plant residues, and the WEOC increased with carboxylic/carbonyl C and decreasing OC/ON ratio of the plant residues. Incubation of OM solutions revealed that the molar (N_2O+N_2)–N/CO_2–C ratio and share of N_2O are influenced by the WEOM's chemical composition.

iii) Suspensions of bulk soils with and without addition of soil-derived POM or MOM were incubated for 24 h under anoxic conditions. Gas emissions and the respective shares of initial WEOC were then used to estimate the contribution of POM and MOM-derived WEOC to total CO_2, N_2O, and N_2O+N_2 production. The results show that POM is generally far more effective in fueling denitrification than MOM. However, contributions of POM and MOM to denitrification can vary widely among soils, depending on physicochemical soil properties (texture, total OC content) and the chemical composition of OM fractions. The results suggest that the denitrification potential of soils is not predictable based on the C distribution over POM and MOM alone. Instead, the source strength of POM and MOM for WEOC plus the WEOC's quality turned out as the most decisive determinants of potential denitrification.

In conclusion, the WEOC in POM, especially in largely undecomposed plant residues, has a much higher potential to support short-term denitrification activity in a typical 'hot spot–hot moment' situation than MOM. However, in OC-rich soils with high clay contents MOM can play an important role in providing organic substrates during denitrification. Overall, the present thesis suggests that WEOC, representing readily bioavailable OM, is a straightforward indicator of the denitrification potential of soils. Future research should aim at better understanding of mutual effects of WEOM and POM during litter decomposition on denitrification. Additional studies focusing on the contribution of OM fractions to denitrification in intact soils and under aerobic conditions would give valuable information on the relevance of individual OM fractions for soil denitrification.

Zusammenfassung

Zwar ist bekannt, dass die Verfügbarkeit von organischem Material (OM) einer der wichtigsten Kontrollfaktoren für die Denitrifikation im Boden ist, doch die Rolle der OM-Qualität für räumliche und zeitliche Muster der Denitrifikation ist noch relativ unbekannt. Das übergeordnete Ziel dieser Arbeit war es, zu einem verbesserten Verständnis der Relevanz verschiedener funktioneller OM-Fraktionen für die Denitrifikation im Boden beizutragen. Obwohl viele Studien auf einen wichtigen Beitrag von wasser-extrahierbarem OM (WEOM) zur Denitrifikation hinweisen, gibt es keine Studie, die sich systematisch mit der Rolle der molekularen Zusammensetzung und Konzentration von WEOM für die Denitrifikation im Boden beschäftigt. Darüber hinaus ist derzeit nicht bekannt, inwieweit partikuläre und mineralassoziierte Fraktionen des OM (POM, MOM) leicht verfügbaren organischen Kohlenstoff, d. h. wasserextrahierbaren organischen Kohlenstoff (WEOC), für Denitrifikationsreaktionen bereitstellen. Die drei konkreten Ziele dieser Arbeit waren

(i) die Untersuchung des Einflusses der durch unterschiedliche Düngungsregime verursachten OM-Zusammensetzung des Bodens auf die potentielle Denitrifikation, das $N_2O/(N_2O+N_2)$-Produktverhältnis und die Häufigkeiten funktioneller Gene der Denitrifikation (Kapitel 2),

(ii) die Untersuchung des potenziellen Effekts von landwirtschaftlich wichtigen Pflanzenrückständen auf die Denitrifikation mit besonderem Augenmerk auf WEOM (Kapitel 3), und

(iii) die Untersuchung von Kurzzeiteffekten von POM und MOM auf die potentielle Denitrifikation und die Abschätzung des Beitrags von aus POM und MOM stammendem WEOC zur Denitrifikation und CO_2-Produktion von drei landwirtschaftlich genutzten Oberböden (Kapitel 4).

i) Die potenzielle Produktion von N_2O-, N_2- und CO_2 unter anoxischen Bedingungen wurde für Proben eines Haplic Chernozem aus sechs Düngungsparzellen des Statischen Düngungsexperiments Bad Lauchstädt (Deutschland) in Abhängigkeit von ihren OM-Eigenschaften und Genhäufigkeiten von Denitrifikanten bestimmt. Die Emissionen von stickstoffhaltigen Gasen und CO_2 standen in einem engen Zusammenhang mit WEOC, der

wiederum mit freier POM korrelierte. Begrenzte OM-Quellen begünstigten eine mikrobielle Gemeinschaft, die effizienter in der Ressourcennutzung ist, d. h. vollständig denitrifizierende Organismen (*nosZ I*).

ii) Es wurden die Effekte von Rückständen verschiedener landwirtschaftlich wichtiger Pflanzenarten (Weidelgras, Mais und Luzerne) auf die potenzielle Denitrifikation und CO_2-Produktion bestimmt. Hierbei wurden Pflanzenteile (Wurzeln, Stängel und Blätter) mit einer großen Spanne an C/N-Verhältnissen und unterschiedlicher molekularer Zusammensetzung einbezogen. Darüber hinaus wurden zwei verschiedene Partikelgrößen und drei Auswaschungsstufen von Weidelgrasrückständen sowie WEOM-Lösungen mit äquivalenten C-Konzentrationen verglichen, die aus Maisstroh und Weidelgrasblättern sowie Maiswurzelexsudaten hergestellt wurden. Die Produktion aller Gase sowie das molare (N_2O+N_2)-N/CO_2-C-Verhältnis standen in direktem Zusammenhang mit dem WEOC-Gehalt der Pflanzenrückstände, wobei der WEOC-Gehalt mit dem Anteil an Carboxy-C/Carbonyl-C und abnehmendem OC/ON-Verhältnis der Pflanzenrückstände zunahm. Die Inkubation von OM-Lösungen zeigte, dass das molare (N_2O+N_2)-N/CO_2-C-Verhältnis und der Anteil von N_2O von der chemischen Zusammensetzung des WEOM beeinflusst werden.

iii) Bodensuspensionen mit und ohne Zugabe von aus dem Boden stammendem POM oder MOM wurden für 24 h unter anoxischen Bedingungen inkubiert. Die Gasemissionen und die jeweiligen Anteile an der initialen WEOC-Gesamtmenge wurden dann verwendet, um den Beitrag von aus POM und MOM stammenden WEOC zur gesamten CO_2-, N_2O- und N_2O+N_2-Produktion abzuschätzen. Die Ergebnisse zeigen, dass POM im Allgemeinen viel effektiver Denitrifikation antreibt als MOM. Die Beiträge von POM und MOM zur Denitrifikation können jedoch, je nach physikochemischen Bodeneigenschaften (Textur, Gesamt-OC-Gehalt) und chemischer Zusammensetzung der OM-Fraktionen, stark variieren. Die Ergebnisse deuten darauf hin, dass das Denitrifikationspotenzial von Böden nicht allein auf Grundlage der C-Verteilung auf POM und MOM vorhersagbar ist. Stattdessen erwiesen sich die Eignung von POM und MOM als Quelle für WEOC sowie die Qualität des WEOC als die entscheidenden Bestimmungsfaktoren der potenziellen Denitrifikation.

Zusammenfassend lässt sich sagen, dass WEOC in POM, insbesondere in leicht zersetzten Pflanzenrückständen, ein wesentlich höheres Potenzial besitzt, die kurzfristige Denitrifikationsaktivität in einer typischen 'hot spot-hot moment'-Situation voranzutreiben, als WEOC in MOM. In OC-reichen Böden mit hohen Tongehalten kann MOM jedoch eine wichtige Rolle bei der Bereitstellung organischer Substrate während der Denitrifikation spielen. Insgesamt deutet die vorliegende Arbeit darauf hin, dass WEOC, das leicht bioverfügbares OM repräsentiert, ein einfacher und direkter Indikator für das Denitrifikationspotenzial von Böden ist. Zukünftige Forschung sollte darauf abzielen, die wechselseitigen Effekte von WEOM und POM während der Streuzersetzung auf die Denitrifikation besser zu verstehen. Zusätzliche Studien, die sich mit dem Beitrag von OM-Fraktionen zur Denitrifikation in intakten Böden und unter aeroben Bedingungen beschäftigen, würden wertvolle Informationen über die Bedeutung einzelner OM-Fraktionen für die Denitrifikation im Boden liefern.

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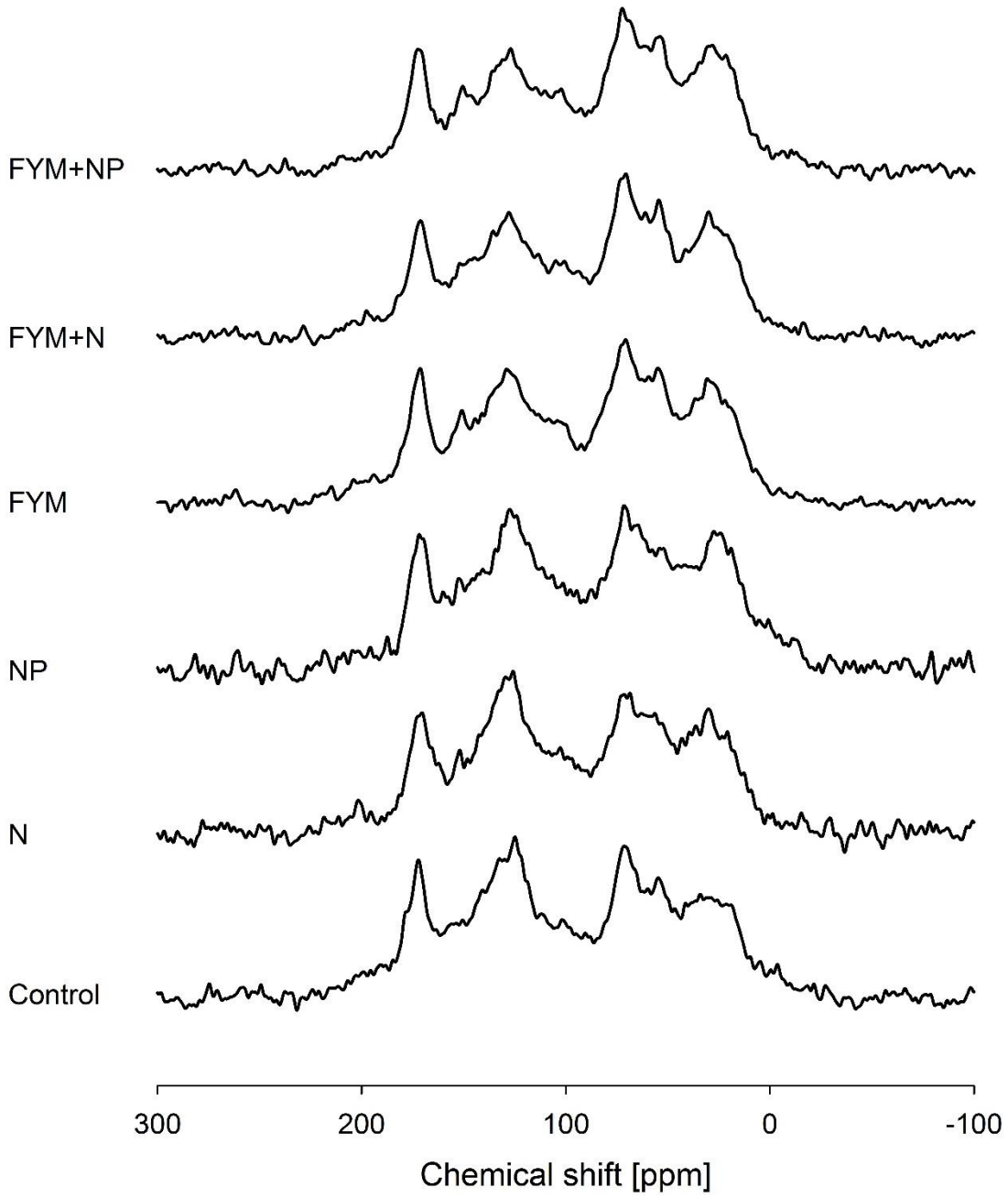
Appendix

Appendix 1 Amounts of fertilizers applied in the Static Fertilization Experiment Bad Lauchstädt (since 1995) (Surey et al., 2020a; Chapter 2).

Crop	Fertilization	N	NP	FYM[3]+N	FYM[3]+NP
Sugar beets[1]	N [kg ha^{-1}]	100 + 70	100 + 70	100 + 50	100 + 50
	P [kg ha^{-1}]	-	60	-	12
Spring barley[1]	N [kg ha^{-1}]	40 + 40	40 + 40	30 + 30	30 + 30
	P [kg ha^{-1}]	-	-	-	-
Potatoes	N [kg ha^{-1}]	140	140	120	120
	P [kg ha^{-1}]	-	60	-	12
Winter wheat[1]	N [kg ha^{-1}]	50 + 50	50 + 50	40 + 40	40 + 40
	P [kg ha^{-1}]	-	-	-	-
Silage maize[2]	N [kg ha^{-1}]	140	140	120	120
	P [kg ha^{-1}]	-	60	-	12

[1] The mineral N fertilization has been divided into two applications
[2] Silage maize replaces root crops since 2015
[3] Farmyard manure (FYM) has been applied (30 t ha^{-1}) every second year with root crops (sugar beets, potatoes) and since 2015 with maize cropping



Appendix 2 Solid-state ^{13}C-CPMAS NMR spectra of OM in bulk soils of the Static Fertilization Experiment (control, mineral N and NP, farmyard manure – FYM, and FYM + mineral N or NP) (Surey et al., 2020a; Chapter 2).

Appendix 3 PCR mastermixes and thermocycling protocols (Surey et al., 2020a; Chapter 2).

nirS

Component	Volume [µl]	Final concentration
Sensimix[1]	10	
Mg^{2+}	1.2	1.2 µM
Forward primer	2	1 µM
Reverse primer	2	1 µM
BSA	3	150 ng/µl
DNA	1.5	
Water	0.3	
Total	20	

[1] SensiMix™ SYBR® & Fluorescein Kit, Bioline, London, UK

Time [min]	Temperature [°C]	Number of cycles
10:00	95	
0:30	95	40
0:30	58.5	
0:30	72	
5:00	72	
∞	8	

nirK

Component	Volume [µl]	Final concentration
Sensimix[1]	10	
Mg^{2+}	1.2	1,2 µM
Forward primer	1	0.5 µM
Reverse primer	1	0.5 µM
BSA	3	150 ng/µl
DNA	1	
Water	2.8	
Total	20	

[1] SensiMix™ SYBR® & Fluorescein Kit, Bioline, London, UK

Time [min]	Temperature [°C]	Number of cycles
10:00	95	
0:30	95	35
0:30	58	
1:00	72	
5:00	72	
∞	8	

nosZ I

Component	Volume [µl]	Final concentration
Sensimix[1]	10	
Mg^{2+}	1.2	3 µM
Forward primer	2	1 µM
Reverse primer	2	1 µM
BSA	3	150 ng/µl
DNA	1.25	
Water	0.55	
Total	20	

[1] SensiMix™ SYBR® & Fluorescein Kit, Bioline, London, UK

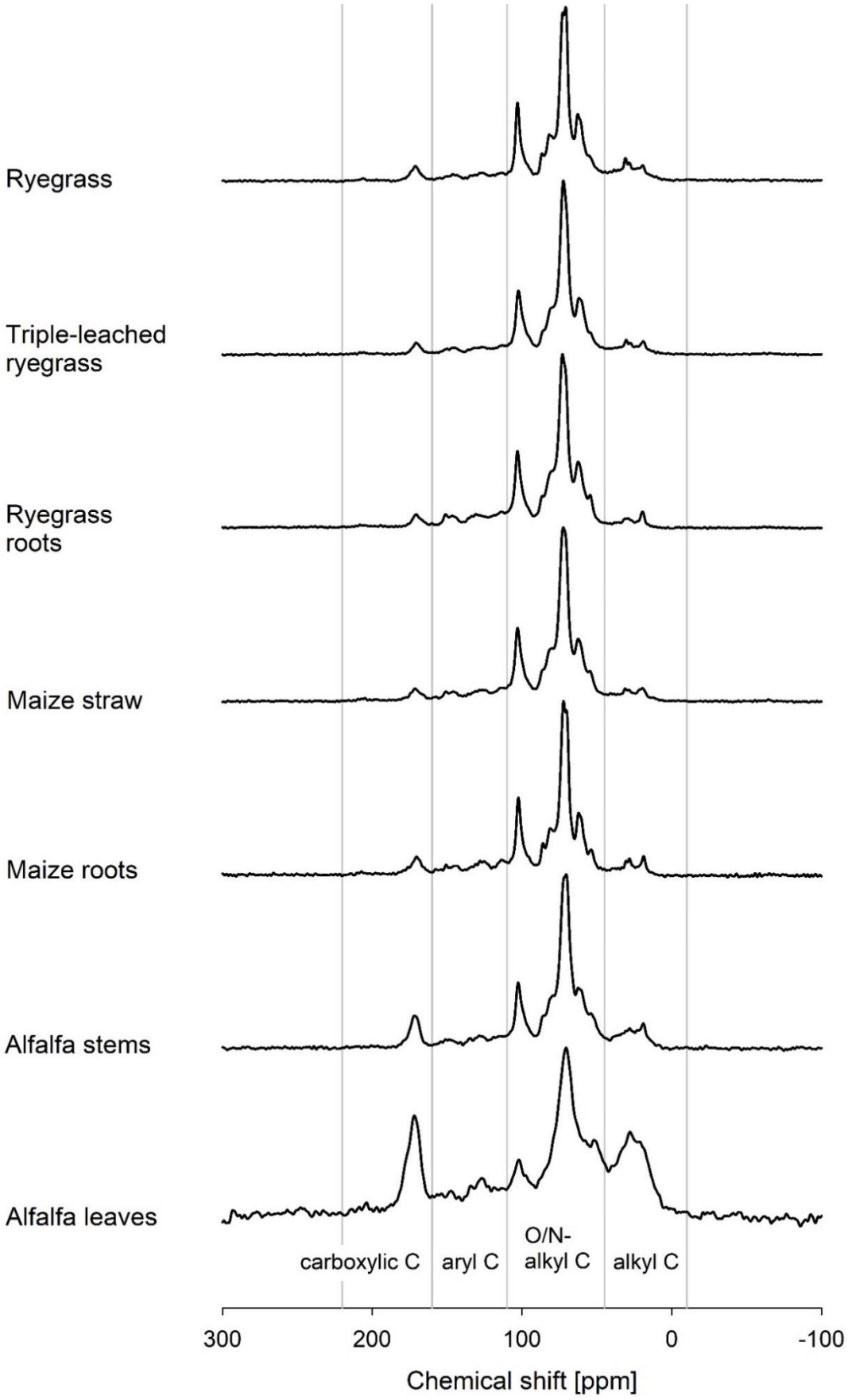
Time [min]	Temperature [°C]	Number of cycles
15:00	95	
0:15	95	
0:30	65	6
0:30	72	
0:15	80	(data aquisition)
0:15	95	
0:15	60	40
0:15	72	
0:15	80	(data aquisition)
∞	8	

nosZ II

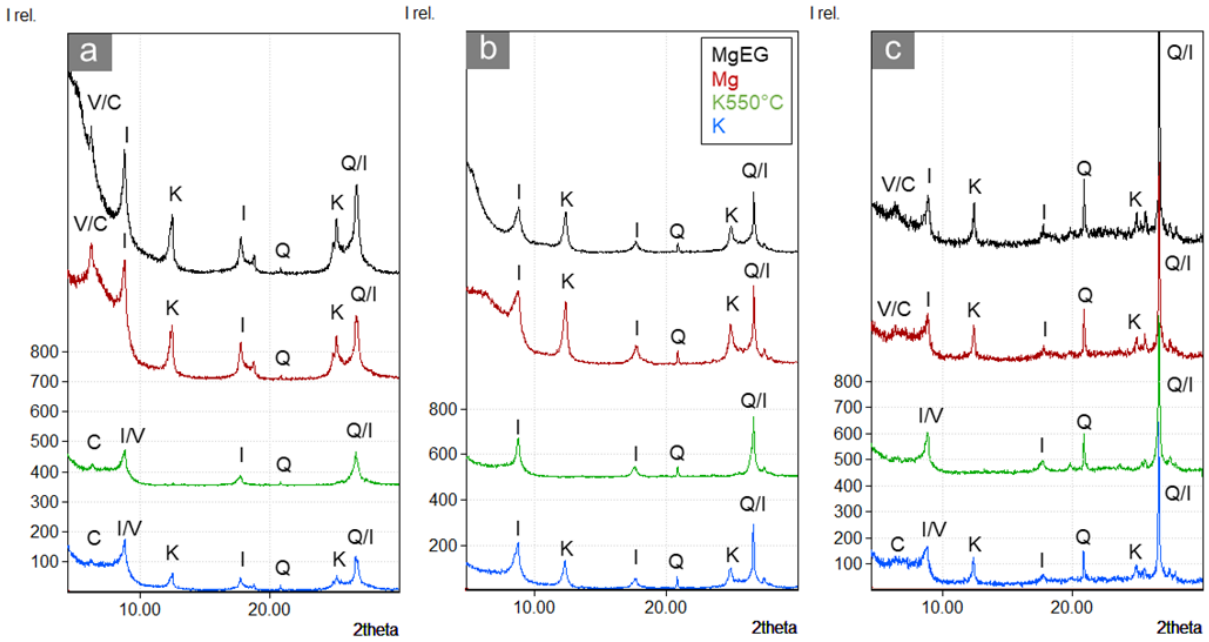
Component	Volume [µl]	Final concentration
Sensimix[1]	10	
Mg^{2+}	1.2	3 µM
Forward primer	1.6	0.8 µM
Reverse primer	1.6	0.8 µM
BSA	3.6	180 µg/ml
DNA	2	
Total	20	

[1] SensiMix™ SYBR® & Fluorescein Kit, Bioline, London, UK

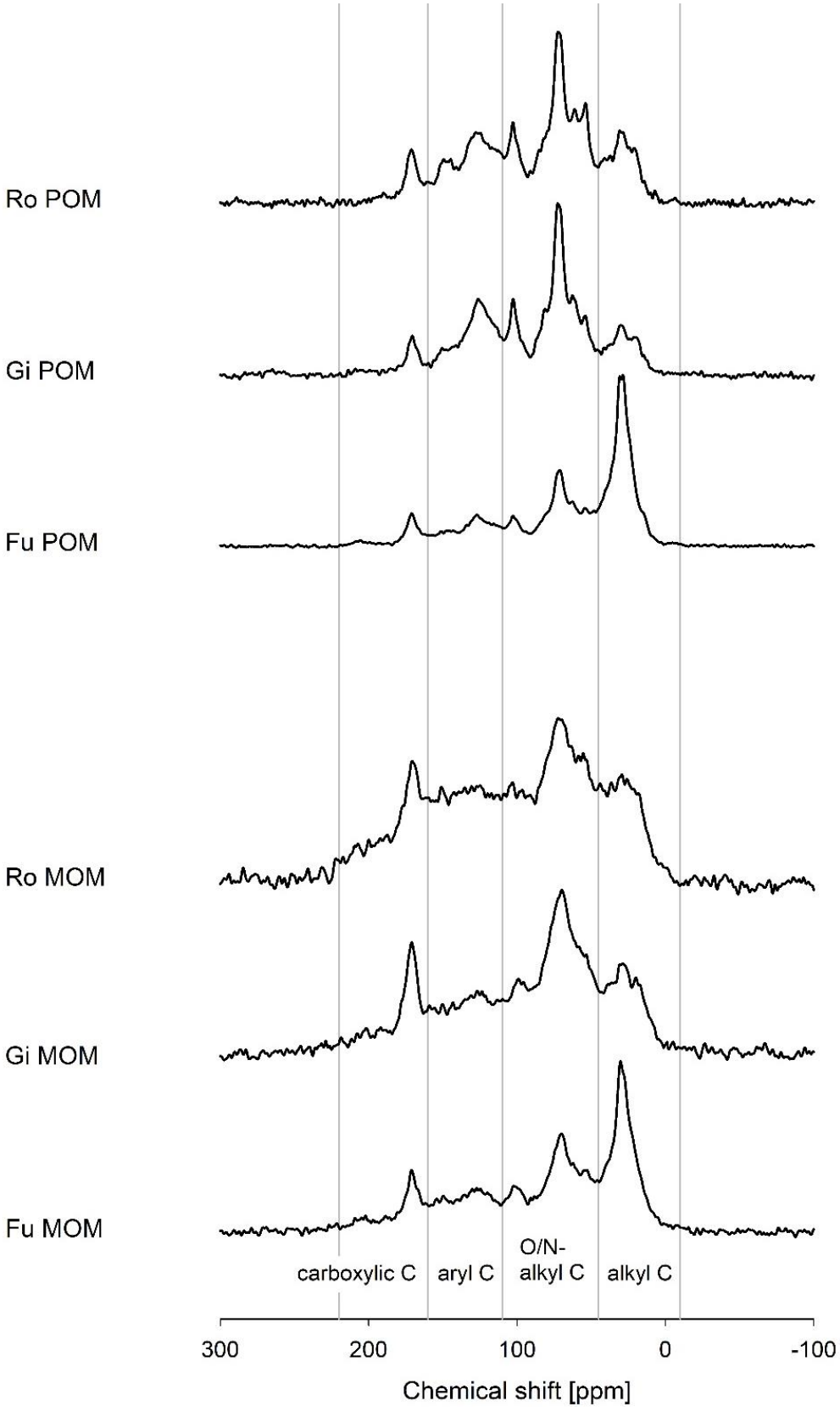
Time [min]	Temperature [°C]	Number of cycles
5:00	95	
0:30	95	
1:00	54	35
1:00	72	
10:00	72	
∞	8	



Appendix 4 Solid-state ^{13}C-CPMAS NMR spectra of plant residues (Surey et al., 2020b; Chapter 3).



Appendix 5 X-ray diffraction patterns of oriented clay specimens from studied topsoil material (a = Rotthalmünster, b = Giessen, c = Fuhrberg) (Surey et al., 2021; Chapter 4). Diffractograms were recorded using an X-ray diffraction device (D5005, Siemens AG, Karlsruhe, Germany) with Cu Kα-radiation (λ = 1.541 nm) in stepscan mode with a step size of 0.02 °2θ, fixed slits, and 10 s counting time. Clay samples were analyzed after removal of organic matter (by hydrogen peroxide) and pedogenic Fe oxides (by dithionite-citrate-bicarbonate extraction), and exposure to the following four treatments: Mg-saturated and solvated in ethylene glycol (MgEG), magnesium-saturated (Mg), potassium-saturated and heated to 550 °C (K550°C), and potassium-saturated (K). Peaks of identified minerals were labeled as follows: V = vermiculite, C = chlorite, I = illite, K = kaolinite, and Q = quartz.



Appendix 6 Solid-state ^{13}C-CPMAS NMR spectra of particulate and mineral-associated organic matter (POM, MOM) obtained by density fractionation from soils collected at Rotthalmünster – Ro, Giessen – Gi, and Fuhrberg – Fu (Surey et al., 2021; Chapter 4). A mixture of free and occluded POM (according to their mass contributions to bulk soils) was used to represent total POM.

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Publikationsliste *(List of publications)*

Surey R., Kaiser K., Schimpf C. M., Mueller C. W., Böttcher J. and Mikutta R., (2021): Contribution of particulate and mineral-associated organic matter to potential denitrification of agricultural soils. *Frontiers in Environmental Science* 9, https://doi:10.3389/fenvs.2021.640534.

Surey R., Schimpf C. M., Sauheitl L., Mueller C. W., Rummel P. S., Dittert K., Kaiser K., Böttcher J. and Mikutta R. (2020): Potential denitrification stimulated by water-soluble organic carbon from plant residues during initial decomposition. *Soil Biology and Biochemistry* 147, https://doi.org/10.1016/j.soilbio.2020.107841.

Surey R., Lippold E., Heilek S., Sauheitl L., Henjes S., Horn M. A., Mueller C. W., Merbach I., Kaiser K., Böttcher J. and Mikutta R. (2020): Differences in labile soil organic matter explain potential denitrification and denitrifying communities in a long-term fertilization experiment. *Applied Soil Ecology* 153, https://doi.org/10.1016/j.apsoil.2020.103630.

Lebenslauf *(Curriculum Vitae)*

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Datum: Unterschrift:

_____ _____

Eidesstattliche Erklärung *(Declaration under Oath)*

Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.
I declare under penalty of perjury that this thesis is my own work entirely and has been written without any help from other people. I used only the sources mentioned and included all the citations correctly both in word or content.

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