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Shifts in *Nitrobacter*- and *Nitrospira*-like nitrite-oxidizing bacterial communities under long-term fertilization practices

Shun Han[a], Luyang Zeng[a,b], Xuesong Luo[a,b], Xiang Xiong[a,b], Shilin Wen[c], Boren Wang[c], Wenli Chen[a,*], Qiaoyun Huang[a,b,**]

[a] State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan, 430070, China
[b] Key Laboratory of Arable Land Conservation (Middle and Lower Reaches of Yangtze River), Ministry of Agriculture, College of Resources and Environment, Huazhong Agricultural University, Wuhan, 430070, China
[c] Hengyang Red Soil Experimental Station, Chinese Academy of Agricultural Sciences, Hengyang, 421001, China

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ABSTRACT

Nitrite-oxidizing bacteria (NOB) are key players in the second step of nitrification, which is an important process in the soil nitrogen (N) cycle. However, the ecology of nitrite oxidizers and their response to disturbances such as long-term fertilization practices are scarcely known in agricultural ecosystems. We used samples from a Red soil subject to a long-term chemical and organic fertilization experiment, including control without fertilizer (CK), swine manure (M), chemical fertilization (NPK), and chemical/manure combined fertilization (MNPK) treatment, to explore how agricultural practices impact the community structure, abundance, and potential activity of nitrite oxidizers (PNO). The abundance of *Nitrobacter* was significantly increased in the M and MNPK plots, whereas the abundance of *Nitrospira* was significantly reduced in the M and NPK treatment plots and less inhibited in the MNPK treatment. The PNO showed a similar trend to that for *Nitrobacter* abundance. The diversity of *Nitrobacter* increased in the M-treated plots, while that of *Nitrospira* increased in the M and MNPK plots and decreased in the NPK plots. Non-metric multidimensional scaling (NMDS) revealed that the *Nitrobacter*- and *Nitrospira*-like NOB community was shift in these four fertilization treatments. Redundancy analysis showed that pH + SOC (soil organic carbon) and pH + TN (total nitrogen) significantly explained the variation in the composition of *Nitrobacter* and *Nitrospira*, respectively. In addition, the *Nitrospira*/*Nitrobacter* abundance ratio and community structure of *Nitrobacter*- and *Nitrospira*-like NOB are responsible for the changes of soil PNO. Collectively, these data suggest that the nitrite-oxidation process in the red soil is possibly controlled by both *Nitrospira* and *Nitrobacter*-like NOB, which were shaped by pH + TN and pH + SOC, respectively.

1. Introduction

Nitrification, the microbiological oxidation of ammonia (NH_4^+) to nitrite (NO_2^-) and subsequently to nitrate (NO_3^-), influences the fate of nitrogen (N) in terrestrial systems. The first limiting step of nitrification, NH_4^+ oxidation to NO_2^-, is mediated by ammonia-oxidizing bacteria (AOB) of the β- and γ-*Proteobacteria,* ammonia-oxidizing archaea (AOA) of the *Thaumarchaeota* (Kowalchuk and Stephen, 2001; Leininger et al., 2006; Schleper and Nicol, 2010; Norton and Stark, 2011). The second step of nitrification, NO_2^- oxidation to NO_3^-, is catalyzed by nitrite-oxidizing bacteria (NOB), which play an important role in the biogeochemical N cycle in many terrestrial ecosystems such as soils (Prosser, 1989; Daims et al., 2015). NOB are broadly distributed among the α-, β-, γ-, and δ-*Proteobacteria* as well as the *Nitrospira* classs (Gould and Lees, 1960; Teske et al., 1994; Alawi et al., 2007; Sorokin et al., 2012). Comammox *Nitrospira* complete oxidation of ammonium to nitrate for the nitrification (Daims et al., 2015; van Kessel et al., 2015).

In recent years, many studies have focused on the ecology of AOA and AOB in a broad range of soil environments (Kowalchuk and Stephen, 2001; Webster et al., 2005; Chu et al., 2007; Chen et al., 2008; Shen et al., 2012; Bertagnolli et al., 2016; Zhang et al., 2017). Ammonia oxidation is often assumed to be the rate-limiting step of nitrification as nitrite generally does not accumulate in the environment. In fact, nitrite oxidation can also become the limiting step for nitrification in disturbed soil systems (Gelfand and Yakir, 2008; Roux-Michollet et al., 2008). In view of the importance of nitrite oxidizers, it is urgently needed to decipher the ecological response of nitrite oxidizer community to any

* Corresponding author. State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan 430070, China.
** Corresponding author. State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan 430070, China.
E-mail addresses: wlchen@mail.hzau.edu.cn (W. Chen), qyhuang@mail.hzau.edu.cn (Q. Huang).

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type of environmental disturbances, such as different fertilization regimes in agricultural soils.

Nitrobacter-like NOB are considered to be the key functional players within the NOB community, exhibiting high activity levels in tillage/no-tillage agricultural systems with high N availability (Attard et al., 2010). In addition, soil potential nitrite oxidation activity (PNO) is strongly positively correlated with the abundance of *Nitrobacter*-like NOB as well as weakly negatively correlated with the abundance of *Nitrospira*-like NOB. Possible changes were also observed for the abundance and community of *Nitrobacter* and *Nitrospira* in agricultural soils in response to tillage practices. In contrast, fertilization causes rapid shifts in the structure of the *Nitrobacter*-like NOB community, which dominates nitrification in forest soils (Wertz et al., 2011). The application of manure in a Merzenhausen agricultural soil was found to have decreased the diversity of *Nitrobacter* community in the rhizosphere (Ollivier et al., 2013). Nitrite oxidation in surface agricultural soils may be predominantly driven by *Nitrospira* spp. (Ke et al., 2013). Han et al. (2017) showed that *Nitrospira* might be more responsive than *Nitrobacter* in soils under a rapeseed-rice rotation. In addition, the *Nitrospira*-like NOB community was found to be significantly shaped by soil pH, moisture, and NH_4^+ content, whereas the *Nitrobacter*-like NOB community was not. In acidic forest soils, long-term fertilization increased AOB and *Nitrobacter*-like NOB abundances but did not influence AOA and *Nitrospira*-like NOB abundances (Wertz et al., 2011). However, the responses of the community structure and diversity of NOB to long-term fertilization regimes and their links with nitrite oxidation activities in agricultural soils are still not well understood. The ways in which NOB populations respond to changes in soil pH, specifically the acidification of terrestrial environments, have rarely been investigated.

In this study, samples from a Red soil in the Qiyang Long Term Soil Experimental Station, Hunan Province, China was used to investigate the ecological effects of long-term fertilization practices on the *Nitrobacter*- and *Nitrospira*-like bacterial community and their potential activity. Quantitative PCR (qPCR) and high-throughput sequencing of marker genes were performed to investigate the abundance, community diversity, and population composition of *Nitrobacter*- and *Nitrospira*-like NOB in the soil. We hypothesized that (1) chemical fertilization may have a negative impact on the activity and diversity of NOB; (2) manure would have a positive effect on NOB community structure, as it provides both inorganic and organic nutrients for NOB and does not lead to acidification; (3) the combined use of manure and chemical fertilizers would represent a balanced fertilization treatment, inhibiting soil acidification and balancing the negative effect of chemical fertilizers on NOB activity and diversity.

2. Material and methods

2.1. Experimental site and sampling

The experimental site was located at the Qiyang Red Soil Experimental Station (26°45′N, 111°52′E), Hunan Province, China. This site represents a typical agricultural region of subtropical China. It has a subtropical monsoon climate with an annual rainfall of 1300 mm and annual average temperature of 18 °C. A long-term fertilizer experiment was established in 1990 with a winter wheat (*Triticum aestivum* L.) and summer maize (*Zea mays* L.) rotation system, including three replicates of four treatments in a randomized plot design: control without fertilizers (CK); swine manure (M); chemical fertilization (nitrogen, phosphate, and potassium fertilizers, NPK); and chemical/organic combined fertilization (nitrogen, phosphate, potassium, and swine manure fertilizers, MNPK). The NPK-treated soil was severely acidified. The nitrogen fertilizer was applied as urea or swine manure at 300 kg N ha^{-1}, phosphate (P) as a single application of superphosphate [$Ca(H_2PO_4)_2$] at 53 kg P ha^{-1}, and potassium (K) as potassium chloride (KCl) at 100 kg K ha^{-1}.

Soil samples were collected at a depth of 0–20 cm in November 2016. Six soil cores (approximately 5 cm in diameter) were taken from each plot and mixed to form one composite sample. Samples were placed in a sterile plastic bag for transport to the laboratory within 24 h after collection. Each soil sample was divided into three portions. One portion was used for DNA extraction and stored at −80 °C, another was stored at 4 °C for measuring PNO, and the third was air-dried at room temperature for analysis of soil chemical properties.

2.2. Soil chemical analytical procedures

Soil total carbon (TC) and total nitrogen (TN) contents were analyzed using a Vario Max element analyzer (Elementar Vario PYRO cube and Isoprime100, Germany). Soil exchangeable ammonium (NH_4^+-N) and nitrate (NO_3^--N) contents were determined on a FIAstar 5000 Analyzer (Foss Tecator, Denmark) after extraction from fresh soil with 2 M KCl (w/v, 1:5). Soil pH was determined at a soil/water ratio of 1:2.5, and soil organic content (SOC) was determined by the $K_2Cr_2O_7$ oxidation method.

2.3. Assays for determination of PNO

PNO was determined using the method described by Wertz et al. (2007), modified from Smorczewski and Schmidt (1991). Briefly, samples of fresh soil (5 g equivalent dry mass) were incubated with 50 ml of a solution of $NaNO_2$ (5 μg of N-NO_2 g^{-1} dry soil) dissolved in autoclaved deionized water for 30 h with gentle shaking (180 rpm) at 28 °C. During incubation, 2.0-ml aliquots of the suspensions were sampled at 0, 4, 8, 12, 24, and 30 h and centrifuged (5000 rpm for 5 min). The supernatants were filtered (0.2-μm pore size) and analyzed for nitrite (N-NO_2^-) concentration on a spectrophotometer (L6, Shanghai, China) at 520 nm using Griess reagent at room temperature (Smorczewski and Schmidt, 1991). Levels of nitrite generally decreased linearly with time throughout the first 12 h until nitrite was depleted. Rates were calculated from the linear decrease and taken as the PNO.

In this study, according to the literature, we used nitrite solution to measure the potential value directly without considering the ammonia oxidation process (Smorczewski and Schmidt, 1991; Wertz et al., 2007; Attard et al., 2010). The nitrite produced from ammonia oxidation process during incubation was not taken into account.

2.4. Soil DNA extraction

DNA was extracted from 0.5 g soil using Lysing MatrixB tubes (Bio-101) as described previously by Griffiths et al. (2000). Humic acids, a PCR inhibitor, were removed from the soil DNA using DNA-EZ Reagents M Humic Acid-Be-Gone B (Sangon Biotech, Shanghai, China). Purification was performed according to the manufacturer's instructions. The quality and concentration of DNA were determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.5. Measurement of Nitrobacter- and Nitrospira-like NOB abundance by qPCR

QPCR assays were conducted using an ABI7500 FAST Real-time PCR system with the *nxrA* primers F1norA and R2norA (Attard et al., 2010) for *Nitrobacter*-like NOB and the *nxrB* gene primers *nxrB*169f and *nxrB*638r (Pester et al., 2014) for *Nitrospira*-like NOB. The 20-μl PCR reaction mixtures contained 10 μl SYBR Premix Ex Taq II (2×) (Takara, Bio Inc., Shiga, Japan), 1.0 μl of a 10 mM solution of each primer, 6.0 μl DEPC-treated water, and 2.0 μl soil extract (diluted to 5 ng/μl) or 2.0 μl standard plasmid. A standard curve was generated using ten-fold serial dilutions of a plasmid containing a copy of the target gene. The following program was used for the *nxrA* gene: 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s. For the *nxrB* gene, the program