RESEARCH ARTICLE

Stimulation of thaumarchaeal ammonia oxidation by ammonia derived from organic nitrogen but not added inorganic nitrogen

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Abstract

Ammonia oxidation, the first step in nitrification, is performed by autotrophic bacteria and thaumarchaea, whose relative contributions vary in different soils. Distinctive environmental niches for the two groups have not been identified, but evidence from previous studies suggests that activity of thaumarchaea, unlike that of bacterial ammonia oxidizers, is unaffected by addition of inorganic N fertilizer and that they preferentially utilize ammonia generated from the mineralization of organic N. This hypothesis was tested by determining the influence of both inorganic and organic N sources on nitrification rate and ammonia oxidizer growth and community structure in microcosms containing acidic, forest soil in which ammonia oxidation was dominated by thaumarchaea. Nitrification rate was unaffected by the incubation of soil with inorganic ammonium but was significantly stimulated by the addition of organic N. Oxidation of ammonia generated from native soil organic matter or added organic N, but not added inorganic N, was accompanied by increases in abundance of the thaumarchaeal *amoA* gene, a functional gene for ammonia oxidation, but changes in community structure were not observed. Bacterial *amoA* genes could not be detected. Ammonia oxidation was completely inhibited by 0.01% acetylene in all treatments, indicating ammonia monooxygenase-dependent activity. The findings have implications for current models of soil nitrification and for nitrification control strategies to minimize fertilizer loss and nitrous oxide production.

Introduction

The efficiency of the utilization of applied nitrogen fertilizers in many agroecosystems is low owing to microbially mediated nitrification, the conversion of ammonia, via nitrite, to nitrate. Whereas ammonium ions adsorb to anionic particles that dominate in soil, nitrate is readily leached or denitrified, resulting in loss of up to 70% of applied N fertilizer from managed ecosystems worldwide (Raun & Johnson, 1999). The consequent annual economic losses, estimated at $15.9 billion (Raun & Johnson, 1999), are compounded by nitrate pollution of groundwaters and nitrogen loss through the denitrification of nitrate (Ryden *et al.*, 1984; Subbarao *et al.*, 2006). Denitrification and nitrification are also the major biological sources of the greenhouse gas, nitrous oxide (Galloway *et al.*, 2008; Ravishankara *et al.*, 2009; Schlesinger, 2009). Strategies to reduce nitrification-associated loss of nitrogen fertilizer are therefore increasingly driven by the desire to reduce nitrous oxide production (Di & Cameron, 2002; Subbarao *et al.*, 2006; Singh & Verma, 2007) and employ chemically synthesized or crop-derived inhibitors (Wolt, 2004; Subbarao *et al.*, 2006, 2009; Zakir *et al.*, 2008).

All commercial inhibitors have been tested against ammonia oxidizing bacteria, particularly *Nitrosomonas europaea* (Subbarao *et al.*, 2006), but there is strong evidence that thaumarchaeal ammonia oxidizers (Brochier-Armanet *et al.*, 2008; Spang *et al.*, 2010) can contribute significantly to soil nitrification. Thaumarchaeal

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amoA genes are ubiquitous in soil (Schleper *et al.*, 2005), *amoA* genes and gene transcripts of thaumarchaeal groups 1.1a, 1.1a-associated and 1.1b frequently outnumber those of betaproteobacterial ammonia oxidizers (Leininger *et al.*, 2006; Prosser & Nicol, 2008), their abundance and diversity can respond more to changes in environmental factors such as temperature (Tourna *et al.*, 2008) and pH (Nicol *et al.*, 2008), and thaumarchaeal growth is inhibited by acetylene, an inhibitor of ammonia oxidation (Offre *et al.*, 2009; Gubry-Rangin *et al.*, 2010). Thaumarchaeal lineages 1.1c and 1.3 dominate archaeal communities in acidic peat soils (Oline *et al.*, 2006; Bomberg & Timonen, 2007; Kemnitz *et al.*, 2007; Lehtovirta *et al.*, 2009; Stopnišek *et al.*, 2010) but are not known to possess *amo* gene homologues.

Knowledge of the physiology, including inhibition, of thaumarchaeal ammonia oxidizers is limited by the small number of cultivated strains (Könneke *et al.*, 2005; De la Torre *et al.*, 2008; Hatzenpichler *et al.*, 2008; Lehtovirta-Morley *et al.*, 2011; Tourna *et al.*, 2011) but the marine, cultivated, archaeal ammonia oxidizer, *Nitrosopumilus maritimus*, has a much higher affinity for ammonia than bacterial ammonia oxidizers (Martens-Habbena *et al.*, 2009). Ammonia concentrations in soil are several orders of magnitude greater than in marine environments, but this finding has led to suggestions that thaumarchaea may dominate ammonia oxidation in low-ammonium environments, and there is evidence that bacteria dominate ammonia oxidation in heavily fertilized soils (Di *et al.*, 2009, 2010) or soils exposed to long-term pollution by municipal wastewater rich in ammonium (Höfferle *et al.*, 2010). There is also debate regarding carbon metabolism within the thaumarchaea. Betaproteobacterial soil ammonia oxidizers are obligate chemoautotrophs, and there is evidence of autotrophic thaumarchaeal ammonia oxidation in marine (Wuchter *et al.*, 2003; Herndl *et al.*, 2005; Ingalls *et al.*, 2006) and terrestrial (Zhang *et al.*, 2010) environments, but also for heterotrophic or mixotrophic archaeal growth (Ouverney & Fuhrman, 2000; Hallam *et al.*, 2006; Teira *et al.*, 2006). Thaumarchaeal growth can occur in the presence of root exudates, rather than following inorganic fertilizer addition (Chen *et al.*, 2008), without the incorporation of inorganic carbon (Jia & Conrad, 2009) and in response to added organic fertilizer (Schauss *et al.*, 2009). In addition, the recently isolated soil thaumarchaeal ammonia oxidizer, *Nitrososphaera viennensis*, grows mixotrophically on ammonia and pyruvate (Tourna *et al.*, 2011). The potential for mixotrophic growth within at least some thaumarchaeal ammonia oxidizers therefore has implications for distinct ecological niches for soil ammonia oxidizers.

In many soils, nitrification rate is controlled by the rate of ammonia release following the mineralization of organic matter (Booth *et al.*, 2005). Mineralization control potentially involves effects of ammonia concentration (slow release of ammonia rather than high concentrations following inorganic N fertilization) and carbon metabolism, if growth is mixotrophic. There is evidence that addition of inorganic ammonia to soil does not stimulate archaeal ammonia oxidation (Stopnišek *et al.*, 2010; Verhamme *et al.*, 2011), suggesting niche specialization, with archaeal and bacterial ammonia oxidizers, respectively, utilizing organic and inorganic sources of ammonia. If true, the consequences for fertilizer strategies are considerable and we therefore tested this hypothesis by investigating the influence of both inorganic and organic N on nitrification rate and thaumarchaeal and bacterial ammonia oxidizer growth in soil microcosms. The findings generate a new model for thaumarchaeal and bacterial nitrification with implications for nitrogen fertilization strategies.

Materials and methods

Soil microcosms and analysis

The influence of inorganic and organic forms of nitrogen on nitrification and abundance and community structure of archaeal ammonia oxidizers was determined in microcosms containing soil from Ljubljana marsh, Slovenia. This soil has an acidic pH (4.5, range 3.8–4.7), an organic carbon content of 45%, a C : N ratio of 16.5 : 1, and high water holding capacity (8 g H_2O g^{-1}) (Ausec *et al.*, 2009). Soil was sampled in January 2010 (long-term experiment) and January 2011 (short-term experiment) from the upper 30-cm soil layer at three locations, approximately 3 m apart. Samples were combined, sieved (mesh size 8 mm) and stored at 4 °C prior to establishing microcosm experiments. Soil gravimetric water content was determined after drying 10-g subsamples at 105 °C for 24 h.

Microcosms consisted of 250-mL flasks containing 40 g wet soil amended with 4 mL filter-sterilized (pore size 0.2 μm) deionized water (control) or 4 mL of inorganic or organic N solutions, in sterile deionized water (NH_4Cl (AnalaR, BDH Chemicals, UK), urea (VWR, BDH Prolabo, Poole, UK), L-glutamic acid (Sigma, Germany) or yeast extract (Oxoid, UK). Nitrogen compounds were added to soil to give a final concentration of 300 μg N g^{-1} and a soil moisture content equivalent to 65% of water holding capacity. Flasks were closed with a butyl rubber stopper and sealed with a metal cap, and acetylene was added to the headspace of selected microcosms, to a final concentration of 10 Pa (0.01%), which has previously been shown to inhibit nitrification completely in this soil (Stopnišek *et al.*,

2010). This experiment was performed in parallel with a stable isotope probing experiment with ^{13}C-CO_2. The headspace of selected microcosms was therefore adjusted to 5% (v/v) ^{12}C-CO_2, which has previously been shown to have no significant effect on thaumarchaeal or bacterial ammonia oxidation (Zhang *et al.*, 2010). Triplicate microcosms for each treatment were incubated at 27 °C for 14 days, and soil (6 g) was sampled from each flask at 0, 4, 7, 10 and 14 days. Microcosms were resealed after each sampling, and the acetylene and CO_2 headspace concentrations were re-established. To determine short-term changes, following inorganic and organic N amendment, an additional microcosm experiment was performed for 4 days with soil sampled from the same site 1 year later. Experimental design was identical, except that microcosms contained 80 g soil, were amended with 8 mL of water or solutions containing respective nitrogen compounds, resulting in soil moisture equivalent to 67% water holding capacity, and soil samples (6 g) were removed at intervals of 12 h.

A portion of soil sampled from each microcosm was processed immediately (within 1 h) for colorimetric analysis of ammonium and combined nitrite and nitrate concentration by flow injection analysis (FIA star 5010 Analyser, Tecator) or continuous flow analyser (FlowSys; Alliance Instruments, Salzburg, Austria) after extraction from 4 or 3 g of soil, for the 14- and 4-day experiments, respectively, with 1 M KCl (1 : 8, soil : KCl), and data are presented as $\mu g\ N\ g^{-1}$ ($\mu g\ N\ g^{-1}$ dry soil) (lower detection limits approximately 1 $\mu g\ N\ g^{-1}$). Previous studies (Stopnišek *et al.*, 2010) have shown that nitrification in this soil does not lead to the accumulation of nitrite, with nitrite concentration always at or below the detection limit. Data are therefore presented as nitrate concentration g^{-1}, for simplicity. For the 14-day experiment, the remainder of the soil was stored at −20 °C for molecular analysis. The pH of each microcosm was measured initially and after incubation for 14 days or every 12 h, for 14-day and 4-day experiments, respectively, in soil suspensions in dH_2O (1 : 2, soil/water) after thorough mixing and settling for 30 min (Thomas, 1996).

Nucleic acids were extracted, for molecular analysis, from 0.5 g of soil from the 14-day experiment as described by Griffiths *et al.* (2000) and modified by Nicol *et al.* (2005). Lysing Matrix B (MP Biomedicals, Solon, OH) was used for lysis with tubes shaken on a Vortex Genie 2 (Scientific Industries, NY) for 2 min at maximum speed. For use in PCR reactions, concentration and purity of extracted DNA was determined using a Nanodrop 1000 (Thermo Fisher Scientific, Inc., Waltham, MA) and diluted 140- to 480-fold to reduce the influence of potential PCR inhibitors.

Quantitative PCR enumeration of *amoA* genes

Quantification of thaumarchaeal *amoA* genes was performed using primers crenamoA23F and crenamoA616R (Tourna *et al.*, 2008). Standard curves were generated from a 10^7–10^1 dilution series (in duplicate) of known amounts of a 1610-bp PCR product containing *amoA* and amplified from soil fosmid 54d9 (Treusch *et al.*, 2005) using newly designed, specific primers Orf37-38f (5′-ACA TTG CCT TCT CGT TGT GCA TTT -3′) and Orf39-40r (5′-TCG CAT GAA TCA CGT TCC CCA-3′). PCR reactions were performed in 20-μL reaction mixtures containing 1× Power Sybr Green PCR Master Mix (Applied Biosystems, CA), 1 μM each primer, 0.2 mg mL^{-1} BSA and 10 ng of soil DNA (5 μL). Amplification was performed in an ABI 7900 HT Fast real-time PCR system (Applied Biosystems, CA) with initial denaturation of 95 °C for 10 min, 40 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, followed by a final extension of 72 °C for 10 min and melting curve analysis (60–95 °C). qPCR amplification efficiency of thaumarchaeal *amoA* was 85% with $r^2 > 0.99$ and slope −3.74. PCR products were confirmed by standard 1% agarose gel electrophoresis. Quantification of bacterial *amoA* genes was performed using primers amoA-1F and amoA-2R (Rotthauwe *et al.*, 1997) as described by Stopnišek *et al.* (2010), and abundance was below the detection limit.

Denaturing gradient gel electrophoresis (DGGE) analysis

DGGE analysis of total thaumarchaeal 16S rRNA genes and *amoA* genes was performed with primers and thermocycling conditions described by Nicol *et al.* (2008). PCR amplification of thaumarchaeal 1.1c 16S rRNA genes was performed as described for total thaumarchaeal 16S rRNA genes (Nicol *et al.*, 2008), using primer FFS-200F (Jurgens & Saano, 1999) for primary PCR amplification. DGGE gels were prepared as described previously by McCaig *et al.* (2001), without the use of Gelbond PAG film (FMC BioProducts, Rockland, ME) and with linear gradients of denaturants, as described by Nicol *et al.* (2008). Gels were electrophoresed in a DCode Universal Mutation Detection System (Bio-Rad) with 6.5 L of 1× TAE buffer at a constant temperature of 60 °C for 16 h at 75 V. Gels were stained with a solution containing 5× TAE and 3.3× SYBR Green I dye (Molecular Probes, OR) for 1 h before imaging on a UV-transilluminator (UVItec, Cambridge, UK). Analysis of DGGE gels (Supporting Information, Figs S1 and S2) was performed using BIONUMERICS software (Applied Maths NV, Belgium).

Statistical analysis

Data exploration and graphical representation were performed in R language and environment for statistical computing, version 2.11.1 (R Development Core Team, 2010). Nitrification rate was determined using linear models. Pairwise differences in nitrate concentration, ammonium concentration and abundance of *amoA* genes between amendments were determined with two-tailed Student's *t*-tests assuming unequal variances and multiple-sample comparisons were performed using one-way analysis of variance test (*aov*), implemented in R. Comparisons per amendment were determined with repeated measures ANOVA or paired two-sample Student's *t*-test. The values reported in this study are mean ± standard error of triplicate microcosms.

Results

Influence of inorganic and organic N amendments on nitrification

Net nitrification rate was determined as the rate of increase in nitrate concentration, assuming zero-order kinetics ($0.966 < r^2 < 0.999$), during the incubation of soil microcosms for 14 days after addition of 300 μg N g^{-1} inorganic or organic N (Fig. 1a). In the absence of acetylene, nitrate production in control microcosms (8.34 ± 0.12 μg NO_3^--N g^{-1} day^{-1}) resulted from the oxidation of ammonia released through the mineralization of native soil organic N. Amendment with ammonium did not increase net nitrification rate and, in fact, led to a small but significant decrease to 7.05 ± 0.15 μg NO_3^--N g^{-1} day^{-1} ($P = 0.002$). In contrast, nitrate production in microcosms amended with organic N (urea, glutamate and yeast extract) was biphasic, with significant stimulation from 0 to 7 days (16.5–17.7 μg NO_3^--N g^{-1} day^{-1}) ($P < 0.001$) and lower rates between 7 and 14 days. Rates between 7 and 14 days for organic N and control treatments were not significantly different ($P > 0.05$) but were significantly greater than those in ammonium-amended microcosms ($P < 0.028$). Nitrate yield after 14 days was highest following amendment with glutamate, slightly but significantly lower for urea and yeast extract amendments ($P < 0.004$) and significantly lower for control and ammonium-amended microcosms ($P < 0.001$). Nitrate yield following ammonium amendment was significantly lower than in control microcosms ($P = 0.029$).

Acetylene completely inhibited nitrate production in all microcosms (Fig. 1b), and changes in ammonium concentration (Fig. 1d) are equivalent to rates of the mineralization of native soil organic matter and added organic N. Thus, ammonium concentration increased continuously ($P < 0.001$) in acetylene-treated control microcosms (Fig. 1d) at rates similar ($P = 0.77$) to net nitrification

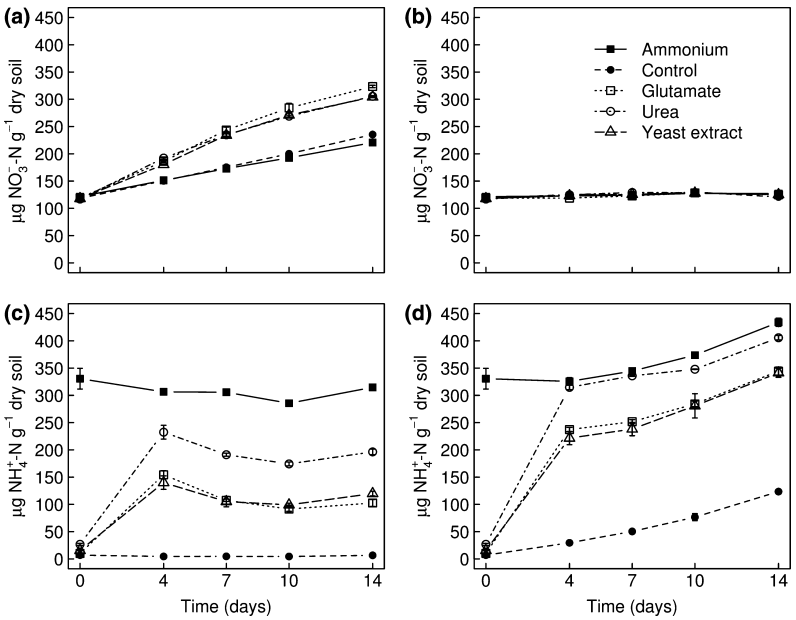

Fig. 1. The influence of inorganic and organic nitrogen compounds on nitrification in soil microcosms incubated at 27 °C for 14 days. Changes in nitrate (a, b) and ammonia (c, d) concentrations in the absence (a, c) and presence (b, d) of the nitrification inhibitor acetylene. Data are means and standard errors calculated from triplicate microcosms for each treatment. Error bars are not visible where smaller than the symbol size.

rate in non-inhibited microcosms and respective nitrate and ammonium yields at day 14 were similar ($P = 0.93$). There was therefore no evidence for loss of nitrate through denitrification or other processes. Similar changes were seen in ammonium-amended microcosms. Amendment increased initial ammonium concentration, and subsequent increases through mineralization were similar ($P = 0.08$, rates between 4 and 14 days) to nitrate production in ammonium-amended, non-inhibited microcosms. Mineralization of urea was complete by the first sampling point (day 4), and the subsequent increase in ammonium reflected mineralization of native soil organic N. Mineralization of glutamate and yeast extract also occurred within 4 days but was incomplete (77% and 72%, respectively).

In non-inhibited microcosms, changes in ammonium concentration (Fig. 1c) represent the balance between mineralization and nitrification rates. In control microcosms, nitrification was at least equal to mineralization rate, and ammonium concentration remained low ($P = 0.24$). Ammonium amendment increased the ammonium concentration, which did not change significantly ($P = 0.74$) during incubation, providing further evidence for the nitrification of ammonium derived from mineralization, rather than added inorganic N. Mineralization of added organic N exceeded nitrification, and ammonium concentration increased rapidly after incubation for 4 days, following amendment with urea, glutamate and yeast extract ($P < 0.01$). Differences between ammonium concentration in non-acetylene (Fig. 1c) and acetylene-treated microcosms (Fig. 1d) represent maximum nitrification rates. Ammonium concentration then decreased between days 4 and 10, before increasing at 14 days to a rate corresponding to the mineralization of native soil organic matter. Thus, remarkably, inorganic N generated by the mineralization of added organic N was nitrified, but added inorganic N was not.

Changes in soil pH following nitrogen amendment

Soil pH_{H2O} was determined at the beginning and end of the 14-day incubation, to assess potential effects of pH changes on nitrification rate (Fig. 2). All changes were within a small pH range (3.6–4.3) and are unlikely to have significantly affected nitrification rates. Addition of ammonium chloride reduced initial soil pH by 0.12 pH units, while addition of organic N compounds led to increases of 0.02–0.14. Initial pH was greatest and lowest following amendment with glutamate and ammonium, respectively. Nitrification led to a significant decrease ($P < 0.019$) in soil pH in all microcosms, with largest (0.38 ± 0.009) and smallest (0.27 ± 0.038) decreases after amendment with urea and ammonium, respectively, and final pH after amendment with glutamate was significantly higher than

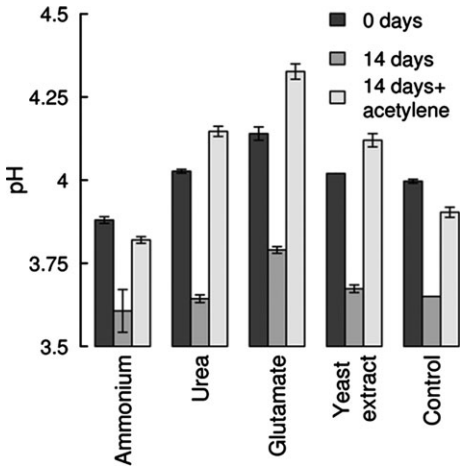

Fig. 2. Soil pH (in dH_2O) at the beginning (0 days) and end (14 days) of incubation of soil microcosms amended with inorganic and organic nitrogen compounds in the absence or presence of acetylene. Data are means and standard errors calculated from triplicate microcosms for each treatment.

for other amendments ($P < 0.036$). In acetylene-inhibited microcosms, pH increased significantly ($P < 0.013$) after amendment with organic N and was highest after glutamate amendment. A small but significant decrease ($P < 0.027$) in soil pH was observed at day 14 in control and ammonium-amended microcosms.

A second microcosm experiment was performed to determine whether the initial increases in ammonium production and nitrification rate were due to increases in pH. Changes in ammonium and nitrate concentration after incubation for 4 days were qualitatively similar to those in the original microcosm study, but with small quantitative differences, most likely arising from use of different soil samples and more frequent sampling. Ammonium production from organic amendments was maximal after 0.5 days (urea) or 1 day (glutamate and yeast extract) and subsequently (days 2–4) decreased to concentrations similar to those in the original experiment (Fig. 3a and b). Ammonium production led to small increases in soil pH, which returned to values < 4 by day 4 (Fig. 3c). The maximum pH observed was 4.41 ± 0.02, 1 day after amendment with glutamate. Nitrate concentration did not change significantly during the initial increases in ammonia and pH but increased only after incubation for $\geq$ 3.5 days, and only in microcosms amended with organic nitrogen.

Influence of inorganic and organic N amendment on *amoA* abundance and community structure

Growth of archaeal ammonia oxidizers during nitrification was estimated by quantification of thaumarchaeal *amoA*

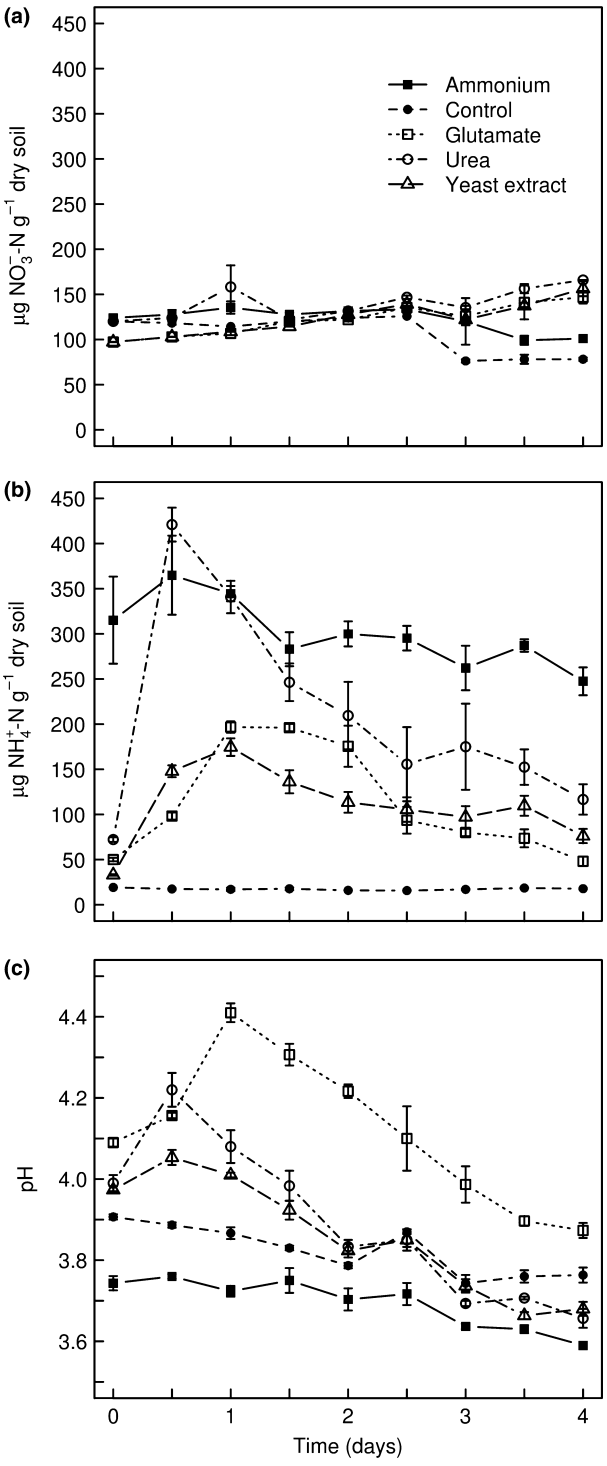

Fig. 3. The influence of inorganic and organic nitrogen compounds on nitrification in a short-term (4-day) soil microcosm experiment: changes in nitrate concentration (a), ammonium concentration (b) and soil pH (in dH_2O) (c). Data are means and standard errors calculated from triplicate microcosms for each treatment. Error bars are not visible where smaller than the symbol size.

genes and is expressed as gene abundance μg^{-1} DNA, to facilitate comparison between microcosms. Abundance of *amoA* genes ranged between 1 and 20 $\times$ 10^6 g^{-1}. Variability in abundance data between microcosms was greater than that for ammonium and nitrate concentrations, partly because of the smaller sample size used for analysis, but abundance generally corresponded to nitrate production and yield. *amoA* gene abundance did not change significantly with time during incubation of acetylene-inhibited microcosms for any amendment ($P > 0.58$) (Fig. 4b), although significant differences between some treatments were observed at days 4 and 14. Gene abundance increased significantly ($P < 0.03$) with time in control and organic N-amended non-inhibited microcosms (Fig. 4a), except those amended with glutamate ($P = 0.67$), where variability was particularly high. However, abundance in glutamate-amended microcosms was significantly higher than all other amendments at days 4 and 10 ($P < 0.03$), except for urea at day 4 ($P = 0.07$). Generally, *amoA* abundance increased 2.2- and 3.5-fold after 14 days in control and glutamate-amended microcosms, the latter showing an initial increase. Abundance increased later after amendment with urea and yeast extract (2.6- and 3.6-fold, respectively). Archaeal *amoA* abundance did not change significantly with time following amendment with ammonium ($P = 0.87$) but was significantly lower at day 14 than all other amendments ($P < 0.034$), except glutamate ($P = 0.27$).

There was no evidence of changes in thaumarchaeal community structure assessed by DGGE analysis of *amoA* genes (Fig. S1), amplified from DNA extracted from soil after incubation for 0, 7 and 14 days and total thaumarchaeal and thaumarchaeal Group 1.1c 16S rRNA genes after incubation for 0 and 10 days (Fig. S2). Bacterial *amoA* genes were not detected in standard end-point PCR and were below the detection limits for bacterial qPCR analysis (10^4 μg^{-1} DNA).

Discussion

Bacterial *amoA* genes could not be detected in the acid soil investigated and assumed ammonia monooxygenase-dependent (i.e. acetylene-sensitive) nitrification was therefore dominated by archaea, as observed previously (Stopnišek *et al.*, 2010). Thaumarchaeal *amoA* abundance was high and generally increased during nitrification, and the soil therefore provides an ideal system to investigate the influence of different forms of ammonia supply on their activity. Five supply routes were investigated: addition of inorganic ammonium, as ammonium chloride, and supply through the mineralization of four organic sources: native soil organic matter, yeast extract, glutamic acid

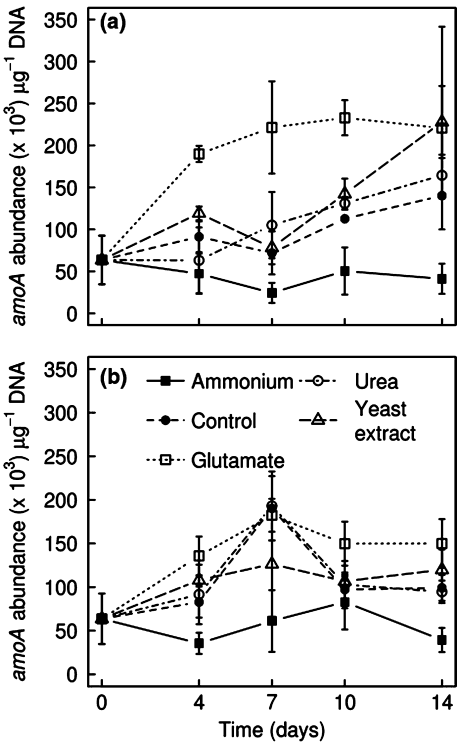

Fig. 4. The influence of inorganic and organic nitrogen compounds on thaumarchaeal *amoA* gene abundance in soil microcosms incubated at 27 °C for 14 days in the absence (a) and presence (b) of acetylene. Data are means and standard errors calculated from triplicate microcosms for each treatment, except for ammonium-amended microcosms at day 7 ($n = 2$).

and urea, in order of increasing expected mineralization rate. All amendments would generate equivalent amounts of ammonium if mineralization were complete.

Mineralization

The dynamics of ammonium production through mineralization was determined following acetylene inhibition of nitrification. There was an underlying increase in ammonium concentration in all amendments through the mineralization of native organic matter. Mineralization rates in unamended and ammonium-amended soils were similar, suggesting that none of the added ammonium was assimilated and that mineralization in this soil was not nitrogen-limited. After 4 days (in the 14-day microcosm experiment), approximately 75% of nitrogen in glutamate and yeast extract was present as ammonium. Yeast extract contains a complex mixture of organic compounds, and complete mineralization may not have occurred within the course of this experiment. However, complete conversion of glutamate within 14 days was expected, and incomplete conversion of organic N compounds to ammonium may reflect assimilation of added organic N by heterotrophs. The initial 'flush' of ammonium was followed by increases due to mineralization of native soil organic matter. Mineralization was accompanied by small increases in pH, because of production of ammonia, while the lower pH following ammonium chloride addition was because of ion exchange between NH_4^+ and H^+. The pH of control microcosms did not change significantly prior to ammonia oxidation. Nitrification was completely inhibited in acetylene-treated soils, and there were no statistically significant temporal changes in *amoA* abundance.

Nitrification

Nitrification was driven solely by mineralization. All ammonium produced from native soil organic matter was converted to nitrate, with a small decrease in pH and a small increase in *amoA* abundance. There was no evidence for the oxidation of added inorganic ammonium, with similar changes in nitrate concentration and pH in control microcosms, and no detectable change in *amoA* abundance. The flush of ammonium from mineralized organic matter was accompanied by increases in nitrate concentration, but not all released ammonium was converted by day 4, presumably because nitrification potential was lower than mineralization rate. Ammonium therefore accumulated; some was nitrified between days 4 and 10, but subsequent nitrification appears to be due to the oxidation of ammonium derived from native organic matter. Nitrification was accompanied by a small decrease in soil pH and an increase in *amoA* abundance but was incomplete. Remarkably, therefore, accumulated inorganic ammonium generated from mineralized organic N was nitrified, while added inorganic ammonium was not.

Ammonia oxidation rates following nitrogen amendments may have been influenced by changes in soil pH, but there was no evidence for such effects. Increases in pH following the mineralization of organic nitrogen were transitory and by day 3.5 most treatments were within 0.13 pH units and all were within 0.27 pH units, because of buffering by the soil. This included soils amended with inorganic ammonium, in which pH decreased slightly, but significant nitrification was not detectable in any treatment until 4 days, after pH had stabilized. The recognized explanation for lack of ammonia oxidation at low pH of cultivated bacterial ammonia oxidizers is reduced ammonia availability, through ionization. Estimated ammonia concentrations at pH values of 3.63 and 3.76 (the pH range for most treatments at day 3.5), after amendment with 300 µg NH_4^+-N g^{-1}, are 0.74×10^{-3} and 1.0×10^{-3} µg NH_3-N g^{-1}, respectively. These differences would have negligible influences on specific growth rate.

Community structure of thaumarchaeal ammonia oxidizers did not change with any of the treatments, possibly because of the relatively short incubation period compared with other studies (Stopnišek *et al.*, 2010). This is consistent with findings of Jia & Conrad (2009) and Gubry-Rangin *et al.* (2010), who observed no significant changes in thaumarchaeal community structure in the presence or absence of inorganic fertilizer, despite a small change in the abundance. However, the existence of archaeal and bacterial ammonia oxidizers, in this soil (highly organic, low pH), which cannot be targeted with the primers used in this and many other studies (Leininger *et al.*, 2006; Agogue *et al.*, 2008; Tourna *et al.*, 2008; Zhang *et al.*, 2010), cannot be ruled out. Previous phylogenetic analysis of the archaeal 16S rRNA gene fragments from this soil (Stopnišek *et al.*, 2010) indicates that the *amo*-containing archaeal lineages belong to groups 1.1b and 1.1a-associated but comprise only a small proportion of the total archaeal community, which is dominated by groups 1.1c, 1.3 and 'deep peat' lineages (Stopnišek *et al.*, 2010). Variability in abundance data was high, but there was evidence for increases in thaumarchaeal *amoA* abundance associated with ammonia oxidation and for a small reduction in soils amended with inorganic ammonium.

Implications for niche separation and nitrification control strategies

The findings suggest that thaumarchaea preferentially oxidize ammonia generated by the mineralization of organic N. This may be due to high affinity for ammonia, as found for *N. maritimus* (Martens-Habbena *et al.*, 2009), particularly if ammonia oxidation rate exceeds mineralization rate. However, it does not explain the lack of oxidation of inorganic ammonia, unless thaumarchaea are also inhibited by high ammonia concentration, for which there is currently little evidence. A more compelling explanation is that thaumarchaea utilize ammonia localized at the site of organic N mineralization, enabling rapid oxidation of ammonia when and where it is produced, and preventing significant accumulation. An attractive mechanism would be mixotrophic growth (Ouverney & Fuhrman, 2000; Hallam *et al.*, 2006; Teira *et al.*, 2006; Jia & Conrad, 2009; Tourna *et al.*, 2011), with sequential intracellular generation and oxidation of ammonia, as proposed for pH-independent, urea-based ammonia oxidation (De Boer *et al.*, 1989; Burton & Prosser, 2001). Our findings do not exclude this mechanism, but mineralization was unaffected by acetylene inhibition and would therefore need to be independent of energy generation from ammonia oxidation. It also does not explain the oxidation of inorganic ammonia accumulated after mineralization. Alternatively, 'trapping' of ammonia may result from close physical association between mineralizers and ammonia oxidizers and immediate oxidation of ammonia as it is produced. This is consistent with evidence of nitrification within aggregates (De Boer *et al.*, 1991) and biofilms (Allison & Prosser, 1993) at low pH and with associated models for nitrification in acid soils (De Boer & Kowalchuk, 2001). Intriguingly, inorganic ammonium that accumulated when mineralization exceeded nitrification was oxidized, when added inorganic ammonium was not. Localized trapping of ammonia explains the former, although trapping was not complete, as oxidation stopped after incubation for 10 days, resulting in biphasic nitrate production. This suggests that some of the organic N-derived ammonium entered the same 'pool' as added inorganic ammonium and became unavailable, for example by immobilization through cation exchange or diffusion from the site of production. The proposed mechanisms provide an explanation for ammonia oxidation in other acid and neutral soils (Offre *et al.*, 2009; Gubry-Rangin *et al.*, 2010) in which ammonia oxidation is driven by mineralization, and potential niche separation of thaumarchaeal and bacterial ammonia oxidizers.

Oxidation of ammonia generated by the mineralization of added organic nitrogen, but not of inorganic ammonium, also has significant implications for fertilization and nitrification inhibitor strategies. Regardless of potential mechanisms, oxidation by thaumarchaea of ammonia generated from organic, but not inorganic N, influences prediction and control of nitrification in agroecosystems, to reduce fertilizer loss and nitrous oxide production. Control of nitrification in thaumarchaea-dominated soils by existing inhibitors has not been tested, but there are reports of differential inhibition of bacterial and thaumarchaeal ammonia oxidation (Schauss *et al.*, 2009). More importantly, nitrification inhibitors have been developed to inhibit the oxidation of ammonia supplied in inorganic form. Oxidation of ammonia supplied through mineralization in this low pH soil and the suitability and efficacy of chemical inhibitors may be affected in a similar manner.

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