Ammonia-limited conditions cause of Thaumarchaeal dominance in volcanic grassland soil

Running title: Ammonia-limited conditions cause Thaumarchaeal dominance

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Abstract

The first step of nitrification is carried out by ammonia-oxidizing bacteria (AOB) and archaea (AOA). It is largely unknown, by which mechanisms these microbes are capable of coexistence and how their respective contribution to ammonia oxidation may differ with varying soil characteristics. To determine how different levels of ammonium availability influence the extent of archaeal and bacterial contributions to ammonia oxidation, microcosm incubations with controlled ammonium levels were conducted. Net nitrification was monitored and ammonia oxidizer communities were quantified. Additionally, the nitrification inhibitor allylthiourea (ATU) was applied to discriminate between archaeal and bacterial contributions to soil ammonia oxidation.

Thaumarchaeota, which were the only ammonia oxidizers detectable at the start of the incubation, grew in all microcosms, but AOB later became detectable and grew as well. Low and high additions of ammonium increasingly stimulated AOB growth, while AOA were only stimulated by the low addition. Treatment with ATU had no effect on net nitrification and sizes of ammonia-oxidizing communities suggesting that the effective concentration of ATU to discriminate between archaeal and bacterial ammonia oxidation is not the same in different soils. Our results support the niche differentiating potential of ammonium concentration for AOA and AOB and we conclude that ammonium limitation can be a major reason for absence of detectable AOB in soil.
Introduction

Ammonia oxidation in soil is driven by two groups of microorganisms: ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). It is unknown however, how significant the contribution of AOA to ammonia oxidation is under different environmental conditions in soil. Current research points to the specification of distinct niches for both groups of ammonia oxidizers. Such niche separation would result in differential contribution of AOA and AOB to the ammonia oxidation process in dependence of environmental conditions. Generally it is now assumed that AOA may be largely responsible for ammonia oxidation under oligotrophic, acidic, oxygen-deprived and elevated temperature conditions (Hatzenpichler, 2012; Prosser & Nicol, 2012). However, usually both AOA and AOB are found within a soil habitat and the ratio of AOA to AOB as well as the application of specific inhibitors have therefore been employed to assess the contribution of AOA to total ammonia oxidation in soil (Akiyama et al., 2013; Dai et al., 2013; Di et al., 2010; Gong et al., 2013; Kleineidam et al., 2011; Lehtovirta-Morley et al., 2013; Leininger et al., 2006; Nardi et al., 2013; Taylor et al., 2013; Zeglin et al., 2011). One of the nitrification inhibitors with a promising potential for the discrimination between archaeal and bacterial ammonia oxidation is the copper-chelating, sulfur-containing allylthiourea (ATU). From as early as 1946 the inhibitory effect of ATU on soil nitrification has been described (Lees, 1946) and it has been used extensively in nitrification research since (Bedard & Knowles, 1989). The mechanism of ammonia oxidation inhibition by copper-chelating agents has been proposed to involve a reversible reaction with nucleophilic amino acids close to the copper in the active center of the ammonia monooxygenase (Hyman et al., 1990). Recently, it could be demonstrated in soil and pure culture that AOA are less inhibited by ATU at
concentrations that completely prevent bacterial ammonia oxidation (Hatzenpichler et al., 2008; Jung et al., 2011; Mosier et al., 2012; Santoro & Casciotti, 2011; Shen et al., 2013a; Taylor et al., 2010, 2013).

Making use of a volcanic soil from Iceland that contains negligible numbers of AOB (Daebeler et al., 2012), we studied the niche-differentiating potential of ammonium availability by incubating soil microcosms at *in situ*, slightly elevated and elevated ammonium concentrations. Since this soil, as all andosols, has a relatively high cation exchange capacity (unpublished data) a reason for the low AOB abundance may be strong binding of ammonia to soil particles and hence low substrate availability for ammonia oxidizers. Such a condition is thought to benefit AOA. By theory, incubation with higher concentrations of ammonium should then stimulate AOB activity and growth, which can be ideally monitored in the AOA-dominated soil utilized here. We hypothesized that (i) elevated ammonium levels in soil microcosms will promote the growth of AOB and the decline of AOA:AOB ratios and that (ii) AOA will be the dominant ammonium oxidizers in low and in *in situ* ammonium level microcosms. Additionally, the effect of ATU on net nitrification and growth of ammonia oxidizers at the three ammonium levels was investigated. Here, we specifically hypothesized that (iii) the stimulatory effect of ammonium concentration on AOB growth will be suppressed by 100 µM ATU.

**Material and Methods**

*Soil microcosms*

The effects of ammonium concentration and allylthiourea (ATU) on nitrification and ammonia oxidizer community dynamics were determined in microcosms containing
volcanic grassland soil taken from Grændalur valley (64° 1’ 7” N, 21° 11’ 20” W), Iceland, in May 2012. The soils physico-chemical properties are described in a previous study (Daebeler et al., 2012). Samples were taken from the top 10 cm accounting for spatial variation by taking subsamples within a radius of 2 m. After pooling the samples they were stored at 4°C for 2 weeks. Before further analysis and incubation, soil was mixed and sieved through a 3 mm sieve. In order to determine the water content of field-fresh soil, the weight loss in 10 g of soil was measured before and after drying at 60°C for 3 days. Field-fresh soil had a moisture content of 0.67 ml ∙ g wet soil⁻¹ and a water-holding capacity (WHC) of 2.63 ml ∙ g dry soil⁻¹; the latter was determined according to Wilke (2005).

The incubation was conducted in triplicate microcosms consisting of sterile 250 ml bottles containing 10 g of field-moist, sieved soil after letting the soil recover from mixing and sieving for 2 days at 25°C in loosely capped bottles. Soil was amended with ammonium by equally dropping 0.5 ml of ammonium sulfate solutions containing 0, 1.5 and 15 g (NH₄)₂SO₄ L⁻¹ onto the soil to obtain final amounts of 0, 48 and 480 µg NH₄⁺—N ∙ g dry soil⁻¹. Further, microcosms are referred to as ‘NA’ for no ammonium microcosms (0 µg NH₄⁺—N ∙ g soil⁻¹), ‘LA’ for low ammonium (48 µg NH₄⁺—N ∙ g dry soil⁻¹) and ‘HA’ for high ammonium microcosms (480 µg NH₄⁺—N ∙ g dry soil⁻¹). The inhibitory effect of ATU was tested in microcosms receiving 0.5 ml of ammonium sulfate solutions that additionally contained 167 mg ATU L⁻¹ resulting in additions of 48 and 480 µg NH₄⁺—N ∙ g dry soil⁻¹ and in a final concentration of 100 µM ATU in the moist soil (respective microcosms further referred to as ‘LA+ATU’ for low ammonium with ATU and ‘HA+ATU’ for high ammonium with ATU). Concentrations of 100 µM ATU have been reported to completely inhibit bacterial ammonia oxidation in pure culture (Shen et al.,
2013b; Jung et al., 2011), while affecting archaeal ammonia oxidation and abundance in soil and pure culture to a much lesser extent (Lehtovirta-Morley et al., 2013).

Microcosms were incubated for 28 days in the dark at 25°C with loose caps to allow for air exchange. Ammonium sulfate treatments were renewed by addition of 0, 30 and 241 
µg NH$_4^+$—N g· dry soil$^{-1}$ on a weekly or bi-weekly basis according to soil ammonium measurements. ATU treatments were similarly refreshed by addition of 0 and 52 µM ATU. Soil moisture content was monitored by weight and kept constant at 65% WHC by addition of demi-water or respective volumes of ammonium sulfate solutions at times of treatment renewal.

Triplicate microcosms were destructively sampled on days 0, 7, 14, 21 and 28 of incubation. Soil for extraction and analysis of DNA was immediately stored at -20°C. The remaining soil of each microcosm (~ 8 g) was stored at -20°C until used for extraction and determination of inorganic nitrogen. In brief, 15mL of 1M KCl were added to the soil sample of one destructively sampled microcosm, followed by shaking at room temperature for one hour at 200 rpm and centrifugation at 5000 rpm for 20 min. KCL extracts were used for pH measurements and then stored at -20°C until analysis of ammonium and combined nitrite plus nitrate contents by a continuous flow auto-analyzer (SA-40, Skalar Analytical BV, The Netherlands). The detection limits for the ammonium and nitrite + nitrate measurements were 30 µg N/L corresponding to 0.72 µg N per g of dry soil.

**qPCR amplification of bacterial and archaeal amoA genes**

At the start of the experiment and after 14 and 28 days of incubation, total nucleic acids were extracted from 0.5 g soil of the destructively sampled microcosms according to
Lueders et al. (2004). Quantification of archaeal and bacterial amoA genes from total DNA extracts was performed in duplicate on the samples from three replicate microcosms as described previously (Daebeler et al., 2012) using the primer set ArchamoA-1F/ArchamoA-2R (Francis et al., 2005) for AOA and amoA-1F/amoA-2R (Rotthauwe et al., 1997) for AOB. The amount of DNA template used per reaction ranged from 1-10 ng ∙ µl⁻¹ for all qPCR assays and all samples were analyzed in duplicates. Efficiencies of qPCR runs ranged between 0.96 and 1.00 and did not differ significantly between runs of AOA and AOB assays. Each product was analyzed by melting curve and only samples with melting curve profiles consistent with the standards were used for analysis. Additionally we checked amplicons for correct length by gel electrophoresis. The detection limit for both qPCR assays was 160 gene fragments ∙ g dry soil⁻¹. Assuming that the soil ammonia-oxidizing communities did not grow or shrink within the time from start of the incubation to the first sampling at day 0 (approx. 30 min), we only performed qPCR analysis with samples from the NA microcosms for this time point.

Statistical analysis

All statistical analysis was conducted in R ver. 3.0.2 (R Core Team, 2013). Graphics were produced with package ggplot2 (Wickham, 2009). Differences in ammonia and nitrite plus nitrate concentrations and qPCR data were compared by ANOVA analysis followed by Tukey’s HSD Post Hoc tests using the aov function in R. The relationship of the ratio of archaeal to bacterial amoA genes with the nitrite + nitrate concentration was assessed by an exponential regression using least squares.
Results

Changes in mineral N concentrations

Only microcosms given the higher dosage of ammonium maintained ammonium levels that were measurable throughout the 28-day incubation (Figure 1A). There was a significant positive effect of ammonium addition on the measured concentration of ammonium. In the microcosms that had received low amounts of ammonium, ammonium could only be detected on day zero of the incubation, despite additions of 10 µg NH$_4^+$ - N per g soil every week. Larger additions of ammonium to the HA and HA+ATU microcosms elevated the concentration in the microcosms. The addition of 80 µg NH$_4^+$ - N per g soil on day 14 led to an increase of ammonium concentrations in the HA, but not in the HA+ATU microcosms on day 21. The resulting difference between these two treatments on day 21 was significant. After another seven days of incubation however, this difference was gone and the ammonium concentrations in both treatments were comparable to those before addition on day 14. The NA microcosms, which did not receive ammonium additions, never contained measurable amounts of ammonium.

Nitrite + nitrate concentrations were low in all microcosms at the beginning of the incubation and significantly increased towards the end in all treatments (Figure 1B). The ammonium treatment significantly increased the accumulation of nitrite + nitrate, more so in the high ammonium microcosms. There was no significant effect of ATU addition on the accumulation of nitrite + nitrate during the entire incubation period.

Changes in archaeal and bacterial amoA gene abundance
Thaumarchaeal amoA gene abundance increased by almost an order of magnitude from the beginning of the incubation to day 28 in all treatments except HA (Figure 2A). The LA microcosms contained significantly more archaeal amoA genes than the HA microcosms on day 28, but none of the treatment microcosms were significantly different from the NA microcosms on day 28. There was however no significant effect of the ATU treatment on archaeal amoA gene abundance at any given time point. At the onset of the incubation bacterial amoA genes were not detectable (Figure 2B). Numbers remained below the detection limit in the NA microcosms at all times of the incubation, while both the HA and HA+ATU microcosms contained $8.5 \cdot 10^4 \pm 1.1 \cdot 10^4$ and $7.1 \cdot 10^4 \pm 7.9 \cdot 10^3$ bacterial amoA genes per g dry soil on day 14, respectively. On the same day of the incubation there was no bacterial amoA detectable in the LA and LA+ATU microcosms however. On day 28 the LA and LA+ATU microcosms contained detectable numbers of bacterial amoA genes for the first time. The numbers of bacterial amoA genes in the HA and HA+ATU microcosms had decreased from day 14 to day 28 however. There was no significant effect of the ATU treatment on bacterial amoA gene abundance.

**Discussion**

*Dynamics of net nitrification*

The elevated ammonium availability in the HA and HA+ATU microcosms secured by the larger addition of ammonium sulfate stimulated the process of net nitrification already within the first week of the incubation and likely led to a condition of unlimited ammonia availability for the nitrifiers. The ammonium values being slightly higher than expected in
HA and HA+ATU microcosms immediately after addition on day 0 of the incubation can likely be attributed to the adsorption properties of the soil.

On a lower level, also LA and LA+ATU microcosms showed elevated ammonium contents on day 0, but afterwards was not distinguishable from the NA microcosms, most likely due to an immediate uptake of all added ammonium by the ammonia oxidizers and the ammonia-assimilating heterotrophic microbial community. Very similar patterns of ammonium and nitrite + nitrate concentrations over time were observed by Verhamme and colleagues (2011) in soil incubations amended with 25% more ammonium than in our study. It remains unclear, why nitrite + nitrate concentrations in the HA+ATU microcosms dropped from day 14 to 21. Although denitrification in anoxic or suboxic microsites cannot be excluded, it would have been the same for the HA and the HA+ATU microcosms. Possibly, the drop at day 21 constitutes a lag in activity of nitrifiers due to ATU addition, since concentrations rose again to expected values on day 28. It becomes apparent that N mineralization must have taken place in the NA microcosms when looking at the balance of mineral nitrogen (Figure 3).

The loss of ammonium and accumulation of nitrite + nitrate was well balanced in both LA and LA+ATU microcosms with no difference caused by the ATU addition. It thus seems reasonable to assume that the fate of the added ammonium was mainly oxidation. Unfortunately, the large standard errors that result from the calculation of lost ammonium and accumulated amounts of nitrite + nitrate do not allow for interpretation of differences between average lost ammonium and accumulated nitrite + nitrate in HA/HA+ATU microcosms and LA/ LA+ATU microcosms. It is thus impossible to speculate on the amounts of assimilated and not-nitrified ammonium for example in relation to AOB and AOA growth.
Effect of ammonium addition to soil microcosms

As shown for fresh soil in a field study comprising the same sampling site (Daebeler et al., 2012), no bacterial ammonia oxidizers were detectable when the soil was incubated without addition of ammonium, while at the same time net nitrification occurred and we clearly observed growth of the thaumarchaeal community. It is therefore plausible to attribute the ammonia oxidation indirectly observed via net nitrification in the NA microcosms to the activity of AOA. This is in contrast to many other soils where AOB are suggested to have a more important role in nitrification than AOA (e.g. Jia & Conrad 2009; Morimoto et al. 2011; Xia et al. 2011).

Confirming our hypothesis of promoted AOB growth by ammonium addition, AOB communities, but not AOA communities, were significantly stimulated showing a shorter lag time in the HA and HA+ATU microcosms than in the LA and LA+ATU microcosms. AOA growth however, was significantly inhibited by the higher ammonium addition. Consequently, the ratio of archaeal amoA to bacterial amoA dropped significantly in the ammonium-amended microcosms and we found an overall negative exponential relationship between the ratio of archaeal to bacterial amoA genes with the concentration of nitrite + nitrate (see Figure 4). This could suggest an increasing contribution of AOB to nitrification AOB as ammonium concentrations increased, or ammonia oxidation by AOA uncoupled from growth in the later stages of the incubation.

The first explanation is in accordance with numerous previous studies that have accredited AOB the prevailing importance for ammonia oxidation under conditions of elevated ammonium (e.g. Di et al., 2010; Fan et al., 2011; Taylor et al., 2010; Verhamme et al., 2011). Additionally, we show that even in a soil were AOB exist in
such low numbers that they are not detectable \textit{in situ}, they can grow to become a significant part of the soil microbial community and likely contribute to nitrification when relieved from intrinsic limitations, in our case via addition of ammonium. These observations lead to the conclusion that, indirectly or directly, the low availability of ammonium in this Histic Andosol limits the abundance and activity of AOB and is the main reason for the numeric dominance of AOA. Interestingly, other soils in which AOB could also not be detected did not show an increased bacterial ammonia-oxidizing community with the application of organic or inorganic N (Levicnik-Höfferle \textit{et al.}, 2012; Lu & Jia, 2013). These soils were however acidic and the availability of ammonia as a substrate for AOB will have increased to a much lesser degree than with the application of ammonium sulfate to the neutral pH soil studied here.

The ammonium treatment also impacted the archaeal ammonia-oxidizing community, albeit less severely. On the one hand, the low additions of ammonium significantly stimulated AOA growth, but higher ammonium additions had an inhibitory effect similar to a previous study (Verhamme \textit{et al.}, 2011). On the other hand, these differences were rather small and all ammonium-amended microcosms did not differ with respect to archaeal \textit{amoA} gene copies from non-amended NA microcosms at the end of the incubation. These findings are well in line with previous environmental and pure culture studies showing a preference of AOA for low NH$_4^+$ concentrations (Di \textit{et al.}, 2010; Hatzenpichler \textit{et al.}, 2008; Lehtovirta-Morley \textit{et al.}, 2011; Pratscher \textit{et al.}, 2011). It is further possible, that AOA growth was largely coupled to ammonification through N mineralization of organic N. The study of Levicnik-Höfferle and colleagues (2012) nicely demonstrates the possible dependence of AOA on organically derived ammonia and supports this possibility.
Our hypothesis of AOA dominance over AOB in the NA, LA and LA+ATU treatments was confirmed, even though AOA were in fact the dominant ammonia-oxidizers in all microcosms including the HA and HA+ATU treatments. However, this does not necessarily imply, as is often concluded, that Thaumarchaea contributed accordingly to the process of ammonia oxidation as the negative relation of the ratio of archaeal to bacterial amoA genes with the concentration of nitrite + nitrate shows (see Figure 4). Quite contrastingly, we may assume that the contribution of AOA to total nitrification was no larger than under the NA conditions. In these microcosms AOB were not detectable and the AOA community size was comparable to those microcosms that did receive additional ammonium. As discussed above, we can however not exclude that AOA actively nitrified more in HA and HA+ATU than in low ammonium microcosms, uncoupled from the observed increased growth. To verify or dismiss this scenario, future studies should specifically addressing the active nitrifying community, e.g. via functional gene transcript analysis.

We do not know why both archaeal and bacterial amoA gene abundance dropped from day 14 to 28. This was observed for both ammonia oxidizers independent of treatments and we hence assume that the microcosms may have provided sub-optimal conditions for ammonia oxidizer growth after 14 days. Moisture content of the soil and oxygen concentrations will unlikely have played a role as they were controlled for, but we cannot exclude other factors negatively influencing ammonia oxidizers such as growth of grazers or depletion of nutrients other than ammonia.

Effects of ATU addition to soil microcosms
Most surprisingly, the treatment of soil microcosms with 100 µM ATU did not show any effect on the size of AOB and AOA communities disproving our hypothesis of suppressed AOB activity through ATU. Whereas pure cultures of AOB are always shown to be sensitive to ATU at concentrations between 0.4 - 86 µM (Ginestet et al., 1998; Shen et al., 2013a; Hooper & Terry, 1973), it may well be that ATU applied in the same concentration range does not have the same effect when applied to soil where a large part of the inhabiting microbial community will be able to degrade the inhibitor. Furthermore, the spatial structure of soil does not allow for a completely homogenous distribution of solvents in this range of concentration, possibly leaving parts of the soil matrix uninfluenced as recently discussed in Lehtovirta-Morely and colleagues (2013). These authors even observed a stimulation of archaeal amoA gene abundance by addition of 100 – 1000 ng ATU per g soil to an AOA-dominated soil, while the accumulation of nitrite was inhibited. Nevertheless, it has been demonstrated that in other soils a concentration of 100 µM ATU partly inhibits nitrification and decreases the activity of AOB, but not AOA (Taylor et al., 2010, 2013). It thus appears that the effect of ATU on soil nitrifiers in their habitat is not only highly dependent on the concentration applied, but possibly also on edaphic properties like spatial structure, microbial community composition and soil nutrient status. Therefore, initial tests may be required to confirm that a concentration of 100 µM ATU in a specific soil is sufficient to discriminate between ammonia oxidation by AOA and AOB. It will be promising for future studies to follow the fate of ATU (adsorption, degradation, consumption) in a specific soil in order to fully be able to assess the effects of this inhibitor in soil. Furthermore, it would be interesting to test whether ATU may possibly interfere with ammonium measurements. This possibility seems the most likely explanation for the
insignificantly, but consistently lower levels of ammonium in the HA+ATU microcosms as compared to the HA microcosms.

**Conclusion**

Although fresh soil material and all NA microcosms did not contain detectable numbers of AOB, bacterial ammonia oxidizers did become active and grew by more than two orders of magnitude when the conditions were favorable, i.e. through elevated ammonium availability. Moreover, AOA growth was significantly negatively affected by higher ammonium additions. Clearly, this shows that major reasons for the numerical dominance of AOA in the *in situ* and NA soils are direct or indirect effects of ammonium limitation of AOB. It further confirms the importance of ammonium concentration as a niche-differentiating factor between AOA and AOB. The insensitivity of ammonia-oxidizing bacterial and archaeal communities to ATU points to the possibility that ATU will affect soil nitrification and ammonia oxidizers in strong dependence of intrinsic soil characteristics. Therefore, it seems that the same effective concentration of ATU can neither be applied to discriminate between archaeal and bacterial contributions to ammonia oxidation in all soils nor to compare the specific contributions of both groups between different soils.

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**Figure 1.**

Changes in ammonium (A) and nitrite plus nitrate (B) concentrations in soil microcosms incubated at 25°C for 0, 7, 14, 21 and 28 d. NA, no added ammonium treatment; HA, high ammonium treatment with initial addition of 480 µg NH₄⁺ - N per g dry soil; HA+ATU, same as before with further addition of ATU to 100 µM in the pore water; LA, low ammonium treatment with initial addition of 48 µg NH₄⁺ - N per g dry soil; LA+ATU, same as before with further addition of ATU to 100 µM in the pore water. Treatments were renewed as indicated by arrows and numbers in panel A showing time points and concentrations of ammonium additions [µg NH₄⁺ - N per g soil]. Data plotted are mean values and standard errors are derived from triplicate microcosms destructively sampled at each time point.

**Figure 2.**

Abundance of archaeal (A) and bacterial (B) amoA gene fragments in soil microcosms after incubation for 0, 14 and 28 days. NA, no added ammonium treatment; HA, high ammonium treatment with initial addition of 480 µg NH₄⁺ - N per g dry soil; HA+ATU, same as before with further addition of ATU to 100 µM in the pore water; LA, low ammonium treatment with initial addition of 48 µg NH₄⁺ - N per g dry soil; LA+ATU, same as before with further addition of ATU to 100 µM in the pore water. Data plotted are mean values and standard errors are derived from triplicate microcosms destructively sampled at each time point. Dashed lines indicate the detection limit. Same lower case letters above the bars indicate no significant difference in means between treatments at day 14; same upper case letters above the bars indicate no significant difference in means between treatments at day 28; (p > 0.05).
Figure 3

Values of ammonium loss and nitrite plus nitrate accumulation based on measurements over the time course of the entire incubation for all microcosms. NA, no added ammonium treatment; LA, low ammonium treatment with initial addition of 48 µg NH4+ - N per g dry soil; LA+ATU, same as before with further addition of ATU to 100 µM in the pore water; HA, high ammonium treatment with initial addition of 480 µg NH4+ - N per g dry soil; HA+ATU, same as before with further addition of ATU to 100 µM in the pore water. Data shown are values and standard errors derived from twelve microcosms per treatment.

Figure 4

Fit of a least square, exponential regression to the relationship of the ratio of archaeal to bacterial amoA gene copies per g dry soil with the concentration of nitrite + nitrate [µg N per g dry soil]. Where no bacterial amoA genes could be detected the value of the qPCR detection limit was used to conservatively estimate the ratio. Dotted lines depict the 95% confidence intervals. The equation of the regression line (y = ab^x), significance values of the equation terms (p_a = p value for a; p_b = p value for b) and the correlation coefficient (r) are given in the graph.