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REGULAR ARTICLE

Rhizosphere processes in nitrate-rich barley soil tripled both N_2O and N_2 losses due to enhanced bacterial and fungal denitrification

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Abstract

Background and aims Plants can directly affect nitrogen (N) transformation processes at the micro-ecological scale when soil comes into contact with roots. Due to the methodological limitations in measuring direct N_2 losses in plant-soil systems, however, the effect of rhizosphere processes on N_2O production and reduction to N_2 has rarely been quantified.

Methods For the first time, we developed a robotic continuous flow plant-soil incubation system (using a $He+O_2+CO_2$) combined with N_2O ^{15}N site preference approach to examine the effect of plant root activity (barley – *Hordeum vulgare L.*) on: i) soil-borne N_2O and N_2 emissions, ii) the specific contribution of different pathways to N_2O fluxes in moist soils (85% water holding capacity) receiving different inorganic N forms.

Results Our results showed that when a nitrate-based N fertiliser was applied, the presence of plants tripled both N_2O and N_2 losses during the growth period but did not alter the $N_2O/(N_2O+N_2)$ product ratio. The ^{15}N site preference data indicated that bacterial denitrification was the dominant source contributing to the observed N_2O fluxes in both nitrate and ammonium treated soils, whereas the presence of barley increased the contribution of fungal N_2O in the nitrate treated soils. During the post-harvest period, N_2O and N_2 emissions significantly

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increased in the ammonium-fertilised treatment, being more pronounced in the soil with a senescing root system.

Conclusion Overall, our study showed a significant interaction between rhizosphere processes and N forms on the magnitude, patterns, and sources of soil borne N_2O and N_2 emissions in moist agricultural soils.

Keywords Denitrification · Nitrous oxide · Nitrogen cycling · Rhizosphere · Plant

Introduction

Nitrogen (N) is the plant nutrient element that most often limits primary production in terrestrial ecosystems and has been introduced into the biosphere mainly as reactive N through the chemical and biological fixation of dinitrogen (N_2) (LeBauer and Treseder 2008). Denitrification is the most important process that removes reactive N from the biosphere and returns it to the atmosphere, which includes all or parts of the sequential reduction of nitrate (NO_3^-) to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O) and N_2. Whereas N_2 is the ultimate end product of denitrification, other intermediate gaseous forms of N, such as N_2O, can also be produced through denitrification. The increase in atmospheric N_2O concentrations is of growing concern, since N_2O has been considered not only to be a potent greenhouse gas (GHG), but also be the most important destroyer of stratospheric ozone in the twenty-first century (Ravishankara et al. 2009). Denitrification is an anaerobic process which occurs mostly in anoxic microsites in unsaturated soils and requires readily available carbon (C) as an electron donor (Weier et al. 1993). Much of this C originates from plant roots either through root exudation or root turnover.

The presence of roots also induces physical and chemical changes in the rhizosphere soil, which subsequently influences N transformation processes such as nitrification and denitrification, and ultimately N_2O emissions (Guyonnet et al. 2017). For example, strong competition for soluble N occurs between plant roots and microorganisms (Jones et al. 2013). Further, the continuous uptake of nutrients by plants often leads to the development of strong depletion zones around roots (Kuzyakov and Xu 2013). Root exudate compounds could also alter the structure and activity of soil microbial communities (Shi et al. 2011). For instance, it has been found that many types of plants can secrete organic substances that inhibit nitrification in the rhizosphere (Subbarao et al. 2009, 2013; Sun et al. 2016; Coskun et al. 2017). On the other hand, authors have hypothesized that plant root exudates can stimulate denitrification rates by i) providing organic C for denitrifiers and/or ii) creating O_2 depleted microenvironments arising from roots and microbial respiration (Bakken 1988; Hayashi et al. 2015). Furthermore, it has been also suggested that root exudation may also alter/modify fungal and bacterial denitrification in the rhizosphere (Philippot 2002; Broeckling et al. 2008).

Understanding the extent to which plants can influence N_2O emissions and alter the $N_2O/(N_2O + N_2)$ product ratio is of particular importance when estimating N budgets, for improving N use efficiency in agriculture and developing effective GHG mitigation strategies. However, there have been relatively few studies on this topic, with those undertaken often coming to contradictory conclusions. For instance, some studies have reported a reduced N_2O/N_2 ratio with plants (Stefanson 1972, wheat with a sealed Helium system; Vinther 1984, spring barley with acetylene inhibition method), while others have shown no consistent effect (Smith and Tiedje 1979, corn with acetylene inhibition method; Klemedtsson et al. 1987, barley with acetylene inhibition method). Additionally, due to methodological problems associated with the direct measurement of N_2 production, most of the previous studies had used the classical acetylene inhibition method, which is now considered unsuitable for quantifying denitrification rates due to a range of inevitable artefacts such as catalytic NO decomposition (Groffman et al. 2006; Nadeem et al. 2013).

Recent decades have seen the development of several continuous flow soil-core incubation systems using a Helium (He) atmosphere to allow a more complete evaluation of denitrification processes in soil (Cardenas et al. 2003; Wang et al. 2011; Senbayram et al. 2018). These systems facilitate a direct and high-accuracy measurement of soil N_2O and N_2 emissions as compared to the acetylene inhibition approach (Weier et al. 1993) and the ^{15}N isotope labelling approach (Cai et al. 2001). Additionally, new developments in analysis of natural abundance isotopic signatures of N_2O can be used to examine the microbial pathways of N_2O production (Toyoda and Yoshida 1999). The N_2O ^{15}N site preference (SP), i.e. the intramolecular distribution of the N isotopes in the central (α) and peripheral (β)

positions in the linear asymmetric N_2O molecule, has been proved to be a useful non-invasive tool to differentiate sources of N_2O production pathways (Decock and Six 2013).

In this study, we conducted an incubation experiment in a modified robotic, continuous-flow, plant incubation system (PRoFLOW) with a $He+O_2+CO_2$ mixed atmosphere equipped with LED plant light to enable the direct measurement of soil N_2O and N_2 emissions at high temporal resolution. Additionally, the SP approach was used to distinguish the processes contributing to N_2O emissions and their responses to rhizosphere processes. For our experiment, we used an intensively managed moist agricultural soil and a common cereal crop (barley) to examine whether a growing plant in the soil would: i) stimulate N_2O and N_2 emission; ii) directly affect the $N_2O/(N_2O+N_2)$ product ratio; iii) influence the sources of N_2O emission; and iv) whether different N fertiliser (NO_3^- and NH_4^+ based) would interfere with the plant impact on N_2O and/or N_2 fluxes.

Materials and methods

Soil

In vegetable fields, much more organic and inorganic fertilizers are applied per unit area as compared to non-vegetable cropping systems causing serious environmental problems. Thus, the soil used in the incubation study was sampled from a vegetable field (Chinese cabbage-*Brassica rapa* L.) near the Changshu Agro-ecological Experimental Station of the Chinese Academy of Sciences, Jiangsu Province, China (31°33′5”N, 120°42′38″E).. The vegetable field (mainly leafy vegetables, e.g. pak choi and Chinese cabbage) is planted 4–5 times a year and receives circa 1044 kg N ha^{-1} yr^{-1} as a combination of mineral fertiliser and manure. The soil (clay 22.6%, silt 42.3%, sand 35.1%) was a typical Wushan Soil (Hydragric Anthrosol according to FAO 1998) developed from lacustrine sediments of the Taihu Lake with a pH (0.01 M $CaCl_2$) of 6.1, containing 1.3% organic C. During soil sampling, the top 2 cm soil layer was removed for homogeneity (removing fresh plant residues from the top layer) and the soil was collected from a depth of 2–10 cm. Subsequently, the soil was air-dried and sieved through a 4 mm mesh and then stored at 4 °C. Prior to performing the experiment, the soil was re-wetted to ca. 85% water holding capacity (WHC; equivalent to 28% gravimetric water content) and placed in the experimental vessels (0.9 kg dry soil) for 21 days to allow the soil to equilibrate and to reduce the initial mineral N content. The NO_3^- and NH_4^+ concentrations were 0.82 ± 0.43 and 2.94 ± 0.47 mg N kg^{-1} soil, respectively, at the time of seeding.

Robotic continuous flow plant-soil incubation system

The incubation experiment was performed in a modified robotic continuous flow plant incubation system (PRoFLOW) using a He (80%) + O_2 (20%) + CO_2 (400 ppm) mixed atmosphere under LED plant light supply at Thünen Institute of Climate-Smart Agriculture in Braunschweig, Germany (Fig. 1; Fig. S1) (Senbayram et al. 2018). Transparent acrylic glass cylinders with an inner diameter of 140 mm and 150 mm height were used as incubation vessels. At the bottom of each vessel, a polyamide filter membrane (EcoTech, Bonn, Germany - hydrophilic; pore size 0.45 μm) was used for adjusting the soil moisture and sampling soil water. The experiment consisted of six treatments ($n = 3$): i-ii) non-fertilised control treatment unplanted (B-CK) or planted with barley (P-CK) with no N addition; iii-iv) applied with 230 mg KNO_3-N kg^{-1} soil (equivalent to 136.5 kg N ha^{-1}) unplanted (B-KN) or planted with barley (P-KN); and v-vi) applied with 230 mg $(NH_4)_2SO_4$-N unplanted (B-AS) or planted with barley (P-AS). Briefly, soil was packed into each vessel with a bulk density of 1.25 g cm^{-3} (equivalent to 0.9 kg dry soil per pot). After 3 weeks of pre-incubation (85% WHC), in the respective treatments (P-CK, P-KN and P-AS), 12 barley seeds (*Hordeum vulgare* L. *c.v.* Onia; KWS SAAT SE, Einbeck, Germany) were sown into each vessel. The incubation vessels were then sealed and the atmospheric air in the vessels replaced by a pure He/O_2 mixture (to remove any CO_2, NO, N_2O or N_2 in the soil pores or headspace) by applying a vacuum from the top and filling with He/O_2 mixture in three cycles that were completed within 8 h. Subsequently, the headspace of each vessel was flushed continuously with a gas mixture of He, O_2 and CO_2 at a flow rate of ca. 25 ml min^{-1}. The temperature of the incubation room was set to 20 °C during the pre-incubation period and 59 days of incubation. After germination (14 days after seeding), N fertilisers were applied as a solution (50 ml) from the top of each vessel using the outlet opening. The same procedure was also applied

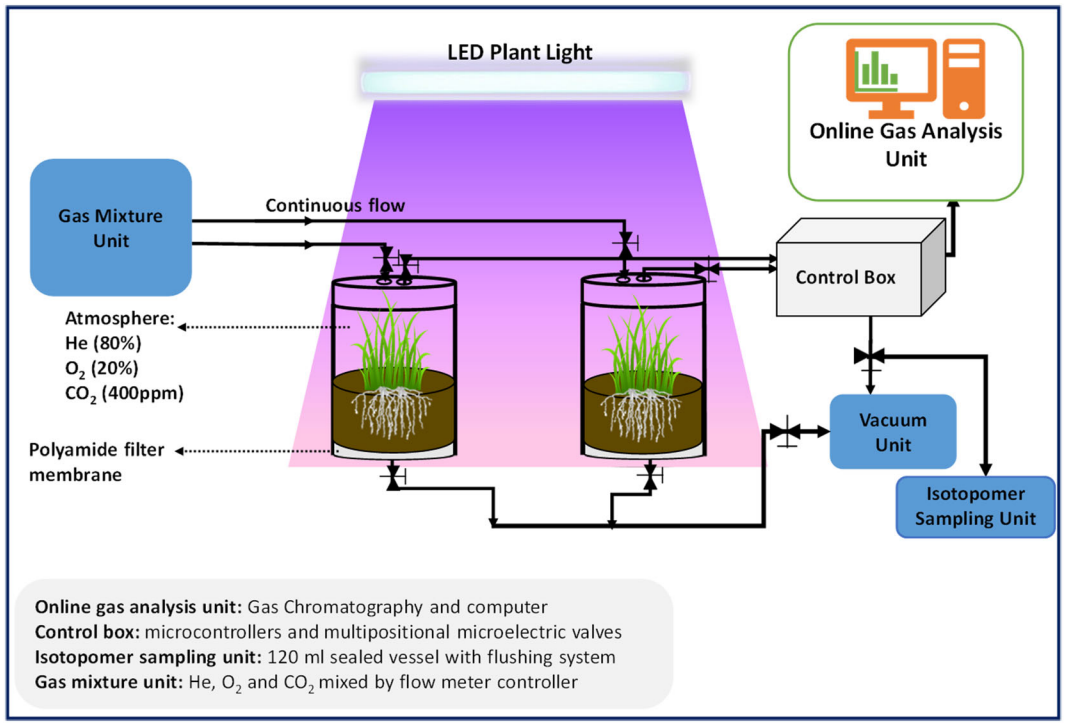

Fig. 1 Simplified diagram of the robotised continuous flow incubation system (PRoFLOW) used in the experiment. The system consists of 18 airtight acrylic glass cylinders and is controlled by an Arduino-based microcontroller unit (Arduino Mega 2560 R3 equipped with two 16-position relays). This control unit adjusts the position of VICI valves, gives signals to the GC (start/stop method) and the computer (start and stop data acquisition)

to the non-fertilised control treatment (B-CK, and P-CK) using distilled water (50 ml) in place of the N fertiliser. The final soil moisture at seeding was calculated as ca. 85% WHC. The airflow from each plant/soil cuvette was directed sequentially to a gas chromatography system (GC-2014-Shimadzu Scientific Instruments) by two multi-positional micro-electric valves (VICI, Houston, USA), where the gas sample was analysed by a He ionization detector (HID) for N_2, and O_2 and an electron capture detector (ECD) for N_2O quantification. Three-standard gases (containing i) 10 ppmv N_2 and 0.4 ppmv N_2O, ii) 50 ppmv N_2 and 1.0 ppmv N_2O, and iii) 100 ppmv N_2 and 100 ppmv N_2O inHe) were measured at the end of each cycle for calibration. Each gas sampling cycle (19 pots (including one empty vessel to check for any background noise or contamination), 3 reference gases and carrier gas ((He (80%) + O_2 (20%) + CO_2 (400 ppm)) - to check for any potential leaks)) was completed in 5.5 h. Outlet gas concentration of each pot was measured three times a day during the plant growth period, and at least one time a day during the post-harvest period. N_2 and N_2O emission rates were calculated using the commonly employed dynamic flux chamber approach (Senbayram et al. 2018) and the equation:

$$E_m = (C_o – C_e)Q/A, \quad (1)$$

where E_m (kg N_2O-N or N_2-N ha day^{-1}) is the daily flux rate, C_o and C_e (kg N_2O-N or N_2-N m^{-3}) are the outlet concentration of the sample pot and empty vessel respectively, Q is the flushing flow rate (m^3 d^{-1}), and A is the enclosed emission area (ha). Here, an empty vessel was measured at the end of each cycle to ensure that there is no leak in the system. The outlet N_2 concentration of the empty vessel was below 2 ppm N_2 throughout the experiment (see Supp. Figure 2). Light was supplied with LED labs (100 W, B.E.S.T. Agro GmbH, Germany), keeping the light intensity in a 14 h photoperiod at a minimum of 350 μmol m^{-2} s^{-1} photosynthetically active photon flux density (PPFD) at the top of the plant canopy. The plant shoots were harvested by cutting at ground level 24 days after sowing and their dry weight determined

after oven drying (80 °C, 48 h). The root system was left in the soil at harvest. The total N content of the plant dry matter was determined using a NA 1500 elemental analyser (Carlo Erba, Milano, Italy).

Mineral N analysis

Soil samples were collected from each vessel at plant harvest (24 days after sowing) with a soil core sampler (2 cm in diameter) and at the end of the incubation period (36 days after harvest). The soil samples were extracted with 2 M KCl (1:5 *w/v*) by shaking for 1 h. The KCl extracts were subsequently filtered through a Whatman 602 filter paper and stored at −20 °C until analysis. The concentrations of NH_4^+ and NO_3^- in soil extracts and soil solution were measured using a continuous flow colorimetric autoanalyser (Smartchem 200S/N1104238, WESTCO, France).

Isotope analysis and N_2O source partitioning

Additional gas samples for isotopic analysis were taken from each incubation vessel by attaching pre-vacuumed 120-ml serum bottles to the outlets in flow-through mode for around 2 h (Wu et al. 2017). The N_2O $\delta^{15}N^{bulk}$, $\delta^{15}N^{\alpha}$ and $\delta^{18}O$ isotope signatures were then determined by analysing *m/z* 44, 45 and 46 of intact N_2O^+ molecular ions, and *m/z* 30 and 31 of NO^+ fragment ions (Toyoda and Yoshida 1999) on an isotope ratio mass spectrometer (DELTA V PLUS, Thermo Fisher Scientific, Bremen, Germany). Pure N_2O (Westfalengas; purity >99.995%) was used as internal reference gas. The SP value of the produced N_2O (SP_0), i.e. prior to its partial reduction to N_2, was calculated using a Rayleigh-type model, assuming that isotope dynamics followed closed-system behaviour (Lewicka-Szczebak et al. 2014). The model can be described as follows:

$$SP_{N2O-r} = SP_0 + \eta_r \ln\left(\frac{C}{C_0}\right) \quad (2)$$

In this equation, SP_{N2O-r} is the SP value of the remaining substrate (i.e. N_2O), SP_0 is the SP value of the initial substrate, η_r is the net isotope effect (NIE) associated with N_2O reduction, and C and C_0 are the residual and the initial substrate concentration (i.e. C/C_0 expresses the $N_2O/(N_2O + N_2)$ product ratio). For the SP source partitioning approach, the end-member values (SP_{fD}) were defined as 37‰ for nitrification and fungal denitrification, and − 5‰ (SP_D) for bacterial denitrification and nitrifier denitrification (Toyoda et al. 2017) (see Supplementary material for further details of the calculation).

Due to the overlapping SP signatures between nitrification and fungal denitrification as well as between bacterial denitrification and nitrifier denitrification, distinguishing the N_2O produced by those pathways based on SP values is impossible (Lewicka-Szczebak et al. 2014; Toyoda et al. 2017). Thus, $f_{D\text{-}SP}$ and $f_{fD\text{-}SP}$ represent the contribution of bacterial denitrification+nitrifier denitrification and nitrification+fungal denitrification, respectively, to the total N_2O release calculated based on the SP_0 values. However, in the B-KN and P-KN treatments, the specific experimental conditions were set up to favour denitrification, i.e. i) N was applied in the form of NO_3^-; ii) initial soil NH_4^+ content was below the detection limit (<3 mg NH_4^+-N kg^{-1} soil) with constantly low NH_4^+ content during the incubation (Table 1); and iii) high soil moisture (85% WHC). Therefore, the contributions of nitrification and nitrifier denitrification were assumed to be negligible in KNO_3 treated soils (see Discussion). Thus, only the most plausible scenario (heterotrophic bacterial denitrification vs. fungal denitrification) was considered for the SP_0 source partitioning calculation in the B-KN and P-KN treatments.

Calculations and statistical analysis

Cumulative emissions and the share of bacterial and fungal denitrification was calculated by linear interpolation between measured N_2O emissions and SP_0 values. Differences in cumulative N_2O, N_2 emissions, the $N_2O/(N_2O + N_2)$ ratio and soil mineral N content and the interactions were examined using a two-way analysis of variance (ANOVA-significant difference post-hoc tests at a 5% significance level) by SPSS 21 (SPSS Inc., Chicago, IL, USA).

Results

Soil moisture, mineral N and plant N

At plant harvest (day 24), the soil water content (initial ca. 85% WHC) was slightly lower in the planted soil (78.4 ± 1.6% WHC) than in the bare soil (82.9 ± 3.3%

Table 1 Soil nitrate (NO_3^-) and ammonium (NH_4^+) concentrations (mg N kg^{-1} dry soil), plant biomass (kg dry matter ha^{-1}) and plant N content at plant harvest (day 24) and at the end of the experiment (day 60) in non-amended control (B-CK = unplanted soil, and P-CK = planted soil), $(NH_4)_2SO_4$ amended (B-AS = unplanted soil, and P-AS = planted soil), and in KNO_3 amended (B-KN = unplanted soil, and P-KN = planted soil) treatments (n = 3). Means denoted by a different letter in the same column differ significantly according to the Tukey's HSD post-hoc tests at α = 0.05. The constant value of 0.58 can be used to convert the mineral N concentration unit to kg N ha^{-1}

Treatment	N_{min} at plant harvest		N_{min} at end of the experiment		Plant parameters	
	NO_3^- (mg N kg^{-1} dry soil)	NH_4^+ (mg N kg^{-1} dry soil)	NO_3^- (mg N kg^{-1} dry soil)	NH_4^+ (mg N kg^{-1} dry soil)	Biomass (kg dry matter ha^{-1})	Plant N content (%)
B-CK	0.9 ± 0.3^b	4.6 ± 2.4^b	1.1 ± 0.3^c	4.4 ± 3.6^c	–	–
B-AS	3.4 ± 2.6^b	175.6 ± 34.2^a	57.8 ± 21.1^b	91.8 ± 29.7^a	–	–
B-KN	136.6 ± 18.6^a	3.8 ± 0.5^b	134.4 ± 24.7^a	0.1 ± 0.0^c	–	–
P-CK	0.6 ± 0.2^b	2.4 ± 1.0^b	0.4 ± 0.0^c	2.5 ± 0.6^c	589 ± 105^a	$2.9\% \pm 0.1^c$
P-AS	1.9 ± 1.4^b	165.2 ± 35.8^a	95.0 ± 8.0^b	31.0 ± 12.5^b	481 ± 124^a	$3.7\% \pm 0.1^b$
P-KN	71.0 ± 24.8^b	8.6 ± 3.4^b	73.5 ± 11.5^b	7.7 ± 7.3^c	503 ± 68^a	$4.4\% \pm 0.1^a$

WHC) ($P < 0.05$; data not shown). The concentrations of soil NH_4^+ and NO_3^- in the B-CK and P-CK treatments were below 5 mg N kg^{-1} soil throughout the incubation period (Table 1). The soil NO_3^- concentrations in the B-KN treatment were significantly higher than in all other treatments at harvest (day 24). Here, the soil NO_3^- concentrations were two-fold higher in the B-KN compared to the P-KN treatment. No significant difference in the NH_4^+ concentration was found between the B-AS and the P-AS treatment at plant harvest.

At the end of the post-harvest phase, the soil NH_4^+ concentrations decreased markedly in the P-AS and B-AS treatments compared to the concentrations at harvest date, being more pronounced in the P-AS treatment (Table 1). In contrast, the soil NO_3^- and NH_4^+ concentrations in the B-KN and the P-KN treatments were similar to those at plant harvest. At the end of the incubation, the soil NO_3^- and NH_4^+ concentrations were the highest in the B-KN and the B-AS treatments, respectively. No significant difference was found for plant dry matter yield among treatments, whereas the plant N concentrations were significantly higher in the P-AS and P-KN treatments compared to the P-CK treatment (Table 1).

Emissions of N_2O and N_2

The daily N_2O fluxes in the B-CK and P-CK treatments were relatively stable and low (<3 g N ha^{-1} d^{-1}) during the entire experimental period (Fig. 2a, b), which was similar in all other treatments prior to the mineral N amendment. The N_2O flux increased immediately after $(NH_4)_2SO_4$ addition in both, planted and unplanted soil, at the same order, and then decreased gradually until plant harvest. Similarly, application of KNO_3 triggered N_2O fluxes in both, planted and unplanted soil; however, the increase was more dramatic compared to the $(NH_4)_2SO_4$ treatments and was more pronounced, especially in the P-KN treatment. The emission of N_2O peaked at day 19 in the P-KN treatment (with 298 ± 116 g N ha^{-1} d^{-1} maximum daily flux) and then decreased gradually, whereas N_2O fluxes in the B-KN treatments increased gradually until plant harvest (with 142 ± 67 g N ha^{-1} d^{-1} maximum daily flux).

During the post-harvest period, N_2O emissions in both, the B-AS and the P-AS treatment, increased gradually, whereas the observed N_2O emission increased more rapidly in the planted compared to the unplanted soil. In contrast, in the P-KN treatment, the N_2O flux remained constant for 1 day and then sharply decreased to almost background levels after harvest, whereas N_2O fluxes in the B-KN treatment remained high until day 29 and then decreased gradually (Fig. 2c, d). Overall, the cumulative N_2O emissions followed the order: P-KN > B-KN > P-AS>B-AS>B-CK > P-CK (Table 2).

Fluxes of N_2 in the non-fertilised treatments were below detection limits. Similar to N_2O, the N_2 fluxes immediately increased to detectable levels after application of $(NH_4)_2SO_4$, with the effect being more pronounced in the unplanted soil than in the planted soil (Fig. 2c, d). During the plant growth period, the emissions of N_2 were slightly higher in the B-AS treatment

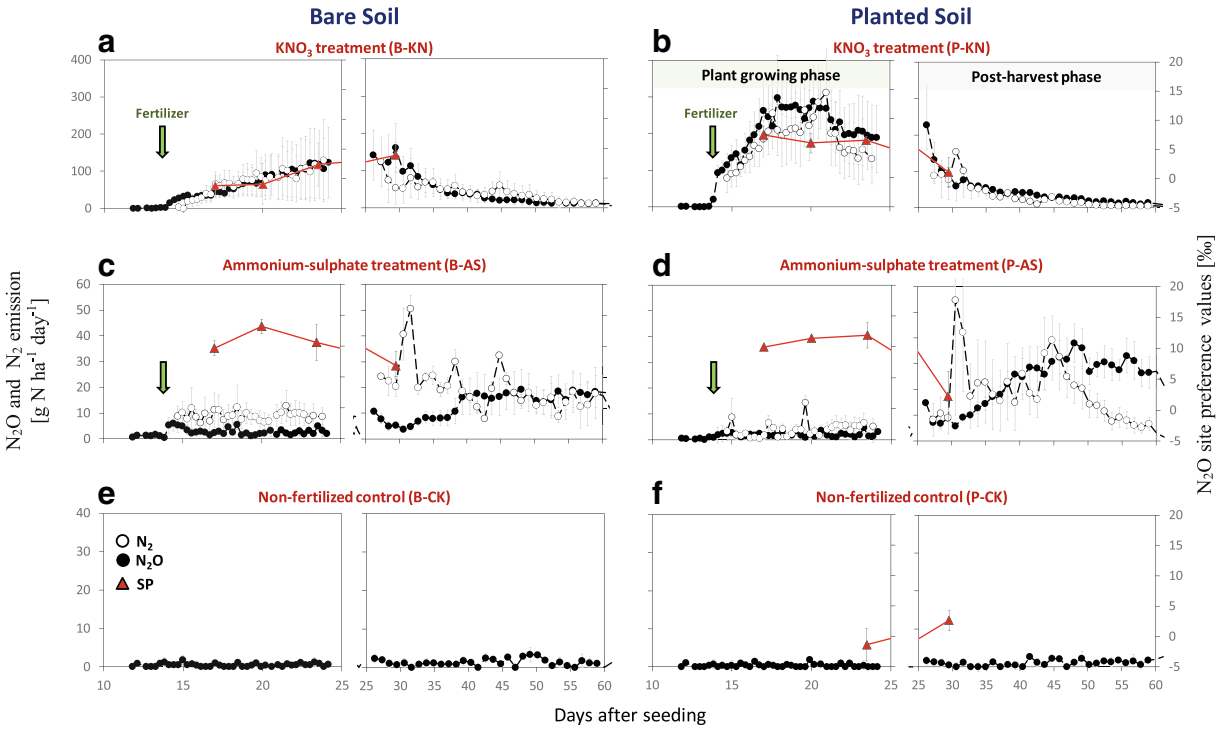

Fig. 2 Daily emissions of N_2O, N_2, and site preference (SP_0) values during the incubation period (60 days) in KNO_3 amended (B-KN = unplanted soil, and P-KN = planted soil), ammonium-sulphate amended (B-AS = unplanted soil, and P-AS = planted soil), and in unamended control (B-CK = unplanted soil, and P-CK = planted soil) treatments ($n = 3$). Emission of N_2 in control soils (B-CK and P-CK) were below detection limits and therefore the data was not presented. Error bars show the standard error of each treatment ($n = 3$). The arrows show the time of fertiliser application and the green dotted line denotes the point at which the plants were harvested. The legend is the same for all panels

(4–12 g N ha^{-1} d^{-1}) than in the P-AS treatment (1–7 g N ha^{-1} d^{-1}). During the post-harvest period, N_2 emissions in the P-AS treatment increased gradually until day 46 and decreased afterwards. In the B-AS treatment, however, the N_2 flux increased sharply during the post-harvest period and then decreased gradually towards the end of the experiment. In the KNO_3 treated soil, N_2 emissions followed almost the same trend as the N_2O fluxes, i.e. they increased over time, being more pronounced in the planted compared to the unplanted soil and then decreased over time. The peak in N_2 fluxes equated to a rate of 245 ± 81 g N ha^{-1} d^{-1} in the P-KN treatment and 95 ± 59 g N ha^{-1} d^{-1} in the B-KN treatment.

During the plant growth period, the cumulative total N flux ($N_2O + N_2$) in the P-KN treatment was almost three times as high as in the B-KN treatment, while it was twice as high as when taking the whole incubation period (pre- and post-harvest phase) into account (Table 2). Until plant harvest, the $N_2O/(N_2O + N_2)$ product ratio ranged between 0.33 and 0.67, being the highest in the B-KN and P-KN treatments and the lowest in the B-AS treatment (N_2 fluxes were below detection limits in the B-CK and P-CK treatments). Here, the ratio was significantly lower in the B-AS treatment compared to the P-KN and B-KN treatments ($P < 0.05$); however, over the whole incubation period no significant differences in the $N_2O/(N_2O + N_2)$ product ratio were observed between any treatments.

N_2O SP_0 values and source partitioning

During the plant growing phase, the N_2O SP_0 values over all treatments ranged from −1.4 to 13.2‰, being the lowest in the P-CK treatment (−1.4‰ ±2.7) and the highest in the B-AS treatment (13.2‰ ±1.2; Fig. 1). The N_2O SP_0 values were more or less constant before harvesting in all treatments (except for a slight increase in the B-KN treatment), indicating relatively stable N_2O sources in each treatment. No plant effect was observed in soils treated with $(NH_4)_2SO_4$. However, in the KNO_3 amended planted soil (P-KN treatment), N_2O SP_0 values

Table 2 Cumulative emissions of N_2O, N_2, NO and CO_2 during plant growth (11–24 days after sowing) and during the whole incubation period (11–60 days after sowing) in non-amended control (B-CK = unplanted soil, and P-CK = planted soil), $(NH_4)_2SO_4$ amended (B-AS = unplanted soil, and P-AS = planted soil), and in KNO_3 amended (B-KN = unplanted soil, and P-KN = planted soil) treatments ($n = 3$). Means denoted by a different letter in the same column differ significantly according to Tukey's HSD post-hoc tests at $\alpha = 0.05$. "–"indicates below detection limit

Treatment	Plant growth phase				Total emission			
	N_2O (g N ha^{-1})	N_2 (g N ha^{-1})	$N_2O + N_2$ (g N ha^{-1})	Ratio ($N_2O/(N_2O + N_2)$)	N_2O (g N ha^{-1})	N_2 (g N ha^{-1})	$N_2O + N_2$ (g N ha^{-1})	Ratio ($N_2O/(N_2O + N_2)$)
B-CK	19 ± 7^{b}	–	–	–	104 ± 27^{b}	–	–	
B-AS	37 ± 13^{b}	105 ± 50^{b}	141 ± 43^{b}	0.33 ± 0.07^{b}	558 ± 76^{b}	756 ± 49^{b}	1314 ± 46^{b}	0.42 ± 0.05^{a}
B-KN	717 ± 385^{b}	667 ± 441^{b}	1384 ± 614^{b}	0.67 ± 0.22^{a}	3838 ± 1728^{b}	1994 ± 1030^{ab}	5831 ± 2642^{ab}	0.72 ± 0.16^{a}
P-CK	17 ± 3^{b}	–	–	–	79 ± 25^{b}	–	–	–
P-AS	32 ± 9^{b}	42 ± 17^{b}	74 ± 26^{b}	0.46 ± 0.05^{ab}	826 ± 345^{b}	656 ± 212^{b}	1482 ± 492^{b}	0.49 ± 0.14^{a}
P7-KN	2219 ± 650^{a}	1901 ± 614^{a}	4121 ± 1196^{a}	0.54 ± 0.04^{a}	8795 ± 3313^{a}	3019 ± 774^{a}	$11{,}814 \pm 4065^{a}$	0.73 ± 0.02^{a}

were significantly higher than in the unplanted soil. After harvest, the SP_0 values sharply decreased in the B-AS, P-AS and P-KN treatments, whereas the SP_0 values slightly increased in the P-CK and B-KN treatments.

To estimate the share of each N_2O emitting process on the observed N_2O fluxes, source partitioning based on a two end-member model was used. The very low SP_0 values in the P-CK and B-KN treatment suggest that almost all of the emitted N_2O originated from bacterial denitrification, whereas the significantly higher SP_0 values in the $(NH_4)_2SO_4$ treatments compared to KNO_3 treated soils indicated a lower share of bacterial denitrification derived N_2O. Assuming that the contribution of nitrification to N_2O emissions was minor in the KNO_3 amended treatments (due to the constant low soil NH_4^+ content and high soil moisture), the increase in SP_0 values over time in the B-KN treatment would imply an increase in fungal denitrification activity over time. Therefore, the higher SP_0 values in the P-KN treatment compared to B-KN treatment from the beginning of the incubation indicated a higher contribution of fungal denitrification in the presence of growing plants. After harvest, the decreasing SP_0 values in the B-AS, P-AS and P-KN treatments indicated an increased share of bacterial denitrification in the total N_2O production. During the plant growing period the share of bacterial denitrification in P-KN (74% ±2.5) was significantly lower compared to the B-KN treatment (92% ±4.0; Fig. 3).

Discussion

Plant effects on N_2O, N_2 emissions and $N_2O/(N_2O + N_2)$ product ratio

In this study, the three-fold higher N_2O and N_2 fluxes in the KNO_3 treated soil containing plants was most likely due to the stimulation of bacterial denitrification, which was confirmed by the low N_2O SP_0 values. Plant roots can affect denitrification in many ways, however, of most importance is the stimulation of microbial activity, growth of C and N transformations in the rhizosphere (Hayashi et al. 2015; Guyonnet et al. 2017). In cereal plants, typically 5% of the net C fixed in photosynthesis is lost into the soil and enters the soil microbial community (Farrar et al. 2003). In the present experiment, the rhizosphere effect on denitrification can be partly

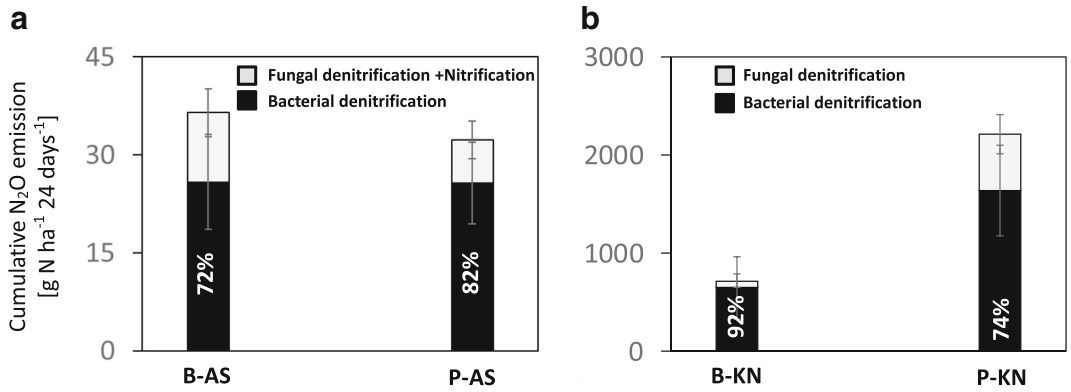

Fig. 3 Contribution of fungal (+nitrification in $(NH_4)_2SO_4$ treatment) and bacterial denitrification derived N_2O emissions to the cumulative N_2O fluxes during vegetation period (0–24 days) in $(NH_4)_2SO_4$ amended (B-AS = unplanted soil, and P-AS = planted soil) treatments (Panel A), and in the KNO_3 amended (B-KN = unplanted soil, and P-KN = planted soil) treatments (Panel B). Error bars show the standard error of each treatment ($n = 3$)

attributed to the possible depletion of O_2 due to root and symbiont respiration (e.g. mycorrhizas) and partly to the stimulation of microbial respiration by rhizodeposition (Bakken 1988; Hayashi et al. 2015). However, if O_2 depletion was the dominant factor, we would have expected a lower nitrification rate and lower nitrification-derived N_2O (i.e. lower SP values) in the $(NH_4)_2SO_4$ supplied soil containing plants in comparison to the bare soil during the plant growing period, which was not the case (Fig. 1 and Table 1). Therefore, we assume that root exudates and root turnover (delivering additional electron donor to denitrifiers) rather than O_2 depletion played a more important role in stimulating denitrification in our study. Nevertheless, more research is required to quantify the extent to which root exudates and O_2 depletion are responsible for the observed increase in denitrification and N-derived gas emissions in the root zone.

The effects of plants and the underlying mechanisms regulating the amount of end-product N_2 produced during denitrification are not fully understood due to a lack of suitable techniques for accurately quantifying N_2 emissions. Using hermetically sealed soil-plant growth chambers with an argon atmosphere, Stefanson (1972) first reported that growing plants (*Trifolium subterraneum* and *Lolium rigidum*) decreased the $N_2O/(N_2O + N_2)$ product ratio in the planted treatments, while the ratio increased when NO_3^- was supplied to the plants. Henry et al. (2008) reported that the composition of root exudates may also affect the $N_2O/(N_2O + N_2)$ ratio of denitrification, where artificial exudates with more sugar appeared to promote more N_2O reduction. The latter reflects the general assumption that labile C from root exudates and O_2 depletion in the root zone may alter the $N_2O/(N_2O + N_2)$ ratio (Hayashi et al. 2015). On the other hand, it is commonly accepted that NO_3^- is preferred over N_2O during denitrification processes if it is sufficiently available at the denitrifying microsites (Blackmer and Bremner 1978; Baggs et al. 2003; Smith 2010). Our recent studies (Senbayram et al. 2018; Wu et al. 2018) illustrate that the soil NO_3^- concentration is likely to be the predominant factor that directly regulates the denitrification end products. Conversely, O_2 availability and available C appear to mainly influence the $N_2O/(N_2O + N_2)$ ratio when soil NO_3^- concentrations fall under a so called site-specific threshold value (35–50 mg N kg $soil^{-1}$) (Weier et al. 1993; Senbayram et al. 2012; Qin et al. 2017). The NO_3^- concentrations in both the B-KN and the P-KN treatments were constantly higher than the aforementioned NO_3^- threshold values (>50 mg N kg $soil^{-1}$; Table 1), which likely explain why no significant effect of growing plants on the $N_2O/(N_2O + N_2)$ product ratio was observed in the KNO_3 treated soil.

When ammonium based N fertilizers are used, ammonium oxidation is the first and rate-limiting step in the nitrogen cycle. As far as we are aware, interaction effect of nitrogen form and rhizosphere processes on denitrification and the $N_2O/(N_2O + N_2)$ product ratio has not yet been studied. In the present study, emission rates of N_2O and N_2 in the $(NH_4)_2SO_4$ amended planted and bare soil were significantly lower compared to the KNO_3 treatments. We ascribe this to the limited nitrification activity under the given experimental conditions.

During the plant growth period, the total N flux in the B-AS compared to the P-AS treatment was one-fold higher (significant ($p < 0.05$) when analysed separately). We attribute this to an increased plant uptake of NO_3^- or to the enhanced N immobilization due to root exudates inducing growth of the microbial community in the planted soil (Smith and Tiedje 1979; Kuzyakov and Razavi 2019), which depleted the soil NO_3^- level and decreased the nitrogenous gas emission through denitrification. This information is particularly important and can be used to implement mitigation techniques in planted soils. For example, nitrogen fertilization practices that avoid a NO_3^- build up in the root zone (e.g. split N application, nitrification inhibitors, and slow release fertilizers) are likely to mitigate both N_2O and N_2 emissions (Guyonnet et al. 2017; Senbayram et al. 2012).

During the post-harvest phase, the higher total gaseous N fluxes with lower SP_0 values suggested that bacterial denitrification (likely triggered by NO_3^- production through nitrification) was responsible for the observed increase in nitrogenous gas fluxes in both B-AS and P-AS treatments. Moreover, slightly lower soil moisture with higher soil NO_3^- content at harvest indicates stimulated nitrification activity causing more rapid increase in $N_2O + N_2$ emissions in the P-AS compared to the B-AS treatment (Table 1). These suggest that harvesting of the plants (i.e. removal of plant N uptake), stimulated nitrification activity due to a lower soil moisture in the P-AS treatment and the delivery of more NO_3^- to the denitrifying microsites (opposite effect compared to the rhizosphere effect during plant growth) and thus triggered gaseous N loss.

Plants have also been found to directly emit N_2O produced by the plants themselves (Lenhart et al. 2019) or to serve as a conduit for the transport of N_2O produced in the soil to the atmosphere (Chen et al. 1999). In this study, the N_2O emission rates before and immediately after harvesting showed no immediate decrease in all the planted treatments, indicating that the contribution of plant-emitted N_2O was insignificant in this study. This is probably due to the barley plants being in the very early stages of growth prior to harvest, and thus a reduced ability to convey or produce N_2O in comparison to mature plants with hollow stems (Chang et al. 1998). It should also be noted that we studied the initial stages of plant development (first basal N application period) where plant N uptake was limited, and where competition between roots and soil microorganisms for N is limited. Our approach was designed to minimise excess root growth in small vessels, and also to better reflect field conditions when a first basal N dressing is applied to cereals. Further studies (with larger vessels) should focus on the later growth stages of the barley crop (e.g. second N dressing period), when more competition between plant roots and microbial activity occurs for both water and nutrient uptake.

Sources of N_2O as affected by growing plants

Increasing evidence suggests that actively growing plants in moist soils play a critical role not only on controlling the rate of denitrification but also on the composition of the microbial population (Guyonnet et al. 2017; Langarica-Fuentes et al. 2018). For example, Broeckling et al. (2008) and De Graaff et al. (2010) showed that labile soil C inputs by root exudates could increase the metabolic activity and gene abundance of both fungi and bacteria. Several studies have also reported that the addition of labile C to soil can induce a shift in microbial community structure (e.g. increase the fungal-to-bacterial biomass ratio) leading to enhanced fungal N_2O production (Laughlin and Stevens 2002; Hayden et al. 2012; Senbayram et al. 2018; Zhong et al. 2018). Our observations support this as the SP_0 values were higher in the P-KN treatment (up to 8.5‰) compared to the B-KN treatment suggesting enhanced fungal denitrification in the presence of plants alongside the delivery of additional labile C substrate to the denitrifying hotspots in the planted soil. This indicates that root exudates not only enhanced the rate of microbial activity by supplying additional electron donors, but also modified the actively N_2O producing microbial community (Figs. 2 and 3).

In the current study, we presume that the enhanced fungal denitrification derived N_2O in the presence of plants (only in KNO_3 applied soils) was likely due to the available C supplied by root exudates. As shown in our recent study, application of organic C would enhance fungal denitrification over bacterial denitrification specifically in soils with a high NO_3^- content (Senbayram et al. 2018). On the other hand, with the increase in incubation time, significant decreases in SP_0 in P-KN treatment (in parallel to the decreasing trend in $N_2O + N_2$ emission) indicates a clear shift from fungal to bacterial denitrification, whereas both emission rates and SP_0 values remained constant for a longer period in the

B-KN treatment. As fungi lack the N_2O reductase enzyme (Laughlin and Stevens 2002; Shoun et al. 2012), the present study suggests that the faster depletion in soil NO_3^- in the P-KN treatment (converted mainly to $N_2O + N_2$) overrode the assumed stimulatory effect of rhizodeposition on fungal denitrification, leading to an increase in bacterial denitrification (decrease in SP_0 values). In our previous study, we showed for the first time that labile C has a major impact on fungal denitrification as well as being dependent on soil NO_3^- level (Senbayram et al. 2018). Our present study supports this view, especially in the planted soils. Therefore, we may conclude that any practices that lower soil NO_3^- content (e.g. nitrification inhibitors (Wu et al. 2017), using ammonium based fertilizers (Senbayram et al. 2009) and/or split N application (Lebender et al. 2014) may mitigate N_2O loss and total gaseous N fluxes in planted moist soils.

We also acknowledge that the SP_0 source partitioning approach employed here (especially when NH_4^+ is used as an N source) provides a rough source estimation of emitted N_2O. This is due to the i) overlapping SP_0 signals of different processes (Decock and Six 2013); ii) variability of isotopologue enrichment factors of N_2O reduction (Lewicka-Szczebak et al. 2014); and iii) variation in SP_0 signals between different microbial strains (Toyoda et al. 2017). Nevertheless, assuming that endmember values and enrichment factors were identical in all treatments, there would be still significant differences between treatments and thus our conclusions would be still valid.

N_2O and N_2 emissions as affected by roots in the post-harvest period

In the post-harvest period, the N_2O and N_2 fluxes both gradually decreased in the NO_3^- treatments (P-KN and B-KN), while the NO_3^- contents show only minor changes when compared to the values at harvest and at the end. This implies that the decrease is most likely due to the depletion of available C in soil that limits denitrification. Previous research indicates that excision of shoots does not cause instant death of cereal roots, but rather they can remain active for 7–14 days after photosynthetic activity has ceased (Marella et al. 2017). Root activity during this period is fuelled by the progressive autolysis of the root cells and exhaustion of any remaining internal C stores. In this scenario, exudation is also expected to decline due to i) a decrease in passive exudation due to the low concentration of solutes in the cytoplasm; ii) a cessation of C losses associated with active root growth; and iii) no more unloading of C from the phloem into the apoplast (Jones et al. 2013; Paterson et al. 2005). The sharp decline of N_2O and N_2 emission in the P-KN treatment in the post-harvest phase can thus be attributed to the response of reduced supply of root C to the soil microbial community (Dilkes et al. 2004). On the other hand, the small N_2O emission increase observed immediately after cutting in both the P-AS and P-KN treatments could be due to a pulse of root exudation following defoliation due to shifts in internal root C partitioning (Paterson et al. 2005).

Removal of the shoots induces senescence of the root system potentially leading to a large input of C into the soil via root turnover. However, the constant low N_2O and N_2 emission observed in the NO_3^- treatments during the entire period of post-harvest indicates that root integrity is not yet lost 26 days after plant harvest.

As evidenced by the decreased NH_4^+, increased NO_3^- content and extremely low SP values during post-harvest period (Table 1; Fig. 1), the significant increase of N_2O and N_2 fluxes in the NH_4^+ treated treatments is likely attributed to the ongoing nitrification, denitrification and/or nitrifier denitrification. N_2O and N_2 emissions in the P-AS treatment increased more rapidly than in the B-AS treatment, which is possibly due to the NH_4^+ released by root autolysis. Root autolysis induces the breakdown of cellular proteins to create keto acids for use in respiration, while the cleavage of the amine groups leads to the accumulation of NH_4^+ within the cell which is subsequently excreted into the soil to prevent cytotoxicity (Bingham and Rees 2008; Saglio and Pradet 1980). This may have provided an additional source of substrate for nitrifier denitrification to produce N_2O and N_2, as evidenced by the significant decreased SP values in both the P-KN and P-AS treatments in this period (Fig. 2).

Conclusions

Our results clearly show that in moist soils with a moderately high NO_3^- content (ca. >50 mg N kg^{-1} dry soil), the root system of an actively growing barley plant stimulates greater losses of both N_2O and N_2 through denitrification but without affecting the $N_2O/(N_2O + N_2)$ product ratio. The stimulation of microbial activity by rhizodeposition plays a key role in the observed

increase in denitrification activity, whereas the N_2O SP_0 approach suggests growing plants may alter the contribution of fungal-to-bacterial denitrification-derived N_2O in NO_3^- rich moist soils. In conclusion, we suggest that the assessments of N budgets and GHG emissions especially in agricultural ecosystems must pay more attention to the decisive influence of the rhizosphere on N_2O and N_2 emissions and its interaction with different forms of N fertiliser.

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