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Selective inhibition of ammonia oxidising archaea by simvastatin stimulates growth of ammonia oxidising bacteria

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ABSTRACT

The desire to understand and distinguish the relative growth and activity of ammonia oxidising archaea (AOA) and ammonia oxidising bacteria (AOB) in soil nitrification has increased the search for selective inhibitors of these two groups. This study aimed to investigate the potency and specificity of simvastatin as a specific AOA inhibitor in pure cultures and in soil and to determine the effect of AOA inhibition on both ammonia oxidation activity and growth of AOB, under the hypothesis that AOB growth is higher when competition for NH_4^+ from AOA is removed. Simvastatin selectively inhibited pure cultures of all tested AOA at concentrations of 8–100 μM. In soil microcosms incubated for 21 days with low and high NH_4^+ concentrations, AOA but not AOB were selectively inhibited by simvastatin in both acidic (pH 4.5) and near-neutral (pH 6.5) soils. Additionally, growth of AOB significantly increased at both NH_4^+ concentrations following inhibition of AOA by simvastatin, suggesting that competition for substrate between AOA and AOB is a key factor restraining AOB growth in NH_4^+-limited soils. Simvastatin can therefore be used as a selective AOA inhibitor to investigate kinetic characteristics of AOB in soils and to study competition between AOA and AOB in complex environments.

1. Introduction

Ammonia oxidising archaea (AOA) and bacteria (AOB) and more recently discovered complete ammonia oxidisers (comammox) perform the initial step of nitrification (oxidation of ammonia to nitrite). There is evidence that AOA and AOB have different preferences for ammonia source and concentration, with AOA being favoured when NH_4^+ is generated by mineralisation of organic N, while AOB prefer supply of high concentrations of inorganic NH_4^+ (Di et al., 2009; Hink et al., 2018; Höfferle et al., 2010; Levičnik-Höfferle et al., 2012; Stopnišek et al., 2010; Verhamme et al., 2011). This was previously explained by higher ammonia affinity and sensitivity of AOA compared to AOB (Prosser and Nicol, 2012), but recent studies have challenged this theory following the isolation of several *Ca.* Nitrosocosmicus strains of AOA (Jung et al., 2016; Lehtovirta-Morley et al., 2016a; Sauder et al., 2017) that can grow at high ammonium concentrations, similar to those supporting growth of typical soil AOB. Recent studies have also failed to find major differences between AOA and AOB affinities for NH_3 (Hink et al., 2017a; Kits et al., 2017). In addition, stimulation of AOA growth has recently been demonstrated at high soil ammonium concentration following specific inhibition of AOB (Hink et al., 2018). These findings suggest that both AOA and AOB are able to grow at both low and high NH_4^+ concentrations, while competition for ammonia may be the major factor differentiating growth of AOA and AOB in soil.

Soil pH is another key factor controlling niche specialisation of both AOA and AOB (Aigle et al., 2019; Gubry-Rangin et al., 2011, 2015; 2018; Nicol et al., 2008). While AOA can dominate ammonia oxidation in both acidic and neutral soils (Gubry-Rangin et al., 2010; Zhang et al., 2010, 2012), information on AOB growth and activity in acidic soils is limited. However, the potential activity of AOB at low pH is indicated by the presence of AOB phylotypes in acidic soils and laboratory cultivation and isolation of AOB from such soils (Aigle et al., 2019; Allison and Prosser, 1991; Carnol et al., 2002; De Boer et al., 1995; Jiang and Bakken, 1999; Long et al., 2012; Nicol et al., 2008; Petersen et al., 2012; Wertz et al., 2012), including recent isolation of an acid-tolerant AOB strain (Hayatsu et al., 2017). Accumulating evidence for physiological adaptation of AOB to low pH and potentially similar substrate affinities of soil AOA and AOB provide the basis for hypotheses that i) AOB growth

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occurs in soils of low pH and/or low NH_4^+ concentration and ii) alleviation of AOA competition for ammonia will stimulate AOB growth in soil.

Testing these hypotheses requires an inhibitor of AOA that does not inhibit AOB. Several nitrification inhibitors have been tested for selective inhibition of AOA or AOB and there is strong evidence that the aliphatic alkyne 1-octyne is an efficient inhibitor of AOB, which does not reduce AOA activity and growth in both pure culture and soil (Hink et al., 2017b, 2018; Taylor et al., 2013). Several selective inhibitors of AOA have also been tested, but the majority of these compounds are unsuitable due to either weak inhibitory effect or high toxicity to AOB (Shen et al., 2013). PTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide) is currently the most commonly used inhibitor for archaeal ammonia oxidation in pure culture (Kozlowski et al., 2016; Martens-Habbena et al., 2015; Shen et al., 2013) and environmental samples (Duan et al., 2018; Meinhardt et al., 2018; Sauder et al., 2017). This inhibitor scavenges NO, an important intermediate of archaeal ammonia oxidation (Walker et al., 2010; Yan et al., 2012). However, PTIO showed no or incomplete inhibition of AOA nitrification in several environmental samples, including soil (Fu et al., 2018; Sauder et al., 2016), and might affect the activity and growth of AOB, following evidence that NO may also be an obligate intermediate for ammonia oxidation by AOB (Caranto and Lancaster, 2017). Therefore, an alternate AOA inhibitor with distinct mechanism of action is needed to replace PTIO.

Statins are a class of molecules that interfere with archaeal cell membrane biosynthesis by inhibiting 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, a crucial enzyme in the mevalonate pathway (Lam and Doolittle, 1992; Miller and Wolin, 2001). Statins do not inhibit growth of bacteria, which use an alternative pathway for cell membrane biosynthesis (Jain et al., 2014). Statins have been demonstrated as effective inhibitors of several archaea, including methanogens from animal and human intestines (Gottlieb et al., 2016), but have not been tested on AOA in culture or in soil. The initial aim of this study was therefore to assess the effect of a commercially available statin derivative drug, simvastatin, on the specific growth rate of pure cultures of several AOA and AOB and on soil communities. The second aim was to test the hypotheses stated above, that AOB growth is stimulated in the absence of AOA growth, regardless of ammonium concentration and soil pH. This was investigated by analysis of nitrification rate and changes in growth and activity of AOA and AOB in microcosms containing soils of different pH and in the presence and absence of urea and simvastatin.

2. Materials and methods

2.1. Inhibition of ammonia oxidisers in laboratory culture

The effect of simvastatin was assessed on growth of four AOA strains, *Candidatus* Nitrosotalea devanaterra (Lehtovirta-Morley et al., 2011), *Candidatus* Nitrosotalea sinensis (Lehtovirta-Morley et al., 2014), *Nitrososphaera viennensis* (Tourna et al., 2011) and *Candidatus* Nitrosocosmicus franklandus (Lehtovirta-Morley et al., 2016a), and three AOB strains, *Nitrosomonas europaea* (ATCC, 19718), *Nitrosospira multiformis* (ATCC 25196) and *Nitrosomonas eutropha*. *Ca.* N. devanaterra and *Ca.* N. sinensis were cultivated in freshwater medium (FWM) at pH 5.0 as described by Lehtovirta-Morley et al. (2011), modified by adding 2.5 mM final concentration 2-(N-morpholino) ethanesulfonic acid buffer (MES buffer) (pH 5.35) and 4 mM sodium bicarbonate. *Ca.* N. franklandus and *N. viennensis* grew in FWM at pH 7.5 as described by Lehtovirta-Morley et al. (2016a) and Tourna et al. (2011), respectively. *N. europaea*, *N. multiformis* and *N. eutropha* were cultivated in Skinner and Walker medium at pH 7.9 (Skinner and Walker, 1961). Cultures were incubated for 4–17 days in 100-ml Duran bottles containing 10 ml of liquid medium. Autoclaved growth media were supplemented with HPLC-grade simvastatin (Sigma-Aldrich, UK), dissolved in dimethyl sulfoxide (DMSO), to reach final simvastatin concentrations of 0, 8, 16, 32 or 100 μM. The amount of DMSO was adjusted to give a final concentration of 0.0098% (for simvastatin concentrations of 8, 16 and 32 μM) or 0.17% (v/v) (for a simvastatin concentration of 100 μM). Potential toxicity of DMSO was assessed in cultures supplemented with DMSO at 0.0098% or 0.17%. All cultures were incubated in triplicate in the dark without shaking and 100 μl of culture was sampled daily for assessment of growth through changes in nitrite concentration.

Ammonia oxidiser maximum specific growth rate was calculated as the slope of semi-logarithmic plots of temporal increases in nitrite concentration. Effects of a range of simvastatin concentrations (8–100 μM) were quantified as the proportional decreases in specific growth rate in comparison to the control (culture with same DMSO concentration).

2.2. Inhibition in soil microcosms

Microcosms consisted of 250-ml serum bottles containing agricultural soil at pH 4.5 or 6.5 collected from field plots at SRUC, Craibstone, Scotland (grid reference NJ872104). Details of the sampling sites and soil characteristics were described by Kemp et al. (1992). Triplicate microcosms were constructed for each soil with the following combined treatments: i) with or without urea amendment, ii) with or without addition of simvastatin and iii) incubation under 5% (v/v) $^{12}C–CO_2$ or isotopically labelled $^{13}C–CO_2$ in the headspace gas, resulting in a total of 8 treatments. The urea in $^{13}C–CO_2$ amended microcosms was 99 atom % ^{13}C-labelled (Sigma-Aldrich). Soil microcosms were established in sterile 250-ml serum bottles containing 23 g equivalent dry soil and sterile distilled water to achieve an initial moisture content of 30% (w/w, with respect to soil dry weight). For the urea treatment, water was replaced by urea solution to provide an initial concentration of 200 μg urea-N g^{-1} dry weight soil. Urea was rapidly converted to ammonium and ammonium-N concentration was monitored weekly in 1-g soil samples and microcosms were further supplemented with urea at the beginning of the third week to restore a concentration of 200 μg N g^{-1} soil. Simvastatin was added by thoroughly mixing solid Acros Organics™ simvastatin (Thermo Fisher Scientific, USA) with soil at a mass ratio of 12.5 mg g^{-1} dry weight soil before construction of the microcosms. This proportion is based on a preliminary experiment performed on the pH 4.5 soil (see supplementary results and Fig. S1). All bottles were sealed with a butyl rubber stopper and aluminium cap during incubation in the dark at 28 °C for 21 days. Bottles were aerated by removing caps at 3–4-day intervals to ensure adequate O_2 supply for nitrification and were re-supplied with $^{13}C–CO_2$ to avoid dilution by $^{12}C–CO_2$ gas originating from soil respiration. All microcosms were destructively sampled after incubation for 21 days and collected soils were frozen at – 80 °C before use.

2.3. Chemical analysis

Nitrite concentration in samples of growth media was measured colorimetrically as described by Lehtovirta-Morley et al. (2011) and the increases in NO_2^- concentration (μM) over time during exponential growth were used to calculate the maximum specific growth rate of AOA and AOB. Soil NH_4^+ and NO_x^- (NO_2^- plus NO_3^-) concentrations were determined by colorimetric analysis as previously described using 1 g of soil (Hink et al., 2018).

2.4. DNA extraction and quantification of amoA genes

DNA extraction was performed on 0.5 g soil as described by Griffiths et al. (2000). The quantity and quality of DNA extracts were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific). The abundance of archaeal and bacterial *amoA* genes was estimated by quantitative PCR (qPCR) of the DNA extracts (diluted to 2–10 ng μl^{-1}) using the primer pairs amoA23f/amoA616r (Tourna et al., 2008) and amoA1F/amoA2R (Rotthauwe et al., 1997), respectively. qPCR

conditions and standards were as described in Hink et al. (2017b). To test whether comammox also contributed to nitrification and were stimulated when AOA growth is inhibited, the abundance of comammox *amoA* gene was estimated in 20-μl reaction mixes consisting of 10 μl iQ™ SYBR® Green Supermix, 0.4 μg BSA, and 0.5 μM each of the primers Ntsp-amoA 162F/359R (Fowler et al., 2018). qPCR conditions were optimised as: 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min followed by measurement of fluorescence. The standards for comammox qPCR contained an equal-molar mix of 23 out of 40 sequenced clones of PCR amplicons by Ntsp-amoA 162F/359R, to cover the degeneracy of the primers as much as possible, and a dilution series containing 10^1–10^8 genes per assay was used for quantification of all genes. The efficiencies of the AOA, AOB and comammox *amoA* qPCR assays were 0.89–1, 0.82–0.91 and 0.93–1.02, respectively, with R^2 values > 0.99. Melting curve analysis and standard agarose gel electrophoresis were used to assess amplification specificity. Growth of ammonia oxidisers was estimated as the difference between initial and final gene abundances for each sample. Relative contributions of growing AOA and AOB to nitrification were then estimated using reported maximum specific cell activities of AOA (0.57 fmol NH_3 cell^{-1} h^{-1}) and AOB (23 fmol NH_3 cell^{-1} h^{-1}) (Prosser and Nicol, 2012).

2.5. Stable isotope probing

Isopycnic density gradient centrifugation was performed on the extracted DNA from microcosm samples after incubation for 21 days as previously described (Zhang et al., 2010). Briefly, 1 μg of DNA was mixed with CsCl solution (dissolved in TE buffer, 1.710 g ml^{-1} density) in an 8-ml quick-seal polyallomer tube (Beckman Coulter, USA) before centrifugation in a MLN-80 rotor (Beckman Coulter) at 100,00 *g* (45,000 rpm) for 60 h at 20 °C. DNA in each tube was then separated into 15 fractions (500 μl each). DNA in each fraction was precipitated by incubating at 4 °C overnight with 1 ml polyethylene glycol 6000 and 10 μg glycogen (Thermo Fisher Scientific) followed by centrifugation at 16, 000 *g* for 45 min. The pelleted DNA was washed by 1 ml 70% ethanol and re-suspended in 30 μl sterile water. The archaeal, bacterial and comammox *amoA* genes in each DNA fraction (fractions 2–14) were then quantified by qPCR. Autotrophic growth of ammonia oxidisers was determined by comparing ^{12}C–CO_2 and ^{13}C–CO_2 incorporation profiles, i.e. when the relative gene abundance was higher in ^{13}C–CO_2- than ^{12}C–CO_2-amended treatments in the "heavy fractions" (with buoyant density of 1.721–1.739 g ml^{-1} for AOA, 1.733–1.755 g ml^{-1} for AOB and 1.730–1.750 for comammox). The abundances of growing AOA, AOB and comammox were estimated as the proportions of cells incorporating ^{13}C–CO_2 multiplied by the total corresponding *amoA* gene abundance, estimated by qPCR in each sample. The relative contributions of autotrophic AOA and AOB to nitrification were estimated using the reported maximum specific cell activity of ammonia oxidisers described above, but the absence of cultivated terrestrial comammox prevented their inclusion.

2.6. Statistical analysis

All statistical analyses were performed on Statistics 23 (SPSS, Chicago, IL, USA). For pure cultures, one-way ANOVA was performed on the proportional decrease in maximum specific growth rates with simvastatin concentration as a fixed factor. For soil microcosms, two-way ANOVA was performed to assess the effect of NH_4^+ concentration and inhibitor on soil nitrification rate (measured as NO_x^- production after incubation for 21 days) and growing AOA, AOB and comammox abundances (calculated as the difference between initial and final gene abundance). Tukey HSD multiple *post-hoc* tests were used to assess the significance of the differences among the means.

The relative proportion and abundance of ^{13}C-labelled archaeal, bacterial and comammox *amoA* genes in heavy fractions were analysed by independent Student's *t*-tests between corresponding microcosms in the absence and presence of simvastatin.

3. Results

3.1. Effect of simvastatin on ammonia oxidiser growth in liquid culture

Inhibition of ammonia oxidation activity of four AOA and three AOB isolates by simvastatin was determined by calculating the proportional decrease in maximum specific growth rate in medium supplemented with a series of simvastatin concentrations. Ammonia oxidation activity of *Ca.* N. devanaterra and *Ca.* N. sinensis was completely inhibited by simvastatin at a concentration of 8 μM (Fig. 1), with no detectable increases in nitrite concentration after incubation of simvastatin-amended cultures for 17 and 11 days, respectively. *Ca.* N. franklandus and *N. viennensis* growth were not inhibited at 8–32 μM simvastatin but were inhibited at the higher concentration of 100 μM after incubation for 10 and 7 days, respectively (Fig. 1). In contrast, growth of the three AOB strains was not inhibited at any simvastatin concentration tested in this study (8–100 μM) after incubation for 4 days (Fig. 1). Growth of all AOA and AOB isolates tested was not inhibited by 0.0098 or 0.17% (v/v) DMSO (Fig. S2).

3.2. Effect of simvastatin on nitrification and ammonia oxidiser growth in soil

Ammonium concentration remained low (<10 μg N g^{-1} dry soil) during incubation of microcosms containing non-amended agricultural soil with pH 4.5 or 6.5 (Fig. S3) and nitrification (measured as NO_x^- production) occurred at rates of <55 and <30 μg N g^{-1} dry soil, respectively (Fig. 2a and b). Nitrification rate was not significantly affected by addition of simvastatin (Fig. 2a and b). In urea-fertilised soils, NH_4^+ concentration increased rapidly after urea supplementation, due to the rapid urea hydrolysis and release of ammonium, and CO_2-assimilation (Fig. S3) and nitrification rate were greater than in the unfertilised soils (Fig. 2a and b). Simvastatin supplementation significantly increased and decreased NO_x^- production in urea-amended pH 4.5 and pH 6.5 soils, respectively (Fig. 2a and b).

Growth of AOA occurred in both soils in the absence of simvastatin and was not affected by urea amendment (Fig. 2c and d). Growth of AOA was not detectable in soil microcosms after addition of simvastatin, except in urea-amended pH 4.5 soil, where growth was significantly reduced (Table 1 and Fig. 2c and d). AOB growth was not detected in either unfertilised soil, in the presence or absence of simvastatin. However, AOB growth was significant in urea-supplemented soils and, importantly, was significantly greater in microcosms amended with simvastatin (Fig. 2e and f). Comammox growth was not detected under any conditions in either soil (Table 1 and Fig. S4).

DNA-SIP confirmed selective inhibition of AOA growth. Autotrophic AOA growth was detected in all soils in the absence of simvastatin (Fig. 3), but was not detected in microcosms amended with simvastatin, with the exception of urea-amended pH 4.5 soil, in which simvastatin supplementation significantly reduced the relative abundance of ^{13}C-labelled AOA from 10.2% to 1.5% (Fig. 3). Integration of these percentages with AOA total abundance indicated that some autotrophic growth occurred in the absence of simvastatin in both soils (Table 1). In both soils, autotrophic growth of AOB was not decreased or inhibited by simvastatin addition. DNA-SIP provided evidence for AOA growth in both unamended and urea-amended pH 4.5 soil, and growth of AOB in unamended simvastatin-treated soil (Fig. 3 and Table 1). DNA-SIP also indicated potential autotrophic growth of comammox in urea fertilised pH 4.5 soil, following inhibition of AOA growth by simvastatin (Fig. 3 and Table 1).

The estimation of relative contributions of AOA and AOB, either by the autotrophic ammonia oxidiser abundance (^{13}C-labelled genes in heavy fractions) or by the total increase in ammonia oxidiser abundance after incubation for 21 days, indicated unaccounted sources of

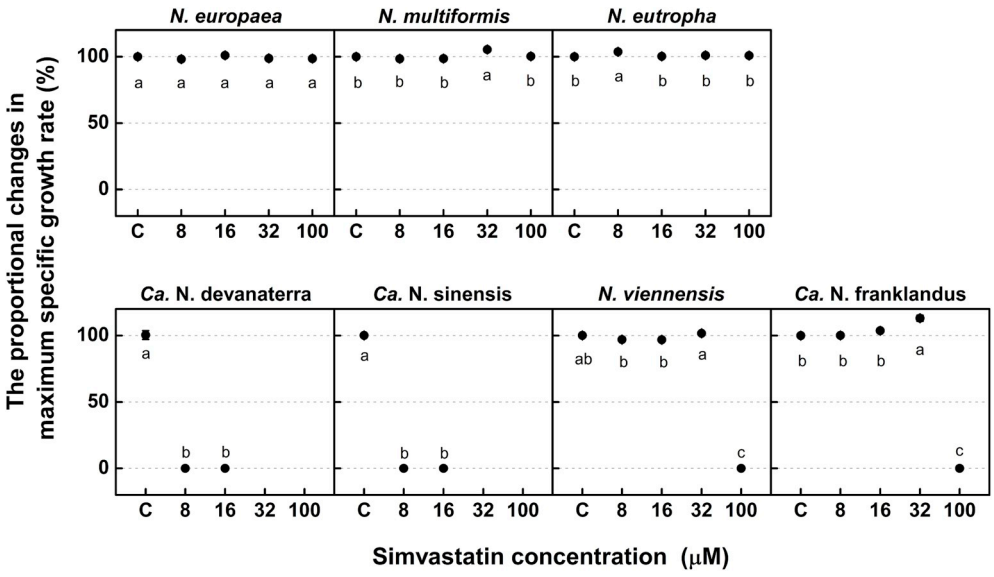

Fig. 1. The proportional changes in maximum specific growth rate of ammonia oxidiser cultures without inhibitor (C) or in the presence of simvastatin at concentrations of 8, 16, 32 and 100 μM. Activity of *Ca.* N. devanaterra and *Ca.* N. sinensis was not detectable at 8 and 16 μM of simvastatin and higher concentrations were not investigated. Error bars represent standard errors of means from triplicate cultures and the different letters in the graphs indicate significant differences ($p \leq 0.05$) within each plot.

nitrification in low NH_4^+ soils after inhibition of AOA growth (Fig. S5), suggesting growth of other ammonia oxidisers.

4. Discussion

This study is the first to investigate the inhibitory effect of simvastatin on ammonia oxidisers and provided evidence for selective inhibition of AOA growth in all pure cultures tested at varying concentrations that do not affect AOB growth (Fig. 1). This is consistent with previous inhibition by statins of many non-AO archaeal isolates (Cabrera et al., 1986; Gottlieb et al., 2016; Lam and Doolittle, 1992; Matsumi et al., 2007; Wendoloski et al., 2001; Zheng et al., 2012). The minimum inhibitory concentration (MIC) of simvastatin for the two acidophilic Thaumarchaeota (8 μM) was similar to that for the Euryarchaeota

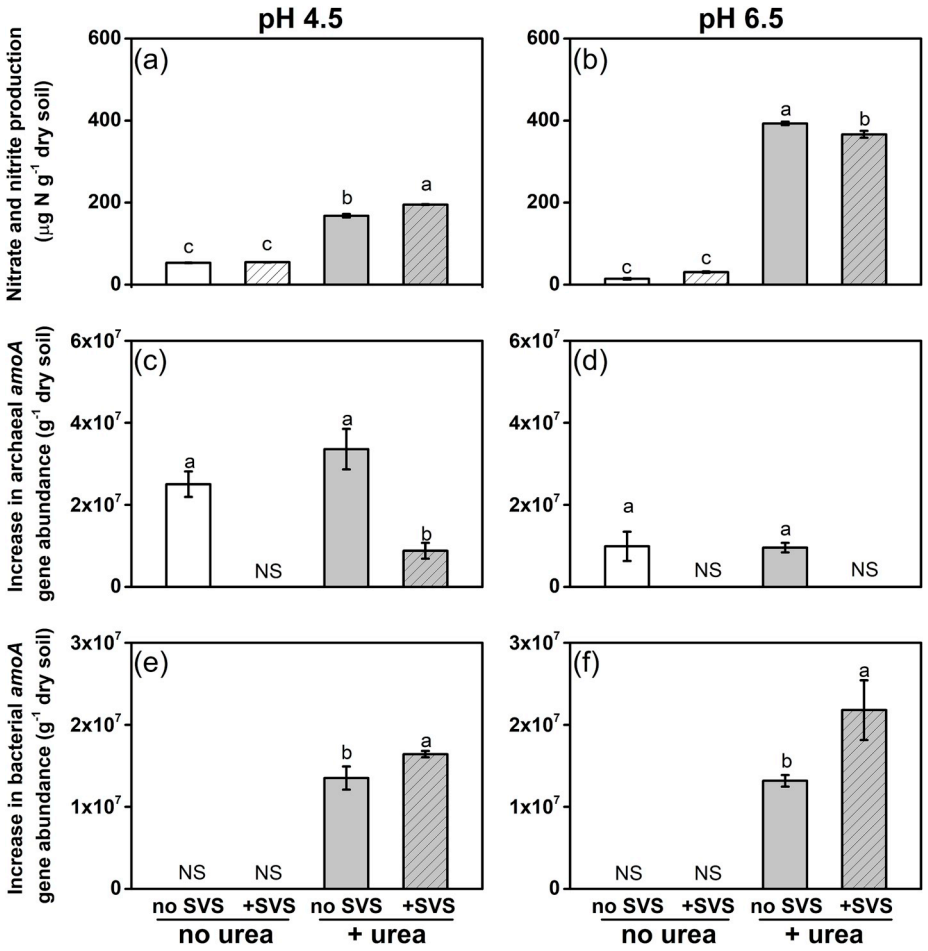

Fig. 2. Increases in nitrite plus nitrate (NO_x^-) concentration (a–b) and archaeal (c–d) and bacterial (e–f) *amoA* gene abundances after incubation of microcosms containing soils of pH 4.5 and 6.5 for 21 days. "no SVS" and "+SVS" refer to incubation of soil microcosms in the absence and presence of simvastatin, respectively, while "no urea" and "+ urea" refer to soils without and with urea fertilisation, respectively. Error bars represent standard errors of means from triplicate microcosms, with different letters above bars indicating a significant difference ($p \leq 0.05$) within each plot. "NS" refers to no significant increase after microcosm incubation.

Table 1
Estimated abundance of growing AOA, AOB and comammox (*amoA* genes g^{-1} dry soil). The abundance was either calculated as the increased abundance of total AOA, AOB or comammox *amoA* genes after incubation (qPCR) or estimated as the abundance of ^{13}C-labelled *amoA* genes in heavy fraction (SIP). "no SVS" and "+SVS" refer to incubation of soil microcosms in the absence and presence of simvastatin, respectively, while "no urea" and "+ urea" refer to soils without and with urea fertilisation, respectively. "NS" indicates absence of significant increase. Symbol * indicates significant difference between "no SVS" and "+SVS" treatments based on *t*-test statistics.

	AOA		AOB		comammox	
	qPCR	SIP	qPCR	SIP	qPCR	SIP
pH **4.5 (no urea)**						
no SVS	*2.5 × 10^7	*3.9 × 10^6	NS	*8.1 × 10^4	NS	NS
+SVS	NS	NS	NS	*1.7 × 10^5	NS	NS
pH **4.5 (+ urea)**						
no SVS	*3.4 × 10^7	*6.2 × 10^6	*1.4 × 10^7	1.2 × 10^7	NS	NS
+SVS	*8.8 × 10^6	*4.7 × 10^5	*1.6 × 10^7	1.4 × 10^7	NS	*2.7 × 10^4
pH **6.5 (no urea)**						
no SVS	*9.9 × 10^6	*8.6 × 10^6	NS	NS	NS	NS
+SVS	NS	NS	NS	*2.4 × 10^5	NS	NS
pH **6.5 (+ urea)**						
no SVS	*9.6 × 10^6	*2.3 × 10^6	*1.3 × 10^7	1.2 × 10^7	NS	NS
+SVS	NS	NS	*2.2 × 10^7	1.7 × 10^7	NS	NS

Thermococcus kodakaraensis (5 μM) (Matsumi et al., 2007), while the MIC observed for the two neutrophilic thaumarchaeotal strains tested (100 μM) was much higher than the highest reported archaeal inhibitory concentration for a wild-type archaeal strain (16 μM for the crenarchaeotan *Sulfolobus islandicus*, Zheng et al., 2012). The reasons for different sensitivities to simvastatin in AOA strains are not known, but may be due to differences in membrane composition, as simvastatin inhibits the rate-limiting enzyme of the pathways producing the main component (isoprenoid) of the archaeal lipid layer (Delmotte and Delmotte-Plaquee, 1953; Sirtori, 2014). All AOA membranes studied contain a characteristic glycerol dialkyl glycerol tetraether lipid, crenarchaeol, that is unique to Thaumarchaeota (Elling et al., 2017; Schouten et al., 2013), but the proportion of crenarchaeol is much higher in all tested neutrophilic AOA (including *N. viennensis*) than the acidophilic *Ca.* Nitrosotalea devanaterra strains (Lehtovirta-Morley et al., 2016b). This suggests that the majority of thaumarchaeotal genera (except *Ca.* Nitrosotalea) would be inhibited at similarly high concentrations (>100 μM), considering the phylogenetic divergence of the *Ca.* Nitrosotalea genus (Gubry-Rangin et al., 2015; Macqueen and Gubry-Rangin, 2016). However, additional screening of other AOA is required to generalise this finding. The mechanism of action of simvastatin on AOA is different from that of previous inhibitors of ammonia oxidation in AOA and AOB, inhibiting membrane synthesis rather than using a competitive substrate (CO, methane and n-alkynes (McCarty, 1999; Ruser and Schulz, 2015; Taylor et al., 2013)) to inhibit oxidation of ammonia to hydroxylamine, deactivating the AMO enzyme (DCD, DMPP and ATU (McCarty, 1999; Ruser and Schulz, 2015)), or preventing the conversion of hydroxylamine to nitrite by inhibiting the hydroxylamine dehydrogenase or acting as a nitric oxide scavenger (PTIO, caffeic acid and methylene blue hydrate (Sauder et al., 2016)).

Autotrophic growth of AOA was detected in all soil microcosms in the absence of simvastatin irrespective of NH_4^+ concentration (Fig. 3), demonstrating that growth of AOA in soil is not inhibited by high ammonium concentration (>100 μg N g^{-1}) and supporting previous physiological and ecological studies on high NH_4^+ tolerance of AOA (Hink et al., 2017b, 2018; Jung et al., 2016; Lehtovirta-Morley et al., 2016a; Sauder et al., 2017). Urea amendment increased growth of both AOA and AOB (Fig. 3). AOB dominated autotrophic ammonia oxidation in soils with high NH_4^+ concentrations, while AOA appeared to be the more active ammonia oxidisers in soils with low NH_4^+ concentrations (Fig. 3). These results collectively support previous findings (Hink et al., 2018), suggesting that niche differentiation is not due to inability of AOA or AOB to grow under high and low concentrations, respectively, but rather to differences in the ability of AOA and AOB to compete for NH_4^+ within the soil.

Further support for this proposal comes from microcosms in which ammonia was supplied through mineralisation of native soil organic nitrogen. In the absence of simvastatin, this favoured growth of AOA, which is consistent with previous studies (Di et al., 2010; Hink et al., 2018; Levičnik-Höfferle et al., 2012; Stopnišek et al., 2010; Verhamme et al., 2011). This has been explained by resultant lower ammonium concentration and higher ammonia substrate affinity of AOA, as indicated by early studies of cultivated isolates (Prosser and Nicol, 2012), but more recent studies indicate that cultivated representatives of soil AOA and AOB have similar K_m values (Hink et al., 2017a, 2018; Kits et al., 2017) and direct evidence for niche specialisation through differences in high NH_3 affinity is therefore lacking. However, this study suggests that preferential growth of AOA could be due to higher NH_3 affinity, but estimation of ammonia affinity on a larger range of AOA strains is still required, in particular for the strains thriving in acidic soils such as those affiliated to the *Nitrosotalea* lineage. Although there was no detectable increase in AOB abundance in these unamended soils, some autotrophic AOB growth was demonstrated by DNA-SIP in pH 4.5 but not pH 6.5 soil, demonstrating that DNA-SIP provided greater sensitivity than changes in *amoA* abundance when determining AO growth. Interestingly, DNA-SIP demonstrated that growth of AOB was higher in each unamended soil when AOA were inhibited by simvastatin. Thus, when relieved of competition from AOA, AOB but not comammox growth was significantly stimulated and nitrification rate was unaltered at low ammonium concentration, indicating that low substrate affinity was a potential limiting factor for AOB but not comammox growth. Inhibition of AOA also stimulated AOB growth in urea-amended soils where NH_4^+ concentration was high (Fig. 2e and f), while AOA activity in urea-amended soils in the absence of simvastatin (Fig. 3) provided further evidence for the activity of, and competition between AOA and AOB at both high and low ammonium concentration.

Surprisingly, DNA-SIP also provided evidence for AOB growth in unfertilised pH 4.5 soil even without inhibition of AOA (Fig. 3 and Table 1), which was significantly stimulated following a urea fertilisation, indicating physiological adaptation of AOB to grow at low pH. Analysis of the betaproteobacterial *Nitrosospira amoA* genes present in this soil showed that a single phylogenetic cluster was highly abundant (Aigle et al., 2019) and strains affiliated to this phylogenetic cluster have been isolated from acidic soils, including *Nitrosospira* sp. III7, O13, O4 and AHB1 (Aakra et al., 1999; De Boer and Laanbroek, 1989; Jiang and Bakken, 1999). In addition, a survey of 48 UK soils over a range of pH (3.5–9.0) showed that AOB phylotypes within this cluster were frequently detected in low pH soils (Aigle et al., 2019; Laverman et al., 2001; Nugroho et al., 2007). Therefore, this study provides evidence for AOB-mediated ammonia oxidation in acidic terrestrial ecosystems, possibly through pH-independent urea hydrolysis (Burton and Prosser, 2001).

Comammox were detected in both soils but did not increase in abundance during incubation at either high or low NH_4^+ concentration, and the inhibition of AOA stimulated primarily autotrophic growth of AOB, suggesting that competition for NH_4^+ is not the most critical factor in differentiating comammox from other ammonia oxidisers in soils. Comammox *amoA* was 43-fold more abundant in pH 6.5 than in pH 4.5 soil and their abundance was lower than that of AOA and either similar

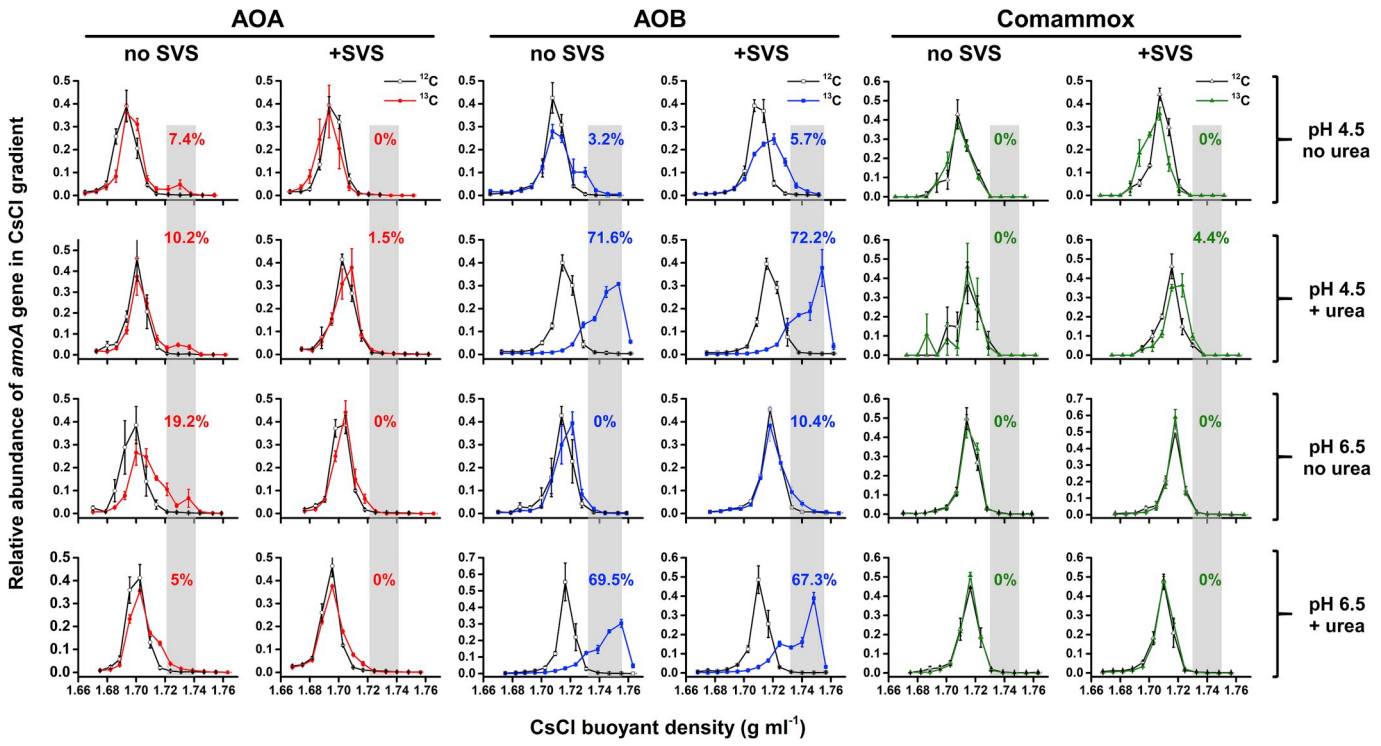

Fig. 3. Distribution of the relative abundance of archaeal, bacterial and comammox *amoA* genes in CsCl gradients of DNA for ^{13}C–CO_2- and ^{12}C–CO_2-treated microcosms. "no SVS" and "+SVS" refer to incubation of soil microcosms in the absence and presence of simvastatin, respectively, while "no urea" and "+ urea" refer to soils without and with urea fertilisation, respectively. The plotted values are the relative abundances of archaeal, bacterial or comammox *amoA* genes in each fraction as a proportion of total abundance across the gradient. Vertical error bars represent standard errors of the relative abundances from triplicate microcosms. Horizontal error bars represent standard errors of buoyant density of the same order fraction from triplicate microcosms. The shaded region represents the 'heavy DNA' CsCl fractions, enriching ^{13}C-enriched ammonia oxidiser DNA, at buoyant densities in the ranges of 1.721–1.739, 1.733–1.755 and 1.730–1.750 g ml^{-1} for AOA, AOB and comammox, respectively. The percentage within each grey bar indicates the relative abundance of ^{13}C-labelled *amoA* genes for each incubated soil.

to or lower than AOB abundance in pH 4.5 soil (Fig. S4). In pH 6.5 soil, comammox abundance was in the same order of magnitude as AOA, and either similar to or greater than that of AOB in the urea-amended and unfertilised soils, respectively. This is in agreement with growth pH preference (pH 7.0–7.8) of known cultivated comammox strains (Daims et al., 2015; van Kessel et al., 2015) and suggests that soil pH might be an important factor controlling comammox populations (Hu and He, 2017). Nonetheless, potential growth of comammox occurred in pH 4.5 soil following urea fertilisation and selective inhibition of AOA (Fig. 3 and Table 1), suggesting existence of acidophilic or acid-tolerant comammox. Further identification of these comammox phylotypes will be required, but testing of the primers specifically targeting either clade A or clade B comammox *amoA* genes (Pjevac et al., 2017) resulted in too many non-specific PCR products in both pH soils for qPCR analysis.

While these results indicated that simvastatin is an efficient inhibitor of AOA in soil, its low water solubility might limit its application in some environments. High concentrations can be achieved by dissolution in organic solvents such as DMSO, as used here, or ethanol for use in liquid cultures without significant toxicity to ammonia oxidisers or other microbes, facilitating studies in liquid culture or aquatic environments. However, variation in sensitivity of different ammonia oxidiser phylotypes to nitrification inhibitors (Shen et al., 2013) and the complex nature of soils with varying ammonia oxidiser abundance and community composition necessitate preliminary testing before use in new systems. Similarly, concentrations required for inhibition of nitrification in environmental samples are often at least 10-fold greater than those inhibiting laboratory cultures of ammonia oxidisers, e.g. ATU and acetylene (Martens-Habbena et al., 2015; Offre et al., 2009). Such high concentrations of simvastatin may not be achievable in soil solution without use of potentially toxic levels of DMSO and, in this study, required direct mixing of solid simvastatin with soil (without preliminary dissolution). This strategy was effective in inhibiting AOA growth in soils and might be explained by the lipophilic nature of simvastatin which can be directly absorbed through cell membrane. However, preliminary testing of both additional pure cultures of AOA and AOB and of new experimental systems is encouraged to determine optimal concentrations and delivery mechanisms.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2019.107673.

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