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Responses of bacterial and archaeal ammonia oxidizers to soil organic and fertilizer amendments under long-term management

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Abstract

Ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA) co-exist in soil, but their relative distribution may vary depending on the environmental conditions. Effects of changes in soil organic matter and nutrient content on the AOB and AOA are poorly understood. Our aim was to compare effects of long-term soil organic matter depletion and amendments with labile (straw) and more recalcitrant (peat) organic matter, with and without easily plant-available nitrogen, on the activities, abundances and community structures of AOB and AOA. Soil was sampled from a long-term field site in Sweden that was established in 1956. The potential ammonia oxidation rates, the AOB and AOA *amoA* gene abundances and the community structures of both groups based on T-RFLP of *amoA* genes were determined. Straw amendment during 50 years had not altered any of the measured soil parameters, while the addition of peat resulted in a significant increase of soil organic carbon as well as a decrease in pH. Nitrogen fertilization alone resulted in a small decrease in soil pH, organic carbon and total nitrogen, but an increase in primary production. Type and amount of organic matter had an impact on the AOB and AOA community structures and the AOA abundance. Our findings confirmed that AOA are abundant in soil, but showed that under certain conditions the AOB dominate, suggesting niche differentiation between the two groups at the field site. The large differences in potential rates between treatments correlated to the AOA community size, indicating that they were functionally more important in the nitrification process than the AOB. The AOA abundance was positively related to addition of labile organic carbon, which supports the idea that AOA could have alternative growth strategies using organic carbon. The AOB community size varied little in contrast to that of the AOA. This indicates that the bacterial ammonia oxidizers as a group have a greater ecophysiological diversity and potentially cover a broader range of habitats.

Keywords: ammonia oxidation, AOB, AOA, *amoA* gene, agricultural soil

1. Introduction

Ammonia oxidation is an integral part of the global nitrogen cycle and it determines the balance between reduced and oxidized forms of nitrogen (N). The process and organisms involved have been extensively studied since ammonia oxidation has both environmental and economical concerns. It can result in leaching of nitrogen from soil to aquatic ecosystems or emissions of the greenhouse gas nitrous oxide, both resulting in nitrogen loss from agricultural soil. However, the process can also be beneficial for nitrogen removal from polluted water and sewage. Ammonia-oxidizing bacteria (AOB), belonging to the *Beta-* and *Gammaproteobacteria*, were reported already in the late 1800's (Winogradsky, 1890), while it took a further 100 years to discover the other group of ammonia oxidizers; i.e. the ammonia oxidizing archaea (AOA) belonging to the Crenarchaeota (Venter et al., 2004; Könneke et al., 2005; Treusch et al., 2005). Both groups employ the same functional *amoA* gene, encoding the α-subunit of ammonia monooxygenase, which catalyzes the first step in ammonia oxidation. However, the bacterial and archaeal genes are sufficiently divergent to be distinguished by their sequences.

Evidence has been presented that both AOB and AOA are key players in ammonia oxidation in agricultural soils (Jia and Conrad, 2009; Offre et al., 2009), but there have been ambiguities concerning the relative importance of these different groups. Following the discovery of the AOA, a number of reports have shown that they are significantly more abundant than the AOB in soils of different origins (Leininger et al., 2006; He et al., 2007; Chen et al., 2008). However, also the contrary has been shown (Boyle-Yarwood et al., 2008). Abundance alone is not sufficient to determine the relative contribution of either group towards the ammonia oxidation process. Tourna et al. (2008) suggested that non-thermophilic Crenarchaeota played a role in soil nitrification since the community structure of active AOA

changed in relation to temperature during nitrification, but this was not the case for the AOB. In agreement, Offre et al. (2009) showed that of the two groups, only AOA were growing during active nitrification in a microcosm experiment. However, it has also been shown that although AOA were more abundant than AOB, bacteria rather than archaea were more active ammonia oxidizers in agricultural soil (Jia and Conrad, 2009). In addition, functional redundancy between the two groups has been suggested, since ammonia oxidation was taken over by the AOA when the AOB were suppressed by antibiotics (Schauss et al., 2009). These contrasting results imply that the relative importance of AOB and AOA may vary in arable soil depending on the environmental conditions, with one or the other being more competitive under a given set of conditions. Since the AOB and AOA belong to separate phylogenetic domains, with different cell metabolic and biochemical processes, they could theoretically respond differently to the microenvironmental conditions in soil. Recently, Erguder et al. (2009) summarized the environmental conditions related to the dominance of AOA and suggested potential niches for AOA. Nevertheless, the relatively wide ecophysiological diversity, known at least for the AOB, would also support overlaps between the groups. This could explain the contrasting results that have been described for the effects of some soil parameters, such as pH (Nicol et al., 2008; Hallin et al., 2009) and fertilizers (He et al., 2007; Shen et al., 2008; Hallin et al., 2009; Jia and Conrad, 2009) on AOB and AOA.

The objective of this study was to assess responses of bacterial and archaeal ammonia oxidizers to soil organic matter quality, as well as nitrogen and organic carbon content. The effects of these soil factors on the occurrence and abundance of AOB versus AOA are poorly investigated (Erguder et al., 2009). Our hypothesis was that differences in soil organic matter quality would have an impact on the abundance, composition and activity of the ammonia oxidizing communities, since organic carbon content and soil C:N ratios are known to affect both plant growth as well as the general microbial community structure and activity

(Marschner et al., 2003; Lejon et al., 2007). Nitrogen fertilization in the form of nitrate was not expected to have a direct effect on the ammonia oxidizing community, but rather an indirect effect due to increased primary production that increase energy input to the ecosystem and accelerates N-cycling. For this study, we took advantage of the long-term environmental treatment approach (Reed and Martiny, 2007), which has increased potential for revealing possible soil factors as drivers on the AOB and AOA communities. We sampled from a field trial established in 1956 that is located at the Swedish University of Agricultural Sciences (SLU) in Ultuna, Sweden. Samples were chosen from treatments representing soils with either organic matter depletion or labile or more recalcitrant organic matter, and with different nitrogen contents resulting in correspondingly high and low primary production values. In addition, we studied the rates of ammonia oxidation to be able to compare the soil's potential to oxidize ammonia in relation to the size and composition of the genetic potential, i.e. the abundance of *amoA* genes in ammonia oxidizing bacteria and archaea. The rates of ammonia oxidation were correlated to the AOB and AOA community sizes and structures in the different treatments and the correlations were explored to test for possible niche differentiation between the AOB and AOA communities and their relative importance for ammonia oxidation.

2. Materials and Methods

2.1. Field site, soil sampling and chemical analysis

Soil was sampled from the Ultuna long-term soil organic matter experiment in Uppsala, Sweden (Kirchmann et al., 1994). The field experiment was established in 1956 to study the effect of different organic and inorganic fertilizers on soil properties and crop yields. The soil

117 is a clay loam, classified as a Eutric Cambisol. The field trial has three independent replicate

118 blocks, where each block comprises different treatments randomized in plots of 2 x 2 m

119 separated by wooden frames. Six treatments in triplicate were used in this study: calcium

120 nitrate ($Ca(NO_3)_2$), straw amendment, straw amendment with $Ca(NO_3)_2$, peat amendment,

121 peat amendment with $Ca(NO_3)_2$ and unfertilized plots not subjected to any additional

122 fertilization but a yearly addition of 22 kg phosphorus and 35-38 kg potassium ha^{-1}, as in all

123 of the treatments. Straw and peat were applied as 8000 kg ha^{-1} ash free organic matter every

124 second fall, while inorganic N-fertilization (80 kg N ha^{-1}) was applied yearly in the spring.

125 From 1956 to 1999, spring-sown cereal dominated the field site, but since 2000, all plots were

126 planted with maize (*Zea mays*). From each plot, 10 soil cores (2 cm diameter, 20-cm depth)

127 were sampled in-between plant rows in the time-span between harvest and tillage September

128 2002. During this period, the weather conditions were normal for the region with a mean

129 temperature of 10ºC and no precipitation (http://celsius.met.uu.se). The cores were mixed into

130 one composite sample per plot, resulting in a total of 18 samples. Each sample was sieved (4-

131 mm mesh) and thoroughly mixed before it was stored at -20°C until analysis.

132 Data on total soil nitrogen (Tot-N), soil organic carbon (Org-C), $pH_{(H2O)}$ and crop yield

133 were kindly provided from the monitoring program of the site by Lennart Matsson at The

134 Department of Soil and Environment, Swedish University of Agricultural Sciences, Uppsala

135 (Table 1). Soil moisture was determined as gravimetric water content by drying 10 g soil at

136 105°C for 24 h. Crop yield was determined as dry weight of total harvested green biomass

137 after drying at 105°C for 24 h and this value was used as a proxy for plant primary

138 production. To compensate for year-to-year variations due to weather conditions, we used the

139 average crop yield from 2000 to 2006.

140

141 *2.2. Potential ammonia oxidation activity*

The potential ammonia oxidation (PAO) rate was measured in triplicate as accumulated nitrite according to a short incubation, chlorate inhibition technique (Belser and Mays, 1980; Torstensson, 1993) (ISO 15685). In brief, 100 ml of 1 mM potassium phosphate buffer (pH 7.2) containing 0.4 mM (di-)ammonium sulphate and 15 mM sodium chlorate was added to 25 g soil and the soil slurries were incubated on a rotary shaker at 25°C for 6 hrs. Aliquots of 2 ml were taken once every hour and the nitrite concentration was determined by flow injection analysis (FIA, Tecator, Höganäs, Sweden). The potential ammonia oxidation rates were calculated by linear regression of the accumulated NO_2^- - N per g dry weight soil over time.

2.3. DNA extraction

DNA was extracted in duplicate from 500 mg of soil from each sample using the FastDNA SPIN for Soil Kit according to the manufacturer's instructions. The two resulting DNA extractions per sample were pooled before further analysis. DNA concentration was measured using a Qubit fluorometer (Invitrogen, USA).

2.4. Real-time PCR quantification of Bacteria and Crenarchaeota

Real-time quantitative PCR of bacterial 16S rRNA genes was performed to estimate the abundance of the total bacterial community using primer pairs 341F (5'-CCTACGGGAGGCAGCAG-3') (Muyzer et al., 1995) and 534R (5'-ATTACCGCGGCTGCTGGCA-3') (Muyzer et al., 1996) with slight modifications of the reverse primer sequence according to López-Gutiérrez et al. (2004) generating a 194 bp

fragment. The crenarchaeal 16S rRNA genes were quantified using primer pairs Crenar771F (5'-ACGGTGAGGGATGAAAGCT-3') and Crenar957R (5'-CGGCGTTGACTCCAATTG-3') (Ochsenreiter et al., 2003) generating a 228 bp fragment. Quantification was based on the fluorescence intensity of the SYBR Green dye and reactions for each sample were carried out in an ABI7900HT thermal cycler. The reactions were performed in a total volume of 20 µl using Absolute QPCR SYBR Green Rox Abgene/Thermo (Bio-Medicine), 1 µM of each primer, 10 ng of soil DNA and T4Gp32 (QBIOgene, France) which was added to each reaction to reach final concentrations of 0.025 µg μl^{-1}. The bacterial 16S rRNA gene fragments were amplified using an initial denaturation step at 95°C for 15 min, followed by 35 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C and 30 s at 80°C. The crenarchaeal 16S rRNA gene fragments were amplified using the same program as for the bacteria, except the annealing temperature was 55°C. Data were retrieved at 80°C and all reactions were finished with a melting curve starting at 80°C with an increase of 0.5°C up to 95°C to verify amplicon specificity. The PCR reaction runs had an efficiency of 110 and 90% for the Bacteria and Crenarchaeaota, respectively. Standard curves were obtained using serial dilutions of linearized plasmids (pGEM-T, Promega) containing cloned 16S rRNA genes amplified from *Pseudomonas aeruginosa* (PAO1) for bacteria and cloned crenarchaeal 16S rRNA genes from soil (r^2=0.99 for both standard curves). Controls without templates resulted in values lower than the detection limit or negligible values. Inhibitory effects on PCR performance were tested for all samples by running a PCR with a known amount of circular plasmid mixed with a known amount of DNA, as well as samples with a known amount of circular plasmid mixed with water. The measured cycle threshold (C_t) values for the different samples were compared with those measured for the controls with water and the differences in C_t-values were negligible.

192 *2.5. Real-time PCR quantification of amoA genes for AOB and AOA*

193

194 Real-time quantitative PCR of *amoA* genes was performed to estimate the abundance of
195 the ammonia oxidizing bacterial and archaeal communities, respectively. The primers amoA-
196 1F (5'-GGGGTTTCTACTGGTGGT-3') and amoA-2R (5'-
197 CCCCTCKGSAAAGCCTTCTTC-3') were used for bacteria generating a 491 bp fragment
198 (Rotthauwe et al., 1997); and CrenamoA23f (5´-ATGGTCTGGCTWAGACG-3´) and
199 CrenamoA616r (5´-GCCATCCATCTGTATGTCCA-3´) were used for archaea generating a
200 628 bp fragment (Tourna et al., 2008). Quantification was based on the fluorescence intensity
201 of the SYBR Green dye and reactions for each sample were carried out in a Bio-Rad IQ5
202 thermal cycler. The quantification of *amoA* genes was performed in a total volume of 20 µl
203 using DyNAmo Flash SYBR Green qPCR Kit (Finnzymes), 0.5 µM of each primer, and 10
204 ng of soil DNA. Bovine serum albumin (BSA) was added to reach final concentrations of 800
205 ng μl^{-1}. The fragments for the AOB and AOA were amplified using an initial denaturation
206 step at 95°C for 15 min, followed by 35 cycles of 15 s at 95°C, 30 s at 55°C and 30 s at 72°C
207 and 30 s at 80°C. Data was retrieved at 80°C and all reactions were finished with a melting
208 curve starting at 60°C with an increase of 0.5°C up to 95°C to verify amplicon specificity.
209 The PCR reaction runs had an efficiency of 90 and 94% for the AOB and AOA, respectively.
210 Standard curves for the AOB and AOA were obtained using serial dilutions of linearized
211 plasmids (pGEM-T, Promega) containing cloned *amoA* genes amplified from environmental
212 clones (r^2=0.99 for both standard curves). Controls without templates resulted in undetectable
213 values in all samples and inhibitory effects on PCR performance were tested as described
214 previously for the 16S rRNA genes and were negligible.

215

216 *2.6. T-RFLP of amoA genes for AOB and AOA*

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65

For T-RFLP, PCR amplification of the *amoA* genes for AOB and AOA were done in triplicate for each individual DNA extract using the same primers as above, except that the forward primer was labeled at the 5´end with hexachlorofluorescein. Each reaction was performed in a total volume of 25µl containing 20 ng of soil DNA, 2.5 µl 10X PCR buffer, 0.4µM of each primer, 200 µM (each) deoxyribonucleoside triphosphate, 1.25 U of Taq DNA polymerase and BSA was added to reach final concentrations of 1600 ng μl^{-1}. The PCR was performed in a GeneAmp PCR System 9700 machine according to Rotthauwe et al. (1997) for the AOB, with the modification of only running 30 cycles, and according to Sahan and Muyzer (2008) for the AOA.

For T-RFLP, the replicated PCR reactions for each DNA extract were pooled, divided in three portions and digested by three different restriction enzymes in separate reactions. The AOB were digested with 10 U of the restriction enzymes *Aci*I, *Hae*III, and *Rsa*I and the AOA with 10 U of *Aci*I, *Alu*I and *Mbo*I during 2 h according to the instructions provided by the manufacturer (New England Biolabs, USA). The enzymes were selected based on *in silico* restriction analysis of 50 *amoA* sequences from AOB and AOA, respectively. The TRF patterns were evaluated using the software Peak Scanner v.1.0 (Applied Biosystems). Fragments smaller than 50 bases and TRFs contributing with <0.5% of the total signal were excluded from the subsequent statistical analysis.

2.7. Statistical analyses

Pairwise comparisons between treatments regarding soil properties, crop yield, PAO rates and community size were done using either the Student's t-test or the Mann-Whitney test depending on whether the data was normally distributed or not at $P<0.05$ and $P<0.001$.

Pairwise correlations between soil properties, crop yields, PAO rates and abundance of AOB and AOA communities were done using Pearson's correlation coefficient (Pearson, 1896). All the above-mentioned analyses were performed using XLSTAT Version 2008.6.07 (Addinsoft^{TM}, New York, USA). To evaluate the correspondence between dissimilarity matrices for the AOB and AOA community structures, soil properties (pH, Org-C, Tot-N, C:N ratio and Soil moisture), potential ammonia oxidation rates and abundances of *amoA* genes, Mantel's test (Mantel, 1967) with a Monte Carlo simulation with 999 randomizations was conducted using PC-ORD version 5.10 (MjM Software, Oregon, USA). For this purpose, the values for the soil properties, rates and gene abundances were transformed to dissimilarity matrices using Euclidian distance measure, whereas the community composition dissimilarity matrices were obtained by Bray-Curtis distance measure.

The community structures of AOB and AOA derived from T-RFLP fingerprints (calculated as relative abundance) were analysed by non-metric multidimensional scaling (NMS) using PC-ORD version 5.10 (MjM Software, Oregon, USA). Data matrices of community fingerprints were arc-sinus square root transformed and the Bray-Curtis distance measure was used to generate dissimilarity matrices. Abundances of AOB and AOA, soil properties, PAO rates and crop yields were incorporated into the analysis through the use of bi-plot ordinations, where variables were combined into a secondary matrix and plotted as vector fits against community composition ordinations. The data in the second matrix were relativized by dividing values within each variable by column totals. The NMS was run using a random starting configuration, a maximum of 250 iterations and an instability criterion of 0.00001 and performed on 200 runs with the real data and 200 runs with randomized data to test for the null hypothesis. For a two-dimensional solution with the lowest possible stress value, a final run using the best starting configuration from the first run was performed.

3. Results

3.1. Soil factors and crop yields

The different long-term soil amendments had an effect on the soil chemical properties (Table 1). In comparison to 1956 when the experiment started, the soil pH remained relatively stable in most of the treatments except in the plots amended with peat with or without N-fertilization, which had a significantly lower pH. When studying changes in other soil factors by comparison to values present at the start of the experiment, the unfertilized control and the N-fertilized plots had less Org-C, the straw amended treatment had Org-C at the same level, while all other treatments resulted in a 21 to 60% increase in the amount of Org-C. With respect to Tot-N values during the course of the experiment, the N-fertilized treatment, the plots with straw amendment alone and the unfertilized treatment all resulted in a decrease, whereas the other treatments resulted in an increased Tot-N in the soil. Addition of organic material resulted in a significant increase in the C:N ratio in the soil, with peat amendment resulting in the highest C:N ratio. The unfertilized soil and the N-fertilized soil had the lowest C:N ratios. Soil moisture largely followed the same trend as the soil org-C and C:N ratios with the highest values in the peat treatments and the lowest in the unfertilized control. Crop yield was used as a measure of primary production and as expected, N-fertilization resulted in a significantly higher yield compared to organic amendments alone or to no fertilization (Table 1).

3.2. Potential ammonia oxidation activity

291 The different treatments clearly affected the potential ammonia oxidation rates in the soil
292 (Fig. 1). The rates varied between 1.1 and 10.6 ng NO_2^- - N g^{-1} dw min^{-1} with the peat
293 treatment displaying the lowest activity and straw with N-fertilization the highest activity.
294 The straw treatment resulted in rates that were equal to those in the N-fertilized treatment.
295 Peat amendment with N-fertilization had rates of activity that were comparable to the
296 unfertilized plots.

297

298 *3.3. Community sizes of Bacteria, Crenarchaeota, AOB and AOA*

299

300 The bacterial 16S rRNA gene abundance, reflecting the size of the total bacteria
301 community, showed minimal variation between treatments and was in the range of 6.9×10^9 –
302 1.9×10^{10} gene copies g^{-1} dry weight soil (Fig. 2a). The plots that received organic
303 amendments combined with N-fertilization had significantly larger bacterial communities
304 than the other treatments. The crenarchaeal 16S rRNA gene abundance, reflecting the size of
305 the total crenarchaeal community was lower than that of the bacteria in all treatments, having
306 a crenarchaeal:bacterial 16S rRNA ratio of 1 to 4%. Similar to the situation for the bacteria,
307 the abundance of Crenarchaeota did not vary much between treatments and was in the range
308 of 1.4×10^8 – 6.4×10^8 gene copies g^{-1} dry weight soil (Fig. 2a). Only the treatment with
309 straw and N-fertilization contained a significantly larger crenarchaeal community when
310 compared to the other treatments and a 5 fold difference was found between that treatment
311 and the peat treatment. A direct comparison of 16S rRNA gene copies between treatments
312 should, nevertheless, be interpreted with caution, since the gene copy number can vary
313 between 1 and 12 per bacterial genome (Fogel et al., 1999).
314 The *amoA* gene abundance, reflecting the AOB community size, was in the range of $1.5 \times$
315 10^7 – 4.6×10^7 gene copies g^{-1} dry weight soil and relatively unaffected by treatments (Fig.

2b). The only significant difference in AOB abundance was between the N-fertilized plots and the plots with peat amendment, with peat resulting in a slightly higher abundance. The number of *amoA* gene copies corresponding to the AOA community showed more variation between treatments and was in the range of $8.1 \times 10^6 - 1.8 \times 10^8$ copies g^{-1} dry weight (Fig. 2b). The AOA abundance was significantly lower in the peat treatment compared to all other treatments, except peat with N-fertilization. The two treatments with organic amendment combined with N-fertilization were significantly different from each other with the highest abundance of AOA in the straw and N-fertilized plots. As for 16S rRNA genes, the comparison of *amoA* gene abundance should also be interpreted with caution, since the gene copy number per genome may vary.

In all treatments, but the two different peat treatments, the community size of the AOA was significantly ($P<0.05$) higher than the AOB with an AOA:AOB ratio that ranged from 3.7 to 4.6 (Fig. 2a). In the peat treatment, the AOB were significantly more abundant than the AOA with a AOA:AOB ratio of 0.3, while the two groups were equally abundant in the plots with peat plus N-fertilization. The peat treatment also harboured a crenarchaeal community that had a significantly lower fraction of AOA *amoA* genes to crenarchaeal 16S rRNA genes as compared to the other treatments (Fig. S2, supplementary material). The AOA *amoA* gene abundance varied between 6 to 32% of to the crenarchaeal 16S rRNA gene abundance, whereas the fraction of AOB *amoA* genes compared to the bacterial 16S rRNA gene abundance varied only between 0.2 to 0.3% with a significant difference between the peat and peat with N-fertilization treatment (Fig. S1, supplementary material).

3.4. Treatment effects on community structures of AOB and AOA

The T-RFLP profiles generated by each enzyme of the amplified *amoA* genes from the AOB and AOA communities consisted of two to five dominant and several minor peaks. When combining profiles from the three enzymes the average number of peaks was 32 and 39 for AOB and AOA, respectively. The NMS ordination revealed that the community structure of AOB and AOA differed depending on the treatment (Fig. 3). Differences in community structure among the samples for both the AOB and AOA community were supported by low final stress values (7.7 and 7.0), the Monte Carlo test ($P<0.005$; 200 permutations) and the strong correlation between distances in the 2-dimensional ordination space and the original space ($r^2=0.95$ and $r^2=0.94$). The peat amendment had the largest impact on the AOB community structure (Fig. 3a). Plots with either peat or straw with N-fertilization each harboured different AOB communities, but no separation was seen among the N-fertilized, unfertilized and straw amended plots. Also for the AOA the peat amendment had the largest effect on the community structure (Fig. 3b). In addition, a separation was observed between samples with and without N-fertilization in the AOA community that were not seen for the AOB.

3.5. Environmental factors controlling AOB and AOA communities

The ordinations were used to explore possible correlations between the community structure, community size, PAO rates and soil properties (Fig. 3). For the AOB communities, the bi-plot showed that the separation of samples was mainly explained by the soil pH and C:N ratio along axis 1 (Fig. 3a). The pH co-varied with PAO rates in the NMS. The same variables seen for the AOB explained the separation of the samples based on the AOA community structure (Fig. 3b). However, primary production (yield) and Tot-N in the soil were important factors related to the separation of samples along axis 2 for the AOA. Again

pH co-varied with PAO rates, which also co-varied with the number of *amoA* gene copies of AOA, indicating that the abundance of these genes are related to ammonia oxidation activity.

The differences among treatments in the AOB and AOA community structures, measured with Mantel's test, were significantly correlated to each other ($r=0.87$; $P<0.001$). The differences in both the AOB and AOA communities correlated to the dissimilarities in soil C:N ratios ($r=0.57$; $P<0.001$ and $r=0.52$; $P<0.001$; Tab. 2) and soil organic carbon ($r=0.21$; $P<0.05$ and $r=0.20$; $P<0.05$; Tab. 2) and the differences in PAO rates measured in the different plots ($r=0.46$, $P<0.001$ and $r=0.51$, $P<0.001$; Tab. 2), but only the community structure of AOA was significantly correlated to the abundance of the corresponding community ($r=0.58$, $P<0.001$; Tab. 2). Differences in pH and soil moisture were not significantly correlated to differences in either AOB or AOA community structure. Pairwise correlations using Pearson's correlation coefficient (Tab. 3) revealed that the PAO rates were correlated positively to pH ($r=0.72$, $P<0.001$) and negatively to Org-C and C:N ratio ($r=-0.50$; $P<0.05$ and $r=-0.66$, $P<0.05$). The PAO rate was also significantly correlated to the abundance of the AOA ($r=0.76$; $P<0.001$), but not to the AOB. Interestingly, among the soil chemical parameters, the size of the AOB community correlated to the Tot-N ($r=0.51$, $P<0.05$;), whereas the AOA size was correlated to soil pH, ($r=0.67$, $P<0.05$) and negatively related to Org-C and C:N ratio ($r=-0.60$, $P<0.05$ and $r=-0.73$, $P<0.05$).

4. Discussion

The ammonia oxidizing archaeal and bacterial communities detected at the site were impacted differently by long-term treatments with either labile or more recalcitrant organic matter and by different levels of N-content. The results are based on one sampling occasion and we cannot rule out temporal variations due to seasonal effects. However, the site has

390 stable soil parameters that have been documented since the 1990's confirming long-term
391 differences in the soil properties. In agreement with most studies, the AOA dominated in
392 abundance over the AOB in the majority of the treatments and the AOA:AOB ratios were in
393 the same range as reported by others for agricultural top soils (Leininger et al., 2006; He et
394 al., 2007; Chen et al., 2008; Shen et al., 2008; Hallin et al., 2009: Jia and Conrad, 2009).
395 However, the AOA:AOB ratios alone do not provide sufficient information to determine
396 which of the two groups is more significant for ammonia oxidation at this site. Therefore, we
397 assessed potential ammonia oxidation rates in the differently treated soils and correlated this
398 evidence of function to the size of the AOB and AOA *amoA* gene pool to determine which
399 community was likely to be mainly responsible for ammonia oxidation. A large range in
400 potential ammonia oxidation rates was observed among the treatments, with N-fertilization
401 and addition of the more readily degradable straw in relation to peat having the most positive
402 effects. In another long-term fertilization study, He et al. (2007) studied the nitrification
403 process by assessing potential ammonia oxidation activity and found that both AOB and AOA
404 abundances were correlated to this process. In our study, only the *amoA* gene abundances in
405 the AOA community were significantly correlated to the potential ammonia oxidation rates,
406 suggesting that the AOA were the main contributors to ammonia oxidation. These findings
407 agree with previous results we obtained from other fertilizer treatments at the field site
408 sampled at the same occasion (Hallin et al., 2009) where the abundance of AOA, but not of
409 AOB, was significantly correlated to potential ammonia oxidation rates. Others have shown
410 that changes in the transcriptional activity of archaeal ammonia oxidizers were correlated to
411 nitrification activity and that only archaeal ammonia oxidizers grew during active nitrification
412 (Tourna et al., 2008; Offre et al., 2009). Prosser and Nicol (2008) commented that a greater
413 AOA community size could be unrelated to ammonia oxidation, since AOA may have
414 alternative growth strategies. In agreement, Jia and Conrad (2009) reported that the AOA

community size increased with incubation time in soil from an N-fertilization experiment when nitrification activity was completely inhibited by acetylene. This indicates that ammonia oxidation alone does not sustain growth of the archaeal populations and supports the hypothesis that AOA are mixotrophs or heterotrophs (Hallam et al., 2006; Tourna et al., 2008; Jia and Conrad, 2009).

The response of AOA to changes in soil organic carbon is poorly understood (Erguder et al., 2009), but this has previously been shown to affect AOB communities (e.g. Innerebner et al., 2006; Enwall et al., 2007). In the present study, the AOA community size was negatively correlated to the soil organic carbon content and the C:N ratio. This negative correlation could be due to the competition of ammonia oxidizers with N-demanding heterotrophs for available ammonium and oxygen since the latter would be favoured under high C:N ratios. The peat treatment, having the highest C:N ratio and the lowest pH, had a significantly lower abundance of both AOA and Crenarchaeota compared to the other treatments. However, the proportion of AOA within the crenarchaeal community was significantly lower in the peat treated soil indicating a stronger negative effect on the AOA and not just a general decrease in Crenarchaeota. This was also the only treatment where the AOB exceeded the AOA abundance. The type of organic matter may therefore play a role in AOA abundance, for example if the AOA community is favoured by access to more labile soil organic matter for mixotrophic or heterotrophic growth. In support of this hypothesis, recent findings have shown that plant derived organic substrates stimulated archaeal *amoA* transcript levels (Chen et al., 2008) and that AOA were prevalent at higher levels than AOB in the rhizosphere (Herrmann et al., 2008). However, the effects shown in the peat treated soils might also be due to effects of lower pH, since the AOA abundance increased significantly with increasing soil pH. Thus, it is not possible to distinguish between the impact of organic matter and pH on AOA abundance in the peat soil. An increase in AOA abundance with increasing pH has

440 previously been observed for *amoA*-containing archaea (He et al., 2007; Hallin et al., 2009;
441 Jia and Conrad, 2009) and Crenarchaeota populations in general (Weijers et al., 2006; Pearson
442 et al., 2008). However, Nicol et al. (2008) found a negative relationship between the
443 abundance of archaeal *amoA* genes and soil pH. Archaeal *amoA* genes have been found in a
444 variety of environments with pH ranging from 2.5 to 9.0 showing a wide ecological and
445 phylogenetic diversity within AOA (Erguder et al., 2009). Contradicting results concerning
446 the effect of pH on AOA abundance reported from various studies could be explained by
447 differences in the physiological diversity in the archaeal communities present in different
448 soils.

449 In contrast to the AOA, the AOB community size varied little between treatments, despite
450 the relatively large differences in soil properties at the field site. Several other studies have
451 also reported no significant differences in community sizes of AOB between fertilized and
452 unfertilized soil plots (Bruns et al., 1999; Phillips et al., 2000; Hallin et al., 2009). In addition,
453 the AOB fraction of the total bacterial community showed only minor differences between
454 treatments and was within the same range as previously reported; between 0.003 and 0.9%
455 (He et al., 2007; Shen et al., 2008). Other studies have also shown that the AOB community is
456 more stable in terms of size than the AOA with regard to changes imposed by varying
457 environmental conditions (Chen et al., 2008; Hallin et al., 2009). In another study, the AOA,
458 and not the AOB, were affected by zinc contamination and the AOB restored nitrification in
459 the contaminated soil (Mertens et al., 2009). Differences in bacterial *amoA* gene copies and
460 transcript copies were also smaller than those for archaeal *amoA* genes in soils with pH
461 varying between 4.9 and 7.5 (Nicol et al., 2008). Based on these reports and our results we
462 hypothesize that the AOB community as an entity is more resilient than the AOA community,
463 irrespective of the observed differences in the AOB and AOA community composition
464 demonstrated in our study as well as in the work by Nicol et al. (2008). This would imply that

the AOB have greater ecophysiological diversity than the AOA and thereby have the potential to cover a broader range of habitats. This is further supported by studies in which only the AOB and not the AOA were detected in certain soil samples (Boyle-Yarwood et al., 2008; Hallin et al., 2009) and to our knowledge the contrary has not yet been shown.

Long-term treatment effects were also observed on the composition of the AOB and AOA communities and both groups were, in contrast to their community sizes, affected in similar ways. The differences in the AOA and AOB community structures among treatments were correlated to differences in ammonia oxidation activities, but the underlying explanation for the link between structure and activity is likely the pH differences. Soil pH was a strong driver behind separation of the communities in the ordinations, but also soil carbon content and the C:N ratio. Due to the expected competition with heterotrophs for substrates under high C:N ratio conditions, as discussed above, we propose that competition selects for AOB and AOA communities with high affinities for ammonia. The AOA community structure was also linked to differences in soil nitrogen content and plant yield. The correlation to yield could be an indirect effect of changed soil properties, but could also indicate the stimulation of plants on AOA communities, as discussed in other studies and mentioned above (Chen et al., 2008; Herrmann et al., 2008). An effect of N-fertilization was seen in the separation of the ammonia oxidizing communities along axis 2 in the ordinations, demonstrating that nitrate addition, although not the direct substrate for ammonia oxidizers, has an indirect effect on community structure. Several studies have discussed the effect of N-fertilization on AOB and AOA communities (Phillips et al., 2000; Avrahami et al., 2002; Enwall et al., 2007; He et al., 2007; Shen et al., 2008; Jia and Conrad, 2009) but since the type of fertilizer applied affects additional soil parameters, e.g. soil pH, sweeping generalisations are difficult to make.

5. Conclusions

The results from this study demonstrate that long-term N-fertilization with calcium nitrate, as well as organic amendments with peat or straw, both with and without N-fertilization affect the potential for ammonia oxidation and the size and composition of the AOA community, and to a lesser degree the AOB community structure and size. The differential relationships between soil properties and AOB and AOA community size also suggest niche differentiation between the two groups present at the field site. We propose that although the AOB and AOA co-exist and both can be important for ammonia oxidation in soil, the AOB community size appear to be more stable across conditions and could therefore cover a broader range of habitats.

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Figure legends

Fig. 1. Potential ammonia oxidation (PAO) rates measured for the different treatments (mean ± standard deviation, n = 3). The same letters above the bars indicate treatments without significant differences ($P<0.01$). Treatments: N= $Ca(NO_3)_2$, S=straw, SN=straw + $Ca(NO_3)_2$, P=peat, PN=peat + $Ca(NO_3)_2$, UC=unfertilized control.

Fig. 2. Size of bacterial and crenarchaeal communities in the different treatments (mean ± standard deviation, n = 3). a) Number of copies of the 16S rRNA gene from the total bacterial community (grey bars) and from the crenarchaeal community (black bars) per gram dry weight soil. b) Number of copies of the *amoA* gene from the bacterial community (grey bars) and from the archaeal community (black bars) per gram dry weight soil. The ratios of the crenarchaeal to bacterial 16S rRNA gene copies and the AOA to AOB *amoA* gene copies are shown in boxes above the bars in the respective figure. The same letters above the bars within the bacterial and archaeal community, respectively, indicate treatments without significant differences ($P<0.05$). Treatments: N= $Ca(NO_3)_2$, S=straw, SN=straw + $Ca(NO_3)_2$, P=peat, PN=peat + $Ca(NO_3)_2$, UC=unfertilized control.

Fig. 3. Non-metric multidimensional scaling analysis of effects of the different treatments on a) the AOB community structure and b) the AOA community structure. Both were determined by T-RFLP analysis of *amoA* genes. The abundance of *amoA* for AOB (qAOB) and AOA (qAOA), as well as the potential ammonia oxidizing rate (PAO), crop yield (Yield) and soil properties (pH, Total N, Total Organic C, C:N ratio and Soil moisture) were incorporated in the analysis using vector fitting with ordination scores. Only those with $r^2>0.2$ are included and the arrow length is proportional to the strength of correlation. ▲=$Ca(NO_3)_2$, ◇=straw,

662 ◆=Straw + $Ca(NO_3)_2$, ▽=peat, ▼=peat + $Ca(NO_3)_2$, △=unfertilized control. Stress values

663 (*S*) are indicated in the respective figure.

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Figure

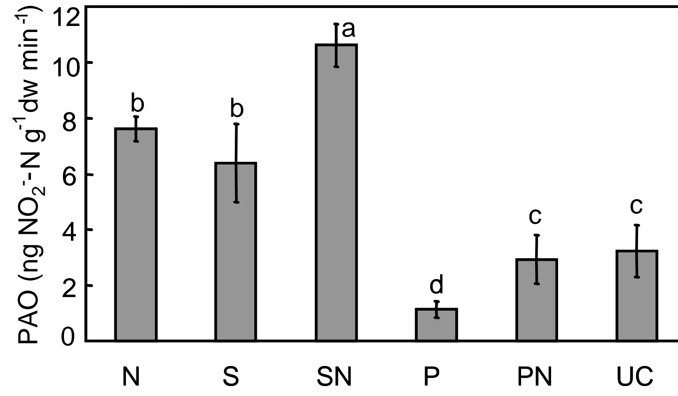

Figure 1

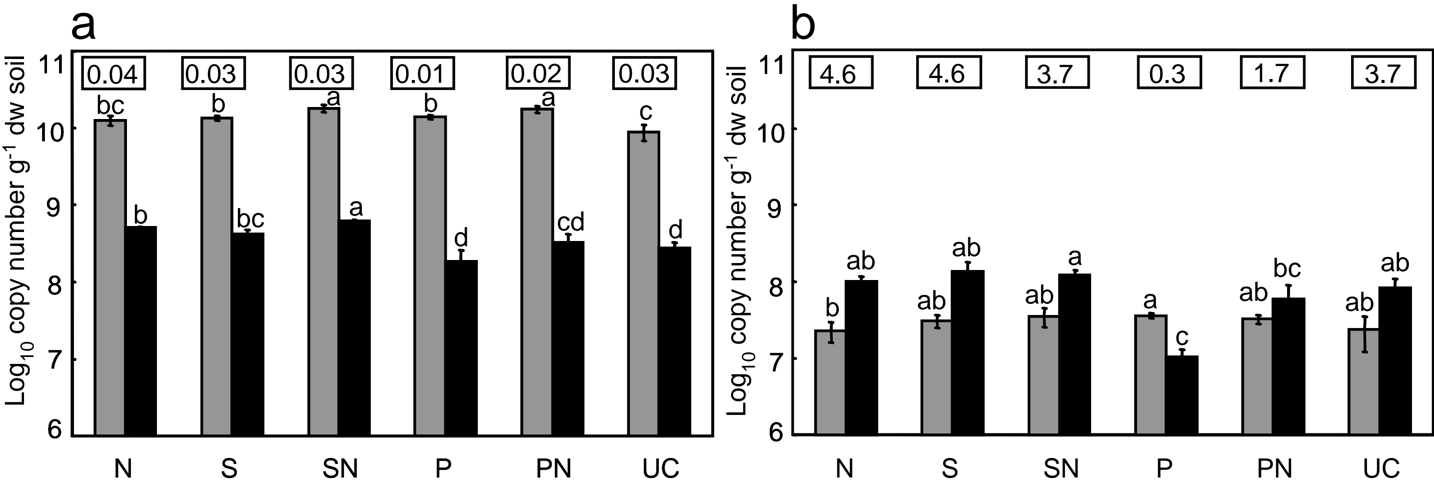

Figure 2

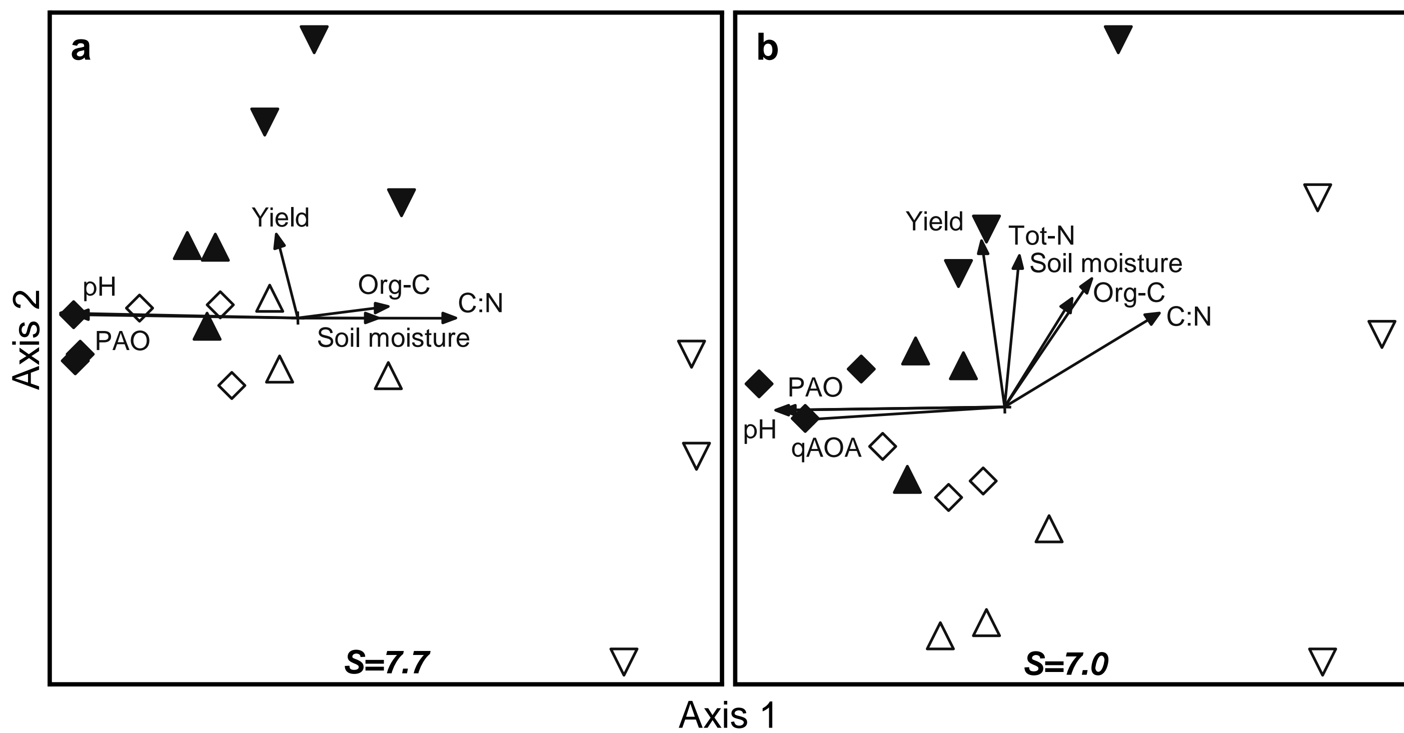

Figure 3

Tables

Table 1. Soil properties and crop yields for different fertilizer treatments in the Ultuna long-term soil organic matter experiment (mean ± standard deviation, n=6). Values followed by the same letter (a-f) within columns are not significantly different (*P*>0.05).

Treatment	Fertilizer regime	pH[1,2]	Org-C[1] (% of dw)	Tot-N[1] (% of dw)	C:N	Crop yield[3] (kg dw ha^{-1})	Soil moisture (%)
N	$Ca(NO_3)_2$	6.6±0.3[a]	1.3±0.04[e]	0.14±0.002[c]	9.6±0.2[d]	7630±3270[b]	17.3±2.0[abc]
S	Straw	6.4±0.2[ab]	1.5±0.06[d]	0.14±0.003[d]	10.3±0.2[c]	4470±1810[dc]	16.8±0.4[b]
SN	Straw and $Ca(NO_3)_2$	6.5±0.4[ab]	1.9±0.10[c]	0.18±0.009[b]	10.6±0.1[b]	8840±3880[ba]	17.2±1.0[b]
P	Peat	5.6±0.4[d]	3.2±0.32[b]	0.18±0.010[b]	17.7±0.9[a]	5640±1910[c]	19.1±0.6[a]
PN	Peat and $Ca(NO_3)_2$	6.0±0.2[c]	3.8±0.29[a]	0.22±0.011[a]	17.6±0.5[a]	10430±3020[a]	19.7±0.6[a]
UC	Unfertilized control	6.2±0.3[b]	1.1±0.05[f]	0.12±0.004[e]	9.6±0.1[d]	4100±1830[d]	16.3±0.4[c]

Abbreviations: dw, dry weight; Org-C, total soil carbon; Tot-N, total soil nitrogen.

[1]When the experimental site was established in 1956, the soil pH was 6.5 and the total organic C and total N were 1.5% and 0.17% of the soil dry weight, respectively.

[2] (n=9)

[3]Total green biomass

Table 2. The standardized Mantel statistics (*r*) from Mantel tests of correlations between dissimilarity matrices of community structure of ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea AOA, soil properties (pH, soil organic carbon [Org-C], total nitrogen [Tot-N], C:N ratio and soil moisture), potential ammonia oxidation rates (PAO) and abundance of AOB and AOA. Soil properties, PAO rates and community abundance dissimilarities were calculated using Euclidian distances, while community structure dissimilarities were calculated using Bray-Curtis' distances. (***P<0.001; *P<0.05; NS, not significant at P>0.05; ND, not determined)

	pH	**Org-C**	**Tot-N**	**C:N**	**Soil moisture**	**Crop yield**	**PAO**	**AOB abundance**	**AOA abundance**	**AOB structure**
AOB structure	NS	0.21*	NS	0.57***	NS	NS	0.46***	NS	ND	-
AOA structure	NS	0.20*	NS	0.52***	NS	NS	0.51***	ND	0.58***	0.87***

Table 3. Pearson correlations (*r*) between soil properties (pH, soil organic carbon [Org-C], total nitrogen [Tot-N], C:N ratio and soil moisture), crop yield, potential ammonia oxidation rates (PAO) and abundance of ammonia oxidizing bacteria (AOB) and archaea (AOA). (***P<0.001; *P<0.05; NS, not significant at P>0.05)

	pH	**Org-C**	**Tot-N**	**C:N**	**Soil moisture**	**Crop yield**	**PAO**	**AOB abundance**
PAO	0.72***	-0.50*	NS	-0.66*	NS	NS	-	NS
AOB abundance	NS	NS	0.51*	NS	0.48*	NS	NS	-
AOA abundance	0.67*	-0.60*	NS	-0.73*	-0.58*	NS	0.76***	NS

Supplemental material

Responses of bacterial and archaeal ammonia oxidizers to soil organic and fertilizer amendments under long-term management

Ella Wessén, Karin Nyberg, Janet K Jansson and Sara Hallin

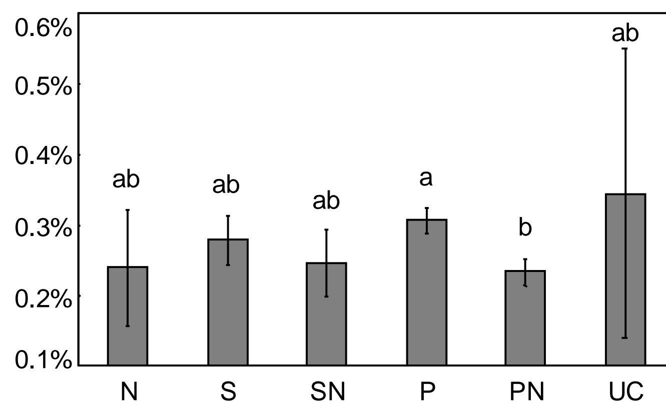

Fig. S1. Relative abundance of the AOB in the different treatments (mean ± standard deviation, n = 3) shown as percentage of the total bacterial 16S rRNA gene copies. The same letters above the bars indicate treatments without significant differences (P<0.05). Treatments: N= $Ca(NO_3)_2$, S=straw, SN=straw + $Ca(NO_3)_2$, P=peat, PN=peat + $Ca(NO_3)_2$, UC=unfertilized control.

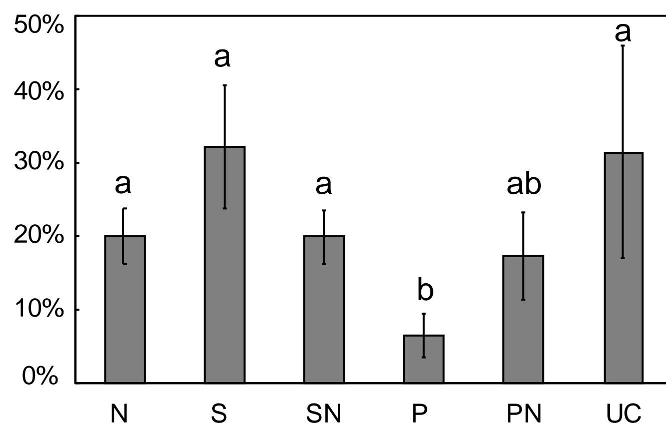

Fig. S2. Relative abundance of the AOA in the different treatments (mean ± standard deviation, n = 3) shown as percentage of the total Crenarchaeal gene copies. The same letters above the bars indicate treatments without significant differences (P<0.05). Treatments: N= $Ca(NO_3)_2$, S=straw, SN=straw + $Ca(NO_3)_2$, P=peat, PN=peat + $Ca(NO_3)_2$, UC=unfertilized control.