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Vertical distribution and activity of anaerobic ammonium-oxidising bacteria in a vegetable field

Li-dong Shen [a,b,*], Hong-sheng Wu [a,b], Xu Liu [b], Ji Li [b]

[a] Collaborative Innovation Center on Forecast and Evaluation of Meteorological Disasters, Jiangsu Key Laboratory of Agricultural Meteorology, College of Applied Meteorology, Nanjing University of Information Science and Technology, Nanjing 210044, China
[b] Department of Agricultural Resource and Environment, College of Applied Meteorology, Nanjing University of Information Science and Technology, Nanjing 210044, China

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ABSTRACT

Anaerobic ammonium oxidation (anammox) is an important nitrogen removal pathway in aquatic systems. However, the significance of anammox in nitrogen removal in terrestrial ecosystems, particularly in the agricultural ecosystems, is currently not well known. Here, we studied the vertical distribution and activity of anammox bacteria in a vegetable field (0–100 cm; 10-cm intervals) in Southeastern China. Three different genera of anammox bacteria were detected by Illumina-based bacterial 16S rRNA gene sequencing, including *Candidatus Kuenenia*, *Candidatus Brocadia* and *Candidatus Jettenia*, with *Candidatus Brocadia* being the dominant one. Quantitative PCR of hydrazine synthase genes showed that the anammox bacterial abundance ranged between 7.8×10^4 and 1.1×10^6 copies g^{-1} dry weight at different depths of soil cores, with higher values being observed in the upper soils (0–40 cm). Stable isotope labeling experiments showed that the potential rates of anammox varied between 1.1 and 17.5 nmol N_2 g^{-1} dry weight d^{-1} at different depths of soil cores, with higher rates being found in the subsurface soils (10–30 cm). The anammox process contributed 1.4–18.4% (relative to denitrification) to dinitrogen gas production in soil. Pearson correlation analyses suggested that the ammonium concentration in soil might have an important impact on the vertical distribution of anammox bacterial abundance ($r = 0.725$) and activity ($r = 0.452$) in the examined soil cores. The results of the present study showed that the importance of anammox in nitrogen removal in vegetable fields cannot be neglected, and indicated that the subsurface soils are the preferred habitats for anammox bacteria.

1. Introduction

Previously, heterotrophic denitrification was identified as the only important microbial-mediated process converting fixed nitrogen to atmospheric dinitrogen gas (N_2) in the environment. The discovery of anaerobic ammonium oxidation (anammox), which ammonium can be oxidised via nitrite under anoxic conditions with N_2 as the end product (Mulder et al., 1995; Strous et al., 1999), enriched the content of the microbial nitrogen cycle in nature. There are a total of five different genera of anammox bacteria have been described to date, including *Candidatus Kuenenia* (Schmid et al., 2000), *Candidatus Brocadia* (Strous et al., 1999; Kartal et al., 2008), *Candidatus Anammoxoglobus* (Kartal et al., 2007), *Candidatus Jettenia* (Quan et al., 2008; Ali et al., 2015) and *Candidatus Scalindua* (Schmid et al., 2003; van de Vossenberg et al., 2013).

The anammox process has been proven to be an important pathway for nitrogen removal in the ocean, which is responsible for approximately 50% of the N_2 production (Arrigo, 2005; Brandes et al., 2007). Presently, the occurrence and activity of anammox bacteria have been reported in a variety of aquatic systems, such as deep-sea sediments (Schmid et al., 2007; Song et al., 2013; Bale et al., 2014), marine oxygen minimum zones (Kuypers et al., 2005; Lam et al., 2009; Brabandere et al., 2014), estuarine sediments (Dale et al., 2009; Hu et al., 2012a; Hou et al., 2013), river sediments (Hu et al., 2012b; Sun et al., 2014; Zhou et al., 2014), lake sediments (Yoshinaga et al., 2011; Zhao et al., 2013; Zhu et al., 2013) and lake water columns (Schubert et al., 2006; Hamersley et al., 2009; Wenk et al., 2013).

As compared with these reported aquatic systems, the activity and significance of anammox bacteria in terrestrial realm is not well studied. Soils are a heterogeneous and complex habitat which contains different types of microenvironments. The anoxic environments can be found in the bulk soils (within soil macro-aggregates), the rhizosphere soils because of respiration of plant roots and microorganisms, and the soil-groundwater table interface including its fluctuation zone (Humbert et al., 2010). The anoxic environments (particularly the oxic/anoxic interface) in soil could be favourable for anammox bacteria (Humbert et

* Corresponding author at: Collaborative Innovation Center on Forecast and Evaluation of Meteorological Disasters, Jiangsu Key Laboratory of Agricultural Meteorology, College of Applied Meteorology, Nanjing University of Information Science and Technology, Nanjing 210044, China.
E-mail address: shenld@nuist.edu.cn (L. Shen).

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al., 2010). Thus far, several studies have reported the distribution and activity of anammox bacteria in freshwater wetland soils (Humbert et al., 2010, 2012; Zhu et al., 2013; Naeher et al., 2015). Agriculture is one of the dominant forms of land management, and agricultural ecosystems cover approximately 40% of the terrestrial surface of the Earth (Power, 2010). Agricultural ecosystems are very important man-made ecosystems and essential to human life, which can provide human with food, forage, bioenergy and pharmaceuticals (Power, 2010). Nitrogen fertilisation is crucial for achieving high yields of field crops. In the meantime, it can also cause serious environmental problems (e.g. eutrophication of lakes and rivers resulting from agricultural run-off). Accurate quantification of various nitrogen removal pathways is of great importance for better understanding of the fate of inorganic nitrogen in agricultural soils, which may provide basis for development of potential management strategies for reducing nitrogen loss in agricultural ecosystems (Yang et al., 2015). Until now, a few studies have reported the occurrence and activity of anammox bacteria in paddy soils, and the anammox process was reported to contribute ~37% to N_2 production in soil (Zhu et al., 2011a; Sato et al., 2012; Shen et al., 2014; Yang et al., 2015), suggesting that this process can be served as an important nitrogen loss pathway in paddy fields. Vegetable fields are characterised with intensive nitrogen fertilisation for promoting crop yield. In China, the total rate of nitrogen fertilisation during individual vegetable growing seasons varied between 300 and 700 kg N ha^{-1} and even more in some cases (Zheng et al., 2004; He et al., 2009). Theoretically, the high rate of nitrogen fertilisation can lead to an increasing nitrogen load in vegetable soils, and thus could make them as suitable habitats for nitrogen-utilising microbes (e.g. anammox bacteria and denitrifiers). Humbert et al. (2010) provided the molecular evidence of anammox bacteria in a wide range of soil environments, which include the agricultural soils and samples associated with nitrophilic or nitrogen-fixing plants. Recently, Shen et al. (2015) reported the anammox activity in the subsurface soils of vegetable fields. It is still unknown whether anammox bacteria are active or not at different depths of vegetable soils. Therefore, the quantitative importance of the anammox process in removing nitrogen in vegetable fields is still poorly known.

The primary objective of the present study was to investigate the vertical distribution, abundance and activity of anammox bacteria in a vegetable field in Southeastern China to better understand the importance of anammox in removing nitrogen in vegetable fields. Different depths of vegetable soils (0–100 cm; 10-cm intervals) were selected and analysed in the current study.

2. Materials and methods

2.1. Soil sample collection

Soil samples were obtained from a vegetable field in Nanjing City, Jiangsu Province (China), and the field was planted with cabbage (Fig. S1). The field is affected by periodic flooding, which the bulk soil experiences periodic water saturation because of irrigation and rainfall. Nitrogen fertilizer is typically applied as ammonium nitrate, or ammonium sulphate. In this study, five intact soil cores (5-cm diameter and 100-cm depth) were collected in October 2014, and sliced at 10-cm intervals in the field. The soil cores were placed in sterile plastic bags and transported to the laboratory within 3 h, and subsequently divided into three individual parts. The first part was incubated in vials immediately after arrival at the laboratory to determine the potential anammox rates and denitrification rates, and the second part was stored at 4 °C for physicochemical analyses within one week. The last part was stored at −20 °C for later molecular analyses.

2.2. Chemical analyses

Soil ammonium and nitrate were extracted from the collected soil cores by using 2 M KCl as described in Shen et al. (2013), and their concentrations were determined according to the previously reported studies (Dorich and Nelson, 1984; Kempers and Zweers, 1986). The soil pH was determined after mixing the soil with denoised water at a ratio of 1:2.5 (soil/water). The total nitrogen (TN) concentration in soil was determined using the FOSS Kjeltec™2300 analyser (FOSS Group, Sweden), and the organic carbon (OrgC) concentration in soil was determined by the $K_2Cr_2O_7$ oxidation method (Bao, 2000). The water content in soil was determined by oven drying overnight at a temperature of 110 °C. All the physicochemical parameters were determined in triplicate in this study.

2.3. Isotope tracer experiments

The potential rates of anammox and denitrification were measured according to the previously reported methods (Risgaard-Petersen et al., 2004; Engström et al., 2005). Briefly, approximately 5 g of fresh soil was transferred into the He-flushed glass vial (30 ml), together with 20 ml of degassed deionised water. The soil slurries were pre-incubated for 2 d to remove the residual NO_x^- and dissolved oxygen. The slurries were then divided into three groups amending different nitrogen compounds: (i) 100 μM $^{15}NH_4Cl$ (^{15} N at 99.6%), (ii) 100 μM $^{15}NH_4Cl$ plus 100 μM $Na^{14}NO_3^-$, and (iii) 100 μM $Na^{15}NO_3^-$ (^{15}N at 99.6%). Incubation of the slurries was stopped by injecting 50% $ZnCl_2$ solution into the vials. Three replicates per sample of each treatment group were performed. The potential rates of anammox and denitrification were calculated based on the production of $^{29}N_2/^{30}N_2$ within the headspace of vials (measured by a gas chromatograph-mass spectrometer; Agilent 7890/5977A inert MSD; Agilent, United States) amended with $^{15}NO_3^-$ only using the equations reported by Yoshinaga et al. (2011).

2.4. DNA isolation

Soil DNA was extracted using a Power Soil DNA kit (Mo Bio Laboratories, Carlsbad, California, USA) according to the manufacturer's instructions. Approximately 0.25 g homogenised soil was used for the isolation of DNA. The quality and the concentration of the extracted DNA were evaluated and determined on a NanoDrop spectrophotometer (ND-1000; Isogen Life Science, the Netherlands).

2.5. Illumina-based 16S rRNA gene sequencing

High-throughput sequencing of total bacterial 16S rRNA genes was performed on the Illumina MiSeq platform. Briefly, the V3-V4 region of bacterial 16S rRNA genes was amplified from the soil DNA extracts with the primer 319f (5′-ACTCCTACGGGAGGCAGCAG-3′) and primer 806r (5′-GGACTACHVGGGTWTCTAAT-3′) according to the previously reported studies (Fadrosh et al., 2014; Shen et al., 2016). Briefly, the PCR reaction (30 μl) mixtures contained 1.0 μl of forward primer (10 mM), 1.0 μl of reverse primer (10 mM), 5.0 μl of DNA sample, 0.5 μl of dimethyl sulphoxide, and 15.0 μl of Phusion High-Fidelity PCR Master Mix with HF Buffer. The thermal program cycle was performed with an initial melting step for 30 s at 98 °C, followed by 30 cycles of denaturation at 98 °C for 15 s, annealing at 58 °C for 15 s and elongation at 72 °C for 1 min. The PCR products were purified and then subjected to Illumina MiSeq sequencing (300 bp paired-end protocol). The average length of the recovered bacterial 16S rRNA gene sequences was 435 bp. Analysis of Mi-Seq sequencing data was conducted using Quantitative Insights Into Microbial Ecology (QIIME, www.qiime.org) as described in Shen et al. (2016). High-quality anammox bacterial 16S rRNA gene sequences were confirmed by aligning these sequences with GenBank database using the BLAST search engine. Each operational taxonomic unit (OTU) was defined by 97% 16SrRNA gene sequence identity.