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Use of Aliphatic *n*-Alkynes To Discriminate Soil Nitrification Activities of Ammonia-Oxidizing Thaumarchaea and Bacteria

Anne E. Taylor,[a] Neeraja Vajrala,[b] Andrew T. Giguere,[a] Alix I. Gitelman,[c] Daniel J. Arp,[b] David D. Myrold,[a] Luis Sayavedra-Soto,[b] Peter J. Bottomley[a,d]

Department of Crop and Soil Science,[a] Department of Botany and Plant Pathology,[b] Department of Statistics,[c] and Department of Microbiology,[d] Oregon State University, Corvallis, Oregon, USA

Ammonia (NH_3)-oxidizing bacteria (AOB) and thaumarchaea (AOA) co-occupy most soils, yet no short-term growth-independent method exists to determine their relative contributions to nitrification *in situ*. Microbial monooxygenases differ in their vulnerability to inactivation by aliphatic *n*-alkynes, and we found that NH_3 oxidation by the marine thaumarchaeon *Nitrosopumilus maritimus* was unaffected during a 24-h exposure to ≤20 μM concentrations of 1-alkynes C_8 and C_9. In contrast, NH_3 oxidation by two AOB (*Nitrosomonas europaea* and *Nitrosospira multiformis*) was quickly and irreversibly inactivated by 1 μM C_8 (octyne). Evidence that nitrification carried out by soilborne AOA was also insensitive to octyne was obtained. In incubations (21 or 28 days) of two different whole soils, both acetylene and octyne effectively prevented NH_4^+-stimulated increases in AOB population densities, but octyne did not prevent increases in AOA population densities that were prevented by acetylene. Furthermore, octyne-resistant, NH_4^+-stimulated net nitrification rates of 2 and 7 μg N/g soil/day persisted throughout the incubation of the two soils. Other evidence that octyne-resistant nitrification was due to AOA included (i) a positive correlation of octyne-resistant nitrification in soil slurries of cropped and noncropped soils with allylthiourea-resistant activity (100 μM) and (ii) the finding that the fraction of octyne-resistant nitrification in soil slurries correlated with the fraction of nitrification that recovered from irreversible acetylene inactivation in the presence of bacterial protein synthesis inhibitors and with the octyne-resistant fraction of NH_4^+-saturated net nitrification measured in whole soils. Octyne can be useful in short-term assays to discriminate AOA and AOB contributions to soil nitrification.

For about a century, most ammonia (NH_3) oxidation in soils was thought to be carried out by chemolithoautotrophic ammonia-oxidizing bacteria (AOB). In 2005, the nitrification paradigm changed with the discovery of another type of microorganism from the phylum *Thaumarchaeota* that performs NH_3 oxidation (1). Molecular techniques have shown that ammonia-oxidizing *Thaumarchaeota* (AOA) are widely distributed in soils throughout the world (2, 3). AOA are usually more numerous in soil than AOB, and in some soils, AOB are present at levels below the detection limit of quantitative PCR (qPCR) (4, 5). This has led to speculation about the extent to which AOA contribute to soil nitrification (6, 7). AOA may be more metabolically versatile than AOB, with some cultured AOA growing at acid pH (8), scavenging NH_4^+ at low concentrations (9), and showing mixotrophic growth on a combination of pyruvate and NH_4^+ (10), and an AOA soil population has been shown to convert organic N sources to NO_3^- (11). The evidence for AOA contributing to soil nitrification has arisen from enrichment approaches involving long incubations (4 to 6 weeks) of soil in the laboratory, where NH_3 oxidation was accompanied either by the incorporation of $[^{13}C]CO_2$ into thaumarchaeal DNA (12–14) or by acetylene preventing an increase in the number of copies of the ammonia monooxygenase subunit A gene (*amoA*) of AOA (15, 16), supported either by mineralization of soil organic N or by repeated amendments of small amounts of NH_4^+.

Our goal has been to develop short-term assays (≤48 h) that are growth independent and directly measure the potential rates of soil nitrification attributable to either AOA or AOB. We have taken advantage of the fact that acetylene specifically and irreversibly inactivates ammonia monooxygenase (AMO) of AOB and is known to inhibit NH_3 oxidation by AOA (15, 17, 18). After exposure and removal of acetylene (6 h), the recovery of NH_3-oxidizing activity in soil slurries was monitored with or without antibiotics targeted at bacterial protein synthesis to discriminate between the relative contributions of AOA and AOB to the recovered nitrification potential (RNP) activity. We showed that after the removal of acetylene, there was a lag before nitrite (NO_2^-) plus NO_3^- began to accumulate in both AOA- and AOB-dominated soils, suggesting the need for *de novo* protein synthesis after irreversible inactivation. We showed that AOA dominate the nitrification potentials (NPs) of soil samples taken from pastures (19) and that AOB dominate the NPs of wheat-cropped soils recently fertilized with inorganic N. In addition, we found that the relative contributions of AOA and AOB to NPs differed across the cropped and fallowed phases of a 2-year winter wheat rotation and were affected by the time since N fertilization and by seasonal soil conditions (20). In the search for a strategy that would be more practical for unsaturated whole soils (WSs), we reasoned that whereas monooxygenases (MOs) generally have a broad substrate range, the ranges of some are more restricted than those of others (21–24). For example, AMO of *Nitrosomonas europaea* has a broad substrate range for *n*-alkanes (C_2 to C_8) and is inhibited by aliphatic

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Address correspondence to Anne E. Taylor, anne.taylor@oregonstate.edu.
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n-alkynes of the same chain lengths (25). In contrast, other Cu-containing membrane-bound monooxygenases, such as methane MO (MMO) and the Cu-containing alkane MO of *Nocardioides* sp. strain CF8, have a more restricted alkane substrate range ($\leq C_6$) (26–29). Given that the AOA AMO also falls into the Cu-containing AMO/particulate MMO (pMMO) family (30), we hypothesized that it might show a different sensitivity than the bacterial AMO to the effects of aliphatic *n*-alkynes of different chain lengths (C_2 to C_9). This article reports on the results of studies carried out to assess the effects of *n*-alkynes on the NH_3-oxidizing activity of AOA and AOB in pure culture, in soil slurries, and in whole-soil incubations.

MATERIALS AND METHODS

Chemicals. *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer, vanadium chloride, spectinomycin dihydrochloride, 1-allyl-2-thiourea (ATU), and NH_4Cl were obtained from Sigma-Aldrich (St. Louis, MO). Kanamycin sulfate was obtained from EMD Biosciences, Inc. (La Jolla, CA). Linear aliphatic 1-alkynes (C_3 to C_9) were obtained from Sigma-Aldrich, and acetylene (C_2) was obtained from Airgas (Radnor, PA).

Pure culture experiments. (i) Sensitivity to C_2 to C_9 alkynes. *N. europaea* was grown as described previously (25), and cell aliquots were added to growth medium supplemented with 1 mM NH_4^+ and preequilibrated for 30 min with a 5 μM aqueous concentration (C_{aq}) of each alkyne (C_2 to C_9; prepared as described in the supplemental material). NO_2^- concentrations were determined colorimetrically as described previously and monitored for 24 h (31). *Nitrosopumilus maritimus* cultures were grown and harvested by filtration onto a 0.2-μm-pore-size nylon membrane (18) and incubated in synthetic *Crenarchaeota* medium (SCM) with 1 mM NH_4^+ with or without 20 μM alkyne (C_2 to C_9) without shaking at 30°C. NO_2^- accumulation was followed for ~24 h. See the supplemental material for experimental details.

(ii) Octyne sensitivity. *N. europaea* or *Nitrosospira multiformis* cell suspensions were added to 30 mM TES buffer (pH 7.2) plus 1 mM NH_4^+ that had been preequilibrated with octyne (0, 1, 2, or 5 μM) for 30 min. Cell suspensions were shaken at room temperature (~23°C), and NO_2^- concentrations were monitored for ~24 h. *N. maritimus* cultures were incubated in SCM medium plus 1 mM NH_4^+ with octyne (0, 20, 50, or 100 μM) at 30°C, and NO_2^- accumulation was followed for ~24 h.

(iii) Inactivation of AMO by alkynes and protein synthesis-dependent recovery. Aliquots of *N. europaea* and *N. multiformis* cell suspensions were exposed to either 4 μM octyne or 6 μM acetylene for 2 h and then degassed to remove the alkynes (see the supplemental material). In the case of *N. maritimus* cultures, cells were incubated on the filter membrane in SCM medium plus 1 mM NH_4^+ with acetylene (30 μM) at 30°C. After 6 h, the filter membrane was placed in fresh SCM medium plus 1 mM NH_4^+ in the absence of acetylene. Recovery of NH_3 oxidation was monitored for 48 h by following the accumulation of NO_2^-. In some AOB treatments, the bacterial protein synthesis inhibitors kanamycin (*N. europaea* experiments) and spectinomycin (*N. multiformis* experiments) were added at a final concentration of 200 μg/ml to prevent resynthesis of AMO by AOB. Non-alkyne-treated controls with or without antibiotics were included to demonstrate that antibiotics were not immediate inhibitors of NH_3-oxidizing activity.

Response of soil nitrification to alkynes. (i) Response of NPs to inhibitors. NPs were determined on soil samples as described previously (19). See the supplemental material for descriptions of the soils and soil sampling. NP treatments included ATU (100 μM) and C_2 to C_9 1-alkynes. NP controls were comprised of soil suspensions to which acetylene was added to evaluate the possibility of heterotrophic nitrification and also the significance of the NO_3^- sink. There was no significant change in background NO_3^- concentrations in the controls containing acetylene (data not shown), indicating that all NO_2^- plus NO_3^- accumulation was due to nitrification and that there was no significant NO_3^- consumption. The details of the recovered nitrification potential (RNP) assay have been described previously (19, 20). In some treatments, the bacterial protein synthesis inhibitors kanamycin (800 μg/ml) and spectinomycin (200 μg/ml) were added to prevent resynthesis of AMO by AOB. The fraction of RNP that is recovered in the presence of bacterial protein synthesis inhibitors, i.e., antibiotics (RNP_{ab}), is considered to be due to AOA.

(ii) Response of net nitrification in whole soil to octyne during 21 to 28 days of incubations. Corvallis pasture (CP) and Corvallis cropped (CC) soils were incubated under three NH_4^+ levels established by adding anhydrous NH_3 gas to achieve 0, 2, and 20 μmol/g soil NH_4^+. Three treatments were imposed at each NH_4^+ level: (i) no alkyne amendment, (ii) amendment with acetylene (6 μM soil solution concentration, C_{aq}), and (iii) amendment with octyne (2 μM apparent C_{aq}). The treated soils were incubated at ~23°C and opened weekly to allow gas exchange and sampling of soil for DNA extraction and to determine NH_4^+ and NO_2^- plus NO_3^- concentrations. See the supplemental material for further details.

(iii) Response of net nitrification in whole soil to octyne during short (2-day) incubations. Soils were incubated with or without octyne and with or without NH_4^+. Supplemental distilled H_2O was added to achieve a soil water content approximating 0.6 of the maximum water-holding capacity. Aliquots of anhydrous NH_3 gas which supported the maximum rate of nitrification were added (see Display S1b in the supplemental material). Soils were incubated under three treatments: (i) with no alkyne, (ii) with acetylene (6 μM C_{aq}), and (iii) with octyne (2 μM apparent C_{aq}) at ~23°C for 2 days. At the end of the incubation, NO_2^- plus NO_3^- concentrations were determined (see the supplemental material).

Nucleic acid analysis. DNA was extracted from soil using a MoBio PowerSoil (Carlsbad, CA) extraction kit, quantified using a NanoDrop ND-1000 UV-visible spectrophotometer (Thermo Scientific, Rockwood, TN), and stored at −80°C. qPCR of the AOA and AOB *amoA* genes was performed as described previously (20). See the supplemental material for more information.

Statistics. To determine whether the rate of NO_2^- plus NO_3^- accumulation was different between treatment conditions, analysis of variance was performed using the slopes from simple linear regressions of the repeated measurements versus time within each incubation replicate. The AOA/AOB ratios of the no-alkyne treatment and the treatments with octyne were log transformed, and the data were fit with a linear mixed-effects model in R (http://www.r-project.org/) with a random effect for the sample and fixed effects for the inhibitor, NH_4^+, and time of sampling, using additional R code, attributable to Christopher Moore (http://blog.lib.umn.edu/moor0554/canoemoore/2010/09/lmer_p-values_lrt.html), to calculate *P* values. Other comparisons between treatments with or without octyne in the presence and absence of NH_4^+ were done with a two-tailed Student's *t* test. See the supplemental material for further details.

RESULTS

Differential sensitivity of AOA and AOB cultures to C_2 to C_9 1-alkynes. To determine if AOA and AOB had differential sensitivity to alkynes of various chain lengths, we compared the NH_3 oxidation response of *N. europaea* and *N. maritimus* (an AOB and an AOA, respectively) to C_2 to C_9 1-alkynes in suspensions with 1 mM NH_4^+ (Fig. 1A and B). Nitrification by *N. europaea* was severely inhibited by 5 μM C_{aq} of C_3 and C_4 alkynes and completely inhibited by the remaining alkynes. In contrast, whereas all NO_2^- production by *N. maritimus* was prevented by 20 μM C_2 to C_5 alkynes, 20 μM C_6 and C_7 inhibited only about 50% of *N. maritimus* activity and 20 μM C_8 and C_9 had no effect on NO_2^- accumulation.

Differential sensitivity of AOA and AOB cultures to C_8. Further work with pure cultures was focused on C_8 (octyne) and compared the effective concentration range of octyne on NH_3

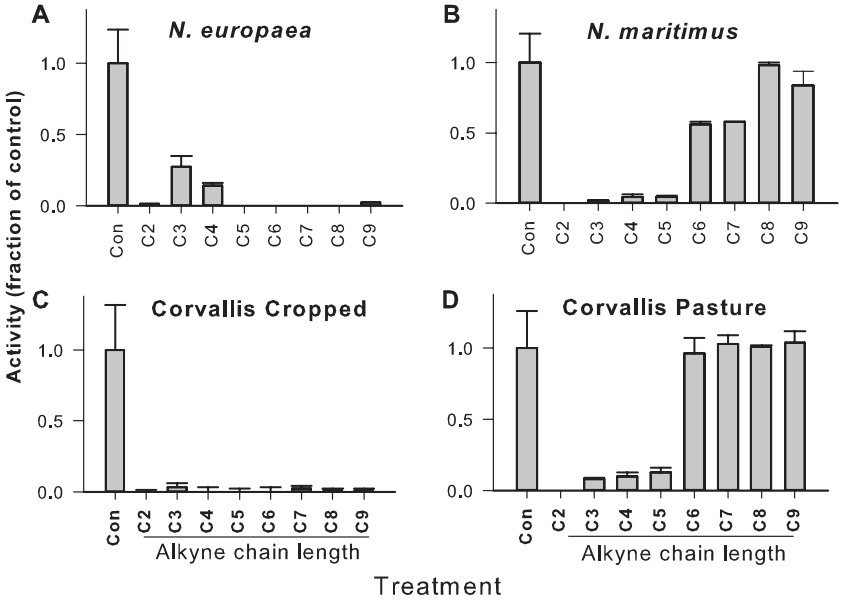

FIG 1 (A and B) Response of NO_2^- production to C_2 to C_9 1-alkynes by *N. europaea* (A) and *N. maritimus* (B) cultures. *N. europaea* and *N. maritimus* were incubated with 5 μM and 20 μM (C_{aq}) each alkyne, respectively, in the presence of 1 mM NH_4^+. (C and D) Effects of C_2 to C_9 alkynes on net NO_2^- plus NO_3^- production by slurries of Corvallis cropped (AOB-dominated) soil (C) and Corvallis pasture (AOA-dominated) soil (D) incubated with 2 and 4 μM (apparent C_{aq}; see the information in the supplemental material) each alkyne, respectively, in the presence of 1 mM NH_4^+. See Materials and Methods for further experimental details. NO_2^- (A and B) or NO_2^- plus NO_3^- (C and D) production in the presence of each alkyne was compared with that of a no-inhibitor control (Con). Error bars represent the SD of the mean ($n = 3$).

oxidation by the AOB *N. europaea* and *Nitrosospira multiformis* with that by *N. maritimus*. Production of NO_2^- by *N. europaea* and *N. multiformis* was completely inhibited by 1 to 5 μM C_{aq} octyne, and there were no significant increases in NO_2^- production after 2 h ($P \geq 0.07$; Fig. 2). In contrast, the rate of NO_2^- production by *N. maritimus* was unaffected by up to 20 μM octyne for at least 20 h (data not shown). Higher concentrations of octyne did not have an immediate negative effect on NO_2^- production, but at between 4 and 20 h of incubation in the presence of 50 or 100 μM octyne, NO_2^- accumulation was only 56 or 12%, respectively, of that for the no-octyne control.

Although the C_2 alkyne acetylene is well-known to be an irreversible inactivator of AMO in *N. europaea*, the mode of inhibition of AMO in *N. maritimus* is unknown; therefore, recovery of nitrification by *N. maritimus* after 6 h of acetylene exposure was evaluated (see Fig. S1 in the supplemental material). NO_2^- accumulated immediately in an *N. maritimus* culture that had not been pretreated with acetylene, but there was a delay of approximately 5 h before NO_2^- began to accumulate in the culture that had been acetylene treated, suggesting the need for *de novo* protein synthesis after acetylene inactivation. There was no NO_2^- production in *N. maritimus* cultures incubated continuously in the presence of acetylene.

The alkynes C_5 to C_9 are known to inhibit *N. europaea*, but it has not been unequivocally proven that the longer-chain alkynes are irreversible inactivators or merely inhibitors of AMO (25). Recovery of nitrification by *N. europaea* and *N. multiformis* after exposure to octyne (4 μM C_{aq}) was followed by treatment with or

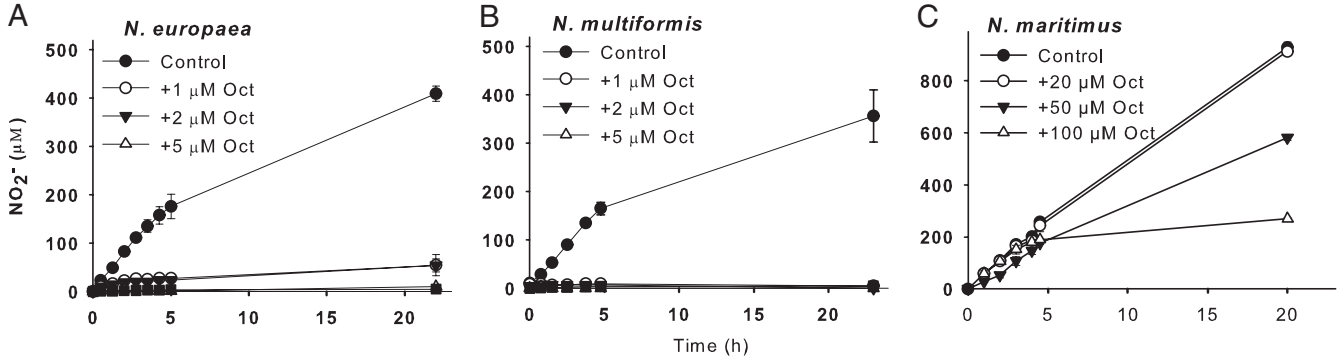

FIG 2 Sensitivity of NO_2^- production by *N. europaea* (A), *N. multiformis* (B), and *N. maritimus* (C) to different octyne (Oct) concentrations. *N. europaea* and *N. multiformis* cultures were suspended in 30 mM TES buffer (pH 7.2) with 1 mM NH_4^+. *N. maritimus* cells were suspended in SCM medium with 1 mM NH_4^+ (pH 7.5). The loss of linearity of NO_2^- production by *N. europaea* and *N. multiformis* after 5 h was likely due to a low affinity for NH_4^+. See Materials and Methods for further experimental details. Controls contained no octyne. Error bars represent the SD of the mean ($n = 3$).

without bacterial protein synthesis inhibitors and compared with recovery from acetylene inactivation (see Fig. S1 in the supplemental material). Both *N. europaea* and *N. multiformis* showed a 6- to 8-h lag before NO_2^- began to accumulate in octyne- and acetylene-treated cultures, and this was prevented by bacterial protein synthesis inhibitors. Interestingly, whereas kanamycin (200 μg/ml) prevented recovery of *N. europaea*, *N. multiformis* proved to be insensitive to kanamycin, and spectinomycin (200 μg/ml) was used to prevent protein synthesis. Neither kanamycin nor spectinomycin affected the initial rates of NO_2^- production in controls that were not inactivated by acetylene or octyne, proving that these bacterial protein synthesis inhibitors are not direct inhibitors *per se* of NO_2^--producing activity.

Effects of alkynes on nitrification potentials of soil slurries. The effects of C_2 to C_9 1-alkynes on net NO_2^- plus NO_3^- production were evaluated in two soils that were previously shown with the RNP assay as having NPs dominated by either AOA (Corvallis pasture [CP] soil) or AOB (Corvallis cropped [CC] soil) (19, 20). Samples of these soils were treated with C_2 to C_9 alkynes (each at 2 μM apparent C_{aq}; see the supplemental material) in soil slurry NP assays (Fig. 1C and D). In the AOB-dominated CC soil, there was no significant NO_2^- plus NO_3^- production in treatments with alkynes ($P > 0.05$). However, in AOA-dominated CP soil, whereas C_2 completely inhibited NO_2^- plus NO_3^- production, there was residual accumulation of NO_2^- plus NO_3^- in the presence of alkynes C_3 to C_5 but no significant effect of alkynes C_6 to C_9 compared with that of the control ($P > 0.1$). C_6 to C_9 alkynes did not inhibit NO_2^- plus NO_3^- accumulation in CP soil when tested at 4 μM apparent C_{aq} (Fig. 1D).

Effects of octyne on long-term (21- to 28-day) soil incubations. We evaluated the effects of octyne on net NO_2^- plus NO_3^- accumulation and changes in AOA and AOB *amoA* gene copy numbers during long-term (21 to 28 days) whole-soil (WS) incubations of both CC and CP soils amended with either (i) no supplemental NH_4^+, (ii) low NH_4^+ (2 μmol/g soil), or (iii) high NH_4^+ (20 μmol/g soil).

After 21 days of incubation, microcosms of CC soil that received no NH_4^+ or a low NH_4^+ addition showed no significant differences in the rates of NO_2^- plus NO_3^- accumulation with or without an apparent octyne C_{aq} of 2 μM ($P > 0.3$; Fig. 3A and B), suggesting that all nitrification in those NH_4^+ treatments was octyne resistant (AOA). However, in the high-NH_4^+ treatment, there was a significant difference in the rates of NO_2^- plus NO_3^- accumulation between treatments with and treatments without octyne ($P < 0.001$; Fig. 3C), indicating an increased contribution to nitrification by AOB (octyne sensitive). While there was a statistically nonsignificant response of the octyne-resistant rates of nitrification to NH_4^+ treatments ($P > 0.4$), NO_2^- plus NO_3^- accumulation in the no-alkyne treatments (AOA plus AOB) increased 6- and 14-fold over that in the unamended treatment in response to low and high NH_4^+, respectively ($P < 0.001$). The rate of NO_2^- plus NO_3^- accumulation in the no-alkyne treatment treated with high NH_4^+ was significantly greater than the rates of nitrification in the treatments with no NH_4^+ and low NH_4^+ ($P < 0.001$). Soil microcosms treated with acetylene did not accumulate NO_2^- plus NO_3^- in response to any NH_4^+ treatment and showed no increases in either AOA or AOB *amoA* gene copy numbers. During the 21-day incubation of CC soil, there was convincing evidence of differences between the log(AOA/AOB) response to the octyne and the no-alkyne treatments ($P < 0.0001$). For example, AOA *amoA* gene copy numbers increased in response to no-added-NH_4^+ and low-added-NH_4^+ treatments with or without octyne (Fig. 3D and E), whereas AOB *amoA* gene copy numbers increased only in response to the high-NH_4^+ treatment minus octyne (Fig. 3F). In the treatments with or without octyne, there was also strong evidence of the effect of an interaction between NH_4^+ treatment and time on the log(AOA/AOB). Specifically, there was convincing evidence of an increase in the log(AOA/AOB) in response to the low-NH_4^+ treatment from 0 to 21 days ($P < 0.0001$) and strong evidence of increases in log(AOA/AOB) from 7 to 21 days in both the no-NH_4^+ and high-NH_4^+ treatments ($P = 0.006$ and 0.01, respectively). There was sufficient net nitrification in CC soil to account for the increase in AOA *amoA* gene abundance observed by qPCR. In octyne-treated incubations with either low or high NH_4^+, ~3.3 μmol NO_2^- plus NO_3^-/g soil accumulated, and this accumulation was accompanied by an AOA *amoA* cell yield of 2.8×10^7 to 7.7×10^7 cells/μmol NH_4^+. This yield compares favorably with the cell yields measured in cultures of *N. maritimus* (1), *Nitrosocaldus yellowstonii* (32), and pyruvate-assisted *Nitrososphaera viennensis* (1.6×10^7 to 4.7×10^7 cells/μmol NH_4^+ [10]) and with the AOA yield measured in soil microcosms incubated with additions of low NH_4^+ (2.3×10^7 to 2.9×10^7 cells/μmol NH_4^+ [16]).

During the 28-day incubation of CP soil, microcosms that received no NH_4^+ addition expressed the same rate of net NO_2^- plus NO_3^- accumulation with or without octyne (see Fig. S2a in the supplemental material), whereas NO_2^- plus NO_3^- accumulation in microcosms amended with low or high NH_4^+ was significantly greater in the no-alkyne treatments than in the treatments including octyne ($P < 0.001$; see Fig. S2b and c in the supplemental material). The octyne-resistant (AOA) rates of nitrification increased 5- and 6-fold in response to low and high NH_4^+, respectively ($P < 0.001$), whereas in the no-alkyne treatment (AOA plus AOB), the rates of NO_2^- plus NO_3^- accumulation increased significantly (6- and 14-fold) over the unamended rate in response to low and high NH_4^+, respectively ($P < 0.001$). Soil microcosms treated with acetylene did not accumulate NO_2^- plus NO_3^-, and there were no increases in AOA and AOB *amoA* gene copy numbers. During the 28-day incubation of CP soil, there was strong evidence of a difference in the log(AOA/AOB) response between the octyne and the no-alkyne treatments ($P = 0.007$). In the no-alkyne treatment, AOB *amoA* gene copy numbers increased 10- and 100-fold in response to low and high NH_4^+, respectively (see Fig. S2e and f in the supplemental material), whereas the AOA *amoA* gene copy number did not change in either the no-alkyne or the octyne treatment. As a consequence, log(AOA/AOB) ratios were lower in the soil treated with high NH_4^+ than in the soil not treated with NH_4^+ ($P = 0.03$; see Fig. S2d in the supplemental material). Because there were no detectable AOA *amoA* gene copy number increases in CP soil, an alternate approach was taken to demonstrate that the AOA community of this soil could synthesize protein and express octyne-resistant NO_2^- plus NO_3^--producing activity. CP soil was acetylene inactivated and subjected to an RNP experiment where soil slurries were exposed to a combination of antibiotics targeted at bacterial protein synthesis (RNP_{ab} assay; kanamycin and spectinomycin, 800 and 200 μg/ml, respectively), octyne (RNP_{oct} assay; 2 μM apparent C_{aq}), or a combination of antibiotics and octyne ($RNP_{ab\ +\ oct}$). Rates of RNP_{ab}, RNP_{oct}, and $RNP_{ab\ +\ oct}$ were the same (0.36 ± 0.03 μmol NO_2^- plus NO_3^-/g soil/day), demonstrating that AOA in CP soil are

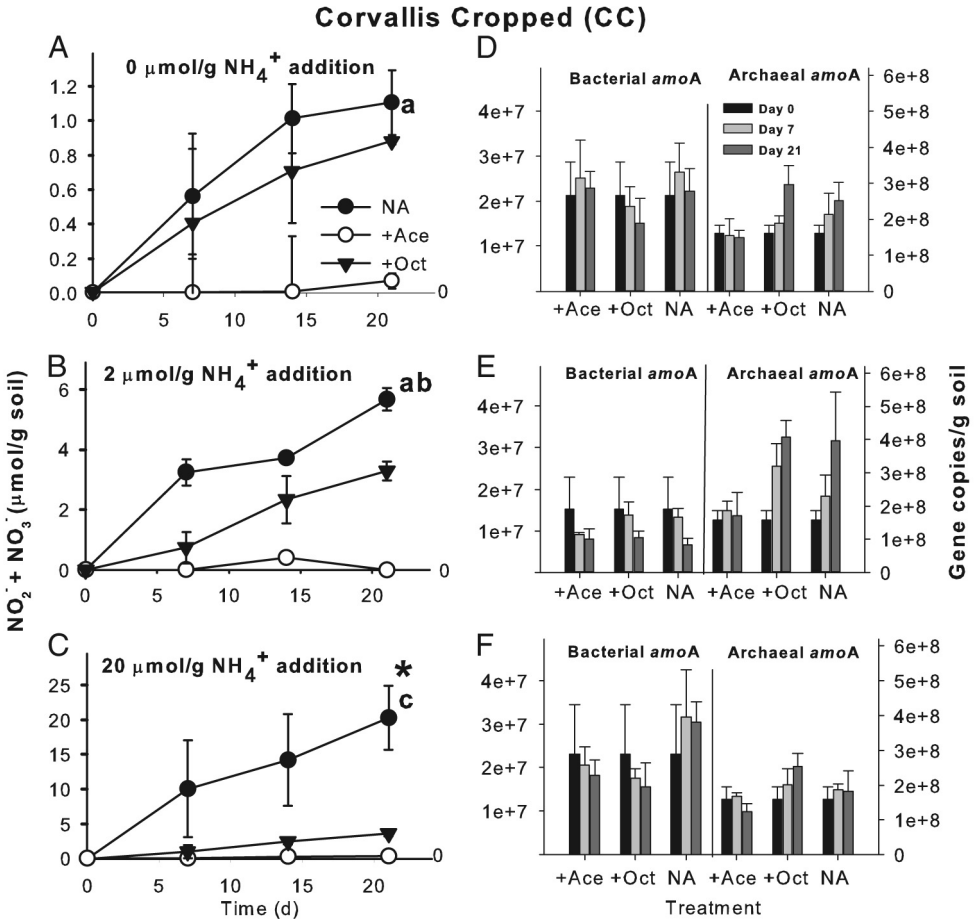

FIG 3 Comparison of the effects of alkyne (acetylene [+Ace], octyne [+Oct]), and no-alkyne ([NA]) treatments on net NO_2^- plus NO_3^- accumulation and on AOA and AOB *amoA* gene copy numbers during incubations of CC soil amended with no NH_4^+ (A and D), low NH_4^+ (B and E), or high NH_4^+ (C and F) (0, 2, and 20 μmol NH_4^+/g soil, respectively). (A, B, and C) NO_2^- plus NO_3^- accumulation over the time course of the experiment. Error bars represent the SD of the mean NO_2^- plus NO_3^- concentration ($n = 3$). d, day. (D, E, and F) AOB and AOA *amoA* gene copy numbers/g soil. Error bars represent the SD of the average number of copies of the *amoA* gene/g soil from triplicate qPCRs for each treatment ($n = 3$). *, NO_2^- plus NO_3^- accumulation was significantly different in the no-alkyne and octyne treatments within an NH_4^+ treatment ($P < 0.001$). Different lowercase letters indicate that NO_2^- plus NO_3^- accumulation was significantly different in the no-alkyne treatment between different NH_4^+ levels ($P < 0.001$). There was no significant difference in the rate of NO_2^- plus NO_3^- accumulation in the octyne treatment between different NH_4^+ levels.

able to synthesize AMO proteins and resume NO_2^- plus NO_3^- production in the combined presence of octyne and antibiotics. Considering the AOA yield observed in the CC soil microcosms, it is unlikely that a statistically significant increase of this magnitude could be measured over the background AOA *amoA* gene copy number abundance.

The fraction of octyne-sensitive soil nitrification correlates with the fraction sensitive to other AOB inhibitors. The effect of octyne on net NO_2^- plus NO_3^- production was evaluated in cropped and noncropped soils collected from several agricultural research stations located across Oregon. These soils had a range of pHs, textures, and sizes of AOA and AOB populations (see Table S1 in the supplemental material). The cropped soils were primarily cropped to winter wheat and had histories of being routinely fertilized with inorganic N fertilizers. Noncropped soils were collected from under diverse native vegetation of either conifer, sage brush, or grasses, had no history of cultivation or N fertilization, and were close in proximity to the cropped soils. The effects of octyne on NO_2^- plus NO_3^- accumulation in NP slurries were compared with the effects of 100 μM ATU and also with the RNP response with or without bacterial protein synthesis inhibitors. The sensitivities of NPs to octyne and ATU over the wide range of soils were strongly and positively correlated (Fig. 4A; $r^2 = 0.938$), showing a range of sensitivities from entirely sensitive to completely resistant. The fraction of RNP that was insensitive to antibiotics (RNP_{ab}/RNP) correlated positively and strongly ($r^2 = 0.905$) with the fraction of NP that was insensitive to octyne (NP_{oct}/NP; Fig. 4B). The cropped soils, which had the lowest AOA/AOB ratios (≤31), were more sensitive to antibiotics, ATU, and octyne than the noncropped soils, suggesting that AOB had a greater potential to contribute to nitrification in these soils. Noncropped soils showed a wide range of responses, with two soils, CP and Madras rangeland (MR), expressing AOA-dominated nitrification (AOA/AOB ratios ≥ 400), and two noncropped soils, Klamath woodlot (KW) and Pendleton grassland (PG) (AOA/AOB ratios, 45 and 60, respectively), showing an intermediate effect of octyne, ATU, and RNP_{ab}, suggesting that both AOA and AOB populations were capable of contributing to NPs.

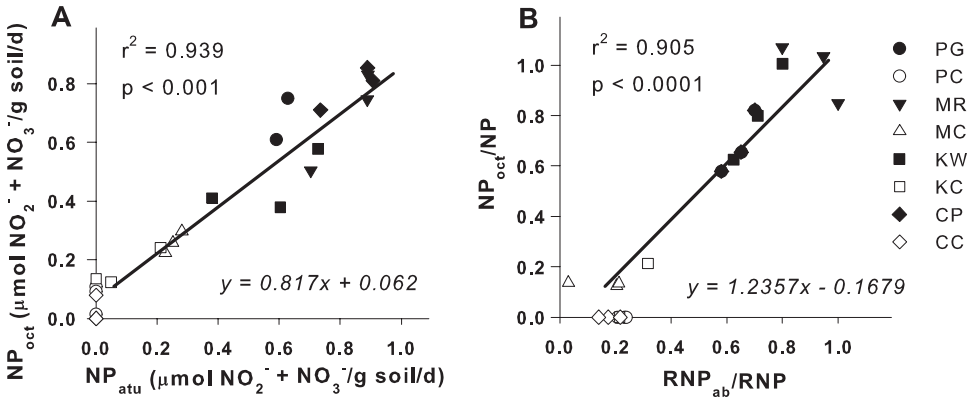

FIG 4 Correlation among three methods used to assess the relative contributions of AOA and AOB to soil slurry NPs of diverse Oregon soils. (A) Correlation between the rates of net NO_2^- plus NO_3^- production that were resistant to octyne (NP_{oct}) versus resistant to 100 μM ATU (NP_{atu}). (B) Correlation between the fractions of the RNPs that were insensitive to bacterial protein synthesis inhibitors (RNP_{ab}/RNP) versus the fractions of activity that were resistant to octyne (NP_{oct}/NP). Open and closed symbols represent cropped and noncropped soils, respectively. PG, Pendleton grassland; MR, Madras rangeland; KW, Klamath woodlot; CP, Corvallis pasture; PC, Pendleton cropped; MC, Madras cropped; KC, Klamath cropped; and CC, Corvallis cropped. See Table S1 in the supplemental material for site locations and soil characteristics.

A comparison of the effects of octyne on nitrification in WS and soil slurry (NP) assays. The effects of octyne (2 μM apparent C_{aq}) on nitrification were compared between WS and NP assays (see Display S1a in the supplemental material). Unsaturated WS samples (soil moisture, ~0.6 of saturation) were incubated in sealed bottles supplemented with sufficient anhydrous NH_3 gas (2 to 20 μmol NH_4^+/g soil; see Display S1b in the supplemental material) to support the maximum rate of nitrification. The fraction of the WS maximum rate of net NO_2^- plus NO_3^- production that was insensitive to octyne (WS_{oct}/WS) was strongly and positively correlated with the fraction of nitrification that was resistant to octyne in NP assays (NP_{oct}/NP; $r^2 = 0.884$). This study confirmed that octyne has the potential to differentiate the contributions of AOA and AOB to soil nitrification in short-term (2 days) WS incubations.

From assay of potential to *in situ* measurement of net nitrification. The effect of octyne on the NH_4^+-saturated rates (NH_4^+-sat, potential rate) of nitrification in WS was compared with the rates generated without addition of NH_4^+ (NH_4^+-0; *in situ* rate; Table 1). In the NH_4^+-0 treatment of all noncropped soils, significant amounts of net NO_2^- plus NO_3^- accumulated over the 2-day incubation ($P < 0.05$), and there was no significant difference between soils treated or not treated with octyne ($P > 0.8$), indicating that AOA contributed all nitrification under this treatment. In the majority of cropped soils, insufficient NO_2^- plus NO_3^- accumulated in the NH_4^+-0 treatment to draw any inference about effects with or without octyne. Addition of NH_4^+-sat led to significantly higher rates of octyne-sensitive NO_2^- plus NO_3^- production by AOB (total nitrification rate − octyne-resistant rate) in all soils except CP soil ($P < 0.05$) and resulted in ≤0.14 of the WS rate being contributed by AOA in the cropped soils. In the noncropped soils, NH_4^+-sat significantly increased octyne-resistant rates of NO_2^- plus NO_3^- production ($P < 0.05$), and a large fraction (0.62 to 0.96) of the NH_4^+-sat rate remained due to AOA. In contrast, in cropped soils treated with NH_4^+-sat, the rate of nitrification contributed by AOA increased significantly ($P < 0.05$) only in CC soil.

DISCUSSION

In this study, we established that the octyne method could be used in relatively short assays (24 to 48 h) in both soil slurries and whole-soil microcosms. The soils exhibited a wide range of nitrification rates, and nitrification was completely inactivated by acet-

TABLE 1 Rates of net NO_2^- plus NO_3^- accumulation in response to NH_4^+-0 and NH_4^+-sat by AOA and AOB for 2 days of incubation in WS[d]

	Rate of net NO_2^- plus NO_3^- accumulation (μg N/g soil/day)[e]			
	NH_4^+-0		NH_4^+-sat	
Soil	AOB	AOA	AOB	AOA
Noncropped				
PG	0.2 (0.2)	1.2 (0.3)[c]	3.9 (0.9)[a]	6.1 (0.4)[a]
MR	0.0 (0.7)	1.0 (0.1)[c]	0.7 (0.1)[a]	6.5 (0.1)[a]
KW	0.1 (0.0)	0.7 (0.1)[c]	3.0 (0.5)[a]	4.7 (0.5)[a]
CP	0.0 (0.3)	1.1 (0.3)[c]	0.5 (0.3)	9.2 (0.3)[a]
Cropped				
PC	0.0 (0.3)	0.4 (0.3)	6.2 (1.0)[b]	0.9 (0.3)
MC	0.0 (0.4)	1.1 (0.3)	12.3 (3.2)[b]	1.0 (0.3)
KC	0.3 (0.2)	1.2 (0.3)[c]	7.0 (0.5)[b]	0.8 (0.3)
CC	0.1 (0.2)	0.0 (0.0)	18.9 (2.1)[a]	0.4 (0.1)[a]

[a] Significant increase in net NO_2^- plus NO_3^- accumulation of both octyne-resistant (AOA) and octyne-sensitive (AOB) activities in NH_4^+-sat versus NH_4^+-0 treatments ($P \leq 0.05$).
[b] Significant increase in net NO_2^- plus NO_3^- accumulation of octyne-sensitive (AOB) activities in NH_4^+-sat versus NH_4^+-0 treatments ($P \leq 0.05$).
[c] Significant increase in net NO_2^- plus NO_3^- accumulation in NH_4^+-0 treatments ($P < 0.05$) and no significant difference between treatments with or without octyne ($P > 0.05$).
[d] See Display S1b in the supplemental material for NH_4^+-sat concentrations and Table S1 in the supplemental material for site locations and soil characteristics. AOA are octyne resistant, and AOB are octyne sensitive (total nitrification − nitrification by octyne-resistant AOA). PG, Pendleton grassland; MR, Madras rangeland; KW, Klamath woodlot; CP, Corvallis pasture; PC, Pendleton cropped; MC, Madras cropped; KC, Klamath cropped; and CC, Corvallis cropped.
[e] Values in parentheses are standard deviations of the means ($n = 3$).

ylene, making it unlikely that heterotrophic nitrification accounted for the octyne-resistant activity. Yet, the fraction of nitrification that was octyne resistant ranged widely and correlated well with that detected by other short-term methods used to assess the contributions of AOA activity, including ATU resistance and the RNP assay with antibiotics. Furthermore, in long-term soil incubations, octyne was as effective as acetylene in preventing AOB proliferation but did not prevent NH_4^+-dependent, acetylene-sensitive AOA growth. However, there is tremendous phylogenetic diversity within the *Thaumarchaeota*, and until more isolates that are representative of this phylum are available, we will not know if octyne universally discriminates between AOA and AOB nitrification activity. Nonetheless, there are some advantages of the octyne method over other published attempts to determine the relative contributions of AOA and AOB. First, whereas all AOB that have been tested are very sensitive to ATU, nitrification by pure cultures and soil AOA shows a range of sensitivities. Recently, it was shown that NO_2^- production by the obligate acidophilic AOA *Nitrosotalea devanaterra* was completely inhibited by 100 μM ATU (33). However, this response to ATU may not be representative of AOA occupying less acidic soils. For instance, "*Candidatus* Nitrososphaera viennensis," isolated from neutral soil and demonstrating optimal growth at pH ~7.5, required 500 μM ATU to stop the majority of nitrification (34). Second, although we have had success in measuring the relative contributions of AOA and AOB to NP in soil slurries by using the RNP assay with or without antibiotics (19), there are several challenges with extending this method into whole soil, including (i) difficulty in distributing aqueous solutions of antibiotics uniformly throughout an unsaturated soil, (ii) antibiotic effectiveness being negated by binding to soil particles (35, 36), (iii) variable antibiotic resistance among AOB (this study), and (iv) incomplete removal of acetylene by degassing.

Although this study was not focused upon elucidating the biochemical details of why octyne does not inactivate AOA AMO, our findings can be placed in context with other findings in the literature describing prokaryotic Cu-containing MOs with substrates with a variable range of *n*-alkane chain lengths that might be linked with sensitivity/resistance to aliphatic *n*-alkynes of different chain lengths. For instance, the chain length range of 1-alkynes that successfully inactivate AMO of *N. europaea* closely mirrors the chain length of linear alkanes (C_1 to C_8) that are AMO substrates (25). The relative insensitivity of *N. maritimus* AMO to C_6 to C_9 alkynes may infer that it has an alkane substrate range more restricted than that of *N. europaea* AMO or that C_6 to C_9 alkynes are much poorer substrates and inactivators. Other investigators have also been interested in the potential of AOA to oxidize hydrocarbons (37). Further studies are needed to examine the hydrocarbon substrate range of thaumarchaeal AMO and place it into context with other members of the Cu-containing AMO/pMMO enzyme family.

By extending the octyne assay to compare pairs of N-fertilized/cropped and unfertilized/noncropped soils, some new insights about octyne-resistant, AOA-dependent soil nitrification emerged. First, there was no effect of octyne on the rate of net NO_2^- plus NO_3^- production measured in noncropped soils incubated without NH_4^+, implying that AOA dominate this low level of indigenous activity. This result is consistent with results previously published in the literature showing that AOA *amoA* gene copy numbers increased in an acetylene-sensitive manner in soils incubated without supplemental NH_4^+ and are reliant upon a background net N mineralization rate of ~1 μg N/g/day (16). Second, we consistently observed that octyne-resistant nitrification in noncropped soils was significantly stimulated 5- to 8-fold (to ~2 to 9 μg N/g/day) by adding NH_4^+. The latter rate of nitrification is similar to that reported by Verhamme et al. (16), who amended a soil with a small amount of NH_4^+ that exclusively stimulated the growth of AOA and supported a rate of nitrification of ~5 μg N/g/day. Our results suggest that some soil AOA could be NH_3 limited *in situ*, despite evidence that some AOA pure cultures have an extremely high affinity for NH_3 (9). It is possible that stimulation of octyne-resistant activity by addition of NH_3 gas is due to its more even distribution throughout the soil fabric, stimulating net NO_2^- plus NO_3^- production by a greater percentage of the soil AOA than that being supported by organic N mineralization. Furthermore, it is also reasonable to speculate that NH_3 gas might be promoting the activity of a functionally distinct group of AOA that differs from the groups responding to organic N mineralization (11).

Another observation with interesting implications was the detection in two noncropped soils (PG and KW soils) of both octyne-resistant and octyne-sensitive NO_2^- plus NO_3^- production stimulated by NH_3 addition. Given the caveat that it remains a possibility that some AOA populations might contain both octyne-resistant and -sensitive phenotypes (see earlier discussion), we interpret this result to indicate that in some noncropped soils active AOB coexist in significant numbers alongside an active AOA population. In contrast, in another noncropped soil (CP soil), our previous RNP assay results (19) and the results of the current octyne assay showed that AOB contributed very little to the short-term net nitrification rate, which was explained by a low AOB abundance ($2.5 \times 10^6 \pm 0.4 \times 10^6$ *amoA* gene copies/g soil). A population of such magnitude could have accounted for ~10% of the observed rate of net nitrification, assuming a rate of 4×10^{-15} mol NH_4^+-N/cell/h (38) and two copies of *amoA* per cell (39, 40). Nonetheless, in the long-term incubation of CP soil with NH_4^+ amendments, an NH_4^+-dependent octyne-sensitive increase in AOB *amoA* copy numbers occurred, suggesting that the small AOB population is viable and proliferates in response to NH_4^+. In contrast, the short-term WS octyne assay showed that all of the NH_4^+-stimulated increase in three of four cropped soils was octyne sensitive and presumably due to AOB. Nevertheless, even though the short-term assays showed an overwhelming dominance of AOB activity after NH_4^+ amendment of cropped soils, an increase in the density of the AOA population that was octyne resistant and stimulated by a small amount of NH_4^+ was detected after about 7 days of incubation. These results clearly emphasize that AOA in the cropped soil and AOB in the noncropped soil are capable of proliferating in response to supplemental NH_4^+ within a short period of time and have the potential to change the relative contributions of AOA and AOB to soil nitrification. Further studies are needed to more accurately define the relationships between NH_4^+ availability and other factors that determine AOA and AOB population sizes, control their activities, and determine their relative contributions to soil nitrification.

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