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Long-term fertilization effects on active ammonia oxidizers in an acidic upland soil in China

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ABSTRACT

The effects of long-term fertilization of acidic soils on ammonia-oxidizing archaea (AOA) and bacteria (AOB) communities and its ecological implications remain poorly understood. We chose an acidic upland soil site under long-term (27-year) fertilization to investigate ammonia oxidizer communities under four different regimes: mineral N fertilizer (N), mineral NPK fertilizer (NPK), organic manure (OM) and an unfertilized control (CK). Soil net nitrification rates were significantly higher in OM soils than in CK, N or NPK soils. Quantitative analysis of the distribution of *amoA* genes by DNA-based stable isotope probing revealed that AOA dominate in CK, N and NPK soils, while AOB dominate in OM soils. Denaturing gradient gel electrophoresis and clone library analyses of *amoA* genes revealed that Group 1.1a-associated AOA (also referred to as *Nitrosotalea*) were the most dominant active AOA population (>92%), while *Nitrosospira* Cluster 3 and Cluster 9 were predominant among active AOB communities. The functional diversity of active ammonia oxidizers in acidic soils is affected by long-term fertilization practices, and the responses of active ammonia oxidizers to mineral fertilizer and organic manure are clearly different. Our results provide strong evidence that AOA are more highly adapted to growth at low pH and low substrate availability than AOB, and they suggest that the niche differentiation and metabolic diversity of ammonia oxidizers in acidic soils are more complex than previously thought.

1. Introduction

The nitrification process is a crucial part of the N cycle, which is required for the mobilization of ammonium in soils. Nitrate, the product of nitrification, may contaminate groundwater and promote denitrification, producing the greenhouse gas N_2O, which is the key process of nitrification in the soil N cycle. The first and rate-limiting step of nitrification is the oxidation of ammonia, which is carried out by chemolithoautotrophic ammonia-oxidizing archaea (AOA) and bacteria (AOB) (Gruber and Galloway, 2008). AOA and AOB have different physiological characteristics, ecological niches and environmental adaptations (Gubry-Rangin et al., 2011; Alonso-Sáez et al., 2012; He et al., 2012; Hu et al., 2013). Key factors affecting the growth, geographical distribution and ecological influence of ammonia oxidizers include pH, substrate concentration and organic matter (He et al., 2012; Prosser and Nicol, 2012), with pH serving as the main determinant in the geographical distribution of ammonia oxidizers in undisturbed soils (Gubry-Rangin et al., 2011; Hu et al., 2013). Compared with AOB, AOA have a higher affinity for ammonia (Martens-Habbena et al., 2009), and these microorganisms have unique metabolic pathways with the potential to use (or to be stimulated by) organic substrates (Alonso-Sáez et al., 2012).

Acidic soils (pH < 5.5) occupy 30% of the earth's ice-free land area. More than half of the acidic soil area is dedicated to agriculture or has potential agricultural uses (Von Uexküll and Mutert,

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1995). The agricultural use of acidic soil often is accompanied by the application of large amounts of organic and inorganic N fertilizers, which results in dramatic changes in soil pH, nitrification substrates and organic matter content. The application of large amounts of inorganic N fertilizers promotes nitrification and further acidifies the soil. The acidification of soils is often dealt with by the addition of organic fertilizers to increase the pH of the soil (Zhong et al., 2010; Saiful Alam et al., 2013), a practice that tends to accelerate soil nitrification and may compound the problem (He et al., 2007). AOA are often considered to be the most abundant group of ammonia oxidizers in acidic soils (Lu et al., 2012; Zhang et al., 2012; Wang et al., 2014). Our understanding of the relative distribution of AOA and AOB in acidic soils, which are managed differently, as well as their relative contribution to the nitrification process, remains limited (Lu et al., 2012; Zhang et al., 2012; Wang et al., 2014).

Long-term fertilization has been shown to affect nitrification and the community abundance and composition of ammonia oxidizers (Chu et al., 2007; He et al., 2007; Shen et al., 2008; Chen et al., 2011; Wu et al., 2011; Saiful Alam et al., 2013). Studies of neutral and alkaline soil have revealed that high levels of N application dramatically alter the community abundance and composition of AOB, but this practice has less of an effect on AOA (Chu et al., 2007; Shen et al., 2008; Wu et al., 2011). However, studies of acidic soils have revealed dramatic changes in the AOA community under long-term fertilization (He et al., 2007; Chen et al., 2011; Saiful Alam et al., 2013). To date, the effect of long-term fertilization on ammonia oxidizers in acidic soil has been examined using total nucleic acids, but the active populations of ammonia oxidizers and their composition remain unknown.

The environmental adaptation, niche differentiation and metabolic diversity between soil AOA and AOB populations seem to be different. Long-term fertilization with mineral fertilizers or organic manure alters soil pH, substrate concentration and organic matter composition, and we hypothesized that these changes may affect the functional populations of AOA and AOB and their contribution to soil nitrification. Thus, we chose a long-term (>27 years) field fertilization experimental site to investigate the responses of active ammonia oxidizers (both AOA and AOB) and their composition to long-term application of mineral fertilizers and organic manure. We used DNA-based stable isotope probing (DNA-SIP) to trace the active ammonia oxidizers, and we employed quantitative-PCR (q-PCR), denaturing gradient gel electrophoresis (DGGE) and clone library methods to determine their abundance and composition.

2. Materials and methods

2.1. Long-term fertilization experiment and soil sampling

The long-term fertilization experimental plots that were sampled are at Jiangxi Institute of Red Soil in China (116°20′24″N, 28°15′30″E) and were described previously (Zhong et al., 2010). The region has a typical subtropical monsoon climate with an annual precipitation of 1537 mm, annual evaporation of 1100–1200 mm, and a mean annual temperature of 17.5 °C. The soil in this region is derived from quaternary red clay and classified as Ultisols and Oxisols (U.S. Soil Taxonomy). A maize (*Zea mays* L.) double-cropping system is maintained from early April to the end of November, and the rest time was in fallow. The long-term fertilization experiment was established in 1986, and maintained for 27 years. Tour treatments in triplicate plots (5.5 by 4 m) separated by concrete wall were investigated in this study: 1) CK treatment (without fertilization), 2) N treatment (amended with mineral N fertilizer), 3) NPK treatment (amended with mineral NPK fertilizer) and 4) OM treatment (amended with organic manure). N, P, K and organic manure were applied as basal fertilization before planting in the form of urea (120 kg N ha^{-1} yr^{-1}), calcium superphosphate (60 kg P_2O_5 ha^{-1} yr^{-1}), KCl (120 kg K_2O ha^{-1} yr^{-1}), and composted pig manure (2000 kg ha^{-1} yr^{-1}), respectively.

Soil samples (approximately 200 g) were performed from triplicate plots of each treatment during the seedling stage for maize growth in April 2013. Bulk soils was collected from the plow layer (0–15 cm) and transported to the laboratory for immediate processing. Soil samples were homogenized through a 2 mm mesh to remove plant material and macroscopic organisms. Each soil sample was divided further into three parts. One part was stored at −80 °C for molecular analysis at a later date; a second part was immediately processed for DNA-SIP microcosms, and a third part was air dried and used for physicochemical analyses.

Soil pH was measured with a water/soil ratio of 2.5 and a 1.0 M KCl/soil ratio of 2.5. Soil organic C (SOC) content was measured using the dichromate oxidation method (Bremner and Jenkinson, 1960) and total N content was determined using the Kjeldahl method (Bremner, 1960). Ammonium and nitrate were extracted with 2.0 M KCl and determined by a segmented flow analyzer (Skalar SAN Plus; Skalar Inc., Breda, The Netherlands). The basic characteristics of soils are listed in Table 1.

2.2. DNA-SIP microcosms

Microcosms for DNA-SIP were constructed as previously described (Xia et al., 2011; Lu and Jia, 2013). For each soil, three SIP treatments were prepared in 6 replicates: $^{13}CO_2$ microcosms, $^{12}CO_2$ microcosms, and $^{13}CO_2$+C_2H_2 (100 Pa) microcosms. The $^{12}CO_2$ treatment was used to assess the ammonia oxidizers assimilation of $^{13}CO_2$ resulting from autotrophic growth. The $^{13}CO_2$+C_2H_2 treatment was used to assess $^{13}CO_2$ assimilation by ammonia oxidizers that is inhibited by C_2H_2 (Berg et al., 1982; Xia et al., 2011). For each treatment, sieved fresh soil at 60% maximum water-holding capacity (equivalent to 5.0 g of dry weight soil, *d.w.s*) was added in a 120 ml serum bottles containing 5% (v/v) of $^{13}CO_2$ (99% enrichment, Engineering Research Center of Stable Isotope, Shanghai, China) or $^{12}CO_2$ (made by acidifying sodium carbonate) with or without 100 Pa C_2H_2. The bottles were capped with black butyl stoppers and incubated at 28 °C in the dark for 8 weeks. The $^{13}CO_2$ and $^{13}CO_2$ + C_2H_2 treatments were amended with 100 μg ^{13}C-urea-N g^{-1} *d.w.s* (99% enrichment, Engineering Research Center of Stable Isotope, Shanghai, China) weekly, while the $^{12}CO_2$ treatment was amended with 100 μg ^{12}C-urea-N g^{-1} *d.w.s* weekly. Each bottle received 800 μg ^{13}C-urea-N g^{-1} *d.w.s* during the 8-week incubation. The headspace of each bottle was flushed weekly with air (80% N_2 and 20% O_2) for 1 min to replenish O_2. Lost water was replaced by the addition of sterilized water, and the $^{13}CO_2$, $^{12}CO_2$ and C_2H_2 were immediately replenished. Destructive sampling was carried out in triplicate after 56 days incubation. Three bottles in 6 replicates were used for determining the pH value and soil moisture content and about 2.0 g of fresh soil was collected from the remaining three replicates and stored immediately at −80 °C for molecular analysis, the rest soil was used for measuring soil ammonium, nitrite and nitrate contents.

2.3. DNA extraction and SIP gradient fraction

Total soil DNA was extracted from 0.5 g soil using a bead beating as described in the manufacturer's instructions (MP Biomedicals, Cleveland, OH, USA). Purity and quantity of DNA were checked using a Nanodrop ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA extracts were stored at −20 °C until use.