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J.W. Munroe, I. McCormick, W. Deen, and K.E. Dunfield

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Munroe, J.W.[1], I. McCormick[2], W. Deen[3], K.E. Dunfield[4*]

1. Present address: Jake Munroe, Ontario Ministry of Agriculture and Rural Affairs, 63 Lorne Ave. E, Suite 2B, Stratford, Ontario, Canada, N5A 6S4
2. Present address: Ian McCormick, FarmStart, 5420 Hwy 6 N., Guelph, Ontario, Canada, N1H 6J2
3. Department of Plant Agriculture, University of Guelph, 50 Stone Road E., Guelph, Ontario, Canada, N1G 2W1
4. School of Environmental Sciences, University of Guelph, 50 Stone Road E., Guelph, Ontario, Canada, N1G 2W1

*author of correspondence:
K.E. Dunfield
Email: dunfield@uoguelph.ca
Phone: 519 824-4120 x 58088
Fax: 519 824-5730

Abbreviations: AOA, ammonia-oxidizing archaea; AOB, ammonia-oxidizing bacteria; qPCR, quantitative polymerase chain reaction; cDNA, complementary DNA; CC, continuous corn; RC, corn-corn-soy-wheat (red clover); NT, no-till; CT, conventional tillage

Core Ideas

- No-till showed trend of higher archaeal and AOA abundance across growing season
- Expression of archaeal *amoA* gene was undetected in our neutral pH agricultural soil
- AOA were, on average, more than 10-fold more abundant than AOB
- Abundance and gene expression of AOB, but not AOA, decreased with soil depth

Abstract

Ammonia oxidizing bacteria (AOB) and archaea (AOA) both mediate soil nitrification and may have specialized niches in the soil. Little is understood of how these microorganisms are affected by long-term crop rotation and tillage practices. In this study, we assessed abundance and gene expression of AOB and AOA under two contrasting crop rotations and tillage regimes at a 30-year old long-term experiment on a Canadian silt loam soil. Continuous corn (*Zea mays* L.; CC) was compared with a rotation (RC) of corn, corn, soybean (*Glycine max* L.), winter wheat (*Triticum aestivum* L.) under-seeded with red clover (*Trifolium pretense* L.), with conventional tillage (CT) and no-till (NT) as sub-plot treatments. Soil sampling was performed during the first corn year at four time-points throughout the 2010 season and at three discrete depths (0-5, 5-15, and 15-30 cm). Overall, AOA abundance was found to be more than 10× that of AOB, though AOA transcriptional activity was below detectable levels across all treatments. Crop rotation had a marginally significant effect on AOB abundance, with 1.3× as many gene copies under the simpler CC rotation than the more diverse RC rotation. More pronounced effects of depth on AOB abundance and gene expression were observed under NT versus CT management, and NT supported higher abundances of total archaea and AOA than CT across the growing season. We suggest that AOB may be more functionally important than AOA in this high-input agricultural soil, but that NT management can promote enhanced soil archaeal populations.

1. Introduction

Ammonia oxidation, the rate-limiting step in nitrification, was until recently believed to be performed exclusively by autotrophic ammonia oxidizing bacteria (AOB). The discovery of ammonia oxidizing archaea (AOA) (Venter et al., 2004; Treusch et al., 2005; Schleper, 2010) has initiated a re-assessment of nitrification processes within a range of ecosystems. Given the impact of nitrification on nitrate leaching potential and nitrous oxide production in agro-ecosystems, an improved understanding of management effects on abundance and gene expression of ammonia oxidation is needed. Quantitative molecular techniques have shown AOA to be abundant in soil (He et al., 2007; Adair and Schwartz, 2008; Leininger et al., 2006; Prosser and Nicol, 2012). However, in an agricultural soil, Jia and Conrad (2009) showed that despite greater abundance of AOA, AOB were more active. Soil pH tends to influence AOA and AOB populations, where increasing pH has been associated with increasing AOB activity (Nicol et al., 2008). Other distinguishing physiological characteristics that may discriminate between AOA and AOB in managed soil environments have been documented (Hatzenpichler, 2012; Prosser and Nicol, 2012). For example, AOA and AOB are believed to predominate in low and high ammonia soils, respectively (Prosser and Nicol, 2012). Cultivated AOA have been found to have a higher substrate affinity than AOB (Martens-Habbena et al., 2009), as well as a lower tolerance to ammonium (NH_4^+) levels (Hatzenpichler, 2012). Micro and mesocosm studies have demonstrated enhanced growth and activity of AOB in soils amended with high levels of inorganic ammonium (Jia and Conrad, 2009; Pratscher et al., 2011). Prosser and Nicol (2012) have proposed that ammonia status of soils be considered on the basis of intermittent increases in concentration due to fertilization, i.e. a high ammonia soil is one that recently received inorganic fertilization. Additionally, growth of AOA has been linked to NH_3 derived from organic materials, whereas NH_3 from ammonium or

urea fertilizers typically supports preferential growth of AOB over AOA (Hatzenpichler, 2012). Stopnišek et al. (2010) and Levičnik-Höfferle et al. (2012) observed close interactions between soil organic N mineralizers and AOA in soil. Thus, the source of NH_3, along with its concentration, is believed to govern niche adaptation of AOA and AOB.

Previous studies on AOA and AOB in agro-ecosystems have focused on long-term fertilizer amendments (Wessén et al., 2010; Zhang et al., 2012) and short-term responses to N inputs (Schauss et al., 2009), but little is known about the effects of crop rotation and tillage practices.

Quantification of gene and transcript abundance of AOA and AOB under various long-term crop rotation and tillage combinations can reflect how N is being transformed and complement information on pathways of potential N loss from the system. Soils managed under more complex crop rotations have been shown to have enhanced structural properties (Munkholm et al., 2013), which can lower risk of N losses by both nitrate leaching and nitrous oxide production. And though tillage practices have shown contradictory results with respect to emission of nitrous oxide, under best practices, no-till management have been shown to reduce nitrous oxide emissions (Wagner-Riddle et al., 2007).

Crop rotation is used to maintain soil fertility and to suppress pests (Davis et al., 2012). More diverse rotations have been found to increase yield and yield stability across a wide range of soil types and climatic conditions (Raimbault and Vyn, 1991; Légère et al., 2011; Davis et al., 2012; Munkholm et al., 2013; Gaudin et al., 2015). Soils under crop rotation tend to contain higher concentrations and qualities of soil organic matter, increased structural stability, increased quantities of microbial biomass and activities (Moore et al., 2000; Munkholm et al, 2013), and enhanced soil enzyme activities (Dick, 1992). Additionally, the inclusion of legumes in rotation,

such as red clover, contribute N to the soil system and to the N requirements of the succeeding crop (Gaudin et al., 2013).

A majority of studies demonstrate higher soil microbial biomass under no-till versus conventional tillage systems (Wardle, 1995), which Kladivko (2001) attributes in part to cooler, wetter conditions and lesser fluctuations in temperature and moisture under no-till. Due to lack of soil disturbance, long term no-till management also results in stratification of organic carbon and N, (Edwards et al., 1992; Zibilske et al., 2002; Gál et al., 2007) but the impact that this may have on AO populations has not been investigated.

The objective of our study was to investigate the long-term effects (30 years) of tillage and rotation on the abundance and gene expression of AOA and AOB throughout a growing season. By contrasting a simple monoculture with a relatively complex rotation, and a strict no-till system with a conventional moldboard plow system, we intentionally sought to investigate magnified differences in management regimes. We hypothesized AOA to have a greater overall abundance under the more complex, legume-based rotation, due to a higher amount and diversity of plant-derived organic N. Under no-till we predicted reduced AOB-to-AOA abundance and transcriptional activity with depth, in accordance with N stratification under such systems and respective affinities of each group for NH_3. Finally, we hypothesized to observe increased abundance and gene expression of AOB, but not AOA, following an early season N fertilization event, regardless of tillage type or crop rotation.

2. Materials and Methods

2.1 Site description and experimental design

Samples were collected from the University of Guelph's Elora Research Station long-term rotation and tillage trial, established in 1980 and located in Elora, Ontario (43°39' N, 80°25' W,

elevation 376 m). The soil on site is a well-drained Woolwich silt loam and classified as a Gray Brown Luvisol (CSSC, 1998) or Albic Luvisol (WRB, 2006). At 0-15 cm, soil texture was comprised of 29% sand, 52% silt, and 19% clay, organic carbon 26.9 g kg^{-1}, and total N 2.4 g kg^{-1} (Jayasundara et al., 2007). Soil pH range was 7.48 – 7.51 (Table S1).

The experiment was designed as a randomized block split-plot with four replicates. The main plot treatment, crop rotation, included seven different rotation sequences. In this study, we collected data on two crop rotations: a corn (*Zea mays* L.) monoculture (CC), and corn-corn-soybean (*Glycine max* L.)-winter wheat (*Triticum aestivum* L.) with an under-sown red clover (*Trifolium pretense* L.) cover crop (RC). The red clover was frost-seeded into winter wheat in early spring and terminated in the fall after wheat harvest (by plowing in CT plots and by herbicide in NT plots). At the time of sampling, in 2010, both crop rotations were planted in their first year of corn. Plot treatment was tillage, and each rotation treatment included no-till (NT) and conventional tillage (CT), with mouldboard plowing to a depth of 15-20 cm as primary tillage under CT. Secondary tillage in CT plots involved two passes with a field cultivator and packer within 1 day of seeding. Fertilizer inputs were the same amongst all rotation and tillage treatments for corn and consisted of 157 kg ha^{-1} of 5-20-20 (5% N, 20% P_2O_5, and 20% K_2O) applied at planting on May 7, 2010, and a second application of 150 kg N ha^{-1} as urea-ammonium-nitrate (UAN; 28% N) side-dressed in a band between corn rows on June 18, 2010. Nitrogen fertilization of other crops in the RC rotation is described in Jayasundara et al., 2007. Corn was harvested at full maturity on October 19, 2010 and yield was measured.

2.2 Soil sample collection

Soil sample collection dates were selected based on tillage and fertilization events. Samples were taken on May 3 (pre-cultivation and seeding, which occurred May 6 and 7, respectively),

June 30 (post-nitrogen side-dress application of June 18), October 12 (pre-corn harvest and plowing, which occurred October 18 and 19, respectively), and on November 23, 2010 (post-corn harvest and plowing). Soil moisture and ambient air temperature values from each sampling date are provided in Table S2. All samples were acquired using a 5 cm diameter soil core and were separated at depth increments of 0-5 cm, 5-15 cm, and 15-30 cm. Four subsamples per plot were collected along a diagonal transect. Subsamples were separated by depth increment and composited by plot. Nucleic acids were preserved in the field by placing two grams of fresh soil from each collected sample immediately into 5 mL of Lifeguard Soil Preservation Solution (MoBio Laboratories, Inc. Carlsbad, CA). Remaining soil was stored at -20^{o}C for nutrient analyses.

2.3 Nucleic acid extraction and reverse transcription

Nucleic acids were extracted within 30 days of sampling, as described in Nemeth *et al*., 2013. Total RNA and DNA were extracted from soils according to manufacturer's protocol using the RNA PowerSoil® Total RNA Isolation Kit and DNA Elution Accessory Kit (MoBio Laboratories, Inc. Carlsbad, CA). Both RNA and DNA were eluted using RNase and DNase-free water. DNA concentration and purity was checked using NanoDrop 8000 spectrophotometry (NanoDrop Technologies, Wilmington, DE). Isolated DNA was stored at -80^{o}C. Isolated RNA was immediately converted to complementary DNA (cDNA) via a reverse transcription reaction (Promega, Madison, WI). Ten µL of RNA was used per reaction, which contained random primers and the reverse transcriptase enzyme. Conversions were performed in triplicate for each sample. A control reaction was performed using nuclease-free water instead of reverse transcriptase to verify the absence of DNA. cDNA was stored at -80^{o}C until further analysis.

2.4 Quantitative analysis of genes and transcripts

A quantitative polymerase chain reaction assay (qPCR) was performed to estimate abundance of AOB, AOA, total bacterial and total archaeal DNA and their respective transcripts. Primer pairs that target a segment of *amoA* (bacterial *amoA*), *crenamoA* (archaeal *amoA*), *bac16S* (bacterial), and *arch16S* (archaeal) genes were used, respectively (Rotthauwe et al., 1997; Tourna et al., 2008; Fierer et al., 2005; Kemnitz et al., 2007). Primer sequences can be found in Table S3. All qPCR assays were performed with a Bio-Rad iQ5 detection system (Bio-Rad Laboratories, Mississauga, ON). Optimized cycling profiles were used from the aforementioned citations, with the exception of *amoA*, for which the thermocycle profile included an annealing temperature of 57 °C and was adapted from Glaser et al. (2010), and *bac16S*, which was altered from Fierer et al. (2005) to 5 minutes at 95 °C, followed by 30 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds, and a final 10 minutes at 72 °C.

DNA extracts were tested for inhibition by dilution and appropriate dilutions were used for quantitative PCR. For each 1 μL template (approx. 5 ng of DNA, 1.5 ng cDNA), reactions were performed in a total volume of 25 μL per sample, with 12.5 μL of 2x SYBR Green Supermix, 10 μM (10 pmol μL^{-1}) each forward and reverse primers, 1 μL T4g32, and RNase and DNase free water. To reduce inhibitory effects of soil humic acid during the qPCR process, 150 ng μL^{-1} T4 gene 32 protein (Applied Biosystems, Life Technologies Corp., Carlsbad, CA, USA) was included (Kreader, 1996). Gene copy quantification was determined by fluorescence intensity of SYBR Green dye. Melt curve analyses were performed for each respective gene to verify amplicon specificity. Polymerase chain reaction runs had average efficiencies of 90.5, 89.4, 105.9, 94.2% and average R^2 values of 0.995, 0.990, 0.996, and 0.996 for bacterial and archaeal *amoA*, and bacterial and archaeal *16S* genes, respectively. Each measurement was performed in triplicate and

no-template controls in each run had undetectable amplification.

Serial dilutions of non-linearized plasmid DNA containing the target genes were used to construct standard curves. PCR products of bacterial and archaeal *amoA* and *16S* genes were cloned into One Shot® Top10 *Escherichia coli* competent cells using TOPO TA cloning kits (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Products were amplified using plasmid specific primers, visualized by gel electrophoresis, and sequenced to verify the correct and complete sequence (Laboratory Services Department at the University of Guelph).

2.5Nutrient analyses

Inorganic nitrogen was extracted from soil samples using 2 M KCl. Extracted nitrate (NO_3^--N) and ammonium (NH_4^+-N) concentrations were determined colourimetrically via segmented flow analysis (AutoAnalyzer-3, Seal Analytical, Mequon, WI) according to methods described in Drury et al., 1991 and converted to mg kg^{-1} dry soil.

2.7 Statistical analyses

Data was analyzed using Statistical Analysis Software for Windows version 9.2 (SAS Institute, Cary, NC). Statistical differences were determined amongst and between different treatments, sample times, and sample depths. All data were analyzed in a generalized linear mixed model (Proc GLIMMIX). Data were adjusted with a natural logarithm transformation and analyzed with a normal distribution assumption. Sample time, as well as block and block interactions were set as random variables. Independent and interactive fixed effects were analyzed for crop rotation, tillage, sample time, and soil depth. Statistical significance was accepted at $P < 0.05$, and P-values < 0.10 were considered marginally significant. Multiple means comparisons of gene counts were analyzed by least square means, using a Tukey adjustment ($\alpha = 0.05$). Due to missing data for bacterial *amoA* transcripts, data from depth increments of 0-5 and 5-15 cm, not 15-30 cm, were

analyzed to determine statistically significant differences amongst sampling dates. Treatment means and standard errors presented in figures were determined using non-transformed data.

3. Results

3.1 Rotation effects

Crop rotation did not affect AOA abundance, but did have a marginally significant effect on AOB abundance (Fig. 1). The CC rotation had a larger AOB community (3.1×10^6 gene copies g^{-1} dry soil) than the RC rotation (2.3×10^6 gene copies g^{-1} dry soil) (Fig. 1a; $P = 0.06$). When bacterial *amoA* gene abundance was normalized by bacterial *16S* gene abundance, the rotation effect was significant ($P = 0.03$; Table S4). The CC and RC rotations did not differ for AOA, with 6.1×10^7 and 6.5×10^7 archaeal *amoA* gene copies g^{-1} dry soil, respectively. A difference was not detected in mean bacterial *amoA* gene expression levels, which were 1.3×10^4 and 1.8×10^4 gene copies g^{-1} dry soil for CC and RC rotations, respectively (Fig. 1c). No archaeal *amoA* gene expression was detected in any samples.

3.2 Tillage and depth effects

Bacterial and archaeal amoA gene abundance overall were unaffected by tillage. The same was true for total bacteria and archaea (Table S4). Bacterial amoA transcript abundance, however, were significantly higher under no-till than conventional tillage ($P = 0.05$; Table S4). Community size of AOA and AOB, as well as gene expression of AOB, varied by depth according to tillage treatment when pooled by rotation and sampling dates. Under no-till, bacterial amoA gene abundance was significantly lower at 15-30 cm depth than at 0-5 and 5-15 cm (Fig. 2a; $P < 0.01$ for both). Similarly, mean bacterial amoA transcript levels under no-till decreased with depth, with a higher mean value at 0-5 cm than 15-30 cm depth (Fig. 2c; $P = 0.002$). No statistically significant differences existed, however, amongst depth increments for bacterial amoA gene abundance or expression under conventional tillage. Abundance of AOA, alternatively, was found to increase

234 with depth, exclusively under conventional tillage. Mean archaeal amoA gene copy number was
235 greater at 15-30 cm depth than 0-5 cm (Fig. 2b; P = 0.05). Statistically significant differences in
236 archaeal amoA gene abundance amongst depth categories did not exist under no-till.

237 *3.3 Tillage and seasonal effects*

238 Total bacterial and archaeal populations decreased during the 2010 growing season up until
239 October 12 under both no-till and conventional tillage (Fig. 3a & b). The archaeal population
240 rebounded at the November 23 (Post-Plow) time-point, while overall bacterial abundance at
241 November 23 did not differ significant from the previous two time-points. Total bacterial
242 abundance at May 3 under no-till and conventional tillage was significantly greater than June 30
243 no-till (P = 0.02 and 0.01, respectively) and October 12 no-till (P = 0.02 and 0.04, respectively).
244 Total archaeal abundance was significantly greater at May 3 (no-till and conventional tillage) and
245 November 23 under no-till than conventional tillage on June 30 (P = 0.01, 0.01 and 0.02,
246 respectively). Archaea showed a trend of greater abundance under no-till than conventional tillage
247 management at all four sampling dates (Fig. 3b).

248 Ammonia oxidizing bacteria population levels rose slightly from May 3 to June 30,
249 dropped substantially at October 12, and rebounded slightly at November 23 (Fig. 3c). Mean AOB
250 abundance on October 12 under NT was significantly lower than that of May 3 (P < 0.0001),
251 November 23 (P = 0.01), and June 30 (P = 0.01). When bacterial *amoA* was normalized to bacterial
252 *16S* gene abundance June 30 abundance was marginally (P = 0.05; Table S4) greater than that of
253 May 3. No statistically significant differences existed between tillage types at any time-point for
254 AOB. There were no statistically significant differences between sampling dates for bacterial
255 *amoA* transcript levels (Table S4), likely due to large variability of data. Ammonia oxidizing
256 archaea showed a similar pattern to that of total archaea throughout the season: abundance dropped

from May 3 to October 12, but partially rebounded at the November 23 time-point (Fig. 3d). No statistically significant differences existed amongst time-points for AOA; however, when normalized by archaeal *16S*, mean abundance on June 30 differed significantly from May 3 (P = 0.02). Again, AOA showed a trend of greater abundance under no-till than conventional tillage management at all four sampling dates (Fig. 3d), though there were no statistically significant differences in mean AOA abundance at individual time-points between tillage types.

3.4 Soil nutrient concentrations

Mean soil ammonium and nitrate concentrations did not differ significantly by rotation or tillage type (data not shown). Nitrate-nitrogen, but not ammonium, varied significantly amongst sampling dates when averaged across rotation, crop rotation, and tillage (Table S5). The June 30 sampling date had a NO_3^- concentration that was more than 2-3x greater than all other sampling dates: May 3 (P = 0.04); October 12 (P = 0.01); and November 23 (P = 0.01). Both Pre-Tillage (May 3) and Pre-Plow (October 12) NO_3^- mean concentrations were greater than that of November 23 (P = 0.01 and 0.02, respectively; Table S5).

4. Discussion

4.1 Evidence of niche specialization between ammonia oxidizing bacteria and archaea

As has been the case in many other studies on agricultural soils (Leininger et al., 2006; He et al., 2007; Jia and Conrad, 2009; Wessén et al., 2010), we found AOA to be more than 10-fold as abundant as AOB. Despite this finding, archaeal *amoA* transcripts were not detectable. AOB transcripts, however, were detected in all samples, and found to largely mirror AOB abundance. It is important to note that the analysis for AOA and AOB was performed on the identical cDNA samples, therefore, we are confident that the lack of AOA gene expression was not an artifact of PCR inhibition. It could be possible that the primers designed by Tourna et al. 2008 target a sub-

population of AOA that are not active under our soil conditions. Overall, our results suggest that despite a substantial AOA population, AOBs were more transcriptionally active in this slightly basic, intensively managed agricultural soil. Jia and Conrad (2009) determined ammonia oxidation to be explained by AOB rather than AOA in a neutral pH agricultural soil upon addition of fertilizer NH_4^+, despite larger AOA populations. Nicol et al. (2008) observed decreasing AOA and increasing AOB transcriptional activity with increasing pH along a gradient of seven soils. Additionally, changes in nitrification rates in their study were most closely related to AOB gene abundance and expression across all soils (Nicol et al., 2008). However, both archaeal *amoA* gene and transcript abundance was greater than that of bacterial *amoA* in all soils studied (Nicol et al., 2008). In contrast, Gubry-Rangin et al. (2010) found a significant positive relationship between nitrification rate and growth of AOA, but not AOB, in a microcosm study in two acidic agricultural soils.

The absence of detectable archaeal *amoA* transcripts suggests that that AOA existed in the soil in high numbers, but were not actively oxidizing ammonia. This finding contrasts that of Leininger et al. (2006), who detected levels of archaeal *amoA* gene expression to be greater than or equivalent to those of AOB across three different soils. Interestingly, these particular soils were grassland pasture land, not soil under annual crop cultivation (Leininger et al., 2006). In our annual crop rotation, high input agricultural soil, AOA may utilize alternative energy acquisition strategies aside from ammonia oxidiation. Genes indicative of mixotrophy have been identified in several ammonia oxidizing archaea (Hatzenpichler, 2012). A high nitrogen-input soil environment, such as the one in our study, is likely one in which AOA were less competitive than AOB for ammonia substrate, given the preference of AOA for low NH_4^+ concentrations (Hatzenpichler, 2012). Offre et al. (2009) determined that ammonia oxidation by archaea was more

303 important than that of AOB in a microcosm study without external nitrogen input. Indeed, this may
304 be the case for soils in which the nitrification process is fuelled solely by continuous N
305 mineralization of soil organic matter; however, our agricultural soil had a high nitrogen input.
306 Additionally, nitrification has been shown to be governed primarily by AOA under acidic soil
307 conditions, whereas neutral and alkaline soils tend be dominated by AOB (Jia and Conrad, 2009;
308 Gubry-Rangin et al., 2010; Shen et al., 2012).

309 Bacterial and archaeal ammonia oxidizer abundance varied substantially in relation to soil
310 depth and these distributions were affected differently by tillage for each group. In general, AOB
311 abundance and gene expression decreased from 0-30 cm depth, while AOA abundance increased.
312 This finding is similar to that of Leininger et al. (2006), who observed decreasing AOB and
313 constant AOA gene copy numbers with depth, in both fertilized and unfertilized agricultural soils.
314 Hatzenpichler (2012) also state that archaeal: bacterial *amoA* ratios increase with soil depth. Di et
315 al. (2010) found AOB abundance to decrease with soil depth in a grazed pasture soil, and attributed
316 the phenomenon to higher levels of nitrogen input to the topsoil. They observed a larger archaeal
317 than bacterial ammonia oxidizer community in the subsoil, which was explained as a consequence
318 of decreased N concentrations at lower depths (Di et al., 2010; Hatzenpichler, 2012). Indeed, our
319 data also show decreased N (data not shown), and decreased AOBs, lower in the profile. The fact
320 that ammonia oxidation alone does not sustain AOA growth (Tourna et al., 2008; Wessén et al.,
321 2010), may also explain differences in relative AOA and AOB population levels at lower, more
322 oligotrophic soil depths.

323 We observed a significant decrease in AOB abundance and gene expression with depth
324 under no-till management, but not under conventional tillage. Stratification of total nitrogen and
325 organic carbon in the top 30 cm is a well-established phenomenon under no-till (Gál et al., 2007;

Dimassi et al., 2013). Although we did not measure total nitrogen, our findings may reflect the magnified stratification of N that commonly occurs under long-term no-till. In contrast, AOA abundance did not change with depth under no-till, and in fact increased with depth under conventional tillage. These patterns, both overall and in terms of individual tillage systems, suggest niche differentiation between AOA and AOB.

4.2 Management effects on ammonia oxidizing bacteria and archaea

When averaged across depths, sampling dates, and tillage treatments, we hypothesized that the RC rotation would support a larger AOA population than the CC rotation. Growth of AOA is associated with continuous low-level ammonia supply via organic matter mineralization (Offre et al., 2009), which we predicted to be enhanced following a red clover cover crop. The data, however, did not show any difference between the contrasting rotations for AOA. Instead, AOB abundance, when quantified relative to total bacteria, was found to be greater under the CC rotation. Differences in soil mineral N concentrations in the season of sampling did not explain this phenomenon. Fertilization history of the two crop rotation treatments may have played a role. AOB are known to be well adapted to conditions common to cultivated soils (Zeglin et al., 2011), which include regular disturbance, influxes of inorganic N fertilizer, and high availability of N in soil (Cavagnaro et al., 2008). Since the establishment of the experiment in 1980, fertilizer N has been applied according to general yield goal recommendations for the region (Raimbault and Vyn, 1991; Jayasundara et al., 2007). As a consequence of a higher frequency of corn, a high N-demanding crop, the CC rotation received an average of 640-680 kg fertilizer N ha^{-1} per four year cycle, while the RC rotation received an average of only 410-430 kg fertilizer N ha^{-1} per four year cycle. Specifically, growth of bacterial but not archaeal ammonia oxidizers is associated with nitrification activity after high levels of ammonium input (Di et al., 2009; Jia and Conrad, 2009;

Verhamme et al., 2011). It is possible that after 30 years of receiving an extra 60 kg ha^{-1} N annually, the CC rotation may have selected for AOBs compared to the RC rotation. Alternatively, differences in crop residue and root composition between crop rotation treatments, in particular C: N ratios, are another potential driver of AOB abundance.

Interestingly, at all sampling dates we observed a trend towards higher total archaeal abundance under no-till management. This pattern existed for AOA as well, but was not observed for total bacteria or the AOB community. Most organisms have a greater abundance under no-till management than conventional tillage (Kladivko, 2001), although this is not true in all cases. For example, abundance of Astigmatid mites has been shown to be negatively affected by tillage in just under 50% of studies, while over 50% show minimal to large increases in abundance under tillage (Wardle, 1995). However, in the majority of cases bacteria and fungi are negatively affected by tillage in agro-ecosystems (Wardle, 1995). Relatively little is known regarding the effects of tillage on archaea; the same is true for ammonia oxidizing archaea. Organisms from the Thaumarchaeota phylum, to which AOA belong, were found by Dorr de Quadros et al. (2012) to be more abundant under long-term no-till than conventional tillage management. Souza et al. (2013) observed consistently higher archaeal abundance under no-till versus conventionally tilled fields under a 13-year experiment – as determined by a shotgun sequencing approach – and proposed archaea to be strong indicators of soil quality. Our results provide further indication that no-till management promotes enhanced abundance of archaea, and suggest that AOA community size may increase in response to reduced tillage.

AOB population sizes are known to increase in response to nitrogen fertilization in agricultural soils (Hermansson and Lindgren, 2001; Okano et al., 2004; Cavagnaro et al., 2008; Di et al., 2010). We therefore predicted an increase in AOB abundance and gene expression following

the application of side-dressed nitrogen fertilizer. Ammonia oxidizing archaea, conversely, do not typically increase in abundance after fertilizer N application. We hypothesized that AOA abundance would remain similar or decrease on June 30 relative to May 3 pre-fertilization levels. Indeed, AOA levels did not change from May 3 to June 30, after fertilization. While bacterial amoA gene abundance was not significantly different between these time points either, AOB were enriched on June 30 relative to total bacteria. This was reflected in a marginally greater AOB: total bacteria ratio Post-Fertilization (June 30) than Pre-Tillage (May 3). Accordingly, inorganic N soil levels were highest at the June 30 sampling date, which followed N side-dressing. Di et al. (2010) found AOB to grow substantially after addition of a urine-N substrate in a grassland soil, whereas AOA grew solely in soils that did not receive substrate. In a microcosm study, Verhamme et al. (2011) concluded that AOA growth was not strongly correlated to different rates of ammonia oxidation; AOB exhibited the dominant response to high soil ammonium levels. Additionally, seasonal changes in soil moisture and ambient air temperature likely had an influence on the populations.

Given that the side-dressed N fertilizer applied at our site contained a substantial portion of both urea and ammonium, we expected a response from AOB. Relative to total bacteria, we observed a marginal increase in AOB abundance. This very well could be explained by enhanced substrate availability and preferential growth conditions for ammonia oxidizing bacteria over their archaeal counterparts. The effect of changing soil moisture and temperature with the progression of the growing season stands as a potential confounding factor when considering AOB abundance alone. Importantly, however, bacterial *amoA* gene abundance followed the seasonal pattern of AOA and total bacterial and archaeal communities at all time points except that date immediately following the application of inorganic N fertilizer. Overall, our data suggest that AOB, not AOA,

were the dominant ammonia oxidizing microorganisms in these cropping systems, particularly following fertilizer N input.

Conclusions

The effects of 30-years of crop rotation and tillage on AO abundance and gene expression supports the notion that AOB are functionally more important than AOA in neutral pH, high-input agricultural soils. Interestingly, despite high abundance of AOA, we did not detect archaeal amoA gene expression. Throughout the season, we found that tillage influenced archaeal, but not bacterial populations, no-till plots showed a pattern of increased total archaeal and AOA abundance relative to conventional tillage. Distributions of AOB and AOA differed by depth, to our knowledge, we are the first to show accentuated differences in AOB abundance and transcriptional activity along a soil depth profile under long-term no-till versus conventional tillage management. Finally, we anticipated a pronounced increase in AOB abundance and transcriptional activity following an in-season application of inorganic nitrogen. Although this was not the case, comparison with seasonal changes in total archaeal and bacterial abundance suggest that AOB and AOA populations responded differently to nitrogen application.

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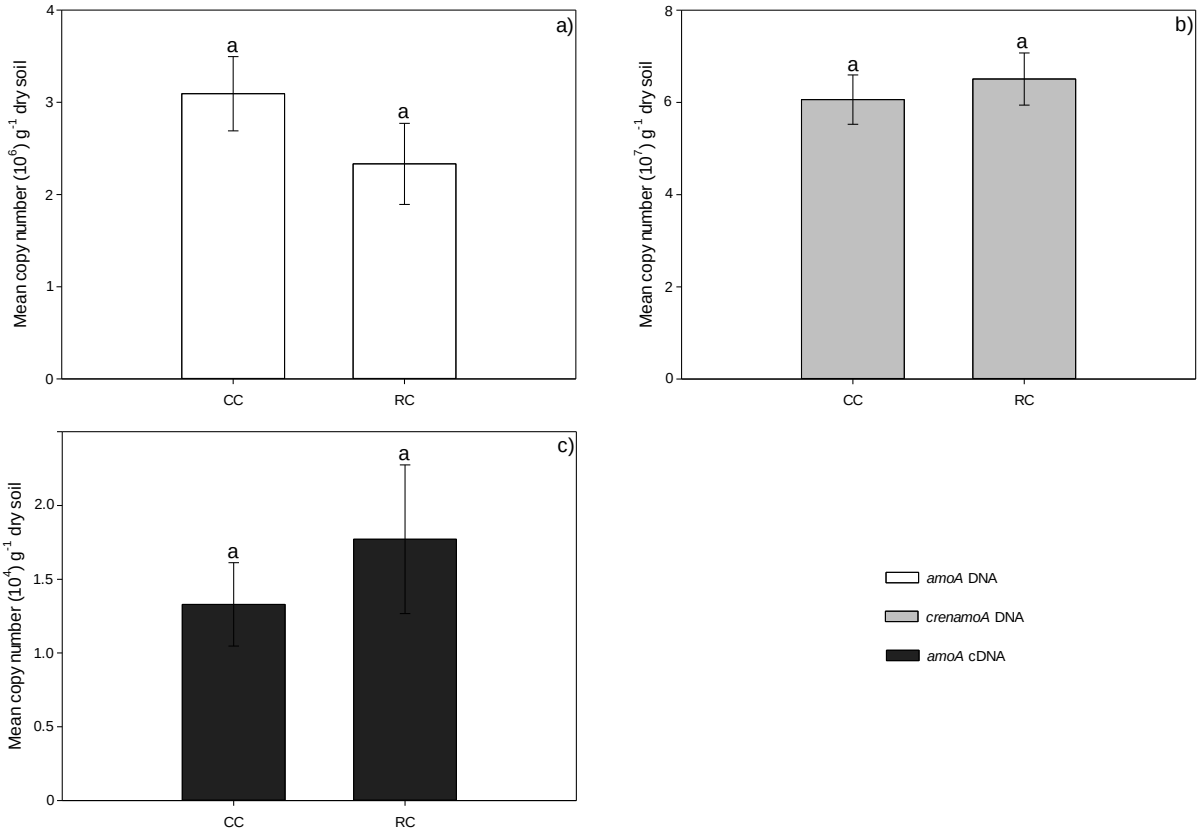
Figure Captions

Figure 1. Mean number of copies of a) the bacterial *amoA* gene, b) the archaeal *amoA* gene, and c) bacterial *amoA* transcripts per gram of dry soil under continuous corn (CC) and corn-corn-soy-wheat-red clover (RC) rotations. Data averaged across tillage types (no-till and conventional tillage) and depth increments (0-5 cm, 5-15 cm, and 15-30 cm). The same letters above bars within each graph represent a non-statistically significant difference ($P < 0.05$) in mean copy number between crop rotation treatments. Statistically significant difference ($P < 0.1$) is indicated by *. Bars represent standard error of the mean (n=4).

Figure 2. Mean number of copies of a) the bacterial *amoA* gene, b) the archaeal *amoA* gene and c) bacterial *amoA* transcripts, per gram of dry soil for no-till and conventional tillage sub-treatments at 0-5, 5-15, and 15-30 cm depth increments. Data averaged across rotation treatments and sampling dates at each depth increment. The same lower case letters beside bars within the no-till sub-treatment and same upper case letters within the conventional tillage sub-treatment represent non-statistically significant ($P < 0.05$) differences in mean copy number amongst depth increments. Bars represent standard error of the mean (n=4).

Figure 3. Mean number of copies for a) the bacterial *16S*, b) archaeal *16S*, c) bacterial *amoA*, and d) archaeal *amoA* genes per gram of dry soil by sampling date, under no-till (NT) and conventional tillage (CT) sub-treatments. Data averaged across rotation treatments and depth increments (0-5 cm, 5-15 cm, and 15-30 cm). Time-points represent pre-spring tillage and planting (May 3), post-nitrogen side-dress application (June 30), pre-plowing (October 12), and post-plowing (November 23). Bars represent standard error of the mean (n=4). The same letters between time-points represent non-statistically significant ($P < 0.05$) differences in mean gene abundance (no-till and conventional tillage pooled). Regression lines provided only to demonstrate trends of data over time.



612

613 **Fig 1**

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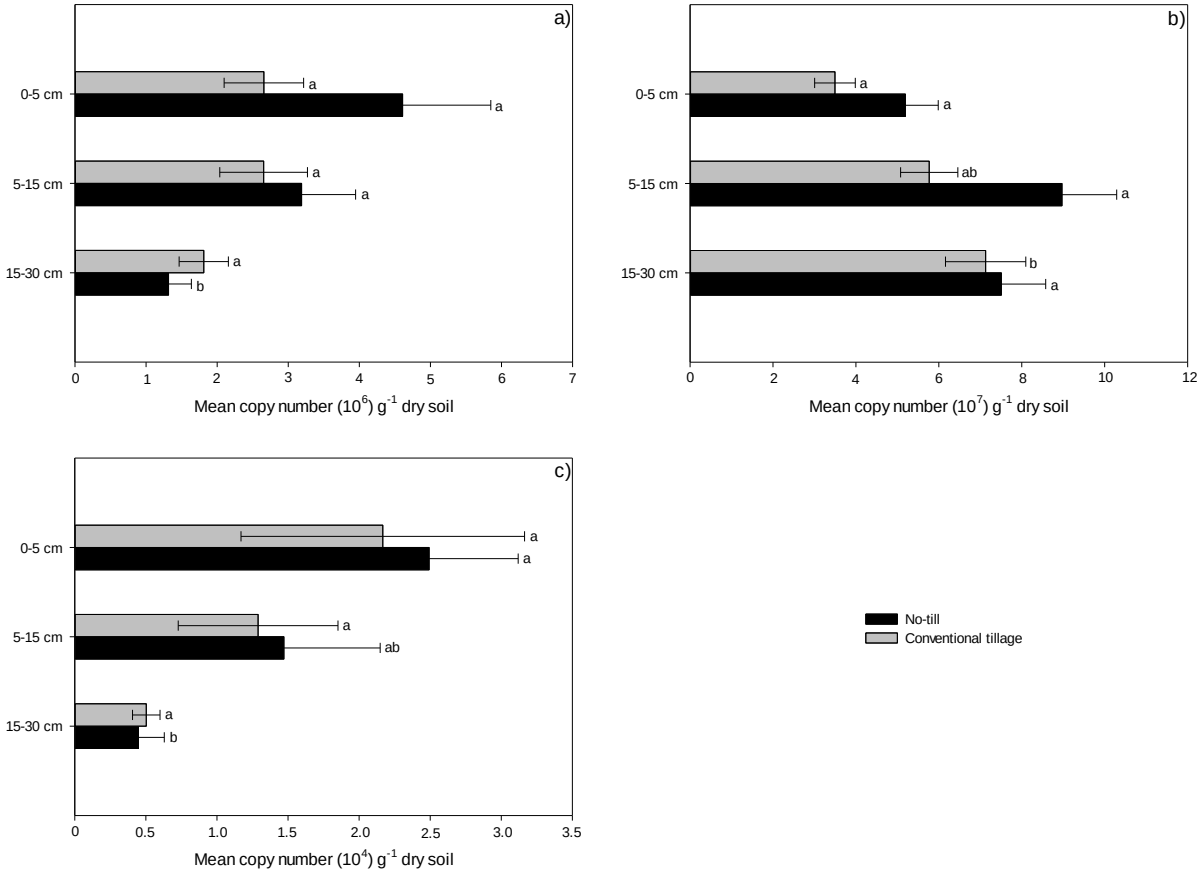

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616 **Fig 2**

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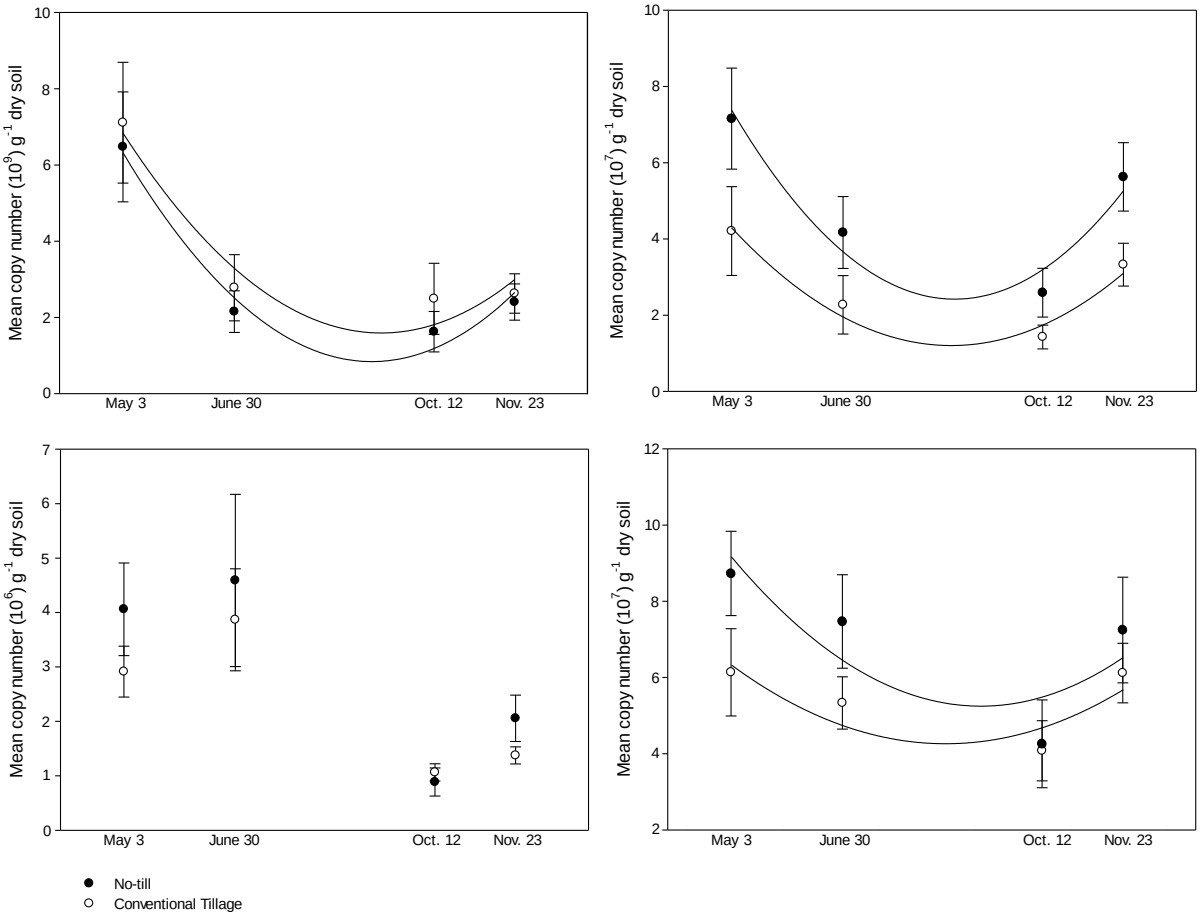
Mean copy number (10^9) g^-1 dry soil
Mean copy number (10^7) g^-1 dry soil
Mean copy number (10^6) g^-1 dry soil
Mean copy number (10^7) g^-1 dry soil
May 3
June 30
Oct. 12
Nov. 23
No-till
Conventional Tillage

Fig 3

Table S1. Mean soil pH (n=4) under main plot rotation treatments, 2010.

Crop rotation	Soil pH
Continuous corn	7.51
Corn-Corn-Soybean-Winter Wheat (Red Clover)	7.48

Table S2. Soil moisture and ambient surface air temperature on sampling dates.

Sampling Date	Soil Moisture (%)	Temperature (°C)
Pre-tillage (May 3)	22.1	18.1
Post-fertilization (June 30)	16.4	15.2
Pre-plowing (October 12)	18.5	8.9
Post-plowing (November 23)	16.4	4.8

Table S3. Sequences, target genes, and associated citations for primer pairs used.

Primer	Sequence (5'-3')	Target	Citation
amoA-1F	GGGGTTTCTACTGGTGGT	Ammonia monooxygenase gene	Rotthauwe et al., 1997
amoA-2R	CCCCTCKGSAAAGCCTTCTTC		
crenamoA-23F	ATGGTCGGCTWAGACG	Ammonia monooxygenase gene	Tourna et al., 2008
crenamoA-616R	GCCATCCATCTGTATGTCCA		
bac16S-338F	ACTCCTACGGGAGGCAGCAG	16S small ribosomal gene	Fierer et al., 2005
bac16S-518R	ATTACCGCGGCTGCTGG		
arch16S-364F	CGGGGYGCASCAGGCGCGAA	16S small ribosomal gene	Kemnitz et al., 2007
arch16S-934R	GTGCTCCCCCGCCAATTCCT		

Table S4. Long term rotation, tillage, sampling date and depth effects on bacterial and archaea *amoA* and *16S* gene abundance, as well as bacterial *amoA* transcript abundance, in the 2010 season. Statistical significance at $P < 0.05$ indicated by bold text and $P < 0.1$ by italicized text.

	df	Bacterial *amoA* gene abundance	Archaeal *amoA* gene abundance	Bacterial *16S* gene abundance	Archaeal *16S* gene abundance	Bacterial *amoA* transcript abundance*	Bacterial *amoA*: *16S* gene abundance	Archaeal *amoA*: *16S* gene abundance
		Pr (>F)	Pr (>F)	Pr (>F)	Pr (>F)	Pr (>F)	Pr (>F)	Pr (>F)
Rotation	1	*0.0591*	*0.0723*	0.1242	0.6444	0.4780	**0.0285**	0.5445
Tillage	1	0.2412	0.2370	0.1702	0.1000	**0.0467**	0.2262	0.2685
Date	3	**<0.0001**	0.1455	**<0.0001**	**0.0007**	0.2628	**0.0115**	**0.0132**
Depth	2	**<0.0001**	**0.0215**	**0.0082**	**0.0050**	**0.0211**	**0.0112**	**0.0010**
Rotation × Tillage	1	0.3514	0.3776	**0.0445**	0.5243	0.1357	0.5441	0.1158
Rotation × Date	3	0.6483	*0.0590*	0.1351	0.1871	0.1201	0.8914	0.8246
Rotation × Depth	2	0.4569	0.1095	0.3651	0.1330	0.6110	0.9124	0.7705
Tillage × Date	2	0.2093	0.3016	0.2296	0.4864	0.6281	0.7594	0.9201
Date × Depth	6	0.1072	0.7356	**0.0007**	**0.0386**	**0.0132**	0.9875	0.1788
Depth × Tillage	2	**0.0011**	0.1904	0.2292	0.4078	0.4404	**0.0251**	0.4816
Rotation × Tillage × Date	3	0.5883	0.5221	0.5027	0.1890	0.4013	0.7382	0.4417
Rotation × Date × Depth	6	0.1344	0.1249	**0.0128**	0.5046	0.2751	0.5707	0.7109
Rotation × Depth × Tillage	2	0.3897	**0.0293**	0.5809	0.5436	0.5223	0.1671	0.0891
Tillage × Depth × Date	6	**0.0021**	0.4600	**0.0077**	0.1946	n/a	**0.0425**	0.5833
Rotation × Tillage × Date × Depth	6	0.6356	**0.0067**	*0.0633*	0.5578	n/a	0.0719	0.4615

Table S5. Soil ammonium- and nitrate-nitrogen values (mg kg^{-1} dry soil) averaged across rotation treatments, tillage sub-treatments, and across depth increments at each sampling date. Different lower case letters represent a statistically significant ($P < 0.05$) difference between sampling dates. Standard error of the mean is provided after ± symbol.

Sampling Date	NH_4^+ (mg kg^{-1} dry soil)	NO_3^- (mg kg^{-1} dry soil)
May 3	2.64 ± 0.54 a	6.70 ± 1.02 b
June 30	5.70 ± 1.35 a	14.49 ± 2.36 a
October 12	2.03 ± 0.43 a	5.04 ± 0.56 b
November 23	2.35 ± 0.41 a	3.84 ± 0.43 c