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Short communication

Denitrification-dependent anammox activity in a permanently flooded fallow ravine paddy field

Sheng Zhou [a,*], Sodbilig Borjigin [b,1], Shohei Riya [b], Masaaki Hosomi [b]

[a] Eco-environmental Protection Research Institute, Shanghai Academy of Agricultural Sciences, No.1000 Jinqi Road, Shanghai 201403, China
[b] Institute of Engineering, Tokyo University of Agriculture & Technology, 2-24-16 Naka-cho, Koganei, Tokyo 184-8588, Japan

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ABSTRACT

It is vital to understand the nitrogen dynamics in ravine paddy fields due to their nitrogen removal capacity, providing a potential method for non-point source pollution treatment. This study measured bacterial anaerobic ammonium oxidation (anammox) and denitrification activity using a nitrogen isotope pairing technique (IPT) in fallow ravine paddy soil that is permanently flooded with seepage water from an upland vegetable field. The inhibition experiment explored the correlation between anammox activity and the denitrification processes, using chlorate to inhibit the first step of denitrification.

Denitrification activity decreased with increasing paddy soil depth; with anammox activity showing a similar trend, but with even greater declines in activity. In addition, when nitrate reduction to nitrite was inhibited, anammox activity exponentially decreased, likely to be due to a reduction in the intermediate NO_2^-. Furthermore, the rate of reduction in anammox activity was significantly greater than the reduction in denitrification. This study presents the first direct exploration of the correlation of denitrification with anammox activity in ravine paddy fields receiving nitrate polluted water.

1. Introduction

Anaerobic ammonium oxidation (anammox) is an important process in the microbial nitrogen cycle, where NH_4^+ (ammonium) is oxidized with NO_2^- (nitrite) under anaerobic conditions (Strous et al., 1998). The discovery and potential contribution of anammox bacteria to N_2 production is important, as this system of nitrogen transformation affects the entire global nitrogen budget. N_2 production from anammox activity has been detected in a wide range of ecosystems from marine sediments (Thamdrup and Dalsgaard 2002; Kuypers et al., 2003), and estuarine/tidal river sediments (Trimmer et al., 2003; Meyer et al., 2005), to freshwater ecosystems (Schubert et al., 2006; Hamersley et al., 2009) using a ^{15}N isotope labeling technique. Depending on different environmental conditions, there is a range in the reported contributions of anammox to total N_2 flux (Terada et al., 2011), for example only a minimal contribution was found at the Thames River estuary (Trimmer et al., 2003), although an approximate contribution of 70% was established at the Danish belt seaway (Thamdrup and Dalsgaard 2002).

The Anammox reaction is defined as $NO_2^- + NH_4^+ \rightarrow N_2 + 2H_2O$. NO_2^- supply is a key limiting factor of the process, as NH_4^+ is usually abundant in anoxic sediment, although NO_2^- can be provided through the oxidation of NH_4^+ to NO_2^- by ammonium-oxidizing bacteria in aerobic environments, or from NO_3^- reduction by denitrifying bacteria in anoxic environment (Meyer et al., 2005). The association between anammox activity and the denitrification processes suggests that denitrifying bacteria may be a primary source of NO_2^- for anammox bacteria, due to high levels of denitrification activity in anoxic freshwater sediments (Hou et al., 2013). This is supported by previous studies, which have shown that NO_2^- used by anammox bacteria is produced as an intermediate during the first step of the denitrification process in anoxic sediment (Zhou et al., 2014), suggesting that the relative contribution of anammox activity to total N_2 production is likely to be dependent on the rate of denitrification. However, the relationship between anammox activity and denitrification has not been sufficiently investigated in agricultural environments.

This study investigates the relationship between anammox and denitrification in abandoned ravine paddy fields. Un-cultivated paddy fields, can potentially function as wetlands for water quality purification and nitrogen removal (Tabuchi et al., 1993; Abe et al., 2014). Before anammox activity was confirmed, denitrification was thought to be the sole driver of microbial N_2 production processes in paddy fields (Kuroda et al., 2005; Zhou and Hosomi, 2008). More

* Corresponding author.
E-mail addresses: zhous@outlook.com, szhou@saas.sh.cn (S. Zhou).
[1] Present address: Inner Mongolia Tech-Luck Environmental Production and Energy Saving Co., Ltd., Hohhot 010070, China.

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recently, anammox bacterial activity has been confirmed in paddy fields, establishing a new system for nitrogen removal (Zhu et al., 2011; Sato et al., 2012; Yang et al., 2015), however, more information is needed about the contribution of anammox activity to total N_2 production, as well as the anammox pathway mechanisms in paddy field environments compared with marine or estuary environments.

In Southern China, anammox activity has been reported to contribute between 4 and 37% of N_2 production in paddy field soil with high ammonia concentrations caused by manure slurry application and the association of both archaeal and bacterial nitrification to anammox is major progress in understanding the mechanisms of anammox activity (Zhu et al., 2011). Studies have recently found anammox bacteria to be present in 12 typical paddy soils in southern China, where anammox rates ranged from 0.27 to 5.25 nmol N g^{-1} soil h^{-1}, contributing 0.6–15% to soil N_2 production (Yang et al., 2015). However, nitrogen transformations in paddy fields continuously receiving an excess of nitrate from polluted water may differ from paddy fields applied with high ammonia manure, but may show some similarities to nitrate-polluted river sediment.

Ravine paddy fields are important agricultural components in Japan, as well as hill areas of Southwest China and South East Asia. The paddy fields are usually located among mountains and are permanently flooded with seepage water from adjacent plateaus. When nitrogen fertilizers are used to support agricultural production on adjacent plateaus, the ground water that seeps into the ravine paddy fields is usually highly polluted with nitrates (Kuroda et al., 2005). It is vital to understand the nitrogen dynamics in ravine paddy fields in order to potentially harness the nitrogen removing ability of paddy fields, providing a specific method of non-point source pollution treatment. Recently, DNA clone library analysis of the microbial community in the ravine paddy field used in the present study, revealed DNA related various species of the *Candidatus genus*, *C. brocadia fulgida*, *C. brocadia anammoxidans* and *C. kuenenia stuttgartiensis*, suggesting that this specific population of anammox bacteria may be highly relevant in nitrogen removal via the anammox pathway in ravine paddy fields (Sato et al., 2012). However, there is little information known about anammox microbial activity and denitrification in ravine paddy fields or how anammox activity is affected by the enhancement or inhibition of denitrification, therefore, the present study investigates the relationship between anammox and denitrification in a fallow ravine paddy field environment.

2. Materials and methods

2.1. Study site and sampling method

The study site of the ravine paddy field (35°59′07.05″N, 140°16′23.92″E) was located in Ibaraki prefecture on the Kanto plains of Japan, which has been maintained as an experimental paddy field for nitrate removal studies for 20 years. The ravine paddy field is continuously flooded with seepage water from an upland vegetable field on the adjacent plateau. An experimental plot of ravine paddy field (25 m × 1.4 m) surrounded with a protective plastic frame was used for this study, which had been maintained in an uncultivated and non-vegetative state without any additive fertilization since 1991. However as stated, seepage water continuously flows into the fallow ravine paddy field containing a high concentration of NO_3^- (Kuroda et al., 2005), originating from the fertilizers and manure applied to the cultivated upland paddy fields (Fig. 1).

Surface water and soil samples were collected within an approximate 1 m distance from the seepage water inlet at the

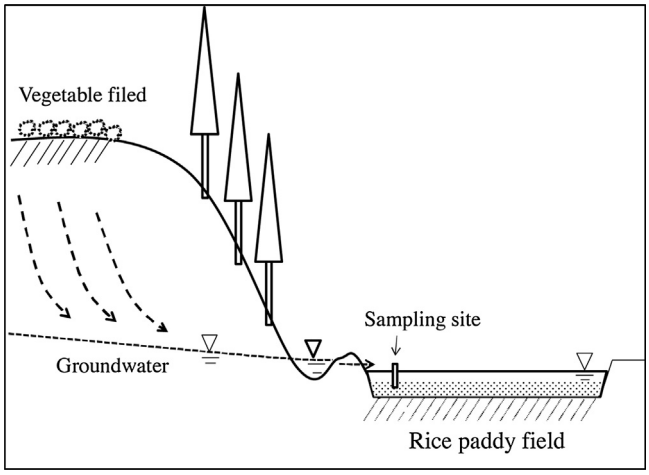

Fig. 1. Schematic diagram of ravine rice paddy fiel.
revised from Sato et al. (2012)

experimental site (Fig. 1). Triplicate soil samples collected to a depth of 10 cm using a plastic core sampler in both June and October 2012. Samples were sliced into 2-cm-thick segments, from 0–10 cm below the surface after the overlying surface water was removed. The soil samples from the same depths (0–2, 2–4, 4–6, 6–8, and 8–10 cm) from triplicate core soil samples were pooled and homogenized. Each homogenized sample according to depth was then further subdivided into duplicate replicates (Amano et al., 2007; Rich et al., 2008). These samples were then used to establish the anammox/denitrification activity profiles according to soil depth in June and October investigation. In addition, additional triplicate soil samples were collected from a depth of 0–5 cm at the same sampling site in October 2012, for chlorate inhibition analysis. Soil samples used for activity assessment were immediately placed in a 250 mL glass bottle (Duran Co. Ltd., Germany), which was tightly sealed and stored at 4 °C in the dark until experiments took place within a week. All accompanying surface water samples were collected at the same time as soil samples, for water quality analysis. All samples were placed in 50 mL tubes (As one Co. Ltd., Japan) and held at 4 °C during transport to our laboratory (2 h), where the water samples were filtered and frozen (−20 °C) for later analysis (Hamersley et al., 2009). In addition, soil samples collected for pore water quality analysis were centrifuged for 10 minutes at 10,000 rpm, with the liquid supernatant of soil samples then stored at −20 °C. Both surface water and pore water samples were analyzed within 1 week from sample collection, for the following major parameters: NO_3^-, NO_2^- and NH_4^+. Ion content was measured using an ion chromatograph (ICS-90, Dionex, Sunnyvale, USA) with an anionic column (IonPac@ AS12A) or cationic column (IonPac@ CS12A).

2.2. Measurement of anammox and denitrification

Anammox and denitrification activities at different soil depths were examined in duplicate by anaerobically incubating soil slurries combined with ^{15}N-labeled or non-labeled NH_4Cl and $NaNO_3$ (Amano et al., 2007; Yoshinaga et al., 2010; Zhou et al., 2014). There are three steps involved in the assessment of anammox and denitrification activity: (1) substrate pre-incubation; (2) the addition of ^{15}N labeled NH_4^+ or NO_3^-; (3) analysis of $^{29}N_2$, $^{30}N_2$ and calculation of anammox and denitrification activity. The detailed method of incubation, measurement and calculation methods used in this study are given in the previous publication (Zhou et al., 2014), with