1 **Type of contribution:** research paper

2 **Number of text pages:** 27

3 **Number of tables & figures:** 2 tables and 8 figures

4 **Title:** Ammonia-oxidizing bacteria are more responsive than archaea to nitrogen
5 source in an agricultural soil.

6 **Authors:** Yang Ouyang¹, Jeanette M. Norton^{1*}, John M. Stark², Jennifer R
7 Reeve¹ and Mussie Y. Habteselassie³

8 ¹*Department of Plants, Soils and Climate,* ²*Department of Biology, Utah State*

9 *University, Logan, UT;* ³*Department of Crop & Soil Sciences, University of Georgia -*

10 *Griffin, Griffin, GA*

11 **Corresponding author,*

12 *4820 Old Main Hill AGS 352*

13 *Logan Utah 84322-4820*

14 *Tel. /fax: +1 435-797-2166 /+1 435-797-3376*

15 *E-mail address: jeanette.norton@usu.edu*

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Ammonia-oxidizing bacteria are more responsive than archaea to nitrogen source in an agricultural soil.

Abstract

In the majority of agricultural soils, ammonium (NH_4^+) is rapidly converted to nitrate (NO_3^-) in the biological ammonia and nitrite oxidation processes known as nitrification. The often rate-limiting step of ammonia oxidation to nitrite is mediated by ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA). The response of AOA and AOB communities to organic and conventional nitrogen (N) fertilizers, and their relative contributions to the nitrification process were examined for an agricultural silage corn system using a randomized block design with 4 N treatments: control (no additional N), ammonium sulfate (AS) fertilizer at 100 and 200 kg N ha⁻¹, and steer-waste compost (200 kg total N ha⁻¹) over four seasons. DNA was extracted from the soil, and real-time PCR and 454-pyrosequencing were used to evaluate the quantity and diversity of the *amoA* gene which encodes subunit A of ammonia monooxygenase. Soil pH, nitrate pools, and nitrification potentials were influenced by ammonium and organic fertilizers after the first fertilization, while changes in AOB abundance and community structure were not apparent until after the second fertilization or later. The abundance of AOA was always greater than AOB but was unaffected by N treatments. In contrast, AOB abundance and community structure were changed significantly by ammonium fertilizers. Specific inhibitors of nitrification were used to evaluate the relative contribution of AOA and AOB to nitrification. We found that AOB dominantly contributed to potential nitrification activity determined at 1 mM ammonium in soil slurries and nitrification potential activity was higher in soils treated with ammonium fertilizers relative to control soils. However, AOA dominated gross nitrification activity in moist soils. Our result

suggests that AOB activity and community are more responsive to ammonium fertilizers than AOA but that *in situ* nitrification rate is controlled by ammonium availability in this agricultural soil. Understanding this response of AOA and AOB to N fertilizers may contribute to improving strategies for the management of nitrate production in agricultural soils.

Keywords: Nitrification, ammonia oxidizing archaea, ammonia oxidizing bacteria, nitrogen fertilizers, *Nitrosospora*, octyne.

1. Introduction

Nitrification, the biological oxidation of ammonia to nitrite and subsequently to nitrate, influences the fate of N in terrestrial systems and often promotes the loss of nitrate from soils. In soils, the first step of autotrophic nitrification is mediated by ammonia oxidizing bacteria (AOB) of the *Betaproteobacteria* and ammonia oxidizing archaea (AOA) of the *Thaumarcheota* (Norton, 2011; Schleper and Nicol, 2010). Since both AOA and AOB contain the ammonia monooxygenase enzyme, the *amoA* gene is frequently used as a molecular marker to explore the abundance and diversity of ammonia oxidizers. Leininger et al (2006) first revealed that AOA were quantitatively dominant in a variety of soils from diverse ecosystems. The abundance and communities of AOA and AOB and their relative contributions to soil nitrification are influenced by complex factors in agricultural soils (Giguere et al., 2015; Taylor et al., 2012).

Comparisons of N dynamics under organic N sources versus mineral fertilizers have consistently shown changes in N transformation rates and associated microbial communities (Burger and Jackson, 2003; Chu et al., 2007; Geisseler and Scow, 2014; Habteselassie et al., 2006b; Habteselassie et al., 2013; Reeve et al., 2010; Shi and Norton, 2000). AOB and AOA have been found to co-exist in most agricultural soils

and may have functional differences in their response to nitrogen management (Di et al., 2009; Habteselassie et al., 2013; Offre et al., 2009; Xia et al., 2011). The application of mineral fertilizers or urea changed the abundance and composition of AOB, but it did not significantly influence the AOA community in several agricultural soils (Ai et al., 2013; Shen et al., 2008; Wang et al., 2009; Xia et al., 2011). In contrast, AOA growth was detected when ammonia was supplied by mineralization of organic N derived from composted manure or soil organic matter (Jiang et al., 2014b; Levicnik-Hoefferle et al., 2012; Offre et al., 2009; Schleper, 2010). However, these studies were conducted in microcosms or after long term (>5 years) field fertilization experiments. We have limited information about how AOA and AOB population and community respond to mineral and organic fertilization temporally in the field.

While previous studies have shown that the archaeal *amoA* is often more abundant than bacterial *amoA* in soils (Leininger et al., 2006; Schleper and Nicol, 2010; Shen et al., 2012), the rate of ammonia oxidation may or may not be linked directly to current AOA and AOB populations (Myrold et al., 2014; Nicol et al., 2008). Xia et al. (2011) found AOB dominantly contributed to nitrification activity in an agricultural soil by using a stable isotope probing technique. However, AOA were found to be the more dominant players in nitrification in other agricultural soils (Offre et al., 2009; Zhang et al., 2010). Taylor et al. (2010) developed a short-term assay based on the recovery of the nitrification potential (RNP) after inhibition with acetylene in the presence and absence of bacterial protein synthesis inhibitors. They discovered that in recently N fertilized and cropped soils the majority of RNP activity was due to AOB, and that in pasture and grassland soils, RNP was due primarily to AOA or to a mixture of AOA and AOB. Recent findings by Taylor and colleagues (2015; 2013) showed that AOA pure cultures were more resistant to the C8 alkyne

inhibitor, 1-octyne, in comparison to AOB, and that therefore octyne can be used to distinguish AOA and AOB contributions to soil nitrification in short-term assays. These differential inhibitors allow us to assess the relative contribution of AOA and AOB to nitrification.

In the present study, our goal was to investigate effects of conventional and organic N sources on AOA and AOB populations and community composition in a replicated field experiment over 4 years. Concurrently, we assessed nitrification rates and the relative contribution of AOA and AOB under these contrasting N treatments. We hypothesized that the AOA and AOB communities and their relative contributions to nitrification would respond differentially to these contrasting N sources. Our expectation was that AOB abundance and activity would increase in response to ammonium fertilizer while AOA would increase in response to an organic N source. Understanding the relative contribution of AOA and AOB to nitrification may contribute to improving strategies for the management of nitrate production in agricultural soils.

2. Materials and methods

2.1 Experimental field plots and soil sampling

Field plots were established in 2011 at the Utah Experiment Station Greenville farm located in North Logan, Utah (41°45' N, 111°48' W). The soil is an irrigated, very strongly calcareous Millville silt loam (Coarse-silty, carbonatic, mesic Typic Haploxeroll). Previously, the field was used for conventional small grain production with the annual application of 70 kg N ha⁻¹ as urea. The experimental design is a randomized complete block with four blocks and four nitrogen treatments: control (no N fertilization), ammonium sulfate (AS 100 and 200 kg N ha⁻¹), and steer-waste

compost (200 kg total N ha⁻¹). Each plot is 3.8 m wide and 9.1 m long with a 4.6 m alley between each plot and a 1.2 m alley between each block. Composted steer manure (obtained from Miller Co. Hyrum, UT) was analyzed for moisture and N content immediately before application to determine the mass needed to supply the 200 kg total N ha⁻¹ rate. Treatments were surface applied in May of each year (2011 - 2014), incorporated by tilling immediately after application, and silage corn was planted within one week after treatment application as previously described (Habteselassie et al., 2006a).

Soil samples were collected in May (0-30 cm depth) and August (0-15 cm depth) from 2011 to 2014. We collected 0-30 cm soil depth in May before tillage to coincide with standard practice for fertility determinations and to assess winter leaching of nitrate. In August, we sampled the 0-15 cm soil depth approximately 90 days after fertilization. Fertilizers are tilled into this depth and nitrification activity is generally higher in this surface layer (Habteselassie et al., 2006a; 2006b). Six soil cores were randomly taken from each plot, composited and mixed, placed on ice, and brought to the laboratory for immediate processing.

2.2 Soil chemical properties

Soil ammonium and nitrate were extracted immediately after sampling with 2M KCl (1:5 of soil:solution by mass). Soil ammonium and nitrate were measured with a flow injection analyzer (QuikChem 8500, methods 12-107-06-1-A, 12-107-04-1-J Lachat Instrument, Loveland CO). The soil moisture was determined by drying soils at 105°C for 24 h. Soil samples were then sieved (2.0 mm) and stored at 4°C or air-

dried for other measurements. Soil pH was determined on a 1:2 soil-water suspension.

Soil organic C and total N were determined by dry combustion (Primacs^{SLC} for organic carbon, Primacs^{SN} for total N, Skalar, Inc, GA, USA).

2.3 Nitrification potential and recovered nitrification potential assays

Nitrification potential was determined by the shaken soil slurry method as described previously (Hart et al., 1994; Norton and Stark, 2011). Briefly, 15 g sieved fresh soil was placed into a 250 ml flask, and 100 ml 1mM phosphate buffer (pH=7.2) containing 1 mM NH_4^+ was added to the flask. Flasks were shaken for 24 h at 200 rpm and sampled four times (2, 4, 22, 24 h) after the beginning of shaking. The concentrations of nitrite (NO_2^-) and nitrate (NO_3^-) were measured with a flow injection analyzer (QuikChem 8500 method 10-107-04-1-C Lachat Instruments, Loveland CO).

We measured the recovery of nitrification potentials (RNP) as described by Taylor et al (2010; 2012). Briefly, 9 g of moist soil were added to 60 ml of 30 mM TES buffer (pH 7.2) with 1 mM NH_4^+ in 150 ml bottles with caps fitted with gray butyl stoppers. Soil slurry was then exposed to acetylene (0.025 KPa) for 6 h. Acetylene was removed by placing the soil slurries under a vacuum and degassing for 6 min. After degassing, all bottles were shaken and incubated with caps loosened at 30°C. Once acetylene is removed, AMO may be synthesized again allowing nitrification to resume and nitrite and nitrate to accumulate after a delay of approximately 24 h. Thus the RNP was calculated by assessing the rate of nitrite and nitrate accumulation 24 to 48 h after acetylene removal. In some samples, two

bacterial protein synthesis inhibitors, kanamycin (at a final concentration of 800 $\mu\text{g/ml}$) and spectinomycin (at a final concentration of 200 $\mu\text{g/ml}$) were used together to prevent synthesis of ammonia monooxygenase (AMO) by AOB, and thus the recovery in these samples is due to AOA (RNP_{AOA}). RNP of AOB was calculated as the difference between total RNP and RNP_{AOA} .

Nitrification potential with and without 4 μM aqueous concentration (C_{aq}) of 1-octyne was measured to distinguish the contribution of AOA and AOB to NP, using the modified method of Taylor and colleagues (2013). Briefly, 4.5 g sieved soil samples were placed in bottles with caps fitted with butyl stoppers, and then 30 ml of 30 mM TES buffer with ammonium sulfate (1.0 mM final $\text{NH}_4^+\text{-N}$) was added. Bottles were shaken for 2 h to equilibrate the soil and solution at 30°C. One group of these soil slurries was then exposed to 4 μM C_{aq} of octyne, and the other group served as a control. Five ml aliquots were sampled two times (2 and 24 h) after the beginning of shaking. The aliquots were centrifuged at 8000 g for 8 min, and the supernatants were frozen until analysis. The concentration of $\text{NO}_2^- + \text{NO}_3^-$ was measured with a flow injection analyzer as above. The nitrification potentials were determined by linear regression of $\text{NO}_2^- + \text{NO}_3^-$ accumulation over time. Nitrification activity in the presence of octyne was considered to be contributed by AOA (which are octyne-resistant), and the difference between nitrification activity with and without octyne was considered to be contributed by AOB (octyne-sensitive).

2.4 Gross and net nitrification rates

Gross nitrification rates were determined in laboratory incubations for soil

sampled in August 2011 and 2014. Three well-mixed 40 g dry-weight equivalent subsamples were weighed into plastic specimen cups. Then, 1.6 ml of a $^{15}\text{NO}_3^-$ solution (containing 3.33 mM K^{15}NO_3 at 99 atom % ^{15}N) was added to the soils and carefully mixed, creating a final soil water content of 0.18 kg kg^{-1} . The quantity of ^{15}N added approximately doubled the soil NO_3^- pool. Immediately following soil mixing, one subsample was harvested and extracted with 2 M KCl to determine NO_3^- concentration and ^{15}N enrichment at time-0. The other two subsamples were placed in 1-L Mason jars with lids containing butyl rubber septa and with 1 ml water at the bottom of the jar to minimize loss of moisture from the soil. Octyne was immediately added to the subsample in one of the jars after the lid was sealed (final concentration was $4 \mu\text{M C}_{\text{aq}}$). Jars were incubated for 48 h at 25°C before extraction in 2M KCl. Soil $\text{NO}_2^- + \text{NO}_3^-$ were measured as described above. The extracts were prepared for ^{15}N analyses using a diffusion procedure described in Stark and Hart (1996), and the ^{15}N enrichment was measured by continuous-flow direct dry combustion and mass spectrometry with an ANCA 2020 system (Europa Scientific, Cincinnati, OH). Gross nitrification rates were calculated using the equation of Norton and Stark (2011). Net nitrification was determined by a 21-day incubation. Fifteen grams of moist soil (0.18 kg kg^{-1} water content) in a plastic specimen container was placed in a 1-L Mason jar with 1 ml water at the bottom of it to minimize loss of moisture from the soil. Soil was extracted before and after incubation and $\text{NO}_2^- + \text{NO}_3^-$ accumulation determined over the incubation time.

2.4 Soil DNA extraction and real-time quantitative PCR

Soil samples were brought from the field to the laboratory, and immediately a 10 g sample of soil was frozen at -80 °C until DNA extraction. Soil DNA was extracted following the MoBio Power Soil DNA isolation protocol (MoBio Laboratories Inc, Carlsbad, USA) using 0.25 g moist soil. DNA extracts were quantified by using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Quantitative PCR of AOA and AOB *amoA* genes was performed using the SsoAdvanced SYBR Green Supermix and a CFX CONNECT Real-Time PCR Detection System (Bio-Rad laboratories, Hercules, CA, USA). Primers amoA19F and amoA643R were used to quantify AOA *amoA* gene abundance, while primers amoA189F and amoA2R were used to quantify AOB *amoA* gene abundance (Habteselassie et al., 2013; Leininger et al., 2006; Okano et al., 2004). Standard curves were constructed with plasmids containing cloned *amoA* products from genomic DNA of *Nitrosospira multiformis* ATCC 25196 or from environmental DNA. The 25 µl reaction mixture contained 12.5 µl SsoAdvanced SYBR Green Supermix (Bio-Rad laboratories, Hercules, CA, USA), 0.5 µM of both reverse and forward primers, and 10 µl of 10-fold diluted soil DNA extract. Amplifications were carried out as follows: an initial denaturation step of 95°C for 10 min, 40 cycles of 95°C for 45 s, 60°C for 1 min for AOB or 58°C for AOA, and 72°C for 45 s, and a final extension step of 72°C for 10 min. Fluorescence intensity was read during the 72°C step of each cycle. Reaction efficiencies ranged from 99%-105%, and R² values ranged from 0.990-0.999 for AOB. For AOA, efficiencies ranged from 98-106% and R² values ranged from 0.992-0.999.

2.5 Pyrosequencing and bioinformatic analysis

Sequencing of *amoA* amplicon libraries was accomplished using the 454 sequencing system (Roche Life Sciences, Branford, CT). Amplicons of AOA and

AOB *amoA* genes were obtained with the same primers as mentioned above, but 10 bp barcodes and linkers were added to each forward primer. The 25 µl PCR reaction mixture contained 1 × PCR buffer (MgCl₂ plus), 0.2 mM dNTPs, 1 µM of each forward and reverse primers, 0.05 U of enzyme (FastStart High Fidelity Enzymes Blend (Roche Life Sciences, Branford, CT), and 2 µl of template DNA. Amplification was carried out as follows: an initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min, and a final extension step of 72°C for 10 min. The PCR products were checked by agarose gel electrophoresis, and cleaned by Agencourt AMPure XP (Beckman Coulter, Inc.). The DNA concentration of the purified PCR product was measured using the Quant-iTTM PicoGreen dsDNA BR Assay Kit (Invitrogen) according to the manufacturer's protocol. Finally, the purified PCR products were pooled in an equal mole concentration, and then sequenced unidirectionally from the forward primer on a GS FLX+/XLR70 Instrument.

The bioinformatic analysis was performed using QIIME (Caporaso et al., 2010). Raw pyrosequencing reads were split based on the barcodes, and the sequences of low quality (quality score < 25, length < 350 bp) were removed. The obtained sequences were further denoised (Reeder and Knight, 2010), and then submitted to RDP pipeline using the FrameBot tool to fix frame shifts (Wang et al., 2013). The remaining quality-screened sequences were clustered into operational taxonomic units (OTU) based on 90% sequence identity. The 90% similarity level was chosen because AOB pure cultures with about 90% *amoA* identity have ~97% identity of 16S rRNA genes (Norton et al., 2002). The longest sequence from each OTU was chosen as a representative sequence. AOA *amoA* representatives were trimmed and aligned to an existing high-quality *amoA* database (Pester et al., 2012) using the ARB programs

(Ludwig et al., 2004). AOB *amoA* representatives were also trimmed and aligned with reference sequences, which were retrieved from GenBank. Phylogenetic trees were constructed by neighbor-joining using a Kimur 2-parameter distance with 1000 bootstrap replicates with MEGA 6 (Tamura et al., 2013).

2.6 Statistical analysis

Statistical analysis of the effect of N source on soil properties and *amoA* gene copy numbers were completed using a repeated measures analysis of variance (ANOVA) with the Proc Mixed model in SAS 9.2 (SAS Institute, Inc., Cary, NC, USA). Treatment and year were used as fixed effects and block as a random effect. Data were log transformed as necessary to meet normality assumptions. Two-way ANOVA was used to analyze effects of treatment and year on AOA and AOB *amoA* relative abundance. Two-way ANOVA was used to analyze effects of treatment and 1-octyne on gross and potential nitrification rates in soils sampled in Aug 2014. One-way ANOVA was used to evaluate the relative contribution of AOB to RNP and NP among treatments. P values <0.05 were considered to be statistically significant. To carry out the beta-diversity analysis, the AOA and AOB libraries were first normalized to 359 and 219 reads respectively (fewest sequences per sample) and then weighted UniFrac distance matrices were produced using QIIME. PCA and two-way PerMANOVA were conducted to visualize and assess the weighted Unifrac distances matrices in *vegan* package of R software (<http://www.r-project.org>).

3. Results

3.1 Soil chemical properties

Soil N treatments showed no significant effects on soil organic C over four years (Fig.1A and table S1) although soil organic C was higher in soils receiving compost in

2014 when the year was considered separately. Total N was affected by both treatment and year (Fig.1B). Soil pH was lowest in the AS200 treatment in all four years ranging from 8.11 to 8.15 (Fig.1C), while control and compost treatments had a relative higher soil pH (8.15-8.35). Soil extractable NH_4^+ was not influenced by treatment in either May and Aug soil samples over four years (Fig.1D and Table S1). Soil extractable nitrate showed significant treatment and year effect in soils sampled both in May and Aug (Fig. 1E, 1F and Table S1). AS200 often had a higher extractable nitrate than other treatments over four years due to excessive nitrate not used by plants.

3.2 Nitrification potential and recovered nitrification potential

Nitrification potentials reflect the short-term rate of nitrate formation in a well-mixed system supplied with 1 mM NH_4^+ . The actual solution concentration of ammonium during the assay is closer to 0.5 mM due to soil adsorption. Repeated ANOVA analysis showed that both treatment and year significantly affected NP, and there was also a treatment and year interaction effect (Table S1). As early as August 2011, NPs were significantly elevated by AS or compost treatments compared with control (Fig.2). Over all four years, AS200 had the highest NP. Compost treatment had a significantly higher NP than control treatment in 2011 and 2013.

Like the NP assay, recovered nitrification potential measured nitrate production contributed by AOA or AOB under 1 mM NH_4^+ in a well-mixed system. The RNP assay indicated that both AOB and AOA were involved in ammonia oxidation in August 2013 (Fig.3A). RNP_{AOA} showed no significant differences among the

treatments, and averaged $5.63 \text{ mg N kg}^{-1} \text{ d}^{-1}$. However, the RNP_{AOB} were higher in AS treatments, ranging from 5.59 to $20.08 \text{ mg N kg}^{-1} \text{ d}^{-1}$. RNP_{AOA} and RNP_{AOB} contributed almost equally to total RNP in control soils, while RNP_{AOB} contributed more (above 70%) to total RNP in soil from the fertilized treatments.

The soil nitrification potential assays with the differential inhibitor 1-octyne showed similar results to the RNP assay for August 2013 soil samples (Fig.3B). Generally, RNP_{AOA} was significantly correlated with octyne-resistant NP ($r=0.56$, $p=0.02$), but octyne-resistant NP was lower than RNP_{AOA} averaging $3.0 \text{ mg N kg}^{-1} \text{ d}^{-1}$. Therefore, the NP assay with octyne suggested that octyne-sensitive NP (i.e. AOB) contributed dominantly (74-88%) to NP under all treatments. ANOVA analysis also indicated that octyne-sensitive NP showed a higher fraction of total NP in N added treatments (Fig.3B). The NP measured in May 2014 from 0-30 cm depth soil (Fig 3C) was about 50% of NP in Aug 2013 from 0-15 cm depth soil (Fig 3B), and the octyne-resistant NP ranged from 1.3 to $2.3 \text{ mg N kg}^{-1} \text{ d}^{-1}$ in May 2014. Again, octyne-sensitive NP dominated under all treatments. NP assay in August 2014 soil samples showed the similar result as August 2013 soil samples (Fig. 4A).

Net and gross nitrification rates, measured in whole soils without NH_4^+ addition, were elevated after four years repeated fertilization and cropping (Table 1). There was no significant treatment effect in Aug 2011. In Aug 2014, both net and gross nitrification rates were higher in AS and compost treatments, but net and gross rates showed no difference between AS and compost treated soils. Under ambient NH_4^+ condition, almost all of the gross nitrification rate was due to octyne-resistant activity

(Fig.4B). Octyne-resistant gross nitrification rates were higher in N-addition treatments.

3.3 Abundance of AOA and AOB

In May 2011 before treatment application, the copy numbers of *amoA* from AOA and AOB were $4.1 \times 10^7 \text{ g}^{-1} \text{ soil}$ and $3.9 \times 10^6 \text{ g}^{-1} \text{ soil}$, respectively (Fig.5A and 5B). AOA *amoA* numbers were significantly affected by year in May and August soil samples, respectively (Fig. 5A and 5C; Table S2). While treatment showed no significant effects on AOA *amoA* numbers, AOA *amoA* numbers were higher than AOB for all time points. In contrast, AOB *amoA* numbers were significantly affected by both treatment and year (Table S2). After the first fertilization, AOB *amoA* numbers showed no significant difference among treatments (August 2011 and May 2012; Fig 5B and 5D). However, after the second fertilization, AOB numbers were higher in the AS200 treatment. This effect lasted through the third and fourth years, over all sampling times and depths. The ratios of AOA to AOB *amoA* copy numbers, varying from 2.9 to 158.8, were strongly affected by treatment and year, and were higher under control and compost treatments than under AS treatments after the second fertilization (Table 2, Table S2). AOB *amoA* copy numbers (but not AOA) were significantly correlated with NP rates ($p < 0.001$) (Fig S1).

3.4 Community structure of AOA and AOB

The structure of AOA and AOB communities was revealed by 454-pyrosequencing analysis of *amoA* genes for Aug 2011 and 2013 samples. Totals of 22,925 and 25,903 high-quality raw sequence reads were obtained for AOB and AOA,

respectively, with 27 and 19 unique OTUs respectively (90% identity cut off).

Consensus representative sequences for the most abundant OTUs are available in GenBank KU291532-54.

Phylogenetic analysis showed that the dominant AOB OTUs were affiliated with the *Nitrosospira* Cluster 3 lineage (Fig.6A). The relative abundance of AOB OTUs was not significantly affected by treatment in 2011, but the five most abundant OTUs (BamoA14, BamoA25, BamoA26, BamoA27, BamoA4) were all significantly affected by treatment in 2013 (Fig.6B). For example, BamoA14, BamoA27 and BamoA4 were higher under AS treatments, while BamoA25 and BamoA26 were higher under control and compost treatments in 2013 soils. Most of AOB OTU relative abundances were significantly affected by year. For example, BamoA10, BamoA15, BamoA23, and BamoA26 were significantly decreased from 2011 to 2013 under all treatments. *Nitrosomonas* (BamoA11, 0.17% of total sequences) was present in 2011 under all treatments, but it was below detection in 2013. AOB community structure was different between 2011 and 2013 and treatment changed AOB community structure in 2013 as revealed by weighted UniFrac distance matrices (Fig.8). Two-way PerMANOVA further confirmed that AOB community structure was significantly affected by year ($p=0.001$) and treatment ($p=0.05$).

All of AOA *amoA* OTUs were affiliated with the *Nitrososphaera* cluster (Fig.7A). Most of AOA *amoA* gene sequences were grouped into AamoA OTU5 (44.1% of total sequences) and AamoA OTU12 (44.0% of total sequences) (Fig.7B). AamoA12 was classified into *Nitrososphaera* subcluster 1.1, while AamoA5 could not

be classified into a stable *Nitrososphaera* subcluster although it was consistently associated with a group including the soil fosmid 54d9. AamoA7, which was the third most abundant OTU, belongs to *Nitrososphaera* subcluster 4.1. The relative abundance of AamoA5 decreased, but AamoA12 increased from 2011 to 2013. However, none of AOA OTU relative abundances were significantly affected by treatment. AOA community structure as revealed by weighted UniFrac distance matrices (Fig.8) and two-way PerMANOVA also showed AOA community composition was not affected by treatments ($p=0.33$) or year ($p=0.18$).

4. Discussion

In this four-year field study, we started with soil from plots with a consistent agricultural management history. We observed significant effects of the new N management on soil total N concentrations, pH, nitrate pools, nitrification activities, ammonia oxidizers populations and communities. Soil pH, nitrate pools, and nitrification potentials were quickly changed by mineral and organic fertilizers after the first fertilization, while changes in ammonia oxidizers populations and communities occurred after the second fertilization or later. Our observations suggest that NP is an useful indicator of community wide nitrification activity, and reflects changes in active nitrifiers and their abundance over time at a non-limiting substrate concentration, while substrate availability controls rates of nitrification under field conditions as indicated by gross nitrification rate measurements.

AOA and AOB abundance and community composition response to contrasting N sources

Overall, we observed higher abundance of AOA compared to AOB, similarly to observations of Leininger et al. (2006) in various agricultural soils. We found ratios of AOA to AOB were higher in the control and compost treated soils after repeated fertilization, both treatments have the majority of their ammonium supplied through mineralization of organic nitrogen (Habteselassie et al., 2013). In this study, AOA abundance fluctuated over four years, but did not respond to ammonium or organic N fertilizers. This is in contrast to previous studies in agricultural soils (Ai et al., 2013; Jiang et al., 2014b), which reported that AOA abundance increased when ammonia was supplied as mineralized organic N derived from composted manure or soil organic matter over more than 10 years. In our study, we did not observe a significant increase in AOA abundance but these changes may develop with additional years or with higher amounts of manure applications.

In this study, AOA *amoA* OTUs were all affiliated with *Nitrososphaera*, which is commonly detected in agricultural soils (Bates et al., 2011; Pester et al., 2012). Most of AOA *amoA* gene sequences were equally distributed into two *Nitrososphaera* clades (together 88.1% of total sequences). This pattern of low richness of AOA is a common observation when soils are sampled at a single location (Jiang et al., 2014a). One of our abundant OTUs (AamoA 5) was associated with the soil fosmid 54d9, which is widely distributed in global soils (Alves et al., 2013; Bates et al., 2011). To date, this group of AOA does not have any pure culture representatives. Another abundant AOA OTU (AamoA12) was classified into *Nitrososphaera* subcluster 1.1, which are often abundant in soils, and includes cultured representatives *Nitrososphaera gargensis* and *Nitrososphaera viennensis* (Hatzenpichler et al., 2008; Tourn et al., 2011) .

Ammonium fertilizer treatments strongly stimulated AOB growth rather than AOA

with repeated fertilizations. This is consistent with previous studies in agricultural soils (Ai et al., 2013; Habteselassie et al., 2013; Shen et al., 2008; Wu et al., 2011). AOB are favored by inputs of mineral ammonium or urea sources and high concentrations of N. Field and soil microcosm experiments have previously shown significant increases in AOB abundance under high ammonium concentration (Jia and Conrad, 2009; Okano et al., 2004; Taylor et al., 2012; Verhamme et al., 2011), while AOA abundance was not affected (Di et al., 2009, 2010). However, we observed that AOB abundance was not increased after a single fertilization when measured near the end of the season in August and in the following May, even though NP was stimulated. Our observations indicate that pre-planting fertilization with soluble ammonium fertilizers (e.g application of ammonium sulfate) changes the growth and community of AOB but not significantly in one season. Continuous corn management would stimulates nitrification in the field, possibly increase the N loss in the soil, and thus reduce the N use efficiency for plants.

The AOB communities were mainly affiliated with the *Nitrosospira* cluster 3 in this study, a finding consistent with other studies that have shown the dominance of this group of AOB in a number of agricultural soils (Chu et al., 2007; Habteselassie et al., 2013; Phillips et al., 2000). Tournia et al. (2010) suggested that *Nitrosospira* cluster 3 often outcompetes other *Nitrosospira* under high ammonium conditions. This is true for most AOB OTUs (including BamoA14 and BamoA27), which were favored by ammonium fertilization in the current study. However, from 2011 to 2013, BamoA26, our most abundant OTU, significantly decreased under AS treatments. This OTU is

related to the 3A cluster containing *Nitrosospira* sp. Wyke8 (Webster et al., 2005) see Fig. 6A, which was also observed to be inhibited by high ammonium concentrations. Interestingly, we detected *Nitrosomonas* (0.17% of total sequences) in 2011 under all treatments, but it was below detection in 2013. *Nitrosomonas* have often been detected in manure treated soils (Fan et al., 2011) and in liquid waste treated soils (Habteselassie et al., 2013). Our results suggest that soil *Nitrosomonas* may not be favored by fertilization and conventional corn management.

Amplicon pyrosequencing revealed that the community composition of AOB was significantly altered by three years but not one year of contrasting N treatment, while AOA community was less responsive to N treatment. Previous microcosm studies for agricultural soils did not show any AOB community shifts under ammonium treatment with 4-6 weeks of incubation (Avrahami et al., 2002; Mendum et al., 1999), while AOB community composition was altered after relatively longer incubations (Avrahami et al., 2003). We observed shifts in the AOB community under AS fertilization after the third year of treatment. Other studies that investigated the effect of long-term field fertilization on ammonia-oxidizer communities showed contrasting results. Some studies indicated repeated fertilization significantly altered AOB community composition (Ai et al., 2013; Chu et al., 2007; Wu et al., 2011), while others have shown changes in AOA community composition as well (Gubry-Rangin et al., 2010; He et al., 2007). The temporal aspect of AOB community shifts in the field that we observed suggest that responses to are likely after 2-3 years of ammonium fertilizer applications.

Contribution of AOA and AOB to nitrification activity

In our study, nitrification potentials range from 5-31 mg N kg⁻¹ d⁻¹, which falls within the range of 2–45 mg N kg⁻¹ d⁻¹ found in many agricultural soils (Ai et al., 2013; Fortuna et al., 2003; Habteselassie et al., 2006a; Taylor et al., 2010). Since NP is measured under excess substrate and without diffusional limitations, it is often thought to reflect the legacy of substrate availability (Fortuna et al., 2003). We found that AS200 treatment had a much higher NP than compost treatment in all years, even though the compost treatment received the same total N. The slow release of ammonium from the compost does not stimulate NP to the same extent as applications of ammonium fertilizers. Gross nitrification measures the actual rate of conversion of NH₄⁺/NH₃ to NO₃⁻, and is often limited by substrate availability (Booth et al., 2005). We found that the highest gross nitrification rates are in the compost treated soils where mineralization increases substrate availability and mineralized ammonium stimulates rates of nitrification under low ammonium ambient conditions. We found that gross nitrification rate was only 1-11% of the NP, but also reflected the effects of fertilization (Table 1). Net nitrification rate was about 60% of the gross nitrification rate (Table 1), indicating an important role for other nitrate consumptive processes.

Interestingly, nitrification potentials were elevated by mineral and organic fertilizers after the first fertilization, while AOA and AOB *amoA* gene abundance showed no significant difference among treatments at this time point. Our *amoA* gene abundance measurement was based on soil DNA, while the rate of ammonia oxidation may be more related to the relationship among transcription, translation and enzyme

function (Myrold et al., 2014; Nicol et al., 2008). Our observation suggests a proteomic approach might be appropriate for explaining short-term changes in nitrification activity especially for the AOB.

In this study, NP rates significantly correlated with AOB *amoA* copy numbers, but not with AOA abundance over four years of repeated N treatments (Fig. S1). The RNP after acetylene inactivation in the presence and absence of bacterial protein synthesis inhibitors suggested that AOB were responsible for approximately 69-76% of nitrifying activity in fertilized soils. The soil slurry approach with or without 1-octyne also indicated that AOB contributed approximately 82-91% to NP in N treated soils. This is consistent with several previous studies (Taylor et al., 2010; Taylor et al., 2012; Xia et al., 2011). Xia et al. (2011) found AOB dominantly contributed to nitrification activity (about 76%) in an agricultural soil with the addition of 100 mg $\text{NH}_4^+\text{-N kg}^{-1}$ soil every week. Taylor et al. (2010) discovered that in recently N fertilized cropped soils, the majority of RNP activity was due to AOB. A subsequent study has shown the relative contributions of AOA and AOB to RNP were affected by cropping treatments, the time since N fertilization, and soil conditions in the field (Taylor et al., 2012). Assuming each AOB cell has 2.5 *amoA* gene copies, and using the octyne-sensitive NP, we calculated ammonia oxidation rate of AOB was about 41 fmol NH_3 oxidized $\text{cell}^{-1} \text{h}^{-1}$. This rate is comparable to ammonia oxidation rates (4-23 fmol NH_3 oxidized $\text{cell}^{-1} \text{h}^{-1}$) of AOB pure cultures (Belser and Schmidt, 1980; Jiang and Bakken, 1999). We observed that AOA also play a role in nitrification potential, especially in control and compost treated soils. Assuming each AOA cell has one

amoA gene copy, and using our estimate of octyne-resistant nitrification potential at 30 °C, we calculated ammonia oxidation rate of AOA was about 0.2 fmol NH₃ oxidized cell⁻¹ h⁻¹. This is similar to specific rates found for AOA pure cultures (Hatzenpichler et al., 2008; Konneke et al., 2005; Tourna et al., 2011) and estimated rates from soils (Jia and Conrad, 2009).

Three months after fertilization, readily available ammonium was consumed, and the majority of ammonium was supplied through mineralization of organic N. Since AOA may have a higher affinity for ammonia than AOB (Martens-Habbena et al., 2009), we expected AOA might dominate *in situ* nitrification, and this hypothesis was confirmed by the findings that the addition of 1-octyne did not inhibit gross nitrification rate in whole soils sampled in August. Taylor et al. (2013) discovered that in cropped soil AOB dominantly contributed to the nitrification activity under saturated ammonium conditions, while AOA activity dominated without ammonium addition based on whole soil octyne inhibition assays. This is consistent with other studies that show that AOA dominate nitrification activity when ammonia is produced by mineralization of soil organic matter (Gubry-Rangin et al., 2010; Offre et al., 2009; Zhang et al., 2010).

The contrasting results of octyne inhibition of nitrification activity under ambient and nitrification potential conditions suggests that ammonium concentration and diffusional supply is a key factor determining the relative contribution of AOA and AOB to measured nitrification. Immediately following ammonium fertilizations AOB activity increases and growth may occur. AOA are still functioning and at low

concentrations of ammonium such as found in August they are responsible for a higher relative amount of the gross nitrification activity. We also observe that at about 1 mM concentrations AOA are still responsible for about 20% of the nitrification potential (Fig 3B,C). but at these concentrations their capacity to respond to additional ammonium is likely saturated Therefore, AOA's relative contributions to nitrification activity decreases rapidly with increasing ammonium availability such as occurs in recently fertilized soils (Giguere et al., 2015).). While inhibition of nitrification activity may occur at high ammonium concentrations (i.e. > 5 mM (Tourna et al., 2010; Tourna et al., 2011 Koper et al., 2010; Prosser and Nicol, 2012)) we did not reach these levels of ammonium in the current study. Inhibition at high ammonium levels may further affect the relative contributions to nitrification of AOA and AOB and this effect requires further investigation especially under field conditions. Our observations suggest controlling the activity of AOB in the field immediately after the application of mineral N fertilizers would be an effective strategy to reduce and delay nitrification and therefore improve the synchronization of N mobility with crop N uptake. However, our study also showed that AOA contributed most to nitrification at low ammonium availability which is typical over a longer portion of the season. Practices to control AOA activity would need to be an extended effort, and may be more important under organic N management. Further studies examining seasonal changes in nitrification kinetics and the role of AO communities in this experimental system are in progress.

In conclusion, our investigation of AOA and AOB population and community composition under mineral and organic N sources for 4 years in the field, found that

one fertilization did not significantly alter the abundance and community composition of AOB and AOA, but significantly elevated nitrification potential. After repeated fertilization, N treatment significantly affected abundance and community composition of AOB, but had little effect on AOA. By evaluating the relative contribution of AOA and AOB to NP after 3 or 4 years of repeated N treatments, we found that AOB dominantly contributed to potential nitrification. However, this dominant role of AOB in NP does not indicate the same role for *in situ* nitrification, and AOA dominated gross nitrification activity at low levels of ambient ammonium. Our results suggests that AOB activity and community are more responsive to ammonium fertilizers than AOA in agricultural soil, and practices to control nitrification should focus attention on AOB, especially in the short term after application of ammonium fertilizers.

Acknowledgements

We would like to thank Danielle Barandiaran, Brittany Johnson, Cory Ortiz, Marlen Craig Rice, Lili Song for laboratory and field assistance. This research was supported by USDA NIFA Award 2011-67019-30178 and Utah Agricultural Experiment Station, Utah State University, and approved as journal paper number 8842.

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Table 1. Net and gross nitrification rates in August 2011 and 2014.

Treatments	NNR*		GNR*		NNR/GNR		GNR /NP	
	2011	2014	2011	2014	2011	2014	2011	2014
Control	0.14	0.36 a	0.19	0.50 a	0.77	0.71	0.02	0.08 b
AS100	0.20	0.49 b	0.19	0.67 ab	1.09	0.73	0.01	0.05 a
AS200	0.17	0.54 b	0.38	1.00 b	0.45	0.54	0.02	0.05 a
Compost	0.15	0.53 b	0.63	0.99 b	0.24	0.53	0.05	0.11 b

Abbreviation: NNR-Net nitrification rate, GNR-Gross nitrification rate, NP-nitrification potential. Different letters within a column indicate significantly different treatment means within the year.

*Unit: mg N kg⁻¹d⁻¹.

Table 2. Ratios of AOA to AOB *amoA* copy numbers from May 2011 to Aug 2013
(mean values, $\pm$ SD, N=4, lowercase letters indicate significant difference among
treatments, P<0.05).

Sample Time	Ratios of AOA to AOB <i>amoA</i> copy number			
	Control	AS100	AS200	Compost
May-2011	10.1 a	13.3 a	16.4 a	20.3 a
May-2012	7.0 a	3.2 a	6.9 a	8.0 a
May-2013	65.6 a	27.1 a	23.5 a	126.4 a
May-2014	122.3 b	18.0 a	15.6 a	94.5 b
Aug-2011	2.8 a	2.9 a	4.8 a	4.0 a
Aug-2012	41.6 b	7.7 a	4.5 a	30.7 b
Aug-2013	36.0 b	9.6 a	6.5 a	34.7 b
Aug-2014	158.8 b	37.1 a	14.4 a	109.9 b

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6A

Figure 6B

Figure 7A

Figure 7B

Figure 8

Figure legends

Fig.1. Soil organic C (**A**), total N (**B**), soil pH (**C**), extractable ammonium (**D**), and extractable nitrate (**E**) in August, and extractable nitrate (**F**) in May for four N treatments (control (no N fertilization), ammonium sulfate (AS 100 & 200 kg N ha⁻¹), and compost (200 kg N ha⁻¹)). Error bars represent standard errors (n=4). Different lowercases above the bars indicate a significant difference among treatments in a specific year (p<0.05), based on repeated measures ANOVA.

Fig. 2. Nitrification potentials in August over four years. Nitrification potential rate was measured by soil slurry assay supplemented with 1 mM NH₄⁺. Error bars represent standard errors (n=4). Different letters above the bars indicate a significant difference among treatments in a specific year (p<0.05), based on repeated measures ANOVA.

Fig.3. Recovery of nitrification potential (RNP) in Aug-2013 soil samples (**A**), nitrification potential (NP) with and without 1-octyne in Aug-2013 (**B**), and in May-2014 (**C**). Percentages above the bars indicates the relative contribution of AOB to total RNP or NP. Different uppercases indicate a significant difference in relative contribution of AOB among treatments (p<0.05), based on one-way ANOVA.

Error bars represent standard errors (n=4). Different letters above the bars indicate a significant difference in total NP or RNP among treatments (p<0.05), based on one-way ANOVA.

Fig. 4. Effect of N treatment and 1-octyne on nitrification potential (**A**), and gross nitrification rate (**B**) in soils sampled in August 2014. Nitrification potential rate was measured by soil slurry assay supplemented with 1 mM NH_4^+ . Gross nitrification rate was determined by using N^{15} pool dilution technique with soils incubated at 25 °C for two days. Error bars represent standard errors (n=4). Different lowercases indicate a significant difference among treatments without exposure to octyne ($p < 0.05$), based on one-way ANOVA. Different uppercase letters indicate a significant difference among treatments with soil samples exposed to octyne ($p < 0.05$), based on one-way ANOVA.

Fig. 5. Abundance of AOA and AOB *amoA* gene copy numbers ($\log_{10}$ transformed) across four N treatments. Error bars represent standard errors (n=4). Different letters above the bars indicate a significant difference among treatments in a specific year ($p < 0.05$), based on repeated measures ANOVA.

Fig. 6. **A**) Neighbor joining tree for representative AOB partial *amoA* OTUs (representatives with relative abundance > 0.1%). OTUs from this study are shown in bold. The scale bar represents 5% nucleic acid sequence divergence, and bootstrap values (>50%) are showed at branch points. **(B)** The relative abundance of partial AOB *amoA* OTUs (relative abundance > 0.1%) among four N treatments in Aug-2011 and Aug-2013 soil samples. Error bars represent standard errors (n=4).

Fig.7. A) Neighbor joining tree for AOA partial *amoA* OTU (representatives with relative abundance > 0.1%). AOA *amoA* OTU representative sequences from this study are shown in bold. The scale bar represents 5% nucleic acid sequence divergence, and bootstrap values (>50%) are showed at branch points. **B**). The relative abundance of partial AOA *amoA* OTUs (relative abundance > 0.1%) among four N treatments in Aug-2011 and Aug-2013 soil samples Error bars represent standard errors (n=4).

Fig. 8. Principal coordinate analysis based on the abundance of all AOA and AOB *amoA* gene OTUs (weighted UniFrac). For this analysis, The AOA and AOB *amoA* OTUs were normalized to 359 and 219 reads per sample, respectively. Shapes denote treatment (square=Control, circle=AS100, up triangle=AS200, star=Compost). Fill denote sampling time (open=2011, solid=2013).