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Variable responses of ammonia oxidizers across soil particle-size fractions affect nitrification in a long-term fertilizer experiment

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ABSTRACT

We used soils from a 33-year fertilizer experiment and performed high-throughput pyrosequencing analyses targeting the ammonia monooxygenase alpha subunit (*amoA*) gene (also qPCR and terminal restricted fragment length polymorphism (T-RFLP)) to determine whether and how the composition and structure of ammonia-oxidizing microbes in different particle-size fractions (>2000, 2000–200, 200–63, 63–2 and 2–0.1 μm) have changed in response to chronic inorganic (NPK) and organic (NPKM) fertilizer additions under a rice-wheat rotation. We found fertilization and particle-size fractions had strong effects on abundance and structure of ammonia-oxidizers and potential nitrification activity (PNA) ($P < 0.05$) which was significantly enhanced under NPKM across all soil fractions. PNA was higher in clay fraction and lowest in silt fraction which was largely correlated to total N and C:N ratio. Aggregated boosted tree emphasized the role that total N and C:N ratio play on ammonia oxidizer abundance. Pyrosequencing data revealed that the *Nitrososphaera* and *Nitrosospira* were the dominant clusters of ammonia-oxidizing archaea (AOA) and bacteria (AOB), respectively. Even though AOA population was hundreds of times greater than AOB, it seemed AOB responded more sensitively to ambient shifts which was convinced by NMDS analyses based on Bray-Curtis distance of TRFs and also of OTUs from 454 pyrosequencing.

1. Introduction

Soils consist of particles of sand, silt and clay which are bound into aggregates of various sizes by organic and inorganic agents (Tisdall and Oades, 1982). The different soil particle-size fractions provide spatially heterogeneous microclimatic conditions for microorganisms, which are characterized by differences in organic matter composition (Davinic et al., 2012), water potential and oxygen concentrations and predation pressure (Balesdent et al., 2000; Muruganandam et al., 2010; Ranjard and Richaume, 2001). Investigation of microbial distributions and their response to environmental conditions and artificial fertilizations across different particle-size fractions are of great significance to the understanding of bioavailability and microbial transformation of nitrogen nutrients in agricultural soils (He et al., 2007; Ling et al., 2014). However, molecular ecological studies from the perspective of soil particle fractions are still limited.

Nitrification, as an essential component of the global nitrogen cycle, is a microbially regulated process converting ammonia to nitrate via nitrite and leads to changes in plant nitrogen availability, nitrate leaching and greenhouse gas N_2O emissions (Galloway et al., 2008). The first step involving conversion of ammonia to nitrite is often assumed to be rate limiting and performed by two distinct groups of prokaryotes: ammonia-oxidizing bacteria (AOB) affiliated within the β- and γ-Proteobacteria (Purkhold et al., 2000), and recently discovered ammonia-oxidizing archaea (AOA) within the Thaumarchaeota phylum (Brochier-Armanet et al., 2008). It has been observed that both AOA and AOB are key players in ammonia oxidation in agricultural soils (Jia and Conrad, 2009; Offre et al., 2009). Conventionally, the widespread presence of AOB and observation of nitrification activity at low pH values have led to speculation that autotrophic nitrifying activity is dominated by AOB belonging to the genera *Nitrosospira* and *Nitrosomonas* under acidic conditions (Kowalchuk and Stephen, 2001). Furthermore, the

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growth of AOB has been linked to nitrification activity following amendment with high levels of ammonium, either directly as mineral fertilizer or as urea which is rapidly hydrolysed to ammonium (Di et al., 2009; Jia and Conrad, 2009). In contrary, after the discovery of AOA, it was observed that majority of acidic soils contained higher AOA abundance than AOB (He et al., 2007; Zhang et al., 2011), implying the ability of AOA ecotypes to adapt to low-pH soils. Even though AOA outnumber AOB in many ecosystems (Leininger et al., 2006; Adair and Schwartz, 2008; Wessén et al., 2010), this dominance does not always equate to AOA contributing to ammonia oxidation more than AOB (Jia and Conrad, 2009; Di et al., 2009; Adair and Schwartz, 2011). It remains unclear why the abundance of AOA is often unrelated to ammonia oxidation rates (Shen et al., 2008; Wessén et al., 2010). The reason probably is ammonia oxidation may depend not only on population size but also on community composition due to differential substrate affinities and ecological sensitivities among and within the AOA and AOB (Kowalchuk and Stephen, 2001; Bollmann et al., 2002; Schleper and Nicol, 2010). However, our collective knowledge and understanding of the relative role of AOB and AOA in nitrification is still very limited and inconsistent and mostly derived from bulk soil condition. Information at the particle-size fraction level is especially not fully understood.

The Yangtze River Delta in eastern China, covering a total of 3.6 million hectares of agricultural land, has been the most intensive rice (*Oryza sativa* L.) with wheat (*Tritucum aestivum* L.) cropping system for several centuries (Zhong et al., 2016). There are complex interactions between soil biota and abiotic conditions at different stages of above-ground ecosystem succession and below-ground soil development (Schulz et al., 2013). Fertilization, which is widely used to improve soil fertility and crop yield, strongly influences soil biochemical biological properties. The effects of fertilization on the activity and community structure of AOA and AOB, which are ubiquitous in soils and aquatic environments, has recently been emphasized (Cavagnaro et al., 2008; Shen et al., 2008; Verhamme et al., 2011; Wang et al., 2009). These ubiquitous soils also experienced intensive management, including irrigation and fertilizer inputs, both in agricultural and urban residential areas (Warren et al., 1996; Davies and Hall, 2010). However, most investigations have been conducted just on a bulk soil scale or in short-term experiments; therefore, there is still little information available regarding different soil particle-size fractions on ammonia oxidation in agricultural soils subject to long-term fertilization.

In the same experimental field tested in this study, soil particle-size fraction played pronounced role in mediation of the degree to which long-term fertilization drove soil microbial community and extracellular enzyme activities to different changes (Zhang et al., 2015; 2016). However, specific difference existed among AOA and AOB communities in heterogeneous particle-size fractions remained unclear so far. So in the present study, we focused our attention on the distribution and community diversity of ammonia oxidizers using soils of a 33-year fertilizer experiment under typical rice-wheat rotation in China. Specifically, the ammonia oxidizers community composition and structure were analyzed through terminal-restriction fragment length polymorphism (T-RFLP) and 454-pyrosequencing to answer the following questions: (i) What are the dominant ammonia oxidizer microbes (AOA or AOB) and their taxa in this yellow–brown paddy soil? Whether ammonia oxidizers are same or distinct in different particle-size fractions and fertilizer treatments? (ii) How does the abundances and structure of ammonia oxidizers in different particle-size fractions respond to long-term chemical and organic fertilizer input? Are those variations consistent with the changes of nutrient availability and other biochemical characters? (iii) Do both AOA and AOB respond sensitively to ambient shifts? What are the main soil factors structuring the pattern of soil ammonia oxidizers communities?

2. Materials and methods

2.1. Site description and design

The long-term field fertilizer experiment was initiated in 1981 at South Lake station (30°37′N, 114°20′1″E), Hubei Province, China, where rice–wheat rotation is the common cropping system. The site is located in the northern subtropical to middle subtropical transitional geographic climate zone with an annual average total accumulated temperature of 5189.4 °C (>10 °C/day) and precipitation of 1300 mm. The tested yellow–brown paddy soil belongs to the soil order of Udalfs with clay loam texture (USDA soil classification). At the beginning of the experiment, the soil had a pH (H_2O) of 6.3, organic matter of 27.43 g kg^{-1}, total N, P, K of 1.801 g kg^{-1}, 1.004 g kg^{-1} and 30.22 g kg^{-1}, respectively. The concentrations of available P and K were 5.0 mg kg^{-1} and 98.5 mg kg^{-1}. Three treatments (three replicates each) were randomly implemented in 9 plots (40 m^2 each) under a rotation of winter wheat and rice. Treatments consisted of soil without fertilizer (control, CK), fertilizer N, P and K (NPK), organic manure plus fertilizer N, P and K (NPKM). Chemical fertilizers were applied as annual rate of 150 kg N ha^{-1}, 75 kg P_2O_5 ha^{-1}, 150 kg K_2O ha^{-1}. The N, P and K were applied as urea, superphosphate and potassium chloride, respectively. For the NPKM treatment, the same rate of chemical fertilizers as NPK treatment was used in addition to 22,500 kg ha^{-1} per year. Organic manure was applied as pig manure (H_2O ~69%) with properties of 15.1 g kg^{-1} N, 20.8 g kg^{-1} P_2O_5 and 13.6 g kg^{-1} K_2O.

Sixty percent of chemical fertilizers were applied to rice and the other 40% were applied during the wheat season, while organic manure was applied equally (50:50) to the two crops. All fertilizer P and K and manure during the wheat season and the rice season were applied once as basal dressing. Meanwhile 40% of fertilizer N was applied as a basal fertilizer, 40% during tillering stage and 20% during booting stage in rice season. The amounts of N fertilizer applied to wheat were 50% as basal fertilizer, 25% for overwintering period and 25% during the jointing stage. Manure and mineral fertilizers were evenly broadcasted onto the soil surface and immediately incorporated into the plowed soil (0–20 cm depth) by tillage before sowing.

2.2. Soil sampling and particle-size fractionation

Undisturbed soil samples from the three replicates of each treatment were collected 1 week before wheat harvesting in May 17th, 2014 and rice harvesting in September 20th, 2014. Three soil cores (5 × 10 × 18 cm) were collected at a depth of 0–20 cm from each plot. Moist soils were gently broken apart along the natural breakpoints and passed through a 5-mm sieve to remove visible organic debris. The 5-mm sieve was used rather than a 2-mm sieve because of the unique viscid characteristic of the paddy soil. If soils were forced through a 2-mm sieve, the natural structure of the soil would be destroyed. After thorough mixing, the field-moist soil was used for particle-size fractionation.

Soil samples were dispersed by low-energy sonication and the particle size fractions were separated as described by Stemmer et al. (1998). Briefly, the soil–water suspension was dispersed by low-energy sonication (output energy of 0.2 kJ/g) and subsequently fractionated by a combination of wet sieving and repeated centrifugation to avoid disruption of microaggregates. Finally, five fractions were obtained for each sample: large macroaggregates (>2000 μm), coarse sand-sized fraction (2000–200 μm), fine sand-