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Stimulation of heterotrophic nitrification and N_2O production, inhibition of autotrophic nitrification in soil by adding readily degradable carbon

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Abstract

Purpose This study aimed to test the hypothesis that readily degradable Carbon (C) has contrasting effect on soil N autotrophic and heterotrophic nitrification, can stimulate nitrous oxide (N_2O) emission. The knowledge can improve our understanding of the effect of readily degradable C on soil N nitrification and the related N_2O emission.

Materials and methods ^{15}N tracing technique along with acetylene inhibition was used to determine the effect of different doses of glucose-C addition on the rates of total nitrification (n_{tot}), autotrophic nitrification (n_a), heterotrophic nitrification (n_h), and N_2O production in two soils. Soils were collected from Glenormiston (GN) and Terang (TR), Victoria, Australia and incubated at soil moisture content of 60% water-filled pore space (WFPS) and at 25 °C.

Results and discussion The addition of mixed C and N substrates with wide C/N ratio (> 25) promoted heterotrophic nitrification by 2.84- to 3.33-folds but inhibited autotrophic nitrification by 30.4–54.8%, thereby resulting in high n_{tot} and NO_3^- accumulation compared with the soil samples under the control treatment. The mechanism of glucose inhibition of n_a might be caused by increasing the microbial immobilization of NH_4^+ and not by affecting the gene copy numbers of ammonia-oxidizing archea and ammonia-oxidizing bacteria. The glucose addition stimulated N_2O production in soil, which might be caused by promoting heterotrophic nitrification and denitrification.

Conclusions The stimulating effect of degradable C application on the contribution of heterotrophic nitrification to total nitrification, NO_3^- accumulation, and N_2O production should be considered, especially in soils with low pH and high organic C content.

Keywords ^{15}N tracing · Acetylene · Denitrification · Glucose · Nitrification · Nitrous oxide

1 Introduction

Nitrification in soil leads to nitrate (NO_3^-) leaching, gaseous nitrous oxide (N_2O) production, and up to 50% loss of nitrogen (N) availability for plants (Beeckman et al. 2018). NO_3^- leaching results in eutrophication of ground (drinking) water and may cause health problems. Meanwhile, N_2O is an ozone-depleting and atmosphere-threatening greenhouse gas that is approximately 300 times more potent than CO_2.

NO_3^- ion is produced through nitrification in soils via two pathways. One pathway is autotrophic nitrification (n_a), which is the oxidation of NH_4^+ to NO_3^- by chemo-lithotrophic ammonium (e.g., ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacteria (AOB)) or nitrite-oxidizing bacteria (Stein and Klotz 2016; Fig. 1). The other pathway is heterotrophic nitrification (n_h) wherein a wide phylogenetic range of heterotrophic bacteria, fungi, and archea are capable of nitrification without gaining energy from the process, thereby using monomeric organic carbon (C) compounds for growth (Stein and Klotz 2016; Li et al. 2018; Fig. 1). Nitrification by

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heterotrophic microbes is ignored in most studies regarding autotrophic nitrifiers. However, large contribution of heterotrophic nitrifying microbes to nitrification in acidic forest soil is still proposed or debated (Zhang et al. 2011, 2015; Nelissen et al. 2012; Chen et al. 2017). Moreover, the recently discovered complete ammonia oxidizers (comammox) within the *Nitrospira* genus can directly convert NH_4^+ to NO_3^- (Daims et al. 2015; Kessel et al. 2015; Fig. 1). To date, a technique that can be used to investigate the importance of comammox to nitrification is unavailable (Hu and He 2017). Meanwhile, techniques, such as standard soil incubation protocol with and without acetylene (C_2H_2) (Garrido et al. 2002) or ^{15}N isotope enrichment approach combined with ^{15}N tracing model (Cheng et al. 2015, Zhang et al. 2015), can be used to distinguish the contribution proportion of autotrophic and heterotrophic nitrification to total nitrification.

Autotrophic nitrification is the dominant production process of NO_3^- and N_2O in agricultural soils. However, growing evidence shows that heterotrophic nitrification may act as the predominant pathway for producing NO_3^- in soils in environments not facilitated by autotrophic nitrification (Cai et al. 2010). Heterotrophic nitrification occurs in grassland and forest soils and is generally negligible in cropland (Müller et al. 2004; Nelissen et al. 2012; Zhang et al. 2015; Chen et al. 2017). Several environmental factors affect autotrophic nitrification (Hart et al. 1994; Sahrawat 2008). Glucose is an essential ingredient of root exudates, is a major C source for microorganisms, and is easily degraded in soil. Previous studies shown that readily degradable C, such as glucose, can stimulate NH_4^+ immobilization by soil microorganisms, thereby possibly decreasing NH_4^+ availability temporarily depending on the availability and application rate of external C (Kaye and Hart 1997). Thus, readily degradable C might inhibit autotrophic nitrification by reducing NH_4^+ via immobilization (Freppaz et al. 2007; Ma et al. 2016). However, in terrestrial ecosystems, the availability and quality of C often limit the growth and activities of heterotrophic microbes (Ilstedt and Singh 2005). Readily degradable C might facilitate heterotrophic nitrification by providing sufficient C for the growth of heterotrophic bacteria, fungi, and archea (Yokoyama et al. 1992; Zhu et al. 2015). The contrasting effects of degradable C on autotrophic and heterotrophic nitrification might lead to discrepant results on the effect of the application of C on N nitrification and NO_3^- accumulation in soil. Previous results about the effect of adding exogenous organic C on gross N nitrification and NO_3^- accumulation in soils are controversial (Cheng et al. 2012; Emeterio et al. 2014; Zhao et al. 2018), and to date, studies that independently examine the effect of readily degradable C on the contribution of autotrophic and heterotrophic nitrification to total nitrification are scarce.

Despite the identification of various abiotic and biotic N_2O forming processes (Fig. 1), N_2O is believed to be produced mainly by ammonia oxidizers (AOA and AOB) and nitrite-oxidizing bacteria responsible for autotrophic nitrification (Hink et al. 2017) and heterotrophic denitrifying microorganisms responsible for denitrification (Baggs 2011; Butterbach-Bahl et al. 2013; Hu et al. 2015); the former process is the main contributor of N_2O under aerobic conditions, and the latter is dominant under anaerobic conditions (Liu et al. 2017). Moreover, evidence shown that the AOA N_2O yield relative to nitrite produced was half that of AOB, likely due to additional enzymatic mechanisms in the latter (Hink et al. 2017). Several previous studies reveal that the N_2O production rate in soil is stimulated by the application of readily degradable C possibly due to increasing denitrification (Wang et al. 2005; Cheng et al. 2012; Ameloot et al. 2016; Mehnaz et al. 2018). However, increasing evidence shows that heterotrophic nitrification of organic N might play an

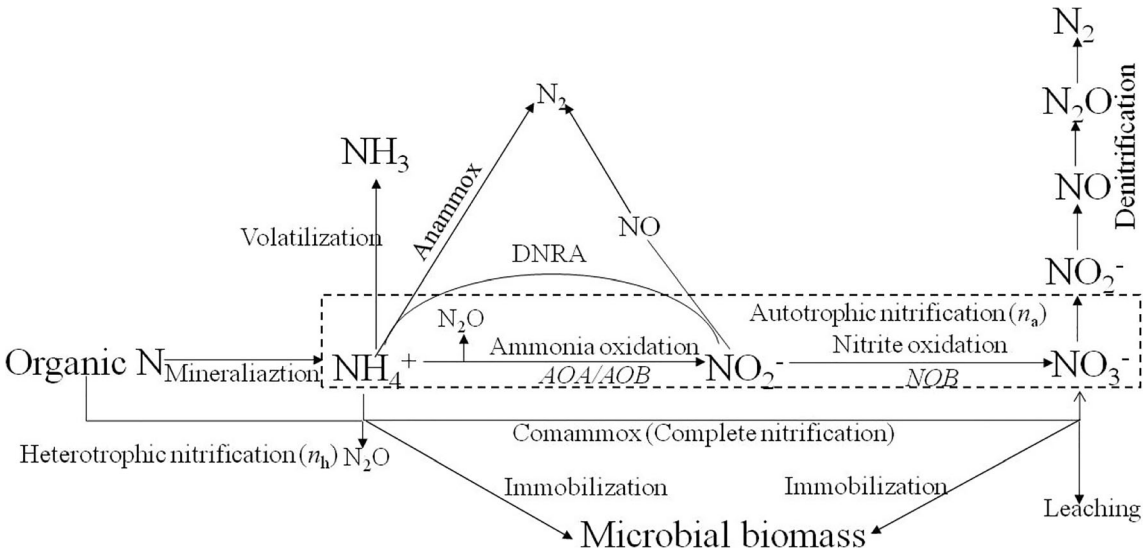

Fig. 1 N transformation in soils (Revised from Hu et al. 2017)

important role in N_2O emission from soils, especially acidic soils (Zhang et al. 2015). Readily degradable C might facilitate heterotrophic nitrification, thereby possibly promoting N_2O production.

In the present study, ^{15}N tracing technique along with acetylene (C_2H_2) inhibition method was used to determine the effects of different doses of glucose addition on total N nitrification rate (n_{tot}), N_2O production rate, and the relative contributions of autotrophic and heterotrophic nitrification to total nitrification. We hypothesized that glucose addition has contrasting effect on soil autotrophic and heterotrophic nitrification, and can stimulate N_2O emission. The findings from this study can improve our understanding of the effect of readily degradable C on soil nitrification and are beneficial for making mitigation strategies to reduce the negative effect of nitrification on the environment.

2 Materials and methods

2.1 Soil properties and pretreatment

Soil samples were from two typical pastures in Glenormiston (GN, 38.18° S, 142.97° E) and Terang (TR, 33.73° S, 84.43° E) in Victoria, Australia. At each site, ten replicate samples collected from the upper 10 cm because most of the feeding roots in pastures lie in this active zone. Soils were thoroughly homogenized and transported on ice to a laboratory. Roots and stones were removed, and the soil was sieved at 2 mm before being air-dried. The apparent density of the sieved soil was determined. Table 1 shows the physical and chemical properties of the soil.

2.2 Experimental design

A 7-day aerobic soil laboratory study was conducted, and destructive sampling was initiated on five occasions (2 h, 1 day, 2 days, 4 days, and 7 days). Fresh soil samples (weight equivalent of 60 g of oven-dried soil) were packed into 500-mL plastic vials to an average packing density of 0.71 g cm^{-3}. The vials were kept at 25 °C in the dark during the entire incubation period. The following eight treatments (four replicates per treatment) were included in this study:

1) $NH_4Cl + K^{15}NO_3$ (Control);
2) $NH_4Cl + K^{15}NO_3$ + Glucose-1 (NC_1);
3) $NH_4Cl + K^{15}NO_3$ + Glucose-2 (NC_2);
4) $NH_4Cl + K^{15}NO_3$ + Glucose-3 (NC_3);
5) $NH_4Cl + K^{15}NO_3$ + 0.1% *v*/*v* C_2H_2 (NA);
6) $NH_4Cl + K^{15}NO_3$ + 0.1% *v*/*v* C_2H_2 + Glucose1 (NAC_1);
7) $NH_4Cl + K^{15}NO_3$ + 0.1% *v*/*v* C_2H_2 +Glucose 2 (NAC_2);
8) $NH_4Cl + K^{15}NO_3$ + 0.1% *v*/*v* C_2H_2 + Glucose 3 (NAC_3).

A total of 320 vials (8 treatments × 4 replicates × 5 destructive sampling time × 2 soils) were obtained. For each treatment, 100 mg N kg^{-1} soil (50 mg N kg^{-1} as NH_4^+ and 50 mg N kg^{-1} as NO_3^-; NO_3^- has 10% ^{15}N) was applied evenly to the surface of the soils in sample jars using a syringe and a wide bore needle. For glucose treatments, three levels of C application dose were 1000 (C1, low dose), 2500 (C2, medium dose), and 5000 mg C kg^{-1} (C3, high dose) soils. Thus, the corresponding C/N ratio of the applied mixed C and N substrates were 10, 25, and 50. The C and N treatments were applied on day 0. An equivalent volume of MilliQ water instead of glucose was used in non-glucose treatments. The soil water content of all treatments was 60% water-filled pore space (WFPS). For each C_2H_2 treatment, C_2H_2 (0.5 mL) was injected using an air-tight syringe, replacing the corresponding volume of headspace air in the vial. A 0.1% *v*/*v* concentration was achieved in the C_2H_2 treatments. The water loss during incubation was replenished by weighing every 3 days by adding MilliQ water, and C_2H_2 was replenished as well.

2.3 Soil analyses and nitrification rates

The soil samples in the four replicates of each treatment were destructively sampled on 2 h, 1 day, 2 days, 4 days, and 7 days after treatment application. A subsample (2 g) of soil was

Table 1 Properties of soils

Soil	Organic matter (%)	Organic C (%)	Total N (%)	C/N	pH (1:5 water)	CEC (c mol kg^{-1})	Clay (< 2 μm, %)	Silt (2–60 μm, %)	Sand (60–2000 μm, %)	Soil texture	NH_4^+–N mg kg^{-1}	NO_3^-–N mg kg^{-1}
Terang (TR)	7.9	4.60	0.5	9.3	5.50	7.67	8	63	29	Sandy loam	12.3	6.90
Glenormiston (GN)	10.0	5.90	0.6	9.8	6.00	24.0	11	53	36	Sandy loam	12.2	14.9

extracted from each vial on day 0, day 4, and day 7 for molecular analysis and stored in a freezer at − 80 °C prior to DNA extraction. The remaining soil was extracted with 2 M KCl (1:5 = soil:solution) by shaking for 1 h. Extracts were filtered using quantitative filter paper (Whatman 42) and were kept at − 20 °C prior to analysis in a segmented flow analyzer (Skalar, SAN^{++}). The ^{15}N enrichment of NO_3^-–N was determined after micro-diffusion, as reported by Saghir et al. (1993) with the following modification: an acidified filter paper disk was used instead of a Petri dish of acid to absorb NH_3; moreover, analysis was performed using an isotope ratio mass spectrometer (Hydra 20–20, SerCon, Crewe, UK). The details of soil DNA extraction and quantitative PCR methods were reported in our previously published paper (Lan et al. 2018).

We calculated the rates of n_{tot} using standard isotope dilution equations as follows (Hart et al. 1994):

$$n_{tot} = \frac{\left(\left([NO_3^-]_0 - [NO_3^-]_t\right)/t\right) \times \log(APE_0/APE_t)}{\log\left([NO_3^-]_0/[NO_3^-]_t\right)},$$

where t represents time (days), APE_0 denotes the atom % ^{15}N excess of NO_3^- pool at time 0, APE_t is the atom % ^{15}N excess of NO_3^- pool at time t, APE is the atom % ^{15}N enrichment of an N pool enriched with ^{15}N minus the atom % ^{15}N enrichment of that pool prior to ^{15}N addition, $[NO_3^-]_0$ is the total NO_3^- concentration (mg kg^{-1}) at time 0, and $[NO_3^-]_t$ is the total NO_3^- concentration (mg kg^{-1}) at time t.

Autotrophic nitrifiers were assumed to be completely inhibited by 0.1% v/v C_2H_2. Therefore, for the control treatment, $n_{tot} = n_a + n_h$; for the C_2H_2 treatments, $n_h = n_{tot}$.

The headspace gas for N_2O analysis was extracted from the 500-mL vials using gas-tight syringes at 1, 2, 4, and 7 days after the application of treatments. Prior to collection of gas samples, the vials were opened to ensure that the N_2O concentration in the headspace was at ambient level. On each sampling day, 20-mL gas samples were collected 12 h after vial closure. Each sample was transferred into a pre-evacuated exetainer (Exetainer®, Labco Ltd., Lampeter, Ceredigion, UK). The samples were analyzed for N_2O concentrations using a gas chromatograph (Agilent 7890) with an ECD detector.

2.4 Statistical analyses

Comparisons of n_{tot}, n_{net}, n_a, n_h, N_2O flux, and gene copy numbers of AOA and AOB among treatments were performed through one-way ANOVA. Redundancy and correlation analyses were performed to investigate the relationships between N_2O production rate and NO_3^-–N concentration and nitrification rate. All statistical analyses were conducted using SPSS software version 19.0 for Windows (SPSS Inc., Chicago, IL USA). Differences were considered significant at $P < 0.05$ level.

3 Results

3.1 Dynamics of NH_4^+, NO_3^- pools, and $^{15}NO_3^-$ APE

As shown in Fig. 2, the concentration of NH_4^+ of GN and TR soils decreased in all treatments, although to a varying degree (Fig. 2a and c). Moreover, the decrease in NH_4^+ concentration in C_2H_2 treatments was smaller than that in non-C_2H_2 treatments. The addition of medium and high dose of glucose accelerated the decrease in NH_4^+ concentration, whereas the addition of low dose of glucose had no such effect. Among all treatments in both soils, NC2 and NC3 exhibited the largest decrease in NH_4^+ concentration, from 62 mg N kg^{-1} d^{-1} at day 0 to nearly zero at day 7 (Fig. 2a and c). The concentration of NO_3^- generally increased with the incubation time in all treatments, except in NC2 and NC3 where the NO_3^- concentrations were relatively stable during days 4–7 (Fig. 2band d). The increase in NO_3^- concentration was higher in samples under non-C_2H_2 treatments than that in samples under C_2H_2 treatments. Furthermore, the increasing dose of applied glucose resulted in increasing NO_3^- concentration regardless of the soils and C_2H_2 treatments (Fig. 2b and d). The highest accumulated NO_3^- concentrations were in the samples under NC2 and NC3 treatments, whereas the lowest were in the samples under NA and NAC1 treatments. The dynamics of NO_3^- was negatively correlated with NH_4^+ ($NO_3^- = -0.8338NH_4^+ + 99.343$, $R^2 = 0.7043$, $P < 0.05$).

The changes in $^{15}NO_3^-$ APE under different treatments are shown in Fig. 3. When the NO_3^- pool was labeled, the $^{15}NO_3^-$ APE decreased with incubation time under all treatments for GN and TR soils. The extent of decrease in $^{15}NO_3^-$ APE was amplified when the dose of applied glucose was increased; however, such effect was weakened by C_2H_2. The smallest decrease in $^{15}NO_3^-$ APE was observed in samples under NA and NAC1 treatments for GN and TR soils, whereas the largest decrease was observed in TR soil under NC3 and GN soil under NAC3, NC3, and NC2 treatments (Fig. 3).

3.2 Nitrification and NO_3^- consumption rates

The variations in the calculated average n_{net} and n_{tot} were between 2 mg N kg^{-1} day^{-1} and 11 mg N kg^{-1} day^{-1} in GN and TR soils under different treatments (Table 2). Generally, n_{tot} was higher than the corresponding n_{net} (Table 2), and both were significantly correlated with each other ($n_{tot} = 1.2241n_{net} - 0.2389$, $R^2 = 0.9542$, $P < 0.01$). n_{net} and n_{tot} were largest in both soil samples under NC2 and NC3 treatments, and smallest in samples under NA and NAC1 treatments ($P < 0.01$, Table 2). No significant difference in n_{net} and n_{tot} was found between NC2 and NC3, NA and NAC1, and among control, NC1, NAC2, and NAC3 (Table 2, $P > 0.05$).

The rates of n_a in control and NC1 were similar (Table 2); n_a was significantly inhibited by 30.3–34.7% in GN soil and

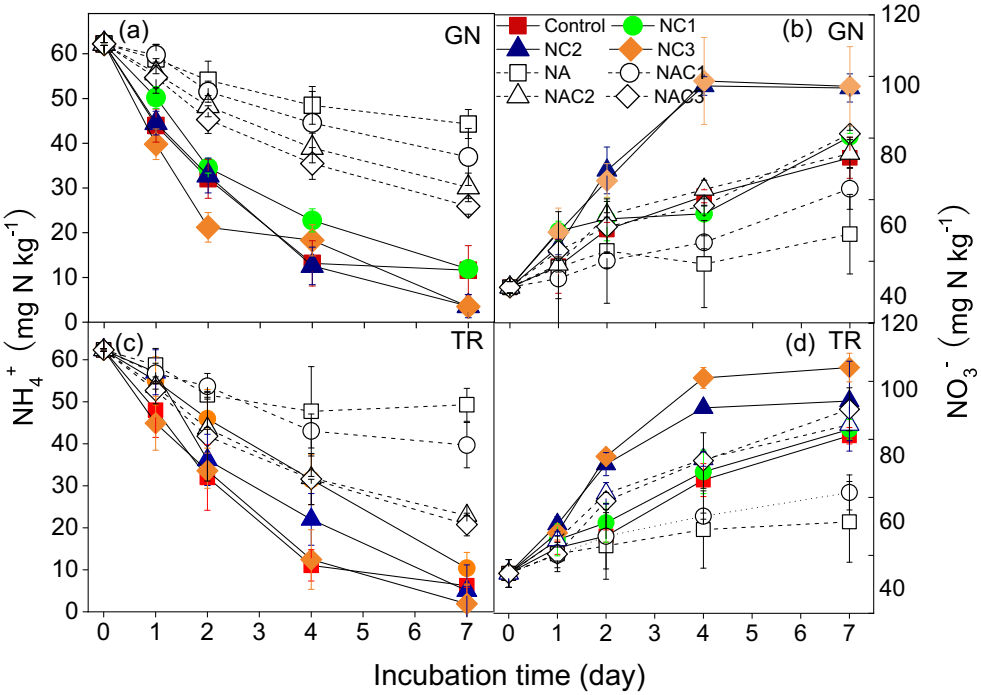

Fig. 2 Dynamics of NH_4^+ and NO_3^- concentrations under different treatments during 7 days of incubation. Control: NH_4Cl + $K^{15}NO_3$; NC_1: NH_4Cl + $K^{15}NO_3$ + Glucose-1; NC_2: NH_4Cl + $K^{15}NO_3$ + Glucose-2; NC_3: NH_4Cl + $K^{15}NO_3$ + Glucose-3; NA: NH_4Cl + $K^{15}NO_3$ + 0.1% *v*/*v* C_2H_2; NAC_1: NH_4Cl + $K^{15}NO_3$ + 0.1% *v*/*v* C_2H_2 + Glucose1; NAC_2: NH_4Cl + $K^{15}NO_3$ + 0.1% *v*/*v* C_2H_2 + Glucose 2; NAC_3: NH_4Cl + $K^{15}NO_3$ + 0.1% *v*/*v* C_2H_2 + Glucose. Error bars indicate standard errors of four replicates

42.8–54.8% in TR soil after high-dose glucose application. Conversely, the rates of n_h were significantly increased by 2.84- to 3.21-folds in GN soil and 2.85- to 3.33-folds in TR soil after high-dose glucose application. In the control treatment, the ratios of n_a to n_{tot} (P_{na}) were 57.1% in GN soil and 62.5% in TR soil; the ratios of n_h to n_{tot} (P_{nh}) were 42.9% in GN soil and 37.5% in TR soil (Table 2). However, P_{na} decreased by approximately 20%, whereas the ratio of n_h to n_{tot} (P_{nh}) increased by approximately 80% under high-dose glucose treatment.

Table 2 shows the NO_3^- consumption rates (C_{NO3-}), which was calculated by subtracting n_{net} from n_{tot}. The rates under low-dose glucose treatment were comparable with those under control. Under medium-dose and high-dose glucose treatments, C_{NO3-} increased by 1.9- to 3.3-folds in GN soil and 1.3- to 2.4-folds in TR soil compared with samples under the control treatment. However, the TR soil under NAC2 was an exception, in which C_{NO3-} decreased by 33% compared with soil under the control treatment.

3.3 N_2O production rate

The changes in N_2O production rates in GN and TR soils under NA and NAC1 treatments were constant during incubation; whereas the rates of N_2O production in samples under other treatments reached the peak during the first 2 days but decreased as the incubation proceeded (Fig. 4a, b). With the addition of high-dose glucose significantly stimulated N_2O production ($P < 0.01$), especially in the first half of the incubation; however, such effects were not observed in low-dose glucose treatments (Fig. 4a, b). The highest N_2O production rates were observed in GN and TR soils under NC2 and NC3 and the lowest rates were under NA treatment. Furthermore, N_2O production rates were inhibited in samples under C_2H_2

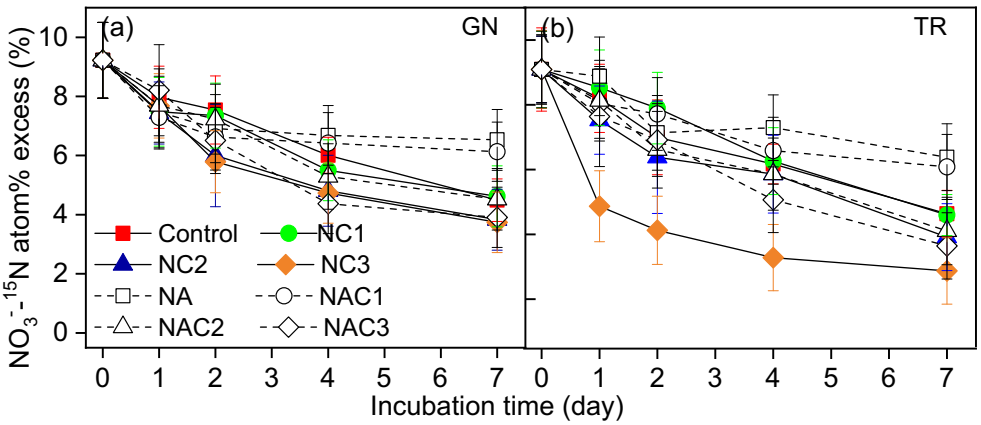

Fig. 3 ^{15}N atom % excess of NO_3^- pools in GN and TR soils under different treatments during 7 days of incubation. Error bars indicate the standard errors of four replicates

Table 2 Time-weighed averaged N nitrification rates (mg N $kg^{-1}d^{-1}$) and the proportion of autotrophic nitrification and heterotrophic nitrification to total nitrification (%)

[a]Treatments	GN						TR					
	[b]n_{net}	n_{tot}	C_{NO3-}	n_a	n_h	P_{na} (%)	P_{nh} (%)	n_{net}	n_{tot}	C_{NO3-}	n_a	n_h	P_{na} (%)	P_{nh} (%)
Control	5.26(0.3)	6.01(1.0)	0.76(0.1)	3.43(0.3)	2.58(0.3)	57.1(3.5)	42.9(2.3)	5.94(0.5)	6.80(0.6)	0.87(0.1)	4.35(0.3)	2.46(0.2)	32.5(4.2)	37.5(2.5)
NC1	6.11(0.9)	6.43(0.5)	0.32(0.1)	3.54(0.3)	2.89(0.3)	55.1(5.1)	44.9(4.0)	6.21(0.3)	6.43(0.7)	0.22(0.0)	3.45(0.4)	2.98(0.3)	58.6(3.5)	42.4(3.4)
NC2	8.09(0.9)	9.56(0.9)	1.47(0.2)	2.24(0.4)	7.33(0.6)	23.4(2.9)	76.6(3.9)	7.43(0.9)	9.49(0.7)	2.06(0.2)	2.49(0.1)	7.00(0.5)	13.7(1.0)	86.3(5.5)
NC3	8.16(0.8)	10.7(0.5)	2.52(0.2)	2.39(0.2)	8.29(0.7)	22.4(3.0)	77.6(5.2)	8.88(0.7)	10.2(0.9)	1.33(0.1)	2.01(0.2)	8.20(0.9)	20.3(1.3)	79.7(6.8)
NA	2.16(0.5)	2.58(0.4)	0.42(0.1)	[c]nd	nd	nd	nd	2.22(0.2)	2.46(0.4)	0.24(0.0)	nd	nd	nd	nd
NAC1	2.57(0.3)	2.89(0.3)	0.32(0.1)	nd	nd	nd	nd	2.63(0.3)	2.98(0.2)	0.35(0.0)	nd	nd	nd	nd
NAC2	5.43(0.8)	7.33(0.5)	1.90(0.1)	nd	nd	nd	nd	6.41(0.5)	7.00(0.5)	0.59(0.0)	nd	nd	nd	nd
NAC3	6.23(0.7)	8.29(0.6)	2.05(0.1)	nd	nd	nd	nd	7.08(0.8)	8.20(0.5)	1.12(0.1)	nd	nd	nd	nd

[a] Treatments: Control: NH_4Cl+$K^{15}NO_3$; NC_1: NH_4Cl+$K^{15}NO_3$+ Glucose-1; NC_2: NH_4Cl+$K^{15}NO_3$+ Glucose-2; NC_3: NH_4Cl+$K^{15}NO_3$+Glucose-3; NA: NH_4Cl+$K^{15}NO_3$+0.1% *v/v* C_2H_2; NAC_1: NH_4Cl+$K^{15}NO_3$+0.1% *v/v* C_2H_2+Glucose1; NAC_2: NH_4Cl+$K^{15}NO_3$+0.1% *v/v* C_2H_2+Glucose 2; NAC_3: NH_4Cl+$K^{15}NO_3$+0.1% *v/v* C_2H_2+Glucose

[b] n_{net}: Net N nitrification rate; n_{tot}: Total N nitrification rate (autotrophic nitrification + heterotrophic nitrification); C_{NO3-}: NO_3^- consumption rate; P_{na}: proportion of autotrophic nitrification to total nitrification; P_{nh}: proportion of heterotrophic nitrification to total nitrification

[c] nd: Not determined

[d] Values in brackets are standard deviations ($n = 4$)

treatments compared with those under non-C_2H_2 treatments. N_2O production rate was significantly correlated with n_{net} ($P < 0.05$, Fig. 5a), n_{tot} ($P < 0.05$, Fig. 5b), and average NO_3^- concentration ($P < 0.05$, Fig. 5d), but negatively correlated with NH_4^+ concentration ($P < 0.05$, Fig. 5c).

3.4 AOA and AOB gene copy numbers

The gene copy numbers of AOA and AOB were observed to be induced by the application of N fertilizer in the two test soils under all treatments (Fig. 6). AOB was more predominant than AOA in both test soils. The addition of C_2H_2 significantly inhibited the AOB gene copy numbers ($P < 0.01$) but exhibited no such effect on the AOA gene copy numbers compared with those samples under the control treatment. By contrast, glucose application had no effect on the gene copy numbers of AOA and AOB.

4 Discussion

4.1 Effects of glucose C on autotrophic and heterotrophic nitrification rate

In this study, when the same amount of $K^{15}NO_3$ was applied, the ^{15}N APE of NO_3^- was significantly lower in samples under glucose treatments than those in samples under non-glucose treatments; it was also lower in samples under high-dose glucose treatments than in samples under low-dose treatments. Indicating that the total nitrification probably be promoted by the application of glucose. Consistently, the calculated n_{tot} values were higher in samples under high-dose glucose treatments than in samples under low-dose and non-glucose treatments (Table 2), thereby confirming the above deduction.

However, the application of glucose exhibited a contrasting effect on n_h and n_a in the two soils; such glucose addition resulted in increased n_h but decreased n_a (Table 2). AOA and AOB were involved in the first step of autotrophic nitrification. We tested the gene copy numbers of AOA and AOB in this study and observed that AOA and AOB were remarkably increased after N addition (Fig. 6). Moreover, the gene abundance of AOB was significantly inhibited by the application of C_2H_2, which could be attributed to the nitrification in arable soil with neutral and alkaline pH or the high levels of NH_4^+, mainly followed by the dynamics of AOB abundance rather than AOA abundance (Di et al. 2009; Li et al. 2018). AOA was predominant in soils with low pH and high NO_3^- level (Hu et al. 2013). However, no remarkable difference in AOA and AOB abundance was found between glucose and non-glucose treatments. Therefore, the mechanism of the reduced n_a by glucose might not be caused by the abundance of AOA and AOB genes. However, we measured AOA and

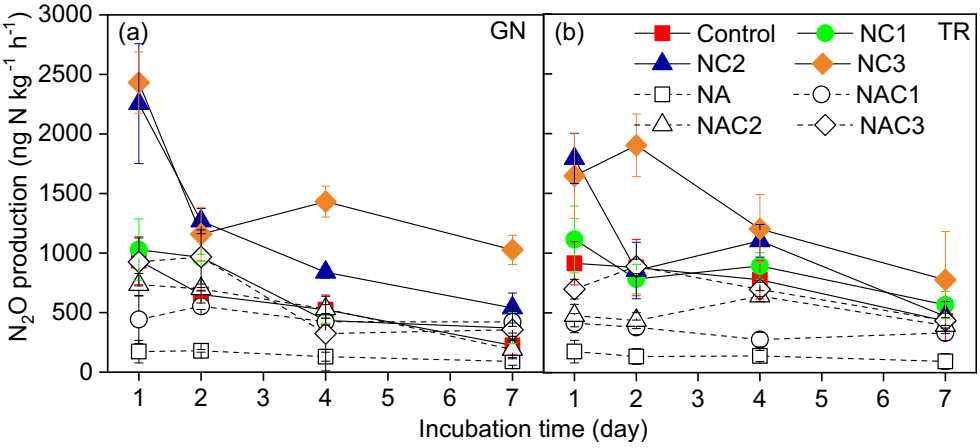

Fig. 4 Changes in N_2O production rates under different treatments during 7 days of incubation. Error bars indicate the standard errors of four replicates

AOB *amoA* gene abundances on the basis of soil DNA. To reveal the influence of glucose on the AOA and AOB activity, measuring active communities using soil RNA is highly desirable in future studies. Moreover, evidence shown that, addition of readily degradable C can stimulate the short-term N immobilization by soil microorganisms (Ma et al. 2016; Yu et al. 2016; Mehnaz et al. 2018). Stoichiometric theory states that the transition of microbes from optimum growth to nutrient limitation is at a critical substrate C/N threshold ratio of approximately 20–25 (Schimel and Weintraub 2003; Sinsabaugh et al. 2013), which was reflected in the requirement of additional N to proliferate soil microorganisms when the C/N ratio of available substrates exceeded this threshold (Manzoni et al. 2008; Mooshammer et al. 2014; Cheng et al. 2017). The C/N ratios of the applied mixed substrate were 10 (NC1 and NAC1), 25 (NC2 and NAC2), and 50 (NC3 and NAC3) in the present study. Therefore, according to the above theory, the difference in autotrophic nitrification rate among treatments may partly because the different extent in the decreasing of NH_4^+ availability temporarily by immobilization, thereby reducing N substrates in different levels for autotrophic nitrification. In consistent, results from Hanan et al. (2016) also suggesting that, under some circumstances organic C may decelerate N cycling if it promotes immobilization and limits NH_4^+ supply to nitrifiers.

Our results showed that heterotrophic nitrification, which accounts for 37.5–42.9% of total nitrification, played roles similar to that of autotrophic nitrification in the total nitrification under the control treatment; While, P_{nh} increased by approximately 80% after the application of high-dose glucose (Table 2). The results confirmed the test hypothesis that heterotrophic nitrification was stimulated by glucose application,

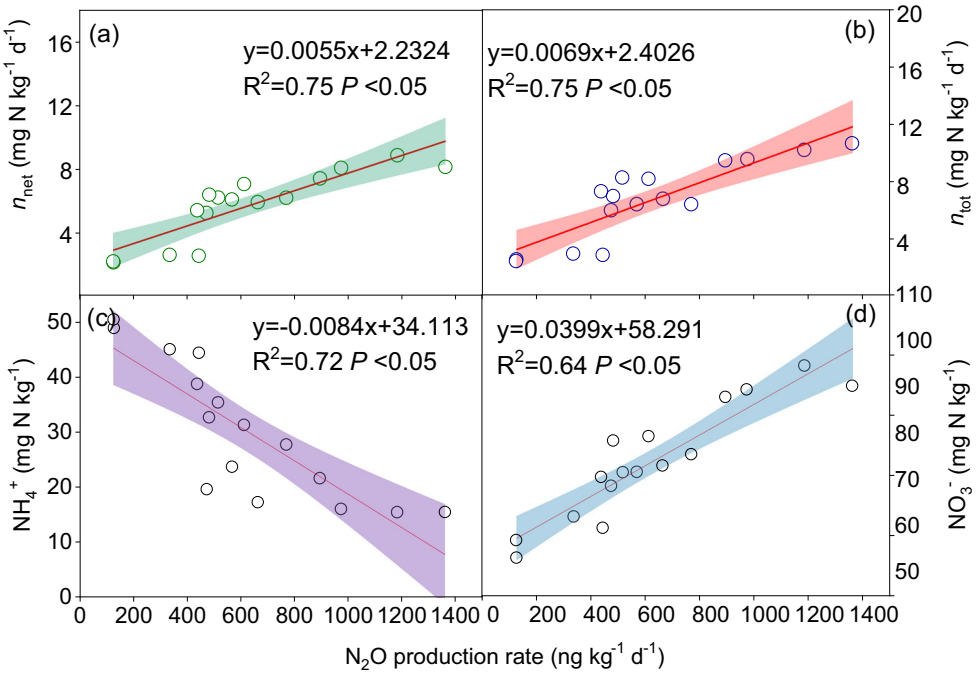

Fig. 5 Relationships between average N_2O production rate and **a** n_{net}, **b** n_{tot}, **c** NH_4^+, and **d** NO_3^-–N. Dashed curves correspond to 95% confidence interval for linear regression

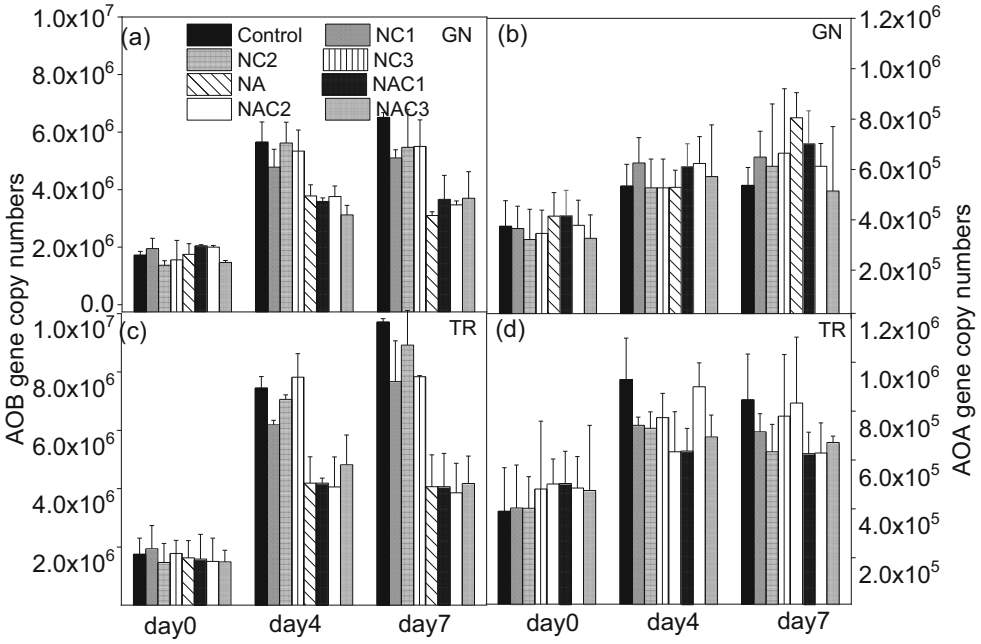

Fig. 6 Changes in AOA and AOB *amo*A gene copy numbers in GN and TR soils after 7 days of incubation. Error bars indicate the standard errors of four replicates

and which possibly by providing sufficient C for the growth of heterotrophic bacteria, fungi, and archaea (Yokoyama et al. 1992; Zhu et al. 2015). Zhang et al. (2014) concluded that substrate was more important than pH in controlling heterotrophic nitrification in acidic forest soils in Eastern China. Liu et al. (2015) reported that NO_3^- production was mainly heterotrophic in an acidic dairy soil with high organic content in Australia. The pH of the two test soils were below 6.0, and the organic matter content was as high as 7.9% and 10% in TR and GN soils, respectively (Table 1), which were suitable for the growth of heterotrophic bacteria and fungi (Zhang et al. 2014, 2015). However, even when soil contained large amounts of organic C, if mostly were complex polymers (Poeplau and Don 2013), such as lignin, cellulose, and humic acids, then organic C is not easily available for microorganisms (Fontaine et al. 2003). Therefore, the availability and quality of C often limit the growth and activities of heterotrophic microorganisms. Hence, in this study, the addition of glucose C might facilitate the growth of heterotrophic microorganisms and thus accelerate heterotrophic nitrification.

While nitrification by heterotrophic organisms has been known for some considerable time, the lack of suitable marker genes has resulted in much less attention into their contribution in natural systems in comparison to autotrophic organisms (Li et al. 2018).

The heterotrophic nitrifiers were not investigate in the current study, therefore, further targeted studies are required to elucidate the unknown mechanisms by which glucose C applications control soil heterotrophic nitrifiers.

Our results showed that the accelerating effect of glucose on heterotrophic nitrification was greater than its inhibition effect on autotrophic nitrification, thereby increasing the total nitrification, which further resulted in higher NO_3^- accumulation in samples with glucose treatment than those without. However, Zhao et al. (2018) revealed that crop residues reduced net nitrification rates by depressing the rates of gross autotrophic nitrification and stimulating the rates of NO_3^- immobilization in purple soil. Emeterio et al. (2014) observed a suppression of nitrification potential with the addition of *Lolium* extract and with phenolics added in combination with glucose. Cheng et al. (2012) found that incorporation of wheat straw suppressed the rates of gross nitrification by 32.2%; conversely, the addition of a biochar produced from wheat straw had no influence in nitrification. Consistently, Yao et al. (2011) reported that the addition of charcoal had no significant effect on net nitrification, but charcoal significantly increased soil basal respiration and altered C substrate utilization in the two Scottish soils. The discrepancies in the results among the studies could be attributed to different qualities, application rates, and timing of organic substrates, as well as various soil types and different levels of soil indigenous N.

4.2 Effects of glucose C N_2O emission

In the present study, we observed an enhanced N_2O production by glucose addition. Several previous studies reveal that soil N_2O production rate is stimulated by the application of readily degradable C, possibly due to increasing denitrification (Wang et al. 2005; Cheng et al. 2012; Ameloot et al. 2016; Mehnaz et al. 2018). The availability of C might support denitrification and N_2O emission, whereas C addition may depress denitrification by reducing the conversion of NH_4^+ to NO_3^-, thereby reducing the amount of substrate for

denitrification. Moreover, high availability of C can promote the reduction of N_2O to dinitrogen, because C availability often limits the final reductive stage of denitrification (Morley and Baggs 2010; Loick 2016). In this study, the soils with water content of 60% WFPS were incubated at 25 °C, which was favorable for nitrification but not for denitrification. We observed that the N_2O production was positively correlated with average NO_3^- concentration, gross nitrification, and net nitrification but was negatively correlated with the average NH_4^+ concentration (Fig. 6). In our previous study, we differentiated the contribution of autotrophic nitrification, heterotrophic nitrification, and denitrification to N_2O production in soils from the same pastures under the same incubation conditions; we found that denitrification accounting for approximately 30% of soil N_2O production and heterotrophic nitrification accounting for 20–30% of N_2O emissions in the same pasture soils played roles similar to those of autotrophic nitrification in N_2O emission (Lan et al. 2018). In the present work, we could not disregard that glucose C addition increased N_2O emission through denitrification because increased NO_3^- consumption was observed (Table 2). However, the possibility that the enhanced N_2O production was through heterotrophic nitrification could not be disregarded in the above correlation analysis. Heterotrophic nitrifiers are known to produce N_2O, and alternative processes can explain the large contribution of organic N as a source for N_2O production (Papen et al. 1989; Baggs 2011; Prosser and Nicol 2012). Therefore, the addition of exogenous organic C addition stimulated N_2O emission probably through NO_3^- denitrification and heterotrophic nitrification, especially when applying wide C/N ratio organic matters in soils with low pH and high organic C content.

5 Conclusions

We observed in the incubation study that the application of mixed C and N substrates with C/N ratio > 25 promoted heterotrophic nitrification but inhibited autotrophic nitrification. The effects on heterotrophic nitrification were greater than on autotrophic nitrification, thereby resulting in higher n_{tot} and NO_3^- accumulation compared with the control treatment. The mechanism of glucose inhibition of autotrophic nitrification might be caused by the increase of the microbial immobilization of NH_4^+ and not influencing the gene copy numbers of AOA and AOB. Our results indicated that the addition of exogenous organic C stimulated N_2O emission might through heterotrophic nitrification and through NO_3^- denitrification, especially when applying organic matters with wide C/N ratio in soils with low pH and high organic C content. Given the limited number of soil samples/sites and the specific conditions applied, in-depth studies are needed to confirm our results. Real environmental conditions should be considered in estimating the effect of exogenous organic C on overall N transformation processes and N_2O pathways. Additional soil types should be selected to establish the underlying mechanisms and factors that influence the N transformation and N_2O production in soil after organic C application.

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