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

Ronny Surey*, Eva Lippoldb, Stefan Heilekc, Leopold Sauheitld, Sina Henjesc, Marcus A. Horn, Carsten W. Muelld and Ines Merbache

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 N2O and N2 as well as CO2 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 (13C-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 (N2O and N2) 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 N2O + N2 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 CO2 and N2O production were well related and up to three-fold larger for FYM-receiving soils than under pure mineral fertilization. The N2 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 N2 production in soils without FYM application, and thus, with less readily decomposable OM, coincided with decreasing abundances of nirS genes (NO2− 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.

1. Introduction

When oxygen (O2) is absent and organic carbon (OC) sources are available, denitrifying microorganisms reduce nitrate (NO3−) via a series of enzymatic steps to NO2−, NO, N2O, and finally N2 (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, N2O exhibits the largest warming potential of all biogenic greenhouse gases (298 times that of CO2) 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 N2O, the N2 production is rarely studied, due to the large background concentrations of N2 in air and water, rendering it difficult to detect N2 release by denitrification (Groffman et al., 2006). Complete denitrification to N2 still results in a net loss of N but has no such effect on climate change as N2O. Consequently, better understanding of factors controlling the N2O/N2 product ratio is of crucial importance for evaluating climatic effects by denitrification.

Denitrification in soil mainly occurs in anoxic microhabitats (‘hot spots’) where enough NO2− 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 CO₂ and N₂O 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., 2016; 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 N₂O 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 N₂ relative to N₂O.

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 N₂O/(N₂O + N₂) 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 N₂O. In addition, we surmise that the denitrifier community (abundances of NO₃⁻ and N₂O reductase genes) is directly linked to the amount and composition of OM fractions or their bioavailability.

2. Materials and methods

2.1. 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 (Supplementary Table S1). 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).

2.2. 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 NaHCO₃ (pH 8.5) solution were shaken for 30 min. After centrifugation (3000 × g) for 10 min (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). Nmin (NO₃⁻ and NH₄⁺) 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 CaCl₂ (1:5 wt./v). Air-dried and sieved bulk soils were analyzed for total C und N (TN) with a Vario Max Cube (Elementar Analyselectric GmbH, Langenselbold, Germany). Inorganic C was not detectable (Vario Max Cube, Elementar), therefore, total C was assumed to represent OC. The difference TN – Nmin 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. Solid-state ¹³C 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 ¹³C cross-polarization magic angle spinning NMR spectroscopy (¹³C-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 (Supplementary Fig. S1).

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2.4. 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 = IPOM), 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 1 h, the suspension was centrifuged (6800 × g) for 30 min at 20 °C (Cryofuge 8500i). The supernatant with the floating IPOM 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 IPOM 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 IPOM. 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 IPOM 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 (WEN) were determined on fresh soil samples. Briefly, 20 g of soil suspended in 100 ml deionized water were shaken for 1 h. After centrifugation (3000 × g) for 10 min (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).

2.5. Incubation and gas measurements

Bulk soils were anoxically incubated at 25 °C in the dark for seven days, as most N₂O production in soil occurs at timescales of less than two weeks (Kuz yakov 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, and then crimped with an aluminum cap (32 mm; Chroma Globe GbR, Kreuznau, Germany). An O₂-free atmosphere for anoxic incubations was achieved after gas sampling and assuming a microbial metabolism of < 2.5% of added C₅H₇ over seven days (Yeomans and Beauchamp, 1978), the C₅H₇ concentration was high enough (> 5% v/v) to prevent flushing with He and 1 h 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 C₅H₇ over seven days (Yeomans and Beauchamp, 1985), the C₅H₇ concentration was high enough (> 5% v/v) to prevent the reduction of N₂O to N₂ over the entire incubation period (Yeomans and Beauchamp, 1978). To estimate the N₂ production, the produced amount of N₂O was subtracted from the respective amount of N₂O in presence of C₅H₇ (representing N₂O + N₂). The ratio of the two N₂O amounts was used to determine the molar N₂O-N/(N₂O+N₂)-N ratio for each incubation day. Proportional NO₂⁻-N losses as N₂O-N and N₂ were calculated based on cumulative gas emissions within seven days and the initial (natural + added) NO₂⁻-N content. The portion of mineralized OC was derived by relating the cumulative CO₂-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.

2.6. DNA extraction and qPCR assay

Pre-incubated and incubated soil samples were frozen and stored at −20 °C prior to analyses of abundances of NO₃⁻ and N₂O reductase genes. While gene abundances in pre-incubated soils were mainly

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Table 1

<table>
<thead>
<tr>
<th>Fertilization treatment</th>
<th>pHf (CaCl₂)</th>
<th>Total OC (g kg⁻¹)</th>
<th>OC/ON ratio</th>
<th>NO₃⁻N [mg kg⁻¹]</th>
<th>Olsen P [mg kg⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.9–7.4</td>
<td>15.5 ± 0.4b</td>
<td>14.3 ± 0.4a</td>
<td>8.0 ± 1.2b</td>
<td>11.0 ± 5.2d</td>
</tr>
<tr>
<td>N</td>
<td>7.1–7.4</td>
<td>16.0 ± 0.3b</td>
<td>13.6 ± 0.6ab</td>
<td>14.7 ± 3.3ab</td>
<td>4.4 ± 0.9d</td>
</tr>
<tr>
<td>NP</td>
<td>5.5–7.2</td>
<td>16.5 ± 0.5b</td>
<td>13.6 ± 0.5ab</td>
<td>9.8 ± 2.6b</td>
<td>35.4 ± 3.6c</td>
</tr>
<tr>
<td>FYM</td>
<td>6.4–6.9</td>
<td>22.3 ± 0.5a</td>
<td>12.8 ± 0.3bc</td>
<td>13.9 ± 4.6ab</td>
<td>48.1 ± 3.4b</td>
</tr>
<tr>
<td>FYM + N</td>
<td>6.2–6.7</td>
<td>23.3 ± 1.6a</td>
<td>12.6 ± 0.3c</td>
<td>17.8 ± 4.6a</td>
<td>39.3 ± 4.1c</td>
</tr>
<tr>
<td>FYM + NP</td>
<td>6.1–6.7</td>
<td>22.3 ± 0.5a</td>
<td>12.7 ± 0.1c</td>
<td>18.9 ± 3.6a</td>
<td>57.5 ± 4.8a</td>
</tr>
</tbody>
</table>

* Range of replicated samples (n = 4).

3. Results

3.1. Chemical soil properties under different long-term fertilization

Different fertilization over 114 years altered the chemical soil properties (Table 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 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 1). The largest contents of KCl-extractable NO₃⁻N occurred in the manured (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 NO₃⁻N·kg⁻¹ dry soil, respectively. Extractable ammonium (NH₄⁺) was not detectable in any treatment.

3.2. Organic matter composition

The overall contribution of IPOM-OC and oPOM-OC to total soil OC was 1%–3% and 2%–7%, respectively (Table 2). Soils receiving FYM had about twice the content of IPOM (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).

Table 2

<table>
<thead>
<tr>
<th>Fertilization treatment</th>
<th>OC/ON ratio</th>
<th>NO₃⁻N [mg kg⁻¹]</th>
<th>Olsen P [mg kg⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.9–7.4</td>
<td>15.5 ± 0.4b</td>
<td>14.3 ± 0.4a</td>
</tr>
<tr>
<td>N</td>
<td>7.1–7.4</td>
<td>16.0 ± 0.3b</td>
<td>13.6 ± 0.6ab</td>
</tr>
<tr>
<td>NP</td>
<td>5.5–7.2</td>
<td>16.5 ± 0.5b</td>
<td>13.6 ± 0.5ab</td>
</tr>
<tr>
<td>FYM</td>
<td>6.4–6.9</td>
<td>22.3 ± 0.5a</td>
<td>12.8 ± 0.3bc</td>
</tr>
<tr>
<td>FYM + N</td>
<td>6.2–6.7</td>
<td>23.3 ± 1.6a</td>
<td>12.6 ± 0.3c</td>
</tr>
<tr>
<td>FYM + NP</td>
<td>6.1–6.7</td>
<td>22.3 ± 0.5a</td>
<td>12.7 ± 0.1c</td>
</tr>
</tbody>
</table>

* Range of replicated samples (n = 4).
Table 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).

<table>
<thead>
<tr>
<th>Fertilization treatment</th>
<th>WEOC [mg kg⁻¹]</th>
<th>WEON [mg kg⁻¹]</th>
<th>Proportion in total OC [%]</th>
<th>OC/TN ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>iPOM-OC</td>
<td>oPOM-OC</td>
<td>MOM-OC</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>23.0 ± 4.7c</td>
<td>4.4 ± 0.7b</td>
<td>2.1 ± 0.1ab</td>
<td>3.4 ± 1.0bc</td>
</tr>
<tr>
<td>N</td>
<td>28.5 ± 1.9bc</td>
<td>6.9 ± 1.5ab</td>
<td>1.8 ± 0.7ab</td>
<td>6.6 ± 1.4a</td>
</tr>
<tr>
<td>NP</td>
<td>28.8 ± 7.1bc</td>
<td>5.6 ± 0.9ab</td>
<td>1.3 ± 0.3b</td>
<td>2.1 ± 0.5c</td>
</tr>
<tr>
<td>FYM</td>
<td>37.9 ± 2.4ab</td>
<td>8.6 ± 1.7a</td>
<td>3.3 ± 1.1a</td>
<td>4.2 ± 1.1abc</td>
</tr>
<tr>
<td>FYM + N</td>
<td>38.6 ± 4.1ab</td>
<td>7.6 ± 2.3ab</td>
<td>3.3 ± 1.2a</td>
<td>5.5 ± 1.1ab</td>
</tr>
<tr>
<td>FYM + NP</td>
<td>39.8 ± 5.4a</td>
<td>8.7 ± 2.1a</td>
<td>3.2 ± 0.6a</td>
<td>6.0 ± 2.1ab</td>
</tr>
</tbody>
</table>

3.3. Initial and cumulative production of CO₂, N₂O, and N₂

Initial (after one day) as well as cumulative N₂O and CO₂ production within seven days were significantly higher for soils receiving FYM than for soils under pure mineral fertilization and fertilizer deprivation (Figs. 2 and 3). Additional N and P input (FYM + N, FYM + NP) resulted in the highest average cumulative N₂O and CO₂ production (7.3–7.4 mg N kg⁻¹ and 30.6–31.7 mg C kg⁻¹, respectively). The cumulative N₂O 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 (Fig. 2). The maximum cumulative N₂O 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 N₂O production trend as the FYM treatments but on a lower level (Fig. 2). The initial N₂ production was about 25% higher for mineral fertilization than for FYM application but the manured soils released almost 18% more N₂ over the entire seven days than soils receiving mineral fertilization (Fig. 4). There was no significant difference in the initial and cumulative N₂ production among all fertilization treatments, but the average cumulative N₂ production was significantly larger for all manured soils (n = 12) than for soils receiving no FYM application (n = 12). The cumulative amount of N₂ emitted from the control (unfertilized) was substantially less than from the fertilized soils. After seven incubation days, about 40% of the initial (natural + added) NO₃⁻ -N was lost as N₂O and N₂ 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 NO₃⁻-N, respectively. The cumulative CO₂-C production accounted for 23%–49% (non-manured soils) and 64%–82% (manured soils) of the initial WEOC content.

When pooling all fertilization treatments, we found a number of statistically significant relations between gas emissions with soil and OM properties: The initial N₂O and cumulative N₂ emissions over seven days correlated positively with the content of WEOC (r = 0.82 and 0.69, respectively, p < 0.001, n = 24). In addition, the cumulative N₂O emissions were highly correlated to the cumulative release of CO₂.

Table 3. Total release of nitrogenous gases (N₂O + N₂) and molar N₂O/(N₂O + N₂) 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).

<table>
<thead>
<tr>
<th>Fertilization treatment</th>
<th>Cumulative N₂O + N₂ [mg N kg⁻¹]</th>
<th>N₂O/(N₂O + N₂) ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After 1 day</td>
<td>After 7 days</td>
</tr>
<tr>
<td>Control</td>
<td>1.7 ± 0.4c</td>
<td>12.6 ± 0.3c</td>
</tr>
<tr>
<td>N</td>
<td>3.9 ± 0.5b</td>
<td>17.1 ± 1.7bc</td>
</tr>
<tr>
<td>NP</td>
<td>3.9 ± 0.2b</td>
<td>19.2 ± 3.3b</td>
</tr>
<tr>
<td>FYM</td>
<td>5.9 ± 1.3a</td>
<td>25.9 ± 3.5a</td>
</tr>
<tr>
<td>FYM + N</td>
<td>6.0 ± 0.7a</td>
<td>27.2 ± 2.4a</td>
</tr>
<tr>
<td>FYM + NP</td>
<td>5.5 ± 0.5a</td>
<td>26.1 ± 1.5a</td>
</tr>
</tbody>
</table>
the absolute contents of particulate or-
ing the WEOC content (r = 0.75, p < 0.001, n = 24). During seven days of anoxic incubation, the molar N₂O/(N₂O + N₂) ratio decreased for all treatments to 0.2–0.3 (FYM treatments), 0.01–0.1 (mineral fertilization), and 0.00 (control) (Table 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 3). There was no significant difference in denitrided N (25.9–27.2 mg kg⁻¹) within the seven incubation days between manured soils receiving no or additional mineral fertilizers. The cumulative N₂O + N₂ production over the seven days was again positively correlated with the WEOC content (r = 0.75, p < 0.001, n = 24).

3.5. Abundance of denitrifier genes

For pre-incubated soils, the different fertilization regimes had no distinct effect on the abundance of N₂O− reductase genes (nirS and nirK) and the N₂O reductase gene nosZ II (Fig. 6a–d). However, nosZ I (N₂O reductase) genes were significantly more abundant in soils receiving FYM than in soils under other fertilization regimes, even before anoxic incubation (Fig. 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% (Fig. 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 N₂O− reductase genes (nirS + nirK) and N₂O reductase genes (nosZ I + nosZ II) after anoxic incubation than soils receiving either no or only mineral fertilizers (Fig. 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 N₂O reductase gene at the end of incubation.

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, \( p < 0.001, \) \( n = 24; \) Fig. 7a, b). The abundance of \( \text{NO}_2^- \) reductase genes showed no relationship to the \( \text{N}_2\text{O} \) production. Also, the abundances of \( \text{N}_2\text{O} \) reduction genes were not significantly correlated to the production of \( \text{N}_2. \) In contrast, the initial abundances of \( \text{nosZ I} \) were highly correlated with the initial \( \text{N}_2\text{O} \) production \( (r = 0.90, p < 0.001, n = 24). \)

4. 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.

4.1. 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 \( \text{N}_2\text{O}/(\text{N}_2\text{O} + \text{N}_2) \) product ratios (Čuhel et al., 2010). However, except for NP, the soil acidity (pH) varied only slightly between fertilization treatments (Table 1). Accordingly, we observed no distinct relations between soil reaction and \( \text{CO}_2, \text{N}_2\text{O}, \) and \( \text{N}_2 \) 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 N2O and N2 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 IPOM as well as between the WEOC content and the production of N2O and N2 (Fig. 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 IPOM-OC to bulk soil OC suggests that the main part of soluble OC derived from IPOM, despite MOM-C the main portion of bulk soil OC (91%–97%). The observed negative correlation between WEOC and the OC/TN ratio of IPOM 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 CO2 and N2O 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 Brenner, 1993). The low and strikingly invariable WEOC/WEON ratios (4.3–5.5) suggest that those leachable components contained a large proportion of proteaceous 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 (Brenner and Shaw, 1958; Ottow, 2011). When pooling all fertilization treatments, the anoxic OC mineralization (CO2 production) was well related to the N2O production (Fig. 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 N2O/N2 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 N2O over the course of the incubation (Fig. 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 N2O production over the entire incubation period. In contrast, N2O was no longer produced in N-fertilized soils and the control after only a few days, along with the complete depletion of previously accumulated N2O at the end of incubation. The decreasing molar N2O/(N2O + N2) ratio over time was mainly due to continuous production of N2, while the gross N2O formation remained at low level and previously accumulated N2O was gradually reduced to N2. Although the average N2 production after seven days was significantly lower for all non-manured samples than for the FYM treatments, the differences in N2 emission between fertilization regimes were small (Fig. 4). This indicates that the N2O production was more strongly affected by the OM bioavailability than the N2 emission or the total denitrification rate. The loss of 22%–40% of the initial (natural + added) NO3-N as N2O or N2 during incubation and the CO2-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 (N2O + N2) was, therefore, significantly higher in soils with FYM application than in soils with mineral and no fertilization (Table 3). As hypothesized, the accumulation of readily decomposable OM in manured soils, reflected by higher portions of components rich in O/N-alkyl C, IPOM, and WEOC, resulted in increased denitrification potential with increased proportions of N2O. 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.

4.2. 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 (Fig. 6c). This observation may be ascribed to the fact that the higher C availability in manured soils results in faster C mineralization and O2 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 (Fig. 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 (Fig. 6c, d). One explanation for this observation could be that N2O 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 (Fig. 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 (Fig. 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 (Fig. 7b). Henderson et al. (2010) found higher \(nosZ\) I gene abundances in soils (soil loamy till; pH 6) amended with different POM materials than in soil amended with glucose, while the abundance of \(nirS\) gene-bearing denitrifiers (\(P.\ mandelli\) 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 (Fig. 6a). This suggests that the availability of WEOC not only determined the amount of denitrified \(N\) but also the \(N_2O/\left(N_2O+N_2\right)\) product ratio. Correspondingly, gross \(N_2O\) production still continued after seven days, resulting in higher molar \(N_2O/\left(N_2O+N_2\right)\) ratios for manured soils (0.2–0.3) than for soils under mineral fertilization (0.01–0.1; Table 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 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 \(N_2O\) and \(N_2\) 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.

5. 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 \(N_2O/\left(N_2O+N_2\right)\) 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 IPOM (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 IPOM. 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 IPOM 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.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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