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Experimental testing of hypotheses for temperature- and pH-based niche specialization of ammonia oxidizing archaea and bacteria

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Summary

Investigation of niche specialization in microbial communities is important in assessing consequences of environmental change for ecosystem processes. Ammonia oxidizing bacteria (AOB) and archaea (AOA) present a convenient model for studying niche specialization. They coexist in most soils and effects of soil characteristics on their relative abundances have been studied extensively. This study integrated published information on the influence of temperature and pH on AOB and AOA into several hypotheses, generating predictions that were tested in soil microcosms. The influence of perturbations in temperature was determined in pH 4.5, 6 and 7.5 soils and perturbations in pH were investigated at 15°C, 25°C and 35°C. AO activities were determined by analysing changes in *amoA* gene and transcript abundances, stable isotope probing and nitrate production. Experimental data supported major predictions of the effects of temperature and pH, but with several significant discrepancies, some of which may have resulted from experimental limitations. The study also provided evidence for unpredicted activity of AOB in pH 4.5 soil. Other discrepancies highlighted important deficiencies in current knowledge, particularly lack of consideration of niche overlap and the need to consider combinations of factors when assessing the influence of environmental change on microbial communities and their activities.

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Introduction

Niche specialization and differentiation are well-established concepts in ecology and play roles in establishment of plant and animal communities and species coexistence (Harper *et al*., 1961; Schoener, 1974). Although frequently proposed to be important in supporting microbial diversity and determining assembly and composition of microbial communities (Bardgett *et al*., 2002; Nemergut *et al*., 2013; Prosser, 2012), these concepts are difficult to demonstrate in natural environments. Microbial communities are complex and information on physiological characteristics of ecological significance is limited, particularly when communities are characterized by analysis of single genes, such as 16S rRNA genes. Consequently, it is often difficult to identify environmental factors that preferentially select for different phylogenetic groups.

Archaeal and bacterial ammonia oxidisers (AOA and AOB) have been extensively studied utilizing a range of approaches and are a useful model for investigation of niche specialization in microbial communities (Gubry-Rangin *et al*., 2011; Prosser and Nicol, 2012). They coexist in most terrestrial ecosystems studied and perform the same ecosystem function, oxidation of soil ammonia, which has significant impacts on soil biogeochemistry, nitrate pollution, fertilizer loss, nitrous oxide production and control of soil nitrification rate (Hink *et al*., 2018; Prosser and Nicol, 2012). Laboratory cultures of both groups grow chemolithoautotrophically, fixing carbon dioxide and gaining energy through oxidation of ammonia to nitrite. Both groups possess related genes encoding ammonia monooxygenase (AMO), which catalyses the conversion of ammonia to hydroxylamine, and *amoA*,

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encoding subunit A, is the standard marker gene for ammonia oxidisers. Both AOA and betaproteobacterial AOB functional groups are monophyletic lineages that do not contain non-ammonia oxidisers and *amoA* analysis therefore targets a functional group. Physiological characteristics including substrate affinity for ammonia (Kits *et al*., 2017) and maximum specific growth rate ranges (Prosser and Nicol, 2012) of newly enriched or isolated cultures of AOA and AOB from soil are similar. Yet, despite their similarities, AOA and AOB belong to different domains, the Archaea and the Bacteria, and major differences in fundamental aspects of their cell biology and physiology have been seen as potential reasons for niche specialization. For example, they differ in pathways for CO_2 fixation (Könneke *et al*., 2014), cell wall structure (Albers and Meyer, 2011) and pathways for oxidation of ammonia (Kozlowski *et al*., 2016).

Relative abundances, growth rates and activities of AOA and AOB vary between soils and suggest niche specialization but evidence from correlation-based studies suggest only three major differentiating environmental factors (Fig. 1A): (i) activity of AOA is frequently greater in soils with pH < 6, which may be explained by the existence of obligately acidophilic AOA, but not AOB (Gubry-Rangin *et al*., 2010; Zhang *et al*., 2012); (ii) there is evidence, in some soils, for preferential activity of AOA for ammonia supplied through mineralisation and of AOB for amendment with high levels of inorganic N (Di *et al*., 2010; Sterngren *et al*., 2015; Verhamme *et al*., 2011) and (iii) relative contributions of AOA and AOB to nitrification are associated with temperature, with AOA communities possessing mean temperature optima ≥ 10°C higher than AOB communities (Ouyang *et al*., 2017; Taylor *et al*., 2017). Possible explanations for ammonia-supply selection include greater ammonia affinity and ammonia sensitivity of AOA, but physiological support for these explanations is controversial (see Hink *et al*., 2017, 2018; Kits *et al*., 2017; Lehtovirta-Morley *et al*., 2016a). While there is evidence that AOA and AOB have competitive advantages under conditions of low and high ammonia supply, respectively, this does not appear to be due to selective inhibition of AOA at high ammonia concentration. AOA are active at high ammonia concentration if AOB are inhibited (Hink *et al*., 2018), and AOB are active at low ammonia supply if AOA are inhibited (Zhao *et al*., 2020). A recent study also provides evidence for greater tolerance of water stress in soil by AOB than by AOA (Bello *et al*., 2019) and greater activity of AOA at low oxygen concentration (French *et al*., 2012; Qin *et al*., 2017).

Development of hypotheses and predictions

A hypothesis-based approach was used to investigate prokaryote niche partitioning, using ammonia oxidisers as models, determining the differential responses of AOA and AOB to perturbations of two potentially important environmental characteristics, soil pH and temperature. Where possible, hypotheses for physiological differences between AOA and AOB were based on evidence from prior cultivation-based or controlled soil microcosm studies. Table 1 summarizes these hypotheses and the associated citations supporting them. The hypotheses were used to predict activities of AOA and AOB both in the presence (Fig. 1A) and absence (Fig. 1B and C) of competition. This conceptual framework illustrates AO activities as functions of soil pH and temperature in established (non-perturbed) ecosystems. The hypotheses were also used to predict responses of AOA and AOB abundances to soil pH and temperature perturbations and consequent relative activities of AOA and AOB. The ensuing ecosystem function (nitrate production rate) was

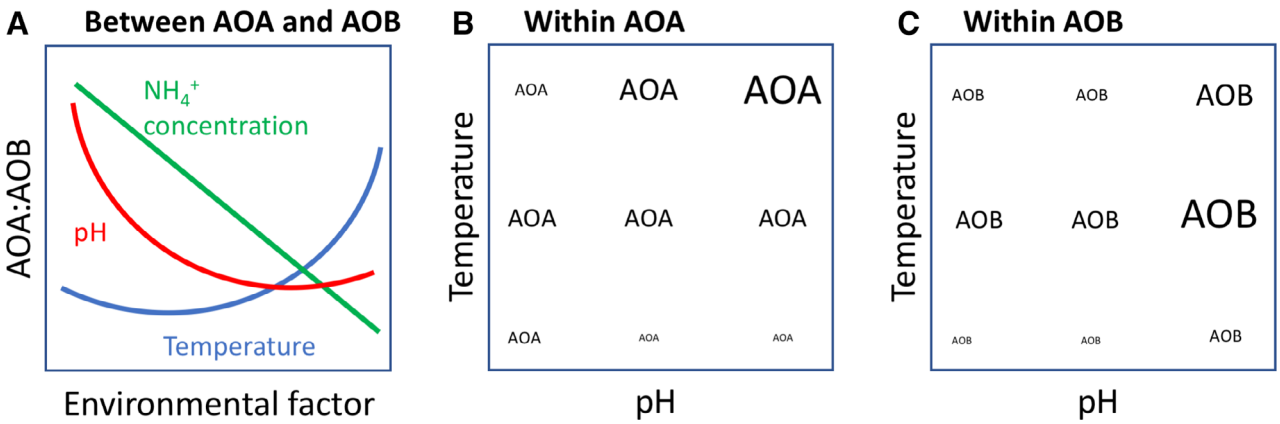

Fig 1. A conceptual model of the influence of several factors on the relative abundance of AOA and AOB in the presence of competition (A) or on their independent absolute abundance within each group (B, C). Font size in panels B and C represents the assumed nitrifier activity in the absence of competition from the other group.

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Table 1. Hypotheses regarding the influence of temperature or pH on AOA and AOB growth and activity, with supporting citations, and semi-quantitative predictions of relative changes.

Factor	Hypotheses	Predicted activities of AOA and AOB
Temperature	H1 AOA and AOB activities increase with temperature (Prosser and Nicol, 2012) but AOB optimal temperature for activity is usually ≤ 30°C (Jiang and Bakken, 1999)	P1a AOA: 15°C < 25°C < 35°C P1b AOB: 15°C < 25°C > 35°C
pH	H2 AOA are active over a wide pH range (Gubry-Rangin *et al*., 2015; Prosser and Nicol, 2012) H3 AOB activity increases with pH (Prosser and Nicol, 2012)	P2 AOA: pH 4.5 = 6 = 7.5 P3 AOB: pH 4.5 < < 6 ≤ 7.5
pH and temperature	H4 Neutrophilic AOA have higher temperature optima than acidophilic AOA (Gubry-Rangin *et al*., 2017)	P4 AOA: at 35°C, pH 4.5 < 6 < 7.5 AOA: at 15°C and 25°C, pH 4.5 > 6 > 7.5
Competition	H5 AOA outcompete AOB when the rate of ammonium supply is low and *vice-versa* (Leininger *et al*., 2006; Verhamme *et al*., 2011) H6 Either AOA or AOB can be active if environmental change reduces activity of their competitor (Hink *et al*., 2018)	P5 AOA:AOB activity ratio decreases as ammonium supply increases P6 AOA or AOB active if competitor activity is low
Community adaptation	H7 Adaptation of AOA to low pH is slower than that to neutral or alkaline pH (Gubry-Rangin *et al*., 2015; Herbold *et al*., 2017; Lehtovirta-Morley *et al*., 2016b; Vico Oton *et al*., 2016)	P7 After pH change, AOA pH 6 > 7.5 > 4.5 at all temperatures

Additional hypotheses and predictions are also presented for the effects of ammonia supply and community adaptation temporal response, both required to fully predict the activity changes.

assumed to be proportional to the total AO activity. The resultant predictions were then tested experimentally in soil microcosms in which both AOA and AOB were present and potentially active (Gubry-Rangin *et al*., 2010; Lehtovirta-Morley *et al*., 2011; Nicol *et al*., 2008; Verhamme *et al*., 2011; Zhang *et al*., 2010). AOA and AOB growth and activity were assessed experimentally using three techniques: temporal changes in abundance of respective *amoA* genes (a direct measure of growth, with relatively large changes in abundance required for detection); temporal changes in *amoA* transcript abundance (providing a measure of transcriptional activity relating to potential ammonia oxidizing activity, but with greater immediacy and sensitivity); and stable isotope probing (SIP) of *amoA* genes in ^{13}C-DNA following incubation with ^{13}C-CO_2 (a direct measure of carbon assimilation and DNA replication in autotrophic AO). These techniques assessed a combination of growth and/or activity, hereafter referred to as 'activity' for simplicity.

Results

The hypotheses (H1–H7) and associated predictions (P1–P7) (Table 1) were tested by investigating changes in AOA and AOB *amoA* gene abundance, *amoA* transcript abundance and ^{13}C-CO_2 assimilation into *amoA* genes (all referred to as 'activity') following changes in temperature while keeping pH constant, or changes in pH while maintaining constant temperature. Detailed statistical analysis of the effects of perturbations on each measure of activity and nitrification rate and temporal effects on abundance and transcriptional activity are presented in Supplementary Tables S1–S6. Predicted changes for every perturbation are presented in Table 2 (temperature perturbation), Table 3 (pH perturbation) and Table 4 (comparison of conditions common to both experiments) as effects on AOA and AOB activities (column 1 and 2), relative activities of AOA and AOB (column 3) and net nitrate production (column 4). Subsequent columns present equivalent experimental data on activities and relative activities, for each activity measure, and on net nitrate production. Further details of data presentation are given in the legend to Table 2.

At 15° and 25°, NH_4^+ concentration was in the range 1–4 μg NH_4^+–N g^{-1} and NO_3^- concentration increased to significantly higher values, indicating active nitrification in all soils resulting from mineralisation of organic nitrogen (Supplementary Tables S5 and S7). At 35°, both NH_4^+ and NO_3^- concentrations increased, again indicating active nitrification but with a greater rate of mineralisation leading to accumulation of NH_4^+ to concentrations in the range 16–72 μg NH_4^+–N g^{-1}.

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Table 2. Temperature perturbation.

Perturbation in temperature	Predicted change in				Gene abundance			Transcript abundance			C-assimilation			Nitrate
	AOA	AOB	AOA:AOB	Nitrate	AOA	AOB	AOA:AOB	AOA	AOB	AOA:AOB	AOA	AOB	AOA:AOB	
A							pH 4.5							
25°C → 15°C	AOA	AOB	AOA:AOB	N	AOA^{0}	AOB^{0}	AOA:AOB	AOA^{0}	AOB^{0}	AOA:AOB	AOA	AOB	AOA:AOB	N
25°C → 25°C	AOA	AOB	AOA:AOB	N	AOA^{0}	AOB^{-1}	AOA:AOB	AOA^{0}	AOB^{-1}	AOA:AOB	AOA	AOB	AOA:AOB	N
25°C → 35°C	AOA	AOB	AOA:AOB	N	AOA^{-1}	AOB^{-1}	AOA:AOB	AOA^{0}	AOB^{-1}	AOA:AOB	AOA	AOB	AOA:AOB	N
B							pH 6							
25°C → 15°C	AOA	AOB	AOA:AOB	N	AOA^{0}	AOB^{0}	AOA:AOB	AOA^{+1}	AOB^{-1}	AOA:AOB	AOA^{+1}	AOB	AOA:AOB	N
25°C → 25°C	AOA	AOB	AOA:AOB	N	AOA^{+1}	AOB^{0}	AOA:AOB	AOA^{+1}	AOB^{-1}	AOA:AOB	AOA^{+1}	AOB	AOA:AOB	N
25°C → 35°C	AOA	AOB	AOA:AOB	N	AOA^{+1}	AOB^{0}	AOA:AOB	AOA^{+1}	AOB^{-1}	AOA:AOB	AOA^{+1}	AOB	AOA:AOB	N
C							pH 7.5							
25°C → 15°C	AOA	AOB	AOA:AOB	N	AOA^{+1}	AOB^{+1}	AOA:AOB	AOA^{0}_{δ}	AOB^{-1}	AOA:AOB	AOA^{+1}	AOB	AOA:AOB	N
25°C → 25°C	AOA	AOB	AOA:AOB	N	AOA^{+1}	AOB^{+1}_{β}	AOA:AOB	AOA^{+1}	AOB^{0}	AOA:AOB	AOA^{+1}	AOB	AOA:AOB	N
25°C → 35°C	AOA	AOB	AOA:AOB	N	AOA^{+1}_{α}	AOB^{-1}	AOA:AOB	AOA^{+1}	AOB^{+1}	AOA:AOB	AOA	AOB	AOA:AOB	N

α. In pH 7.5 soil, AOA gene abundance at 35°C = AOA gene abundance at 25°C and 15°C, but AOA gene abundance at 25°C > gene abundance at 15°C.
β. In pH 7.5 soil, AOB gene abundance at 25°C = AOA gene abundance at 35°C and 15°C, but AOA gene abundance at 15°C > gene abundance at 35°C.
δ. In pH 7.5 soil, AOA transcript abundance at 15°C = AOA transcript abundance at 25°C and 35°C while AOA transcript abundance at 35°C > transcript abundance at 25°C.
Predicted and observed changes in AOA and AOB activity following perturbations in temperature at constant pH. The left column describes the nature of the perturbation, in which three different soils of pH 4.5 (A), 6 (B) or 7.5 (C) were pre-incubated at 25°C and then incubated at either 15°, 25° or 35°C. The columns highlighted in grey present predicted proportional changes in AOA activity (AOA), AOB activity (AOB), activity of AOA versus AOB (AOA:AOB) and net nitrate production (N), with font sizes indicating predicted differences within each column and for each soil pH. The remaining columns indicate the experimentally determined proportional changes in activity, measured as changes in gene abundance, transcript abundance and C-assimilation. Statistically significant differences in proportional activity following perturbation in temperature are represented as differences in font size (within each column and for each soil pH). In addition, superscripts indicate a statistically significant temporal increase (+1), decrease (−1) or no change (0) in *amoA* gene or transcript abundance (Supplementary Tables S3.1 and S3.2) or C assimilation (Supplementary Table S4) ($p \leq 0.1$) following perturbation. (Note that these temporal changes in gene and transcript abundance are based on actual rather than proportional values, regardless of font size.) For example, in panel A, the activity (proportional increase in gene abundance) of AOA is predicted to be greater at 25°C than 15°C, and lowest at 35°C, while AOB are predicted to be more active at 25°C than 15°C and 35°C. AOA activity is predicted to be greater than that of AOB at each temperature and nitrate production is predicted to follow AOA activity (25°C > 15°C > 35°C). In terms of proportional changes in *amoA* gene abundance, experimentally determined AOA and AOB activities were greater at 15°C and 25°C than at 35°C, while AOA and AOB gene abundance was either stable (superscript 0) or decreased (superscript −1). AOA and AOB activities were similar at 15°C and 25°C, but AOA were more active than AOB at 35°C. In terms of transcript abundance, AOA, AOB and AOA:AOB activities did not differ with temperature and activity was either stable (superscript 0) or decreased (superscript −1). No C assimilation was observed at pH 4.5 (absence of superscript), and no significant differences were observed within AOA, within AOB or between AOA and AOB. Finally, nitrate production was greatest at 25°C and lowest at 35°C. Data on proportional activity, and statistical analyses, are presented in Supplementary tables for gene abundance: AOA and AOB (Supplementary Table S1.1) and AOA versus AOB (Supplementary Table S2.1); transcript abundance: AOA and AOB (Supplementary Table S1.2) and AOA versus AOB (Supplementary Table S2.2); C assimilation: AOA and AOB (Supplementary Table S1.3) and AOA versus AOB (Supplementary Table S2.3); and net nitrate production (Supplementary Table S5). Absolute values of gene and transcript abundance are presented in Supplementary Table 3 and NH_4^+ and NO_3^- concentrations are presented in Supplementary Table 6. [Color figure can be viewed at wileyonlinelibrary.com]

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Table 3. pH perturbation.

Perturbation in soil pH	Predicted change in				Gene abundance			Transcript abundance			C-assimilation			Nitrate
	AOA	AOB	AOA:AOB	Nitrate	AOA	AOB	AOA:AOB	AOA	AOB	AOA:AOB	AOA	AOB	AOA:AOB	
A							15°C							
pH 6 > pH 4.5	AOA	AOB	AOA:AOB	N	AOA^{-1}	AOB^{-1}	AOA:AOB	AOA^{0}	AOB^{0}	AOA:AOB	AOA	AOB^{+1}	AOA:AOB	N
pH 6 > pH 6	AOA	AOB	AOA:AOB	N	AOA^{0}	AOB^{0}	AOA:AOB	AOA^{0}	AOB^{0}	AOA:AOB	AOA^{+1}	AOB	AOA:AOB	N
pH 6 > pH 7.5	AOA	AOB	AOA:AOB	N	AOA^{+1}	AOB^{0}	AOA:AOB	AOA^{0} α	AOB^{+1}	AOA:AOB	AOA	AOB	AOA:AOB	N
B							25°C							
pH 6 > pH 4.5	AOA	AOB	AOA:AOB	N	AOA^{0}	AOB^{-1}	AOA:AOB	AOA^{0}	AOB^{-1}	AOA:AOB	AOA^{+1}	AOB^{+1}	AOA:AOB	N
pH 6 > pH 6	AOA	AOB	AOA:AOB	N	AOA^{+1}	AOB^{0}	AOA:AOB	AOA^{0}	AOB^{-1}	AOA:AOB	AOA^{+1}	AOB	AOA:AOB	N
pH 6 > pH 7.5	AOA	AOB	AOA:AOB	N	AOA^{0} β	AOB^{+1}	AOA:AOB	AOA^{+1}	AOB^{0}	AOA:AOB	AOA^{+1}	AOB	AOA:AOB	N
C							35°C							
pH 6 > pH 4.5	AOA	AOB	AOA:AOB	N	AOA^{-1}	AOB^{-1}	AOA:AOB	AOA^{-1}	AOB^{-1}	AOA:AOB	AOA	AOB	AOA:AOB	N
pH 6 > pH 6	AOA	AOB	AOA:AOB	N	AOA^{0}	AOB^{-1}	AOA:AOB	AOA^{+1}	AOB^{-1}	AOA:AOB	AOA^{+1}	AOB	AOA:AOB	N
pH 6 > pH 7.5	AOA	AOB	AOA:AOB	N	AOA^{0}	AOB^{0}	AOA:AOB	AOA^{0}	AOB^{+1}	AOA:AOB	AOA^{+1}	AOB^{+1}	AOA:AOB	N

α. At 15°C, AOA transcript abundance in pH 7.5 = AOA transcript abundance in pH 6 and pH 4.5 soils, but AOA transcript abundance in pH 6 > transcript abundance in pH 4.5 soil.
β. At 25°C, AOA gene abundance in pH 7.5 = AOA gene abundance in pH 4.5 and pH 6, but AOA gene abundance in pH 6 > gene abundance in pH 4.5 soil.
Predicted and observed changes in AOA and AOB activity following perturbations in pH at constant temperature. The left column describes the nature of the perturbation, in which pH 6 soil was pre-incubated at 15°C, 25°C or 35°C and then incubated at the same temperature (15°C, A; 25°C, B or 35°C, C) after adjusting the soil pH to 4.5 or 7.5 or maintaining it at pH 6. See Table 2 legend for description of experimental data. [Color figure can be viewed at wileyonlinelibrary.com]

Perturbations in temperature at each constant soil pH

Incubation temperature was perturbed in soils at each soil pH following pre-incubation at 25°C. For the pH 4.5 soil (Table 2A), AOB activity was predicted to be generally low at each temperature, due to the effects of both low pH (P3) and low ammonium availability (P5) with an optimal activity at 25°C (P1b), while AOA activity was predicted to be greatest at 25°C (P1a, moderated by P4). Gene and transcript abundances for both AOB and AOA remained either constant (no increase, superscript 0) or declined (superscript −1) at the three investigated temperatures in pH 4.5 soil. The former condition suggests a balance between growing/dying and/or active/inactive cells when measuring gene or transcript abundance, respectively, while the latter suggests overall cell death and/or inactivation, respectively. Decreasing abundance at 35°C for these two measures of activity (gene and transcript) suggest important cell death at this temperature, with some evidence that decreases were greatest by the first sample point (1 day) (Supplementary Table S3). Lack of activity is further supported by the C assimilation data, with no detectable assimilation (no superscript) at pH 4.5 at any investigated temperature, even if some C assimilation was observed at 25°C (but without solid replication; see Supplementary Fig. S2A). There was also no evidence of AOB activity with a decrease or maintenance of AOB gene and transcript abundances over time under most conditions, and there was no detectable C assimilation by AOB. Under these conditions, it was therefore not possible to test predictions, although a longer incubation period may have led to detection of activity. While significant changes in activity could not be detected through changes in gene or transcript abundance or C assimilation, net nitrate production followed predictions, and was greatest at 25°C.

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Table 4. Common conditions.

(A) Temperature *vs* pH perturbations at pH 4.5		Predicted change in				Gene abundance			Transcript abundance			C-assimilation			Nitrate
		AOA	AOB	AOA:AOB	Nitrate	AOA	AOB	AOA:AOB	AOA	AOB	AOA:AOB	AOA	AOB	AOA:AOB	
15°C pH 4.5	T	AOA	AOB	AOA:AOB	N	AOA^{0}	AOB^{0}	AOA:AOB	AOA^{0}	AOB^{0}	AOA:AOB	AOA	AOB	AOA:AOB	N
	pH	AOA	AOB	AOA:AOB	N	AOA^{-1}	AOB^{-1}	AOA:AOB	AOA^{0}	AOB^{0}	AOA:AOB	AOA	AOB^{+1}	AOA:AOB	N
25°C pH 4.5	T	AOA	AOB	AOA:AOB	N	AOA^{0}	AOB^{-1}	AOA:AOB	AOA^{0}	AOB^{-1}	AOA:AOB	AOA	AOB	AOA:AOB	N
	pH	AOA	AOB	AOA:AOB	N	AOA^{0}	AOB^{-1}	AOA:AOB	AOA^{0}	AOB^{-1}	AOA:AOB	AOA^{+1}	AOB^{+1}	AOA:AOB	N
35°C pH 4.5	T	AOA	AOB	AOA:AOB	N	AOA^{-1}	AOB^{-1}	AOA:AOB	AOA^{0}	AOB^{-1}	AOA:AOB	AOA	AOB	AOA:AOB	N
	pH	AOA	AOB	AOA:AOB	N	AOA^{-1}	AOB^{-1}	AOA:AOB	AOA^{-1}	AOB^{-1}	AOA:AOB	AOA	AOB	AOA:AOB	N

(B) Temperature *vs* pH perturbations at pH 7.5		Predicted change in				Gene abundance			Transcript abundance			C-assimilation			Nitrate
		AOA	AOB	AOA:AOB	Nitrate	AOA	AOB	AOA:AOB	AOA	AOB	AOA:AOB	AOA	AOB	AOA:AOB	
15°C pH 7.5	T	AOA	AOB	AOA:AOB	N	AOA^{+1}	AOB^{+1}	AOA:AOB	AOA^{0}	AOB^{-1}	AOA:AOB	AOA^{+1}	AOB	AOA:AOB	N
	pH	AOA	AOB	AOA:AOB	N	AOA^{+1}	AOB^{0}	AOA:AOB	AOA^{0}	AOB^{+1}	AOA:AOB	AOA	AOB	AOA:AOB	N
25°C pH 7.5	T	AOA	AOB	AOA:AOB	N	AOA^{+1}	AOB^{+1}	AOA:AOB	AOA^{+1}	AOB^{0}	AOA:AOB	AOA^{+1}	AOB	AOA:AOB	N
	pH	AOA	AOB	AOA:AOB	N	AOA^{0}	AOB^{+1}	AOA:AOB	AOA^{+1}	AOB^{0}	AOA:AOB	AOA^{+1}	AOB	AOA:AOB	N
35°C pH 7.5	T	AOA	AOB	AOA:AOB	N	AOA^{+1}	AOB^{-1}	AOA:AOB	AOA^{+1}	AOB^{+1}	AOA:AOB	AOA	AOB	AOA:AOB	N
	pH	AOA	AOB	AOA:AOB	N	AOA^{0}	AOB^{0}	AOA:AOB	AOA^{0}	AOB^{+1}	AOA:AOB	AOA^{+1}	AOB^{+1}	AOA:AOB	N

Predicted and observed activities in soil at pH 4.5 (A) and pH 7.5 (B) and at three temperatures following temperature (T) or pH perturbations. See Table 2 legend for description of layout of experimental data, with Supplementary Table S6 for details of statistical analysis. [Color figure can be viewed at wileyonlinelibrary.com]

For pH 6 soil (Table 2B), AOA activity was expected to increase with incubation temperature (P1a), while AOB activity was predicted to be optimal at 25°C (P1b), especially due to high AOA competition at 35°C (P6). AOA gene abundance and C assimilation were greater at 25°C and 35°C than 15°C, without any significant difference between 25°C and 35°C, partially supporting P1a; however, AOA transcript abundance did not differ with temperature. There was no evidence of AOB activity, with either no change or a decrease in gene and transcript abundance over time and no detectable C-assimilation, resulting in the inability to test the prediction P1b. Absence of AOB activity at 25°C may have resulted from the competitive advantage of AOA at low ammonium supply rate (P5), and AOB transcript, but not gene, abundance decreased at all temperatures. At 35°C, ammonium produced by mineralisation accumulated, reaching > 40 μg NH_4^+–N g^{-1} soil, but any potential competitive advantage incurred for AOB was offset by the effects of high temperature on AOA and AOB (P1a, P1b). AOA activity was therefore greater than that of AOB in all these pH 6 microcosms, and was responsible for significant increases in nitrate production with temperature, as predicted.

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In pH 7.5 soil (Table 2C), the high rate of mineralisation at 35°C was predicted to reduce the competitive advantage of AOA (P5), increasing AOB activity, while the general effects of temperature on AOA and AOB activity were predicted to lead to greater AOA activity (P1a, P1b). The combined effect of these factors predicted an increase in AOA activity with temperature, optimal AOB activity at 25°C and relative dominance of AO activity by AOA at all temperatures. There was some evidence for increased AOA activity with temperature, with increases in gene abundance (only from 15°C to 25°C) and changes in transcript abundance (only from 25°C to 35°C), partially obeying the predictions. However, C assimilation by AOA was not significant at 35°C, possibly through unusual high variability between replicates of this treatment (Supplementary Fig. S2B). In addition, C assimilation was similar to that at the other two temperatures, despite predictions of higher activity at higher temperature (P1a). AOB gene and transcript abundances partially followed predictions, but were similar at 15°C and 25°C, respectively. In contrast, C assimilation in AOB was not detected at any temperature, again possibly through high variability between replicates (Supplementary Fig. S2B), as C assimilation was significantly higher at 35°C, refuting P1b in those conditions but supporting P5. In general, AOA activity was greater than that of AOB, as predicted, and changes in nitrate production were also as predicted.

Perturbations in pH at constant incubation temperature

pH 6 soil was preincubated at either 15°C, 25°C or 35°C. Incubation was then continued at each preincubation temperature with no pH manipulation or after changing soil pH to pH 4.5 or 7.5. Predictions of the effect of pH perturbation on AO activities considered the time required for adaptation to new conditions, assuming that this would be greater following reduction in pH to 4.5 than following an increase to pH 7.5 (P7). Therefore, AOA activity was predicted to be highest at pH 6 and lower following a pH change to pH 4.5 at all temperatures, with intermediate activity at pH 7.5. AOB activity was predicted to increase with pH at all temperatures (P3).

At 15°C (Table 3A), there was no AOA activity at pH 4.5, as predicted, while C assimilation only occurred at pH 6 despite detection of gene abundance changes at pH 7.5, providing partial support for H7. There was little evidence of AOB activity at 15°C, with significant temporal increases in transcript abundance at pH 7.5 and, surprisingly, C-assimilation at pH 4.5 (Supplementary Fig. S3). Gene abundance of both AOA and AOB declined temporally after reduction in pH to 4.5. These data did not agree with general predictions of greater activity of AOA at all pH values, but the low levels of activity make detailed testing of the hypotheses difficult and longer incubations may be required. Nonetheless, nitrification was detectable and net nitrate production followed predicted behaviour in that activity was lowest at pH 4.5, but there was no significant difference in production at pH 7.5 compared to pH 6.

At 25°C (Table 3B), AOA activity generally followed predictions. Gene and transcript abundance-based activity was not detectable at pH 4.5, presumably through slow adaptation to low pH, but C assimilation occurred. Gene and transcript abundances indicate similar AOA activity at pH 6 and 7.5 but C assimilation was greater at pH 6, as predicted. There was little evidence for AOB activity at pH 4.5 and 6, except for unpredicted C-assimilation at pH 4.5, as observed at 15°C (Supplementary Fig. S3), and evidence for a temporal decline in activity, particularly at pH 4.5. AOB transcriptional activity and C-assimilation were not detectable at pH 7.5, but gene abundance was greatest at pH 7.5, as predicted. Relative activities of AOA and AOB were as predicted at all pH values and nitrate production increased with pH as predicted.

At 35°C (Table 3C), AOA activity measured by all three methods was not detectable at pH 4.5, transcript abundance and C assimilation were observed at pH 6, and C assimilation only was detectable at pH 7.5. Despite no significant differences in C assimilation at pH 6 and pH 7.5, gene and transcript abundances were highest at pH 6, supporting partially the predicted optimal activity of AOA at pH 6 (P7). AOB activity was only detectable at pH 7.5 by changes in transcript abundance and C assimilation, supporting predicted increase in activity with pH, but longer incubation times may be required to detect any activity at other pH values. Greater activity of AOA than AOB was supported at pH 6 but not at pH 4.5 and pH 7.5, where results indicated similar or greater activity of AOB. There was evidence of a temporal decline in activity of both groups at pH 4.5 and of AOB at pH 6. Nitrate production was similar to that at 25°C, and increased with pH, as predicted.

Comparison of changes in the pH and temperature perturbation experiments

Hypotheses also generated predicted differences in AOA and AOB activity under the same final combinations of conditions in the temperature and pH perturbations experiments (pH versus temperature). AOA were predicted to be more active following perturbations in temperature than after a pH perturbation (prediction P7), while greater activity of AOB would occur following pH perturbations (predictions P6 and P7). These predictions, and consequent predictions for nitrate production, are presented alongside experimental data in Table 4 (see

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also Supplementary Table S6 for details of statistical analyses).

At pH 4.5 (Table 4A), there was no evidence of AOA activity except for C assimilation at 25°C in the pH perturbation experiment. Similarly, there was no evidence of AOB activity, except for the unpredicted C assimilation at 15°C and 25°C in the pH perturbation experiments. Nitrate production was greater after perturbations in temperature as predicted.

At pH 7.5 (Table 4B), AOA gene abundance was always greater following temperature perturbations, as predicted. Differences in transcript abundance varied with temperature and greater C assimilation following temperature perturbation was only observed at 15°C. At this pH, differences in AOB activity between the two experiments were rare, the exceptions being changes in gene abundance and transcript abundance at 15°C and C assimilation at 35°C. Nitrate was again greater after perturbation in temperature rather than pH, as predicted, except at 15°C, where there was no significant difference.

Discussion

The aim of this study was to test hypotheses regarding niche specialization and differentiation in microbial functional communities, using AOA and AOB as model organisms. Hypotheses were based on data from a range of previously published microcosm and field studies on niche specialization. Some involved long-term selection of natural communities under different conditions, while others provided information on short-term changes, but the majority considered changes in only one factor. The predictions generated by these hypotheses were tested in microcosms containing soil that had been exposed to long-term shifts in soil pH and relatively short-term pre-incubation at one temperature (25°C) or, for the pH 6 soil, at three temperatures (15°C, 25°C and 35°C). Changes in activity were then investigated during a relatively short-term incubation following changes in temperature or pH. The temperature perturbation experiment was performed on soils that had been exposed to long-term shifts in soil pH values and therefore assessed the versatility of these pH-adapted communities in responding to different temperatures. The pH perturbation experiment assessed the versatility of a single pH-adapted community (pH 6) to changes in soil pH at different temperatures (selecting for distinct communities). The speed and extent of response, and the ability to detect responses, will depend on the versatility of AOA and AOB, but also on the abundance of strains adapted to the new conditions and their growth rate(s). In addition, hypothesis construction highlighted niche overlap and the need to consider the combined effects of pH and temperature. Support for the hypotheses was therefore considered within the context of these and other factors.

Evidence for or against hypotheses

Our hypothesis (H1) that both AOA and AOB activities would increase with temperature (with AOB having an optimal temperature< 30°C) was generally supported. However, few conclusions can be drawn from the temperature perturbations of pH 4.5 soil (Table 2A), since both AOA and AOB activities were undetectable in these samples. There was also evidence of greater death of AOA at 35°C. While the lack of AOB activity at this acidic pH was expected, the lack of AOA activity was surprising, given that their growth at pH 4.5 has been demonstrated previously at this site (Gubry-Rangin *et al*., 2010; Nicol *et al*., 2008) and other acidic soils (Gubry-Rangin *et al*., 2017; Levičnik-Höfferle *et al*., 2012; Stempfhuber *et al*., 2015; Zhang *et al*., 2012). Nevertheless, and at 25°C only, the lack of significant activity may be due to experimental failure on one sample (see Supplementary Fig. S2A), this explanation being supported by a significantly higher nitrate production at 25°C (Table 2A). In any case, the data indicate a lack of low temperature versatility of acidophilic AOA and/or insufficient time to detect activation and selection of different AOA populations, both suggesting a low potential for adaptation within those communities. In pH 6 and 7.5 soils, where AO activity was detectable (Table 2B and C), there was evidence for the predicted increase in activity with temperature but generally not across the whole range, and with some variation for AOB depending on the methodology used to assess activity (see section below). Predictions of temperature effects (arising from H1) were modified to account for additional effects of pH (H7) and ammonium supply (H5) and the results highlight the need for more quantitative information on the relative and combined effects of these factors.

Hypothesis 2 (H2), that AOA are active across a wide range of pH values, could not be tested in the pH perturbation experiment due the potential general effect of the speed of adaptation (H7) of AOA activity at different pH values (P7). In the temperature experiment, hypothesis 2 was partially confirmed with activity observed at pH 6 and pH 7.5 (Table 2A), while significant activity was not detected in the pH 4.5 soil, which may have resulted from delays in activation and/or selection of those present and requirement for additional consideration of temperature (Supplementary Fig. S2A). Therefore, prediction of similar AOA activities at all soil pH values (P2) was not validated as their activity was surprisingly generally low or absent at pH 4.5 (Table 2A).

Hypothesis 3 (H3), which proposes that AOB activity increases with pH, was not supported by the surprising

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evidence of C assimilation at pH 4.5 (Table 3A and B) and no consistent increase in activity with increasing soil pH (Table 3A–C). Predictions arising from this hypothesis are influenced by effects of competition with AOA and ammonium supply. This, again, illustrates the need to consider interactions between factors when considering effects of environmental change.

Hypothesis 4 (H4), that neutrophilic AOA have higher temperature optima than acidophilic AOA, could not be rejected (due to absence of a robust test of the associated predictions with the experimental data), but the results highlighted again the need for greater consideration of the combined effects of environmental factors when considering community changes.

Hypothesis 5 (H5) proposes that AOA will outcompete AOB when the rate of ammonium supply is low (and vice-versa). In our system, ammonium was provided only through organic N mineralisation and, as predicted, led to dominance of AOA over AOB activity in the majority of conditions. This remained true even when ammonium accumulated to concentrations that would be expected to benefit AOB (Verhamme *et al*., 2011), although at 35°C this may also have been due to a preferential growth temperature for AOA. Nevertheless, ammonium accumulation at 35°C in the pH 7.5 soil stimulated AOB to reach similar activity to that of AOA (at least for the C assimilation) (Table 3C). This result highlights once again the importance of multifactorial consideration for ecological predictions.

Hypothesis 6 (H6), which proposes that either group can be active when the activity of the competitor group is reduced, was supported by greater AOB activity where AOA activity is predicted to be low, following reductions in pH to 4.5 at 15°C and 25°C, and of greater AOB activity following changes in pH rather than temperature. The findings therefore provide further evidence that competition between AOA and AOB is due to additional factors, and not only ammonium supply rate or concentration.

The absence and presence of AOA activity following perturbation in pH from pH 6 to 4.5 and 7.5, respectively, supported hypothesis 7 (H7), that AOA adaptation to acidic pH is slower than that to higher pH. Comparison of results from the different perturbations (Table 4) provides further evidence for H7.

Limitations of the experimental system

This study investigated the influence of perturbations in pH and temperature, when some of the evidence on which hypotheses were based derived from data obtained under constant conditions, in either laboratory or field conditions. Several discrepancies between predicted and experimental results strongly suggest the need for longer incubation periods, particularly for pH; temperature responses in surface soil may be rapid, and responses without substantial time for acclimation may be important. Changes in gene abundance require net growth (greater multiplication than death) and, for AO, are unlikely to be detectable for several days, as doubling times of ammonia oxidisers in soil are typically in the order of 1–2 days. C assimilation, measured by DNA-SIP, requires DNA replication and is also a direct measure of growth that is more sensitive and rapid than changes in gene abundance (Tourna *et al*., 2010). In contrast, transcriptional activity may detect early responses to perturbation, but functional activity may persist after cessation of transcriptional activity, if transcripts or translated proteins are stable, making measurement of temporal dynamics essential. Interestingly, with the exception of the pH 7.5 soil incubated at 15°C, nitrate production followed predictions, despite less predictable variations in AOA and AOB activities.

Slow adaptation is likely to be detrimental to ammonia oxidisers in natural soils, for example, through competition for ammonia from plants and other microorganisms. Fast adaptation to perturbation requires the presence and activation of organisms capable of activity under the new conditions, whose success, and detection, will depend on their abundance in unperturbed soil. This, in turn, will depend on their ability to survive non-optimal conditions. The decreases in gene and transcript abundances following some perturbations indicated differing effects of pH and temperature on survival of AOA and AOB. This highlights the need to consider death and survival, rather than just growth, when investigating the influence of environmental change on community composition. The data suggest an interaction between temperature and temporal decreases in activity, and also a more rapid decline immediately after perturbation, possibly due to subpopulations with different survival capacities. These results may therefore inform future studies on the influence of temperature and pH on survival and persistence of AO, but would require directed experimental design. In addition, this study assessed 'total' AOA and AOB communities and their activities, but did not assess functional diversity within these groups.

While variability between microcosms was minimized, variation in responses to pH and temperature perturbations was greater than expected, based on previous experience with similar microcosm systems (Gubry-Rangin *et al*., 2017; Nicol *et al*., 2008), and may reflect the greater range of environmental conditions investigated. This may also indicate stochastic processes in community adaptation and stabilization after perturbation and greater replication may have improved discrimination of treatment effects. The observation of AOB activity, through C assimilation, in pH 4.5 soil was surprising, given the important role of acidophilic AOA in such soils.

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However, the pH minimum for AOB growth can be reduced by 1–2 pH units in attached cells (Allison and Prosser, 1993) and those with urease activity (Burton and Prosser, 2001). Other recent studies have provided evidence for acidic AOB phylogenetic clades (Aigle *et al*., 2019) and even AOB activity at low pH (Zhao *et al*., 2020). If such AOB populations are more versatile with respect to temperature, AOB may have been able to respond immediately after reduction in soil pH, before activation or selection of less versatile AOA.

Conclusions

Niche specialization in AO has received considerable attention in recent years, with information from a range of experimental systems and approaches. Integration of this information in a number of hypotheses and subsequent comparison of predictions and experimental data increased objectivity and avoided biases that are inherent in *post-hoc* comparisons with published results. The general predictions regarding niche specialization with regard to effects of temperature and pH were consistent with experimental data, but critical testing revealed a number of discrepancies. Some discrepancies are likely due to experimental limitations, for example, variability between replicates and differences in the sensitivity and nature of different measures of activity. Other explanations are more fundamental. For example, the study illustrated the need to consider different factors influencing temporal responses, including death rates and survival under unfavourable conditions, initial AO abundances, and growth rates of organisms adapted to new conditions. It also provided evidence for unpredicted growth of AOB in acidic soil. Most importantly, the discrepancies illustrated here highlight the multi-factorial nature of niche specialization and niche overlap and the need to consider the versatility and adaptability of microbial communities with respect to combinations of factors when responding to environmental change.

Materials and methods

Experimental design and microcosm construction

Soil microcosms contained soil sampled on 6 June 2015 from the upper 10 cm of plots of a sandy loam agricultural soil pH gradient (SRUC, Craibstone, Scotland; grid reference NJ872104) maintained at pH values of approximately 4.5, 6 and 7.5 since 1961, with pH values of 4.77, 6.26 and 7.39 at the time of sampling. Soil characteristics are described in Kemp *et al*. (1992). Eight parallel gradients are subject to an 8-year annual rotation of winter wheat, potatoes, spring barley, swedes, spring oat, 3 years of grass with no re-sowing and all receive moderate NPK fertilization before sowing, except for years 2 and 3 of the grass rotation. The crop at the time of sampling was potatoes and the soil had received 100, 66 and 100 kg ha^{-1} of N, P and K, respectively, in April. Soil was sieved (3.35 mm mesh size) within 2 days of sampling and stored at 4°C before construction of microcosms within 9 days. Water content was measured by drying soil subsamples at 100°C for 24 h.

Perturbations in temperature and pH were investigated in soil microcosms using the design illustrated in Fig. 2. The moisture content of soil from the different pH plots (4.5, 6 and 7.5) was adjusted to 30% (w/w). Each soil was then spread to a 3-cm layer in seedling trays (3 kg each) and pre-incubated for 7 days at 15°C (pH 6), 25°C (pH 4.5, pH 6 and pH 7.5) and 35°C (pH 6). Trays were wrapped in plastic bags to prevent evaporation, allowing sufficient headspace for aeration of soil, and were reopened and rewetted after 3 and 7 days to maintain 30% (w/w) water content. For the temperature perturbation experiment, temperature was then perturbed in pH 4.5, 6 or 7.5 soils pre-incubated at 25°C by incubating microcosms for a further 30 days at the same pH and at either 15°C, 25°C or 35°C. For the pH perturbation experiment, the pH 6 soil pre-incubated at either 15°C, 25°C or 35°C was then maintained at pH 6 or adjusted to 4.5 or 7.5 and then incubated in microcosms for 30 days at the same temperature. Soil pH was adjusted by adding the equivalent of 0.02–0.05 g $Ca(OH)_2$ per 10 g of soil to reach a pH of 7.5, and the equivalent of 0.06–0.08 g aluminium sulphate per 10 g soil to reach a pH of 4.5. Aluminium sulphate has been used historically to maintain soil pH at 4.5 in these plots. Although a specific effect of aluminium on AO communities cannot be discounted, we are unaware of evidence for such an effect and acidic soils that do not contain high levels of aluminium select for similar AOA communities (Gubry-Rangin *et al*., 2011) with similar growth and CO_2-assimilation characteristics (Zhang *et al*., 2012). All microcosms were constructed by adding 50 g soil to 250-ml Duran bottles and were incubated in the dark, maintaining moisture content at 30% and maintaining aerobic conditions by opening and re-sealing bottles when sampling or at periods of ≤ 3–4 days.

Triplicate microcosms were established for each of the 18 combinations of temperature or pH change and were sampled non-destructively after 0, 1, 3, 10 and 30 days. Stable isotope probing (SIP) was performed using a parallel set of triplicate microcosms (on the same 18 combinations of temperature or pH change sampled at 0 and 30 days), sealed with butyl rubber stoppers and aluminium caps, in which either $^{13}C–CO_2$ or $^{12}C–CO_2$ (each 99 atm %) was supplied to give a headspace concentration of 5% (v/v) as described previously (Zhang *et al*., 2010). Aeration and replenishment of $^{13}CO_2$ were

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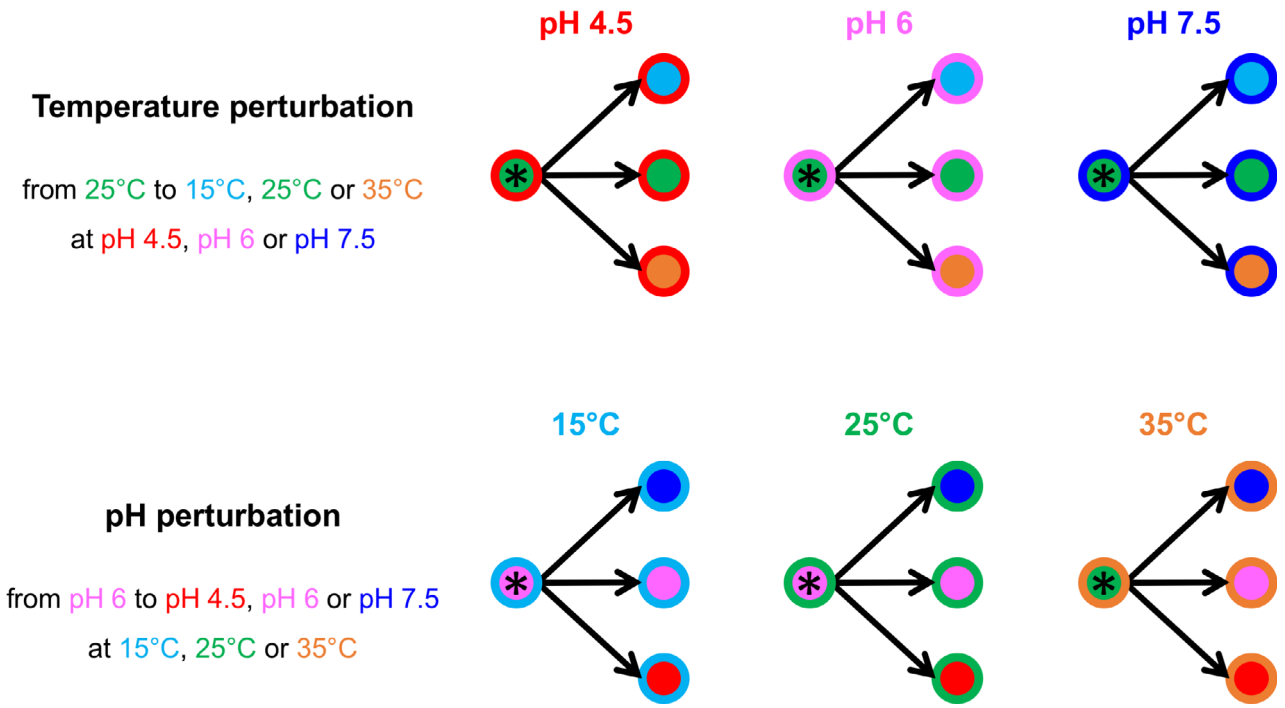

Fig 2. Design of temperature and pH perturbation experiments. For the temperature perturbation experiment, pH 4.5, 6 or 7.5 soils were incubated at 25°C for 7 days. Soil at each pH was then incubated for a further 28 days at either 15°C, 25°C or 35°C. For the pH perturbation experiment, pH 6 soil was incubated at 15°C, 25°C or 35°C for 7 days. Soil pH was then adjusted to 4.5, 6 or 7.5 and the soil was incubated for a further 28 days at either 15°C, 25°C or 35°C. Circle fill colours indicate the value of the factor (pH or temperature) after perturbation and border colours represent the factor (pH or temperature) that was maintained before and after perturbation.

achieved twice weekly by removing caps, to enable air exchange, replacement of caps and reinjection of $^{13}CO_2$ or $^{12}CO_2$.

Chemical and molecular analysis of soil samples

Ammonium, nitrite and nitrate were extracted from soil samples using 1 M KCl and assayed colorimetrically as described in Hink *et al*. (2018). Nitrite was always below the detection limit (2.5 μM) in all samples and nitrification dynamics were determined by analysis of temporal changes in nitrate concentration. Soil pH was measured at each sampling time and demonstrate rapid and stable changes in pH following perturbations (Supplementary Table S8).

DNA and RNA were extracted separately from 0.5 g soil as described by Griffiths *et al*. (2000) and Nicol *et al*. (2005) with the following modifications: centrifugation times were increased to 15 and 45 min, to increase the efficiency of partitioning of soil and cellular debris and then of nucleic acid collection, respectively. For RNA extraction, a mixture of acidified-phenol:chloroform: isoamyl alcohol (125:24:1, pH 4.5, Ambion) was used to increase RNA extraction yield. Remaining DNA was digested using the TURBO DNA-free kit (Ambion) following the manufacturer's instructions. DNA and RNA concentrations and quality were measured using a Nanodrop (ND-1000 Spectrophotometer, Labtech). cDNA was generated from 0.7–1 μg of purified RNA using 50 ng (2.5 μM) of random hexamers (Bioline) and Maxima reverse transcriptase (Thermo Scientific) according to the manufacturer's instructions, with no RNA (water only) and no-RT negative controls included.

For SIP-DNA, ultracentrifugation of DNA from ^{12}C- and ^{13}C–CO_2 incubations in CsCl gradients was performed as described in Zhang *et al*. (2010) with adaptation to an Optima MAX-E Table-top ultracentrifuge (Beckman Coulter). One μg of DNA in 2 μl of water was added to 8 ml CsCl in TE buffer solution (initially prepared with a refractive index (RI) of 1.4003 (1.710 g mL^{-1} buoyant density)) in 8-ml quick-seal polyallomer tubes (16 × 57 mm, Beckman Coulter) and centrifuged in a MLN-80 rotor (Beckman Coulter) at 152,000 *g* (50,000 rpm) for 62 h at 20°C. CsCl density gradients were fractionated into 16 × 500 μl fractions and the buoyant density of each fraction determined indirectly by measuring RI. DNA in 12 fractions (2–13) ranging from 1.670 to 1.750 g mL^{-1} (s.e. 0.005 g ml^{-1}) was recovered by standard PEG precipitation (Zhang *et al*., 2010). Quantitative PCR (qPCR) was used to quantify archaeal and bacterial *amoA* gene abundance in cDNA, DNA and SIP-DNA as described in Gubry-Rangin *et al*. (2010) using AOA and AOB *amoA*

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standards described previously (Thion and Prosser, 2014). Amplification efficiencies for archaeal and bacterial *amoA* qPCR assays ranged from 85% to 95%, with r^2 values > 0.99.

Data analysis

Changes in AO activity in each microcosm were assessed as the proportional changes in *amoA* gene and transcript abundances, calculated as the difference between final and initial values divided by the initial value for each microcosm for the DNA and RNA measurements. For the DNA-SIP analyses, ^{13}C assimilation by AOA and AOB was measured at the final sample point by quantification of *amoA* genes found in ^{13}C–DNA gradients. Specifically, ^{13}C–CO_2 assimilation was calculated as the abundance of *amoA* genes, measured by qPCR, in the ^{13}C–DNA fractions in which *amoA* abundance was higher in ^{13}C- than ^{12}C-microcosms. For archaeal and bacterial *amoA* genes, these comprised fractions with buoyant densities of 1.70–1.71 to 1.75–1.76 g ml^{-1} for AOA and 1.72–1.73 to 1.75–1.76 g ml^{-1} for AOB. The abundance of *amoA* genes in these fractions was estimated following the trapezoidal rule (see Supplementary Fig. S1) for both ^{13}C–DNA and ^{12}C–DNA independently (i.e., in the same range of corresponding fractions in ^{13}C- and the ^{12}C-microcosms). The proportion of AO assimilating CO_2 was then estimated as *amoA* gene abundance relative to the total abundance in the sample. Finally, subtraction of ^{12}C replicate mean proportion from ^{13}C-independent replicate proportion provided the corrected estimation of ^{13}C–CO_2 assimilation for each incubation treatment.

The significance of the effect of each perturbation (temperature or pH) on proportional changes in gene and transcript abundances of AOA or AOB at the end of incubation, and on differences in AOA or AOB ^{13}C assimilation, was independently assessed by one-way ANOVA (low, intermediate or high temperature or pH, for temperature and pH perturbation experiments, respectively), followed by Tukey *post-hoc* tests when overall effects were significant. A similar approach was used to assess significance of differences between relative activities of AOA versus AOB activity (AOA:AOB) for each activity measure. For each temperature-pH combination, significant differences within AOA or AOB activity or in AOA: AOB relative activity were represented by different font sizes within each column in Tables 2–4. Details of statistical analyses are presented in Supplementary Tables S1 and S2.

The comparisons described above compare different treatments but give no information on absolute activity in microcosms over time. In some microcosms, gene and/or transcript abundance decreased during incubation and C assimilation was not significant. For the gene and transcript data, the existence of absolute activity was considered when significant changes of gene and/or transcript abundance occurred over time; these temporal changes were assessed using one-way ANOVA (time, with all five time points used), and Tukey *post-hoc* analysis, and significant ($p < 0.1$) negative, null or positive temporal changes in gene or transcript abundances are represented by superscripts −1, 0 or +1 in Table 2 (see also Supplementary Table S3 for details of statistical analyses). For the C assimilation data, the significance of temporal change was assessed using *t* test comparison of the observed mean proportion C assimilation and a theoretical mean of 0 ($p < 0.1$) (see Supplementary Table S4) and significant C assimilation is represented by a superscript of +1 in Table 2.

Process rate (nitrate production) was also compared using the same statistical analysis as above (ANOVA and Tukey *post-hoc* analysis) by analysing the proportional changes in nitrate concentration during incubation, calculated as the difference between final and initial values divided by the initial value for each microcosm (see Supplementary Table S5).

For all statistical analysis, a significance level of $p < 0.1$ was used, due to the high degree of variability in some treatments. Homoscedasticity and homogeneity of variances were tested for each set of analyses and only the one-way ANOVA of absolute abundance (raw qPCR data) required a log transformation.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Supporting Information.

Appendix S2. Tables.

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