Biology and Fertility of Soils (2022) 58:77–90
https://doi.org/10.1007/s00374-021-01612-x

ORIGINAL PAPER

Inhibitory effect of high nitrate on N_2O reduction is offset by long moist spells in heavily N loaded arable soils

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Received: 28 April 2021 / Revised: 16 November 2021 / Accepted: 19 November 2021 / Published online: 28 December 2021
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Abstract

Numerous interrelated factors (e.g., the labile C, soil NO_3^- concentration, and soil moisture content) are involved in controlling the microbial sources of N_2O and the product stoichiometry of denitrification; however, the interactions among different factors are still poorly understood. Here, a fully robotized continuous flow soil incubation system (allowing simultaneous measurements of N_2 and N_2O fluxes) was employed to investigate the interactive effects of a 51-day duration of moist spell, straw amendment, and the NO_3^- level on the rate and product stoichiometry ($N_2O/(N_2O+N_2)$ ratio) of denitrification in heavily N loaded arable soils (i.e., paddy, vegetable, and orchard soils). The rewetting-induced N_2O emissions mainly originated from bacterial denitrification in all soil types, with a clear shift to fungal denitrification (plus contingent nitrification) over time. The vegetable and orchard soils showed a higher share of bacterial N_2O (62–70%) than that in the paddy soils (50–54%), which may be attributed to more labile-C driven bacterial activity induced by the greater manure and crop residue input therein. Interestingly, the inhibitory effect of high soil NO_3^- on N_2O reduction in these soils was offset by a 51-day-long moist spell, regardless of the amendment of straw. To our knowledge, our study is the first to show that the inhibitory effect of high residual NO_3^- on N_2O reduction is suppressed by a moist spell with a certain duration in heavily N loaded arable soils, suggesting that the water regime history should be considered when optimizing the N fertilizer application timing to mitigate soil N_2O emissions.

Keywords Denitrification · Bacterial N_2O · Soil moisture · Nitrate · Product stoichiometry · ^{15}N site preference

Introduction

The production of N_2O in soils is mainly attributed to biological denitrification and nitrification processes (Giles et al. 2012). The last step of the denitrification pathway (i.e., N_2O reduction to N_2) is the only known biological sink of N_2O (Baggs 2011; Butterbach-Bahl et al. 2013), which determines the $N_2O/(N_2O+N_2)$ product ratio of denitrification. The key controllers of denitrification include the soil pH, oxygen supply and moisture content, and availability of labile C and NO_3^- (Saggar et al. 2013). Depending on the outcomes of the complex interactions among these factors, the $N_2O/(N_2O+N_2)$ product ratio significantly varies from 0 to 1 among different terrestrial ecosystems (Schlesinger 2009). Denitrification in arable soils is commonly limited by labile C, and hence, any management practice that influences labile C availability (e.g., drying-wetting cycles, crop residue amendments, and organic fertilizer applications) can affect the denitrification rate and the associated $N_2O/(N_2O+N_2)$ product ratio (Giles et al. 2012; Saggar et al.

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2013). Although labile C addition can trigger a high denitrification rate, its effects on the $N_2O/(N_2O+N_2)$ product ratio strongly depend on the soil NO_3^- levels (Senbayram et al. 2019; Wei et al. 2020; Wu et al. 2018). This is because high soil NO_3^- concentrations can inhibit N_2O reductase activity, since NO_3^- is preferred over N_2O as a terminal electron acceptor (Firestone 1982; Qin et al. 2017; Senbayram et al. 2012; Weier et al. 1993). Hence, high residual NO_3^- (17–58 mg N kg^{-1} soil) concentrations in arable soils caused by the excessive use of synthetic N fertilizer can increase N_2O emissions, along with the altered $N_2O/(N_2O+N_2)$ product ratios of denitrification (Qin et al. 2017). However, more information is still needed about how the interactions among influencing factors (e.g., labile C, the soil NO_3^- concentration, and the soil moisture content) regulate N_2O production and reduction in N fertilized soils.

The ability to denitrify is widely distributed among bacteria, archaea, fungi, and other eukaryotes (Hayatsu et al. 2008); hence, the soil microbial community exerts substantial effects on the rate and product stoichiometry of denitrification (Yoon et al. 2019). Some bacteria (complete denitrifiers) have all enzymatic pathways and can potentially perform complete denitrification (Philippot et al. 2011), whereas others (incomplete denitrifiers) either lack N_2O reductase and produce only N_2O or are only able to consume N_2O (Shan et al. 2021). It has been reported that adding *Agrobacterium tumefaciens* C58, an incomplete denitrifier lacking the *nosZ* gene, to agricultural soils significantly increased the $N_2O/(N_2O+N_2)$ product ratio (Philippot et al. 2011). Additionally, fungal denitrifiers also have truncated enzymatic pathways for denitrification, with the absence of N_2O reductase encoded by the *nosZ* gene, resulting in N_2O as the terminal product (Shoun et al. 1992). The significant contributions of fungal denitrifiers to soil-borne N_2O emissions have been demonstrated in several studies, wherein the share of fungal N_2O to emitted N_2O varied from 18 to 89% among different terrestrial ecosystems (Chen et al. 2014; Laughlin and Stevens 2002; Senbayram et al. 2018; Zhong et al. 2018). As several studies have shown that the organic C supply in moist soils can increase both the fungal/bacterial biomass ratio and fungal N_2O production (Hayden et al. 2012; Zhong et al. 2018), we hypothesized that fungal denitrification may be a dominant source of N_2O emissions in NO_3^--rich, straw-amended, and moist arable soils.

The intensification of agricultural systems (e.g., higher organic and mineral N fertilizer supplies) may also change the rate and product stoichiometry of denitrification (Hu et al. 2020; Zhou et al. 2018). The Taihu Lake region is one of the oldest agricultural production hotspots in China, with a long history of intensive rice–wheat rotation cultivation. At several locations in the Taihu Lake region, the traditional rice–wheat rotation (receiving 400–600 kg N ha^{-1} $year^{-1}$) has been converted to more intensive vegetable and fruit production systems, and the N fertilizer input has increased twofold to threefold (Wang et al. 2016). Several studies have reported significant N losses by leaching, runoff, and gaseous N (NH_3, NO, and N_2O) in the Taihu Lake region (Xia et al. 2019; Zhu and Chen 2002); however, few studies have explored the sources of soil-emitted N_2O or evaluated the contribution of N_2 losses to the N budget. Meta-analysis of denitrification studies based on the use of the acetylene inhibition technique in upland soils has shown that intensive vegetable and fruit systems are denitrification "hotspots" and that N fertilizer application profoundly changes the rate and $N_2O/(N_2O+N_2)$ product ratio of denitrification therein (Wang and Yan 2016). Since the acetylene inhibition technique often results in significantly underestimated denitrification, until now, the precise N losses through denitrification and the controllers of the $N_2O/(N_2O+N_2)$ product ratio in intensive agricultural soils are still poorly known, partly due to difficulties in directly quantifying N_2 production against the large background N_2 concentration in air.

With the development of continuous flow soil-core incubation systems using helium-based synthetic air, the direct and simultaneous determination of soil-borne N_2 and N_2O fluxes is possible (Cárdenas et al. 2003; Liu et al. 2010; Molstad et al. 2007; Qin et al. 2017; Senbayram et al. 2018; Wang et al. 2011). Together with the analysis of the ^{15}N site preference (SP) signature of soil-emitted N_2O, sources of N_2O production pathways can be largely determined (Decock and Six 2013; Rohe et al. 2017; Toyoda et al. 2017). Here, a fully robotized continuous flow soil incubation system (RoFlow: allowing simultaneous determination of soil-borne N_2 and N_2O emissions) integrated with an analysis of the ^{15}N SP signature of emitted N_2O was employed to investigate the interactive effects of moist spells, straw amendments, and nitrate levels on N_2O sources and the rate and product stoichiometry of denitrification in paddy, vegetable, and orchard soils. The aim of this study was to gain a better understanding of the regulation of N_2O production and reduction during denitrification in heavily N loaded arable soils.

Materials and methods

Soils

Soils were collected from three different sites at the Changshu Agro-Ecological Experimental Station (31°32′N, 120°41′E) of the Chinese Academy of Sciences in Jiangsu Province, China. Characterized by heavy N fertilizer loading, these three different sites represent typical intensive agricultural land use systems in the Taihu Lake region, including (i) paddy fields, (ii) plastic shed vegetable fields, and (iii) orchard (vineyard) fields. The orchard site has been used

for grape cultivation since 2005, and the vegetable site has been cultivated as a plastic shed vegetable field since 2009. Both sites were converted from paddy fields. The experimental soil, which had a silty clay loam texture, was classified as typical Wushan Soil (Hydragric Anthrosols, based on the FAO World Reference Base, 2013) and developed from lacustrine sediments (Table 1). The long-term mean annual air temperature was 17.0 °C, and the mean annual precipitation was 1344 mm. The N fertilizer input rates were approximately 525, 860, and 590 kg N ha^{-1} $year^{-1}$ in the paddy, vegetable, and orchard fields, respectively. The soils were sampled in September after the crops (rice, vegetables, and grapes) were harvested from each location. For homogeneity, the upper 2 cm of soil and roots were removed, and the experimental soil was collected from the first 15 cm below the removed layer. The soil was air-dried, sieved through 4-mm mesh, and stored at 4 °C before being packed into cores. Prior to the experiment, the concentrations of soil exchangeable NH_4^+ were 6.2 ± 0.5, 15.8 ± 1.2, and 8.6 ± 0.4 mg N kg^{-1} soil in the paddy, vegetable, and orchard soils, respectively, whereas the NO_3^- concentrations were 42.1 ± 0.5, 137.8 ± 10.4, and 48.5 ± 0.1 mg N kg^{-1} soil, respectively.

Robotized soil incubation experiment and gas measurements

The incubation experiment was conducted at the Thünen Institute of Climate-Smart Agriculture Braunschweig, Germany, in the RoFlow system using a synthetic air consisting of 80% He and 20% O_2 (Senbayram et al. 2018; Wei et al. 2020). The cylindrical incubation vessels were manufactured with acrylic glass (with inner diameters of 140 mm and heights of 150 mm) equipped with ceramic plates (SOILMOISTURE GmbH, Santa Barbara, CA, USA) at the vessel bottoms to allow the drainage and adjustment of the soil moisture content. The experiment consisted of six treatments ($n = 3$): (i) non-amended paddy soil (Paddy-Control: P-CL), (ii) straw-amended paddy soil (Paddy-Straw: P-ST), (iii) non-amended vegetable soil (Vegetable-Control: V-CL), (iv) straw-amended vegetable soil (Vegetable-Straw: V-ST), (v) non-amended orchard soil (Orchard-Control: O-CL), and (vi) straw-amended orchard soil (Orchard-Straw: O-ST). The soil was mixed by a vertical mixer with wheat straw (0.8% total N and 43% total C) at a rate of 1 g wheat straw kg^{-1} dry soil in the P-ST, V-ST, and O-ST treatments prior to the experiment. Afterwards, 1 kg of dry soil matrix (with or without straw) was packed into each vessel at a density of 1.25 g cm^{-3}. By applying a vacuum from the top of each vessel, the repacked soil cores were flooded from the bottom of the vessels with distilled water and then drained to 27 ± 0.5% gravimetric water content (64% WFPS) by applying a vacuum under the ceramic plate. The incubation vessels were then sealed, and the atmospheric air in the vessels was replaced with a pure He/O_2 mixture (He: 80% and O_2: 20%) 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 a pure He/O_2 mixture in three cycles that were completed within 4 h. The headspace of each incubation vessel was flushed continuously with a pure He/O_2 mixture at a flow rate of approximately 25–30 mL min^{-1}. The temperature of the incubation room was set at 20 °C during the 75 days of incubation. The experiment consisted of four sequential periods: (i) wetting without N addition (Phase I, 51 days), (ii) fertilization with 10 mM KNO_3-N equivalent to 37.5 mg N kg^{-1} dry soil (Phase II, 14 days), (iii) fertilization with 40 mM KNO_3-N equivalent to 150 mg N kg^{-1} dry soil (Phase III, 4 days), and (iv) a completely anoxic period (Phase IV, 6 days). A long period was chosen for Phase I for three reasons: (i) to simulate the wetting effect after a long dry period following crop harvest in each field, (ii) to deplete the residual N, and most importantly, (iii) to accommodate soil microorganisms to the current soil moisture content in order to test the effect of NO_3^- addition on the N_2O reducing processes and the $N_2O/(N_2O + N_2)$ product ratio. For the application of KNO_3 at Phases II and III, 500 mL of the respective KNO_3 solutions (10 or 40 mM KNO_3) was added from the top of each vessel, and the soil matrix was flushed with the corresponding KNO_3 solution and then drained to 27% gravimetric water content (64% WFPS) by applying a vacuum under the ceramic plate to create a homogenous N distribution. Meanwhile, the remaining N_2O and N_2 in the soil matrix were also flushed out during the KNO_3 application, combined with the replacement of the headspace gas. A relatively long period was set for Phase II to investigate the effect of NO_3^--N application and avoid the interference

Table 1 Chemical and physical properties of paddy, vegetable, and orchard soil. Data shown are mean ± standard error ($n = 5$). Means denoted by different letters in the same column differ significantly according to Tukey's HSD post hoc tests at $\alpha = 0.05$

Soil	Exchangeable NH_4^+	NO_3^-	pH	Soil C	Soil N	Clay (< 2 µm)	Silt (2–50 µm)	Sand (> 50 µm)
	mg kg^{-1}	mg kg^{-1}		%	%	%	%	%
Paddy	6.2 ± 0.5c	42.1 ± 0.5b	7.0 ± 1.0a	2.1 ± 0.1a	0.23 ± 0.01a	33.4 ± 0.1b	58.1 ± 0.8a	8.5 ± 0.8a
Vegetable	15.8 ± 1.2a	137.8 ± 10.4a	4.9 ± 0.2b	1.9 ± 0.0a	0.19 ± 0.01a	34.2 ± 0.2a	58.0 ± 0.3a	7.7 ± 0.3a
Orchard	8.6 ± 0.4b	48.5 ± 0.1b	5.3 ± 0.1b	1.9 ± 0.12a	0.22 ± 0.01a	34.6 ± 0.3ab	56.8 ± 0.5a	8.6 ± 0.5a

of residual N on the Phase III determination. In addition, a short anoxic phase IV was conducted by flushing the headspace with 100% He to investigate the interactive effects of the soil moisture content and NO_3^- on the denitrification potential.

The outlet of each incubation vessel was sequentially directed to a gas chromatograph, followed by the determination of the N_2, N_2O, and CO_2 concentrations; the soil exchangeable NH_4^+ and NO_3^- contents in each vessel were measured at the beginning, before the first N dressing (on Day 51), and at the end of the incubation period (see Supplementary Material).

Isotope analysis and partitioning of N_2O sources

For the isotopic analysis, gas samples were collected from each incubation vessel by attaching 120-mL serum bottles to the outlets in the flow-through mode for approximately 1 h (Well et al. 2008). The time points utilized for gas sampling were set according to the concentrations of emitted N_2O and the durations of the different incubation periods. The isotope signatures of N_2O $\delta^{15}N^{bulk}$, $\delta^{15}N^{\alpha}$, and $\delta^{18}O$ were then determined by analyzing *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 (DeltaV, Thermo Fisher Scientific, Bremen, Germany) at the Thünen Institute Braunschweig, Germany (Buchen et al. 2018). The SP value of the produced N_2O (SP_0), i.e., prior to its partial reduction to N_2, was estimated using a Rayleigh-type model, assuming that the isotope dynamics exhibited closed-system behavior (Lewicka-Szczebak et al. 2017). The model can be described as follows:

$$SP_{\mathrm{N2O-r}} = SP_0 + \eta_r \ln\left(\frac{C}{C_0}\right) \quad (1)$$

where $SP_{\mathrm{N2O\text{-}r}}$ is the SP value of the remaining substrate (i.e., residual N_2O), SP_0 is the SP value of the initial substrate (i.e., the N_2O produced before reduction occurred), η_r is the net isotope effect associated with N_2O reduction, and C and C_0 are the residual and initial substrate concentrations (i.e., C/C_0 expresses the $N_2O/(N_2O+N_2)$ product ratio). In this study, an η_r value of −5‰ was used based on previously reported average values (Lewicka-Szczebak et al. 2014). For source partitioning, the end-member values (SP_{fD}) were defined as 37‰ for nitrification and fungal denitrification, and −5‰ (SP_{bD}) for bacterial denitrification (Toyoda et al. 2017). The source partitioning of N_2O production was based on the two-end-member isotopic mass balance equation:

$$SP_0 = SP_D \times f_{bD-SP} + SP_{fD} \times f_{fD-SP} \quad (2)$$

It is not possible to distinguish between the N_2O produced by fungal denitrification and that produced by nitrification with SP analysis because of the overlapping SP signatures from these pathways (Frame and Casciotti 2010; Lewicka-Szczebak et al. 2014; Toyoda et al. 2017). In the equation listed above, $f_{bD\text{-}SP}$ and $f_{fD\text{-}SP}$ represent the contribution of bacterial denitrification and nitrification + fungal denitrification, respectively, to the total N_2O release calculated based on SP_0 values. In this study, specific experimental conditions were set up to favor denitrification (to minimize nitrification-related N_2O emissions), i.e., (i) N was applied in the form of NO_3^-, (ii) the initial soil NH_4^+ content was low at the beginning and below the detection limit (< 0.5 mg NH_4^+-N kg^{-1} soil) before the first N dressing (on Day 51), and (iii) a high soil moisture content was set (64% WFPS). Despite the specific experimental conditions, considering the contingent NH_4^+-N derived from mineralization or dissimilatory nitrate reduction to ammonium processes, the N_2O emissions originating from nitrification or nitrifier denitrification cannot be neglected in the present experiment. Conservatively, only the most plausible scenario (bacterial denitrification vs. fungal denitrification or fungal denitrification plus nitrification) was considered in the SP_0 source partitioning calculation. Nevertheless, nitrifiers' contribution to N_2O emissions should be far less dominant than fungal contribution in our study. Since N_2O from nitrification cannot be distinguished from fungal denitrification due to the overlapping SP_0 signals (Toyoda et al. 2011), fungal denitrification plus nitrification-derived N_2O was referred to as "fungal N_2O or N_2O_{fungal}" for conciseness in this study. Additionally, the N_2O SP_0 values in the O-ST treatment were not determined due to the low signal and limited sampling capacity; thus, the contributions of different sources to the cumulative N_2O emissions in the O-ST treatment were not presented.

Calculations and statistical analysis

The cumulative gas emissions were calculated using linear interpolation between the measured fluxes. Statistically significant differences were evaluated by the general linear model (univariate) using Tukey's honest significant difference and post hoc tests at a 5% significance level with SPSS 21 software (IBM SPSS Statistics, Chicago, IL, USA).

Results

Soil mineral N

Prior to the experiment, the concentrations of soil exchangeable NH_4^+ ranged between 6.2 and 15.8 mg N kg^{-1} soil, and those of soil NO_3^- ranged between 42.1 and 137.8 mg N kg^{-1} soil in the paddy, vegetable, and orchard soils. Shortly before Phase II (fertilization with 10 mM KNO_3 on Day 51), both soil NO_3^- and

exchangeable NH_4^+ contents were below 2 mg kg^{-1} soil in all treatments (Fig. 1). It should be noted that the soil NH_4^+ contents on Day 51, as determined by soil pore water, may have been slightly underestimated due to the immobilization of ammonium in the soil matrix. Nevertheless, considering technological limitations in simultaneously measuring N_2 emissions and soil mineral N, the exchangeable NH_4^+ concentration in the soil pore water could partly represent the ammonium level therein. The soil NO_3^- contents at the end of the 75-day incubation period were higher in the non-amended soils and followed the trend V-CL, P-CL, O-CL, V-ST, P-ST, and O-ST (Fig. 1). The depletion in the soil NO_3^- contents were more pronounced in the orchard soils than in the other soils. The soil exchangeable NH_4^+ content at the end of the incubation was 5.3 ± 1.8 mg kg^{-1} soil in the O-ST treatment, slightly higher than that in the other treatments (Fig. 1).

Daily emissions of N_2O, N_2, and CO_2

The daily fluxes at the different experimental incubation phases are shown in Fig. 2. Shortly after soil rewetting at Phase I, the daily N_2O fluxes increased sharply in all treatments, reaching a maximum around Day 4, and then gradually decreased to zero with different declining rates in each treatment (Fig. 2 (A1 – E4)). Amendment of straw significantly ($P < 0.05$) increased the peak emission rates in all soils, with the effect being more pronounced in the vegetable soil (V-ST treatment) and less pronounced in the paddy soil (P-ST treatment). The maximum daily N_2O emission rates were 442 ± 105, 617 ± 18, and 544 ± 49 g N_2O-N ha^{-1} day^{-1} in the P-ST, V-ST, and O-ST treatments, respectively, whereas they were 304 ± 43, 168 ± 17, and 177 ± 14 g N_2O-N ha^{-1} day^{-1} in the P-CL, V-CL, and O-CL treatments, respectively. The decline in daily N_2O emission rates was more rapid in the paddy soils (i.e., P-CL and P-ST treatments) than in the vegetable and orchard soils.

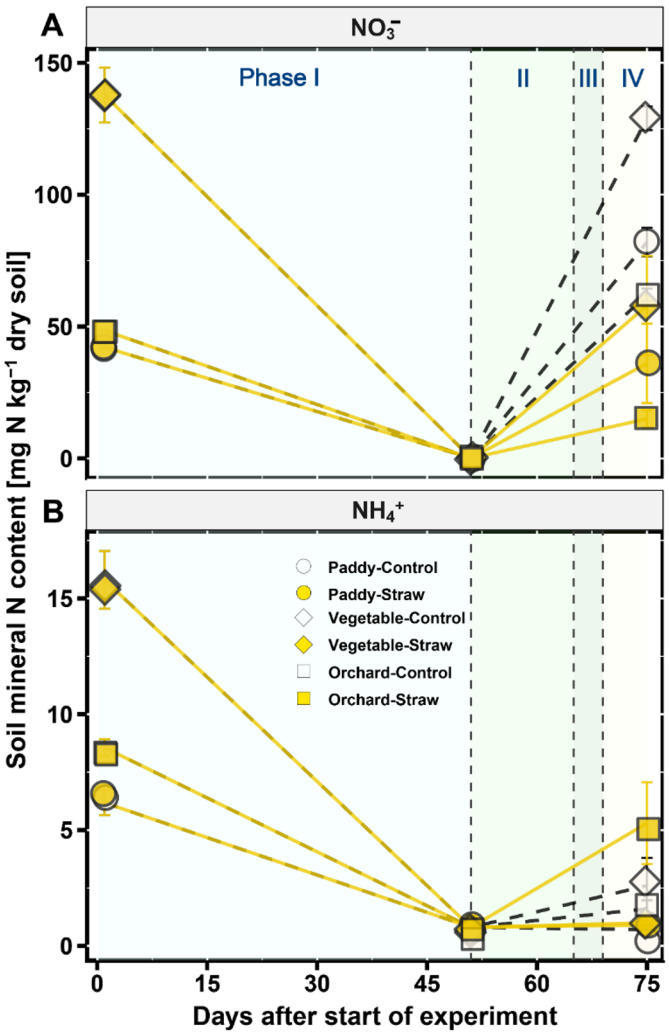

Fig. 1 Soil (**A**) nitrate (NO_3^-) and (**B**) exchangeable ammonium (NH_4^+) concentrations on Day 1, 51 (end of Phase I: wetting (0–51 days)), and 75 (end of the experiment) in non-amended paddy rice soil (Paddy-Control, P-CL), straw-amended paddy rice soil (Paddy-Straw: P-ST), non-amended vegetable soil (Vegetable-Control: V-CL), straw-amended vegetable soil (Vegetable-Straw: V-ST), non-amended orchard soil (Orchard-Control: O-CL), and straw-amended orchard soil (Orchard-Straw: O-ST) treatments. Error bars show the standard error of each treatment ($n = 3$)

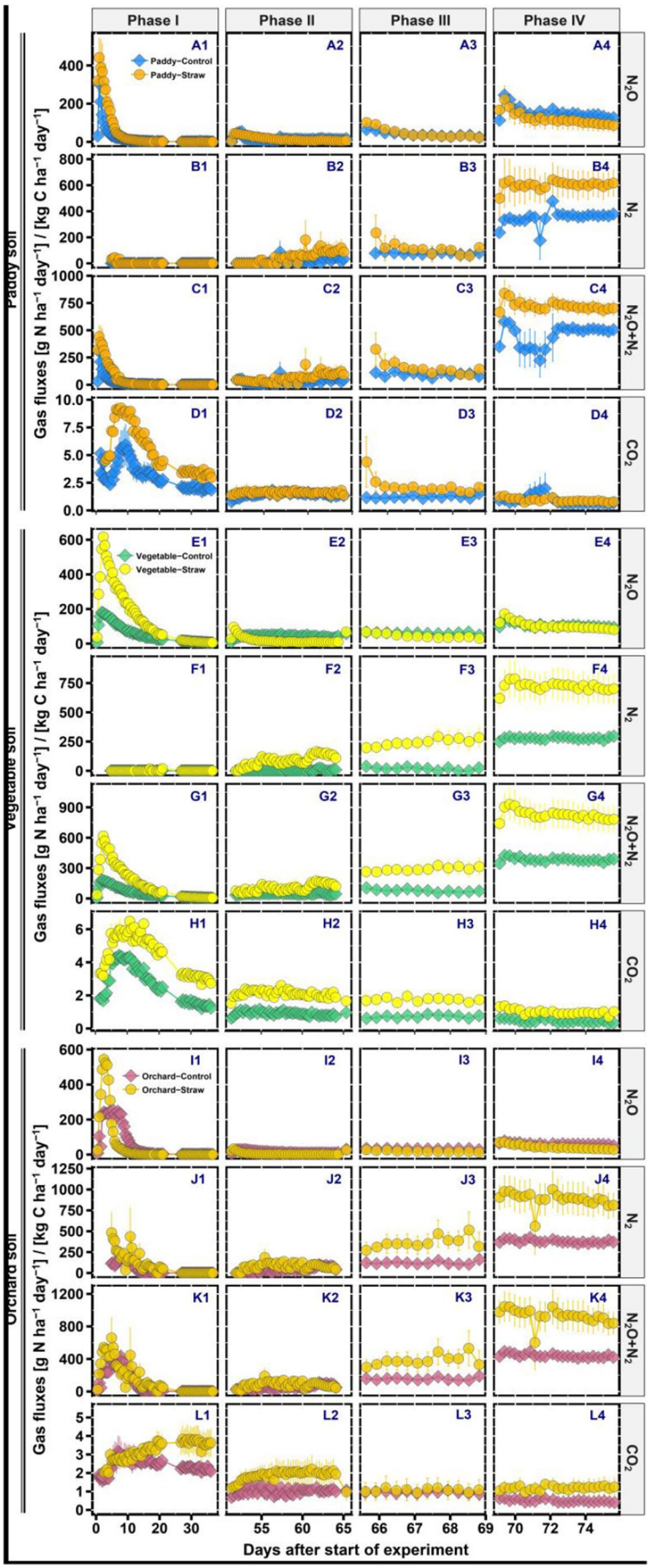

Fig. 2 Daily fluxes of (A1–4, E1–4, and I1–4) N_2O, (B1–4, F1–4, and J1–4) N_2, (C1–4, G1–4, and K1–4) (N_2O+N_2), and (D1–4, H1–4, and L1–4) CO_2 emissions during the various phases (Phase I: wetting (0–51 days), Phase II: 10 mM KNO_3 addition (51–65 days), Phase III: 40 mM KNO_3 addition (65–69 days), and Phase IV: anoxic (69–75 days)) of the experiment in non-amended paddy rice soil (Paddy-Control, P-CL), straw-amended paddy rice soil (Paddy-Straw: P-ST), non-amended vegetable soil (Vegetable-Control: V-CL), straw-amended vegetable soil (Vegetable-Straw: V-ST), non-amended orchard soil (Orchard-Control: O-CL), and straw-amended orchard soil (Orchard-Straw: O-ST) treatments. Error bars show the standard error of each treatment ($n=3$)

Fertilization with 10 mM KNO_3 at Phase II (addition of 37.5 mg N kg^{-1} dry soil) caused another N_2O peak event, and the daily N_2O fluxes reached a maximum on Day 1 after N addition in all treatments, with the highest peak observed in the V-ST treatment and the lowest in the O-CL treatment. The emission rates of N_2O decreased gradually at Phase II in all treatments and were more rapid in the straw-amended treatments. Fertilization with 40 mM KNO_3 at Phase III (addition of 150 mg N kg^{-1} dry soil) caused an immediate increase in N_2O fluxes, with the effect being more prominent in the paddy soils. Here, the N_2O fluxes were almost constant in the non-amended soils but decreased slightly over time in the straw-amended soils. The conditions switching from oxic to anoxic at Phase IV caused a rapid increase in N_2O emission rates in all treatments. The peak N_2O emission rate at Phase IV was the highest in the paddy soils and the lowest in the orchard soils.

The N_2 fluxes at Phase I were extremely low, except for those in the orchard soils. Fertilization with 10 mM KNO_3 at Phase II caused a gradual increase in N_2 emissions, with the effect being more pronounced in the straw-amended soils; this result was coupled with a decrease in N_2O emissions. During Phase II, the peak emission rate of N_2 was the lowest in the V-CL treatment and the highest in the V-ST treatment (up to 63.8 ± 33.6 g N_2-N ha^{-1} day^{-1}). Interestingly, fertilization with 40 mM KNO_3 at Phase III caused a further increase in N_2 fluxes, which then slightly decreased over time in all treatments. The conditions switching from oxic to anoxic environment at Phase IV increased N_2 flux rates drastically, especially in the straw-amended soils. The comparison among different experimental phases revealed that daily N_2 fluxes increased over time and reached the highest level at Phase IV (anoxic conditions) in all treatments. Similarly, the total N flux ($N_2O + N_2$) was the highest in Phase IV and the lowest in Phase II.

The daily CO_2 fluxes were the highest at Phase I, remained relatively low and constant at Phase II and decreased slightly at Phases III and IV (Fig. 2 (D1 − L4)). Remarkably, a sharper daily CO_2 flux peak was observed in the paddy soils than in the other soils. Overall, the daily CO_2 fluxes were higher in the straw-amended soils than in the non-amended soils. In Phase IV, the daily CO_2 fluxes were still one-fold higher in the straw-amended soils than in the non-amended soils.

Cumulative emissions of N_2O, N_2, and CO_2 and the product ratio of denitrification

At the end of Phase I, the cumulative N_2O emissions were significantly ($P < 0.05$) higher in the V-ST treatment than in the other treatments, and the lowest cumulative N_2O emissions were observed in the P-CL treatment (Table S1). During the same period, the cumulative N_2 emissions were significantly ($P < 0.05$) higher in the O-ST treatment than in the other treatments, whereas the cumulative total N fluxes showed the following trend: V-ST, O-ST, O-CL, V-CL, P-ST, and P-CL. In Phase I, N_2O emissions dominated the total gaseous N emissions in all treatments, except in the O-ST treatment. In Phase II (fertilization with 10 mM KNO_3), the highest cumulative N_2O emissions were measured in the V-CL treatment, whereas no significant differences in cumulative N_2 or total N emissions were observed among the different treatments (Table S1). The mean $N_2O/(N_2O + N_2)$ product ratio at Phase II was lower than that at Phase I, specifically in the straw-amended soils. In Phase III (fertilization with 40 mM KNO_3), interestingly, the total N fluxes were dominated by N_2 fluxes with a clear decrease in the $N_2O/(N_2O + N_2)$ product ratio in all soils. Under completely anoxic conditions in Phase IV, cumulative N_2O emissions were surprisingly higher in the paddy soils (i.e., P-CL and P-ST treatments). The cumulative total N emissions were significantly ($P < 0.05$) higher in the straw-amended soils, accompanied by extremely low $N_2O/(N_2O + N_2)$ product ratios (below 0.3) (Table S1).

N_2O SP_0 values and source partitioning

The SP_0 values ranged from − 6 to 6‰ on Day 1 in all treatments, being the lowest in the V-ST treatment (-6 ± 5.8‰) and the highest in the V-CL treatment (6 ± 0.5‰) (Fig. 3). The SP_0 values increased over time in all treatments, with the most rapid increase observed in paddy soils (up to 23.7‰). Amendment of straw caused only a minor increase in the SP_0 values at Phase I. In the paddy and orchard soils, the SP_0 values increased slightly at Phase II, with a sharp decrease on Day 64. Fertilization with 40 mM KNO_3 at Phase III caused a clear increase in the SP_0 values in the paddy soils, whereas the SP_0 values remained almost constant in the other soils. Interestingly, at Phases II–IV, the SP_0 values were significantly ($P < 0.05$) higher in the V-ST treatment than in the V-CL treatment (up to 11‰), whereas they were only slightly higher in the P-ST treatment than in the P-CL treatment (up to 5‰).

The two-end-member source partitioning model was used to calculate the proportion of each N_2O emission process (bacterial and fungal N_2O). During the initial period of the experiment, the observed extremely low SP_0 values indicated that almost all emitted N_2O originated from bacterial denitrification; however, the share of fungal denitrification (plus contingent nitrification)-derived N_2O increased significantly ($P < 0.05$) over time (Figs. 3 and 4). In Phase I, the overall contribution of bacterial denitrification to the emitted N_2O varied from 63 ± 4% (V-CL treatment) to 81 ± 1% (P-ST treatment) (Fig. 4; Table S1) in all treatments. The $N_2O_{bacterial}/(N_2O_{bacterial} + N_2O_{fungal})$ ratio was the highest in the P-ST treatment, indicating a higher share of

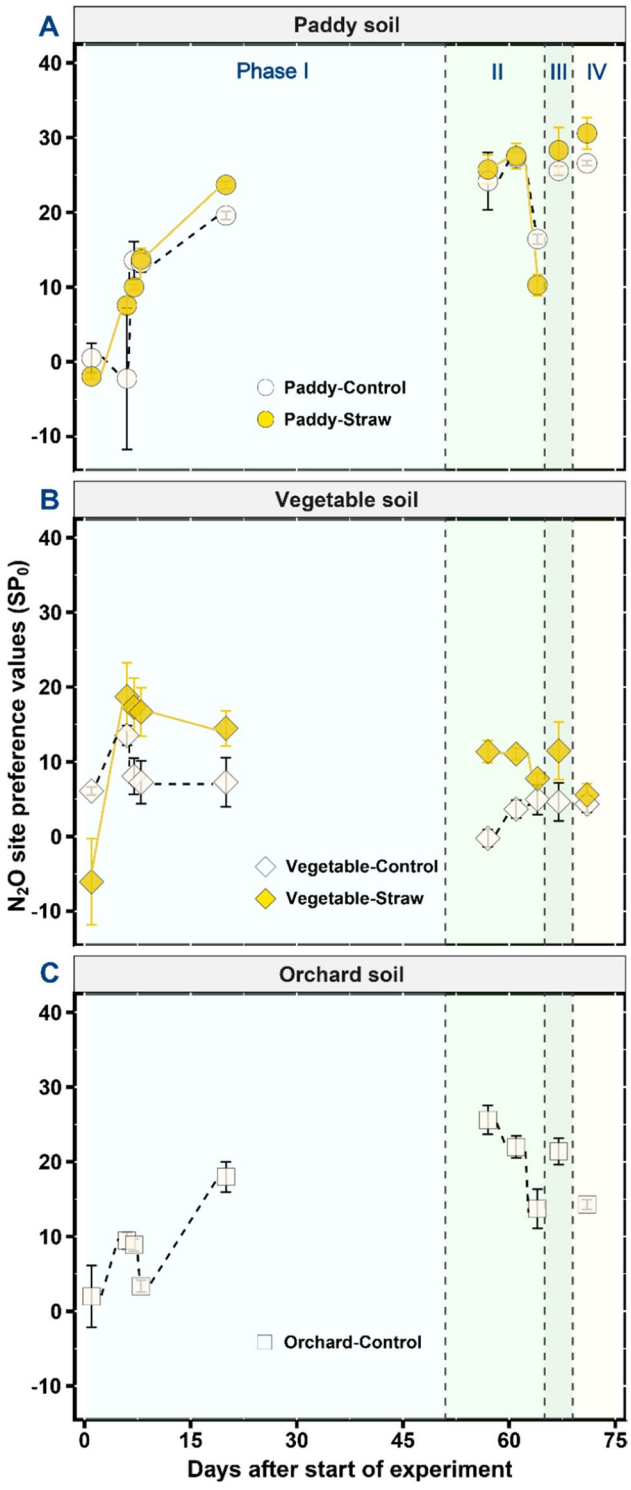

Fig. 3 The N_2O site preference (SP_0) values during the various phases (Phase I: wetting (0–51 days), Phase II: 10 mM KNO_3 addition (51–65 days), Phase III: 40 mM KNO_3 addition (65–69 days), and Phase IV: anoxic (69–75 days)) of the experiment in the P-CL, P-ST (**A**); V-CL, V-ST (**B**); and O-CL (**C**) treatments. The N_2O SP_0 values in the O-ST treatment were not determined owing to the low signal. Error bars show the standard error of each treatment ($n = 3$)

bacterial denitrification. On the other hand, the $N_2O_{bacterial}/(N_2O_{bacterial} + N_2O_{fungal})$ ratio remarkably decreased, specifically in the paddy soils at later phases, indicating a clear shift from bacterial to fungal N_2O. Throughout the entire incubation period, the contributions of bacterial N_2O to the cumulative N_2O emissions were the lowest in the paddy soils ($45 \pm 2\%$ and $54 \pm 6\%$ in the P-CL and P-ST treatments, respectively) and the highest in the vegetable soils ($69 \pm 4\%$ and $71 \pm 6\%$ in the V-CL and V-ST treatments, respectively).

Discussion

Sources of N_2O as affected by the land use type and straw amendment

Increases in N_2O emissions following the wetting of dry soil have been reported in various agricultural systems (Ciarlo et al. 2007; Kessavalou et al. 1998; Kim et al. 2010; Zheng et al. 2000); this is in agreement with the pulse of N_2O emissions observed after rewetting in our study (Fig. 2 (A1, D1, and I1)). The extremely low SP_0 values of the emitted N_2O shortly after the rewetting event (Fig. 3) indicated that almost all of the rewetting-induced N_2O emissions originated from bacterial denitrification in all soils. On the other hand, the clear increase in SP_0 values throughout the incubation indicated that the $N_2O_{bacterial}/(N_2O_{bacterial} + N_2O_{fungal})$ ratio decreased significantly ($P < 0.05$) over time, along with a higher share of fungal N_2O (Table S1). Depending on the soil type, fungi contributed 25 to 55% of the emitted N_2O throughout the entire incubation period. Several incubation studies have illustrated that bacterial denitrification usually dominates shortly after rewetting, whereas in later phases, N_2O sources shift toward the dominance of other microorganisms such as fungi (Henriksen and Breland 2002; Petersen et al. 2020; Senbayram et al. 2018, 2020). This could be attributed to the differences in the growth rate of microbial strains because the development of fungal colonization was reported to be generally slower than that of bacteria (Henriksen and Breland 2002).

Remarkably, the overall share of fungal N_2O throughout the entire incubation period was significantly (one-fold; $P < 0.05$) higher in the paddy soils than in the vegetable and orchard soils (Table S1). The soils in the present study originated from different land-use systems that were converted from paddy fields 12 – 15 years ago, although they exhibited similar total C and N contents (Table 1). The pH values of the vegetable and orchard soils significantly declined relative to that of the paddy soils, implying that excessive input of N fertilizer in such intensive cropping systems has caused obvious soil acidification. Some studies conducted by biocide inhibition techniques have demonstrated that fungi dominated heterotrophic nitrification and N_2O emissions in

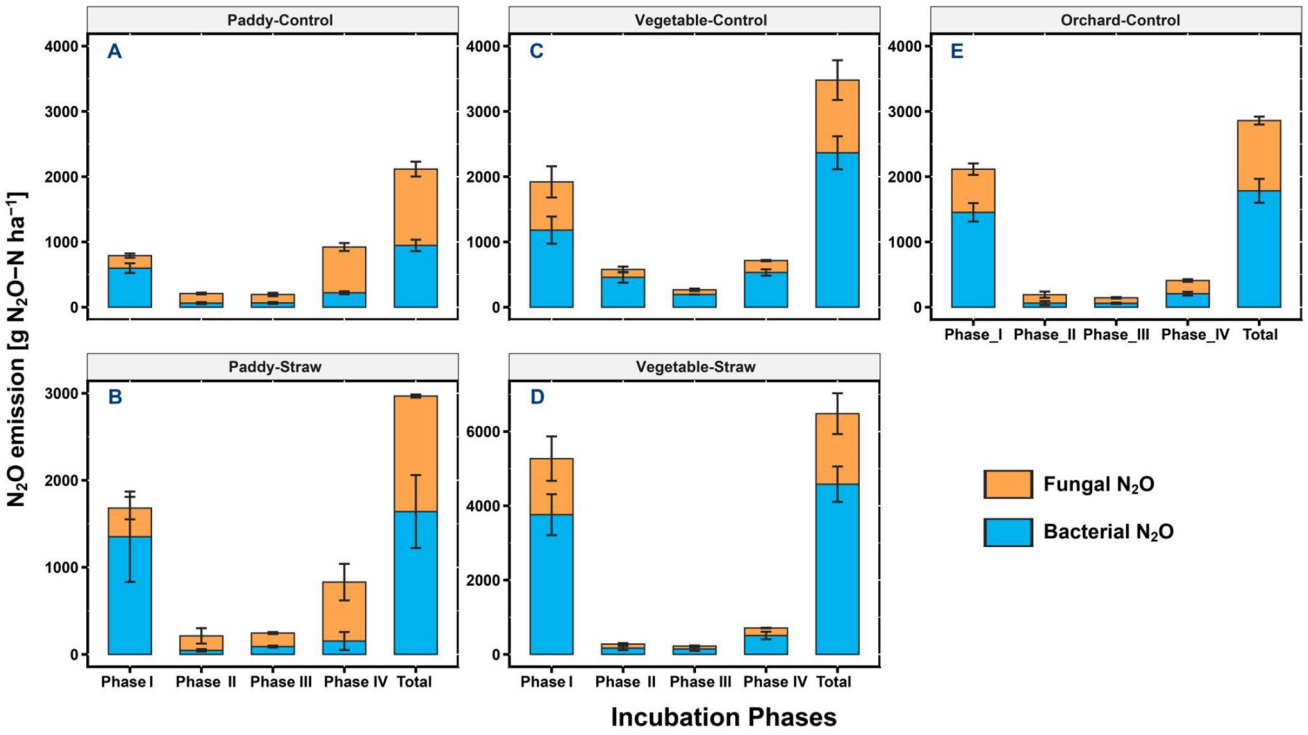

Fig. 4 Contribution of fungal and bacterial denitrification-derived N_2O emissions to the cumulative N_2O fluxes during the various phases (Phase I: wetting (0–51 days), Phase II: 10 mM KNO_3 addition (51–65 days), Phase III: 40 mM KNO_3 addition (65–69 days), and Phase IV: anoxic (69–75 days), Total (0–75 days)) of the experiment in the P-CL (**A**), P-ST (**B**), V-CL (**C**), V-ST (**D**), and O-CL (**E**) treatments. The proportion of N_2O derived from different sources was not calculated and presented owing to the absence of SP_0 values in the O-ST treatment. Error bars show the standard error of each treatment ($n = 3$)

low pH (4.5 – 5.3) forest soils (Chen et al. 2014; Zhu-Barker et al. 2015), in contrast to our results. The reason may be that the biocide inhibition techniques often leads to an overestimation of fungal contribution to soil N_2O emissions (Chen et al. 2014). The observed higher share of bacterial N_2O in the vegetable and orchard soils than in the paddy soils can be attributed to more labile-C-driven bacterial activity (indicated by higher CO_2 fluxes) induced by the greater manure and crop residue input. It is generally believed that agricultural management practices favor bacterial over fungal portions of a microbial community (Ohtonen et al. 1999; van der Wal et al. 2006). The results of the present study were consistent with the aforementioned postulation and suggested that the N_2O-producing microbial community shifted toward a higher proportion of bacteria in the more intensively managed vegetable and orchard soils.

Notably, in Phase I, the contribution of fungi was the lowest in the paddy soils (i.e., P-CL and P-ST treatments) compared with the other soils, indicating a somewhat slower evolution of fungal denitrification (Fig. 4). A lower share of fungal N_2O in the paddy soils than in the vegetable soils was also observed in a short incubation experiment testing similar soils (Ma et al. 2017). On the other hand, the present study clearly showed that the share of fungal N_2O depended on the duration of the incubation time. The contribution of fungal denitrification (plus contingent nitrification) to N_2O emissions increased over time, even with different rates among soils (Fig. 3). After rewetting, the increase in the share of fungal N_2O was slower but to a greater extent in the paddy soils than in the vegetable and orchard soils, which may have been attributed to the adaptation of soil microbes therein under a long-term straw return regime. Indeed, it has been reported that fungi exhibited a slower turnover than bacteria in straw-amended soils (Rousk and Bååth 2007). Our results showed that the contribution of fungal denitrification (plus contingent nitrification) to N_2O emissions (25–50%) was in the same range as that reported for various ecosystems, e.g., 18% measured by Herold et al. (2012) in arable soil, 40–51% in residue-added grassland soils (Zhong et al. 2018), 36–70% in NO_3^--treated coastal sediments (Wankel et al. 2017), and 18% in arable acidic sandy soil (Senbayram et al. 2018). In contrast, Laughlin and Stevens (2002) reported a much greater contribution of fungi to N_2O production (89%) in grassland soils where the soil organic C content was expected to be high. The biocide inhibition techniques used in their study often resulted in an overestimation of fungal contribution to N_2O production (Chen et al. 2014). According to our results,

straw amendment had a minor impact on the $N_2O_{bacterial}/(N_2O_{bacterial}+N_2O_{fungal})$ ratio (albeit it slightly increased at Phase I). Furthermore, the lack of correlation between the $N_2O_{bacterial}/(N_2O_{bacterial}+N_2O_{fungal})$ ratio and CO_2 emissions suggested that the effect of straw amendment on bacterial or fungal N_2O is not straightforward (Table 2).

It needs to be reemphasized that the SP_0 source partitioning approach provides rather rough estimates of the sources of emitted N_2O owing to (i) overlapping SP signals of different N_2O-producing microorganisms, (ii) variability in the isotope enrichment factors of N_2O reduction, and (iii) likely variations in SP signals among different microbial strains (Wu et al. 2019). However, this technique provides useful insights into the effects of NO_3^--N and straw amendment on the production and reduction of N_2O under optimal experimental conditions (e.g., high soil moisture content with low soil NH_4^+ content as in this study). Furthermore, direct measurement of N_2 production enabled calculation of the initial SP values (SP_0) by considering the N_2O reduction fractionation effect (see Method section), thus minimizing the possibility of overestimating fungal denitrification/nitrification. Nevertheless, in our experiment, fungal denitrification may have still been overestimated due to the possible portion of nitrification-derived N_2O related to organic N mineralization during the incubation period and becasue the SP end–member value of heterotrophic bacterial N_2O production could have been lower than the assumed average value of −5‰.

Factors controlling N_2O production and reduction

The highest daily N_2O and CO_2 fluxes occurred shortly after rewetting in Phase I, at which time these fluxes were even higher than those in the anoxic period (Phase IV), thereby showing a predominant rewetting effect on N_2O-producing bacteria and fungi. This pattern may be mostly related to the accumulation of labile C during drying conditions providing more energy sources for denitrifiers, since the moisture content was constant throughout the experiment and additional N (in the form of NO_3^-) was supplied only at Phases II and III. Furthermore, straw amendment increased the N_2O peak emission rate in all soils, supporting the above postulation (Fig. 2 (A, D, and I)). Short-term N_2O pulses after the rewetting of dry soils have been commonly observed (Ruser et al. 2006; Senbayram et al. 2014; Smith and Arah 1990), and such peaks may account for up to 94% of the annual N_2O emissions (Lagomarsino et al. 2016). Straw amendment in conjunction with wetting may further increase N_2O emissions (Table S1). Similarly, Zhou et al. (2020) reported that straw amendment improved the capacity for N_2O production in soils via denitrification, especially after flooding events. The level of rewetting-induced N_2O losses and the associated $N_2O/(N_2O+N_2)$ product ratios were highly variable among different agroecosystems (Firestone and Tiedje 1979; Ruser et al. 2006). In the present study, N_2O fluxes dominated the overall N emissions during Phase I, resulting in high $N_2O/(N_2O+N_2)$ product ratios in all treatments (Table S1). Moreover, the consistently lower N_2 emissions measured in all soil types shortly after rewetting were likely a result of the initially high soil NO_3^- content (> 40 mg N kg^{-1} soil) (Table 1). This observation was

Table 2 Pearson's correlation coefficients between cumulative CO_2 (CO_2), N_2O (N_2O), N_2 (N_2), N_2O+N_2 (N_2O+N_2) emissions, $N_2O/(N_2O+N_2)$ ratio, proportion of bacterial N_2O to total N_2O emissions, and soil NO_3^- content

	CO_2	N_2	N_2O	N_2O+N_2	$N_2O/(N_2O+N_2)$ ratio	B/(B + F) ratio	N_2O/CO_2	N_2/CO_2	$(N_2O+N_2)/CO_2$	Soil NO_3^-
CO_2	1	0.42	0.36	0.51*	−0.25	0.026	−0.035	0.027	−0.13	−0.43
N_2		1	0.10	0.93**	−0.80**	0.41	−0.24	0.90**	0.75**	−0.69**
N_2O			1	0.47	0.40	0.56*	0.71**	−0.08	0.24	−0.02
N_2O+N_2				1	−0.56*	0.51	0.05	0.77**	0.76**	−0.62**
$N_2O/(N_2O+N_2)$ ratio					1	0.31	0.63**	−0.82**	−0.50*	0.75**
B/(B + F) ratio						1	0.54*	0.31	0.50	0.30
N_2O/CO_2							1	0.74	0.50*	0.38
N_2/CO_2								1	0.90**	−0.50*
$(N_2O+N_2)/CO_2$									1	−0.27
Soil NO_3^-										1

B/(B + F) *ratio* bacterial N_2O/(bacterial N_2O + fungal N_2O) ratio, N_2O/CO_2 ratio of cumulative N_2O to cumulative CO_2 fluxes, N_2/CO_2 ratio of cumulative N_2 to cumulative CO_2 fluxes

*Correlation is significant at the 0.05 level

**Correlation is significant at the 0.01 level

consistent with previous findings that NO_3^- is usually preferred over N_2O as a terminal electron acceptor and that N_2O can escape from the soil whenever the NO_3^- supply is greater than the reducing capacity of denitrifiers (Qin et al. 2017; Senbayram et al. 2018; Swerts et al. 1996; Weier et al. 1993). An interesting phenomenon observed in our study was that N_2 emissions increased distinctly over time, causing a lower $N_2O/(N_2O+N_2)$ product ratio (Fig. 2; Table S1). Similar increases in N_2 fluxes and associated decreases in the $N_2O/(N_2O+N_2)$ product ratio over time have been reported repeatedly (Köster et al. 2013; Liu et al. 2010; Mørkved et al. 2007). To investigate the inhibitory effect of high NO_3^- concentrations on N_2O reduction, soils were stepwise amended with NO_3^-, i.e., first flushed with 10 mM KNO_3 solution (equivalent to 37.5 mg N kg^{-1} dry soil at Phase II), and shortly after observing the N_2 peak, flushed with 40 mM KNO_3 solution (150 mg N kg^{-1} dry soil at Phase III) to illustrate the assumed decrease in the N_2O reduction rate. Interestingly, fertilization with 40 mM KNO_3 did not inhibit N_2O reduction and even increased the N_2 fluxes in all soils. In several previous experiments in which N was added shortly after soil rewetting, the results clearly showed that relatively high soil NO_3^- concentrations (over 40–50 mg NO_3^--N kg dry soil) can inhibit N_2O reductase activity, given that NO_3^- is a more preferred terminal electron acceptor than N_2O (Firestone 1982; Qin et al. 2017; Weier et al. 1993). However, our results demonstrated that NO_3^- was not preferentially utilized by denitrifiers over N_2O as a terminal electron acceptor after a long moist spell, likely owing to the adaptive responses (i.e., enzyme activity) of active microorganisms induced by a long moist spell (Fig. 2 (A – J1)). To our knowledge, ours is the first study to demonstrate the effect of NO_3^- on N_2O reduction in such a systematic long-duration experiment, revealing that the drying-rewetting effect on N_2O emissions in heavily N loaded arable soils depends not only on the enhanced availability of C or NO_3^- but also on the level of N_2O-reducing activity. Nevertheless, we speculate that this phenomenon might be related to the change in adaptation of the microbial community composition and enzyme production to the given environmental conditions, where a long and constantly high moisture spell was set at Phase I accompanied by the complete depletion of mineral N toward the end of this phase.

The activity status of the potentially active denitrifying communities fluctuates temporally according to the availability of substrates and electron donors (Holtan-Hartwig et al. 2000). However, our results disagreed with this general assumption and showed that the addition of high levels of NO_3^- (equivalent to 150 mg NO_3^--N kg^{-1} dry soil) did not cause a rapid shift in the active denitrifying community in soils treated with a long moist period along with depleted soil mineral N (Fig. 1A; Fig. 2 (B3, E3, and J3)). Some microorganisms harbor all denitrification enzymes, whereas others either lack N_2O reductases and produce only N_2O (Philippot et al. 2011) or are only able to reduce N_2O (NosZ enzyme) to elemental N_2 (Sanford et al. 2012). Recent studies have identified a previously undescribed *nosZ* clade (a diverse and widespread clade reported as Clade II *nosZ*), and Clade II *nosZ*-possessing microorganisms are more abundant than their typical counterparts (i.e., Clade I *nosZ*-possessing microorganisms) in many ecosystems, underlining their potential role in N_2O consumption in soils (Hallin et al. 2018; Orellana et al. 2014). While the abundance of Clade II *nosZ*-possessing microorganisms is significantly affected by agricultural practices (e.g., the moisture content and C and N supplies) (Domeignoz-Horta et al. 2015; Shan et al. 2021), the conventional primers for Clade I *nosZ* may not capture broader taxonomic coverage, but a new primer set developed by Zhang et al. (2021) and Chee-Sanford et al. (2020) may help in providing greater insight into N_2O reducers. In the present study, we did not perform molecular analysis; however, we hypothesized that the experimental conditions in Phases I and II likely increased the abundance and activity of Clade II *nosZ*-possessing microorganisms, consequently resulting in a lower $N_2O/(N_2O+N_2)$ product ratio. The addition of a high level of NO_3^- at Phase III likely delivered more N_2O (by other denitrifiers) to Clade II *nosZ*-possessing microorganisms (N_2O reducers), causing higher N_2 emissions, as observed in the present study.

Conclusions

The overuse of N fertilizer causing nitrate accumulation in many intensive cropping systems has been widely reported. Previously, many researchers have concluded that high residual nitrate may enhance the share of N_2O emissions from denitrification by inhibiting N_2O reduction to N_2. Our study clearly showed that a long moist spell (typical conditions for intensively irrigated soils) in arable soils significantly suppressed the inhibitory effect of high soil nitrate concentrations on N_2O reduction, as suggested by the relatively high N_2 emissions observed even after the addition of high levels of NO_3^- (equivalent to 37.5 or 150 mg N kg^{-1} dry soil). This result provides direct evidence that the inhibitory effect of high soil nitrate concentrations on N_2O reductions was offset by long moist spells; this should be considered in process-based denitrification models to improve the estimation of N_2O and N_2 losses. Additionally, the rewetting-induced N_2O emissions in arable soils were mainly due to bacterial denitrification, but fungal denitrification (plus contingent nitrification) became more dominant over time following rewetting, indicating a significant role of fungi in N_2O production in

intensively managed arable soils. Moreover, the share of bacterial N_2O increased in soils following land use types converted from paddy to vegetable or orchard fields, which may be attributed to more labile-C driven bacterial activity induced by the greater manure and crop residue input in the vegetable or orchard fields.

Associated content

Details of the gas and soil mineral N content measurements, soil cumulative gas emissions (N_2O, N_2, N_2O+N_2, and CO_2), $N_2O/(N_2O+N_2)$ product ratios, and bacterial N_2O/(bacterial N_2O + fungal N_2O) ratios (Table S1) at Phases I, II, III, and IV in the different treatments are provided.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00374-021-01612-x.

Acknowledgements We thank Martina Heuer and Jennifer Ehe for assistance in the stable isotope analysis, Ute Tambor for assistance in the mineral N analysis, Dr. Dominika Lewicka-Szczebak for assistance in the isotopomer data analysis, and Dr. Roland Fuß for the technical support.

Author contribution Mehmet Senbayram: Conceptualization, Methodology, Investigation, Writing. Zhijun Wei: Investigation, Data curation, Visualization, Writing. Di Wu: Methodology and Formal analysis. Jun Shan: Conceptualization, Writing — review and editing. Xiaoyuan Yan: Writing — review and editing. Reinhard Well: Supervision, Writing — review and editing.

Funding This study was funded by the DFG project (WE 1904/12–1) and key project of the Natural Science Foundation of China (U20A20113). Dr. Jun Shan received financial support from the Scientific Instrument and Equipment Development Project of CAS (YJKYYQ20170058) and the Youth Innovation Promotion Association of CAS (2017362). Dr. Mehmet Senbayram received financial support from the TÜBITAK Project (119R042) and HUBAK Project (21141).

Declarations

Conflict of interest The authors declare no competing interests.

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