Dynamics of ammonia oxidizing archaea and bacteria populations and contributions to soil nitrification potentials

Anne E. Taylor[1*], Lydia H. Zeglin[1], Thomas A. Wanzek[1],

David D. Myrold[1], and Peter J. Bottomley[1,2]

Departments of Crop and Soil Science[1] and Microbiology[2], Oregon State University

*Corresponding author:

anne.taylor@oregonstate.edu
3017 Ag Life Science Building
Corvallis, OR 97331
541-737-4136
541-737-0496 (fax)

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Abstract

It is well known that the ratio of ammonia oxidizing archaea (AOA) and bacteria (AOB) ranges widely in soils, but no data exist on what might influence this ratio, its dynamism, or how changes in relative abundance influences the potential contributions of AOA and AOB to soil nitrification. By sampling intensively from cropped-to-fallowed and fallowed-to-cropped phases of a two year wheat/fallow cycle, and adjacent uncultivated long term fallowed land over a 15-month period in 2010 and 2011, evidence was obtained for seasonal and cropping phase effects on the soil nitrification potential (NP), and on the relative contributions of AOA and AOB to the NP that recovers after acetylene inactivation in the presence and absence of bacterial protein synthesis inhibitors. AOB community composition changed significantly ($P \leq 0.0001$) in response to cropping phase, and there were both seasonal and cropping phase effects on the *amo*A gene copy numbers of AOA and AOB. Our study showed that the AOA:AOB shifts were generated by a combination of different phenomenon: an increase in AOA *amo*A abundance in unfertilized treatments, compared with their AOA counterparts in the N-fertilized treatment; a larger population of AOB under the N-fertilized treatment compared with the AOB community under unfertilized treatments; and better overall persistence of AOA than AOB in the unfertilized treatments. These data illustrate the complexity of the factors that likely influence the relative contributions of AOA and AOB to nitrification under the various combinations of soil conditions and NH_4^+-availability that exist in the field.

Introduction

Ammonia oxidizing archaea (AOA) and ammonia oxidizing bacteria (AOB) co-occupy every agricultural soil that has been examined to date. However, we know little about AOA and AOB population dynamics under field conditions, or how their relative contributions to soil nitrification respond to the combination of cropping treatment and seasonal conditions. It has been observed in laboratory incubations that AOB *amo*A gene abundance increases in soils supplemented with high levels of NH_4^+ (200 – 400 μg N/g soil) (Jia and Conrad, 2009; Verhamme et al., 2011), whereas AOA *amo*A gene abundance increases in soil incubations where NH_4^+ was supplied endogenously from mineralization of organic matter or added in low concentrations (≤ 20 μg N/g soil) (Offre et al., 2009; Zhang et al., 2010; Verhamme et al., 2011). In a previous study we developed an assay that allows us to determine the relative contributions of bacteria and archaea to the nitrification potential (NP) of soil slurries (Taylor et al. 2010). It was shown that whereas the NP of permanent pasture soils was dominated by AOA, the NP of N fertilized cultivated soils under wheat was dominated by AOB, and, that both AOA and AOB contributed to the NP of long term fallowed soils (no tillage or N fertilizer for 19 y) (Taylor et al., 2010). Collectively, these observations suggest that AOA and AOB may occupy different soil niches perhaps controlled by NH_4^+ availability.

In most cropping systems, N fertilization results in a transient pulse of high NH_4^+ concentrations, that is followed by a much longer period of lower NH_4^+ availability dependent on N-mineralization from soil and crop residues (Shi et al., 2004; Norton, 2008). A case can be made that the relative contributions of AOA and AOB to soil nitrification might shift in different phases of crop rotation and during different seasons of the year. Although recent studies have evaluated AOA and AOB population sizes, composition, and/or their relative growth responses

66 in soils recovered from agricultural cropping systems, the soils were often taken from complex
67 crop rotations in multi-year cycles, and sampled in either spring, fall or unspecified times
68 (Tourna et al., 2008; Hallin et al., 2009; Wessen et al., 2010; Wessen et al., 2011; Xia et al.,
69 2011). Clearly, the extent to which the phase of the crop rotation or time of soil sampling might
70 have influenced the results cannot be determined.

71 We have chosen a simple two-year cropping cycle of winter wheat/fallow to test our
72 hypothesis that environmental conditions combined with shifts in NH_4^+ availability will
73 influence the dynamics of AOA and AOB contributions to nitrification. We hypothesized that
74 the relative success of AOA and AOB through the two-year cropped/fallowed cycle will depend
75 upon a combination of the following: (a) differential growth responses of AOA and AOB to the
76 application of fertilizer NH_4^+-N; and (b) differential abilities of the AOA and AOB populations
77 to survive the NH_4^+ limiting conditions that exist over the majority of the two-year cropping
78 cycle, and the associated seasonal shifts in soil conditions.
79

80 **Materials and Methods**

81 **Chemicals.** N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) buffer, neomycin
82 trisulfate salt, and NH_4Cl, were obtained from Sigma (St. Louis , MO). Acetylene was obtained
83 from Airgas (Radnor, PA). Kanamycin sulfate, and gentamycin sulfate were obtained from
84 EMD Biosciences, Inc. (La Jolla, CA). Szechrome NAS was obtained from Polysciences, Inc.
85 (Warrington, PA).

86 **Soils. S**oil samples were collected monthly or bimonthly from field plots at the Oregon State
87 University Hyslop Field Research Laboratory located 16 km north of Corvallis, Oregon (See
88 Supplementary Information for more details about the study site). Beginning in April 2010 we
89 sampled three cropped-to-fallowed plots (CF), which were planted in winter wheat Oct 2009,

fertilized with urea (150 kg NH_4^+-N/acre) in Feb 2010, harvested in Aug 2010, fallowed until Oct 2011, and then replanted to wheat. Three fallowed-to-cropped fields (FC) were sampled, which had grown wheat in 2009, and were fallowed through most of 2010, tilled and planted to wheat in Oct 2010, and fertilized with 150 kg NH_4^+-N/ha (a combination of urea and $(NH_4)_2SO_4$) in Mar 2011. Three long-term fallow fields (LTF) were sampled, which had not been cropped, fertilized, or tilled since 1990 and were colonized by volunteer grasses and forbs, and are mowed twice yearly. Four to five soil samples were recovered to a depth of 10 cm from each field via a random walk process, composited and thoroughly mixed, and brought to the laboratory where it was sieved (4.75 mm) and stored at 4^{o}C (Peterson and Calvin, 1996). Samples of soil (5 – 10 g) were oven-dried at 105^{o}C to determine the water content. 2M KCl extractable NH_4^+ was determined at the Central Analytical Services Laboratory, Department of Crop and Soil Science, Oregon State University by continuous flow analysis using an Alpkem RFA 300 auto-analyzer (Astoria Pacific, Clackamas, OR). Precipitation and soil temperature (0 – 10 cm) data are recorded daily at Hyslop Farm (http://cropandsoil.oregonstate.edu/weather).

Nitrification Potential (NP). NPs with 1 mM NH_4Cl were determined on soil samples within a week of each sampling time as described previously (Taylor et al., 2010). NP rates were determined as nitrite (NO_2^-) plus nitrate (NO_3^-) accumulated over 24 h per g of oven dry soil. An acetylene containing control was also included to ensure that all nitrification activity was acetylene sensitive. See Supplementary Information for additional experimental description.

Recovery of Nitrification Potential (RNP). The details of the RNP assay are described in detail in Supplementary Information. Briefly, acetylene inhibition and RNP steps were carried out at 30^{o}C with 1 mM supplemental NH_4^+ (Taylor et. al. 2010). Acetylene was removed by degassing the soil slurries for 6 min. RNPs in the absence of inhibitors were considered to be the

113 standard (RNP_{total}). In some treatments, the bacterial protein synthesis inhibitor kanamycin was
114 added at a final concentration of 800 μg/ml to prevent resynthesis of ammonia monooxygenase
115 (AMO) by AOB. Any RNP that recovers in the presence of a bacterial protein synthesis
116 inhibitor is likely to be contributed by AOA (RNP_{AOA}). $RNP_{total} - RNP_{AOA}$ is determined to be
117 the contribution of AOB to RNP (RNP_{AOB}).
118 **Nucleic acid analysis.** ***(a) Extraction of nucleic acids.*** Samples of freshly collected and sieved
119 soils for DNA extraction were stored at -20°C. DNA was extracted from frozen samples using a
120 MoBio PowerSoil (Carlsbad, CA) extraction kit, and DNA quantified using a NanoDrop ND-
121 1000 UV-Vis Spectrophotometer (ThermoScientific, Rockwood, TN). Quantifiable DNA was
122 extracted from every soil sample, with higher DNA yields recovered from soils of the LTF
123 (12.9±4.0 μg/g soil) than from soils of the CF and FC treatments (4.4±2.6 μg/g soil).
124 ***(b) Quantitative PCR of the archaeal and bacterial amoA genes***. QPCR of the AOA and AOB
125 *amo*A genes was performed using the HotStart-IT SYBR® Green qPCR Master Mix (USB,
126 Santa Clara, CA) and an ABI 7500 Real Time PCR System (Foster City, CA). Each 20 μL
127 reaction volume included 1 ng template DNA. Primers CrenamoA23f and CrenamoA616r
128 (Tourna et al., 2008) were used to quantify AOA *amo*A gene abundance. Primers (amoA_1R
129 and amoA_2F) and thermal cycler protocols for bacterial *amo*A genes are described elsewhere
130 (Rotthauwe et al., 1997). Standard curves were constructed with 4.6×10^{1} to 4.6×10^{-4} ng
131 *Nitrosomonas europaea* genomic DNA (bacterial *amo*A, efficiency=98±9%, R^2 avg =0.97±0.02)
132 or 54.1×10^{0} to 5.41×10^{-5} ng of '*Candidatus* Nitrosopumilus maritimus' strain SCM1 genomic
133 DNA (efficiency =105±8%, R^2 avg. =0.97±0.01). Archaeal *amo*A standards were also
134 constructed with a TOPO plasmid containing the '*Candidatus* Nitrosopumilus maritimus' strain
135 SCM1 *amo*BAC gene insert to confirm the results obtained with genomic DNA standards. Each

136 reaction was run in triplicate. Copy numbers were standardized to the mass of DNA extracted
137 per g oven dry soil.
138 ***(c) AOA and AOB community composition analysis.*** For terminal restriction fragment length
139 polymorphism (T-RFLP) assays, archaeal and bacterial *amo*A, primer pairs (Arch amoAf /Arch
140 amoAr, and amoA 1F/ amoA 2R), with the forward primer 5'-end 6-FAM-labeled were used to
141 produce PCR products from soil samples of the three field replicates of each field treatment on
142 six sampling occasions (May, Jul, Oct, and Dec 2010, Feb and May 2011). PCR products were
143 purified using a UltraClean PCR Clean-up DNA Purification Kit (Mo Bio, Carlsbad, CA), and
144 restricted with either CfoI, AluI, or TaqI for the bacterial *amo*A gene analysis (Horz et al., 2000;
145 Mintie et al., 2003), and with RsaI and MspI for the archaeal *amo*A gene analysis (Boyle-
146 Yarwood et al., 2008). The digests were purified and fragment lengths and relative abundances
147 were analyzed using an ABI 3100 capillary sequencer and Genotyper® 3.7 (Foster City, CA,
148 USA). For each sample, any fragments comprising < 5% of relative total fluorescence were
149 removed from subsequent analysis. The relative fluorescence abundances of unique terminal
150 restriction fragments (T-RFs) were exported for further analysis.
151 **Statistics.** Repeated measures ANOVA was used to test whether treatment or sampling time had
152 a significant effect on NP, and RNP rates, or AOA and AOB *amo*A abundances. In the case of
153 *amo*A abundances, data were log transformed to meet normality assumptions. Analysis was
154 done with PROC MIXED using a Banded Toeplitz covariance model and least squares means for
155 treatment comparisons (SAS Institute, Inc., Cary, NC). Because the treatment by time interaction
156 was significant for all response variables, the significance of treatments were evaluated at each
157 sampling time and the significance of temporal differences were evaluated within each treatment.
158 The Tukey-Kramer adjustment for multiple comparisons was used to control experiment-wise

159 error rates, with $P \leq 0.05$ chosen to denote significant differences. Complete summaries of the
160 repeated measures ANOVA analyses are contained in Tables S1 and S2. A t-test was used to
161 evaluate the significant difference between NP and RNP_{total} ($p \leq 0.05$), and also between RNP_{AOA}
162 and RNP_{AOB} for a few selected sampling times within a treatment.

163 The structure of the AOA and AOB community composition was investigated with non-
164 metric multidimensional scaling (NMS). Matrices were constructed for the AOA community
165 containing the combined MspI and RsaI T-RFs, and the AOB community matrix containing the
166 combined CfoI and AluI T-RFs. Multiple response permutation procedure (MRPP) was used to
167 test for treatment and temporal differences in community composition. NMS and MRPP were
168 performed using PC-ORD (McCune and Mefford, 1999).

169

170 **Results**

171 **Characterization of study site**. Corvallis OR has cool wet winters and warm dry summers
172 which is reflected in the metadata shown in Figure 1A and B. During this study soil
173 temperatures ranged from 1.7°C (Jan 2010) to 42.8°C (Jul 2010), and total precipitation was >
174 190 cm, with little rain falling during the summer months. Soils in the LTF- plots retained more
175 water (0.18 – 0.38 g /g soil, Figure 1B) than the CF and FC soils (0.09 to 0.24 g /g soil) from Apr
176 2010 to Aug 2010. CF and FC soils were water saturated from Oct 2010 to May 2011, while the
177 better structured LTF soils were unsaturated. CF soils were N fertilized in mid Feb 2010, and
178 FC soils were fertilized in mid Mar 2011, each with 150 kg NH_4^+-N/ha; however, by May 2010
179 (CF) and May 2011 (FC), extractable NH_4^+ levels had returned to the levels in unfertilized soils
180 (Figure 1C). There was a trend for LTF soils to contain a higher level of extractable NH_4^+-N than

the CF or FC soils at all sample times with the exception of the first sampling time post N fertilization (Apr 2010 and Apr 2011). NH_4^+-N accumulated in all three soil treatments between Oct and Dec 2010 before declining in Jan and Feb 2011. During 2010 NO_3^- accumulated in both the CF and FC treatments between Jun and Oct 2010 and reached a higher level in FC than in CF plots (Figure 1D). High extractable NO_3^- concentrations were also measured in FC soils in Apr 2011, yet had declined below the level of detection by May 2011.

Cropping phase and seasonal effects on nitrification potential (NP) activities. The seasonal dynamics of NPs with 1 mM supplemental NH_4^+ are shown in Figure 2.

Cropped/Fallowed (CF). NP rates in CF were highest at the first post N fertilization sampling in Apr 2010 (Figure 2A), and declined significantly (~50%) by Jul 2010. The lowest rate of NP occurred during Jan 2011. In Apr 2011, NP in the CF soils had increased significantly (~2 -fold) above the Jan minimum value, even though this sample was taken 14 months post fertilizer N addition. Subsequently, the NP dropped significantly by Jun 2011.

Fallowed/Cropped (FC). Although FC soils had not received N fertilizer since February 2009, (14 months prior to the start of this study), there was a trend for NP to increase non-significantly between Apr 2010 and May/Jun 2010 (Figure 2B). This was followed by a significant two-fold decline in NP between Jun and Aug. The lowest NP rates were observed during Dec 2010 through Feb 2011. After N fertilization in mid Mar 2011, the NP increased significantly and peaked in Apr at a value ~4-fold greater than the pre-N fertilization rate in Feb, and was significantly greater than the highest NP rates measured in May and June of the previous year (2010) in the fallowed phase.

Long term fallow (LTF). In contrast to the seasonal influences detected in the NPs of CF and FC soils, NP rates in LTF did not change significantly during the course of the study (Figure

2C). Rates of NP in LTF were significantly less than the highest values measured during the cropping phase post N fertilization in either CF (Apr 2010 – Oct 2010) or FC (Apr 2011 – Jun 2011); but, with one exception (CF, Apr 2011), they were not significantly different from the fallowed phase NP rates of CF (Dec 2010 – Jun 2011) and FC (Apr 2010 – Feb 2011), despite LTF having received no N fertilizer for 20 - 21 y.

Cropping phase and seasonal effects on the recovered nitrification potential (RNP). The seasonal dynamics of RNP were determined for each of the three field replicates of CF, FC and LTF soils at each of the sampling times (Figure 3). In most cases RNP_{total} rates were not significantly different ($p \leq 0.05$) than the rates of the NPs, suggesting that the same populations of ammonia oxidizers contributed to both NP and RNP.

Cropped/Fallowed (CF). Both AOA and AOB contributed to RNP at all sampling times indicating the potential of both groups of microorganisms to contribute to ammonia oxidation across all phases and seasons of CF (Figure 3A). Curiously, RNP_{total} was significantly lower than NP in Apr and May 2010, and in Apr 2011 when NPs were at their highest values. The rates of RNP_{total} were highest in Jun 2010 three months after N fertilization, and lowest during Jan 2011. There was a trend for the highest rates of RNP_{AOA} to occur in Jul through Oct 2010. In one case (Jul 2010) RNP_{AOA} was significantly greater ($p \leq 0.05$) than RNP_{AOB} (Table 1). Rates of RNP_{AOB} were significantly higher in Jun 2010 than at any other time (Table S1), and were significantly greater ($p \leq 0.05$) than rates of RNP_{AOA} in both Jun 2010 and Jun 2011. There were no significant differences in the rates of RNP_{AOB} of the remaining samples.

Fallowed/Cropped (FC). The highest rates of RNP_{total} in FC were measured in Apr and May 2011, after N fertilization in Mar (Figure 3B) and the lowest rate occurred in Jan 2011. During the fallowed phase in 2010 the maximum rate of RNP_{AOA} occurred in May, and was

lowest in Jan 2011. At four sampling times prior to N fertilization (Apr, May, and Dec 2010, and Feb 2011), the rate of RNP_{AOA} was significantly greater than the rate of RNP_{AOB} ($p \leq 0.05$, Table 1). After N fertilization in Mar 2011, rates of RNP_{AOA} were significantly greater in Apr and May than in Jan 2011 (Table S1). In 2010, the RNP_{AOB} rate was also greatest in May and declined significantly by Dec 2010. There was a significant increase in the rates of RNP_{AOB} in Apr through June 2011 after N fertilization in Mar 2011. In only two samples (Jul 2010 and Jun 2011), were the rates of RNP_{AOB} significantly greater than the rates of RNP_{AOA} ($p \leq 0.05$).

Long term fallow (LTF). RNP_{total} was no different than NP except in Aug and Dec 2010. Rates of RNP_{AOA} did not change significantly over the study (Figure 3C, Table S1) and were similar in magnitude to all values of RNP_{AOA} measured in FC, and to the majority of the rates in CF (except during Jul and Oct 2010). Rates of RNP_{AOA} were significantly greater than rates of RNP_{AOB} in Jul 2010, Oct 2010, Jan 2011 and May 2011 (Table 1). In LTF there were no significant differences in rates of RNP_{AOB} over the course of the study. However, there was a trend for rates of RNP_{AOB} to be highest between Apr and Aug 2010 when they contributed more to RNP_{total}, and which corresponded with the highest NPs. Rates of RNP_{AOB} were significantly lower than the highest values recorded for CF and FC (Jun and Oct 2010 in CF, and Apr, May and Jun 2011 in FC).

AOA and AOB population dynamics in response to cropping phase and season. With QPCR we compared the sizes of AOA and AOB populations using *amo*A gene copy number as a surrogate for AOA and AOB abundance (Figure 4). AOA and AOB *amo*A were successfully quantified in every sample.

AOA. AOA *amo*A gene copies ranged from being numerically similar to AOB *amo*A gene copies, to two orders of magnitude more abundant (Figure 4). From Apr through Dec 2010

250 there were no significant differences in the AOA *amo*A gene copy abundances among the three
251 treatments (Table S2), and the population densities averaged 3.2±2.1 x10^7 *amo*A copy numbers/g
252 soil. In all treatments the lowest AOA *amo*A copy numbers were measured in Jan 2011, which
253 was followed by significant increases in the AOA *amo*A gene copy numbers in both CF and LTF
254 treatments in Feb 2011. In CF a non-significant upward trend in population size continued into
255 Jun 2011. There were no significant changes in the AOA population of the FC treatment during
256 the same period. The linear regression of AOA *amo*A gene copy number with NP or RNP_{AOA}
257 was insignificant in all treatments (r^2=0.002 - 0.03).
258 *AOB.* AOB *amo*A gene copy abundance was greatest in all treatments in Apr 2010, with
259 a trend for CF to contain the highest population. AOB *amo*A gene abundance subsequently
260 declined throughout 2010 in the three treatments with the decrease becoming statistically
261 significant in Oct 2010 for FC and LTF, and in Dec 2010 for CF (Table S2). The lowest AOB
262 *amo*A gene abundances were measured in Jan 2011, with no differences among the treatments
263 (2.0±1.4 x10^6 *amo*A copy numbers/g soil). After N fertilization of the FC treatment in Mar 2011,
264 the AOB *amo*A abundance was significantly greater in Apr and May than in the non N fertilized
265 CF and LTF treatments, and the increase in AOB *amo*A abundance of FC coincided with a
266 greater RNP_{AOB} contribution to RNP_{total}. The linear regression of AOB *amo*A abundance and
267 NP was significant in CF (r^2=0.5, P<0.001). In LTF both NP and RNP_{AOB} were positively
268 correlated with AOB *amo*A abundance (r^2=0.3, P<0.001).
269 *AOA and AOB community composition.* MspI and RsaI digests of the archaeal *amo*A
270 gene yielded 7 and 5 distinct terminal restriction fragments (T-RFs), respectively. MRPP
271 analysis of the relative abundances of archaeal *amo*A gene T-RFs showed no significant effects
272 among treatments (P = 0.217) or sampling times (P = 0.078) on the composition of the AOA

273 population (Figure S1). T-RFLP analysis detected 9 and 6 distinct T-RFs from AluI and CfoI
274 digests of AOB *amo*A, respectively. Analysis of AOB *amo*A AluI and CfoI TR-Fs showed
275 significant treatment effects on the composition of the AOB population ($P \leq 0.0001$), but no
276 effect of sampling time ($P = 0.6727$). NMS ordination and multiple comparisons made between
277 treatments showed that CF and FC were significantly different from LTF ($P < 0.001$, each). The
278 relative total fluorescence of T-RF AluI200 (74% of AluI T-RFs) and CfoI68 (86% of CfoI T-
279 RFs) were significant biomarkers ($P < 0.05$) for LTF soils, while T-RF distributions in CF and
280 FC were more diverse with up to 9 T-RFs detected and different distributions of AluI200, 220,
281 389 and 491 making up ~ 80% of AluI T-RFs. CF and FC were also significantly different from
282 each other ($P < 0.001$), with AluI389 a significant indicator of soils collected from FC, and
283 CfoI135 a significant indicator of soils collected from CF.
284 Because treatments had significantly different AOB community compositions they were
285 analyzed separately for effects of sampling time. In CF there were no significant differences
286 among sample times ($P = 0.654$). However, in FC May 2011 was significantly different ($P =$
287 0.017) from the other sample times with CfoI135 identified as a significant indicator ($P =$
288 0.0032) of soil collected in May 2011. This coincided with the significantly higher AOB *amo*A
289 gene abundance in post N fertilized FC than in CF or LTF. In LTF there were significant
290 differences between months ($P = 0.008$), primarily due to AluI491 being an indicator of samples
291 collected in December. Restriction digests with TaqI showed that TaqI283 fragments made up ≥
292 95% of the total relative fluorescence of all samples, indicating that AOB populations were
293 dominated by *Nitrosospira* spp. (data not shown).
294
295

Discussion

By sampling intensively over a 15-month period, evidence was obtained for both cropping phase and seasonal effects on the soil NP, on the relative contributions of AOA and AOB to RNP, on the relative abundances of *amo*A gene copies of AOA and AOB, and on AOB community composition. Although it is well documented that the ratio of AOA:AOB ranges widely in soils (Leininger et al., 2006; He et al., 2007; Adair and Schwartz, 2008; Shen et al., 2008; Schauss et al., 2009; Di et al., 2010; Wessen et al., 2010; Zeglin et al., 2011), no data exist on what might influence this ratio, or its dynamism. Our study showed that the AOA:AOB shifts were generated by a combination of different phenomenon: (a) an increase in AOA *amo*A copy numbers in spring 2011 in CF and LTF, compared with their AOA counterparts in FC; (b) a larger population of AOB in spring 2011 under the N fertilized FC treatment compared with the AOB community under CF and LTF treatments; (c) better persistence of AOB between Apr and Oct 2010 in the CF treatment compared with AOB in both the FC and LTF treatments; (d) better overall persistence of AOA than AOB in 2010 in FC and LTF treatments.

These observations raise some interesting questions about the environmental drivers of AOA and AOB growth, as well as about their relative stress tolerances. In regards to growth, the increase in AOA *amo*A abundance that occurred in the fallowed phase of the CF treatment in the late winter-spring of 2011, was accompanied by a significant increase in NP, but not by any significant increase in the rates of RNP_{AOA}, nor by any significant change in the relative contributions of AOA and AOB to RNP_{total}. It is possible this might reflect the limits of sensitivity of the RNP assay to measure statistically significant changes in the relative contributions of AOA and AOB, but might also be supportive of the idea that ammonia oxidation is not the only energy generating metabolism used by AOA for growth under some soil

319 conditions (Jia and Conrad, 2009; Tourna et al., 2011). By contrast, in FC, the significant
320 increase in NP after N fertilization in spring 2011 was accompanied by a significant increase in
321 rates of both RNP_{AOB} and RNP_{AOA}, but with RNP_{AOB} making a greater relative contribution to
322 RNP_{total}, and an AOB *amo*A gene abundance that was significantly greater than in the CF soil;
323 however, there was no significant difference in AOA *amo*A gene abundance between treatments.
324 In sum, our findings have identified soil treatments and seasonal conditions where AOB and
325 AOA activities respond differentially or synchronously, and that are either coupled or uncoupled
326 from changes in their respective population sizes.
327 The different responses of AOA and AOB during the long interval of time in the
328 wheat/fallow cycle where extractable NH_4^+ is at low levels serve to highlight our lack of
329 knowledge of how AOA and AOB respond to the combination of soil stresses and NH_4^+-
330 limiting/starvation conditions under field conditions. There are a small number of publications
331 spanning >30 y which describe how AOB isolates respond to, and recover from NH_4^+ starvation
332 under laboratory conditions. These studies showed that the viability of the marine AOB,
333 *Nitrosomonas cryotolerans*, declined to 1-10% of the initial population after 25 weeks of NH_4^+
334 starvation, (Johnstone and Jones, 1988a; Jones et al., 1988), which fits reasonably well with the
335 25-50-fold decline of the soil AOB populations that occurred over the 32 week period between
336 Apr 2010 and Jan 2011. Additionally, NH_4^+ starved *N. europaea* can immediately oxidize NH_3
337 upon its reappearance and produce *amo* gene transcripts, but there is a delay in production of
338 RuBisCo transcripts (Berube et al., 2007); and recovery of CO_2 fixing activity of NH_4^+ starved
339 *N. cryotolerans* was delayed relative to NH_3 oxidizing activity (Johnstone and Jones, 1988b).
340 Furthermore, a substantial delay in recovery of NH_3 oxidizing activity was caused by irreversible
341 inactivation of AMO by acetylene in 10 d NH_4^+ starved *Nitrosospira briensis* immediately prior

342 to the addition of NH_4^+ (Bollmann et al., 2005), suggesting that *de novo* protein synthesis could
343 be impaired by a relatively short period of NH_4^+ starvation. In this context, it is worth noting
344 that the mean RNP_{AOB} was ≤ 0.4 of RNP_{total} in 9 of 11 samples from the LTF treatment which
345 had not been fertilized for 20 – 21 y, and where NPs and RNPs were generally lower than most
346 values measured in the cropping phases. As a consequence we might speculate that whereas the
347 soil-borne AOA communities maintained the biosynthetic capacity to resynthesize AMO and
348 successfully perform RNP, many of the AOB communities from the same samples had
349 diminished capacity. Whether or not this phenomenon should be regarded as a successful
350 survival strategy by AOB in response to a prolonged period of NH_4^+-limited conditions, or
351 represents a deteriorating physiological state imminently associated with cell death awaits further
352 investigation.
353 A final discussion point relates to the fact that the declines in AOB and AOA population
354 densities of each of the three treatments during 2010 were not accompanied by any major shifts
355 in community composition, thereby providing no evidence for differential resilience among the
356 members of these communities to tolerate limiting NH_4^+ and seasonally-induced soil stresses.
357 From the alternate perspective, neither the significant increase of RNP_{AOA} to fertilizer N (Apr
358 2011, FC) or the steady increase in AOA population density of CF in spring 2011, were
359 accompanied by significant differences in archaeal *amo*A gene community composition between
360 the three treatments, suggesting that AOA community composition was not controlled by NH_4^+
361 availability or cultivation disturbance. In contrast, AOB *amo*A T-RFs shifted in ways that were
362 consistent with NH_4^+ input driving community composition. For example, the T-RF CfoI135
363 which was significantly more abundant throughout 2010 in CF than FC and rare in LTF,
364 increased in relative abundance in FC after fertilizer N addition. In addition, 20 y without

365 fertilizer N applications or tillage has clearly resulted in a significant change in the AOB
366 community of the LTF resulting in dominance by the T-RF AluI200 -a biomarker for
367 *Nitrosospira* cluster 3a (Jia and Conrad, 2009; Mertens et al., 2009; Zeglin et al., 2011). This
368 phylotype has been associated with low N fertility undisturbed soils, and contains members that
369 are growth inhibited by high NH_4^+ levels. However, though the AOB community composition
370 of LTF had changed, RNP_{AOA} made the larger contribution to RNP_{total} in most of the LTF soil
371 samples. Although it remains unclear if the shift in AOB composition has had any impact on the
372 ability of AOB to compete with AOA, it is worth noting, that during May and Jun 2010 AOB
373 contributed significantly to RNP, suggesting that the AOB community in LTF can be
374 competitive with the AOA, even when extractable NH_4^+-N exists < 10 μg/g soil. Clearly,
375 further work is needed to determine what controls the contributions of AOB and AOA to *in situ*
376 soil nitrification, and to determine if certain phylotypes of AOB can compete effectively with
377 AOA.
378
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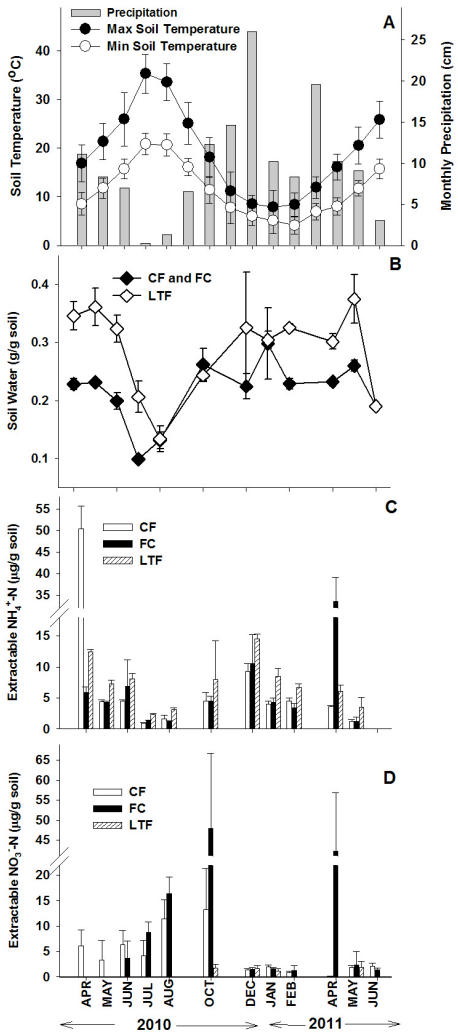


Figure 1. Soil temperature, water content, and mineral-N profiles of CF, FC and LTF treatments during the study period. (A) Monthly precipitation amounts (cm of rainfall) and average minimum and maximum soil temperatures at a depth of 0 - 10 cm. Error bars represent standard deviation of the monthly temperature average. (B) Average soil water content of the three treatments. CF and FC treatments maintained the same soil water contents and each sample time represents an average of the three field replicates from each treatment. The open symbols represent the average of three field replicates from the LTF plots. (C) Average KCl-extractable NH_4^+-N of three field replicates per treatment. (D) Average extractable NO_3^--N of three field

replicates per treatment. Error bars represent standard deviation of average.

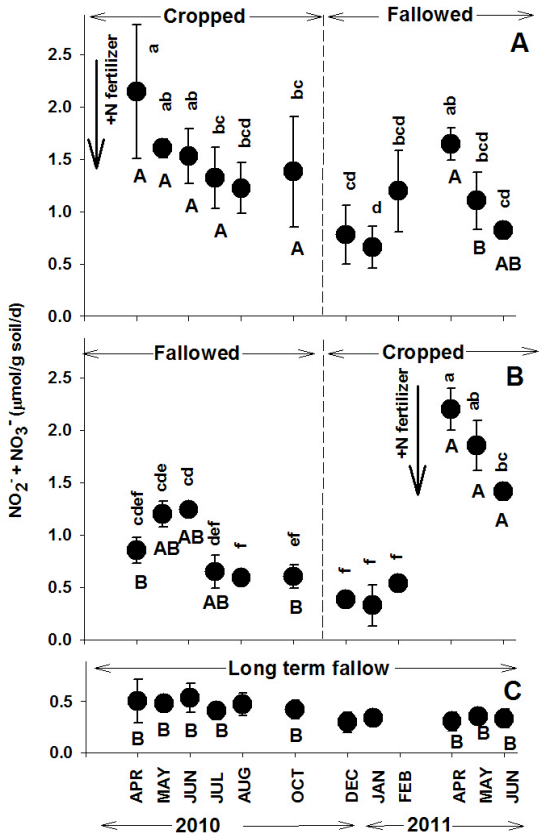


Figure 2. Effects of cropping phase treatment and sampling time on the nitrification potential (NP) rates with 1 mM supplemental NH_4^+ in CF (A), FC (B), and LTF (C) treatments. Bold vertical arrows indicate field applications of 150-kg N fertilizer to CF (Feb 2010) and FC (Mar 2011) treatments. Error bars represent standard deviation of the average NP of three field replicates from each treatment. Lower case letters indicate significance ($p \leq 0.05$) of sampling time within a specific treatment. Values that have lower case letters in common are not significantly different. The absence of lower case letters in LTF indicates no significant difference within sampling times in this treatment. Upper case letters indicate significance ($p \leq 0.05$) between different treatments at the same sampling time. Values that have upper case letters in common are not significantly different. The absence of an upper case letter (Oct, Dec, Jan

498 and Feb) indicates no significant difference between treatments at the same sampling time. See
499 experimental procedures for further details of the statistical analyses.
500

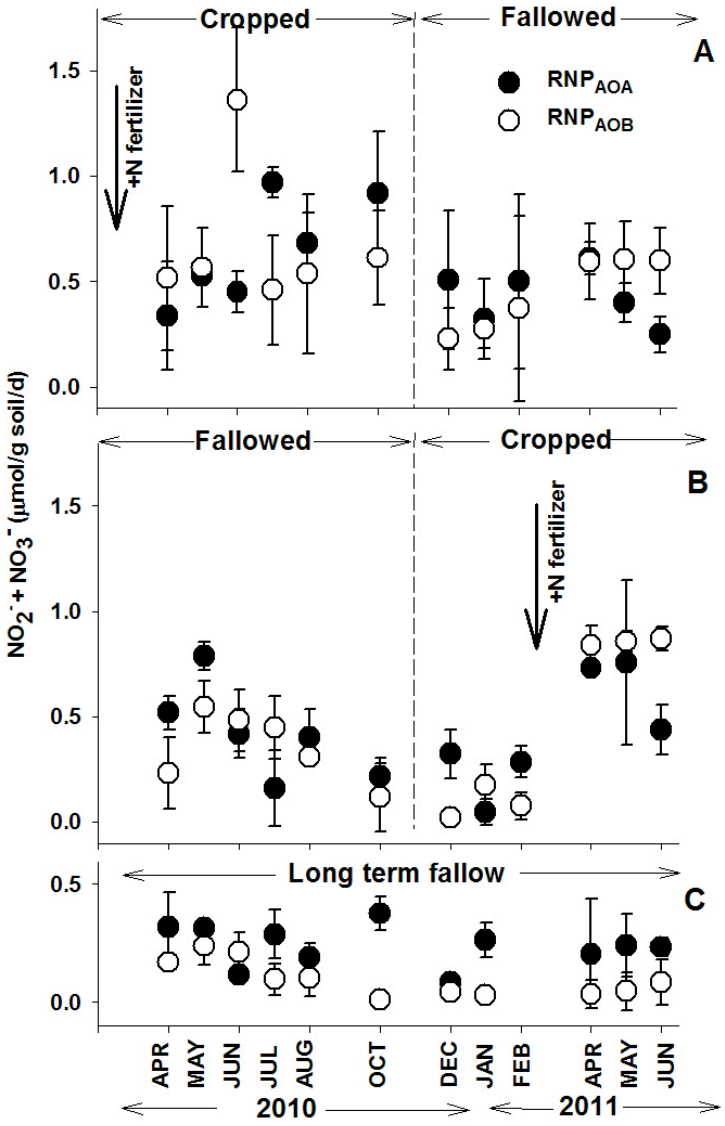
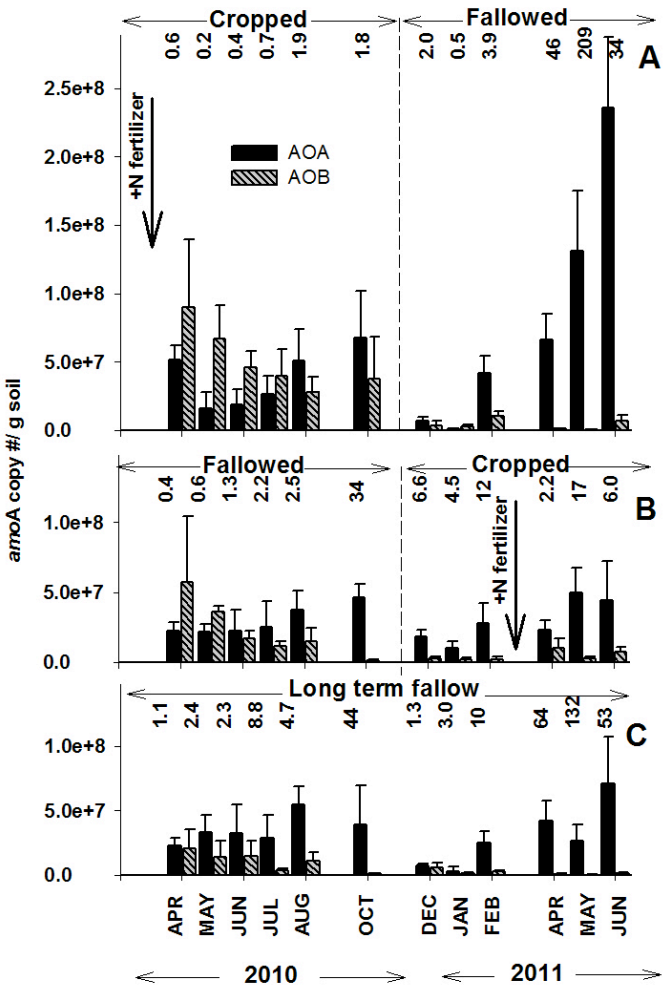


Figure 3. Effects of cropping phase treatment and sampling time on the recovered nitrification potential (RNP) rates contributed by AOA and AOB in CF (A), FC (B), and LTF (C) treatments. Bold arrows indicate field applications of N fertilizer (see Fig. 2). Error bars represent the standard deviation of the average RNP of three field replicates from each treatment. A complete a summary of the statistical analysis of these data is shown in Table S1.



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509 Figure 4. Effects of cropping phase treatment and sampling time on the AOA and AOB *amo*A

510 copy number/g soil in CF (A), FC (B) and LTF (C) treatments. Bold arrows indicate field

511 applications of N fertilizer to CF and FC treatments (see Fig. 2). Error bars represent the

512 standard deviation of the average *amo*A copy number/g soil of triplicate QPCR reactions for

513 each of three field replicates of each treatment. A complete a summary of the statistical analysis

514 of these data is shown in Table S2. Ratios of AOA to AOB *amo*A copy number/g soil are

515 indicated for each treatment.

516

Table 1. A summary of the relative contributions of RNP_{AOB} to RNP_{total} in CF, FC and LTF treatments. Values in parentheses represent the standard deviation of the average of RNP_{AOB}/RNP_{total} of each of the field replicates. Asterisks indicate the times when the rates of RNP_{AOA} and RNP_{AOB} of a specific treatment were significantly different as determined by a t-test ($p \leq 0.05$).

Sample Time	RNP_{AOB}/RNP_{total}		
	CF	FC	LTF
Apr '10	0.60 (0.25)	0.29 (0.17)*	0.37 (0.14)
May '10	0.51 (0.10)	0.41 (0.07)*	0.42 (0.10)
Jun '10	0.75 (0.04)*	0.53 (0.05)	0.63 (0.09)
Jul '10	0.31 (0.12)*	0.75 (0.24)*	0.27 (0.20)*
Aug '10	0.41 (0.20)	0.45 (0.08)	0.34 (0.20)
Oct '10	0.40 (0.14)	0.28 (0.35)	0.02 (0.04)*
Dec '10	0.36 (0.25)	0.08 (0.14)*	0.28 (0.25)
Jan '11	0.48 (0.17)	0.94 (0.32)	0.11 (0.11)*
Feb '11	0.40 (0.44)	0.18 (0.11)*	-
Apr '11	0.49 (0.07)	0.54 (0.06)	0.33 (0.57)
May '11	0.60 (0.05)	0.55 (0.11)	0.17 (0.29)*
Jun '11	0.70 (0.11)*	0.67 (0.04)*	0.23 (0.21)

Supplemental Information

Table S1. A summary of the repeated measures ANOVA of the effect of cropping phase treatment (CF, FC and LTF) and sampling time on the rates of RNP_{AOA} and RNP_{AOB}. Within each specific treatment (CF, FC, or LTF), lower case letters indicate significant differences ($p \leq 0.05$) between sampling times. Values that have lower case letters in common are not significantly different. Upper case letters indicate significance ($p \leq 0.05$) between different treatments at the same sampling time. Values that have upper case letters in common are not significantly different. The absence of an upper case letter indicates no significant difference between treatments at the same sampling time.

Sample Time	RNP_{AOA}			RNP_{AOB}		
	CF	FC	LTF	CF	FC	LTF
Apr '10	c	abc	a	b	cd	a
May '10	abc	a	a	b	bc	a
Jun '10	bc	abc	a	a A	bcd B	a B
Jul '10	a A	c B	a B	b	bcd	a
Aug '10	ab	abc	a	b	cd	a
Oct '10	ab A	c B	a B	b A	cd AB	a B
Dec '10	abc	abc	a	b	d	a
Jan '11	c	c	a	b	cd	a
Feb '11	abc	bc	a	b	cd	a
Apr '11	abc	ab	a	b AB	ab A	B
May '11	c	ab	a	b AB	ab A	B
Jun '11	c	abc	a	b AB	a A	B

Table S2. A summary of the repeated measures ANOVA of the effect of cropping phase and sampling time on $\log_{10}$ transformed AOA and AOB *amo*A gene copy number/g soil. Within each specific treatment (CF, FC, or LTF), lower case letters indicate significance differences ($p \leq 0.05$) between sampling times. Values that have lower case letters in common are not significantly different. Upper case letters indicate significance ($p \leq 0.05$) between different treatments at the same sampling time. Values that have upper case letters in common are not significantly different. The absence of an upper case letter indicates no significant difference between treatments at the same sampling time.

Sample Time	AOA *amo*A $\log_{10}$ copy #/g soil			AOB *amo*A $\log_{10}$ copy #/g soil		
	CF	FC	LTF	CF	FC	LTF
Apr '10	abcd	a	ab	a	a	a
May '10	cd	a	ab	ab	a	ab
Jun '10	bcd	a	ab	ab	ab	ab
Jul '10	bcd	a	ab	abc A	abc AB	abc B
Aug '10	bcd	a	ab	abc	abc	ab
Oct '10	abcd	a	ab	abc A	d B	de B
Dec '10	de	a	b	de	bcd	abc
Jan '11	e AB	a A	c B	de	cd	de
Feb '11	abcd	a	ab	bcd	cd	bcd
Apr '11	abc	a	ab	de B	abcd A	de B
May '11	ab	a	ab	e B	bcd A	e B
Jun '11	a	a	a	cd	abcd	cd

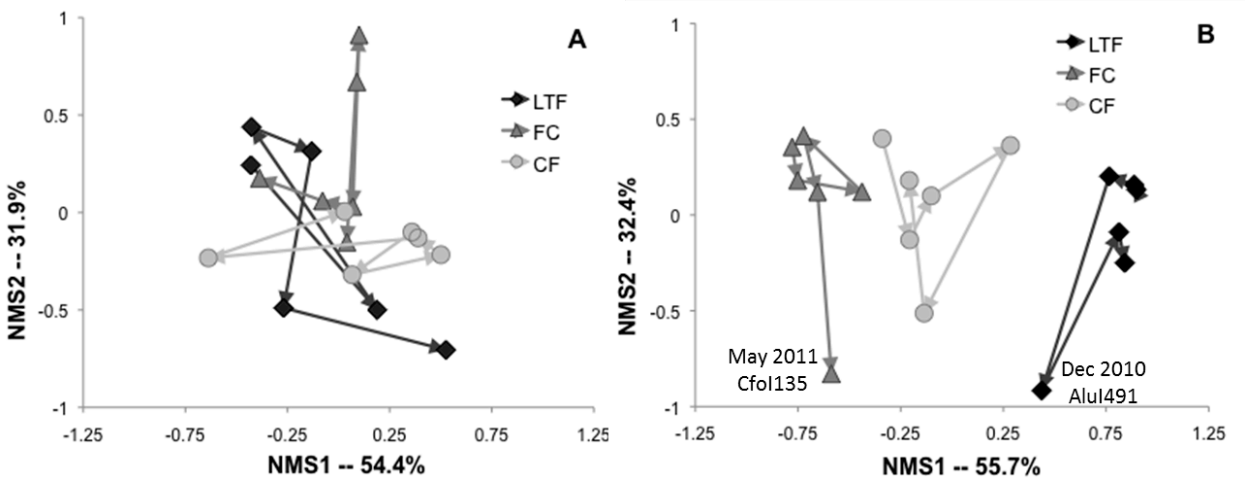

Figure S1. Nonmetric multidimensional scaling (NMS) ordinations of *amo*A gene T-RF relative abundances from the three treatments (CF, FC, and LTF). (A) NMS model of archaeal *amo*A gene community composition of soils sampled on six occasions, May, Jul, Oct, Dec 2010, Feb and May 2011. The direction of arrows indicate the progressive temporal order of sampling times. Archaeal model parameters are stress = 7.645, instability ≤ 0.0001. MRPP confirmed no significant effects among treatments ($P = 0.217$) or sampling times ($P = 0.078$). (B) NMS model of bacterial *amo*A gene communities sampled on six occasions, May, Jul, Oct, Dec 2010, Feb and May 2011. Bacterial model parameters are stress = 20.78, instability = 0.031. The direction of arrows indicate the progressive temporal order of sampling times. CF, FC and LTF were significantly different from each other ($P < 0.001$), and there were significant differences between sampling times in FC and LTF ($P < 0.01$). The sample times that were significantly different are shown with their respective indicator T-RFs in panel B.