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RESEARCH ARTICLE

Effects of different fertilization practices on anammox activity, abundance, and community compositions in a paddy soil

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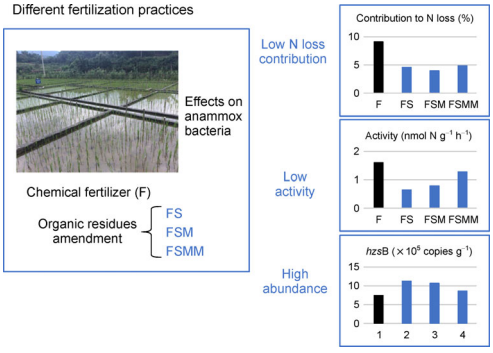
HIGHLIGHTS

- Anammox responded to different fertilization practices.
- Organic residues treated soils contributed lower (4.07%–4.95%) N loss than solely chemical fertilizer (9.13%) in terms of anammox.
- Incorporation of organic residues increased the abundance of anammox bacteria but decreased the activity.
- The anammox activity was not related to functional gene abundance and soil physicochemical properties.

GRAPHICAL ABSTRACT



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ABSTRACT

The return of crop residue and green manure into agricultural soil is known to be important agricultural management strategies, yet how they affect the anammox process remains poorly characterized. A field experiment containing four treatments: chemical fertilizer (F), F plus rice straw (FS), FS plus green manure (FSM), FSM with integrated management (FSMM), was performed to examine the effects of incorporation of rice straw and green manure residues on anammox. The results showed that the anammox activities in FS and FSM treatments (0.65 and 0.80 nmol N g^{-1} soil h^{-1}, respectively) were significantly lower than those in F and FSMM treatments (1.60 and 1.28 nmol N g^{-1} soil h^{-1}, respectively). Anammox contributed 4.07%–4.95% of total N loss in soil incorporated with residues, lower than soil treated with chemical fertilizer only (9.13%), the remaining being due to denitrification. However, the abundance of the *hzs*B gene (the hydrazine synthase β-subunit gene) in FS and FSM treatments (1.13×10^6 and 1.18×10^6 copies g^{-1} soil) were significantly higher than soil using chemical fertilizer only (7.49×10^5 copies g^{-1} soil) while showed no significant difference with FSMM treatment (8.81×10^5 copies g^{-1} soil). Illumina sequencing indicated that *Brocadia* was the dominant anammox genus, following by *Scalindua* and *Kuenenia*. Anammox bacterial diversity was altered after 4-year incorporation of rice straw and green manure, as shown by α-diversity indices. We concluded that rice straw and green manure incorporated with mineral fertilizer reduce N removal from paddy soil in terms of anammox in spite of stimulating anammox bacterial growth.

1 Introduction

A high amount of chemical fertilizer application in rice cultivation has extensively promoted the development of rice

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production but also causes a series of problems such as soil acidification, risk of pollution, and nutrient imbalance (Guo et al., 2010; Liu et al., 2013; Peñuelas et al., 2013; Zhang et al., 2014). China consumed 33% of world N fertilizer (FAO 2014, available at www.fao.org/publications/sofa), and the N use efficiency in the paddy field was 39%, lower than the global average level (Yu and Shi, 2015). Hence, a significant challenge for the sustainable production of rice is the development of alternative fertilizers. In recent years, the return of crop residues and appropriate use of inorganic fertilization to the field are operative pathways for agricultural conservation strategies of rice (Bijay et al., 2008; Yang et al., 2019; Zhou et al., 2020a; Song et al., 2021). While many studies have involved the effects of crop residue return and alternative fertilization on N efficiency or soil microbial structure (Eagle et al., 2000; Spedding et al., 2004; Simmons and Coleman, 2008), studies with regard to the response of anammox bacteria on different fertilization practices in intensively fertilized paddy soil were limited.

The microbially mediated N loss in terrestrial ecosystems is more complex since new processes, such as Sulfammox, Feammox, and NOM-anammox were discovered (Rios-Del Toro and Cervantes, 2019). The anaerobic ammonium oxidation (anammox) is a relatively newly discovered N removal pathway which concerns ammonium oxidation coupled with nitrate reduction and the production of N_2 under anaerobic condition (Thamdrup and Dalsgaard, 2002). Anammox is a promising new process of filling the gaps of N loss that cannot be clarified by ammonia volatilization, nitrous oxide emission, runoff, or leaching (Zhu, 2008). Anammox process was driven by bacteria affiliated to the phylum of *Planctomycetes* (Jetten et al., 2010). The hydrazine synthase β-subunit (*hzs*B) gene was commonly used in the quantitative analysis for the abundance of anammox bacteria (Wang et al., 2012b; Yang et al., 2015). Anammox bacteria prefer stable environments with minimum oxygen conditions (Dalsgaard et al., 2012; Shen et al., 2017), which is characterized by the flooded paddy field. Moreover, exogenous N inputs may stimulate anammox growth and result in subsequent more N loss (Nie et al., 2019). Studies have proposed that anammox bacteria was widely distributed and caused 5%–10% N loss in paddy soils (Zhu et al., 2011; Nie et al., 2015; Yang et al., 2015; Nie et al., 2019). Yet the response of anammox to the return of organic residues is not clear at the moment.

In agroecosystems, various organic materials can apply into paddy fields to increase rice yield (Zhou et al., 2020b). It was recognized that rice residue has adverse effects on rice yield because the rice straw application usually is liable to cause N immobilization (Azam et al., 1991; Verma and Bhagat, 1992; Eagle et al., 2000). Rice straw was characterized by a high C/N ratio and consequently slow release of N. Therefore, rice straw was returned to the field before seeding for decomposing, with a combination of reduced mineral fertilizer to mitigate yield depression (Azam et al., 1991; Adachi et al., 1997). In addition, milk vetch (*Astragalus sinicus* L.), which characterized by high N but low C/N ratio decomposes in the short-term (Zhou et al., 2019), is common green manure incorporated into flooded paddy soil in many two successive rice planting regions (Zhu et al., 2014). Nowadays, the co-incorporation of rice straw and green manure together to paddy fields is a standard practice in rice production (Zhou et al., 2019; Erinle and Marschner, 2020; Zhou et al., 2020a).

The objectives of this study were to investigate the response of anammox on the return of rice straw, incorporation of rice straw, and green manure together, respectively, with a combination of chemical fertilizer. A paddy soil from southern China treated with different practices for 4-year was chosen to examine their differences in the activity, contribution, and role of anammox to N loss. To reveal the effects of improved management practice on anammox, a modification treatment (FSMM) was also performed. Accordingly, a ^{15}N-tracing incubation was operated to assess the rates of anammox, and the q-PCR targeting anammox functional gene was performed to evaluate cell numbers of anammox bacteria. Illumina sequencing analysis was carried out to estimate the community composition and diversity of anammox bacteria.

2 Materials and methods

2.1 Experiment design

A 4-year experimental station which was located in Huilongpu town, Changsha City (112°18′45″E, 28°07′25″N), affiliated to Hunan Soil and Fertilizer Institute, was designed for this study. The soil was a paddy soil developed from river sediments. Before the trial, the main chemical properties of the paddy soil (0–20 cm) were: 6.36±0.38 of pH (soil-water ratio 1:2.5, w/v), 21.60±1.52 g kg^{-1} of total organic carbon; 178.83± 21.68 mg kg^{-1} of alkali-hydrolysable N, 24.67±2.86 mg kg^{-1} of available P, and 94.83±12.24 mg kg^{-1} of available K. A double rice-crop system was conducted with early-season cultivated *Zhongzao 25* and late-season planted *Hyou 518*, respectively. Four treatments were tested: chemical fertilizer (F), F plus rice straw return (FS), FS plus green manure (FSM), and FSM with integrated management (FSMM). For each treatment, the experimental plots were 20 m^2 (4 × 5, m) with three replicates and separated by a ridge of cement fields. The experimental plots were set up with a randomized design (Figure S1). In the F treatment, rice straw was removed, and chemical fertilizer containing 135 kg N ha^{-1} yr^{-1}, 11.01 kg P ha^{-1} yr^{-1}, and 28.00 kg K ha^{-1} yr^{-1} were applied. In residues return plots, reduced fertilizer (114.5 kg N ha^{-1} yr^{-1}, 11.01 kg P ha^{-1} yr^{-1}, and 28.00 kg K ha^{-1} yr^{-1}) was also invested into the fields.

Rice straw (4500 kg ha^{-1}, N 20.55 kg ha^{-1}, P_2O_5 17.7 kg ha^{-1}, K_2O 94.95 kg ha^{-1}, C/N ratio 39.8, respectively, averaged values from multi-year) generated from each plot was chopped into small pieces (3–5 cm in length) then mixed thoroughly prior to returning to corresponding plots before

Table 1 The fertilizer or organic residues for converting amount of N applied into the paddy fields.

Treatment	Fertilizer or organic residue specie(s)	Amount of pure N (kg ha^{-1} yr^{-1})	Total amount of pure N (kg ha^{-1} yr^{-1})
F	Chemical fertilizer	135.00	135.0
FS	Chemical fertilizer	114.45	135.0
	Straw	20.55	
FSM	Chemical fertilizer	114.45	189.0
	Straw	20.55	
	Green manure	54.00	
FSMM	Chemical fertilizer	114.45	189.0
	Straw	20.55	
	Green manure	54.00	

Chemical fertilizer was applied as urea, super phosphate and potassium chloride. Straw was incorporated with rice straw, and green manure was invested with milk vetch, respectively. Decomposition inoculant was added and rotation tillage was conducted in FSMM treatments.

plowing. Chopped green manure (4500 kg ha^{-1} as milk vetch, N 54 kg ha^{-1}, P_2O_5 32.1 kg ha^{-1}, K_2O 81.3 kg ha^{-1}, C/N ratio 13.6, respectively, averaged values from multi-year) was incorporated into the corresponding plots in April before early rice planting (Table 1). In all treatments, 50% urea + 100% superphosphate + 50% potassium chloride was applied as base fertilizer, and the remaining chemical fertilizers were applied as tillering fertilizer of rice growth. An efficient organic matter decomposing inoculant (GB20287-2006) that is able to accelerate the decomposing of organic residues was composed with rice straw and milk vetch in FSMM treatment. Conventional tillage (0–15 cm depth of plowing) was conducted in F, FS, and FSM treatments, while rotation tillage (0–10 cm depth of plowing) was performed in the FSMM treatment. Shallow flooded water was maintained until the late tillering stage of rice growth then drained.

2.2 Soil sampling

Soil samples were collected prior to the early-season rice harvest season in August 2019. Composed soil was mixed thoroughly from five soil cores (0–20 cm) using a cylindrical stainless-steel core sampler from each plot. Mixed soils were placed in two sterilized plastic bags separately as subsamples. The bags were squeezed quickly to remove the remaining air and form an anaerobic environment. One subsample was sealed in a dry ice box for molecular analysis, and another subsample was placed in a box with cracked ice. Both subsamples were quickly brought to the laboratory. A small fraction of soil from another subsample was used for the ^{15}N-isotope tracing incubation experiment, while some fresh soil was used for the determination of moisture, ammonium (NH_4^+), combined nitrate-nitrite (NO_x^-), and alkali-hydrolysable N (AN). The remaining soil was air-dried and sieved (< 2 mm) for the detection of pH, total organic carbon (TOC), total N, active organic carbon (AOC), CEC (cation exchange capacity), and salinity (electricity conductivity). Core soils with steel ring-knifes were sampled for bulk density determination.

2.3 Physicochemical measurements

Soil moisture was measured by oven dry the soil to a constant weight. Soil pH was analyzed at a ratio of 1:2.5 (soil:water). TOC and AOC were determined by the oxidation of $K_2Cr_2O_7$ and $KMnO_4$, respectively. Total N was assayed by a Kjeldahl autoanalyzer (K9860, Hanon, China). Soil ammonium and nitrate were extracted with a KCl solution (2M) at a ratio of 1:5 (soil:KCl) then determined by an auto-analyzer (Technicon Industrial Systems, NY, USA). Salinity was analyzed by a conductivity meter (DDS-11C, INESA, China) in a suspension of 1:5 (soil:water). Available P, CEC, and Fe^{2+} were quantified spectrophotometrically after extraction. Available K was measured by a flame photometer (FP6410, INESA, China) after extracted from NH_4OAc (1M). The detection method of soil bulk density was followed by Lu (2000).

2.4 ^{15}N isotope-tracing technique

Potential anammox and denitrification activity were evaluated simultaneously with an incubation experiment using different ^{15}N-isotope substrates as described by Thamdrup and Dalsgaard (2002) and revised by Hou et al. (2020). According to the principle of N production, anammox and denitrification activity can be distinguished by adding different ^{15}N-amended substrates: (1) $^{15}NH_4Cl$; (2) $^{15}NH_4NO_3$; and (3) $Na^{15}NO_3$, respectively. Briefly, about 3.5 g of fresh soil was transferred to glass vials (20 mL) and saturated with helium-purged deionized water. The soil slurries were pre-incubated for over 24 h to deplete residual NO_3^-, NO_3^- and O_2. Thereafter, all vials were flushed with $^{28}N_2$ and injected with (1) $^{15}NH_4Cl$ at 99.09% (negative control); (2) $^{15}NH_4NO_3$ at 99.09% (positive control); and (3) $Na^{15}NO_3$ at 99.21%, respectively, to a final concentration of 100 μM of inorganic N. All vials were placed in a constant-temperature incubator (Queue Systems,

WV, USA) at 25°C. The reactions were suspended at the defined time point of 0, 3, 6, 12, and 24 h by adding 200 µL of $ZnCl_2$ (7M) solution. Before N_2 determination, the vials were shaken to equilibrate the gas. The content of headspace ^{28}N, $^{29}N_2$, and $^{30}N_2$ was measured by a multi-flow isotope ratio mass spectrometer system (IRMS, Thermo Finnigan Delta V Advantage, Bremen, Germany) coupled with GasBench II. Based on the high precision of IRMS performance, the minimum detectable rate was calculated about 0.001 nmol N g^{-1} h^{-1}. The measurements were completed at the Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences. Linear regression was conducted between the total signal and the amount of produced N_2. According to the raw signal and ratio of $^{28/29/30}N_2$ analyzed by IRMS, the concentration of produced $^{28/29/30}N_2$ both in references and samples could be calculated respectively. The multiple correlation coefficient (R^2) for linear regression of $^{28/29/30}N_2$ concentration was established with R>0.90. The activities of anammox and denitrification and their contributions to N loss were calculated from linear regressions of the fractions of $^{29}N_2$ and $^{30}N_2$ treated with $Na^{15}NO_3$, as described by our previous study (Hou et al., 2020). The equations for calculation and their explanations were shown in Table S1.

2.5 DNA extraction and qPCR

Total soil microbial DNA was extracted from 0.50 g of fresh weight using a FASTDNA SPIN Kit (MP Biomedicals, CA, USA) following the manufacturer's protocols. Extracted DNA was purified with agarose gel (1%). The q-PCR was conducted to assess the cell numbers of total bacteria and anammox bacteria. The total bacterial abundance was evaluated by 16S rRNA gene with primers of 341F and 517R (Roesti et al., 2006). The abundance of the *hzs*B gene was examined with the primers of 396F and 742R (Wang et al., 2012a). The reaction mixture and reaction profiles used for amplification for the target gene were shown in Table 2. Three independent q-PCR assays and three non-template controls were achieved using a qPCR ABI 7500 fast qPCR system. The standard curve was derived from 10-fold serial dilutions of plasmid DNA targeted total bacterial 16S rRNA gene and anammox *hzs*B gene. Meanwhile, the PCR efficiency corresponded from 90% to 110%, and the correlation coefficient value was 0.99. All the runs were accomplished with a melt curve analysis to confirm the specificity of amplification.

2.6 Illumina sequencing

The anammox bacterial 16S rRNA gene was amplified with Amx368F-Amx820R (Schmid et al., 2000) by thermocycler PCR system ABI GeneAmp 9700 for multiplex sequencing. The PCR reactions contained 4 µL 5 × FastPfu buffer, 2 µL dNTPs (2.5 mM), 0.8 µL forward and reverse primers, 0.4 µL FastPfu polymerase, 0.2 BSA, 10 ng DNA and ddH_2O to a final of 20 µL dilutions, were amplificated with 95°C for 3 min, followed by 35 cycles of 30 s at 95°C, 30 s at 58°C, 45 s at 72°C, and 10 min at 72°C. The PCR products were sequenced and preceded on an Illumina MiSeq platform (Majorbio Bio-Pharm Technology Co. Ltd., Shanghai, China).

2.7 Statistical analysis

The original data were processed with Microsoft Excel 2016, and the data were shown as mean with standard error (S.E.). The standard errors were based on the values of three independent replicates. After their normality and homogeneity were assessed, one-way ANOVA (Duncan or Dunnett's T3 test, $p < 0.05$) was conducted to evaluate the differences between treatments. The statistical and correlation analysis was conducted using SPSS 19.0. The figures were created with Sigmaplot 14.0.

3 Results

3.1 Variations of soil properties

After 4 years of different adding approaches of fertilization, the main soil physical and chemical properties were altered significantly (Table 3). Overall, the values of detected chemical properties in soil incorporated with organic materials were higher compared to soil treated with solely chemical fertilizer soil. Soil pH in FSM treatment was significantly higher than F treatment while showed no significant difference with soil treated with FS and FSMM ($p < 0.05$). The content of TOC, AOC, and AN were increased in chemical fertilizer plus organic materials treatments and ranked as FSMM >FSM>FS>F. The TN content in F treatment was significantly lower compared to organic materials plus chemical fertilizer treatments ($p < 0.05$), while no significant difference was

Table 2 Primers, reaction mixture and reaction profiles used for real-time qPCR in this study.

Target gene	Primers	Reaction mixture	Thermal profiles	Reference
Bacterial 16S rRNA	341F 517R	12.5 µL 2× *Taq* Master Mix, 0.5 µL forward and reverse primers, 2.5 µL DNA template, 9 µL ddH_2O	95°C for 30 s, 40 cycles of 95°C for 15 s, 56°C for 34 s, 68°C for 30s	Roesti et al., 2006
*hzs*B	396F 742R	25 µL 2× *Taq* Master Mix, 1 µL forward and reverse primers, 1 µL DNA template, 22 µL ddH_2O	94°C for 5 min, 30 cycles of 94°C at 30 s, 55°C for 30 s, 72°C for 30 s, and 72°C for 10 min	Wang et al., 2012

Table 3 Effects of different treatments on soil properties ($n = 3$).

Treatment	F	FS	FSM	FSMM
pH	6.50±0.19 b	6.57±0.08 ab	6.63±0.18 a	6.57±0.16 ab
Bulk density	1.12±0.09 a	1.12±0.10 a	1.10±0.09 b	1.08±0.09 b
TOC (g kg^{-1})	24.70±1.86 b	25.13±2.94 b	25.90±2.01 ab	26.33±2.39 a
TN (g kg^{-1})	2.54±0.18 b	2.59±0.16 ab	2.66±0.19 a	2.67±0.22 a
AOC (g kg^{-1})	13.37±1.66 b	14.33±1.72 b	14.83±1.82 a	15.40±1.97 a
AN (mg kg^{-1})	166.83±28.08 c	188.07±25.64 b	191.80±23.99 b	208.40±17.98 a
NH_4^+-N (mg kg^{-1})	13.07±0.04 c	15.33±0.07 c	23.35±1.75 b	36.40±1.20 a
NO_x^--N (mg kg^{-1})	0.83±0.04 c	0.90±0.07 c	1.65±0.05 b	2.95±0.15 a
CEC (cmol kg^{-1})	16.73±1.49 b	18.87±1.52 ab	19.37±1.45 a	20.48±1.90 a
Salinity (μS cm^{-1})	219.67±10.30 b	232.67±25.87 a	237.00±12.92 a	239.67±27.29 a
Fe^{2+} (mg kg^{-1})	25.83±2.99 b	27.67±3.56 a	28.40±3.25 a	28.90±3.13 a

TOC, total organic carbon; TN, total nitrogen; AOC, active organic carbon; AN, Alkali-hydrolysable N; CEC, cation exchange capacity. Different letters in the same row indicate significant difference between treatments ($p < 0.05$)

observed in FS, FSM, and FSMM soils ($p>0.05$). The concentrations of ammonium (NH_4^+) and combined nitrate-nitrite (NO_x^-) ranked as FSMM>FSM>FS, while no significant difference was measured between FS and F treatments. No significant difference in salinity and ferrous concentration was observed between organic materials plus chemical fertilizer treatments but significantly higher than solely chemical fertilizer treatment ($p < 0.05$). Soil bulk density in FSM and FSMM treatments was significantly lower than F and FS treatments ($p < 0.05$).

3.2 Activities of anammox and denitrification

The activity of anammox and denitrification was assessed by the detection of N produced from the isotope-tracing incubations, and the results were shown in Fig. 1. In this study, soil samples were incubated with ammonium (NH_4^+) or nitrate (NO_3^-) to a final concentration corresponding to a maximum of lower than 10% of the *in situ* concentration. Moreover, $^{30}N_2$ was undetectable in the slurries amended with $^{15}NH_4^+$ and $^{15}NH_4^+ + {}^{14}NO_3^-$ (Figure S2), suggesting that other processes involved in N loss, like Feammox, did not occur significantly. Hence, the determined activity of anammox and denitrification might not be seriously overestimated. Overall, the total N loss in different fertilization paddy soil produced by denitrification and anammox could be ranked as FSMM>FSM>F>FS. The denitrification activities were much higher than anammox activities in all treatments. In soil treated with FSMM, denitrification activity was significantly higher than those in the F, FS, and FSM treatments ($p < 0.05$). The anammox activities in FS (0.65 nmol N g^{-1} h^{-1}) and FSM (0.80 nmol N g^{-1} h^{-1}) treatments were lower than the values in F (1.60 nmol N g^{-1} h^{-1}) and FSMM soils (1.28 nmol N g^{-1} h^{-1}). The anammox contributed 4.07%–4.95% of total N removal in soil incorporated with organic residues, while 9.13% of N loss could be attributed to anammox in soil treated with chemical fertilizer only.

3.3 Abundance of anammox bacteria and total bacteria

The qPCR assays were performed to estimate the gene copies of total bacteria and anammox bacteria (Fig. 2), respectively. The copy numbers of total bacteria in soil treated with FSMM (1.58×10^8 copies g^{-1}) were significantly higher

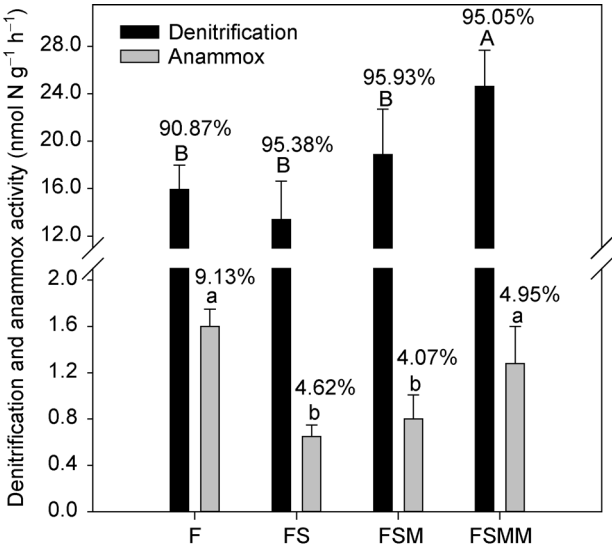

Fig. 1 The anammox and denitrification activity (bar graph) and their contributions (presented above the columns) to total N production by incubation experiment in paddy soil with different fertilization practices in this study. Letters with different labels indicate significant differences between treatments ($p < 0.05$).

than that in FSM treatment ($p < 0.05$). In F and FS treatments, total bacterial numbers showed no significant difference but lower than those in FSM and FSMM soil. However, the anammox bacterial cell numbers showed a different tendency compared with total bacteria. The copy numbers of *hzs*B gene in FS and FSM treatments were 1.18×10^6 and 1.13×10^6 copies g^{-1} soil, respectively, and these values showed no significant difference with FSMM treatment (8.81×10^5 copies g^{-1}) but were significantly higher than those in the F treatment soil (7.49×10^5 copies g^{-1}).

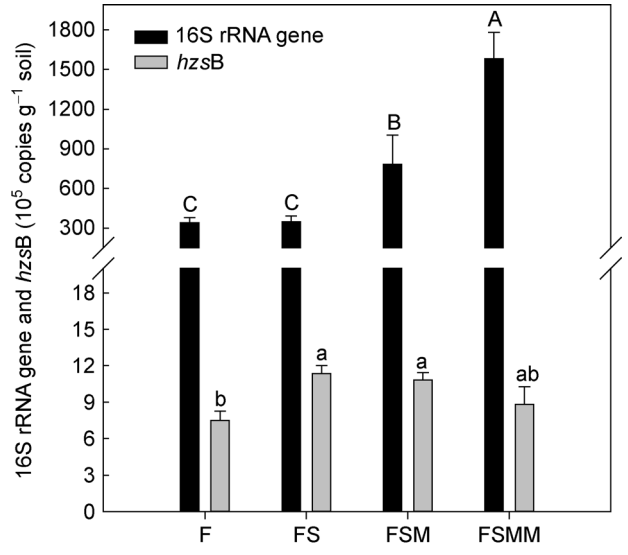

Fig. 2 The abundance of anammox bacteria targeting the *hzs*B gene and total bacteria targeting the 16S rRNA gene in paddy soil under different fertilization practices. Letters with different labels indicate significant differences between treatments ($p < 0.05$).

3.4 Community composition of anammox bacteria

The DNA extracted from four treatments was subjected to Illumina sequencing. The recovered reads that could be aligned with the GenBank database to Planctomycetes genus were affiliated to anammox bacteria. A total of 11 565 reads and 297 effective anammox bacterial sequences were retrieved from the soil (97% similarity). Overall, the community composition of anammox bacteria was similar between different fertilization treatments. Three different genera of anammox bacteria were retrieved, including *Brocadia*, *Scalindua*, *Kuenenia*, the other affiliating to unclassified Planctomycetaceas (Fig. 3). Among the recovered Planctomycetes, *Brocadia* was the most abundant genus, accounting for 75.0%–82.7% of the retrieved sequences. A relatively low abundance of *Scalindua* was detected in all treatments (10.1%–16.1%). Very low fractions of *Kuenenia* (1.1%–5.3%) were identified in four treatments, and the percentage followed the order of F>FS>FSM>FSMM. The percentage of anammox-like bacteria (unclassified) also differed in four treatments.

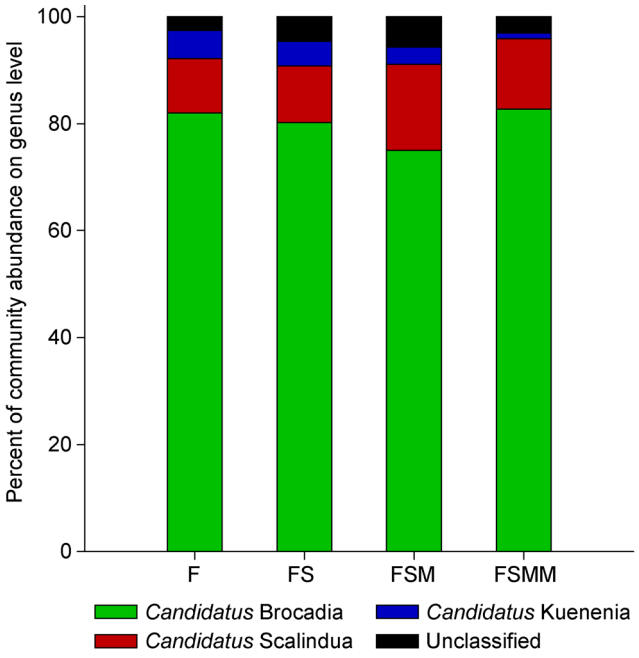

Fig. 3 The community compositions and their relative abundance of anammox bacteria on genus level in paddy soil with different fertilization practices.

The α-diversity was performed to assess the diversity of anammox bacteria between different fertilized paddy soils (Table 4). The Shannon index showed higher values in the FS and FSM soils than in the F and FSMM soils ($p < 0.05$). The Simpson index, which estimates dominant OTUs was significantly higher in FSMM soil than other treatments ($p < 0.05$). Higher values of Chao and ACE indices the FS, FSM, and FSMM soil compared to F treatment soil, indicating the incorporation of organic residues increases anammox bacterial diversity.

4 Discussion

The present study implied that the activity, abundance, community composition, and diversity of anammox bacteria were altered by the combined application of organic amendments and mineral fertilizers. Integrated management combined with organic residues incorporation evidently improved soil properties at the expense of subsequent more N loss produced by denitrification. The N loss produced by anammox was lower in FS and FSM treatments compared to F soils, suggesting the application of crop residues plays an important role in N removal in intensive fertilized paddy soil. These results can provide new information for the understanding of N loss from agricultural soil, which is strongly influenced by management strategies such as different fertilizer regimes.

In the studied paddy soil, anammox contributed approximately 4%–9% of total N loss, which is consistent with what already reported (Yang et al., 2015; Zhu et al., 2015; Nie et al.,

Table 4 Diversity indices of anammox bacteria in the soil sample ($n = 3$).

Treatments	Shannon	Simpson	Chao	ACE
F	3.16±0.45 b	0.09±0.03 b	206.38±4.09 b	216.09±0.95 b
FS	3.73±0.21 a	0.06±0.01 b	243.95±9.21 a	244.72±11.06 a
FSM	3.85±0.11 a	0.05±0.01 b	250.04±15.84 a	254.24±19.68 a
FSMM	3.11±0.54 b	0.19±0.04 a	253.62±17.90 a	243.22±6.71 a

Different letters between individual index indicate significant difference between treatments ($p < 0.05$).

2019). The anammox activity (0.65 to 1.28 nmol N g^{-1} h^{-1}) was comparable to those of a typical paddy soil (0.02–0.77 nmol N g^{-1} h^{-1}) (Bai et al., 2015b), a yellow-gray paddy soil (1.7–2.4 nmol N g^{-1} h^{-1}) (Nie et al., 2018a) and a rice-wheat rotation soil (0.68–2.08 nmol N g^{-1} h^{-1}) (Gu et al., 2017). The anammox activity in FS and FSM treated soils was significantly lower than in F treatment (Fig. 1), indicating that the incorporation of rice straw and green manure decreased anammox activity. Low anammox activity in FS treatments could be explained by the application of rice straw with a high C/N ratio initially, which results in slow N release due to low mineralization (Li et al., 2015; Xu et al., 2021). Moreover, the combined application of mineral and organic fertilizer in soil may have different impacts on soil N dynamics (Zhang et al., 2012). In F and FS treatments, the same total amount of N was applied into the paddy field (Table 1). However, the applying of straw residue may inhibit the ammonium release from the inherent N pool (Eagle et al., 2000; Pan et al., 2017). This was also verified by the lower denitrification activity in FS soil (Fig. 1). The total N loss produced by denitrification and anammox in FSM is higher than in F and FS soils, which could be caused by a higher total amount of N application. Higher denitrification but lower anammox activity in FSM soil indicated that denitrifiers and anammox bacteria respond differently to the incorporation of rice straw and green manure with mineral fertilizer. In addition, the anammox activity in FSMM treatment was significantly higher than in FSM soil (Fig. 1), suggesting rotation tillage has a positive effect on anammox bacterial function. Furthermore, the decomposing inoculant addition in FSMM treatments can accelerate N release rapidly. In paddy ecosystems, the anammox bacterial function may be more sensitive to rapidly released fertilizer. The concentrations of exchangeable ammonium (NH_4^+), and combined nitrate-nitrite (NO_x^-) (Table 3), which were the potential substrates for anammox, were significantly higher in FSM and FSMM soils compared with F and FS soils, suggesting that anammox activity was not correlated with substrate concentrations, consistent with previous studies (Shen et al., 2014; Zhu et al., 2015). Due to different management measures, such as different fertilization or tillage patterns, the anammox has much heterogeneity.

The anammox bacterial abundance (7.49×10^5 to 1.18×10^6 copies g^{-1} soil) was similar to those examined in some other paddy soils (Wang et al., 2012b; Zhu et al., 2011; Shen et al., 2014), indicating that anammox bacteria can adapt to the paddy soil fertilized with different practices. The incorporation of rice straw and green manure in paddy soil increased the abundance and α-diversity of anammox bacteria (Fig. 2, Table 4). The abundance of anammox bacteria was significantly higher in FS and FSM soils than that in F soil (Fig. 2), indicating the incorporation of rice straw and green manure increases anammox bacterial growth. The combined application of organic and mineral fertilizers has a positive effect on soil microbial because soil properties, e.g., organic matter, bulk density, enzyme activities, were improved, while the investment of only mineral fertilizer resulted in decreasing in microbial diversity (Shen et al., 2010; Voisin et al., 2014; Nie et al., 2018b; Zhou et al., 2020a). In the present study, the incorporation of rice straw and green manure improved soil properties (Table 3) that affect microbial communities positively, consistent with what already reported (Li et al., 2019; Zhou et al., 2020a). Moreover, the abundance of total bacteria cells was also higher in FSM and FSMM treatments than in F and FS soils (Fig. 2). It was reported that inorganic fertilizer amended with straw activate biotic process (Pan et al., 2017). A recent study also indicated that the incorporation of green manure and rice straw promoted soil fertility and enhanced microbial biodiversity (Zhou et al., 2020a). In addition, no significant difference of anammox bacterial cells was observed between FSM and FSMM soil (Fig. 2), suggesting rotation tillage and decomposition inoculant have no measurable impact on anammox bacteria growth. Anammox bacteria grow very slow and the doubling time of cell growth is four weeks (Strous et al., 1998). In the studied paddy fields, the interval between tillage and sampling was very long thus may have little or no influence on the anammox growth.

The Pearson correlation analysis showed that the anammox activity was not related to *hzs*B gene abundance as well as soil physicochemical properties (Table S2). The poor correlation between anammox activity, *hzs*B gene copies, and environmental factors could be explained by diverse metabolism pathways of anammox bacteria, resulting in different metabolic rates (Gori et al., 2012; Wang et al., 2012a). Besides, the PCR amplification in this study was relied on DNA level rather than RNA, which may not accurately quantify the active anammox bacteria. Moreover, in paddy soil, some anammox bacteria may be dormant cells (Monballiu et al., 2013; Zhu et al., 2019) due to oxygen permeation from the surface layer (Bai et al., 2015b; Nie et al., 2019), thus low or no activity. The anammox activity in paddy soil depends on the

comprehensive effects of multiple environmental factors.

Illumina sequencing revealed the coexistence of *Brocadia*, *Scalindua*, *Kuenenia,* with *Brocadia* being the most abundant genus, followed by *Scalindua* and *Kuenenia* (Fig. 3). The *Brocadia* genus was the most commonly dominant anammox in many paddy soils (Wang et al., 2012b; Shen et al., 2014; Bai et al., 2015a;Yang et al., 2015; Nie et al., 2018a; Shen et al., 2020), suggesting this genus is likely to be well adapted in paddy soil. In addition, the genus of *Scalindua*, which could only be detected in some paddy soils, such as albic paddy soil (Wang and Gu, 2013), red paddy soils (Li et al., 2016), or a gray yellow paddy soil (Nie et al., 2018a), showed a percentage of 10.1%–16.1% (Fig. 3). These could be explained by the parent material characterized by high salinity (Table 3). A high abundance of *Scalindua* was also detected in marine (Schmid et al., 2007) and freshwater ecosystems, which usually have high salinity (Schubert et al., 2006; Yoshinaga et al., 2011). In this study, the *Scalindua* abundance was more or less correlated with the salinity (Table 3, Fig. 3). Moreover, the *Kuenenia* is sensitive to salinity (Dale et al., 2009), and a small proportion of *Kuenenia* (1.1%–5.3%) was observed in the paddy soil (Fig. 3). The *Scalindua* may be more adaptive anammox bacteria than *Kuenenia* in high salinity paddy soil. Pearson correlation results showed the relative abundance of *Kuenenia*, *Brocadia,* and *Scalindua* were poorly correlated with any soil physicochemical properties (Table S2). The vital driver responsible for shaping the distribution of anammox bacteria in paddy soil has yet to be further explored.

5 Conclusions

Taken together, the activity, abundance, and composition of anammox bacteria altered with different incorporation approaches of organic residues. The abundance of anammox functional gene was increased significantly by the incorporation of rice straw or green manure, whereas anammox bacterial activity was decreased significantly due to the low rate of N mineralization. Integrated management, such as decomposing inoculant and rotation tillage, increased activity of both anammox and denitrification, resulting in subsequent more N loss from paddy soil. The community composition of anammox bacteria was relatively stable under different fertilization practices, with *Brocadia*, *Scalindua*, and *Kuenenia* being identified by Illumina sequencing, while the relative abundance was altered. Future research may aim at exploring the effect of other fertilization management on the anammox process.

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Declaration of interests

The authors declare no known conflict of interest.

Author contributions

Authorship statement: San'an Nie and Hua Wang designed the work. Geng Sun and Mei Sun carried out the ^{15}N-isotope experiment, analyzed the data, and wrote the manuscript with support from all authors. Zunchang Luo, Chao Li, and Xiaoping Xiao were involved in planting and supervised the study. Xiaojing Li and Junjie Zhong participated in soil sampling and soil physicochemical analysis. All authors provided critical feedback and helped shape the research.

Electronic supplementary material

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