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Effects of 44 years of chronic nitrogen fertilization on the soil nitrifying community of permanent grassland

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ABSTRACT

Chronic nutrient addition to grassland soils can strongly influence the composition and abundance of nitrifying microbial communities. Despite the fact that nitrifying microbes play a crucial role in regulating ecosystem nitrogen (N) cycling, our understanding of how long-term N fertilization might influence nitrifying microbial groups is still limited. Here we used soil from a 44-year-old grassland fertilization experiment and performed high-throughput pyrosequencing analyses (and real-time quantitative PCR) to determine whether and how the identity and abundance of nitrifying microbes has changed in response to chronic inorganic (chemical fertilizer) and organic (cattle slurry) N additions. We found that the *amoA* genes of ammonia-oxidizing archaea (AOA) significantly increased under organic N additions, whereas ammonia-oxidizing bacteria (AOB) increased with the addition of inorganic N. Proportional changes of AOA, AOB and nitrite-oxidizing bacteria (NOB) demonstrate that nitrifying phylotypes are influenced by chronic N additions. We also found that AOA/AOB ratios increased with higher application rates of cattle slurry suggesting that AOA may affect N cycling more in soils receiving animal manures, whereas AOB are functionally more important in chemically fertilized soils. Phylogenetic analysis shows that shifts in AOA and AOB community structure occurred through time across N fertilization treatments. For example, (a) fosmid 29i4-like AOA, (b) *Nitrosospira* cluster 3-like AOB, and (c) *Nitrospira*-like NOB dominated nitrifying communities in fertilized soils. Finally, high-throughput pyrosequencing of 16S rRNA genes show that N fertilization (either inorganic or organic) increased the abundance of Actinobacteria in soils while it decreased the abundance of Proteobacteria. Our study is one of the first to show that long-term N additions to soils can greatly affect nitrifying communities, and that phylogenetically and functionally distinct nitrifiers have developed through time in response to chronic N fertilization.

1. Introduction

Grasslands account for approximately 50% of the agricultural land area in Europe and are considered important food production systems to human society (Smit et al., 2008). To maximize biomass production grassland soils receive chronic nutrient additions often in the form of chemical fertilizers (i.e. NPK) or animal manures which greatly contribute to the release of available forms of N for plant uptake and growth (Beauchamp et al., 1989; Reijs et al., 2005). Organic and inorganic nutrient fertilization may however influence the structure of soil nitrifying microbial communities which play a pivotal role in regulating soil N cycling and in maintaining ecosystem sustainability (van der Heijden et al., 2008). Despite the widely acknowledged importance of soil microorganisms, it remains poorly understood how nitrifying microbial guilds ultimately respond to long-term repeated additions of organic and inorganic forms of nitrogen.

Nitrification is a key process in terrestrial ecosystems (Booth et al., 2005) and is generally thought to be executed by key functional microbial guilds, including (1) ammonia-oxidizing bacteria (AOB), (2) archaea (AOA), and (3) nitrite-oxidizing bacteria (NOB). Nitrification occurs through the: (i) the oxidation of NH_3 to NO_2^- by

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AOB and AOA (Stephen, 2001; Leininger et al., 2006), and (ii) the oxidation of NO_2^- to NO_3^- by NOB. These functional guilds use a limited substrate and coexist in the same soil matrix where they have developed distinct physiologies and/or life strategies to adapt to changes in resource availability. Previous studies show that ammonia-oxidizing bacteria (AOB) tend to dominate nitrification activity in neutral soils with heavy ammonium inputs (Jia and Conrad, 2009), whereas the growth of ammonia-oxidizing archaea (AOA) is favored in nutrient-poor environments such as acidic soils with low NH_3 concentrations (Offre et al., 2009; Lu and Jia, 2013). However, AOB and AOA may have evolved more complex lifestyle than previously thought to cope with constantly changing environments. For example, a recent molecular study shows a predominance of AOA in neutral and alkaline soil with high ammonia concentrations (Gubry-Rangin et al., 2011; Hu et al., 2013). This finding is supported by culture studies which have demonstrated that AOA may survive high ammonia concentrations (Tourna et al., 2011; Kim et al., 2012), especially when an organic C source was also added (Tourna et al., 2011). However, these observations were made mostly under controlled experimental conditions and among physiochemically contrasting environments, whereas the response of nitrifying communities to environmental change under field conditions cannot be easily generalized (Prosser, 2012).

Long-term N fertilization experiments provide a unique setting where to address potential human-induced effects on the structure of soil microbial communities (Geisseler and Scow, 2014). In these experiments, N fertilizers are often applied at different levels, and variation in other environmental factors is minimized. Progressive N enrichment may have a profound impact on the structure and activity of different nitrifying guilds (Treseder, 2008). For example, DGGE fingerprinting revealed significant changes in community composition of AOB rather than AOA in an aerobic upland soil (Shen et al., 2008). Real-time quantitative PCR also indicated differential response of AOA and AOB to long-term straw and peat additions to a maize field in Sweden (Wessén et al., 2010). These findings suggest that mixotrophic and/or heterotrophic lifestyles might exist within AOA, whereas AOB might have greater ecophysiological diversity being able to thrive across a broader range of habitats (Wessén et al., 2010). However, these studies often necessitate the use of ammonia oxidizer-specific primers which may have caused the preferential amplification of certain nitrifying phylotypes. Thus it is still not clear whether and how long-term repeated additions of inorganic vs. organic N fertilizer under field conditions might lead to significant changes in nitrifying guilds or not.

High-throughput pyrosequencing of 16S rRNA genes at the whole community level provides a powerful means to address changes in the structure and dynamics of microbial functional guilds in soils (Lu et al., 2012). The use of universal primers enables the detection of each individual microorganism on the basis of its relative frequency in a given microbial community. The proportional change of certain functional guilds could thus be linked with a specific ecological process in the complex soil matrix, without using specific biomarkers. Unlike conventional fingerprinting, high-throughput sequencing is particularly important for the detection of microbial populations that represent only a numerically small fraction of the total microbial community (e.g. soil nitrifiers represent <1% of total microbial community) (Okano et al., 2004). High-throughput sequencing could also circumvent the problem of primer design of phylogenetically diverse NOB in soils (Freitag et al., 2005). The question remains whether and how chronic N additions are associated with shifts in the soil nitrifying community of human-managed soil ecosystems. Here we address this question by performing pyrosequencing analyses on permanent grassland soils where nitrifying guilds have been exposed to 44 years of chronic N enrichment either as inorganic N fertilizer (i.e. NPK) or as organic additions (cattle slurry and at different rates of application).

2. Materials and methods

2.1. Soil samples

We used a long-term grassland fertilization experiment established at Hillsborough, UK (54°27′N, 6°4′W) on clay loam soils in 1970 (Christie, 1987). The experimental design includes six replicates of each of five nutrient treatments: (1) control (CK, no fertilizer or animal slurry applied); (2) NPK fertilizer (CF, 200 kg N (as ammonium nitrate until June 1974 and subsequently as urea) $ha^{-1}Y^{-1}$, 32 kg P ha^{-1} Y^{-1} & 160 kg K ha^{-1} Y^{-1}); (3) cattle slurry applied at LC (50 m^3 ha^{-1} yr^{-1}, 170 kg N ha^{-1}), (4) MC (100 m^3 ha^{-1} yr^{-1}, 340 kg N ha^{-1}) and (5) HC (200 m^3 ha^{-1} yr^{-1}, 680 kg N ha^{-1}). Each of the five nutrient treatments is arranged in three randomized blocks with two replicates of each treatment fully randomized within each block, giving a total of 30 experimental plots (each 29.75 m^2). Soil sampling was performed in February 2012 to a depth of 5 cm. Soil samples from each plot were mixed and homogenized, ground to <2 mm, air-dried and analyzed for pH, Olsen-P, Extractable K, Mg & S. A soil sub-sample was used for molecular analysis in the Institute of Soil Science, Chinese Academy of Sciences for high-throughput pyrosequencing and real-time quantitative PCR.

2.2. Soil properties

Soil properties were determined as previously described (Murphy et al., 2005). Soil pH was determined in 1:2.5 (weight:-volume) ratios of soil with distilled water using a Skalar SP10 automated pH system. Total soil C & N (%) were determined simultaneously by the Dumas method using a LECO CN 2000. Soil available P was extracted using sodium bicarbonate and measured with the molybdenum blue method using a Skalar San++ autoanalyser. Soil available K and Mg were extracted using ammonium acetate, the K concentration was determined using a 410 Sherwood Scientific Flame Photometer while Mg concentration was measured using Atomic Absorption Spectrophotometry (ThermoFisher Scientific iCE 3300). Soil available S was extracted using calcium dihydrogen phosphate monohydrate and S concentration in the extract was determined using Inductively Coupled Plasma Spectroscopy (Varian Liberty series II).

2.3. Nucleic acid extraction and real-time quantitative PCR

Soil nucleic acid was extracted from 0.5 g soil using the FastDNA spin kit for soil (Qbiogene, Inc., Irvine, CA) according to the manufacturer's protocol. DNA purification was conducted with 5.5 M guanidine thiocyanate solution by removing humic substance contamination. The quality and abundance of the extracted DNA was measured by gel electrophoresis (0.8% agarose) and NanoDrop spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE), and stored at −20 °C.

The population size of bacterial and archaeal communities was assessed by quantification of 16S rRNA gene copies in soil samples using the primer pairs 515F-907R (Stubner, 2002) and 771F-934R (DeLong, 1992; Ochsenreiter et al., 2003), respectively. AOA and AOB in soil samples were quantified with the primer pairs of Arch-amoAF/Arch-amoAR (Francis et al., 2005) and amoA-1F/amoA-2R (Rotthauwe et al., 1997), respectively. Real-time quantitative PCR was carried out on a CFX96 Optical Real-Time Detection System (Bio-Rad Laboratories, Inc. Hercules, CA). The DNA templates were

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