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Anammox co-fungi accompanying denitrifying bacteria are the thieves of the nitrogen cycle in paddy-wheat crop rotated soils

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ABSTRACT

Anammox bacteria are the key microbes after denitrifiers in the anaerobic environment. Nitrogen gap cannot be satisfied till date even with the advanced techniques, due to complex microbial network and different pathways. Recently, anaerobic fungi are the concerning point to investigate, which was previously ignored for a long time. Study was conducted with the aim of assessment of an individual and combined contribution of anammox, co-denitrification, and denitrification processes for N losses, under different organic-chemical fertilizers, i.e. 1) control _CK; 2) chemical fertilization _CF; 3) pig manure plus chemical fertilization _PMCF; and 4) straw returned plus chemical fertilization _SRCF). Hybrid techniques of ^{13}C-DNA-Stable isotope and ^{15}N isotopic tracer were used to discriminate the contribution of anammox-co-fungi using antibacterial and antifungal inhibitors. Results showed that fungi are the major culprit in N losses; the overall contribution rate by anammox-co-denitrification was 14.82–29.74%. While in case of individual N losses, fungi were dominating the N losses (3.51–25.60%, AB) than bacteria (7.50–21.80%, AF). The anammox and fungi have a positive correlation with each other's (r = 0.67), principal component analysis (PCA) and correlation analysis validate each other (anammox and fungi), and both showed the same type of attraction to the soil physicochemical properties. However, fungi did not show a significant relationship with NH_4^+-N (r = 0.38). A clone library of ^{13}C-DNA-SIP was constructed, and results showed that denitrifying fungi were very likely belonges to the genera *Agaricus*, *Aspergillus*, *Phycomyces*, *Saitoella*, and *Trichoderma*. Conclusively, we propose that fertilization pattern can change anammox activity and abundance, but fungal activity and community structure undergo changes with organic amendments rather than inorganic fertilizers.

1. Introduction

The nitrogen cycle is no more just a cycle, it's a nitrogen web because of many pathways of nitrogen losses, i.e., denitrification, anammox, and co-denitrification are considered major pathways, while leaching, volatilization, and immobilization are the minor factors (Zumft, 1997; Mulder et al., 1995; Laughlin and Stevens, 2002). The discovery of these pathways has added a new dimension to our understandings of the global N cycle (Amano et al., 2011). Denitrification was considered the leading nitrogen loss pathway comprising the conversion of NO_3^-, NO_2^-, N_2O, and finally N_2. Nitrogen cycle broadened with anammox discovery (Mulder et al., 1995) and anammox process is responsible for N loss, it is a less toxic to the environment due to the formation of N_2 through the reaction of NH_4^+ plus NO_2^- as substrate without the formation of intermediates gasses (NO_x). However, in addition to anammox, another phenomenon called co-denitrification also plays a vital role in the nitrogen cycle (Laughlin and Stevens, 2002; Long et al., 2013).

Denitrification and anammox pathways are largely defined till date, but co-denitrification is lacking, it is as important as others to minimize the N inputs and reduce environmental impact due to toxic gasses. Denitrification process in the soil occurs under anaerobic conditions, and this process performed by denitrifying bacteria, which reduce NO_3^- to N_2 (Zumft, 1997).

According to published literature, N losses due to anammox under long-term fertilization in paddy soils account for 4–37% of applied soil nitrogen (Rahman et al., 2018; Nie et al., 2018; Zhang et al., 2017; Yang et al., 2015). Anammox process carried out by reaction in which

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ammonium reduced to N_2 through hydrazine production, while nitrite being used as substrate under anaerobic condition; the specific process is shown as follow,

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \text{ (No intermediates i. e., } NO_x)$$

Co-denitrification is a process in which one labeled molecule of ^{15}N nitrite ($^{15}NO_2^-$) produced by reduction of labeled $^{15}NO_3^-$ by fungi, combined with a natural N source (i.e., ammonium or hydroxylamine), and this phenomenon called as co-denitrification (d_{CD}), which is totally different from anammox (Laughlin et al., 2010). According to some previous literature, co-denitrification is a complex process in which fungi reduce NO_2^- by utilizing the different types of nitrogen compound as a co-substrate, i.e., ammonium or azide (Tanimoto et al., 1992). Fungi have diverse adaptation mechanism for the respiration; it can use NO_3^- as an electron acceptor in an anaerobic environment (Zhou et al., 2002) and O_2 in an aerobic environment (Laughlin et al., 2010).

Till date, only a few studies explored the effect of co-denitrification to the contribution of N loss in soils (Tanimoto et al., 1992; Laughlin and Stevens, 2002; Spott et al., 2011; Long et al., 2013; Rex et al., 2016; Clough et al., 2017). Recent research revealed that soil moisture had no significant difference between denitrification and co-denitrification (Clough et al., 2017), but elevated NO_2^- showed the impact on co-denitrification in saturated soils. Co-denitrification is also responsible behind the gap in the exact calculation for the N loss via denitrification (Spott et al., 2011). Co-denitrification was leading over the simple denitrification in the soil system, and previously, it was considered that denitrification was the leading pathway for nitrogen loss (Rex et al., 2016). Co-denitrification can be calculated using the following equation (Clough et al., 2017);

$$d_{CD} = -\Delta^{29}Rp_1^2/(-\Delta^{29}Rp_1^2 + \Delta^{29}Rp_1p_2 + q_1p_2 - q_2p_1)$$

whereas; d_{CD} is co-denitrification p1 (0.9963) and q1 (0.0037) are the atom fractions of ^{14}N and ^{15}N in the natural environment, respectively, and p2 and q2 are the fractions of the atoms of ^{14}N and ^{15}N from labeled $^{15}NO_3^-$ source and this reaction considered as co-denitrifying and N_2 produce indirectly.

$$^{14}NH_4^+ + * {}^{15}NO_2^- \rightarrow N_2O \rightarrow N_2$$

(co − denitrification d_{CD}, differs from $^{29}N_2$ * ($^{15}NO_2^-$ reduced by fungi from $^{15}NO_3^-$ tracer)

In previous studies, the contribution of co-denitrification was included in the anammox rates (Maeda et al., 2017; Shoun et al., 2012; Spott et al., 2011), 92% of the microorganisms that participate in co-denitrification are fungi (Laughlin et al., 2010), while bacteria mostly perform denitrification. Anammox, co-denitrification, and denitrification produce N_2 under anaerobic conditions through different mechanisms.

Dinitrogen produced from denitrification passes through a series of intermediate reactions, NO_3^- is the initial substrate and N_2 is the final product. Therefore, nitrogen produced by anammox, co-denitrification and denitrification can be distinguished based on their nitrogen isotope conformations, i.e., $^{29}N_2$ (anammox), d_{CD} (co-denitrification) and $^{30}N_2$ (denitrification), respectively, by utilizing the ^{15}N isotope tracing method. The rates of anammox and denitrification were calculated using the ^{15}N isotope labeling technique (Mulvaney and Boast, 1986) as shown by Thamdrup and Dalsgaard (2002), and co-denitrification rate by fungi calculated according to Clough et al. (2017).

Besides, soil anammox process is driven by bacteria, while co-denitrification and denitrification are driven by both bacteria and fungi to produce N_2 (Shoun et al., 2012). To better understand the soil microbe-mediated nitrogen cycle, it is important to determine the biological sources of nitrogen production in the soil. The contribution of fungi and bacteria in the conversion of inorganic nitrogen to gasses N_2 can be differentiated through antibiotic and antifungal inhibition combined with ^{15}N isotope labeling (Maeda et al., 2017; Long et al., 2013; Laughlin et al., 2010).

DNA-based stable isotope probing (DNA-SIP) is a molecular technique which showed direct evidence of active microbial community through genomic DNA in complex environments (Pepe-Ranney et al., 2016). Over the past ten years, the DNA-SIP technology has drawn extensive attention because of its wide range of applications in microbial ecology and biotechnology. It is one of the most potent tools for coupling the genetic diversity and metabolic diversity of microorganisms (Chen and Murrell, 2010; Radajewski et al., 2000). In recent years, DNA-SIP has also been widely used in microbial ecology and biotechnology for different processes such as soil denitrification (Fan et al., 2014), and microbial decomposition (Pepe-Ranney et al., 2016). Anammox bacteria are generally considered autotrophic bacteria (Jetten et al., 2004), while co-denitrifying fungi are considered heterotrophic microorganisms (Zhu et al., 2015). Thus, ^{13}C-glucose can be used as a substrate and utilization of ^{13}C by co-denitrifying fungi in the soil can be investigated by extracting the incubated ^{12}C- and ^{13}C-labeled DNA. DNA stable isotopes are beneficial because they required in less quantity as substrate and a short time to detect cultivated and uncultivated active microorganism in the environment (Friedrich, 2006).

Stable isotope probing (SIP) technique has become state of the art in microbial ecology for identifying and detecting microorganisms that are actively involved in specific metabolic processes and elemental fluxes taking place in environmental samples to effectively link the taxonomic identity with function (Vogt et al., 2016). After stable isotopes are assimilated in the environmental samples, the metabolism active cells, the label goes into their biomass, including DNA, RNA, lipid, and proteins. These labeled biomolecules serve as biomarkers, which are recovered and analyzed using various techniques, including clone libraries, metagenomics, and next-generation sequencing (Uhlik et al., 2013). According to Radajewski et al. (2000), the 20% assimilation of the ^{13}C-labeled substrate into DNA is enough to differentiate between ^{12}C-DNA and ^{13}C-DNA, and reduction of the error caused by natural ^{12}C-DNA dilution.

The aims of this study are the practical evaluation of ^{15}N isotope tracing and ^{13}C-DNA-SIP technique for the contribution of anammox, co-denitrification, and denitrification processes to the Nitrogen losses in paddy-wheat (crop rotation) soil. Furthermore, to explore the effects of different fertilization treatments on gene abundance, activity, and nitrogen loss caused by anammox, denitrification, and co-denitrifying fungi. Finally, to determine the substrate utilization ($^{15}NO_3^-$ or ammonia) by fungi, and the production of nitrite for anammox in the result of nitrate reduction through fungi during the co-denitrification process (d_{CD}).

2. Materials and methods

2.1. Soil sampling

The study site is located in the Changshu district of Jiangsu province, China (119° 52′ 31.74″ N, 29° 52′ 44.64″ E). The area has a subtropical, humid climate with the monsoon pattern, with an average annual temperature of 15.4 °C, and rainfall of 1054 mm average annually. The site represents a typical agricultural area in subtropical southern China and has been used for rice-wheat crop rotation for five years. Experiment was set up with four treatments having four replicates, divided in different plots of the same size (6 m × 7 m) for each treatment, and every plot had 1 ft strip as plot divider, treatments were as follows: 1) control (CK); 2) chemical fertilizers (CF) with N (Urea), P (P_2O_5), and K (K_2O) fertilizers at rates of 240, 180, and 240 kg ha^{-1}, respectively; 3) pig manure (PM) plus chemical fertilizers (PMCF), PM 6% (6000 kg ha^{-1}) supplemented with N, P, and K fertilizers at rates of 120, 90, and 120 kg ha^{-1} respectively; and 4) straw returning plus chemical fertilizer (SRCF), total straw was returned after every crop harvest plus additional supply of N, P, and K fertilizers at rates of 240, 180, and 240 kg ha^{-1}, respectively. PM, P, and K were applied as base fertilizers (before the wheat was sown), and N was divided into three

parts to avoid losses (50% of total N as basal, 25% at early tillering stage, 25% at panicle initiation). Sampling was done during the wheat season in December 2015; previously, rice was harvested (Wheat-Rice crop rotation previously). Five cores of the soil sample from each fertilizer treatment were collected (top 20 cm) and pooled for individual treatment. Within 2–6 h after collection, the samples were transported on ice to the laboratory. One subsample was incubated immediately after arrival in the laboratory to determine the anammox, co-denitrification and denitrification activities, and remaining was air dried and processed through a 2.0 mm sieve for analyzing its physicochemical properties (Table S2), according to the methods described in Soil and Agricultural Chemistry Analysis (Bao, 2005).

2.2. Experimental design

2.2.1. Soil slurry incubation with antibiotics or antifungals combined with $^{15}NO_3^-$ substrates

Fresh soil collected from individual field treatments (approximately 100 g dried soil), was placed in 100-mL bottles; 60 mL water was added from water channel to keep the soil in a flooded (slurry) condition (three replicates for each treatment). Pre-incubation experiment was performed overnight in the laboratory after flushing and evacuating soil samples with helium gas three times (3 min) in bottles with tight butyl rubber stoppers. The bottles were kept in a rotary incubator (QB-228, Kylin-Bell Lab Instruments, Haimen, China), to remove the previously existed substrate nitrate (including nitrite) and oxygen from the bottles, after 24 h again helium gas flushing and evacuation were done. Inhibitor concentrations were confirmed by pre-experiment in the lab, according to Anderson and Domsch (1973) with some modifications. The optimal rate of streptomycin (3 mg g^{-1}) was used to inhibit the bacterial activity, and cycloheximide (15 mg g^{-1}) was used to inhibit fungal activity, these concentrations were also reported by Laughlin and Stevens (2002). A solution of $K^{15}NO_3^-$ (^{15}N at 99%) about 12 mM concentration was prepared; a volume of 200 µL was added to each sample in 12 mL vials having 2.5 g soil to make it 200 µM (^{15}N) final isotopic concentration approximately. A corresponding volume of $^{15}NH_4^+$ solution was also injected using a 1-mL syringe. Afterwards, for co-denitrification, each soil sample was treated with $^{15}NO_3^-$ follows as (1) no antibiotics or antifungal as a positive control to calculate the baseline levels of $^{29}N_2$, $^{30}N_2$ (treatment NA); (2) cycloheximide at a final concentration of 15 mg g^{-1} (treatment AF) to inhibit fungal activity; and (3) streptomycin at a final concentration of 3 mg g^{-1} (treatment AB) to inhibit bacterial activity. The antibiotic concentrations were the same according to some previous studies (Chen et al., 2015; Herold et al., 2012), and incubation time was selected after some modifications. Each treatment was performed in triplicate, and this procedure was carried out inside an anaerobic box under Ar + H_2 (anoxic) atmosphere. Subsequently, the headspace gas of a vial (12 mL) was replaced with He gas by repeatedly vacuuming and filling with high-purity (99.9%) and incubated for 0, 12, or 24 h in the dark at 25 °C in a mechanical shaker.

After incubation, 200 µL $ZnCl_2$ of 7 M concentration was added to terminate the microbial reaction. Nitrogen gas was mixed by shaking the tubes before the assay; the concentration of N_2 in the incubation tube was measured using a continuous flow isotope ratio mass spectrometer (MAT253 with Gasbench II and autosampler [GC-PAL]; Thermo Fisher Scientific, Waltham, MA, USA). Anammox and denitrification activity rates and their contribution to N_2 production were calculated from the yields of $^{29}N_2$ and $^{30}N_2$ using the method of Mulvaney and Boast (1986) as shown by Thamdrup and Dalsgaard (2002). Moreover, co-denitrification rates were measured according to the equation described by Clough et al. (2017). Quantitative analysis of the *hzs-β* and *18S rRNA* genes were done from each soil sample after incubation using DNA-SIP method.

2.2.2. Soil slurry incubation experiments utilizing ^{13}C-glucose substrates to detect fungal abundance and activity

The organic C sources of ^{13}C-glucose and ^{12}C-glucose (99% atom^{13}C, purchased from the Shanghai Engineering Research Center of Stable Isotopes, Shanghai, China) were added at a final concentration of 0.42 mg g^{-1} into the soil samples after slurry incubation for two weeks in AB (Anti-bacterial) treatment. Each treatment was performed in triplicate. The tubes were sealed with gas-tight butyl rubber stoppers, flushed with helium (He) gas, and re-incubated for two weeks. The soil was analyzed by destructive sampling, and soil heavy isotopic ^{13}C-DNA was isolated by density gradient centrifugation using DNA-SIP technology, as described by Dunford and Neufeld (2010). Quantitative PCR (qPCR) analysis of the *18S rRNA* gene using fungal-specific primers (FF390/FR1) was done on all fifteen layers of DNA, after density gradient centrifugation. The individual DNA samples were divided into fifteen layers according to isotopically weights. All ^{13}C-labeled DNA was further analyzed by constructing clone libraries and sequencing the fungal *18S rRNA* genes.

Co-denitrification was calculated through d_{CD} in AB (antibacterial) treatment using the difference between ^{29}N (anammox) and ^{30}N (total denitrification), according to Clough et al. (2017). Moreover, the ratio of *hzs-β* (anammox) and ^{13}C-*18S rRNA* genes abundance (fungal-specific) under AF (antifungal) and AB (antibacterial) treatments calculated, respectively, to estimate the population ratio of bacteria to fungi to check the activity and abundance relationship.

2.3. Experimental methods

2.3.1. DNA extraction and SIP fractionation

DNA was extracted from 0.5 g (incubated) soil, using the Fast DNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA), according to the manufacturer's protocol. The concentration and purity were estimated using a Nanodrop ND-2000 UV–vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The extracted DNA was stored at −20 °C until analysis.

After extracting the total soil DNA, then separated into the ^{13}C and ^{12}C gradient according to standard protocols of DNA-SIP method with some modifications (Dunford and Neufeld, 2010). Briefly, 2 µg of DNA from ^{12}C and ^{13}C treatments were mixed with a solution of CsCl to form a centrifugal solution having a density of about 1.725 g mL^{-1} and transferred to a 5.1 mL Quick-Seal poly-allomer ultra-centrifugation tube; followed on DNA centrifuged at 190,000 × *g* ultra-high-speed density gradient at 20 °C for 44 h by using a Vti65.2 vertical rotor (Beckman Coulter Inc., Palo Alto, CA, USA). After centrifugation, precisely a total of 340 µL min^{-1} DNA fraction was collected in each 1.5 mL sterilized centrifuge tube using NE-1000 single syringe pump (New Era Pump Systems Inc., Farmingdale, NY, USA) per layer. The refractive index was measured by using an AR200 digital handheld refractometer (Reichert Inc., Buffalo, NY, USA), after that DNA was purified using PEG-6000 and DNA pellet was washed 70% ethanol and dissolved in sterile water, and DNA concentration was re-checked using the (NanoDrop) and stored at −20 °C.

2.3.2. Anammox and fungal qPCR of the hzs-β and 18S rRNA genes

Real-time quantitative PCR (RT-qPCR) analysis was performed on a real-time PCR system using ABI PRISM7500 with the SYBR Premix Ex Taq kit (Takara Bio, Shiga, Japan) to assess the abundance of the *hzs-β* (HSBeta396F/HSBeta742R) and *18S rRNA* genes. Fluorescence qPCR was performed at a final volume of 20 µL containing 1 µL DNA template (1–10 ng), 0.8 µL each primer (10 µM conc.), 10 µL SYBR Premix EasyTaq, and 7.4 µL ddH_2O. Thermal profiles were set up according to primers, specific for each target gene for bacteria and fungi (Table S1). The target gene standards were prepared using positive clones of the *hzs-β* gene, that were selected to isolate plasmid DNA. The concentration of plasmid DNA was confirmed using a NanoDrop 2000 UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) for

the calculation of the *hzs-β*, and functional gene copies. Standard curves were generated with 10-fold serial dilutions of the plasmid DNA. Clones with efficiency and correlation coefficient above 95% and 0.98, respectively, were selected for sequences. Assessment of the diversity indices and operational taxonomic units (OTUs) of *18S rRNA* genes of denitrifying and co-denitrifying fungi was carried out at 98% similarity index using Mothur software.

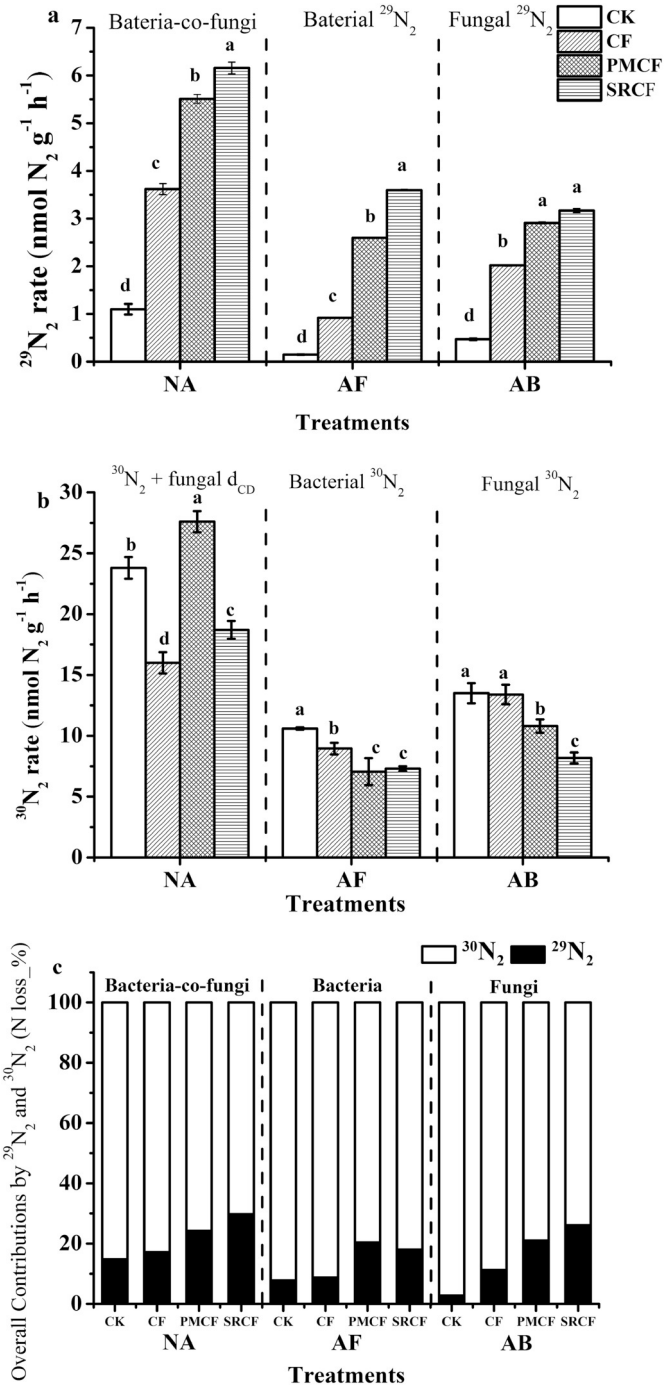

Fig. 1. (a) Represents the individual $^{29}N_2$ anammox rate in (antibacterial (AB) and antifungal (AF) treatments and cumulative anammox rate (Bacteria-co-fungi_NA) under different fertilization treatments (b) represents as like individual denitrification rate (AF and AB treatments) and cumulative denitrification + co-denitrification $_{d_{CD}}$ rate ($^{30}N_2$ + fungal-d_{CD}), (c) represents Nitrogen loss (%) as like individual by bacteria (AF) and fungi (AB), and total loss (%) by both (Bacteria-co-fungi-NA). CK_control, CF_chemical fertilizer, PMCF_pig manure plus chemical fertilizer, and SRCF_straw return plus chemical fertilizer.

2.3.3. PCR, clone libraries, and sequencing of fungal 18S rRNA genes

After PCR amplification of the 18S rRNA (using ^{13}C enriched DNA, 1.70–1.71 g mL^{-1} of CsCl density), the PCR product was purified using the EasyPure PCR Purification Kit (Transgen Biotech, Beijing, China). The purified PCR product was ligated into the T1-sample vector at 4 °C and then transformed into *E. coli* competent cells for transformation, according to the cloning kit manual using the pClone007 Simple Vector Kit (Tsingke Biotech, Beijing, China). White (positive) clones (50) were selected randomly and inoculated on new separate plates for sequencing from the company (Tsingke Biotech, Beijing, China). The sequencing results along with closely related reference sequences were aligned using ClustalW (http://www.ebi.ac.uk/clustalw/). The Results were BLAST on NCBI, and Mothur software was used to cluster co-denitrifying and denitrifying fungal 18S rRNA sequences with ≥98% similarity according to Tedersoo et al. (2015). The sequences which were not highly similar to fungi due to primers biasness (Hagn et al., 2003), were discarded and operational taxonomic units (OTUs) were assigned. Phylogenetic trees of *18S rRNA* gene sequences were constructed using MEGA 5.1 software adopting the neighbor-joining approach with 1000 bootstraps. The *18S rRNA* gene sequences of the co-denitrifying and denitrifying fungi found in this study were deposited in the GenBank database under the accession numbers MF804269-MF804300.

The *hzs-β* (HSBeta396F/HSBeta742R) functional gene was amplified, sequenced, and analyzed by Mothur and MEGA 5.1 software's to establish the phylogeny of *hzs-β* gene sequences. The *hzs-β* gene sequences of the anammox bacteria were deposited in GenBank under the accession numbers KX494112-KX494185 reported in our preceding work (Gu et al., 2017)

2.4. Statistical analysis

The *P* values were determined using a two-way analysis of variance (ANOVA) and Duncan's post hoc test. The relationships between the soil properties and the N-cycling groups were analyzed through Pearson correlation; data represented as the mean ± standard deviation/standard error from three independent replicates (Table S1). All data were analyzed using Excel 2010 and SPSS 20.0. Principal component analysis (PCA) and correlation analysis were performed using SPSS 20.0. Operational taxonomic units (OTUs) were generated by Mothur software, and phylogenetic trees were constructed using MEGA 5.1 software. OriginPro 8.5 was used to draw figures.

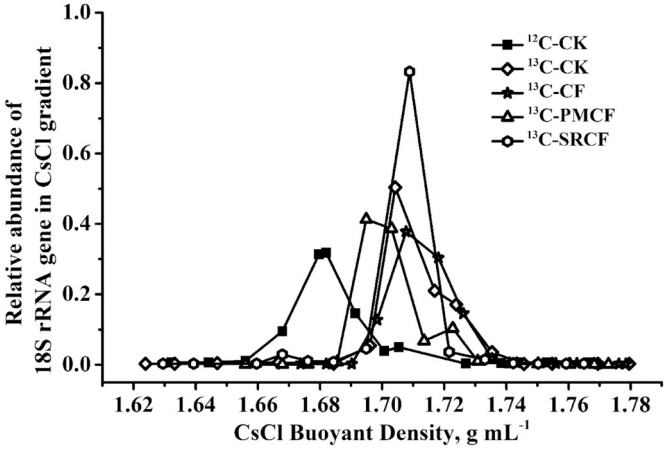

Fig. 2. Distribution of co-denitrifying and denitrifying fungi on the basis of 18S rDNA gene copy numbers across the buoyant density of the DNA isolated from soil microcosms incubated with ^{13}C-glucose and ^{12}C-glucose. CK_control, CF_chemical fertilizer, PMCF_pig manure plus chemical fertilizer, and SRCF_straw return plus chemical fertilizer.

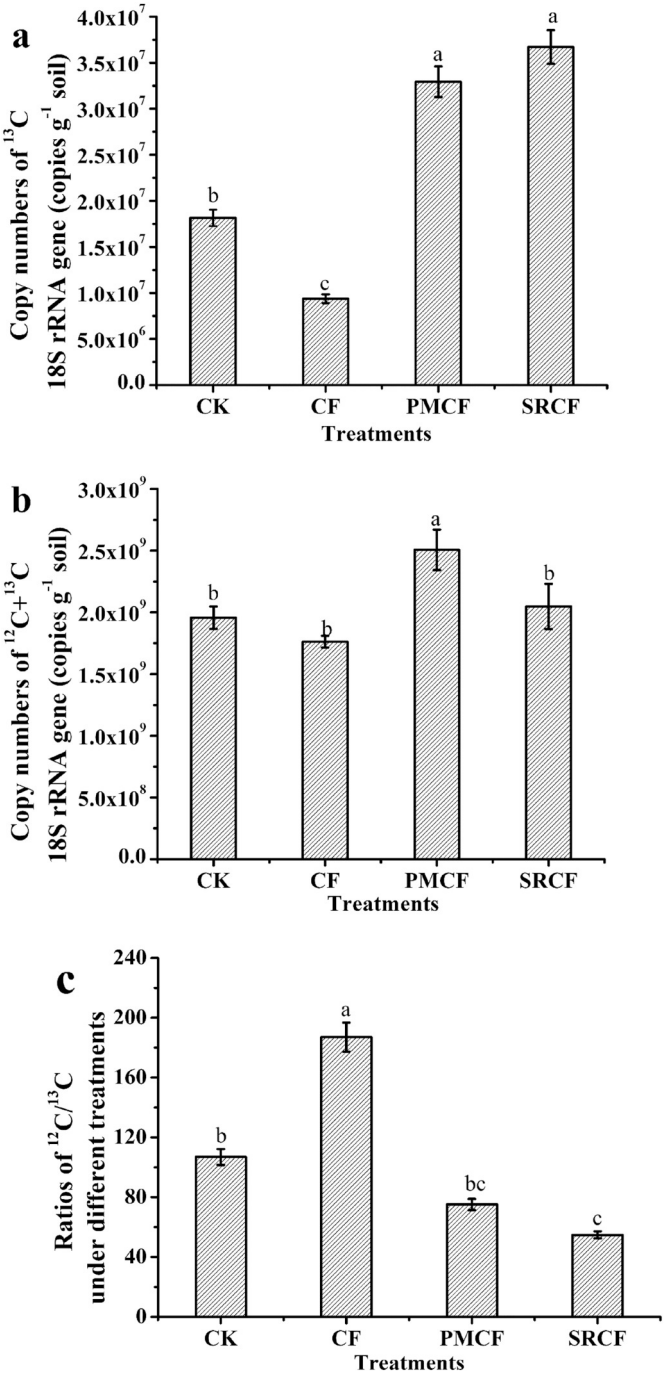

Fig. 3. Abundance of active fungi (co-denitrifying and denitrifying) consuming ^{13}C-18S rDNA and ^{12}C + ^{13}C treated with the antibacterial inhibitor, and ratios of ^{12}C/^{13}C under different treatments after 14 days. CK_control, CF_chemical fertilizer, PMCF_pig manure plus chemical fertilizer, and SRCF_straw return plus chemical fertilizer.

3. Results

3.1. The contribution of anammox, co-denitrification, and denitrification to N_2 production

^{15}N tracer isotopic study showed that the anammox ($^{29}N_2$), co-denitrification (d_{CD}), and denitrification ($^{30}N_2$) processes resulted in a major accumulation of the N in soil slurry, under anaerobic condition. The overall anammox activity (individually and bacteria-co-fungi), based on the rate of $^{29}N_2$ production (Fig. 1a), ranged from lowest 0.15 nmol N_2 g^{-1} h^{-1} in the CK (AF_antifungal) to highest 6.16 nmol N g^{-1} h^{-1} in the SRCF (NA_ Bacteria-co-fungi). The denitrification activity based on the rate of $^{30}N_2$ production (Fig. 1b), as a result of bacterial or fungal activity solitary or by both together, ranged from lowest 7.05 nmol N_2 g^{-1} h^{-1} in the PMCF (AF_bacterial) to highest 27.6 nmol N_2 g^{-1} h^{-1} in the SRCF (NA_$^{30}N_2$ + fungal d_{CD}). Furthermore, while comparing four fertilization treatments (CK, CF, PMCF, and SRCF) using antibacterial and antifungal as control measurement to compare and analyze the individual difference due to denitrification (in AF and AB groups) with co-denitrification ($^{30}N_{2+}$ fungal d_{CD}_NA) (Fig. 1b). The rates in the AB-treated (fungal) groups were higher than that in the AF-treated (bacterial) groups (Fig. 1b). According to ^{15}N stable isotope tracing, the overall contribution rates of anammox by both bacteria and fungi (NA_Bacteria-co-Fungi) in the CK, CF, PMCF, and SRCF treatments were 14.82%, 17.22%, 24.24%, and 29.74% (Fig. 1c), respectively. Moreover, the contribution rates of co-denitrifying fungi to the soil nitrogen losses were 3.51%, 11.20%, 21.25%, and 25.60% in the CK, CF, PMCF, and SRCF treatments, respectively (Fig. 1c_AB_Fungi). The contribution rates of anammox bacteria to the nitrogen removal process were 7.50%, 8.15%, 21.80%, and 18.10%, respectively (Fig. 1c_AF_Bacteria) and the higher rate was found in PMCF (21.80%) by bacteria. Overall, the rate of $^{29}N_2$, and $^{30}N_2$ ($^{30}N_2$ + Fungal d_{CD}_ NA) production activity indicated that fungi play major roles in anammox, co-denitrification, and denitrification (Fig. 1).

3.2. Abundance of anammox bacteria, co-denitrifying and denitrifying fungi in the soil, based on *hzs-β* gene, and ^{13}C-DNA-SIP using fungal 18S rRNA genes

DNA-SIP showed that fungal ^{13}C-DNA extracted from samples mostly enriched in the heavier density of the downstream DNA ranged from 1.70 to 1.71 g mL^{-1}, CsCl Buoyant density, a comparison was made based on qPCR results, after incubation using ^{13}C and ^{12}C-glucose enrichment (Fig. 2). According to qPCR results using combined ^{12}C + ^{13}C *18S rRNA* gene (Fig. 3b), there were no significant differences between fertilization treatments, except for PMCF treatment, which showed the highest gene abundance. The ratio of natural to heavy isotopes (^{12}C/^{13}C) in CF and CK treatments were much higher than those for the SRCF and PMCF groups (Fig. 3c). Moreover, the abundance of anammox (*hzs-β*) bacterial gene (Table 1) in CK _NA (1.08×10^8 copies g^{-1} dry soil) and PMCF (1.08×10^8 copies g^{-1} dry soil) fertilization groups were significantly lower compared to those from the CF (4.02×10^8 copies g^{-1} dry soil) and SRCF (1.53×10^8 copies g^{-1} dry soil) groups. The results of fungal ^{13}C-DNA *18S rRNA* gene (NA treatment) indicated a higher abundance of fungi was present

Table 1
Abundances of anammox bacteria and ^{13}C-*18S rRNA* gene in soils (copies·g^{-1} dry soil.)

Genes	Treatments	CK	CF	PMCF	SRCF
hzs-β	NA	1.08×10^8 (± 2.12×10^6) a	4.02×10^8 (± 1.07×10^7) a	1.08×10^8 (± 1.11×10^5) a	1.53×10^8 (± 1.03×10^7) a
	AF bacteria	3.78×10^5 (± 1.19×10^4) c	8.03×10^6 (± 5.01×10^5) c	2.74×10^7 (± 1.01×10^5) b	9.34×10^7 (± 6.41×10^6) b
18S rRNA	NA	2.19×10^9 (± 1.21×10^7) a	2.29×10^9 (± 1.11×10^6) a	3.70×10^9 (± 1.48×10^7) a	2.17×10^9 (± 1.56×10^7) b
	AB fungi	1.96×10^9 (± 1.13×10^7) a	1.76×10^9 (± 1.63×10^8) a	2.51×10^9 (± 1.73×10^7) b	2.05×10^9 (± 1.33×10^7) c

Note: the different small letters in the same column mean the significant difference at $P < 0.05$. NA_(Control), AF_ antifungal (Bacterial), and AB_ Antibacterial (Fungal), bacterial *hzs-β* and Fungal *18S rRNA* respectively.

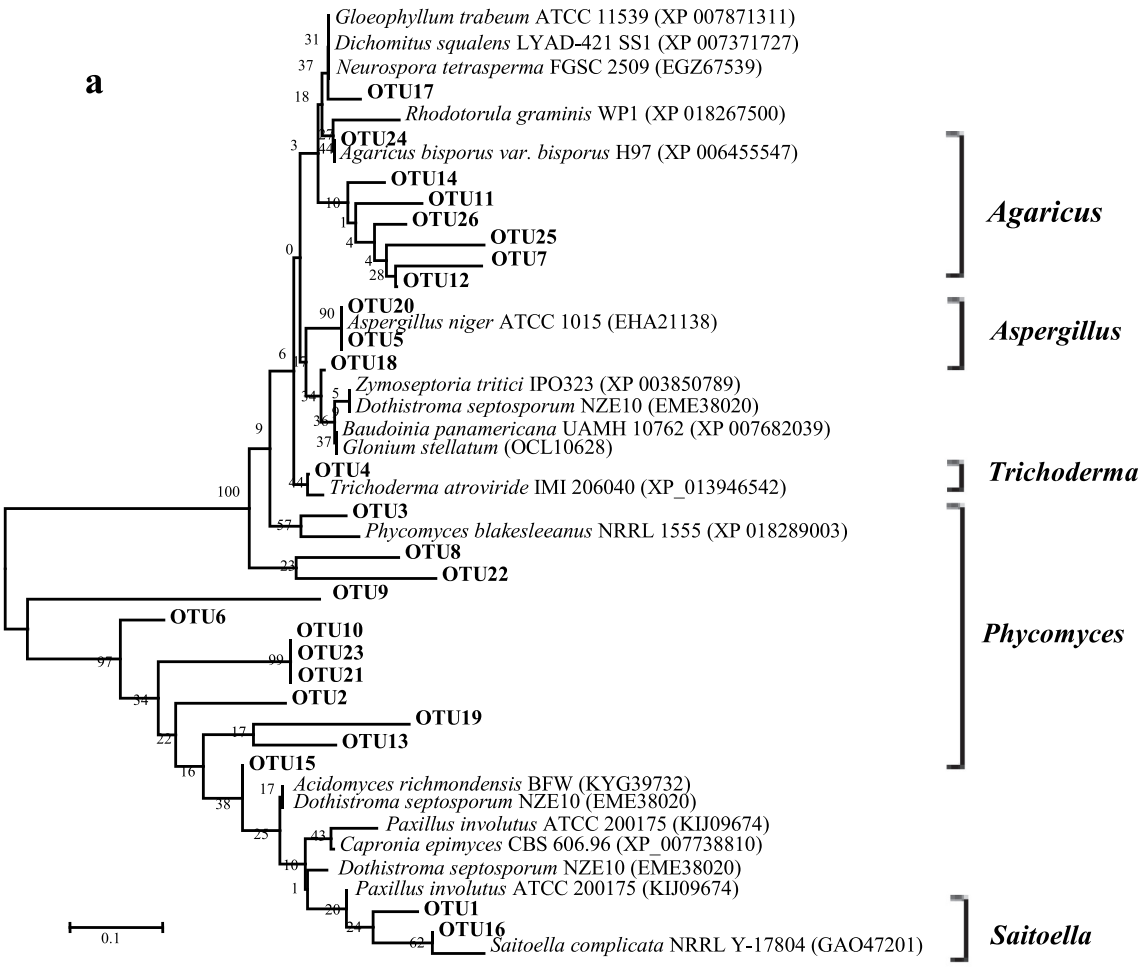

Fig. 4. Neighbor-joining phylogenetic tree on the basis of fungal 18S rDNA gene sequences analysis of co-denitrifying and denitrifying fungi (panel a) and relative abundance of the *Agaricus*, *Aspergillus*, *Phycomyces*, *Saitoella*, *Trichoderma* co-denitrifying and denitrifying fungi detected by the 18S rDNA genes (panel b).

in all treatments and highest fungal abundance was present in PMCF (3.70×10^9 copies g^{-1} dry soil) and lowest in CF. On the other hand, bacterial abundance was lowest in PMCF (1.08×10^8 copies g^{-1} dry soil).

3.3. Community structure and diversity of active fungi and anammox bacteria in the soil

Clone library constructed using ^{13}C-DNA-SIP, *18S rRNA* gene extracted from CK, CF, PMCF, and SRCF samples, we got thirty-two groups of fungal *18S rRNA* gene sequenced from all treatments after

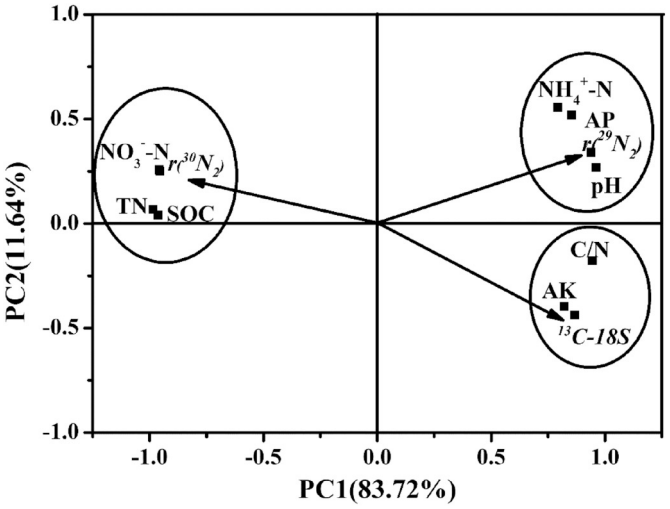

Fig. 5. Shows principal component analysis (PCA plot) comparing $^{29}N_2$ (anammox) and $^{30}N_2$ (Denitrification) production rates, quantitative PCR (qPCR) results of fungi, ^{13}C-18S rDNA revealed strong affinity to the anammox and same attraction to the soil factors as like anammox.

removal of irrelevant sequences amplified due to primer biasness (Hagn et al., 2003), and sequences were clustered into twenty-six OTUs with > 98% similarity (Fig. 4). Moreover, the ^{13}C-labeled fungal *18S rRNA* gene relative abundance of OTUs had high homology with different genera including 30.77% (*Agaricus belongs to phylum Basidiomycota*), 15.38% (*Aspergillus belong to phylum Ascomycota)*, 57.69% (*Phycomyces belongs to phylum Zygomycota*), 19.23% (*Saitoella belongs to phylum Ascomycota*) and 7.69% (*Trichoderma belongs to phylum Ascomycota*) (Fig. 4a) and relative abundance of each genus varied under treatments (Fig. 4b).

We used the clone library of *hzs-β* gene sequences from our previous study as a reference, which was carried out earlier than this study (Gu et al., 2017). An earlier study showed twelve OTUs with 97% similarity index, and the *hzs-β* gene results (Supplementary Fig. S1) showed high similarity with different genera of *Candidatus Brocadia*, Ca. *Jettenia* and Ca. *Scalindua*. The reference Fig. S.1 showed that most OTUs (58.3%) had high homology with Ca. *Brocadia*, 33.3% of OTUs showed high homology with Ca. *Jettenia* followed by 8.3% of OTUs showed high homology with Ca. *Scalindua*. Overall, the results of *hzs-β* gene sequencing showed a high anammox bacterial diversity in the soil samples, of which Ca. *Brocadia* (13.51% of the total sequences), Ca. *Scalindua* (8.11% of the total sequences) and Ca. *Jettenia* (78.38% of the total sequence) were dominant (Fig. S1).

3.4. Correlation analysis of anammox, denitrification, co-denitrifying fungal gene abundance, and soil physico-chemical properties

Correlation analysis of anammox ($^{29}N_2$), denitrification ($^{30}N_2$) production rates, ^{13}C-labeled *18S rRNA* fungal gene abundance, and soil physico-chemical properties are shown in Table 3. The results showed that the rate of $^{29}N_2$ (anammox) was significantly positively correlated with *18S rRNA* gene abundance ($P < 0.05$; $R = 0.676$), while the rate of $^{30}N_2$ (denitrification) production was significantly negatively correlated with *18S rRNA* gene abundance ($P < 0.05$; $R = -0.913$). Furthermore, the correlation about physicochemical properties of the soil (Table 3) verified by principal component analysis (PCA _Fig. 5), correlation and PCA showed that soil pH, available phosphorus, and NH_4^+-N significantly promoted the $^{29}N_2$ (anammox) production rate during anaerobic incubation. However, soil organic carbon, total nitrogen, and NO_3^--N were in favor of denitrification ($^{30}N_2$) (Fig. 5 and Table 3); and more interestingly anammox $^{29}N_2$, available potassium and the C/N ratio significantly promoted the ^{13}C-labeled *18S rRNA* gene abundance (Fig. 5 and Table 3).

The abundance of *hzs-β* functional genes in the AF treatment group (Fungistatic), as well as the abundance of the *18S rRNA* gene bound to ^{13}C-DNA-SIP in the AB treatment group (bacteriostatic), is shown in Table 4. The mean ratio of *hzs-β* abundance in the AF (Fungistatic) group to ^{13}C-labeled *18S rRNA* abundance in the AB (Bacteriostatic) group was 1.92, 4.56, 1.09, and 4.55 in CK, CF, PMCF, and SRCF respectively, and the ratio ranged from lowest to the highest was in PMCF and CF treatments respectively.

4. Discussion

4.1. Contribution of different microorganisms to anammox, co-denitrification, and denitrification processes to nitrogen losses

Exact calculation of nitrogen loss is not easy due to different processes are happening in the environment at the same time by complex microbial community. With the passage of time along with the denitrifying community (Zumft, 1997), anammox bacteria are also considered as thieves of nitrogen loss (Zhang et al., 2017; Gu et al., 2017). Recently, with the findings of Laughlin and Stevens (2002), fungi also play a key role as the second-most contributor to the nitrogen cycle (Boyle et al., 2008). Our results indicated that the denitrification ($^{30}N_2$) losses to the nitrogen removal process under AF_antifungal (bacteria) were slightly lower than that of soil under AB_ antibacterial (fungi) treatment, showing that fungi playing important roles in the co-denitrification process (Fig. 1b). Previous studies (Rex et al., 2018; Cline et al., 2018; Clough et al., 2017; Zhang et al., 2017; Zhu et al., 2015) have been performed to determine the contribution of anammox (co-denitrification) to the nitrogen removal process in agricultural soils as well as in pure culture, which clearly showed that fungi have a significant role in co-denitrification comparatively to the exact denitrification. The reason behind higher fungal co-denitrification is that the fungi have a diverse metabolic system which can perform under a wide range of environment (oxic and anoxic) comparative to denitrifiers (Zumft, 1997), and anammox which are strict anaerobes (Jetten et al., 2004).

Our results showed 3.51–25.60% overall N loss by co-denitrification fungi (CK and SRCF) (Fig. 1c) due to different environment (anoxic)

Table 2
α-biodiversity index of denitrifying and co-denitrifying fungi based on *18S rRNA* gene.

Treatments	Denitrifying and co-denitrifying OTUs	α-Diversity index			Gene copy numbers (copies·g^{-1} dry soil)
		Simpson indices	Shannon-wiener indices	Chao indices	
CK	12 a	0.05 b	2.52 a	31.33 a	1.81×10^7(± 9.07×10^5) b
CF	5 b	*0.00 c	1.61 b	15.00 b	9.37×10^6(± 4.68×10^5) c
PMCF	5 b	0.32 a	1.61 b	9.00 c	3.29×10^7(± 1.64×10^6) a
SRCF	5 b	0.00 c	1.61 b	15.00 a	3.67×10^7(± 1.83×10^6) a

Note: the different small letters (a, b, and c) in the same column mean the significant difference at $P < 0.05$. CK_control, CF_chemical fertilizer, PMCF_pig manure plus chemical fertilizer, and SRCF_straw return plus chemical fertilizer (*0.001c).

and fertilizer management, and this loss was higher than the findings of Lavrent'ev et al. (2009), who said that under favorable conditions fungi could contribute to 8% N_2 loss. A study by Wei et al. (2014) showed the highest contribution rate of co-denitrification by fungi 84%, and only 20% were due to bacterial denitrification. Herold et al. (2012) evaluated co-denitrification in arable soils and showed that fungi could contribute nearly 15% of N loss.

The clear difference between anammox and co-denitrification rate by fungi is due to different factors, i.e., Strous et al. (1997) said that anammox have a higher attraction for substrate and are strictly anaerobic. However, fungi have a different metabolic system which can use O_2 as a substrate in an aerobic environment and NO_3^- in anoxic condition (Laughlin et al., 2010; Takasaki et al., 2004). However, previous studies mostly used $^{29}N_2$ production to determine anammox and co-denitrification contribution rates; there was a lack of research clarifying the separate roles of anammox and co-denitrification in the nitrogen removal process.

With the use of ^{15}N tracer isotope and ^{13}C-DNA-SIP in this study, a strong positive correlation was found between the rates of r($^{29}N_2$) production and the abundance of ^{13}C-labeled *18S rRNA* genes (Table 3). This indicates, that the abundance of ^{13}C-labeled *18S rRNA* genes could be an indicator of the abundance of co-denitrifying fungi in a sample, because the rate of $^{30}N_2$ (denitrification) production was significant negative correlated with *18S rRNA* gene abundance ($P < 0.05$; $R = -0.913$, Table 3) under antibacterial treatment (AB_Fungal). According to literature (Cathrine and Raghukumar, 2009; Takasaki et al., 2004; Laughlin and Stevens, 2002) *Aspergillus* and *Trichoderma* sp., are common in an anaerobic environment and have the ability for denitrification (Fig. 4a).

Soil anammox and denitrification processes are mostly affected by SOC and C/N ratio in the environment, in our study we found that $^{29}N_2$ and *18S rRNA* fungal genes showed a positive correlation with C/N ratio, and this is in line with some previous studies (Wang et al., 2018; Rahman et al., 2018; Gu et al., 2017). Soil denitrification was accelerated under the SOC and this study also showed $^{30}N_2$ and SOC positive correlation ($r = 0.89$) and revealed by many kinds of research that SOC favors denitrification (Tan et al., 2019; Rahman et al., 2018; Wang et al., 2018), and C/N ratio favors anammox (Gu et al., 2017).

The ratio of bacterial *hzs-β* functional genes to the ^{13}C-labeled *18S rRNA* (Fungal) gene varied under treatments and maximum difference was present in CF (4.56), (Table 4). This suggests that fungal activity (Fig. 1) does not correlate with fungal gene abundance (Table 1).

4.2. Anammox, co-denitrifying, and denitrifying microbial abundance and community structure as affected by fertilizer management

Fungal community analysis was performed using ^{13}C-enriched DNA (1.70–1.71 g mL^{-1} of CsCl). Fungal 18S rRNA analysis was performed using PCR with specific primers (Table S1) from (AB_fungal group). Anammox community was analyzed in our previous investigation (Gu et al., 2017) (Fig. S1). According to previous literature, soil nutrients support diverse anammox activity in a variety of environments (Rahman et al., 2018; Nie et al., 2018; Gu et al., 2017). Fertilizers can drive changes in the composition of anammox microbial communities and associated patterns in different soils (Nie et al., 2018; Zhang et al., 2017). However, soil microbes can go under a dormant state when water becomes scarce and low availability of substrate (Lennon and Jones, 2011). Anammox bacteria are sensitive to substrate with slow growth rate (Jetten et al., 2001), and they did not change their community structure in a particular place over a short period of time (Zhao et al., 2018). Anammox and many other microorganisms become inactive under harsh environment and become active when they find suitable conditions, i.e., resupply of water and nutrients (Zhu et al., 2019). To evaluate the microbial diversity and functioning in the environment, combine techniques of molecular analysis and ^{15}N tracer study considered to be best nowadays, because there are many pathways and kinds of microbes working at the same time, so exact estimation needs to use reliable technologies (Pepe-Ranney et al., 2016).

According to the α-diversity analysis based on fungal 18S rRNA genes (Table 2), the difference in the Shannon-Wiener index under fertilized treatments (CF, PMCF, and SRCF-1.61) was not significant, but comparative to CK noticeable difference was present (CK- 2.52 Shannon-Wiener index). The Simpson index showed the highest index in the PMCF treatment (Table 2), suggesting that the addition of pig manure can increase the biodiversity of co-denitrifying fungi because fungi prefer to work in the presence of substrate (Shoun et al., 2012). Some studies investigated the anammox microbial community structure and demonstrated that with application of different fertilizers, soil anammox community affected significantly (Rex et al., 2018; Zhang et al., 2017; Gu et al., 2017), while few researchers focused on the activity of co-denitrifying fungi (Rex et al., 2018; Cline et al., 2018; Maeda et al., 2017).

The fungal *18S rRNA* gene form ^{13}C-DNA-SIP indicated that a lower fungal gene abundance is present in all treatment rather than anammox abundance (Table 1). The lowest fungal *18S rRNA* gene abundance was present in CF (1.96×10^9 copies·g^{-1} dry soil), and highest was in PMCF (2.51×10^9 copies·g^{-1} dry soil) which opposite to the findings of Cline et al. (2018), who said that addition of organic nitrogen decreases fungal population and can change community structure. However, against fungal, the lowest bacterial *hzs-β* gene abundance present in PMCF (2.74×10^7 copies·g^{-1} dry soil), and the highest bacterial gene abundance was in SRCF (9.34×10^7 copies·g^{-1} dry soil). The reason behind lower anammox rate in PMCF treatment could be due to pathogens present in pig manure (Kang et al., 2018) and highest in SRCF due to C/N ratio which favors anammox (Nie et al., 2018). The correlation analysis also enhanced the significance of the C/N ratio for anammox rate (Table 3). In the view of earlier discussion, it's clear evidence that bacteria and fungi use different substrates under different environment, its mean in the presence of organic matter, fungi degrade it and use as substrate (Maeda et al., 2017), while in case of bacteria they preferably like straw for carbon source (Gu et al., 2017).

Additionally, while adopting co-molecular-isotopic study, we found the positive linkage between isotopic results, which correlate the anammox rate $^{29}N_2$ and fungi 18S rRNA gene (Table 3). Clearly, fungi showed a negative correlation with $^{30}N_2$ production under controlled treatment (AB) (Table 3). Previous studies (Pepe-Ranney et al., 2016; Zhang et al., 2017), showed that the DNA-SIP with ^{13}C based isotopes are more reliable to differentiate the active microbial communities in a complex environment. Phylogenetic analysis of the active co-denitrifying fungi showed a high homology with the genera *Agaricus*, *Aspergillus*, *Phycomyces*, *Saitoella*, and *Trichoderma* (Fig. 4a) and (*Agaricus*, *Aspergillus*, and *Trichoderma*) have the ability of denitrification (Wei et al., 2014; Chen et al., 2014; Long et al., 2013). A study by Cathrine and Raghukumar (2009) and Takasaki et al. (2004) revealed that *Aspergillus* sp., are more frequently found to be in soil and have the ability to perform under the anoxic zone, which ultimately signs of co-denitrification in the same environment. A recent study by Maeda et al. (2017) confirmed that *Aspergillus* and *Trichoderma* (Fig. 4a) have the ability to perform the denitrification in pure culture. Inconclusive, on the base of our results and validation of hybrid co-molecular isotopic techniques, this study can be a strong initiative for an understanding of the N losses by anammox, codenitrifying fungi, and denitrifiers through different pathways.

Table 3
Correlation analysis between anammox, denitrification rate,^{13}C labeled fungal *18S rRNA* gene and physico-chemical properties in different treatments.

	r($^{29}N_2$)	r($^{30}N_2$)	^{13}C-18S	pH	TN	NO_3^--N	NH_4^+-N	AK	AP	SOC	C/N
r($^{29}N_2$)	–	−0.806*	0.676	0.997**	−0.909**	−0.819*	0.923**	0.615	0.981**	−0.902**	0.814*
r($^{30}N_2$)	−0.806*	–	−0.913**	−0.846*	0.941**	0.960**	−0.648	−0.940**	−0.678	0.898*	−0.986**
^{13}C-18S	0.676	−0.913**	–	0.730	−0.921**	−0.975**	0.386	0.785	0.526	−0.916**	0.832*
pH	0.997**	−0.846*	0.730	–	−0.938**	−0.861	0.899*	0.661	0.963**	−0.929**	0.847*
TN	−0.909**	0.941**	−0.921**	−0.938**	–	0.983**	−0.705	−0.769	−0.814*	0.993**	−0.900**
NO_3^--N	−0.819*	0.960**	−0.975**	−0.861*	0.983**	–	−0.580	−0.817*	−0.694	0.972**	−0.905**
NH_4^+-N	0.923**	−0.648	0.386	0.899*	−0.705	−0.580	–	0.533	0.952**	−0.674	0.718
AK	0.615	−0.940**	0.785	0.661	−0.769	−0.817*	0.533	–	0.473	−0.694	0.959**
AP	0.981**	−0.678	0.526	0.963**	−0.814*	−0.694	0.952**	0.473	–	−0.815*	0.700
SOC	−0.902**	0.898*	−0.916**	−0.929**	0.993**	0.972**	−0.674	−0.694	−0.815*	–	−0.843*
C/N	0.814*	−0.986**	0.832*	0.847*	−0.900**	−0.905**	0.718	0.959**	0.700	−0.843*	–

TN, total nitrogen; AN, alkali-hydrolyzable nitrogen; AP, available phosphorus; AK, available potassium; SOC, soil organic carbon.
** Significant correlation at 0.01 level (bilateral).
* Significant correlation at 0.05 level (bilateral).

Table 4
Abundances of anammox bacteria (*hzs-β*), co-denitrifying fungi from ^{13}C labeled *18S rRNA* gene in different treatments, as well as the ratio of *hzs-β* and ^{13}C-*18S rRNA* gene (copies·g^{-1} dry soil).

Samples	*hzs-β*_AF	^{13}C-18S rRNA _AB	*hzs-β*/^{13}C-18S rRNA
CK	3.78×10^5 (± 2.09×10^4) c	1.96×10^9 (± 1.13×10^7) a	1.92 a
CF	8.03×10^6 (± 4.49×10^3) c	1.76×10^9 (± 1.19×10^8) a	4.56 b
PMCF	2.74×10^7 (± 1.09×10^6) b	2.51×10^9 (± 1.59×10^8) c	1.09 c
SRCF	9.34×10^7 (± 5.09×10^6) b	2.05×10^9 (± 1.03×10^8) c	4.55 b

Note: the different small letters (a, b, and c) in the same column mean the significant difference at $P < 0.05$. *hzs-β*-AF (only bacterial anammox) and ^{13}C-18Sr RNA (only fungal anammox). CK_control, CF_chemical fertilizer, PMCF_pig manure plus chemical fertilizer, and SRCF_straw return plus chemical fertilizer.

5. Conclusions

Paddy-wheat is the most common crop rotation strategy in the China, and the leading causes of nitrogen losses in this cropping pattern are the denitrification, anammox, and co-denitrification by bacteria and fungi. To fill the gap between input and output, many kinds of investigations have been done, but it cannot be calculated ideally, because the nitrogen cycle is a web of different pathways in itself. In the view of our objectives, we exploit the ^{15}N plus ^{13}C-DNA-SIP to investigate the culprits of nitrogen losses, and their activity and contribution to the nitrogen loss. We found that co-denitrifying fungi along with anammox, denitrifying bacteria are involved in ample amount of nitrogen losses. Fungi showed higher activity in the studied soil treatments due to no limitation of the substrate as like for anammox. So, we postulate that to control nitrogen losses; we need to consider fungi as a major thief for N loss, we need to adopt different fertilizer schemes under both environments along with the use of inhibitors. Finally, in the essence of the utilizing DNA-Stable isotope and ^{15}N tracing, this study could be a strong initiative for further investigations of anammox co-denitrifying fungi in agricultural soil for N assessment and budgeting.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

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