Mitigating N_2O emission from arable soils

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Chapter 1: General introduction

1.1 N_2O emissions from arable lands

The increasing atmospheric nitrous oxide (N_2O) concentration is among the most serious consequences of anthropogenic alteration of the global nitrogen (N) cycle (Bakken and Frostegard, 2017). N_2O was beside carbon dioxide (CO_2), methane (CH_4) the most well-known greenhouse gas (GHG) which was induced by human activity (IPCC, 2013). The global warming potential of N_2O could be 265 time higher than CO_2 on a 100-year basis (Myhre et al., 2013). Moreover, N_2O is considered as the most important ozone destroyer in latest decades (Ravishankara et al., 2009). The atmospheric N_2O concentration in 2017 was 330 ppb and increased by about 21% than pre-industrial level (WMO, 2018). Globally, the largest source of N_2O emissions were soil ecosystems, which was estimated at 6.8 Tg N_2O-N yr^{-1}, comprising 65% of total atmospheric N_2O emission (IPCC, 2006). Among them were 4.2 Tg N_2O-N yr^{-1} were derived from synthetic nitrogen fertilization and its indirect emissions.

The most important indictors of N_2O emission above all is the input of N in the soil and its subsequent availability, therefore emission factors (EF) was commonly used to construct most national GHG inventories (Shcherbak et al., 2014). EF of N_2O is defined as the percentage of fertilizer N that is transformed into N_2O emissions. Intergovernmental Panel on Climate Change (IPCC) suggests that synthetic fertilizer-induced N_2O was round to 1%. The fertilizer application on a global scale is probably to increase, to feed the increasing world population (IPCC, 2006; van Beek et al., 2010). N_2O emissions therefore are likely continue to increase in the coming decades (Reay et al., 2012).

The most widely used synthetic N fertilizers are urea and urea-containing N fertilizers. Urea accounts for about 56% of the global production (Bremner, 2007; International Fertilizer Industry Association, 2013; Suter et al., 2016). Urea is a solid fertilizer with a high N content (46%). It can easily be stored and applied to crops and it can be added to the soil in combination with other N fertilizers. Calcium ammonium nitrate (CAN) was beside urea another important fertilizers. Ammonium nitrate mixed with urea was named urea ammonium nitrate (UAN), which is a liquid

N fertilizer consisting of 50% urea and 50% ammonium nitrate and ranging from 28 to 32% N by weight.

The other side of the coin is the low N use efficiency (NUE). NUE in agriculture was usually lower than 50% by crops (Drury et al., 2017; Galloway et al., 2003; Sun et al., 2015). About 25% of the urea applied to the soil surface is converted to ammonia (NH_3) and volatilized to the atmosphere (FAOSTAT, 2015). NH_3 have an indirect impact on climate change, because of its relation with N_2O, It is thought that about 1–2% of gaseous NH_3 is converted into N_2O (Wulf et al., 2002). Besides, NH_3 is known to cause acidification and eutrophication of both soils and surface (Jongebreur and Voorburg, 1992; Simpson et al., 2012). Therefore, agricultural managements to increase the NUE along with crop yield, is in other way reducing N_2O emissions from agriculture.

1.2 N_2O productions pathways in soil

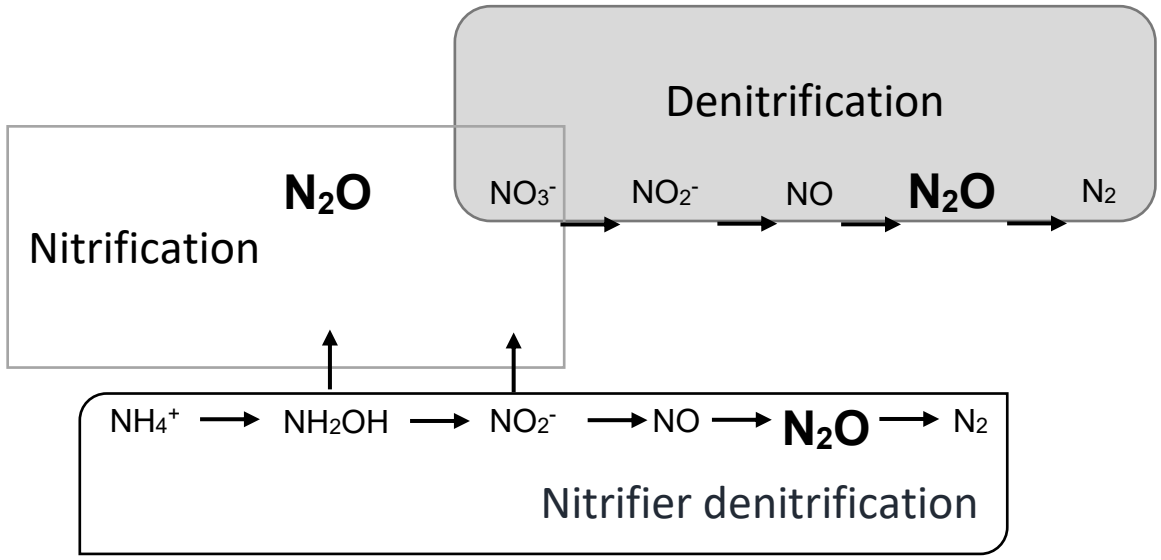

Fig. 1: The most important pathways of nitrous oxide production in arable lands (after Wrage et al., 2001, modified)

The most dominant biological process forming N_2O from mineral N substrates in arable lands are nitrification, denitrification (Bremner, 1997) and nitrifier denitrification (Wrage-Mönnig et al.,

2018). In arable lands, nitrification-related pathways was considered as the principal sources of N_2O emission under water-limited conditions. At higher water contents, denitrification became the major source of soil N_2O emissions (Mathieu et al., 2006). The widely accepted threshold of aerobic and anaerobic conditions was 60% (Menéndez et al., 2012; Volpi et al., 2017). However, the "threshold" may shift by soil types, because soil physical properties, such as soil porosity and pore size distribution, which can affect the diffusion of O_2 into the soil were determined by soil types (Butterbach-Bahl et al., 2013). There are other microbial processes, such as anaerobic ammonium oxidation (annammox) and dissimilatory nitrate reduction to ammonium (DNRA, or nitrate ammonification) are only occasionally important in particular cases.

Nitrification is an aerobic process, which needs the presence of O_2 that performed as a terminal electron acceptor (Zaman et al., 2012). In this process, Ammonium (NH_4^+) was stepwise oxidized to nitrate (NO_3^-) ($NH_3 \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow NO_3^-$). Different groups of prokaryotes was involved in each steps. The first step was ammonia oxidation, was catalyzed by the ammonia monooxygenase (AMO), which was encoded by amoA gene. Two distinctive microbial groups participates ammonia oxidation, namely ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). (Hu et al., 2015). Ammonia-oxidation was considered as rate-limiting step of the whole nitrification process (Kowalchuk and Stephen, 2001). N_2O was normally considered as a byproduct of nitrification (Hu et al., 2015).

Nitrifier denitrification ($NH_3 \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O$) was also ordered to nitrification-related pathways in Hu et al. (2015), as long as the first step of nitrifier denitrification was also ammonia oxidation. Nitrifier denitrification was also supposed to counteract the toxic effect of nitrite (NO_2^-) accumulation during nitrification, and to decrease the competition of NO_2^- removal by nitrite-oxidizing bacteria (NOB) (Beaumont et al., 2004, 2002). Nitrifier denitrification can dominate N_2O production under O_2 limitation or variable O_2-concentrations, and a high NO_2^- concentration might plays a key role of nitrifier denitrification (Wrage-Mönnig et al., 2018). Soil temperature and organic C availability can also affect nitrifier denitrification, however, the mechanisms are not yet fully understood (Wrage-Mönnig et al., 2018).

Denitrification was the most explored biological process involved in N_2O production, it is widely agreed that denitrification was a major source of N_2O emission, especially with a higher soil

moisture. In the denitrification pathway, denitrifying microorganisms use NO_3^- as an electron acceptor and stepwise reduce it to gaseous N_2. N_2O was also considered as intermediaries resulted by incomplete denitrification. Therefore, reduce the N_2O/N_2 ratio of soil denitrification was a possible approach to reduce N_2O emissions (Schlesinger, 2009).

Each step of soil denitrification is regulated by enzymes such as NO_3^-, nitrite (NO_2^-), and N_2O reductase that are encoded by different functional genes carried by microorganisms (Philippot et al., 2007). For instance, the first step was regulated by NO_3^- reductase, periplasmic nitrate reductase is encoded by *nap* and membrane-bound NO_3^- reductase is encoded by *nar* (Bru et al., 2007). NO_2 reductase has two functionally equivalent type, a copper- and a cytochrome cd_1-containing NO_2 reductase are encoded by the *nirK* and *nirS* gene, respectively (Braker et al., 2000; Henry et al., 2004). The final step of denitrification, is catalyzed by N_2O reductase which is encoded by the *nos* gene, controls the reduction of N_2O to N_2. *nos*-mediated N_2O to N_2 reduction is the only known microbial process to reduce N_2O in the biosphere (Jones et al., 2013; Philippot et al., 2007). Thus, increased *nos* abundances in soils may indicate a more complete denitrification and low N_2O/N_2 ratio, and possibly reduced N_2O emission (Jones et al., 2013). Recently findings of microorganisms equipped with *nos* but not the other genes involved in denitrification, can be a valuable contributors to the soil N_2O sink capacity (Jones et al., 2014). Apart bacterial denitrification, fungal denitrification was also reported as a major source of soil N_2O emissions in various of studies (Shoun Hirofumi et al., 2012; Thamdrup, 2012). It was believed to be an important source of N_2O emission, because fungal genomes usually lack the *nos* gene, therefore N_2O was the final product of many fungal denitrifiers (Baggs, 2011; Philippot et al., 2011).

1.3 Enhanced efficiency fertilizers to mitigate N_2O emission

Good agricultural practice is a possible way to maximize N use efficiency, e.g. the correct application techniques, good timing and soil testing to determine the amount of fertilizer required. But agricultural practices always constrained by physical conditions. In last several decades, several enhanced efficiency fertilizers were developed to increase soil N availability and to decrease N loss (Chen et al., 2008; Li et al., 2018). A number of chemical products have been developed to delay the transformation of N in the soil, to better synchronize fertilizer N release with crop uptake (Li et al., 2018) and these can be added to urea and UAN. Two main categories of these slow-release products are urease inhibitors and nitrification inhibitors.

Urease inhibitors are usually added to urea, the aim of urease inhibitors is to reduce the activity of the urease enzyme and slow the rate of urea hydrolysis (Sommer et al., 2004). When urea is applied to the soil, it rapidly hydrolyzes to ammonium carbonate ($(NH_4)_2CO_3$). $(NH_4)_2CO_3$ is unstable and breaks down to NH_3 and CO_2. The NH_3 is either absorbed by the soil or volatilizes. The hydrolysis reaction is catalyzed by urease enzyme. However, urease enzyme can be blocked by urease inhibitors. The conversion of urea to NH_3 was delayed for a period of 1–2 weeks, allowing time for the incorporation of NH_3 into the soil and utilized by plant uptake. The most effective compounds to inhibit urease were phosphoryl amides (Bremner and Chai, 1989; McCarty et al., 1989). from that *N*-(*n*-butyl) thiophosphoric triamide (NBPT) was the most widely used product, and it was proved by many studies that it can effectively prevent the loss of NH_3 (Drury et al., 2017; Liu et al., 2017; Mira et al., 2017; Silva et al., 2017; Tian et al., 2015).

Nitrification inhibitors was intended to decrease the enzymatic activity of NH_3 oxidizing bacteria (Ruser and Schulz, 2015). With the addition of nitrification inhibitors to urea, the conversion of ammonium ions (NH_4^+) to NO_3^- is prevented. Hence, soil NO_3^- leaching and the production of N_2O emissions from denitrification was also prevented. The most extensively studied compounds are nitrapyrin (Belser and Schmidt, 1981; Habibullah et al., 2018; Wolt, 2004; Zacherl and Amberger, 1990), Dicyandiamide (DCD) (Di et al., 2014; Guo et al., 2014; Liu et al., 2017; Zaman et al., 2013) and 3,4-dimethylpyrazol-phosphate (DMPP) (Liu et al., 2015; Pasda et al., 2001; Rose et al., 2018; Shi et al., 2017). In Germany, Piadin (1H-1, 2, 4-triazole and 3-methylpyrazole) and Vizura (containing DMPP) are more often used commercial products. Although only a few studies focused on Piadin, but it has also been shown to be effective in reducing N_2O emissions (Pietzner et al., 2017; Wolf et al., 2014; Wu et al., 2017).

1.4 Impact of plant on N_2O emissions

The presence of plant and its rhizosphere modifies the major factors regulating nitrification and denitrification: carbon, NO_3^- and oxygen. It was estimated that 5-21% of photosynthesis assimilated carbon (C) is released into the soil as root exudates (Derrien et al., 2004; Nguyen, 2003). The intensity of C turnover processes in rhizosphere are estimated to be at least one order of magnitude greater than in the bulk soil (Kuzyakov, 2010). As root-released C served as an electron donor (Philippot et al., 2007), root exudates was also supposed to increase denitrification activity (Bijay-singh et al., 1988). Most of the root exudates were easily available for soil microbes, and

can be metabolized within a few hours (Fischer and Kuzyakov, 2010; Jones et al., 2005; Jones and Kielland, 2002; Kuzyakov and Xu, 2013). Therefore, soil microbial community, for example denitrifying microbes, can be several times greater in rhizosphere, compared to bulk soil (Chèneby et al., 2004; Herman et al., 2006). However, until now, how the diversity of denitrifiers and the expression of denitrification genes are affected by root exudates was still little understood (Henry et al., 2008)

There was an intense competition for mineral N between plant roots and soil microorganisms (Kuzyakov and Xu, 2013). The availability of mineral N in soils is considered as the major factor limiting nitrification and denitrification (Philippot et al., 2007; Saggar et al., 2013). The uptake of ammonium (NH_4^+) by plants can lead to strong depletion zones of NH_4^+ in the rhizosphere (Orcutt, 2000). In contrast, depletion zones of NO_3^- in the rhizosphere are less pronounced, due to its high mobility within most soils (Kuzyakov and Xu, 2013). However, the concentration of NO_3^- in soil can rapidly be decreased by root uptake (Tinker and Nye, 2000). Likewise, regulatory functions of soil NO_3^- on denitrifying soil communities were reported from different ecosystems (Correa-Galeote et al., 2017; Deiglmayr et al., 2006; Enwall et al., 2005). The effect of different soil NO_3^- concentrations on the abundance and diversity of denitrifiers in soils, however, still remains to be elucidated (Correa-Galeote et al., 2017).

The effect of plants on oxygen is more complex (Philippot et al., 2007). On the one hand, oxygen depleting zone emerges in the rhizosphere by respiration of the roots and soil microbes (Bakken, 1988; Hayashi et al., 2015). On the other hand, soil gas exchange and oxygen concentration was increased, due the consumption of water by plant roots (Philippot et al., 2007). How the changed O_2 concentration in rhizosphere affect soil denitrification, still received contradictory conclusions (Chantigny et al., 1996; Klemedtsson et al., 1987; Morley et al., 2008; Prade and Trolldenier, 1988).

1.5 Objectives

The present study aimed mitigating N_2O emission in arable lands. Enhanced efficiency fertilizers were an important approach to achieve this goal, but the effectiveness suffers high uncertainty. Both incubation and field experiment are important tools to evaluate the effectiveness of urease and nitrification inhibitors, but both have their advantages and drawbacks. Hence, our objective are:

1. To evaluate the effectiveness of NBPT, Piadin and a new inhibitor NZONE MAX, on reducing NH_3 and N_2O emissions, under laboratory conditions.
2. To assess the effect of DMPP and NBPT on grain yield and reduction of N_2O emission in a wheat- wheat- oilseed rape rotation system, with a two-year field experiment.
3. With the comparison of unplanted and planted soils, we try to understand how the presence of *Lolium perenne* affect soil C and N dynamics, N_2O emissions, and soil denitrifying communities.

1.6 Experimental concept

The study includes both incubation and field experiments. The two-year field experiment was conducted on Reinshof agricultural research station, University of Goettingen, Lower Saxony, Germany (51°29‘50.3‘‘N, 9°55‘59.9‘‘E). Soil for incubation experiment was also collected from Reinshof research station. Mean annual precipitation: 651 ± 24 mm, mean annual temperature: 9.2 ± 0.1 °C (1981 – 2010, meteorological station at Goettingen, station ID: 1691, Germany's National Meteorological Service). The soil is classified as a Luvisol (IUSS, 2015) and the texture of the topsoil (0–25 cm) was classified into 61% silt, 23% sand, and 16% clay, with a 2% of total C. The bulk density is 1.3 g cm^{-3}, the soil pH was 7.1 ± 0.1 in all measured samples. Gas collection use closed chamber methods. The chamber volume varies in different experiments, but the basic idea is with a closed, air-tight chamber inserted on the soil, soil emitted spur gas for example CO_2, N_2O and CH_4 accumulates in the chamber, we collect the gas samples at 0, 20 and 40 min after the enclosure, then we measure CO_2, N_2O and CH_4 concentration on gas chromatograph (GC), later we use linear regression (Parkin et al., 2012) to calculate the gas flux rates. In all experiments, soil samples were taken with auger, and then stored at -20°C until further analysis. Following parameters were measured later: Water filled pore space (WFPS), soil ammonium (NH_4^+) and nitrite (NO_3^-), soil pH, total C and N, dissolved organic carbon (DOC) analyses and the copy number of bacterial 16S rRNA genes, fungal 18S rRNA genes, *narG, napA, nirK, nirS, nosZ* clade I and *nosZ* clade II.

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Chapter 2: Use of urease and nitrification inhibitors to reduce gaseous nitrogen emissions from fertilizers containing ammonium nitrate and urea

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Use of urease and nitrification inhibitors to reduce gaseous nitrogen emissions from fertilizers containing ammonium nitrate and urea

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ABSTRACT

Nitrogen (N) fertilizers increase agricultural yields, but also lead to the release of the greenhouse gases nitrous oxide (N_2O) and ammonia (NH_3). This not only reduces the efficiency of N use, but also results in climate change and loss of biodiversity. The use of nitrification inhibitors may improve the efficiency of N use and reduce the emission of greenhouse gases. We tested three inhibitors (NZONE MAX, Piadin and *N*-(*n*-butyl) thiophosphoric triamide (NBPT)) added to two common N fertilizers (urea and urea ammonium nitrate (UAN)) and determined emissions of CO_2, N_2O and NH_3 to evaluate the effectiveness of these three inhibitors and to improve our understanding of the soil nitrogen cycle. NBPT effectively reduced NH_3 volatilization by 50% (from 3.0 g NH_3-N m^{-2} in urea alone to 1.4 g NH_3-N m^{-2} in urea + NBPT). Piadin decreased N_2O emissions (from 0.98 g N_2O-N m^{-2} in urea alone to 0.15 g N_2O-N m^{-2} in urea + Piadin and from 0.81 g N_2O-N m^{-2} in UAN alone to 0.39 g N_2O-N m^{-2} in UAN + Piadin) by inhibiting the conversion of NH_4^+ to NO_3^-. However, although Piadin was found to be an effective nitrification inhibitor, the risk of higher NH_3 emissions (from 3.0 g NH_3-N m^{-2} in urea alone to 4.5 g NH_3-N m^{-2} in urea + Piadin) with the addition of Piadin cannot be neglected in environmental and economical evaluations.

1. Introduction

Large-scale inputs of nitrogen (N) fertilizers in agriculture have increased crop yields worldwide, allowing global agricultural production to keep pace with the rapidly growing population (Burney et al., 2010). The global use of N fertilizers is unlikely to decrease while the world's population continues to increase (Bakken and Frostegard, 2017; van Beek et al., 2010). The most widely used synthetic N fertilizers are urea and urea-containing N fertilizers. Urea accounts for about 56% of the global production of N fertilizers (Bremner, 2007; International Fertilizer Industry Association, 2013; Suter et al., 2016). Urea is a solid fertilizer with a high N content (46%). It can be stored and applied to crops easily and it can be added to the soil in combination with other N fertilizers. A common urea-containing fertilizer is urea ammonium nitrate (UAN), which is a liquid N fertilizer consisting of 50% urea and 50% ammonium nitrate and ranging from 28% to 32% N by weight.

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The efficiency of N use is often low (Sun et al., 2015), and typically <50% of the applied N fertilizer can be used by a corn crop owning to environmental and management constraints (Drury et al., 2017). About 25% of the urea applied to the soil surface is converted to ammonia (NH_3) and volatilized to the atmosphere (FAOSTAT, 2015); also, the rate of NH_3 volatilization may be even higher at warm temperatures and under moist soil conditions (Camberato, 2017; Tasca et al., 2011). Such large losses of N not only constitute an economic loss for farmers, but are also an important source of greenhouse gases. NH_3 is known to cause acidification and eutrophication of both soils and surface waters, and may also have an indirect impact on Earth's climate owning to its short lifetime in the atmosphere and its relationships with other climate-relevant gases, such as N_2O (Pietzner et al., 2017). It is estimated that about 1%–2% of volatilized NH_3 is later on converted into N_2O (Wulf et al., 2002). The effect of the emission of N_2O on the atmosphere might be one of the most serious environmental consequences of N fertilizer losses (Bakken and Frostegard, 2017), as it contributes to both global warming and the depletion of the ozone layer (Erisman et al., 2007; Ravishankara et al., 2009). About 70% of N_2O and 90% of NH_3 emissions are caused by agricultural activities (Boyer et al., 2002; Zaman and Blennerhassett, 2010). Therefore, improvement in the efficiency of N use is not only a question for policymakers aiming to meet the demands of the United Nations Framework Convention on Climate Change (the Kyoto Protocol) to estimate anthropogenic greenhouse gas emissions (UNFCCC, 1997), but may also increase profits for farmers.

To increase the efficiency of N use, in addition to good agricultural practices (e.g. the correct application techniques, good timing and soil testing to determine the amounts of fertilizer required, which may be constrained by physical conditions), the use of N stabilizers and nitrification inhibitors may potentially delay detrimental processes such as the volatilization of NH_3, the leaching of nitrate (NO_3^-) and the reduction of N_2O emissions. A number of chemical products have been developed to delay the transformation of N, and these can be added to urea and UAN. These slow-release products are classified as (1) urease inhibitors or (2) nitrification inhibitors (Franzen, 2017):

(1) Urease inhibitors. When urea is applied to the soil, it rapidly hydrolyzes to ammonium carbonate. Ammonium carbonate is unstable and breaks down to NH_3 and CO_2. The NH_3 is either absorbed by the soil or volatilizes. The hydrolysis reaction is determined by the urease enzyme, and urease inhibitors block this enzyme to prevent the conversion of urea to NH_3 for a period of 1–2 weeks, allowing time for the incorporation of urea into the soil by rainfall or other means. Many reports have shown that *N*-(*n*-butyl) thiophosphoric triamide (NBPT) can effectively prevent the loss of NH_3 (Drury et al., 2017; Liu et al., 2017; Mira et al., 2017; Silva et al., 2017; Tian et al., 2015).
(2) Nitrification inhibitors. The enzymatic activity of NH_3 oxidizing bacteria is strongly affected by nitrification inhibitors (Ruser and Schulz, 2015). With the addition of nitrification inhibitors to urea, the conversion of ammonium ions (NH_4^+) to NO_3^- is delayed, possibly also limiting N_2O emissions from soil denitrification. Dicyandiamide (DCD) (Di et al., 2014; Guo et al., 2014; Liu et al., 2017; Zaman et al., 2013) and 3,4-dimethylpyrazol-phosphate (DMPP) (Liu et al., 2015; Rose et al., 2018; Shi et al., 2017) are the most researched compounds and are effective in reducing N_2O emissions. In Germany, however, Vizura (containing DMPP) and Piadin (1H-1, 2, 4-triazole and 3-methylpyrazole) are more often used as nitrification inhibitors, and Piadin has also been shown to be effective in reducing N_2O emissions (Pietzner et al., 2017; Wolf et al., 2014; Wu et al., 2017).

To find new, effective chemical ingredients, novel fertilizer additives should also tested—for example, NZONE MAX (also called a penetrant/nitrogen management aid), which has only been mentioned in a few informal reports. NZONE MAX contains 27.5% alkylarylpolyoxyethylene glycol, 7.25% calcium aminoethylpiperazine and 6.5% calcium heteropolysaccharides. NZONE MAX is an ammonium stabilizer intended to open the exchange sites on the soil colloid and improve the attachment of NH_4^+ to soil colloids. Therefore the loss of N by volatilization, leaching and denitrification can be reduced.

Although there has been a wealth of studies on urease (e.g. NBPT) and nitrification (e.g. DMPP and DCD) inhibitors, new compounds still require research. The effectiveness of inhibitors in reducing NH_3 and N_2O emissions in different types of soil and in different climates is variable. As a result of the complex interactions between N_2O and NH_3 emissions, the mitigation of one gas flux may enhance the emission of another; so, apart from losses by leaching and runoff, both N_2O and NH_3 fluxes need to be considered in environmental evaluations (Ferm et al., 2006; Webb et al., 2010). Therefore, more experimental data about the emissions of NH_3 and N_2O when using new inhibitors are needed. To improve our knowledge of the environmental impact of different inhibitors, we conducted a pot experiment using urea and UAN as N fertilizers, and using NBPT, Piadin and NZONE MAX as N additives, and measured their effects on greenhouse gas emissions. We used analyses of CO_2, N_2O and NH_3 emissions to evaluate the effectiveness of these three chemical additives in improving the efficiency of N use and their environmental impact. Our hypotheses were: (1) the urease inhibitor NBPT can effectively reduce NH_3 emissions; (2) the nitrification inhibitor Piadin can effectively reduce N_2O emissions; and (3) NZONE MAX will decrease NH_3 volatilization and N_2O emissions when used as an additive.

2. Materials and methods

2.1. Soil properties and sample preparation

A loamy loess soil was collected from Reinshof agricultural research station, University of Goettingen, Lower Saxony, Germany (51°29′50.3″N 9°55′59.9″E, 155m asl). The annual mean temperature and mean annual precipitation were 8.5 °C

Table 1
Soil properties (0–25 cm depth) of the soil used in the pot experiments, cited from Roemer et al. (2015).

Clay (%)	Silt (%)	Sand (%)	Organic matter (%)	Bulk density (g cm^{-3})	pH ($CaCl_2$)
16	61	23	2.0	1.30	7–7.2

and 650 mm, respectively. The soil was classified as Luvisol (IUSS, 2015) and the texture of the topsoil (0–25 cm) is described in Table 1 (Römer et al., 2015). It had previously been used for a three-year field rotation consisting of winter barley (*Hordeum vulgare*) (2013–2014), winter oilseed rape (*Brassica napus*) (2014–2015) and winter wheat (*Triticum aestivum*) (2015–2016). The soil was collected on 4th April 2016 and stored in a container for three months before incubation. Before use, the soil was passed through a 2-mm sieve. The soil taken from the field had a moisture content of 30% water-filled pore space (WFPS), which was adjusted to a WFPS of 55% (equivalent to a 60% water holding capacity) at the start of the experiment. White rectangular polypropylene buckets with dimensions of 0.39 m (length) × 0.29 m (width) x 0.27 m (height) and an air-tight lid were used as the incubation system. The soil column therein was 16.5 cm high and consisted of three layers of soil adjusted to a soil bulk density of 1.30 g cm^{-3}. There was a 10-cm headspace above the soil surface when the air-tight lid was closed. The soil was pre-incubated in the buckets at 25 °C for 5 days before the addition of fertilizers. All experiments were conducted under the same controlled environmental conditions.

2.2. Experimental treatments

The experiment consisted of eight treatments (including CK, U, U + NZ, U + P, U + NBPT, UAN, UAN + NZ, UAN + P, described in Table 2) and four replicates. The total amount of N applied to each pot, except the control treatment, was 12 g N m^{-2} (corresponding to 120 kg N ha^{-1}). The calculated amount of fertilizer added to each pot was only 2.066 g of urea or 2.64 ml of UAN and therefore the required amount of inhibitors was very small. The inhibitors were bought in liquid form and diluted according to the manufacturer's recommendations. The fertilizers and diluted inhibitors for each pot were dissolved in 7.5 ml of water and the required volume of liquid was applied evenly to the soil surface using a pipette.

2.3. Gas flux measurements

2.3.1. Measurement of CO_2 and N_2O emissions

Trace gas concentrations of gas samples were analyzed after manual gas sampling from each closed chamber. Lids on the top of the buckets were sealed and samples were taken via silicon stoppers therein. Samples were taken using 60-ml syringes and then 30 mL of gas was transferred into evacuated 12-ml Exetainer vials (Labco, Lampeter, UK). Samples were taken at 0, 20 and 40 min after the chambers had been sealed and measurements were taken each day during the first week, then every two or three days for a period of one month. Gas samples were analyzed on a BRUKER SCION™ 456 gas chromatograph (BRUKER, Bremen, Germany) equipped with electron capture detection for analysis of N_2O, a flame ionization detector for CH_4 and a thermal conductivity detector for CO_2 analysis. Flux rates were calculated with linear or non-linear regression of the gas concentration with time (Parkin et al., 2012; Wang et al., 2013). Cumulative emissions were calculated by linear interpolation.

2.3.2. Measurement of NH_3 emissions

NH_3 emissions were determined by the Dräger tube method (Pacholski et al., 2006) using an X-act 5000 automatic tube pump (Dräger, Kiel, Germany). Four gas collection cylinders were inserted into the soil surface within each bucket and emitted gases were extracted through the tube pump and flushed through NH_3 color indicator-equipped NH_3 absorber tubes (Dräger Safety, Lübeck, Germany). The measured concentrations were converted from ppm into absolute values (kg N ha^{-1}) and the NH_3 fluxes were calculated as reported by Pacholski et al. (2006). Measurements were taken each day during the first week, then every two or three days for a period of one month.

2.4. Additional parameters

On the first day of the experiment, the soil moisture was adjusted to a WFPS of 55% and fertilizer was added. This corresponds to typical spring time moisture conditions when soils tolerate management measures such as fertilizer spreading by

Table 2
Total mineral N (g N m^{-2}) additions and added inhibitors in different treatments.

	CK	U	U + NZ	U + P	U + NBPT	UAN	UAN + NZ	UAN + P
NO_3^--N	0	0	0	0	3	3	3	3
NH_4^+-N	0	12	12	12	9	9	9	9
Added Inhibitors	0	0	NZONE MAX	Piadin	NBPT	0	NZONE MAX	Piadin

CK: control without fertilization, U: urea, NZ: NZONE MAX, P: Piadin, NBPT: N-(n-butyl) thiophosphoric triamide, UAN: urea ammonium nitrate.

agricultural machinery. The moisture decreased to a WFPS of 51% on day 5. Then, simulating a rainfall event, it was adjusted to a WFPS of 80% to stimulate high N_2O emission rates under oxygen depleted soil conditions. By the end of the experiment WFPS had decreased to 60%.

Soil samples were taken before application of fertilizers and at the end of the experiment (30 days later) to determine the soil moisture content and the concentration of mineral N (NO_3^-, NH_4^+). 50 g soil samples were dispersed in 250 ml of 0.0125 mol L^{-1} $CaCl_2$ solution, shaken for 1 h and filtered for later analysis with a San++ continuous flow analyzer (Skalar Analytical, Breda, The Netherlands).

2.5. Calculations and statistical analysis

Emission rates are expressed as arithmetic means ± the standard error of the mean of four replicates. Least significance difference tests were used to check significant pairwise differences among the treatments. Statistical analyses were performed using Statistica 11 (Dell, Round Rock, TX, USA), with $p < 0.05$ as the criterion for a statistical significance.

3. Results

3.1. CO_2 emissions

The time course of the CO_2 emissions showed that all added fertilizers induced a significant increase in respiration before the simulated rainfall/irrigation (Fig. 1A and B). Before irrigation (<55% WFPS), all fertilized treatments had almost the same CO_2 emission rates, and only on day 2 and 3 did they differed from the control treatment. After irrigation to a WFPS of 80%, the CO_2 emissions were much lower, suggesting that the simulated irrigation affected the microbial activity (Fig. 1A and B). The soil respiration rate began to increase again after a few days, and the differences between treatments were more distinct. In the urea series, a reduction in CO_2 emissions only occurred after addition of the nitrification inhibitor Piadin. The addition of NZONE MAX and NBPT did not decrease the emission of CO_2. In the UAN series, neither the addition of Piadin nor NZONE MAX reduced CO_2 emissions. In fact, even slightly higher emission rates were observed (Fig. 1A and B).

The treatment with urea plus Piadin (U + P) resulted in significantly lower cumulative CO_2 emissions (Fig. 2). They were 38% lower than the treatment without Piadin. The other inhibitors did not lead to significant reductions in cumulative CO_2 emissions compared with the N fertilizer treatments without an inhibitor.

3.2. N_2O emissions

N_2O emissions were low in all treatments from the onset of fertilizer treatment to day 5. Upon irrigation the WFPS reached 80% at day 5, N_2O fluxes increased strongly and the emissions from treatments U + P and UAN + P rose to significantly higher levels than those of the other treatments (Fig. 3A and B). Fig. 3 (A and B) shows a remarkable reduction in N_2O emissions in treatments U + P and UAN + P after day 5. Cumulative emissions of N_2O from soil treated with urea alone amounted to 0.98 g N_2O-N m^{-2}, whereas N_2O emission from U + P was only 0.15 g N_2O-N m^{-2}; therefore, the use of Piadin reduced N_2O emissions by >80% (Fig. 4). In the UAN series, the emissions from the UAN + P (0.39 g N_2O-N m^{-2}) treatment was about 48% of that from UAN alone (0.81 g N_2O-N m^{-2}). The cumulative N_2O emissions from U + NBPT (0.67 g N_2O-N m^{-2}) was 31% lower than from the treatment with urea alone (0.98 g N_2O-N m^{-2}) (Fig. 4), although it was not significant at $p < 0.05$. The addition of NZONE MAX did not show any reduction in N_2O emissions in either fertilizer series. The emission rate was higher with urea + NZONE MAX (U + NZ) than with urea alone (Fig. 4).

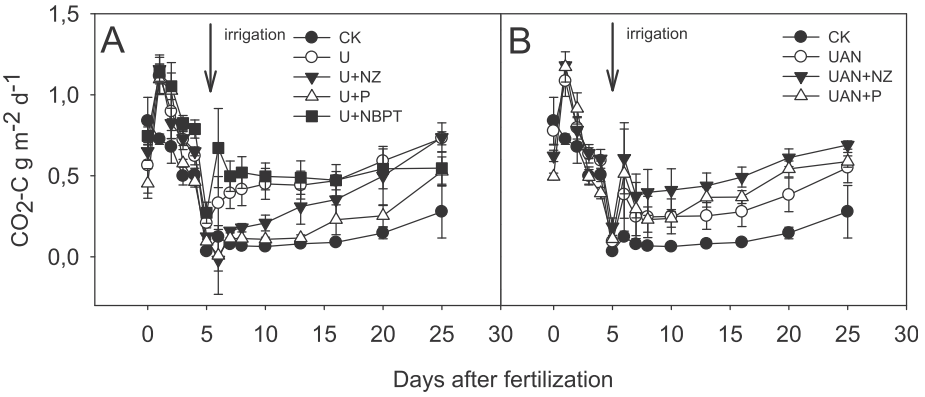

Fig. 1. Time course of CO_2 emissions of different fertilizer treatments. **A**, urea series; **B**, UAN series. Error bars correspond to ±1 SE ($n = 4$). CK: control without fertilization, U: urea, NZ: NZONE MAX, P: Piadin, NBPT: N-(n-butyl) thiophosphoric triamide, UAN: urea ammonium nitrate.

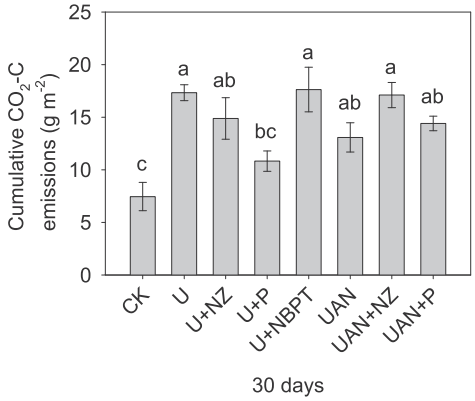

Fig. 2. Cumulative CO_2 emissions of different fertilizer treatments. Error bars correspond to ±1 SE ($n = 4$). Treatments labeled with the same letters did not show statistically differences at the 0.05 probability level. CK: control without fertilization, U: urea, NZ: NZONE MAX, P: Piadin, NBPT: N-(n-butyl) thiophosphoric triamide, UAN: urea ammonium nitrate.

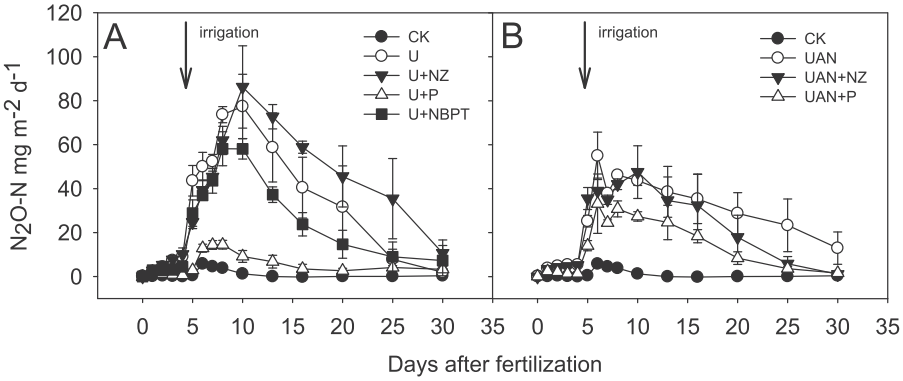

Fig. 3. Time course of N_2O emissions of different fertilizer treatments. **A**, urea series; **B**, UAN series. Error bars correspond to ±1 SE ($n = 4$). CK: control without fertilization, U: urea, NZ: NZONE MAX, P: Piadin, NBPT: N-(n-butyl) thiophosphoric triamide, UAN: urea ammonium nitrate.

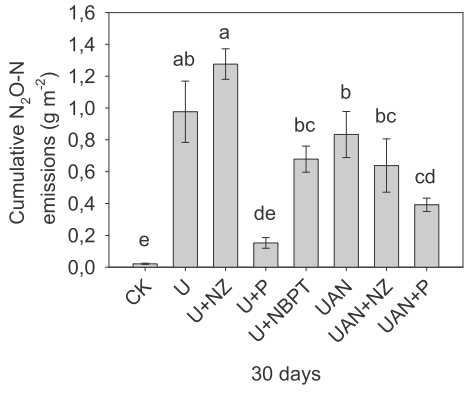

Fig. 4. Cumulative N_2O emissions of different fertilizer treatments. Error bars correspond to ±1 SE ($n = 4$). Treatments labeled with the same letters did not show statistically significant differences at the 0.05 probability level. CK: control without fertilization, U: urea, NZ: NZONE MAX, P: Piadin, NBPT: N-(n-butyl) thiophosphoric triamide, UAN: urea ammonium nitrate.

3.3. NH_3 emissions

Fig. 5 shows that all treatments resulted in a sharp increase in NH_3 emissions after addition of fertilizers. In the urea series, the emissions after the urea alone, U + NZ and U + P treatments showed similar time courses and reached a peak on the third day (Fig. 5A and B). By contrast, the emissions in treatment U + NBPT were much lower, with the peak value on day four. The increase persisted for three days longer than in the other treatments. The peak emission after the U + NBPT treatment was only 0.27 g NH_3-N m^{-2} d^{-1} on day 4, compared with 0.84, 0.84 and 0.96 g NH_3-N m^{-2} d^{-1} at day 3 for the urea alone, U + NZ and U + P treatments (Fig. 5A and B). The time courses of the emissions were similar for the three treatments in the UAN series, with peak values at day 3. The peak emissions in the UAN, UAN + NZ and UAN + P treatments were 0.58, 0.61 and 0.69 g NH_3-N m^{-2} d^{-1}, respectively. In contrast to CO_2 and N_2O fluxes there was no response to the simulated irrigation on day 5 in any treatment.

Cumulative emissions of NH_3 from the soil surfaces of the experimental pots in the urea treatment amounted to 3.4 g NH_3-N m^{-2} in 30 days (Fig. 6), minus the emission of 0.4 g NH_3-N m^{-2} from the control treatment, which was considered as the background emission from the original soil N pool. The emission related to the application of urea alone was therefore about 3 g NH_3-N m^{-2}. In relation to 12 g NH_3-N m^{-2} fertilization, the rate of ammonium volatilization was thus 25% of the applied urea-N. With addition of the urease inhibitor (U + NBPT), the emission was reduced to 1.7 g NH_3-N m^{-2} (the cumulative emission minus the background emission). Therefore, after the treatment with U + NBPT, the cumulative NH_3 emissions were reduced by ca. 50% relative to urea alone.

NH_3 emissions from the Piadin + fertilizer treatment were higher than for urea and UAN alone (Fig. 6). In the urea series, the cumulative emission from the U + P treatment was 4.95 g NH_3-N m^{-2}, i.e. 44% more than after treatment with urea alone (3.42 g NH_3-N m^{-2}). In the UAN series, the cumulative emission of NH_3 after treatment with UAN + P (2.83 g NH_3-N m^{-2}) was 12% higher than after treatment with UAN alone (2.53 g NH_3-N m^{-2}).

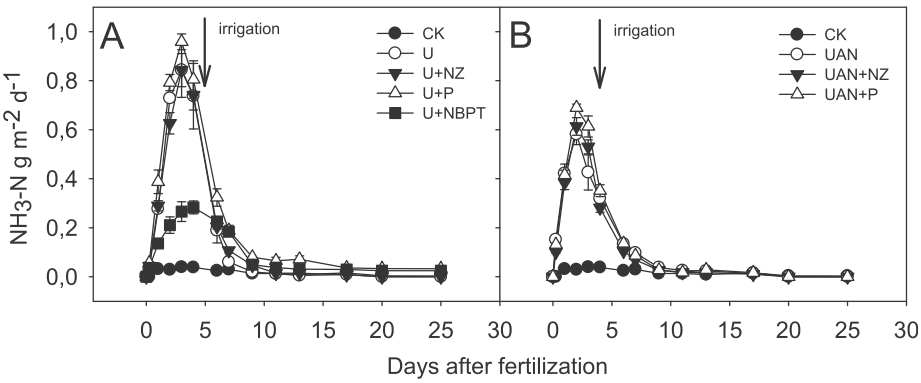

Fig. 5. Time course of NH_3 emissions of different fertilizer treatments. **A**, urea series; **B**, UAN series. Error bars correspond to ±1 SE ($n = 4$). CK: control without fertilization, U: urea, NZ: NZONE MAX, P: Piadin, NBPT: N-(n-butyl) thiophosphoric triamide, UAN: urea ammonium nitrate.

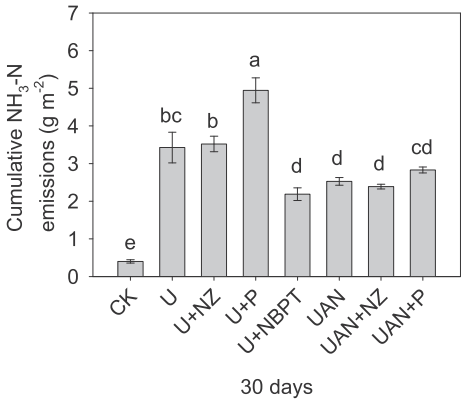

Fig. 6. Cumulative NH_3 emissions of different fertilizer treatments. Error bars correspond to ±1 SE ($n = 4$). Treatments labeled with the same letters did not show statistically significant differences at the 0.05 probability level. CK: control without fertilization, U: urea, NZ: NZONE MAX, P: Piadin, NBPT: N-(n-butyl) thiophosphoric triamide, UAN: urea ammonium nitrate.

3.4. NO_3^--N and NH_4^+-N remaining in the soil after 30 days

The mineral N in the soil samples was determined before the addition of the fertilizers and the concentrations of NO_3^--N and NH_4^+-N were 6.80 and 0.23 g N m^{-2}, respectively (Fig. 7A and B). Thirty days after the addition of 12 g N m^{-2} to all treatments, the remaining soil NO_3^--N ranged from 7.7 g N m^{-2} (U + P) to 14.6 g N m^{-2} (U + NZ) and 2.8 g N m^{-2} in the control treatment (Fig. 7A). The soils treated with U + P showed a lower but not significant NO_3^--N content than those treated with urea alone. The NH_4^+-N remaining after treatment with U + P (1.6 g NH_3-N m^{-2}) was significantly higher than that remaining after the other treatments (<0.5 g NH_3-N m^{-2}) (Fig. 7B).

4. Discussion

4.1. CO_2 emissions

The additional emission of CO_2 from the soils treated with urea fertilizer was a result of two processes: the hydrolysis of urea and induced heterotrophic microbial activity. During hydrolysis of urea, urea is cleaved into NH_3 ($2 \times NH_3$) and carbon dioxide (CO_2) and this goes along with a net increase in the soil pH. In this experiment, the treatment with UAN alone (13.1 g CO_2-C m^{-2}, Fig. 1) resulted in CO_2 emissions that were 25% lower than the treatment with urea alone (17.3 g CO_2-C m^{-2}) ($p < 0.05$). As N in UAN consists of only 50% urea-N that can be hydrolyzed, this figure indicates that, in both treatments, the hydrolysis of urea made a considerable contribution to the volume of CO_2 emitted.

The other source of CO_2 is respiration resulting from the activity of heterotrophic microorganisms, such as the NH_3-oxidizing bacteria population (Kowles, 2018). All treatments showed a surge in the emission of CO_2 after 24–72 h. The soil moisture content was low (55% WFPS) during this time period and the temperature remained constant at 25 °C. Irrigation to a WFPS of 80% on day 5 caused a dramatic decrease in the emission of CO_2, after which the emission of CO_2 increased slowly, with a simultaneous decrease in the WFPS. Therefore it seems that at 55% WFPS conditions were more favorable for microbial respiration than 80% WFPS conditions. The observed decrease in CO_2 emissions after treatment with urea and a nitrification inhibitor has been reported previously (Florio et al., 2016; Maienza et al., 2014; Weiske et al., 2001). The decreased CO_2 emissions after irrigation were mainly from i) disturbed microbial activity and ii) the slower diffusion rate of CO_2 out of the soil with a higher water content.

4.2. N_2O emissions

N_2O emissions were relatively low in all treatments during the first four days of the experiment, before irrigation at day 5. However, the emissions increased rapidly to a high level after irrigation, suggesting that the increase in the soil moisture content (WFPS) from 50% to 55% between days 0 and 5–80% at day 6 was the key driver of N_2O emissions (Cardenas et al.,

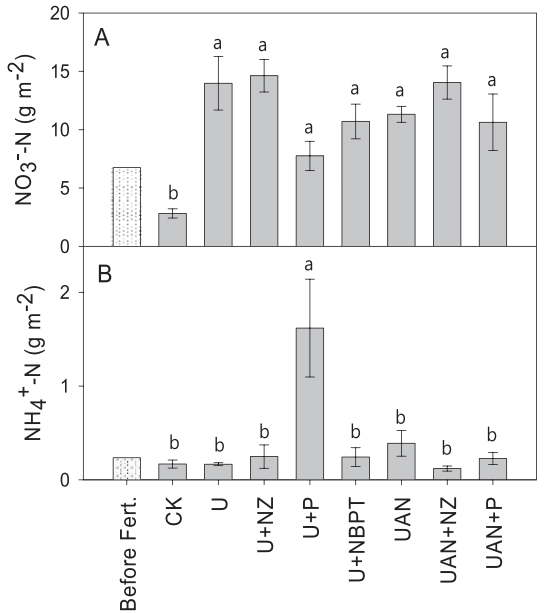

Fig. 7. Nitrate and ammonium present in the soil samples before the application of fertilizer and after 30 days of application for the different treatments. Error bars correspond to ±1 SE ($n = 4$). Treatments labeled with the same letters did not show statistically significant differences at the 0.05 probability level. CK: control without fertilization, U: urea, NZ: NZONE MAX, P: Piadin, NBPT: N-(n-butyl) thiophosphoric triamide, UAN: urea ammonium nitrate.

2017; Yu et al., 2018; Zaman et al., 2013). It is widely accepted that soil moisture has an important impact on N_2O emissions and that a WFPS of 60% is the threshold between aerobic and anaerobic soil conditions (Menéndez et al., 2012). Soil moisture below a WFPS of 60% is unfavorable for the emission of N_2O. Low N_2O emission rates have been observed previously in similar studies reported by Menéndez et al. (2012) and Volpi et al. (2017).

Only a few earlier studies (Pietzner et al., 2017; Wolf et al., 2014; Wu et al., 2017) have evaluated 1H-1,2,4-triazole and 3-methylpyrazole (Piadin) as a nitrification inhibitor. However, the results of these studies were similar to our findings, confirming that Piadin can significantly reduce N_2O emissions. Research has also been carried out on other nitrification inhibitors (e.g. DMPP, DCD and Nitrapyrin), demonstrating their effectiveness in reducing N_2O emissions. As nitrification inhibitors aim to suppress, reduce or delay the oxidation of NH_4^+ to NO_3^- in soils, our observations of reduced N_2O fluxes in the treatments with nitrification inhibitors were probably related to variations in the availability of the substrate (NO_3^-) for denitrification. They may also have been influenced by different contributions from the two major N_2O-forming processes of nitrification and denitrification (Zaman and Nguyen, 2012). In a number of studies (Guo et al., 2014; Yu et al., 2018; Zaman et al., 2013; Zaman and Nguyen, 2012) the time courses of soil NH_4^+ and NO_3^- concentrations after application of fertilizers have shown that treatment with nitrification inhibitors (DMPP or DCD) result in higher NH_4^+ and lower NO_3^- concentrations.

Cumulative emissions of N_2O were high in all treatments in this study, except for the U + P and UAN + P treatments. This suggests that the chosen incubation environment did favor denitrification, probably as a result of the high soil moisture content (80% WFPS), high incubation temperature (25 °C) and high soil NO_3^- content. The N_2O emissions in studies under similar conditions were predominantly from denitrification (Grave et al., 2018; Menéndez et al., 2012; Senbayram et al., 2012; Luo et al., 2008), most likely as a result of limited nitrification due to the low availability of oxygen (Tian et al., 2015). The lowest N_2O emissions in our study were observed in the treatments with the lowest NO_3^- concentrations in the soil (with Piadin treatment), which is seen as further evidence of this assumption.

4.3. NH_3 emissions

The release of large amounts of NH_3 after the application of urea is a serious agricultural problem (Engel et al., 2017; Li et al., 2015; Pacholski et al., 2018; Schraml et al., 2016; Sun et al., 2015; Tian et al., 2015). In this study, the U + NBPT treatment reduced NH_3 fluxes by about 50%, which is in agreement with previously published work (Connell et al., 2011; Drury et al., 2017; Mira et al., 2017; Suter et al., 2013). The meta-analysis of Silva et al. (2017) showed that urea + NBPT reduced 52% losses of NH_3. The trend of reduction was observed in soils over all classes of soil pH, organic carbon content and rate of N addition. Moreover, the addition of NBPT to urea has also been suggested to be effective in increasing crop yields (Drury et al., 2017; Silva et al., 2017).

As UAN is composed of urea and ammonium nitrate in a ratio of 1:1, the volatilization losses of NH_3 from the group of UAN treatments should theoretically be lower than those from the soils treated with the different urea fertilizers. This was confirmed by our results. Although we did not include a UAN + NBPT solution in this study, a number of other studies (Goos, 2012; Grant, 2013; Rajkovich et al., 2017) have shown that the addition of NBPT to UAN can significantly reduce NH_3 losses relative to the application of UAN alone.

By contrast, nitrification inhibitors tend to induce increased NH_3 emissions because NH_4^+ is available for extended periods of time. The addition of Piadin to both groups of N fertilizers increased the cumulative NH_3 emissions by 44% and 12%, respectively, relative to urea or UAN alone. This increase in NH_3 emissions agrees with earlier reports showing that nitrification inhibitor treatments increased NH_3 emissions from 3% to 65% (Fan et al., 2018; Ferm et al., 2006; Lam et al., 2018, 2017; Pan et al., 2016; Qiao et al., 2015; Webb et al., 2010). However, Piadin performed well in reducing N_2O emissions owing to lower NO_3^--N concentrations in the soil. Therefore, the benefit of nitrification inhibitors in reducing N_2O emissions has to be judged against the higher risk of NH_3 volatilization, or additional strategies need to be implemented to reduce NH_3 volatilization.

4.4. Soil NO_3^--N and NH_4^+-N

The remaining mineral N was determined at the end of experiment. It was expected that large amounts of total mineral N (NO_3^--N + NH_4^+-N) would remain in the soil due to the absence of plants utilizing N and the shallow depth of the experiment in the soil layer (16 cm). In addition, N leaching was impeded as a result of the use of water-tight incubation vessels. Consequently, all the treatments (urea alone, U + NZ, U + NBPT, UAN and UAN + NZ) showed residual mineral N of >10 g N m^{-2}. As the total amount of mineral N at the start of the experiment was 19 g N m^{-2} (12 g N m^{-2} fertilizer N and 7 g N m^{-2} initial soil mineral N), more than half of the original amount of N remained in the treated soils. The range of N losses in our experiment was similar to previously reported experiments carried out under similar conditions (Wu et al., 2017; Zaman and Nguyen, 2012). Some of the applied N not recovered as inorganic N was probably taken up by soil microbes and would have been part of the soil organic N pool.

Nitrification inhibitors such as Piadin inhibit the oxidation of NH_4^+ to NO_3^-. In our study, the residual soil NH_4^+-N in U + P treatment was 1.6 g N m^{-2}, whereas in all other treatments it was <0.5 g N m^{-2}. The soil NO_3^- concentration was still low at the end of the incubation period of 30 days, accounting for only 7.7 g NO_3^--N m^{-2}, which was the lowest of all treatments. In the pot experiments of Goos and Johnson (1999) and Sassman (2014), conducted at 25 °C for tests of application rates of 15 g NH_4^--N m^{-2}, the half-life of soil NH_4^+ after the application of urea alone and UAN alone was 2–3 weeks. This is consistent with

our study, in which the conversion of NH_4^+ to NO_3^- was almost complete 30 days after the addition of fertilizer. This process is always clearly delayed in the presence of a nitrification inhibitor (Wu et al., 2017; Yu et al., 2018; Zaman et al., 2013).

4.5. Evaluation of the novel fertilizer additive NZONE MAX

We included the product NZONE MAX because this novel compound has been reported to be a powerful additive, improving the efficiency of N fertilizers by improving the attachment of NH_4^+ to soil colloids and preventing their volatilization. However, we found that NZONE MAX was ineffective in reducing both NH_3 and N_2O emissions with our soil. Goos (2012) and Harrel (2012) reported similar results. Our study clearly confirms that the addition of NZONE MAX to major types of N fertilizer had no effect on the reduction of N losses by volatilization and denitrification and, based on final soil N concentration in our incubation experiment, there was no indication of potential effects on NO_3^- leaching. The impact of that mechanism would likely be dependent on soil texture. A soil with moderate to high clay content and/or organic matter would probably already have sufficient CEC and readily retain ammonium. We refer that product may be more likely to have an impact on emissions in a low CEC soil. Future studies should test if NZONE MAX increases the ammonium sorption capacity (Venterea et al., 2015).

5. Conclusion

This laboratory study shows that NBPT is an effective urease inhibitor and reduces NH_3 volatilization and probably also N_2O emissions. The nitrification inhibitor Piadin was also found to be effective in reducing N_2O emissions. However, the potential of increasing NH_3 volatilization with the use of Piadin or similar nitrification inhibitors should not be neglected. In our study, the novel additive NZONE MAX was found to be unsuitable for reducing greenhouse gas emissions and improving the efficiency of fertilizer use. However, future studies should test this novel additive on soils with a lower clay content or organic matter that limits NH_4^+ attachment on soil colloids. Future studies also need to focus on improving management methods, or on new chemical or biochemical additives.

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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Chapter 3: Use of urease and nitrification inhibitors to decrease yield-scaled N_2O emissions from winter wheat and oilseed rape fields: a two-year field experiment

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Abstract

Nitrogen fertilizers are the major source of nitrous oxide (N_2O) emissions from arable land. The addition of nitrification inhibitors to fertilizers may improve the nitrogen (N) use efficiency and reduce N_2O emissions. However, it is still unclear how crop rotations affect nitrification and urease inhibitors to reduce N_2O emissions. We conducted a field experiment with two-year winter wheat and one-year oilseed rape cultivation in Germany from 2016 to 2017. We applied five different fertilizer treatments: (1) a control treatment without fertilization (N0); (2) calcium ammonium nitrate (CAN); (3) ammonium sulfate nitrate with the nitrification inhibitor 3,4-dimethylepyrazole phosphate (ENTEC); (4) urea; and (5) urea with the urease inhibitor N-(n-butyl) thiophosphoric triamide (UTEC). Crop yield, grain and straw N content, and N_2O fluxes were measured to assess yield-scaled N2O emissions under different treatments. We found that in all fertilized treatments, the aboveground N uptake of wheat after wheat was 199–203 kg N ha−1, which was much lower than that of wheat after oilseed rape (252–271 kg N ha−1). The apparent N recovery of oilseed rape (13–23%) was much lower than in wheat after wheat (63–66%). The enhanced-efficiency fertilizers increased aboveground N uptake by 0–5% compared to fertilizers without inhibitors. The oilseed rape field had the highest yield-scaled N_2O emissions (18.0, 15.1, 16.7 and 15.6 g N_2O-N kg−1 aboveground N uptake in CAN, ENTEC, urea and UTEC, respectively). These results indicate that urease and nitrification inhibitors hold the potential to increase crop yield and reduce N_2O emissions. Oilseed rape straw should be carefully managed to avoid high N_2O emissions. We also

suggest that an optimized N application strategy might be a possible way to increase the efficiency of urease and nitrification inhibitors; for instance, by increasing the N application rate in wheat after wheat, but reducing it in oilseed rape.

Keywords: crop rotation, mineral N concentration, yield, nitrification inhibitors, N fertilization, N_2O emissions

Introduction

Nitrous oxide (N_2O) is a potent greenhouse gas that absorbs longwave radiation (Granli and Bockman, 1994) and has a global warming potential 298 times higher than that of CO_2 on a 100-year timescale (IPCC, 2013; Myhre et al., 2013). N_2O also contributes to the depletion of stratospheric ozone (Ravishankara et al., 2009). The atmospheric concentration of N_2O in 2015 was 330 ppb, 21% higher than pre-industrial levels (WMO, 2018). The uptake efficiency of nitrogen (N) fertilizer is usually <50% (Galloway et al., 2003) and losses of N from fertilizers have both economic and environmental impacts (Fowler et al., 2013; Prather and Hsu, 2010), with agriculture accounting for 60% of global anthropogenic N_2O emissions (Bhatia et al., 2010).

Enhanced-efficiency fertilizers coated with urease or nitrification inhibitors may be used to reduce the losses of N from soils (Lam et al., 2018; Wang et al., 2020). Soil N_2O production relies on nitrate (NO_3^-) and ammonium (NH_4^+) provided by mineral or organic N (N fertilizer) (Ruser et al., 2017). The dominant biological processes that produce N_2O using mineral N substrates in soils are nitrification and denitrification (Bremner, 1997). Urease inhibitors reduce the activity of the urease enzyme and therefore slows the hydrolysis of urea, leading to a decrease in the volatilization of ammonia (NH_3) from soils (Drury et al., 2017; Liu et al., 2017; Mira et al., 2017; Silva et al., 2017). *N*-(*n*-Butyl) thiophosphoric triamide (NBPT) is a very widely used urease inhibitor in arable soils (Tian et al., 2015). Nitrification inhibitors prevent the conversion of NH_4^+ to NO_3^- by decreasing the enzymatic activity of ammonium-oxidizing bacteria, leading to a reduction in nitrification and the emission of N_2O from soils (Hu et al., 2015; Ruser and Schulz, 2015). 3,4-Dimethylpyrazole phosphate (DMPP) (Liu et al., 2015; Rose et al., 2018; Shi et al., 2017) and dicyandiamide (Di et al., 2014; Guo et al., 2014; Liu et al., 2017; Zaman et al., 2013) are very widely used nitrification

inhibitors to reduce N_2O emissions and have been shown to be effective in increasing crop yields and reducing N_2O emissions (Akiyama et al., 2010).

Many incubation studies have focused on the effect of nitrification or urease inhibitors on soil N_2O emissions (Cantarella et al., 2018; Tian et al., 2015). However, short-term incubation studies may lead to a misinterpretation of the environmental effects of N_2O emissions. Firstly, neglecting freeze–thaw emissions would underestimate agricultural N_2O emissions by 17 to 28% (Wagner-Riddle et al., 2017). Secondly, N fertilizers with inhibitors are usually applied in spring, but it is still not clear how long their effects might last. The addition of nitrification inhibitors is assumed to reduce NO_3^- leaching rates and therefore lead to a lower C/N ratio in plant residues that are left after cropping (Pfab et al., 2012). This may accelerate the mineralization of plant residues and promote the release of N_2O in the post-harvest season (Köbke et al., 2018; Walter et al., 2015). Therefore, measurements of N_2O emissions on an annual basis will provide more reliable information to support the mitigation potential of these inhibitors (Pfab et al., 2012). Since N fertilizers coated with nitrification or urease inhibitor can reduce N losses and may have the potential to increase crop yields (Abalos et al., 2014), we suggest that the reduction in yield-scaled N_2O emissions (the annual cumulative N_2O emissions per unit grain yield) from enhanced-efficiency fertilizers would be more significant than the reduction in area-scaled N_2O emissions.

Winter oilseed rape (*Brassica napus* L.) and winter wheat (*Triticum aestivum* L.) are important crops in Germany. The area of winter oilseed rape in Germany in 2018 was twice that in 1990 (FAOSTAT, 2018), and makes up >75% of transport biofuels in Europe (Hamelinek et al., 2014). Winter wheat has the highest yield potential in all cereals, and the cultivated area has reached 26.6% of the total arable land in Germany, which represents an increase by one-third in the last 30 years (DESTATIS, 2015). Continuous cropping of winter wheat could decrease soil fertility, grain yield, and yield stability (de Cárcer et al., 2019; Macholdt et al., 2020). Therefore, winter oilseed rape is recommended as a break crop. The grain yield of oilseed rape preceding winter wheat is usually higher than that of continuous cropping with a cereal (Angus et al., 2015; Sieling and Christen, 2015; Weiser et al., 2018). The N surplus of winter oilseed rape cultivation is higher as a result of its low harvest index and large amount of crop residue (Bouchet et al., 2016; Sieling and Kage, 2010). This high-N surplus can reduce the need for N fertilizer in the following winter wheat season and increase the grain yield. This might be a positive environmental impact on decreasing the

potential yield-scaled N_2O emissions during a winter oilseed rape–winter wheat rotation. However, Sieling and Kage (2010) reported that the large N surplus after the harvest of winter oilseed rape cannot be used by the succeeding winter wheat crop before the winter season, and this large N pool would increase the risk of post-harvest N_2O emissions (Köbke et al., 2018). Therefore, the effect of the preceding crop should also be taken into consideration when assessing annual N_2O emissions.

In this study, we measured soil N_2O fluxes from two adjacent fields that were both grown with a wheat–wheat–oilseed rape rotation. In the first field, the preceding crop was oilseed rape in 2015, and the standing crop during the experiment was winter wheat in 2016 and 2017 (WW2016 and WW2017). In the second field, the standing crop during the measurement period was oilseed rape in 2017 (WR2017), which was preceded by two years of winter wheat in 2015 and 2016. We applied two common fertilizers (calcium ammonium nitrate (CAN) and urea) and two enhanced-efficiency fertilizers (NBPT and DMPP) at each site, and also measured grain and straw yields and their N contents. Our main aim was to verify how crop rotation—here, wheat–wheat–oilseed rape rotation—affects the performance of the two inhibitors in terms of N_2O emissions, crop yield, and yield-scaled N_2O emissions. We hypothesized that the enhanced-efficiency fertilizers would (1) increase the crop yield and (2) reduce N_2O emissions, compared to fertilizers without inhibitors, and (3) oilseed rape fields would have higher N_2O emissions than wheat fields.

Materials and methods

Study site and field treatments

This experiment was conducted at Reinshof, an experimental agricultural station of the University of Goettingen, Lower Saxony, Germany (51° 29′ 50.3″ N, 9° 55″ 59.9′ E). The annual mean temperature and annual precipitation are 9.2 ± 0.1°C and 651 ± 24 mm, respectively (1981–2010, meteorological station at Goettingen, station ID 1691, German Meteorological Service). The soil is classified as a Luvisol (IUSS Working Group WRB, 2006) with a bulk density of 1.3 g cm^{-3} and a pH of 7.0 in the top 20 cm of the soil profile. The soil texture consists of 16% clay, 61% silt, and 23% sand, with an organic C content of 2% (Römer et al., 2015).

The crop rotation of this study was wheat–wheat–oilseed rape in a three-year cycle. Our experiment was conducted in two adjacent fields. For the first field, we conducted our measurements from 3 March 2016 to 2 March 2018, and the field was cultivated with winter wheat (*Triticum aestivum L.*) in both years (WW2016 and WW2017), which was preceded by oilseed rape in 2015. For the adjacent field, we conducted our measurements from 3 March 2017 to 2 March 2018, and the field was cultivated with oilseed rape (*Brassica napus* L.) in 2017 (WR2017), which was preceded by two years of winter wheat in 2015 and 2016. A detailed description of the field management during the experimental period is shown in Fig. 1. Granulated fertilizers were broadcast onto the fields. After harvest, straw and stubble were left in the fields and the soil was ploughed to a depth of 25 cm before next sowing.

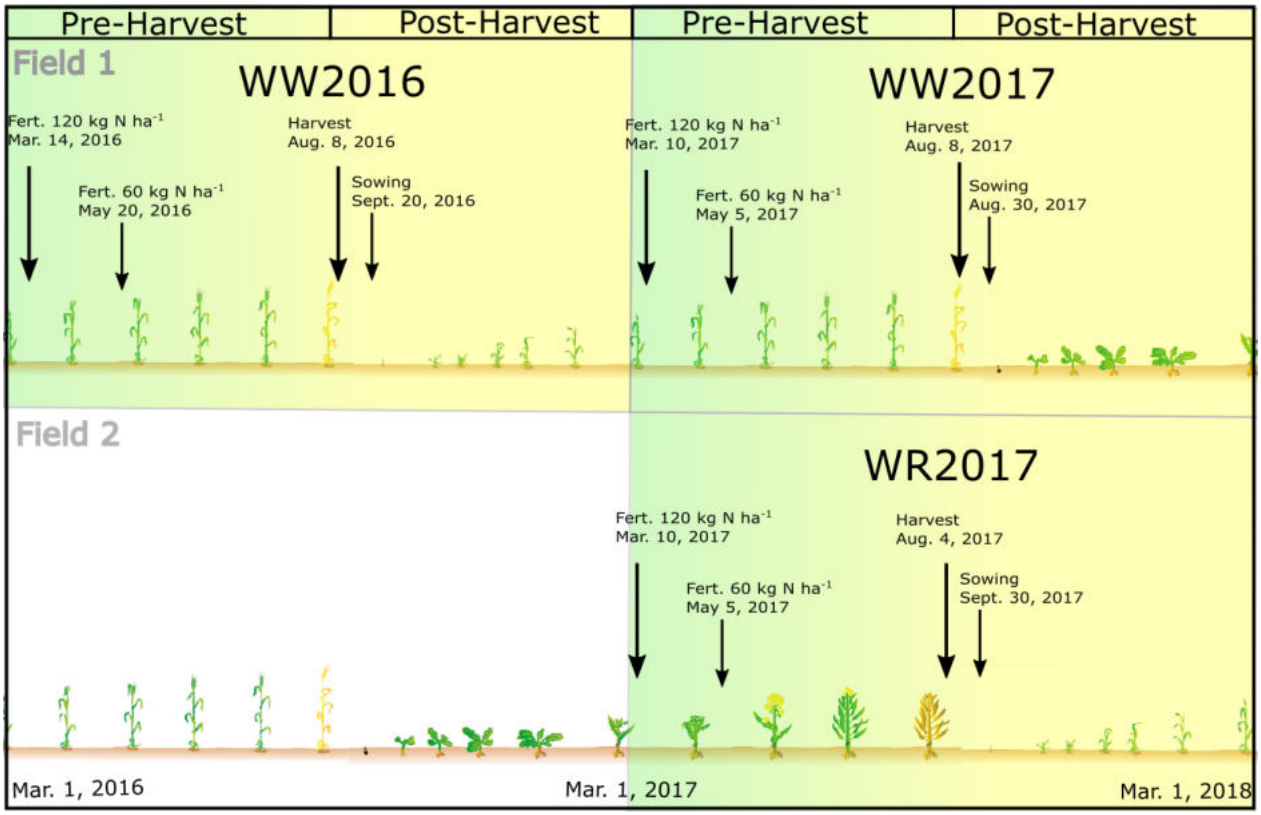

Fig. 1. Timetable and main field management. Measurements began on 1 March and continued for a full year. The winter wheat measurements lasted for two years and the oilseed rape was measured for one year. The two fields were in adjacent locations and had similar management. WW2016 refers to the winter wheat after oilseed rape in 2016; WW2017 to winter wheat after winter wheat in 2017; and WR2017 to winter oilseed rape after two years of winter wheat cultivation in 2017.

Five fertilization treatments were tested: control without N fertilizer input (N0), 180 kg N of CAN (76% NH_4NO_3 and 24% $CaCO_3$, 26% N), 180 kg N of ENTEC26 (18.5% NH_4^+ N and 7.5% NO_3^- N with 0.15% DMPP; BASF, Ludwigshafen, Germany), 180 kg N of urea (NH_2CONH_2, 46% N), and 180 kg of UTEC46 (urea with added NBPT; BASF, Ludwigshafen, Germany). In treatments with fertilizer input, 120 kg of N was applied as basal fertilizer in March (14 March 2016 and 10 March 2017, respectively) and 60 kg of N as top-dressing in May (20 May 2016 and 5 May 2017, respectively). No fertilizer was applied in autumn, which is in accordance with local practice. The treatments were randomized with three replicates, resulting in a total of 15 plots (each with an area of 10 m × 8 m). Each plot was designed with two parts; one was used for the insertion of gas chambers and the collection of soil samples and the other for crop harvest to calculate the crop yield.

Gas sampling and measurement

Gas samples were collected from winter wheat fields using opaque closed chambers. A 0.275 m^2 basal ring frame was inserted into the soil. The chamber was dark inside and wheat plants were included during the growing season. The height of the chamber above ground was 0.35 m and this was increased to 0.75 m as the winter wheat grew taller. A rubber band was used at the joints of the chamber with the ring to avoid leakage. In winter oilseed rape fields, we used rectangular chambers of 0.72 m length, 0.28 m width and 0.15 m hight. The rows were 0.4 m apart, so plants were not included in the oilseed rape gas flux chambers. The gas samples were taken daily for one week after additions of fertilizers, then every two or three days and after one month once each week. The samples were also collected when the levels of emissions were expected to be high due to precipitation or tillage events.

12-mL Labco Exetainers were evacuated the day before sampling. The chambers on the base frames were closed and sealed with the rubber band. Gas in the headspace of the chambers was sampled with 30-mL polypropylene syringes 0, 20 and 40 min after closure. The extracted gas was immediately transferred to the Exetainers before being transported to the laboratory for analysis on a Bruker SCION Model 456 gas chromatograph (Bremen, Germany). An electron capture detector was used to determine the concentration of N_2O. The flux rates were calculated by linear regression of the gas concentration with time (Parkin et al., 2012; Wang et al., 2013). The cumulative emissions were estimated by linear interpolation. The emission factor (EF, N_2O-N emitted as a

percentage of fertilizer applied) was calculated by the annual N_2O emissions and the amount of N fertilizer:

$$\mathrm{EF}(\%) = \frac{\text{Annual } N_2O\text{–}N_{\text{fertilized}} - \text{Annual } N_2O\text{–}N_{\text{unfertilized}}}{\text{Amount of N applied}} \times 100 \quad (1)$$

Auxiliary measurements

Soil samples were taken once a week from a depth of 15 cm in each plot for the full measurement period and used to determine the soil moisture and mineral N (NO_3^- and NH_4^+) content. Briefly, 50 g soil samples were dissolved in 200 mL of 0.0125 M $CaCl_2$ solution, filtered and stored at −20°C. The samples were analyzed using a San++ continuous flow analyzer (Skalar Analytical, the Netherlands). The soil temperature at 5 cm depth was automatically recorded in the field at 30-minute intervals using a temperature logger (LogTag temperature recorder, TRIX-16, Auckland, New Zealand). Winter wheat was harvested after physiological maturity on 7 August 2016 and 9 August 2017, whereas winter oilseed rape was harvested on 4 August 2017. Grain and straw were dried to a constant weight in an oven at 60°C before milling. Total N content of grain and straw was analyzed to determine aboveground N uptake by the crop. Aboveground N uptake of grain and straw were calculated as:

$$\text{Aboveground N uptake} = \text{Yield} \times \text{N content} \quad (2)$$

The apparent N recovery (ARN) was calculated as

$$\mathrm{ARN}\ (\%) = \frac{N_{\text{uptake}}(\text{fertilized}) - N_{\text{uptake}}(\text{unfertilized})}{\text{Amount of N applied}} \times 100 \quad (3)$$

Statistical analysis

Statistical data analyses were performed using the IBM SPSS Statistics 21.0 software package. For soil N_2O fluxes, we first checked for normal distribution with the Shapiro–Wilk test and Levene's test, and found that the data were non-normally distributed and the variance was inhomogeneous. One-way ANOVA was used to test the significance of the differences in treatment on the cumulative N_2O emissions, grain and straw yields, total N content and the yield-scale N_2O emissions. The cumulative emissions were estimated by linear interpolation and the results were tested for variance homogeneity and normal distribution. The mean values were compared via a least-significant-difference test at the 5% level using Tukey's 'honest significant difference' post-hoc test. Spearman's rank correlation tests were performed to determine the relationships between

soil N_2O and WFPS, chamber air temperature, soil NH_4^+ and NO_3^- concentrations (0–15 cm) across WW2016, WW2017 and WR2017.

Results

Climate conditions

The precipitation from 1 March 2016 to 28 February 2017 was 523 mm, whereas the precipitation from 1 March 2017 to 28 February 2018 was 806 mm (Fig. 2a). Heavy rainfall events (>60 mm) occurred three times in 2017 (on 23 June, 24 July and 25 July), whereas the heaviest rainfall in 2016 was only 35 mm (on 24 June) (Fig. 2a). The local mean long-term air temperature is 8.7°C. However, the mean air temperature was 9.5°C in 2016 and 10°C in 2017 (Fig. 2a). The soil water content was regulated by precipitation events and evapotranspiration, ranging from 20 to 80% water-filled pore space (WFPS) during the study period, with no significant difference among the fertilizer treatments crops grown (Fig. 2b). Although in 2017 the spring (about 40% WFPS) was drier compared to 2016 (about 50% WFPS), higher WFPS in summer was observed in summer 2017 than 2016 as a result of the heavy rainfall events (Fig. 2b).

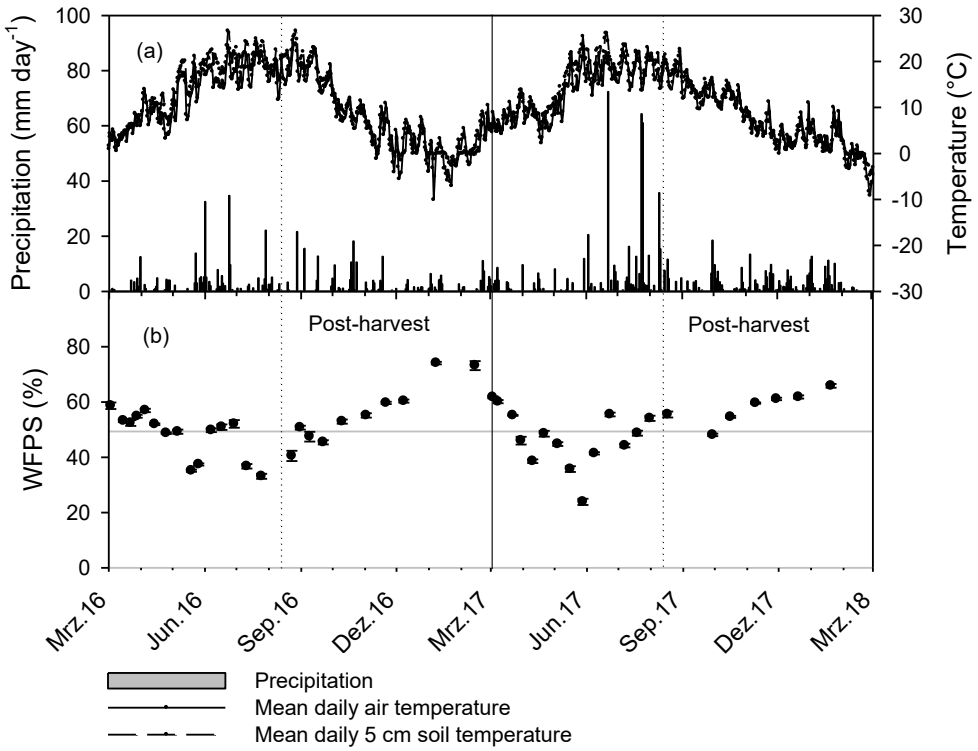

Fig. 2. (a) Mean daily precipitation (mm day^{-1}) (*dark cyan bars*) and mean daily soil temperature (°C) at 5 cm depth (*solid dark red lines*) in the experimental field from March 2016 to March 2018. (b) Mean value of WFPS in unfertilized treatments in the winter wheat field from 1 March 2016 to 1 March 2018. The WFPS data showed no significant difference among the fertilizer treatments and crop fields. Downward vertical dotted arrows indicate harvest events and the red solid horizontal line marks 50% WFPS.

Soil NH_4^+-N and NO_3^--N content

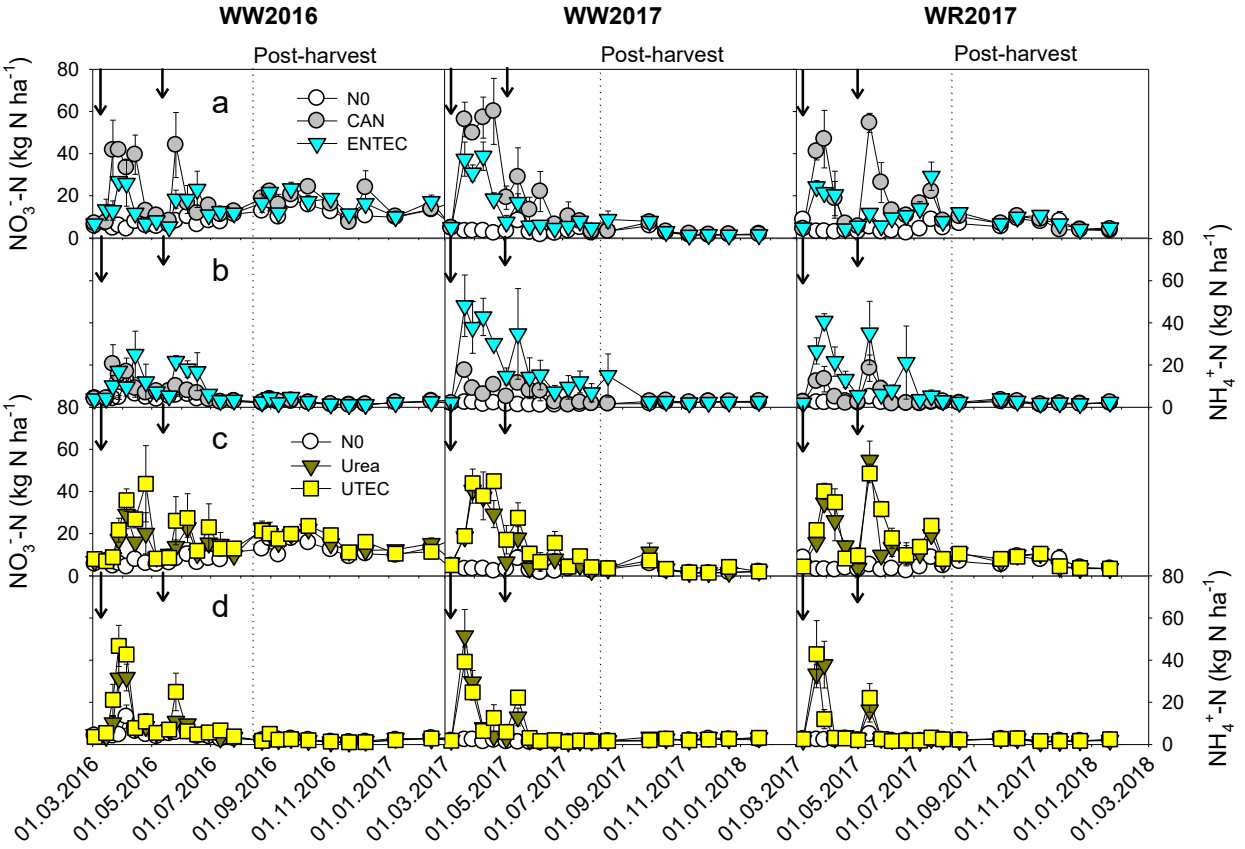

Fig. 3. Seasonal dynamics of (a, c) soil nitrate (NO_3^-) and (b, d) ammonium (NH_4^+) contents (0–15 cm depth) in different fertilizer treatments over two years of winter wheat (WW2016 and WW2017) and one year of winter oilseed rape (WR2017). The symbols show the means of three replicates and the vertical bars show the standard errors of the mean. The downward arrows indicate the addition of fertilizer; larger arrows indicate basal fertilization with 120 kg N ha^{-1}; and smaller arrows indicate the addition of a 60 kg N ha^{-1} dressing. Dotted vertical lines indicate harvest events. WW2016 refers to winter wheat after oilseed rape in 2016; WW2017 to winter wheat after winter wheat in 2017; and WR2017 to winter oilseed rape after two years of winter wheat cultivation in 2017.

The peak soil NO_3^- and NH_4^+ concentrations occurred one week after application of fertilizer in all treatments (Fig. 3). The highest soil NO_3^- and soil NH_4^+ concentrations were 60 kg N ha^{-1} (CAN, WW2016) and 45 kg N ha^{-1} (urea, WW2016), respectively (Fig. 3a, 3b). Smaller soil NO_3^- peaks were found post-harvest in both wheat and oilseed rape fields. Crop residues led to high soil NO_3^- substrates in the post-harvest season and succeeding winter wheat rotations, especially in WR2017 (Fig. 3a, 3c). The background soil NO_3^- concentration (N0 treatment) across the year was >10 kg N ha^{-1} in treatment WW2016, which was clearly higher than in treatment WW2017 (near to zero) (Fig. 3a, 3c).

In most cases, the ENTEC treatment showed lower soil NO_3^- and higher NH_4^+ concentrations than the CAN treatment. Compared to urea (Fig. 3a, 3b), the UTEC treatment showed slightly higher soil NO_3^- concentrations than the urea treatment after each addition of fertilizer in both fields and measurement years (Fig. 3c), although there was no clear difference in soil NH_4^+ concentrations between the urea and UTEC treatments (Fig. 3d).

Nitrous oxide fluxes

The peaks of N_2O emissions were observed around one week after each fertilizer addition (Fig. 4). In addition to the two peak N_2O emissions caused by N top dressing, small peaks were also detected in all treatments during the post-harvest season, especially in the WR2017 treatments (Fig. 4a, b, c and d). The ENTEC treatments all had smaller peak emissions across fields and years than the CAN treatments, especially after the top dressing, and the ENTEC treatment also resulted in lower post-harvest emissions than the CAN treatment, especially in WW2017. There was no recognizable difference between urea and UTEC treatments in all fields.

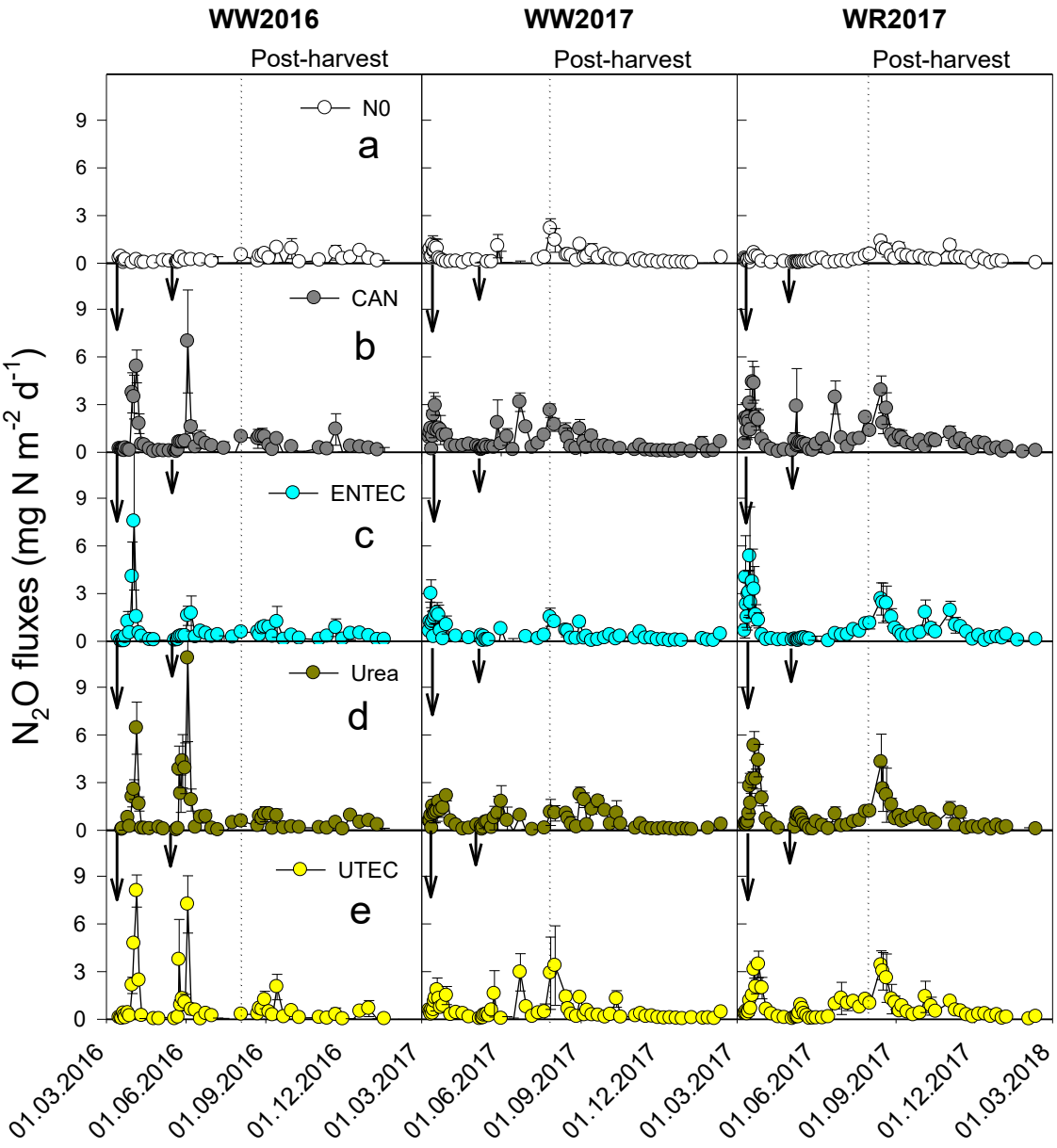

Fig. 4. Seasonal dynamics in nitrous oxide (N_2O) emissions after application of different fertilizers over two years of winter wheat (WW2016 and WW2017) and one year of winter oilseed rape (WR2017). The symbols show the mean of three replicates and vertical bars indicate the standard errors of the mean. Downward arrows indicate the addition of fertilizer; larger arrows indicate basal fertilization with 120 kg N ha^{-1}; and smaller arrows indicate the addition of a 60 kg N ha^{-1} dressing. Dotted vertical lines indicate harvest events. WW2016 refers to winter wheat after oilseed rape in 2016; WW2017 to winter wheat after winter wheat in 2017; and WR2017 to winter oilseed rape after two years of winter wheat cultivation in 2017. (a) N0, (b) CAN, (c) ENTEC, (d) urea and (e) UTEC treatments.

Crop yields and N uptakes

There was a large interannual variation in grain and straw yields of winter wheat fields (Table 1). The wheat after wheat (WW2017) had significantly lower grain and straw yields than the wheat after oilseed rape (WW2016) (Table 1). Grain and straw yields and the aboveground N uptake of winter wheat were higher in the fertilized fields than in unfertilized fields in WW2017. There was no difference in crop yield and aboveground N uptake among the different fertilizers (Table 1).

Fertilization did not increase grain and straw yield in WR2017, but increased the N content compared to the unfertilized treatments. No significant differences in yield and N content were found between fertilized treatments (Table 1). ARN of fertilized treatments were in the range of 41–52% and 63–66% in WW2016 and WW2017, respectively, among the fertilized treatments. However, it was only 13–23% in WR2017 (Table 1).

Area-scaled, and yield-scaled emissions

Cumulative N_2O emissions ranged from 0.6 to 3.03 kg N ha^{-1} yr^{-1} and the mean EF ranged from 0.01 to 1.14% in all fertilizer treatments (Table 2). The cumulative area-scaled N_2O emissions of the unfertilized treatments were 0.60, 0.87 and 0.98 kg N ha^{-1} yr^{-1} in WW2016, WW2017 and WR2017, respectively (Table 2). ENTEC had the lowest cumulative N_2O emissions in fertilized treatments in WW2016 and WW2017 (Table 2). UTEC reduced annual N_2O emissions by 10–30% compared with urea. In all fertilized treatments, post-harvest N_2O emissions ranged from 0.66 to 1.03 in WW2016 and WW2017, yet they were 1.45–2.03 in WR2017 (Table 2). In all fertilized treatments, yield-scaled N_2O emissions in WW2016 and WW2017 ranged between 6.1–9.3 and 4.4–10.5 g N_2O-N kg^{-1} aboveground N uptake, respectively, whereas they were 15.1–18.0 g N_2O-N kg^{-1} aboveground N uptake in WR2017 (Table 2).

Relationships between N_2O fluxes and environmental factors

In all fertilized treatments except for ENTEC, N_2O fluxes were well correlated with air temperature (Table 3). However, ENTEC showed high correlation between N_2O and WFPS in WW2017 and WR2017 ($R^2 = 0.34$, $R^2 = 0.26$, respectively, $p < 0.01$) (Table 3). Significant correlation between NH_4^+ and N_2O were only apparent in UTEC in WW2016 and urea in WW2017 ($R^2 = 0.37$ and 0.30, respectively). For NO_3^- contents, WR2017 showed much higher correlations between N_2O and NO_3^- content ($R^2 = 0.31$–0.43, $p < 0.01$) than WW2016 and WW2017 (Table 3).

Table 1. Grain and straw yield, grain and straw N content, grain, straw, aboveground N uptake and ARN (apparent recovery N) in winter wheat in 2016, winter wheat in 2017, and oilseed rape in 2017. See text for details of the treatments.

Year	Crop	Fertilizer	Yield (t ha^{-1})		N content (%)		Aboveground N uptake (kg N ha^{-1})			ARN (%)
			Grain	Straw	Grain	Straw	Grain	Straw	Total	
2016	Winter wheat	N0	8.4±0.1a	6.3±0.2a	1.75±0.11a	0.49±0.04a	146±11a	31±8a	178±11a	-
		CAN	9.2±0.2a	7.3±0.1b	2.19±0.07b	0.75±0.03b	202±3b	55±1b	257±3b	44
		ENTEC	9.4±0.4a	7.5±0.1b	2.28±0.05b	0.76±0.04b	215±11b	57±4b	271±6b	52
		Urea	9.1±0.2a	8.0±0.7b	2.12±0.03b	0.73±0.02b	193±7b	59±6b	252±11b	41
		UTEC	9.2±0.4a	7.4±0.2b	2.17±0.05b	0.75±0.02b	199±8b	55±3b	254±8b	42
2017	Winter wheat	N0	4.7±0.2a	3.8±0.1a	1.44±0.01a	0.39±0.04a	68±2a	14±1a	83±2a	-
		CAN	7.7±0.2b	5.4±0.3b	2.12±0.05c	0.64±0.02c	164±6b	35±2b	199±6b	64
		ENTEC	7.6±0.1b	5.6±0.6b	2.21±0.02c	0.53±0.02b	169±4b	30±4b	199±5b	64
		Urea	8.2±0.2bc	6.3±0.4c	1.97±0.03b	0.53±0.02b	162±6b	34±1b	196±5b	63
		UTEC	8.3±0.1c	6.1±0.4c	2.03±0.03bc	0.54±0.03b	169±0b	34±4b	203±3b	66
2017	Oilseed rape	N0	3.1±0.2a	5.5±0.3a	3.22±0.07a	0.62±0.04a	101±5a	34±3a	134±6a	-
		CAN	3.1±0.1a	5.5±0.2a	3.75±0.05b	0.99±0.09b	115±1b	54±3b	168±2b	19
		ENTEC	3.4±0.1a	5.9±0.4a	3.69±0.08b	0.84±0.03b	125±7b	50±5b	175±10b	23
		Urea	3.2±0.2a	5.2±0.2a	3.58±0.03b	0.89±0.04b	116±4b	46±1b	161±3b	15
		UTEC	3.2±0.1a	5.3±0.3a	3.62±0.11b	0.82±0.07b	115±2b	43±4ab	158±4b	13

Within each column, different letters indicate significant differences based on LSD test at $p < 0.05$.

Data presented are means ± standard errors; n=3.

Table 2. Cumulative N_2O emissions on either an area-scaled (kg N ha^{-1} yr^{-1}) or grain yield-scaled (g N_2O-N kg^{-1} aboveground N uptake) basis and the direct annual emission factors (EF, %) for the winter wheat in 2016 and 2017 and winter oilseed rape in 2017.

Year	Crop	Fertilizer	Area-scaled N_2O emissions (kg N_2O-N ha^{-1} yr^{-1})			Yield-scaled N_2O emissions (g N_2O-N kg^{-1} aboveground N uptake)	EF (%)
			Pre-harvest	Post-harvest	annual		
2016	Winter wheat	N0	-0.03±0.02a	0.63±0.10a	0.60±0.12a	3.3±0.5a	-
		CAN	0.97±0.23b	0.96±0.12a	1.94±0.28b	7.6±1.0ab	0.74±0.16a
		ENTEC	0.73±0.16b	0.91±0.29a	1.65±0.38ab	6,1±1.2ab	0.58±0.21a
		Urea	1.34±0.45b	1.01±0.12a	2.35±0.55b	9.3±1.7b	0.97±0.31a
		UTEC	0.93±0.15b	0.80±0.07a	1.73±0.25b	6.8±0.8ab	0.67±0.14a
2017	Winter wheat	N0	0.32±0.11a	0.55±0.04a	0.87±0.15a	10.4±1.0b	-
		CAN	1.34±0.05b	0.75±0.14a	2.09±0.19b	10.5±1.4b	0.68±0.11b
		ENTEC	0.54±0.06a	0.35±0.06a	0.89±0.10a	4.4±0.4a	0.01±0.06a
		Urea	0.77±0.27ab	1.03±0.09b	1.80±0.36b	9.2±1.1ab	0.52±0.20b
		UTEC	0.80±0.30ab	0.66±0.11a	1.46±0.40ab	7.2±2.2ab	0.33±0.18ab
2017	Oilseed rape	N0	0.22±0.01a	0.75±0.11a	0.98±0.11a	7.3±0.7a	-
		CAN	1.39±0.03c	1.64±0.19ab	3.03±0.22b	18.0±2.2b	1.14±0.12a
		ENTEC	0.61±0.03b	2.03±0.35b	2.65±0.39b	15.1±2.6b	0.93±0.21a
		Urea	1.09±0.23bc	1.60±0.50ab	2.70±0.72b	16.7±3.6b	0.96±0.40a
		UTEC	1.01±0.02c	1.45±0.36ab	2.46±0.36b	15.6±0.7b	0.82±0.20a

Within each column, different letters indicate significant differences based on LSD test at $p < 0.05$.

Data presented are means ± standard errors; n=3.

Table 3. Spearman correlation coefficients (R^2) between soil N_2O fluxes, chamber air temperature, water-filled pore space (WFPS) and soil NH_4^+ and NO_3^- concentrations (0–15cm) in WW2016, WW2017 and WR2017.

Year	Crop	Fertilizer	Air temp.	WFPS	NH_4^+	NO_3^-
2016	Winter Wheat	N0	0.04 (n=180)	0.35** (n=75)	0.10 (n=75)	0.05 (n=75)
		CAN	0.19*	0.23*	0.03	0.08
		ENTEC	0.18*	0.15	0.13	0.15
		Urea	0.19*	0.22†	0.17	0,20†
		UTEC	0.20**	0.14	0.37**	0.20†
2017	Winter Wheat	N0	0.01 (n=204)	0.11 (n=60)	0.25* (n=60)	0.10 (n=60)
		CAN	0.23**	0.11	0.20	0.13
		ENTEC	0.08	0.34**	0.15	0.02
		Urea	0.11†	0.10	0.30*	0.08
		UTEC	0.12†	0.18	0.16	0.01
2017	Oilseed rape	N0	0.08 (n=204)	0.01 (n=60)	0.04 (n=60)	0.40** (n=60)
		CAN	0.21**	0.13	0.10	0.35**
		ENTEC	0.02	0.26*	-0.14	0.43**
		Urea	0.27**	-0.01	0.24†	0.43**
		UTEC	0.24**	0.06	0.27†	0.31**

† $p \leqslant 0.1$, * $p \leqslant 0.05$, **$p \leqslant 0.01$

Discussion

Crop yields and N uptake

Crop yields showed no difference between fertilizer treatments, but there was a large interannual variability. The variability can mainly be explained by two factors: the climatic conditions and the crop rotation.

The heavy rain events in July 2017 delayed the harvest of the winter oilseed rape and resulted in large yield losses. Winter wheat was also affected by the rainfall events, but the effect was smaller because wheat matured later than oilseed rape. Heavy rainfall often causes NO_3^- leaching (Dai et al., 2016; Errebhi et al., 1998; Xu et al., 2020), and correspondingly the soil NO_3^- content in WW2017 and WR2017 was lower than in WW2016 (Fig. 3). There are many reports for Germany that wheat grown after oilseed rape has higher yields than wheat grown after cereals (Angus et al., 2015; Sieling and Christen, 2015). The ecological reasons for these so-called break-crop benefits include an improved soil structure, better weed control, the suppression of propagation of typical cereal pathogens, and the avoidance of phytotoxic exudates released by harvest residues (Weiser

et al., 2018). The nutritional reason for higher yields is the high surplus of N after oilseed rape crops (Bouchet et al., 2016; Henke et al., 2008), also because the N content of the residual straw was higher in WR2017 than in WW2016 and WW2017. Hence, the mineralization of plant residues provided more mineral N than required for the uptake by wheat of N before winter (Ruser et al., 2017). This may also explain the high background levels of NO_3^- in the succeeding crops of winter wheat.

The enhanced-efficiency fertilizers ENTEC (added with the nitrification inhibitor DMPP) and UTEC (added with the urease inhibitor NBPT) increased aboveground N uptake by only 0–5% relative to the common fertilizers CAN and urea, with no statistical difference. Nauer et al. (2018) and Pfab et al. (2012) also reported only minor benefits to productivity from using urease and nitrification inhibitors. However, two meta-analyses revealed that these enhanced-efficiency fertilizers may increase grain yields by 5.7% (Linquist et al., 2013) or 7.5% (Abalos et al., 2014). Our results do not deny the potential of urease and nitrification inhibitors in increasing crop yields and N uptake, but the increase of grain yield would be minor, whereas climate conditions were also important factors affecting crop yield and aboveground N uptake.

Effect of environmental factors on N_2O emissions

In our study, N_2O fluxes were significantly ($P < 0.05$) correlated with air temperature on most fertilized soils, except for ENTEC (Table 3), which was in line with several other studies (Ding et al., 2019; Kroon et al., 2010; Ni et al., 2012; Shimizu et al., 2013). In 2017, annual N_2O emissions from N0 in both fields were higher than those in the wheat field in 2016, and one reason for this might be the higher annual mean temperature in 2017, which would probably increased the activities of temperature-dependent microbes (Butterbach-Bahl et al., 2013).

In most cases, WFPS was also positively correlated with soil N_2O emissions (Table 3). In summer 2017, both wheat and oilseed rape fields showed higher N_2O emission peaks than in 2016, and one likely reason for this is the higher precipitation in summer 2017. The WFPS in our study was < 60% in most cases and therefore nitrification may have been the dominant process (Baral et al., 2016; Davidson, 1993; Khalil et al., 2002; Scholefield et al., 1997), with water becoming the limiting factor for soil microbes, so the soil N_2O emissions were positively correlated with WFPS (Jäger et al., 2011; Ni et al., 2012).

NH_4^+ contents exhibited a slightly stronger associations with the N_2O fluxes in urea-containing fertilizers than in CAN and ENTEC. The results indicate that nitrification played an important role in urea fertilizers in contributing to N_2O emissions (Fu et al., 2012). It is widely accepted that soil NO_3^- is the dominant factor causing N_2O emissions (Ni et al., 2012; Ruser et al., 2017; Walter et al., 2015; Yan et al., 2018). In our study in WR2017, N_2O fluxes were highly correlated with soil NO_3^- content. The high correlation between N_2O emissions and soil NO_3^- concentrations in WR2017 was mainly attributed to straw mineralization. This was probably related to two factors: (1) compared to wheat residues, the large amount of oilseed rape residues in WR2017 supplied not only NO_3^- to the soil, but also ample organic carbon substrate (Köbke et al., 2018; Mitchell et al., 2013; Senbayram et al., 2012); and (2) the heavy rainfall events in summer 2017 may have accelerated straw decomposition. The high background soil NO_3^- in WW2016 (about 10–15 kg N ha^{-1}, 0–30 cm) and the post-harvest soil NO_3^- in WR2017 (about 10–15 kg N ha^{-1}) can both be explained by the large amount of oilseed rape residues post-harvest, which resulted in high-N surpluses (Köbke et al., 2018; Ruser et al., 2017; Sieling and Kage, 2010).

Effect of urease and nitrification inhibitors on N_2O emissions

Both the area-scaled and yield-scaled N_2O emissions of the enhanced-efficiency fertilizers (ENTEC and UTEC) were numerically lower than N fertilizer without inhibitors (CAN and urea) (Table 2). Such reduction is in agreement with other studies (Hu et al., 2015; Qiao et al., 2015; Silva et al., 2017). Only ENTEC in WW2017 showed impressively lower cumulative N_2O emissions than the other fertilizers ($p < 0.05$). The low cumulative N_2O emissions of ENTEC in WW2017 were mainly attributed to the reduction of N_2O emissions in the pre-harvest season (Table 2). Surprisingly, although ENTEC in WR2017 showed the lowest N_2O emissions in the pre-harvest season, there was no reduction of N_2O emissions in the post-harvest season (Table 2). In fact, N_2O emissions in the post-harvest season of WR2017 were quite high in all fertilized treatments. As mentioned above, the most likely reason for this was the large amount of oilseed rape residues and high soil moisture, which provided N substrate, an energy source, and sufficient water for denitrification (Köbke et al., 2018; Senbayram et al., 2012; Wu et al., 2018). As a result, annual N_2O emissions of the ENTEC treatment in WR2017 did not show a statistical difference with CAN and urea. Therefore, we are able to demonstrate that high post-harvest N_2O emissions may have masked reductions in N_2O emissions by the inhibitor over the full year.

However, in many studies, straw amendment was recommended as a method to reduce N_2O emissions (Huang et al., 2017; Xu et al., 2019; Yao et al., 2017). A key factor of N_2O emissions with straw incorporation is the C:N ratio, or NO_3^- in the soil, in that a higher C:N ratio would lead to more complete denitrification (Hu et al., 2019; Köbke et al., 2018). In WR2017, the yield-scaled N_2O emissions of the fertilized treatments were higher than 15 g N_2O-N kg N aboveground N uptake, which is seen as an indicator of excessive N application (Groenigen et al., 2010). Considering that ARN in WR2017 was much lower than in WW2016 and WW2017, we therefore postulate that the N application rates in the oilseed rape field might have been more than optimal. Reducing the amount of N application in oilseed rape is therefore suggested as possible solution to avoid high post-harvest N_2O emissions. Also, it is necessary to take the N application rate into consideration in straw management strategies (Hu et al., 2019; Yao et al., 2017).

Implications for crop production

Wheat–wheat–oilseed rape is a common crop rotation in Germany. Although this study did not cover a three-year cycle in the same field, it nonetheless provides valuable information for such rotation systems. Firstly, enhanced-efficiency fertilizers have the potential to reduce N_2O emissions, but their effectiveness is influenced by various environmental factors. Therefore, inhibitors should be used with more advanced technology—for example, with more efficient broadcasting or soil injection. Secondly, the risk of N_2O emissions from oilseed residues in the post-harvest season should be carefully assessed. And finally, N application rates should be optimized; our suggestion is to increase the amount of N application in wheat after the wheat and to reduce N fertilization in the oilseed rape.

Conclusions

In this study, we carried out N_2O measurements during two years of winter wheat and one year of oilseed rape. Compared to fertilizers without inhibitors, enhanced-efficiency fertilizers increased the aboveground N uptake by only 0–5%. However, the high surplus of N in oilseed rape also carries a risk of high post-harvest N_2O emissions. Therefore, assessing the benefits of oilseed rape as break crop should also consider the post-harvest management of crop residues. Overall, we conclude that, both ENTEC and UTEC have the potential to increase yield and reduce N_2O emissions in wheat–wheat–oilseed rape rotations, although with the caveat that many

environmental factors (e.g., the accumulative temperature, soil moisture content and soil NO_3^- substrate) contribute to a certain level of uncertainty in the degree to which enhanced-efficiency fertilizers can reduce N_2O emissions. We also recommend an improved fertilization strategy towards gaining a better grain yield and reduced N_2O emissions in the form of increasing the N application level in wheat after wheat and reducing it in the oilseed rape season.

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Chapter 4: The potential of ryegrass as cover crop to reduce soil N_2O emissions and increase the population size of denitrifying bacteria

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The potential of ryegrass as cover crop to reduce soil N_2O emissions and increase the population size of denitrifying bacteria

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Abstract

Nitrogen (N) fertilization is the major contributor to nitrous oxide (N_2O) emissions from agricultural soil, especially in post-harvest seasons. This study was carried out to investigate whether ryegrass serving as cover crop affects soil N_2O emissions and denitrifier community size. A microcosm experiment was conducted with soil planted with perennial ryegrass (*Lolium perenne* L.) and bare soil, each with four levels of N fertilizer (0, 5, 10 and 20 g N m^{-2}; applied as calcium ammonium nitrate). The closed-chamber approach was used to measure soil N_2O fluxes. Real-time PCR was used to estimate the biomass of bacteria and fungi and the abundance of genes involved in denitrification in soil. The results showed that the presence of ryegrass decreased the nitrate content in soil. Cumulative N_2O emissions of soil with grass were lower than in bare soil at 5 and 10 g N m^{-2}. Fertilization levels did not affect the abundance of soil bacteria and fungi. Soil with grass showed greater abundances of bacteria and fungi, as well as microorganisms carrying *narG*, *napA*, *nirK*, *nirS* and *nosZ* clade I genes. It is concluded that ryegrass serving as a cover crop holds the potential to mitigate soil N_2O emissions in soils with moderate or high NO_3^- concentrations. This highlights the importance of cover crops for the reduction of N_2O emissions from soil, particularly following N fertilization. Future research should explore the full potential of ryegrass to reduce soil N_2O emissions under field conditions as well as in different soils.

Highlights

1. This study was to investigate whether ryegrass serving as cover crop affects soil N_2O emissions and denitrifier community size;
2. Plant reduced soil N substrates on one side, but their root exudates stimulated denitrification on the other side;

3. N_2O emissions were lower in soil with grass than bare soil at medium fertilizer levels, and growing grass stimulated the proliferation of almost all the denitrifying bacteria except nosZ clade II;
4. Ryegrass serving as a cover crop holds the potential to mitigate soil N2O emissions.

KEYWORDS

denitrification, perennial ryegrass (*Lolium perenne* L.), soil bacteria, soil CO_2 emissions, soil N_2O emissions

1 | INTRODUCTION

Increasing nitrous oxide (N_2O) concentration in the atmosphere is among the most serious consequences of the anthropogenic alteration of the global nitrogen (N) cycle (Bakken & Frostegard, 2017). In addition to its high global warming potential and long atmospheric lifetime (IPCC, 2013), N_2O has been shown to be the most important emitted compound involved in stratospheric ozone depletion (Ravishankara, Daniel, & Portmann, 2009). The intensive input of mineral N into agricultural soils is one of the crucial factors contributing to soil N_2O emissions (Ju et al., 2009; Song et al., 2018). Denitrification is the predominant N_2O-producing biological process in soils (Bremner, 1997; Hu, Chen, & He, 2015), which is strongly affected by the soil nitrate (NO_3^-) concentration (Köbke, Senbayram, Pfeiffer, Nacke, & Dittert, 2018; Saggar et al., 2013). In the denitrification pathway, denitrifying microorganisms use NO_3^- as an electron acceptor and reduce it to gaseous N_2 in a stepwise manner. Incomplete denitrification results in the emission of gaseous intermediates such as N_2O.

Soil denitrification is regulated by enzymes such as NO_3^-, nitrite (NO_2^-) and N_2O reductases that are produced by microorganisms. In arable soils, plant root architecture and exudation alter soil structure, aeration and biological activity (Bertin, Yang, & Weston, 2003; Kuzyakov & Xu, 2013), as well as soil microbial communities (Berg & Smalla, 2009). The majority of laboratory studies of soil N_2O emissions, however, have not included plants, although it is known that growing plants may increase denitrification activities in soil (Guyonnet et al., 2017; Klemedtsson, Svensson, & Rosswall, 1987). Recent studies investigated how plant and rhizosphere processes affect soil N_2O emissions (Lenhart et al., 2019; Senbayram et al., 2020). On the one hand, plants compete with soil microorganisms for N (Moreau, Bardgett, Finlay, Jones, & Philippot, 2019), on the other hand, plants provide carbon (C) to the soil via root exudates that modulate microbial communities and denitrification activity (Achouak et al., 2019). Apart from effects on the soil N pool, plants consume O_2 and increase soil CO_2 concentrations through root respiration as compared to unplanted soil. It has been estimated that 5% to 21% of all photosynthetically assimilated C is released into the soil in the form of root exudates (Derrien, Marol, & Balesdent, 2004; Nguyen, 2003). Consequently, the C turnover rate in the soil rhizosphere is estimated to be at least one order of magnitude greater than in the bulk soil (Kuzyakov, 2010). It has been suggested that root exudation will increase denitrification (Bijay-singh & Whitchead, 1988), as root-released C can serve as an electron donor (Philippot, Hallin, & Schloter, 2007). Indeed, planted soils are several times greater in density of denitrifiers than unplanted soils (Chèneby et al., 2004; Herman, Johnson, Jaeger, Schwartz, & Firestone, 2006). Growing perennial grasses, such as *Festuca paniculata*, *Bromus erectus* and *Dactylis glomerata* (Guyonnet et al., 2017), barley (*Hordeum vulgare* L.) (Klemedtsson et al., 1987) and maize (*Zea mays* L.) (Mahmood, Ali, Malik, & Shamsi, 1997) has been shown to increase denitrification activities in soil. The stimulation of soil denitrification activity by plants depends on the plant species and soil water content (Bakken, 1988). Furthermore, root exudates have been shown to modulate soil microbial communities (Haichar et al., 2008; Haichar, Santaella, Heulin, & Achouak, 2014).

However, increased denitrification activity does not necessarily mean higher N_2O emissions from soil. Ammonium (NH_4^+) and NO_3^- have different motilities in soil due to the charge-dependent interaction with soil colloids. As a consequence, a depletion zone of NH_4^+ in the rhizosphere can be created by plant root uptake of NH_4^+ as it shows low mobility in most temperate soils (Orcutt, 2000). In contrast, no such depletion zones in the rhizosphere can be expected for NO_3^- due its high mobility in most temperate soils (Kuzyakov & Xu, 2013). The concentration of NO_3^- in soil, however, can rapidly decrease owing to uptake by plant roots (Tinker & Nye, 2000). Therefore, the availability of mineral N in soil is regarded as a major factor limiting denitrification (Philippot et al., 2007; Saggar et al., 2013). The response

of soil N_2O emissions to the application of mineral N fertilizer is exponential rather than linear (Shcherbak, Millar, & Robertson, 2014). Senbayram, Chen, Budai, Bakken, & Dittert (2012) reported that increasing the soil NO_3^- concentration resulted in a higher N_2O/N_2 ratio. The competition for NO_3^- between plants and denitrifiers can result in lower denitrification rates in planted soils (Qian, Doran, & Walters, 1997). Similarly, regulation of denitrifying soil communities by NO_3^- has been reported from different ecosystems (Correa-Galeote et al., 2017; Deiglmayr, Philippot, & Kandeler, 2006; Enwall, Philippot, & Hallin, 2005); however, the effect of the soil NO_3^- concentration on the abundance and diversity of denitrifiers remains to be determined.

Ryegrass is a common cover crop that is used to reduce nitrate leaching (Bergström & Jokela, 2001; Poeplau, Aronsson, Myrbeck, & Kätterer, 2015; Thomsen & Hansen, 2014) and increase soil organic C stocks (Poeplau et al., 2015). The effect of ryegrass on soil N_2O emissions, however, is under-studied. A recent meta-analysis revealed that cover crops have the potential to mitigate N_2O emissions in post-harvest seasons, yet few studies focused on ryegrass (Muhammad et al., 2019). The main aim of this study was therefore to investigate N_2O emissions from soil with ryegrass compared to bare soil under varying fertilizer levels. To achieve this, we used an incubation experiment with two experimental factors: soil planted with grass and unplanted bare soil, each with four levels of N fertilizer addition. Soil N_2O fluxes were determined using the closed-chamber approach. Real-time PCR (qPCR) assays were performed to estimate the abundance of soil bacteria and fungi, as well as microorganisms harbouring genes involved in denitrification. We hypothesized that the presence of grass and the associated belowground modulations would (i) lower soil N_2O emissions at each fertilizer level and (ii) promote the abundance of bacteria, fungi and denitrifiers, as compared to bare soil.

2 | MATERIAL AND METHODS

2.1 | Soil collection

Topsoil (0 to 25 cm) was collected from Reinshof agricultural research station (51°29′50.3"N, 9°55′59.9″E), University of Göttingen, Lower Saxony, Germany. Mean annual precipitation was 651 ± 24 mm and mean annual temperature was 9.2 ± 0.1°C (1981–2010, meteorological station at Göttingen, station ID: 1691, Germany's Meteorological Service). The site had been cropped with winter oilseed rape (*Brassica napus* L.) (2015), winter wheat (*Triticum aestivum* L.) (2016) and winter barley (2017) prior to soil collection on March 23, 2018. The soil was classified as Luvisol (IUSS, 2015) and the texture of the topsoil (0 to 25 cm) was composed of 61% silt, 23% sand and 16% clay. The bulk density was 1.3 g cm^{-3}, the pH was 7.1 ± 0.1, the soil total C concentration was 1.3% and the total N concentration was 0.13%. Following collection, the soil was stored in a polyvinyl chloride (PVC) container for 3 months at room temperature until incubation. Before incubation, the soil was air-dried to 2% gravimetric water content and sieved through a 2-mm mesh to achieve higher homogeneity. PVC cylinders (diameter, 20 cm; height, 20 cm) were used for incubation and sealed with removable lids (height, 5 cm) carrying butyl-rubber septa for headspace gas sampling. Soil moisture was first adjusted to 35% water-filled pore space (WFPS) and soil (equivalent to 4.49 kg dry soil) was filled into the experimental pots in three layers of approximately 3.7 cm each (11 cm in total) for manual compaction to the original bulk density of 1.3 g cm^{-3}, resulting in 4,398 cm^3 of air space (9 cm headspace + 5 cm lid) for gas accumulation when the chambers were closed. The following day, the soil was carefully irrigated in a stepwise procedure to avoid soil compaction and finally adjusted to 60% WFPS.

2.2 | Experimental setup

The experiment was conducted in a fully controlled climate chamber (Fitotron Walk in Plant Growth Room, Type SGR221 LED, Weiss Technik, Leicester, UK). The climate chamber was set to a light intensity of 520 μmol m^{-2} s^{-1} photosynthetically active photon flux density at 25°C air temperature from 6.00 am to 10.00 pm as "day mode" (16 hr), and from 10.30 pm to 6.00 am (8 hr) as "night mode" with no light at 12°C air temperature. The relatively large temperature discrepancy was set in order to mimic conditions close to those in the field.

The experiment consisted of two groups: soil with perennial ryegrass (*Lolium perenne* L.) (DSV AG, Salzkotten, Germany) and bare soil. Each group had four different fertilizer levels (0, 5, 10 and 20 g N m^{-2}, equivalent to 0, 50, 100 and 200 kg N ha^{-1}), resulting in a total of eight treatments. Each treatment was performed in triplicate, yielding a total of 24 pots. Before the first sampling date, grass was sown at a density of approximately 5,000 seeds m^{-2} and pre-incubated for 4 weeks to allow grass establishment in the pots. The treatments with bare soil were treated equally but without plant cultivation. Calcium ammonium nitrate N fertilizer (76% ammonium nitrate (NH_4NO_3) and 24% calcium carbonate ($CaCO_3$)) was applied after dissolution in distilled water. Half of the total N fertilizer was applied after the first collection of soil and gas samples on day 1 (August 3, 2018); the other half

TABLE 1 Total N uptake and C assimilation of grass shoots and roots throughout the experimental period (56 days) at each fertilizer level in soil with grass

	N uptake (g N m^{-2})			C assimilation (g C m^{-2})		
Fertilizer level (g N m^{-2})	Shoot	Root	Total	Shoot	Root	Total
0	2.6 ± 0.1c	1.5 ± 0.2b	4.1 ± 0.1d	58.3 ± 2.6b	58.7 ± 2.8b	117.0 ± 4.4b
5	4.7 ± 0.2c	1.8 ± 0.1a	6.5 ± 0.2c	98.4 ± 4.3a	74.3 ± 0.9a	172.6 ± 4.7a
10	7.4 ± 0.7b	2.0 ± 0.1a	9.3 ± 0.5b	115.8 ± 5.3a	74.8 ± 4.1a	190.5 ± 1.2a
20	11.8 ± 0.7a	2.1 ± 0.1a	13.9 ± 0.5a	123.6 ± 9.2a	68.1 ± 2.6a	191.8 ± 10.8a

Note: Means ± standard errors followed by different lowercase letters indicate significant differences among fertilizer levels within each parameter (one-way ANOVA with Tukey's honestly significant difference (HSD) test or Kruskal-Wallis test with multiple comparison extension) at $p < .05$.

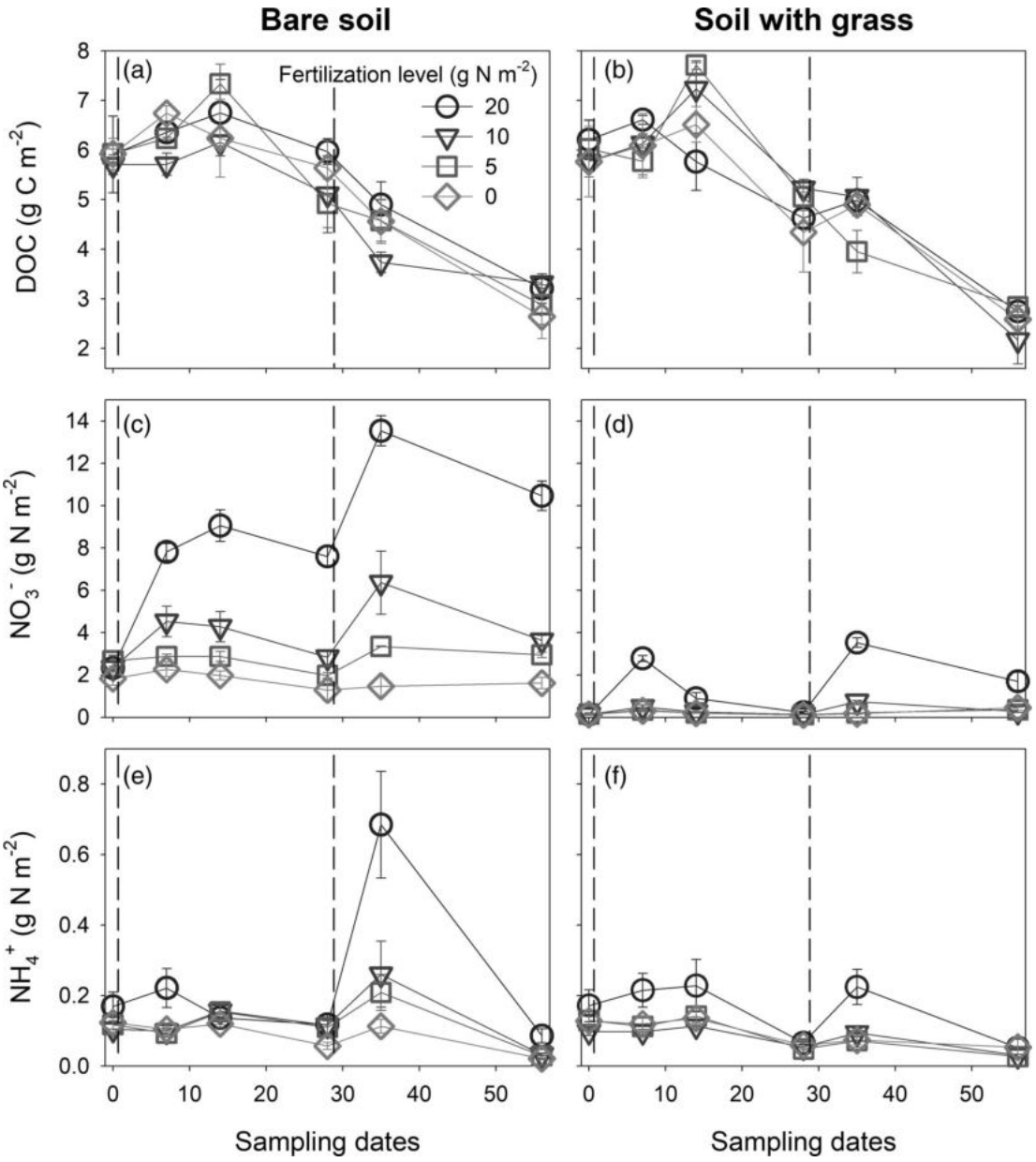

FIGURE 1 Time course of dissolved organic carbon (DOC) concentrations in (a) bare soil and (b) soil with grass, and soil NO_3^--N content in (c) bare soil and (d) soil with grass, and soil NH_4^+-N concentrations in (e) bare soil and (f) soil with grass during the 56 days growing period. Solid lines with points of different grey intensities represent different fertilizer levels (0, 5, 10 and 20 g N m^{-2}); dashed vertical lines indicate fertilization dates (day 1 and 28). Error bars represent the standard error of the mean ($n = 3$)

was applied after 28 days, with a full measuring period of 56 days. After fertilization, soil was irrigated daily, and up to every 2 days in the later period by weighing the pots, to keep the soil moisture at 60 ± 5% WFPS.

Two days before the first fertilization, the grass was cut to a height of 4 cm. Following this, the grass was cut every 2 weeks and the shoot dry matter was determined from air-dried material. At the end of the experiment, the roots were collected as well. Roots were carefully washed, air-dried and weighed. The total C and N of finely ground dry grass shoots and roots were determined on a NA-1500 N elemental analyzer (Carlo Erba, Milano, Italy). Grass N uptake and C assimilation were calculated as:

$$N_{uptake} = DM_{shoot} \times N\text{concentration}_{shoot} + DM_{root} \times N\ \text{concentration}_{root}; \quad (1)$$

and

$$C_{assimilation} = DM_{shoot} \times C\text{concentration}_{shoot} + DM_{root} \times C\ \text{concentration}_{root}; \quad (2)$$

where DM refers to the dry matter of the harvested grass. Apparent N recovery (ARN) was calculated as:

$$\text{ARN}\ (\%) = \frac{N_{uptake}(\text{fertilized}) - N_{uptake}(\text{unfertilized})}{\text{amount of N applied}} \times 100. \quad (3)$$

Gas samples of 25 mL in volume were collected using a syringe inserted in the headspace of sealed lids. Samples were directly transferred to a pre-evacuated 12-mL Exetainer vial (Labco, Lampeter, UK). Gas samples were collected at 0, 20 and 40 min after the pots were sealed. In the first week after each fertilization, gas samples were collected every day to capture the fertilization-induced peaks. In the following 3 weeks, gas samples were taken at larger intervals of 2 to 4 days.

In order to avoid the disturbance of soil structure by soil sampling during the incubation period, we incubated a spare set of pots in parallel to the gas sampling pots for soil sample collection. The setup of these pots was identical to that for pots for gas sampling. Soil samples were taken on day 0 (1 day before the first fertilization), day

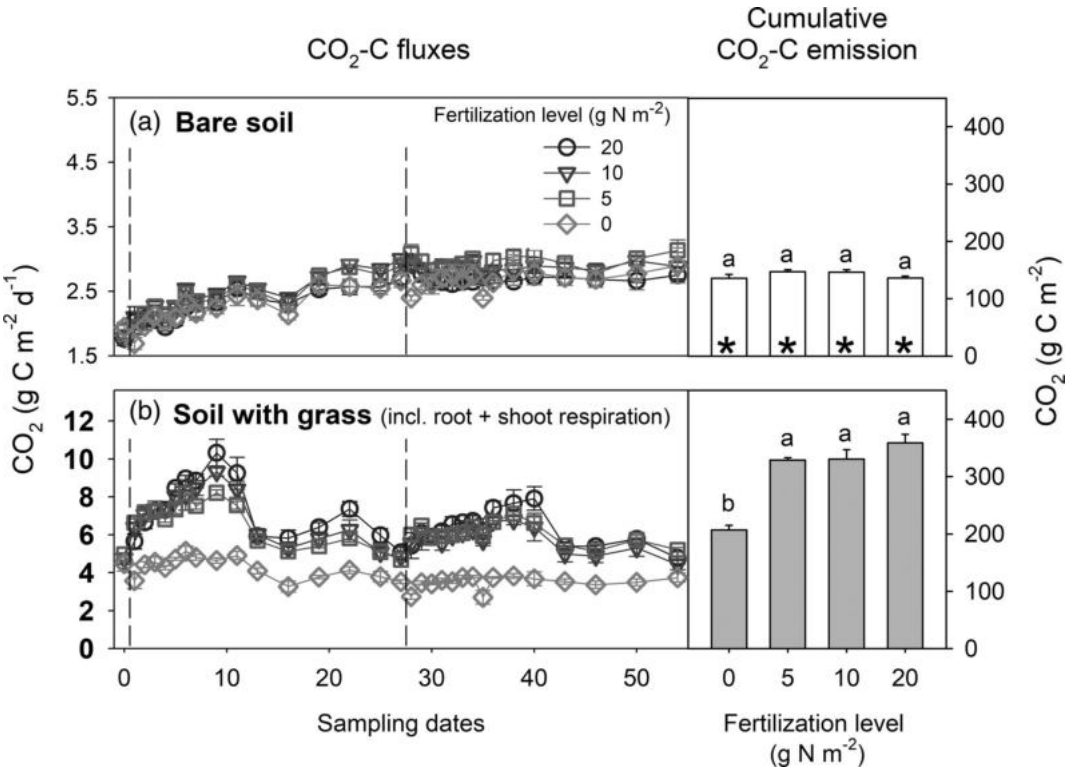

FIGURE 2 CO_2 emission dynamics and cumulative CO_2 emission during the growing period (56 days) from bare soil (a) and soil with grass (b). Error bars represent the standard error of the mean of each treatment ($n = 3$). Solid lines with points of different grey intensities represent different fertilizer levels (0, 5, 10 and 20 g N m^{-2}). Dashed vertical lines indicate fertilization dates (day 1 and day 28). Asterisks indicate significant differences in cumulative CO_2 emission between bare soil and soil with grass at the same fertilizer level (*t*-test or Mann–Whitney *U*-test); lowercase letters indicate significant differences in cumulative CO_2 emission among fertilizer levels within bare soil or within soil with grass (one-way ANOVA with Tukey's honestly significant difference (HSD) test or Kruskal-Wallis test with multiple comparison extension) at $p < .05$

7, day 14, day 28 (before the second fertilization), day 35 and day 56 (final collection of samples). On day 0, day 7 and day 14, soil samples were collected from the first spare pot; on day 35 and day 56, they were collected from the second spare pot. The last soil samples were taken from the pots on which gas measurements were performed. The soil samples (0–11 cm depth) were taken using a 16-mm diameter auger. Remaining holes were filled with reagent glasses (16 mm diameter) to avoid extra water and nutrient losses. Approximately 60 g of fresh soil was taken and sieved through a 2-mm mesh. Soil samples were homogenized and divided for soil NH_4^+ and NO_3^-, and dissolved organic carbon (DOC) analysis. Soil pH, total C and N, and WFPS did not differ among treatments. From the last set of soil samples, aliquots were used for soil DNA extraction and subsequent qPCR analysis.

2.3 | Gas and soil sample analysis

Gas samples were analysed on an Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a thermal conductivity detector for the determination of carbon dioxide (CO_2) concentrations and an electron capture detector for the determination of N_2O concentrations. The flux rates of CO_2 and N_2O were calculated using linear regression of the gas concentration over time (Parkin, Venterea, & Hargreaves, 2012; Wang et al., 2013). Cumulative emissions were calculated by interpolating the values of CO_2 and N_2O emissions.

To determine soil NH_4^+ and NO_3^- concentrations, subsamples (10 g) of sieved fresh soil were extracted by adding 50 mL of 0.0125 M calcium chloride ($CaCl_2$). Mixtures were shaken for 1 hr, filtered (MN615 ¼; pore size, 4–12 μm; Macherey-Nagel, Düren, Germany) and subsequently stored at −20°C until analysis. NH_4^+ and NO_3^- concentrations in the extracts were determined using a San^{++} continuous flow analyzer (Skalar Analytical, Breda, The Netherlands). Soil pH was measured from 10 g of air-dried soil suspended in 50 mL of 0.01 M $CaCl_2$ solution using a pH meter. Total C and N measurements were performed with finely ground air-dried soil using an NA-1500 elemental analyzer (Carlo Erba, Milano, Italy). Prior to the measurement of total C and N, the air-dried soil was fumigated in a hydrogen chloride (HCl) atmosphere

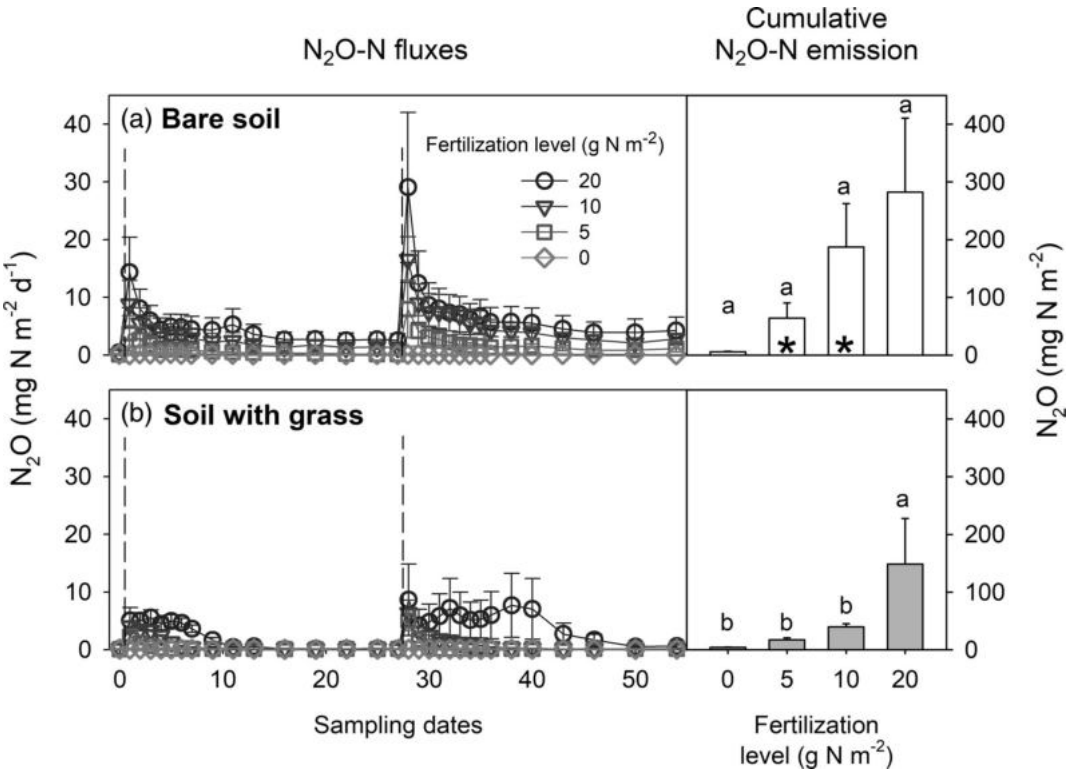

FIGURE 3 N_2O emission dynamics and cumulative N_2O emission during the growing period (56 days) from bare soil (a) and soil with grass (b). Error bars represent the standard error of the mean of each treatment ($n = 3$). Solid lines with points of different grey intensities represent different fertilizer levels (0, 5, 10 and 20 g N m^{-2}). Dashed vertical lines indicate fertilization dates (day 1 and day 28). Asterisks indicate significant differences in cumulative N_2O emission between bare soil and soil with grass at the same fertilizer level (*t*-test or Mann–Whitney *U*-test); lowercase letters indicate significant difference in cumulative N_2O emissions among fertilizer levels within bare soil or within soil with grass (one-way ANOVA with Tukey's honestly significant difference (HSD) test or Kruskal-Wallis test with multiple comparison extension) at $p < .05$

using 3 M HCl for 1 week to remove carbonates (Harris, Horwath, & Kessel, 2001). For DOC measurements, 10 g of fresh soil was extracted using 40 mL of 0.5 M potassium sulphate (K_2SO_4). The solution was shaken for 2 hr and filtered (MN615 ¼; pore size, 4–12 µm; Macherey-Nagel, Düren, Germany). Extracts were stored at −20°C until determination of organic C and total C concentrations using a Total organic carbon/Total inorganic carbon (TOC/TIC) analyser (Multi C/N 2100, Analytik Jena, Jena, Germany).

2.4 | DNA extraction from soil and qPCR

For qPCR analysis, soil was freeze-dried for 72 hr. The freeze-dried material was finely ground using a swing mill (MM400, Retsch, Haan, Germany) for 60 s at 25 Hz. Total DNA was extract from 50 mg ground soil using a modified cetyltrimethylammonium bromide-based protocol (Brandfass & Karlovsky, 2008) as described previously (Beule et al., 2019). Following DNA extraction, the quality and quantity of DNA were examined on 0.8% (w/v) agarose gels stained with ethidium bromide. The extracts were tested for PCR inhibitors as described previously (Guerra, Beule, Lehtsaar, Liao, & Karlovsky, 2020) and diluted 1:50 (v/v) in double-distilled water (ddH_2O) prior to qPCR analysis. We quantified bacterial 16S rRNA and fungal 18S rRNA genes, as well as genes involved in denitrification, namely *narG* and *napA* for NO_3^- reduction, *nirK* and *nirS* for NO_2^- reduction, and *nosZ* clade I and II for N_2O reduction. All reactions were carried out in 4 µL reaction volume (3 µL mastermix +1 µL template DNA or ddH2O for negative controls) on a CFX384 Thermocycler (Biorad, Rüdigheim, Germany). A detailed description of the mastermix composition and thermocycling conditions can be found in Beule et al. (2019).

2.5 | Statistical analysis

All data were tested for homogeneity of variance (Levene's test) and normal distribution (Shapiro–Wilk test). Differences among treatments of cumulative data (N uptake and C assimilation by grass and cumulative CO_2 and N_2O emissions) or data without repeated measurements (soil bacteria, fungi and denitrifiers) were assessed by performing a *t*-test or one-way ANOVA with Tukey's honestly significant difference (HSD) post-hoc test for parametric data, or the Mann–Whitney *U*-test or Kruskal–Wallis test with multiple comparison extension for non-parametric data. Differences among treatments of repeatedly measured data (DOC, NO_3^-, NH_4^+, CO_2 and N_2O fluxes) were analysed using linear mixed effect (LME) models. In the models, either the fertilizer level or the treatment of bare soil versus soil with grass were set as a fixed effect, and sampling date and replicate pot set as random effects. The data were partially $\log_{10}$- or square-root-transformed to meet the criteria for an LME model. Statistical significance was considered as $p < .05$, with marginal statistical significance at $p < .1$. All statistical analyses were performed in R version 3.5.2 (R Core Team, 2018).

3 | RESULTS

3.1 | Grass N uptake and C assimilation

In soil with grass, total plant N uptake, which was calculated by the dry matter of grass shoots and roots, ranged from 6.5 to 13.9 g N m^{-2} in fertilized pots, compared to 4.1 g N m^{-2} in the unfertilized treatment. The ARNs of fertilized treatments were 50% ± 2%. Plant shoot N uptake at the 10 g N m^{-2} fertilizer level was lower than

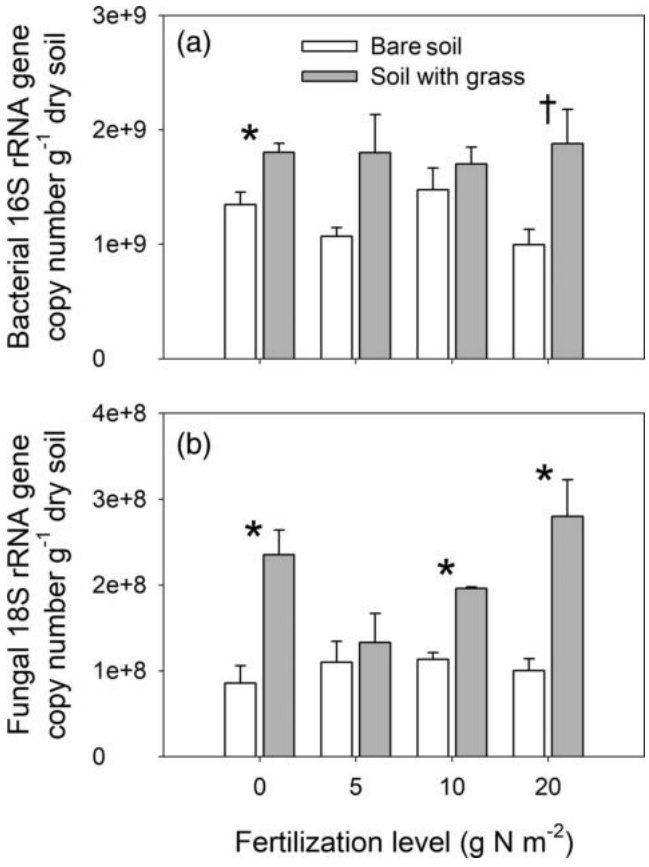

FIGURE 4 Bacterial 16 s rRNA (a) and fungal 18 s rRNA (b) gene copy number per g dry soil in bare soil and soil with grass under different fertilizer levels (0, 5, 10 and 20 g N m^{-2}) at the end of the growing period (day 56). Error bars represent standard error of the mean of each treatment ($n = 3$); asterisks denote differences between bare soil and soil with grass (* $p < .05$); daggers represent marginal differences between bare soil and soil with grass († $p < .1$)

that at 20 g N m^{-2} ($p = 0.006$) and greater than those at 0 and 5 g N m^{-2} ($p < .01$) (Table 1). Plant root N uptake in unfertilized treatments was lower than in the treatments in which 5, 10 and 20 g N m^{-2} were added ($p < .04$) (Table 1). The total N uptake throughout the incubation period (56 days) increased along with increasing fertilizer application ($p < .03$) (Table 1). Shoot C, root C and total C assimilation were greater in fertilized than unfertilized pots ($p < .04$) (Table 1), but did not differ among the 5, 10 and 20 g N m^{-2} fertilization treatments.

3.2 | Soil DOC, NO_3^- and NH_4^+ dynamics during incubation

Dissolved organic carbon was slightly increased in the first 2 weeks, and gradually decreased in the following weeks in both bare soil and soil with grass (Figure 1a, b). DOC concentrations did not differ among bare soil and soil with grass, nor among fertilizer levels (Figure 1a,b). Soil NO_3^--N content in bare soil was always greater than in soil with grass at all fertilizer levels ($p \leq .001$) (Figure 1c,d). Fertilization led to increased NO_3^--N concentrations in bare soil as compared to unfertilized treatments ($p \leq .05$) (Figure 1c). Compared to the background NO_3^- (approximately 2 g of NO_3^--N m^{-2}) in unfertilized bare soil, soil NO_3^--N was close to zero in the unfertilized treatment of soil with grass (Figure 1d). Furthermore, in soil with grass, at the 20 g N m^{-2} fertilizer level, soil NO_3^--N was greater than at all other fertilizer levels ($p \leq .05$) (Figure 1d). When N fertilizer was applied, NH_4^+-N was marginally greater in bare soil than in soil with grass ($p < .1$) (Figure 1e,f).

3.3 | CO_2 and N_2O emissions

The presence of grass strongly enhanced CO_2 emissions compared to bare soil, especially in treatments with fertilizer ($p \leq .003$) (Figure 2). Fertilization had no effect on CO_2 emissions (neither on short-term rates nor on cumulative fluxes) in bare soil (Figure 2a). In soil with grass, however, CO_2 emission rates and cumulative fluxes were increased by fertilizer application ($p \leq .001$) (Figure 2b).

In contrast to CO_2 fluxes, N_2O emissions from bare soil were greater than from soil with grass at each fertilizer level ($p < .05$) (Figure 3). At the 5 and 10 g N m^{-2} fertilizer levels, bare soil showed greater cumulative N_2O emissions than soil with grass ($p < .05$). Cumulative N_2O emissions from soil with grass at the 0, 5 and 10 g N m^{-2} fertilizer levels were lower than at 20 g N m^{-2} ($p < .05$) (Figure 3b).

3.4 | Soil microbial gene abundances in bare soil and soil with grass

At the end of the experiment (day 56), the abundances of bacteria, fungi and denitrification genes in soil were quantified. The fertilization rate did not affect the abundances of bacteria, fungi and denitrification genes (Figure 4, Figure 5). At the 0 and 20 g N m^{-2} fertilizer levels, the soil with grass showed marginally greater bacterial 16S rRNA gene copy numbers than bare soil ($p < .08$) (Figure 4a). Similarly, the number of fungal 18S rRNA gene copies did not differ among fertilizer levels, but were greater in soil with grass than bare soil at the 0, 10 and 20 g N m^{-2} fertilizer levels ($p < .05$) (Figure 4b). The abundance of *narG* was greater in the soil with grass than bare soil at the fertilizer level of 20 g N m^{-2} ($p < .005$) (Figure 5a). At the 0 and 5 g N m^{-2} fertilizer levels, gene copy numbers of *napA* in soil with grass were greater than in bare soil ($p < .06$) (Figure 5b). At each fertilizer level, *nirK* gene copy numbers were greater in soil with grass than in bare soil ($p < .09$) (Figure 5c). The abundance of *nirS* was increased in soil with grass compared to bare soil when 5, 10 or 20 g N m^{-2} of fertilizer was applied ($p < .1$) (Figure 5d). At the fertilization rate of 20 g N m^{-2}, *nosZ* clade I gene copies were marginally greater in soil with grass than in bare soil ($p < .07$) (Figure 5e). No differences between soil with grass and bare soil at any fertilizer level were detected for *nosZ* clade II genes (Figure 5f).

4 | DISCUSSION

4.1 | Soil organic C turnover and CO_2 emissions

The soil microbial community is the main driver of soil respiration and organic C mineralization in bare soils (Li et al., 2018; Liu et al., 2018). The slight increase in DOC in the first 2 weeks may have been due to the rewetting of the dry soil to 60% WFPS (Kalbitz, Solinger, Park, Michalzik, & Matzner, 2000). For example, when Lundquist, Jackson, & Scow (1999) exposed soil to wet–dry cycles, soil aggregates were partly decomposed and their C was found in the DOC fraction. In the first 3 weeks of the experiment, soil CO_2 emissions increased gradually in bare soils, indicating a recovery of the microbial respiration from the rewetted air-dried soil (Figure 2a). As the soil was already pre-incubated for 4 weeks before the application of fertilizer, this may be seen as an indication that the recovery of the soil microbial activity in the bare soil may take approximately 7 weeks under the given conditions. One reason for this

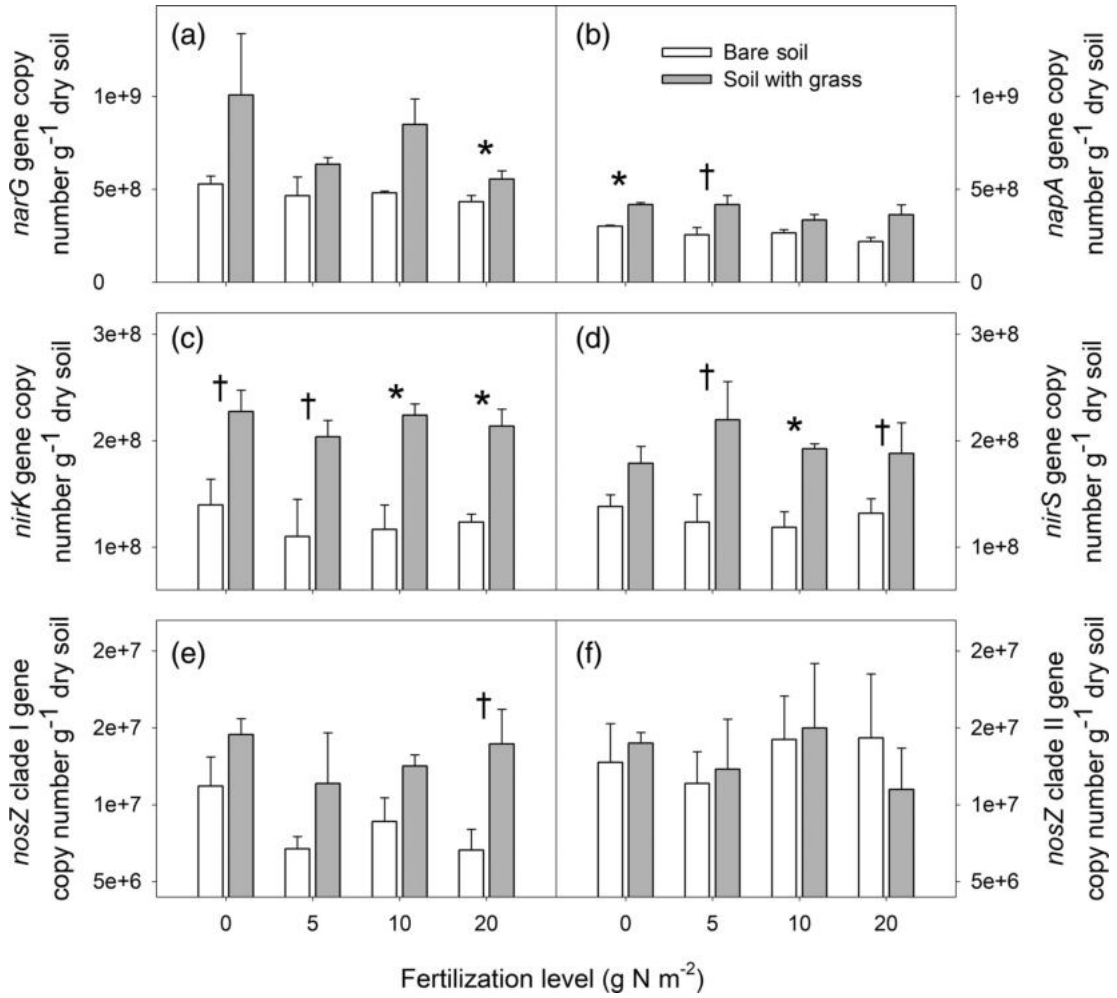

FIGURE 5 *narG* (a), *napA* (b), *nirK* (c), *nirS* (d), *nosZ* clade I (e) and *nosZ* clade II (f) gene copy number per g dry soil in bare soil and soil with grass under different fertilizer levels (0, 5, 10 and 20 g N m^{-2}) at the end of the growing period (day 56). Error bars represent standard error of the mean of each treatment ($n = 3$); asterisks denote differences between bare soil and soil with grass (* $p < .05$). Daggers represent marginal differences between bare soil and soil with grass († $p < .1$)

long recovery period in bare soil may be the limitation of available C. Three weeks after the first fertilization, the stable CO_2 emissions and slow DOC consumption rate may point towards a stabilized soil microbial community.

Pausch & Kuzyakov (2018) reviewed the distribution of C compounds in soil that were released by roots. They concluded that 12% of the assimilated C is emitted from the plant as root-derived CO_2 and 5% is deposited in the rhizosphere. Most plant root exudates have been reported to be readily available to soil microorganisms because they can be metabolized within a few hours (Jones et al., 2005; Kuzyakov & Xu, 2013). Moreover, it is reasonable to expect a greater DOC content in soils with grass than in bare soil given the estimation that 5% of the assimilated C is sequestrated in the rhizosphere (Pausch & Kuzyakov, 2018). However, no such increase was found in our study. Zhang, Li, Wang, & Huang (2018) reported that heavy grazing lowered the C input and decreased C accumulation and total soil organic C contents, due to reduced aboveground tissue (Schönbach et al., 2011), more exposure of the soil surface, and thus increased loss of soil moisture (Y. Zhao et al., 2007) and stimulated compensatory growth of new leaves (W. Zhao, Chen, & Lin, 2008). Therefore, the intensive cutting throughout our experiment is likely to have contributed to the lack of increased soil DOC, and the limitation of available C may restrict denitrification activity and therefore reduce soil N_2O emissions in soil with grass.

4.2 | Soil mineral N and N_2O emissions

The N_2O emissions followed a pattern that was similar to that for soil NO_3^- concentrations. For example, in each of

the eight treatments, the second N_2O emissions peak, which followed the second fertilizer application, was greater than the first peak (Figure 3a,b). Additionally, in contrast to soil with grass, bare soil treatments showed considerably greater N_2O emissions, which lasted over the entire study period. In soil with grass, N_2O emissions fell to nearly zero 2 weeks after each fertilizer application, which was associated with exploited soil NO_3^-. The relationships between soil NO_3^- and N_2O emissions indicate that, under these conditions, soil NO_3^- is the predominant factor controlling soil N_2O emissions (Dong et al., 2018; Ji et al., 2018; Zhou, Zhu, Wang, & Wang, 2017).

At 60% WFPS, both nitrification and denitrification are expected to be important contributors to soil N_2O emissions, as this moisture level is seen as the threshold between aerobic and anaerobic conditions (Köbke et al., 2018; Menéndez, Barrena, Setien, González-Murua, & Estavillo, 2012; Volpi, Laville, Bonari, o di Nasso, & Bosco, 2017). One week post the first fertilization, only 0.2 g N NH_4^+ was found and 7.9 g N NO_3^- were detected in the fertilized bare soil treatment at 20 g N m^{-2} (Figure 1c,e). Because the fertilizer was calcium ammonium nitrate at a ratio of NH_4^+ and NO_3^- N of 1:1, we assume that the vast majority of NH_4^+ was converted to NO_3^- through nitrification. Additionally, we assume that a certain proportion of the added NH_4^+ was released as N_2O during nitrification (Bremner, 1997). After 1 week, nitrification was unlikely to happen because the amount of NH_4^+ (0.2 g N) was low. Although nitrification was not investigated in this study, our observations agree with other incubation studies that more than 50% of the NH_4^+ is converted to NO_3^- within the first week after fertilizer application (Senbayram, Chen, Mühling, & Dittert, 2009; Wu et al., 2017). We anticipate that, in the first week after fertilization, both pathways (nitrification and denitrification) contributed to the observed N_2O emissions. In the following weeks, denitrification is likely to have become the predominant process contributing to N_2O emissions owing to NH_4^+ removal (Figure 1e,f) and lowered oxygen partial pressure induced by root O_2 consumption (Klemedtsson et al., 1987).

Due to root activity, two opposing effects on denitrification are likely to have occurred: (i) O_2 consumption by aerobic root activity (root respiration consuming O_2) (Kuzyakov & Razavi, 2019); and (ii) plant transpiration, leading to drainage of coarse soil pores and thus increased air-filled pore space, which will result in increased oxygen availability. In our study, although water content was adjusted every 1–2 days, soil with grass had about 5% lower WFPS than bare soil prior to irrigation. The loss of water was due to plant transpiration. Therefore, lower soil moisture due to plant transpiration may increase oxygen diffusion into the soil and thereby suppress denitrification (Menéndez et al., 2012; Volpi et al., 2017). Several previous studies reported that the presence of plants would increase denitrification (Guyonnet et al., 2017; Klemedtsson et al., 1987; Mahmood et al., 1997). However, our study was not a perfect proof of the opposite, but at least it provides evidence that the earlier reported promotion of denitrification does not always happen; at least, plants do not always induce higher N_2O emissions.

4.3 | Influence of the presence of plants on soil microbial abundances

The population size and diversity of microbial communities have repeatedly been shown to increase in the presence of plants (Guyonnet et al., 2018; Haichar et al., 2008; Li et al., 2018). In line with this, our results showed that population densities of both soil bacteria and fungi increased with the presence of ryegrass. Considering C-limited conditions in unplanted soil, we assume that root-derived input of easily available C promoted these microbial populations.

Plant root exudates are known to modulate both microbial biomass and community composition (Benizri, Nguyen, Piutti, Slezack-Deschaumes, & Philippot, 2007; Henry et al., 2008; Langarica-Fuentes, Manrubia, Giles, Mitchell, & Daniell, 2018; Zhalnina et al., 2018). However, a limited number of studies have explored how plants influence genes involved in denitrification (Henry et al., 2008; Pivato et al., 2017). We found that, with the exception of *nosZ* clade II, all denitrification genes were promoted in the presence of ryegrass, which may be due to the root exudation of easily available C. Graf (2015) proposed a greater affinity of *nosZ* clade I-carrying microorganisms to root exudates than for those carrying *nosZ* clade II. Our findings agree with the suggestions of Graf (2015): there was a trend showing that *nosZ* clade I genes were greater in soil with grass, whereas *nosZ* clade II showed no preference for bare versus planted soil.

4.4 | Relationship of reduced N_2O emissions and increased denitrifying gene abundances in soil with grass

In our study, N_2O emissions were reduced even though denitrification genes increased under grass. Recovery of N by crops is usually somewhat less than 50% (Fageria & Baligar, 2005). In our study, the high N recovery rate of ryegrass (~50% ARN) indicates that the incubation conditions (60% WFPS, 25°C day temperature and 12°C night temperature) were favourable for plant growth. The

ARN agrees well with the emission factors of bare soil and soil with ryegrass, which were 1.4%–1.8% and 0.5%–0.8%, respectively. Our results indicate that for soil N_2O production, the availability of mineral N was a more important factor than the population size of denitrifiers. It should be mentioned that, due to the limitation of the experimental design, soil samples and gas samples were not taken from the same pots, and N_2O emissions were highly variable. Therefore, it was not possible to correlate N_2O emissions and NO_3^- concentrations in this study.

In soil with grass at the 20 g N m^{-2} fertilizer level, soil NO_3^- concentrations were not depleted by plant uptake and, concurrently, cumulative N_2O emissions at this level were more than twice as high as those at the 10 g N m^{-2} fertilizer level, suggesting that an N input that exceeds the plant's needs can exponentially increase soil N_2O emissions. Previous field studies have shown congruent results (Groenigen, Velthof, Oenema, Groenigen, & Kessel, 2010; Philibert, Loyce, & Makowski, 2012; Shcherbak et al., 2014). The much lower cumulative N_2O emission levels in soil with grass, as compared to bare soil, at the 5 and 10 g N m^{-2} fertilizer levels, were most likely to be due to plant uptake of soil mineral N.

It was recently suggested that soil NO_3^- availability affects denitrifying communities (Deiglmayr et al., 2006; Saggar et al., 2013; Tang et al., 2016). However, our data revealed no link between fertilizer level and denitrification genes. The reason may be the limitation of available C in both bare soil and planted soil. In planted soil, intensive cutting may have limited C input from root exudates into the soil. It should be noted, however, that this observation requires further study, because denitrifiers were only quantified at the end of our experiment. Therefore, potential changes in microbial communities during the course of our experiment may have remained undetected. Our study aimed to explore the potential of ryegrass to reduce soil N_2O emissions under laboratory conditions. We considered the homogenization of the soil as important for a comparable starting point for the development of the soil microbial community. Our incubation study used sieved soil, which altered the soil structure and is likely to have affected the microbial community as compared to the field conditions. The incubation temperature used in the present study was higher than that expected under field conditions, which may have caused greater ammonia (NH_3) volatilization (Bremner, 2007; Forrestal et al., 2016) and higher nitrification and denitrification rates (Bremner, 1997; Saggar et al., 2013). Furthermore, NO_3^- loss by leaching was absent in our study because the incubation pots were not drained. These methodological drawbacks may have led to an overestimation of soil N_2O emissions in our study as compared to field conditions. Follow-up field studies should be carried out to explore the full potential of ryegrass under field conditions and in different soils.

5 | CONCLUSION

Our incubation experiment compared N_2O emissions and population sizes of denitrifying bacteria in soil planted with ryegrass and in bare soil under different N fertilizer levels. We found that 50% of fertilized N was recovered in plant tissues and emissions of N_2O were lower in soil with grass than in bare soil, although the proliferation of denitrifying bacteria in soil with grass was stimulated. We infer that soil mineral N is more related to N_2O emissions than soil denitrifying genes. However, because of the higher potential of denitrification in soil with grass, the risk of high N_2O emissions should also be noted, especially when N fertilizer exceeds the requirements of plants. Altogether, we conclude that ryegrass serving as a cover crop holds the potential to mitigate soil N_2O emissions in soils with moderate or high NO_3^- concentrations. Future studies should focus on how different plant species and their root exudates affect soil N_2O emissions and related soil microorganisms under field conditions and in different soils.

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AUTHORSHIP STATEMENTS

K.D., B.P., S.M. and H.W. designed the experiment, H.W. carried out the incubation experiment, L.B. performed qPCR and the analysis of the gene abundance data, H.W. drafted the manuscript, L.B., S.M., P.K., H.Z., K.D. and B.P. contributed to the final version of manuscript. K.D. supervised the project.

CONFLICT OF INTERESTS

None.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Chapter 5: General Discussion

5.1 Effect of urease and nitrification inhibitors on N_2O emission

We have introduced a popular urease inhibitor NBPT with both incubation and field experiment in our study (Paper I and II). NBPT-added urea was not only intended to decrease soil N_2O emission, but also to reduce NH_3 volatilization, which can account for 25% of urea applied to soil surface (FAOSTAT, 2015). The reduction of NH_3 volatilization was more certain and effective in recently reviewed studies (Dawar et al., 2011; Drury et al., 2017; Liu et al., 2017; Mira et al., 2017; Tian et al., 2015). In our incubation experiment, adding NBPT on urea reduced 50% of cumulative NH_3 emission (Paper I). Considering 1-2% of NH_3 converted to N_2O (Wulf et al., 2002), it counts for 10-20% extra reduction of N_2O than urea alone. Moreover, in field conditions, the reduction of NH_3 volatilization increases N use efficiency, which potentially increases crop yield (Linquist et al., 2013). The reduction of yield-scaled N_2O emission should be more effective.

Direct N_2O emissions reduced by NBPT was also observed in many other studies (Abalos et al., 2012; Dawar et al., 2011; Ding et al., 2011; Singh et al., 2013), though with a higher uncertainty (X. Fan et al., 2018). In our incubation experiment, NBPT reduced 31% of direct N_2O emission, compared with urea alone (Paper I), but the reduction of N_2O emission under field studies was inconsistent, 26%, 19% and 9% of N_2O emission were reduced, under a wheat- wheat- oilseed rape rotation, respectively, and the results were not statistically significant (Paper II). Our postulation was that background NO_3^- substrate in the soil plays an important role on the effectiveness of NBPT. When there was a high NO_3^- content even in unfertilized soil, N_2O emission (induced by soil-borne NO_3^-) masks the effect of NBPT reduced N_2O emission.

Piadin (1H-1, 2, 4-triazole and 3-methylpyrazole) served as a nitrification inhibitor showed its effectiveness on N_2O reduction in a few related studies (Barneze et al., 2015; Pietzner et al., 2017; Wolf et al., 2014; Wu et al., 2017). In our incubation experiment, cumulative N_2O emission was reduced from 0.98 g N_2O-N m^{-2} to 0.15 g N_2O-N m^{-2} (Paper I). The effect of delaying the transformation of NH_4^+ to NO_3^- was also clearly observed. Such astonishing effectiveness was achieved, probably because our experiment condition was very in favor to denitrification (25°C and 80% WFPS). However, it was still worth mentioning that adding Piadin increased NH_3

volatilization by 44%, compared with urea alone. Similar findings were also reported by others (C. Fan et al., 2018; Ferm et al., 2006; Lam et al., 2018, 2017; Pan et al., 2016; Qiao et al., 2015; Webb et al., 2010).

DMPP was also tested in our field experiment using the commercial fertilizer ENTEC (7.5 % N of NO_3^- and 18.5 % N of NH_4^+ with 0.15% DMPP, BASF, Ludwigshafen, Germany) (Paper II). Similar with UTEC (urea + NBPT), no yield increase was found, the reduction of N_2O emission in the wheat- wheat- oilseed rape rotation was 15%, 58% and 13%, respectively. Only in the wheat after wheat season, N_2O was statistically lower in ENTEC. Our postulation that high soil NO_3^- substrate caused by high N surplus of oilseed rape was also valid to explain the ineffectiveness of DMPP on reducing N_2O emissions (in oilseed rape and following wheat fields).

5.2 Effect of crop rotation on yield and yield-scaled N_2O emissions

Our field experiment shows a clear break crop effect (BCB), that wheat after oilseed rape had a higher crop yield than wheat after wheat (Paper II). Though wheat after wheat in 2017 suffers extreme heavy rainfalls during the harvest, which may cause some yield losses. But the obviously higher NO_3^- content in wheat after oilseed rape field is a clear evidence of break crop effect and partly explains its high crop yield. The break crop effect was also well documented in other studies (Angus et al., 2015; Sieling and Christen, 2015; Weiser et al., 2018).

From the plant nutritional aspect, higher crop yield of wheat after wheat was as a result of high N surplus after oilseed rape cultivation. Residues of oilseed rape in post-harvest season provides more mineral N than needed for N uptakes before winter (Ruser et al., 2017). Moreover, residues in post-harvest season provide an impeccable environment for denitrification. It provides not only denitrification-needed NO_3^- substrates, but also sufficient organic C as energy source (Köbke et al., 2018; Ruser et al., 2017). In our study, the climate also additionally provides a high temperature and a high WFPS, which also favors denitrification. The huge amount of N_2O emission at post-harvest season in oilseed rape field in 2017 is consistent with our speculation.

5.3 The presence of plant affect N_2O emissions

In Paper III, we explored how the presence of *Lolium perenne* affect soil C and N cycles and microbial community, by comparing *Lolium Perenne* planted soils and bare soils. Though it is widely agreed that 5-21% of photosynthesis-derived C is released into the soil in the form of root

exudates (Derrien et al., 2004; Nguyen, 2003), we did not find a difference of DOC-content between bare soil and planted soils. The reason could be possibly: i) the majority of root exudates was directly consumed by root and microbes respiration in a few hours (Fischer and Kuzyakov, 2010; Jones et al., 2005; Jones and Kielland, 2002; Kuzyakov and Xu, 2013); ii) intensive cutting decreased organic C accumulation (Zhang et al., 2018).

N_2O emission of soil with grass was presumed to be lower than bare soil, while a large amount of soil NO_3^- can be absorbed by the growth of *Lolium Perenne*. In our study (Paper III), we found a ca. 50% apparent N recovery efficiency in fertilized soil with grass, accompanying with a lower NO_3^- content in soil with grass than bare soil. Not surprisingly, N_2O emission in soil with grass was lower than that in bare soil. Aside from lower NO_3^- in soil with grass, a slightly lower WFPS (55%) during gas measurement, which was caused by plant respiration, might be another reason for lower N_2O emission in soil with grass. However, our results also suggest that the potential of N_2O emission could increase exponentially, when the soil NO_3^- were excessive for plant uptake (Hayashi et al., 2015; Philippot et al., 2013; Uchida et al., 2011). In our study, a 20 g N m^{-2} fertilization level caused more than doubled N_2O emission than a 10 g N m^{-2} fertilization level, and at 20 g N m^{-2} fertilization level, the discrepancy of N_2O emission in soil with grass and bare soil was narrowed, compared with lower fertilized levels.

Root exudates increase soil microbial biomass and community diversity was well documented in various studies (Berg and Smalla, 2009; Guyonnet et al., 2018; Haichar et al., 2008; Li et al., 2019, 2018; Qian et al., 2018). Our results showed that both bacterial 16S rRNA and fungal 18S rRNA gene abundances were increased by the presence of *Lolium perenne*, and soil bacteria may be more favored by plant roots than fungi (Paper III). We also found that rather than other denitrifying genes (*narG, napA, nirK, nirS, nosZ* clade I), nosZ clade II was not stimulated by the presence of *Lolium Perenne*. This is correspondent with Graf et al. (2015), that organisms carrying the *nosZ* clade I have an affinity to plant roots which are not shared by those with *nosZ* clade II. The fact that nitrate and nitrite reductase genes (*narG, napA, nirK* and *nirS*) increases more than nitrous oxide reductase genes (*nosZ*), also indicates that soil with grass has a higher potential of incomplete denitrification, when soil NO_3^- content was not the limiting factor. The incomplete denitrification might lead to a higher N_2O/N_2O+N_2 ratios.

5.4 Approaches for N_2O mitigation in arable lands

This study provide some information on the effectiveness of urease and nitrification inhibitors. But the complexity of soil microenvironment increases its uncertainty. How to improve the effectiveness of enhanced-efficiency fertilizers, still needs more research. From the view of our study, we suggest three possible directions for future research:

1) Investigate on the environmental factors that affect the effectiveness of urease and nitrification inhibitors. In our field experiment, we found that post-harvest residues of oilseed rape leads to high N_2O emission, which masks the effectiveness of urease and nitrification inhibitors (Paper II). For that, appropriate field management (straw removal) could be done, to reduce post-harvest N_2O emission. In other studies, they found urease inhibitors can be more effective to reduce NH_3 volatilization, when the soil was exposed to high risk of NH_3 volatilization, for example high soil temperature and low soil moisture (Grant et al., 1996). Or when the soil suffers high leaching risk, for example sandy soil texture, heavy rainfalls or irrigation after fertilization, nitrification inhibitor could be more effective (Ni et al., 2015).
2) Combine current available inhibitors and additives to improve its effectiveness. The combination of both urea and nitrification is a possible attempt (Drury et al., 2017; Rajkovich et al., 2017; Zaman et al., 2013). Other additives, for example biochar (Cayuela et al., 2014; Palanivell et al., 2017; Roberts et al., 2010), urea coated with zinc sulfate ($ZnSO_4$) (Adotey et al., 2017; Shivay et al., 2008), urea coated with humic acids and zeolites (de Sousa Gurgel et al., 2016), when combined with current available inhibitors, might show a different effectiveness.
3) Research on new inhibitors. Although developing new inhibitors is more likely to face failure, for example NZONE MAX in our study was proved to be ineffective in reducing either NH_3 or N_2O emissions, new compounds is always worth to explore. Some literature shows that Limus® (A formulation containing NBPT and NPPT (N-(n-propyl) thiophosphoric triamide) was an new effective urease inhibitor (Cantarella et al., 2018; Li et al., 2015). A more fascinating inhibitor is 1,9 - decanediol (Sun et al., 2016), which is a newly observed biological nitrification inhibitors (BNI) and might receive more attention in the following years.

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Chapter 6: Summary

Nitrous oxide (N_2O) is an important greenhouse gas which contributes to climate change and ozone depletion. Mineral N fertilizers are one of the most important sources of N_2O emission in agricultural systems. Enhanced-efficiency fertilizers (e.g., N fertilizers with added urease and nitrification inhibitors) represent possible approaches to N_2O emission reduction and improved efficiency of N use. However, their adoption has been limited by the uncertainty of their effectiveness across different ecosystems. The present study aims to evaluate the effectiveness of several inhibitors under various environmental conditions. We found some of them showed their ability to reduce N_2O emissions, for example Piadin and NBPT under laboratory conditions, while some of them are inefficient (NZONE MAX), and some exhibit inconsistent mitigation in N_2O emissions (for example DMPP and NBPT under the wheat- wheat- oilseed rape rotation system). Although the nitrification inhibitor Piadin reduced N_2O emissions from soil, it also increased the risk of higher NH_3 volatilization. Our results reveal the complexity of soil microbial activity in relation to nitrification and denitrification, and provide some references to improve the efficiency of urease and nitrification inhibitors.

These inconsistencies in effectiveness highlights the gap between laboratory and field conditions. One of the most important differences is that incubation experiments do not usually include plants. Our third experiment included both unplanted and planted soils. The results confirm our hypothesis that the presence of *Lolium perenne* increases the activity of microorganisms (probably through the release of root exudates) and lowers N_2O emission through intense competition for mineral N between plant and soil microorganisms. However, further scientific questions have arisen from the results which require investigation. For example: how do root exudates regulate denitrifying communities, are root exudates stimulating or inhibiting nitrification and denitrification; how do plant species (e. g. leguminous and non-leguminous) affect the microbial communities; and how does the application of urease and nitrification inhibitors root exudates? In recent projects, researchers have discovered several compounds released from plant root exudates which have significant nitrification inhibition capacity. These biological nitrification inhibitors may provide an effective method of increasing the efficiency of N use and reducing N_2O emission in future agricultural systems.

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E r k l ä r u n g e n

1. Hiermit erkläre ich, dass diese Arbeit weder in gleicher noch in ähnlicher Form bereits anderen Prüfungsbehörden vorgelegen hat.

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