Linkage of N_2O emission to functional gene abundance in an intensively managed calcareous flu-aquic soil

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Abstract
The linkage between situ N_2O emissions and abundance of functional genes ammonia monooxygenase gene (*amoA*), nitrate reductase gene (*narG*), nitrite reductase genes (*nirS* and *nirK*), N_2O reductase gene (*nosZ*) is not well understood, impeding proposing methods for mitigation in agricultural management. Our work was focusing on this linkage. Combined traditional study method and molecular biological technique and four treatments were involved in: N_0 (Zero N application, straw removal), N_{opt} and CN_{opt} (Improved N_{min} test, straw removal and return respectively), CM (Manure supplementary, chemical fertilizer N based on N balance calculation, straw return). Soil samples were collected on 16th April (reflect long-term N and C management effect), 9th and 14th August (reflect before and after short-term fertilization on 11st August) for biological and chemical properties analysis. We found that the *amoA* gene responed to short-term fertilizer while denitrification genes had no response and annual N_2O emission had significant positive relationships with gene abundance mentioned above. We concluded that strong nitrification triggered by high ammonia concentration after fertilization, nitrifier denitrification or denitrification triggered by strong rainfall or irrigation in normal crop growing days without nitrogen addition were most probably responsible for N_2O emissions. It is critical to reduce *amoA* gene function after urea-based fertilization. Meanwhile, we need to pay attention to enhanced denitrification genes functions in their favourable conditions to produce N_2O when increased SOC due to long-term manure fertilization.

Key Words
nitrous oxide flux, nitrifier and denitrifier abundance, long-term field experiment, nitrogen fertilizer, soil properties

Introduction

N_2O is a powerful long-lived greenhouse gas and has 265-fold stronger warming effect than carbon dioxide in the troposphere on a 100-yr time horizon (Cubasch et al., 2013). It come from both natural and anthropogenic sources and agricultural soils with nitrogen fertilizers or/and manure are mainly anthropogenic sources which contributed up to 66% of current anthropogenic N_2O emissions at global scale (UNEP, 2013). Therefore, agricultural soils play a key role in mitigating anthropogenic N_2O emissions both regionally and globally (Tian et al., 2016). The North China Plain (NCP) is located in northeast China (32–41 °N, 113–120 °E) on the alluvial plain of the Yellow River and has a warm-temperate sub-humid climate with cold winters and hot summers. Soils are calcareous with a pH of 7.5–8.5 and an organic matter content of approximately 1.0–1.5%. The current agricultural practice is a very intensive double-cropping cereal system with irrigated winter wheat and rain-fed summer maize rotations, characterized by applying large amounts of N fertilizer and irrigating with large amounts of groundwater to obtain relatively high yields (Ju et al., 2009). These practices lead to substantial total N_2O emissions in this region, which has become a hotspot of national N_2O emissions with global significance (Zhou et al., 2014).
The exact pathway of N_2O emission is still unclear and few studies have combined situ N_2O emission in the field, abundance of functional genes during nitrification and denitrification together under different nitrogen and carbon management. Relationship between soil chemical and biological properties and N_2O emission, N_2O emission and abundance of functional genes are still not well understood on this low carbon calcareous soil. We examined the following questions. How does *amoA* gene of bacteria response to short-term fertilization? What's the linkage between annual N_2O emission flux and abundance of functional genes including *amoA* of bacteria, *narG*, *nirS*, *nirK*, *nosZ* and 16S rRNA gene? How does long-term nitrogen and carbon management affect this linkage? We hypothesized that short-term fertilization leads to N_2O emission as a result of increased of *amoA* gene abundance; N_2O emission after strong rainfall or irrigation without addition of nitrogen was the result of increased denitrification functional gene abundance. To verify our hypothesis, we carried out the experiment on a long-term field experiment since 2006 in Beijing. Four treatments were involved in this work. Soil samples were collected on16th April, 9th and 14th August respectively. The first two sampling dates were before 10th leaf fertilization of summer maize, 14th August was three days after 10th leaf fertilization. N_2O emission was measured on three dates, as well as soil

chemical properties including ammonium, nitrite, nitrate, pH and soil water content were determined. Soil DNA were also extracted and some downstream biological analysis included the abundance of 16S rRNA, *amoA*, *narG*, *nirS* and *nirK*, and *nosZ* , Illumina-based 16S rRNA gene sequencing from V3 to V4 region. In our study we combined soil properties, N_2O emission, abundance of functional genes and whole bacteria community structure to further explore N_2O emission mechanism on winter wheat-summer maize crop soil of North China Plain.

Methods

N_2O emission measurement

N_2O emissions were measured using the closed static chamber method (Mosier et al., 2006) and detail description in Huang et al.(2013). It was measured on soil sampling day, i.e. on 16th April and 9th August respectively. Daily measurements were carried out for 10 days after the 10th leaf fertilization on 11st August in order to cover the whole N_2O peaking period during this N fertilization event. During the whole crop rotation, daily measurements were also carried out for 10 days after each fertilization event, and 5 days for rainfall or irrigation event; Emissions were measured twice per week and once a week when the soil was frozen(Huang et al., 2013).

Soil molecular analysis

DNA was extracted from the frozen soil using a developed method based on CTAB (Hexadecyl trimethyl ammonium Bromide) method (Griffiths et al. 2000) with some modifications. Diluted DNA (10ng/ul) was used to determine the 16S rRNA, *amoA* gene of bacteria (AOB), *nirS*, *nirK*, *narG* and *nosZ* genes. Real-time PCR were performed on Light cycler 96 system (Swiss, Roche). Each plate included purified plasmid standards and negative controls, also in triplicate. Data analysis was carried out using LightCycler®96 software.

Main Results and Discussion

Although long-term different nitrogen and carbon managements for 6 years from 2006 to 2012 have changed some soil chemical (total nitrogen and organic carbon content, nitrate content) and biological properties (potential nitrification and denitrification) significantly (unpublished), especially for CM treatment, the abundance of total bacteria (Fig.2a) was positively related with soil total nitrogen (P<0.01) and organic carbon content (P<0.05) because manual straw incorporation to the soil could improve soil fertility (Geisseler &Scow 2014, Liang et al. 2015). N_2O flux ($\mu g\ N_2O\text{-}N\ m^{-2}\ d^{-1}$) of 16th April and 9th August (Fig. 1a) were not affected by the soil properties significantly. However, short term fertilization led to N_2O emission on 14th August. N_2O emission had the same trend with *amoA* gene number (Fig.1b) on three sampling dates. Archaea (AOA) and bacteria (AOB) both carry *amoA* gene, but their contribution to N_2O emission is still debated, with previous studies showing that AOA exist widely in some extreme ecological environments but are not functional. In grass and agroecosystems, AOB was thought to be more important for N_2O emissions (He et al. 2007, Leininger et al. 2006, Prosser &Nicol 2008). A positive relationship was also found between abundance of *amoA* gene and annual N_2O emission in our study (Table 1). N_2O flux on 9th August was higher than 16th April because soil average temperature was higher in August (25-30℃) than April (0-5℃) which could influence soil microbial activity (Fig.1a). The other reason was that soil water content (unpublished) was higher on 9th August than 16th April and anaerobic microsites may have existed in concert with the presence of nitrite and nitrate. Little ammonium was detected in the soil on both dates (unpublished), therefore no substrate for nitrification or nitrifier denitrification N_2O emission on 14th August was mainly produced by nitrification from urea hydrolysis and nitrite and nitrate had accumulated , this finding was supported by other previous studied (Cui et al. 2012, Ju et al. 2011, Liu et al. 2011, Sexstone et al. 1985; Smith,1997). On the North China Plain, ammonia-oxidation generates N_2O in an intensively managed calcareous Fluvo-aquic soil and NH_4^+-based fertilizer could lead to N_2O emission in the following 7 to 15 days.

According to Huang (et al, 2013) there are high N_2O emissions after rainfall or irrigation during winter wheat-summer maize rotation system even without fertilization In our study, we could find that the annual N_2O emission of fertilized treatments were higher than N_0, especially for CN_{opt} and CM treatment (Fig. 1b) and influenced by denitrification gene numbers (Fig.4). Crop and soil management practices, such as the application of organic manure and inorganic fertilizers may influence soil microbial biomass and activity (Bohme et al., 2005), and whilst we could find some change on the DGGE fingerprint (unpublished) and PCoA plots (unpublished) based on three distances, this result was consistent with abundance of functional gene numbers. There was a significant correlation between annual N_2O and total nitrogen and organic carbon

content and potential denitrification rate.Annual N_2O emission and functional gene (*narG*, *nirS*, *nirK* and *nosZ*) number ($P<0.01$) (Table 1) during denitrification process also positive related. Significant correlation was also calculated between *amoA* gene number and annual N_2O emission ($P<0.05$). Although *nosZ* gene number (Fig.4d) increased in fertilized treatment, N_2O annual emission did not decrease, this may be due to the increased of *narG*, *nirS* and *nirK* gene numbers which could also explain the increased reduction of N_2O to N_2 by *nosZ* gene.

Table 1. Spearman's rank correlation matrix of annual N_2O emission, some soil properties, abundances of functional genes and 16S rRNA gene

N_2O[a]	1	1.00										
TN[b]	2	0.76**	1.00									
TOC[c]	3	0.66*	0.84**	1.00								
PNR[d]	4	0.53	0.52	0.60*	1.00							
PDNR[e]	5	0.83**	0.76*	0.81**	0.75**	1.00						
amoA	6	0.71*	0.34	0.53	0.73**	0.72**	1.00					
narG	7	0.74**	0.79**	0.78**	0.69*	0.90**	0.61*	1.00				
nirS	8	0.70**	0.82**	0.75**	0.71*	0.83**	0.59*	0.92**	1.00			
nirK	9	0.71**	0.69*	0.84**	0.75**	0.93**	0.77**	0.93**	0.85**	1.00		
nosZ	10	0.72**	0.59*	0.71**	0.75**	0.90**	0.82**	0.92**	0.85**	0.97**	1.00	
16S rRNA	11	0.78**	0.78**	0.80**	0.71**	0.93**	0.68*	0.98**	0.92**	0.96**	0.95**	1.00
		1	2	3	4	5	6	7	8	9	10	11

*$p<0.05$; **$p<0.01$; [a]Annual N_2O emission in 2012-2013 winter wheat-summer maize rotation
[b]Total nitrogen concentration in the soil; [c]Total organic carbon
[d]Potential nitrification rate; [e]Potential denitrification rate

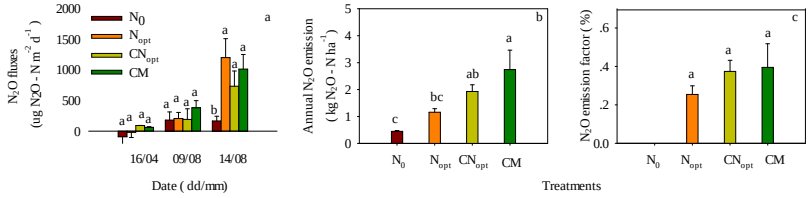

Figure 1. N_2O fluxes on the sampling dates in 2013 (a); N_2O data from the study year in the 2012-2013 winter wheat-summer maize rotation (b); and N_2O emission factor (c). Different letters indicate significant differences ($P < 0.05$) between pairs of treatments.

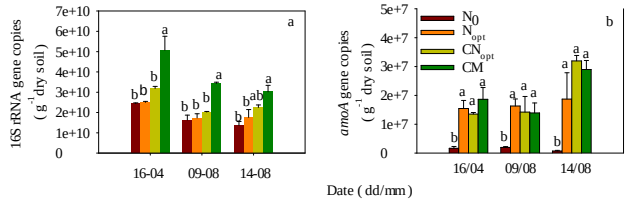

Figure 2. Gene copies of 16S rRNA (a) and ammonia monooxygenase gene (*amoA*) of bacteria (AOB) (b) of different treatments in 0-20cm soil depth in sampling dates in 2013. Different letters indicate significant difference ($P < 0.05$) among treatments

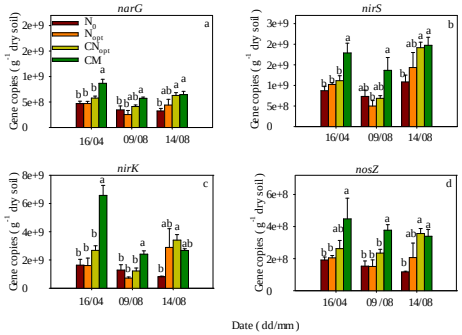

Figure 3. Gene copies of the nitrate reductase gene *narG*, nitrite reductase genes (*nirS)* and (*nirK)* and the N_2O reductase gene (*nosZ)* of different treatments in 0-20cm soil depth in sampling dates in 2013. Different letters indicate significant difference ($P < 0.05$) among treatments.

Conclusion

Our study highlights the linkage of instant high N_2O emission peaks with the function of the bacterial *amoA* gene for nitrification and of annual N_2O emissions and small N_2O pulse after rainfall or irrigation with the function of denitrification genes, providing insight into the mechanism of N_2O production and the factors controlled by distal and proximal drivers in this intensively managed calcareous fluvo-aquic soil. These findings will help to draw the pertinence measures for mitigating N_2O emissions in this hotspot region.

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