Applied Soil Ecology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Nitrogen, season, and tillage management influence ammonia oxidizing bacterial communities in long-term maize

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ARTICLE INFO

Keywords:
Autotrophic nitrification
Ammonia oxidizing bacteria
PCR-DGGE
Community structure

ABSTRACT

Nitrification is the biological oxidation of NH_3 to NO_2^- and NO_3^- for which ammonia-oxidizing bacteria (AOB) are the functionally dominant group in cropland responsible for the rate-limiting step of NH_3 oxidation. Different AOB species have functional differentiation and do not equally contribute to nitrification. This investigation explored if long-term N fertilization and tillage influenced AOB community structure. The study site was a long-term (> 40 years) continuous maize (*Zea mays* L.) experiment with three N fertilization rates (0, 168, and 336 kg ha^{-1}) and either no-tillage (NT) or moldboard plow tillage (PT). We used denaturing gradient gel electrophoresis (DGGE) to analyze PCR-amplified bacterial ammonia monooxygenase genes and detect changes among NH_3-oxidizing bacteria. Tillage, fertilization, and sample season all significantly influenced the AOB community. The AOB were more diverse in NT than PT. The AOB became more diverse with increasing N input and were more diverse in summer than winter samples. *Nitrosomonas*-like and *Nitrosospira*-like groups were identified based on gel migration patterns. Unique bands occurred in different treatments, manifesting environmental selection. The long-term field trial showed soil management consistently influenced AOB communities. Significantly, AOB diversity differed at a small scale within a relatively uniform landscape, even in the presence of long-term management practices.

1. Introduction

Ammonia oxidation has two steps. First, NH_3 is oxidized to hydroxylamine (NH_2OH) by ammonia monooxygenase (AMO):

$$NH_3 + O_2 + 2H^+ + 2\,e \rightarrow NH_2OH + H_2O$$

Second, NH_2OH is oxidized to NO_2^- by hydroxylamine oxidoreductase (HAO) (Arp et al., 2002):

$$NH_2OH + H_2O \rightarrow HONO + 4H^+ + 4e^-$$

Ammonia oxidation is generally regarded as the rate-limiting step (Francis et al., 2007) because NO_2^- rarely accumulates (Kowalchuk and Stephen, 2001). For example, potential NH_3 oxidation in a rice paddy soil was much lower than potential NO_2^- oxidation, implying NH_3-oxidizing nitrifiers regulated the pace of nitrification (Ke et al., 2013).

For slow growing and difficult to culture autotrophic nitrifiers, molecular tools to detect and examine their communities are necessary alternatives to conventional culture techniques. Most often, *amo* genes (encoding AMO) have been used as functional markers to investigate AMO's essential role in NH_3 oxidation. AmoA, one of the three membrane bound polypeptide subunits of AMO, is a 27–30 kDa polypeptide containing the putative active site of AMO (Arp and Stein, 2003; Hyman and Arp, 1992). The functional gene *amoA* is more specific and suited for fine-scale differentiation of AOB (Rotthauwe et al., 1997) than the 16S rRNA gene because similarities among 16S rRNA gene are too great to obtain relatively detailed phylogenetic information (Aakra et al., 2001).

Ammonia-oxidizing archaea (AOA) and AOB coexist in soil and AOA appear to be more abundant than AOB - the AOA/AOB ratios ranging from 1.5 to 232 in different topsoils (0–10 cm) (Leininger et al., 2006). Nevertheless, AOB appear to be the functionally dominant group responsible for NH_3 oxidation in agricultural soil as assessed by fingerprinting of *amoA* genes and DNA stable isotope probing to detect CO_2 assimilation (Jia and Conrad, 2009). In andisols, lowland soil, and yellow soil, AOB were also the major contributors to nitrification; the positive correlation (adjusted R_2 = 0.742) between AOB *amoA* and nitrification activity was much stronger than for AOA *amoA* and nitrification (adjusted R_2 = 0.228) (Morimoto et al., 2011). For these reasons, AOB were the object of our study.

The AOB in soil primarily consist of two major genera, *Nitrosomonas*

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https://doi.org/10.1016/j.apsoil.2018.05.002
Received 6 June 2017; Received in revised form 3 April 2018; Accepted 3 May 2018

(including *Nitrosococcus mobilis*) and *Nitrosospira* (encompassing *Nitrosolobus* and *Nitrosovibrio*) (Head et al., 1993; Stephen et al., 1996). Most physiological studies of AOB are based on *Nitrosomonas europaea,* which is relatively easily grown in the laboratory. However, *Nitrosospira* are the numerical dominant AOB in soils (Bruns et al., 1999).

While it is intuitive nitrification rate should increase with increasing nitrifier density, the correlation between rate and population is inconsistent (Aarnio and Martikainen, 1995) and these two parameters might not change simultaneously (Mendum et al., 1999). The nitrifier community responds to many factors, such as fertilization (Wu et al., 2011), land use (Taylor et al., 2010), temperature (Fierer et al., 2009), and soil pH (Nicol et al., 2008). Not all kinds of nitrifiers make an equal contribution to nitrification. The AOB community structure has been shown to influence potential nitrification rates in a wheat-maize rotation system (Ai et al., 2013). Functional differentiation occurs among different AOB species, so an investigation of the dynamics of nitrifier community structure is valuable.

A study site excluding variables such as vegetation, soil type, and other temporary management practices could focus attention on the effects of N fertilizer and tillage. This has rarely been done for previous studies evaluating nitrifier community diversity. Our experimental context was a long-term (> 40 year) continuous maize study evaluating three N fertilizer levels and tillage vs no-tillage. Therefore, this research could specifically evaluate if tillage and N fertilization influenced AOB community structure. Moreover, for this study site, we already know the AOB population and potential nitrification rates increase with increasing N fertilizer rates; potential nitrification rates are higher in no-till soils than plow tillage soils; and enzyme analysis suggests the AOB communities of different treatments are different (Liu et al., 2017).

Our approach used denaturing gradient gel electrophoresis (DGGE) to assess PCR-amplified bacterial *amoA* genes and determine if the community characteristics among NH_3-oxidizing bacteria in different N fertilization rates and different soil tillage management differed for two different seasons.

2. Materials and methods

2.1. Site design

The field trial from which samples were collected was initiated in 1970 on the Kentucky Agricultural Experiment Station's Maine Chance farm near Lexington KY (N 38°07′24″, W 84°29′50″) on a site with 1 to 3% slope, a southerly aspect, and exhibiting no to slight erosion (Frye and Blevins, 1996). The soil is a moderately weathered Maury silt loam (fine, mixed semi-active, mesic Typic Paleudalf), deep, well-drained, and formed in the residuum of phosphatic Ordovician limestone. It contains no free $CaCO_3$. The clay fraction, dominated by vermiculite, kaolinite, and illite (Frye and Blevins, 1996) contains significant amounts of amorphous Fe and Al oxides (Chung et al., 2008). The site had previously been a bluegrass (*Poa pratensis* L.) pasture for nearly 50 years.

The experiment design is a split-block with four replications, each block is split along the long dimension for the tillage treatments [no tillage (NT) and moldboard plow tillage (PT)] and across the long dimension for each of four N rates (0, 84, 168, and 336 kg N ha^{-1}), resulting in final plot sizes of 5.5 m by 12.2 m for each separate treatment. Tillage and N rate treatments have been maintained on the same plots for the duration of the experiment. Our study did not examine the 84 kg N ha^{-1} rate.

The site is continuously summer cropped to maize (*Zea mays* L.) for grain followed by a winter annual cereal cover crop. The PT treatment consists of moldboard plowing to a depth of 20 to 25 cm in the third or fourth week of April each year, about 1–2 weeks before planting maize. Secondary tillage of plowed plots is performed with two passes of a tandem disk; mixing soil to a depth of 8 cm. Ammonium nitrate is surface broadcast within one week of planting. Maize is harvested in late September or early October each year, and combine-shredded residues are left on the soil surface. The winter cereal is planted over the entire study site with a NT drill after maize harvest without any tillage.

2.2. Climate relative to this research

The climate at the site is temperate, having an average annual rainfall of 110 cm (about 40% falls in summer – May to September), an average annual temperature of 13 °C, and a growing season duration of 175 days (Frye and Blevins, 1996). Relevant precipitation and temperature data for the specific sampling periods were obtained from the University of Kentucky Agriculture Weather Center [wwwagwx.ca.uky.edu]. The monthly total precipitation immediately before sampling was 9.5 cm in December (2011) and 20.2 cm in June (2013). The average bare soil temperature at a 10 cm depth one month before sampling in December was 6.9 °C and 23.1 °C in June.

2.3. Sample collection and processing

Soil samples were collected from the top 15 cm in each plot fertilized at 0, 168, and 336 kg N ha^{-1} using a core sampler (2 cm diam.) in December 2011 and June 2013. On both sampling occasions, ten cores were removed from each plot (5 in row and 5 between rows), composited, and transported from field to lab in sealed plastic bags on ice. Mixed soils were removed from each bag to three Eppendorf tubes as soon as possible and stored at −80 °C for DNA extraction.

2.4. DNA extraction

Total soil DNA was extracted from 0.25 g soil using a MACHEREY-NAGEL NucleoSpin® soil genomic DNA isolation kit (Düren, Germany) according to the manufacturer's instructions, but with the following minor modifications: lysis buffer SL1 was used and centrifuged 2 min to precipitate contaminants; elution buffer SE was incubated at 80 °C before use; DNA was eluted with 50 μL SE (the first elution with 30 μL SE; a second elution with 20 μL SE) and incubated 5 min before centrifuging. The DNA samples were stored at -20 °C.

2.5. PCR amplification

The primer pair *amoA*-1F (5′ -3′ GGG GTT TCT ACT GGT GGT) and *amoA*-2R (CCC CTC KGS AAA GCC TTC TTC) (Rotthauwe et al., 1997) was used to amplify the bacterial *amoA*. When PCR amplifications were performed for subsequent DGGE analysis, a GC (guanosine-cytosine) clamp (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G) was added to the 5′ end of each forward primer. The PCR amplification was performed with a MyCycler™ Thermo Cycler (Bio-Rad Laboratories, Inc.) using Thermo Scientific DreamTaq Green DNA Polymerase (Thermo Fisher Scientific Inc.).

The 25 μL reaction mixture contained 2.5 μL 10X DreamTaq Green Buffer, 2.5 μL dNTP Mix (0.2 mM of each), 0.5 μM of each primer, 0.78 U of DreamTaq DNA Polymerase, and 2 μL of soil DNA template. The thermal profile of the PCR was: 5 min at 95 °C for initial denaturation; 35 cycles consisting of 30 s at 95 °C for denaturing, 30 s at 57 °C for annealing, and 60 s at 72 °C for extension; a final 10 min at 72 °C for extension.

For each gene, a PCR sample was selected at random to confirm the appropriate gene was amplified with these primers. The PCR products were purified with the GeneJET PCR Purification Kit (Thermo Scientific, Waltham, MA), ligated into the pGEM-T vector using pGEM®-T Easy Vector Systems (Promega, Madison, WI), transformed into electrocompetent *Escherichia coli* cells using the Gene Pulser Xcell microbial system (Bio-Rad, Hercules, CA), and cultured on LB agar plate containing carbenicillin (100 μg/mL) with 40 μL of X-Gal (20 ng/mL) and 40 μL of 100 mM IPTG to indicate colonies with the target gene inserted. Plasmid minipreps with the GeneJET Plasmid Miniprep Kit