Use of aliphatic n-alkynes to discriminate soil nitrification activities of ammonia-oxidizing thaumarchaea and bacteria

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Running title: Soil nitrification responses to aliphatic alkynes

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Abstract

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 1-alkynes C_8-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 was obtained that nitrification carried out by soil-borne AOA was also insensitive to octyne. In incubations (21 or 28 d) 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/d persisted throughout the incubation of the two soils. Other evidence that octyne-resistant nitrification was due to AOA included: 1) a positive correlation of octyne-resistant nitrification in soil slurries of cropped and noncropped soils with allyl-thiourea resistant activity (100 μM), and 2), 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.

Introduction

For about a century most ammonia (NH_3) oxidation in soils was thought to be carried out by chemolithoautotrophic NH_3-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 (17). Molecular techniques have shown that NH_3-oxidizing Thaumarchaeota (AOA) are widely distributed in soils throughout the world (20, 27). AOA are usually more numerous in soil than AOB, and in some soils AOB are below the detection limit of quantitative PCR (qPCR) (1, 21). This has led to speculation about the extent to which AOA contribute to soil nitrification (11, 29). AOA may be more metabolically versatile than AOB, with some cultured AOA growing at acid pH (18), scavenging NH_4^+ at low concentrations (22), showing mixotrophic growth on a combination of pyruvate and NH_4^+ (41), and an AOA soil population has been shown to convert organic N sources to NO_3^- (35). The evidence for AOA contributing to soil nitrification has arisen from enrichment approaches involving long incubations (4 – 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 (28, 45, 46), or by acetylene preventing an increase in gene copies of ammonia monooxygenase subunit A (*amo*A) of AOA (26, 44) supported by either mineralization of soil organic N, or by repeated amendments of low 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 AMO of AOB, and is known to inhibit NH_3-oxidation by AOA (14, 26, 42).

After exposure and removal of acetylene (6 h), the recovery of NH_3-oxidizing activity in soil slurries was monitored ±antibiotics targeted at bacterial protein synthesis to discriminate between the relative contributions of AOA and AOB to the recovered nitrification potential activity (RNP). We showed that after the removal of acetylene there was a lag before nitrite (NO_2^-) +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 nitrification potentials (NP) of soil samples taken from pastures (39), and that AOB dominate 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 two year winter wheat rotation, and were affected by the time since N fertilization, and by seasonal soil conditions (38). In the search for a strategy that would be more practical for unsaturated whole soils we reasoned that whereas monooxygenases (MO) generally have a broad substrate range, some are more restricted than others (8, 16, 30, 43). For example, AMO of *Nitrosomonas europaea* has a broad substrate range for n-alkanes (C_2 - C_8) and is inhibited by aliphatic n-alkynes of the same chain lengths (13). In contrast, other Cu-containing membrane-bound monooxygenases such as methane MO and the Cu-containing alkane MO of *Nocardioides* CF8 have a more restricted alkane substrate range ($\leq C_6$) (4, 6, 10, 34). Given that AOA AMO also falls into the Cu-containing AMO/PMO family (32) we hypothesized that it might show different sensitivity than the bacterial AMO to the effects of aliphatic n-alkynes of different chain lengths (C_2 – C_9). This manuscript reports on the results of studies carried out to assess the effects of n-alkynes

on NH_3-oxidizing activity of AOA and AOB in pure culture, and nitrification in soil slurries, and 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 – C_9) were obtained from Sigma-Aldrich, and acetylene (C_2) from Airgas (Radnor, PA).

Pure culture experiments. *(a) Sensitivity to C_2 – C_9 alkynes. N. europaea* was grown as described previously (13) and cell aliquots added to growth medium supplemented with 1mM NH_4^+ and pre-equilibrated for 30 minutes with 5 μM aqueous concentration (C_{aq}) of each alkyne (C_2 – C_9 prepared as described in Supplemental Material). NO_2^- concentrations were determined colorimetrically as described previously and monitored for 24 h (9). *N. maritimus* cultures were grown and harvested by filtration onto a 0.2 μm pore size nylon membrane (42) and incubated in artificial seawater (SCM) medium with 1mM NH_4^+ ± 20 μM alkyne (C_2 – C_9) without shaking at 30 ^oC. NO_2^- accumulation was followed for ~24 h. See Supplemental Material for experimental details.

(b) 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 pre-equilibrated with octyne (0, 1, 2 or 5 μM) for 30 minutes. Cell suspensions were shaken at room temperature (~23^oC), and NO_2^- concentrations monitored for ~24 h. *N. maritimus* cultures were incubated in SCM medium plus 1mM NH_4^+ with octyne (0, 20, 50, or 100 μM) at 30 ^oC, and NO_2^- accumulation followed for ~24 h.

(c) 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 Supplemental Material). In the case of *N. maritimus* cultures, cells were incubated on the filter membrane in SCM medium plus 1mM NH_4^+ with acetylene (30 μM) at 30 ^oC. After 6 h the filter membrane was placed in fresh SCM medium plus 1mM 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) or 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 ±antibiotics were included to demonstrate that antibiotics were not immediate inhibitors of NH_3-oxidizing activity.

Response of soil nitrification to alkynes. *(a) Response of nitrification potentials to inhibitors.* NPs were determined on soil samples as described previously (39). See Supplemental Material for description of soils and soil sampling. NP treatments included ATU (100 μM) and C_2 - 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 plus acetylene controls (data not shown), indicating all NO_2^- +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 (38, 39). In some treatments, the bacterial protein synthesis inhibitors kanamycin (800 μg/ml) plus spectinomycin (200 μg/ml) were added to prevent

resynthesis of AMO by AOB. The fraction of RNP that recovers in the presence of bacterial protein synthesis inhibitors (RNP_{ab}) is considered to be due to AOA.

(b) Response of net nitrification in whole soil to octyne during 21 – 28 d incubations. Corvallis pasture (CP) and 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) plus acetylene (6 μM soil solution concentration, C_{aq}), and (iii) plus octyne (2 μM apparent C_{aq}). Treatments were incubated at ~23^{o}C and opened weekly to allow for gas exchange and sampling of soil for DNA extraction and to determine NH_4^+ and NO_2^-+NO_3^- concentrations. See Supplemental Material for further details.

(c) Response of net nitrification in whole soil to octyne during short (2 d) incubations. Soils were incubated ±octyne, and ±NH_4^+. Supplemental dH_2O was added to achieve a soil water content approximating 0.6 of maximum water holding capacity. Aliquots of anhydrous NH_3 gas were added which supported the maximum rate of nitrification (Display S1b). Soils were incubated under three treatments: (i) no alkyne, (ii) plus acetylene (6 μM C_{aq} in soil solution), and (iii) plus octyne (2 μM apparent C_{aq} in soil solution) at ~23^{o}C for 2 d. At the end of the incubation NO_2^-+NO_3^- concentrations were determined (See Supplemental Material).

Nucleic acid analysis. DNA was extracted from soil using a MoBio PowerSoil (Carlsbad, CA) extraction kit, and quantified using a NanoDrop ND-1000 UV-Vis Spectrophotometer (ThermoScientific, Rockwood, TN) and stored at -80^{o}C. QPCR of the AOA and AOB *amo*A genes was performed as described previously (38). See Supplemental Material for more information.

Statistics. To determine whether the rate of NO_2^-+NO_3^- accumulation was different between treatment conditions, ANOVA was performed using the slopes from simple linear regressions of the repeated measurements versus time within each incubation replicate. AOA:AOB ratios of no alkyne and plus octyne treatments were log transformed, and the data were fit with a linear mixed effects model in R (http://www.r-project.org/) with random effect for sample, and fixed effects for 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 ±octyne in the presence and absence of NH_4^+ were done with a two-tailed Student's t-test. See Supplemental Material for further details.

Results

Differential sensitivity of AOA and AOB cultures to C_2 – 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 AOA, respectively) to C_2 – C_9 1-alkynes in suspensions with 1 mM NH_4^+ (Figure 1a and b). Nitrification by *N. europaea* was severely inhibited by 5 µM C_{aq} of C_3 – 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 of C_2 – C_5 alkynes, 20 µM of C_6 and C_7 only inhibited about 50% of *N. maritimus* activity, and 20 µM of C_8 and C_9 had no effect on NO_2^- accumulation.

Differential sensitivity of AOA and AOB cultures to octyne, C8. Further work with pure cultures was focused on C_8 (octyne), and compared the effective concentration range

of octyne on NH_3-oxidation by the AOBs *N. europaea* and *Nitrosospira multiformis*, with *N. maritimus*. Production of NO_2^- by *N. europaea* and *N. multiformis* was completely inhibited by 1 - 5 μM C_{aq} octyne, and there were no significant increases in NO_2^- after 2 h ($p \geq 0.07$, Figure 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 between 4 - 20 h incubation in the presence of 50 or 100 μM octyne, NO_2^- accumulation was only 56 or 12%, respectively, of 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 acetylene exposure was evaluated (Figure S1). NO_2^- accumulated immediately in a *N. maritimus* culture that had not been pre-treated 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 $C_5 – C_9$ 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 (13). Recovery of nitrification by *N. europaea* and *N. multiformis* after exposure to octyne (4 μM C_{aq}) was followed ±bacterial protein synthesis inhibitors and compared with recovery from acetylene inactivation (Figure S1). Both *N. europaea* and *N. multiformis* showed a 6 – 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 these bacterial protein synthesis inhibitors were not direct inhibitors *per se* of NO_2^- producing activity.

Effects of alkynes on nitrification potentials of soil slurries. The effects of C_2 - C_9 1-alkynes were evaluated on net NO_2^-+NO_3^- production in two soils that were shown previously with the RNP assay as having NPs dominated by either AOA (Corvallis Pasture, CP) or AOB (Corvallis Cropped, CC) (38, 39). Samples of these soils were treated with C_2 - C_9 alkynes (each 2 μM apparent C_{aq}, see Supplemental Material) in soil slurry NP assays (Figure 1c and d). In the AOB dominated CC soil there was no significant NO_2^-+NO_3^- production in treatments with alkynes ($p > 0.05$). However, in AOA dominated CP soil, whereas C_2 completely inhibited NO_2^-+NO_3^- production, there was residual accumulation of NO_2^-+NO_3^- in the presence of alkynes C_3 - C_5, but no significant effect of alkynes C_6 - C_9 compared with the control ($p > 0.1$). C_6 - C_9 alkynes did not inhibit NO_2^-+NO_3^- accumulation in CP soil when tested at 4 μM apparent C_{aq} (Figure 1d).

The effects of octyne on long-term (21 – 28 d) soil incubations. We evaluated the effects of octyne on net NO_2^-+NO_3^- accumulation and changes in AOA and AOB *amo*A gene copies during long-term (21 – 28 d) 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^+ additions (20 μmol/g soil).

After 21 d of incubation, microcosms of CC soil that received no or low NH_4^+ addition showed no significant difference in the rates of NO_2^-+NO_3^- accumulation ±2 μM apparent C_{aq} octyne ($p > 0.3$, Figure 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^-+NO_3^- accumulation between ±octyne treatments ($p < 0.001$, Figure 3c) indicating an increased contribution to nitrification by AOB (octyne-sensitive). While there was a statistically nonsignificant response of octyne-resistant rates of nitrification to NH_4^+ treatments ($p > 0.4$), NO_2^-+NO_3^- accumulation in the no alkyne treatments (AOA+AOB) increased 6- and 14-fold over the unamended treatment in response to low and high NH_4^+, respectively ($p < 0.001$). The rate of NO_2^-+NO_3^- accumulation in the no alkyne high NH_4^+ treatment was significantly greater than the rates of nitrification in the no and low NH_4^+ treatments ($p < 0.001$). Soil microcosms treated with acetylene did not accumulate NO_2^-+NO_3^- in response to any NH_4^+ treatment, and showed no increases in either AOA or AOB *amo*A gene copies. During the 21d incubation of CC, there was convincing evidence of differences between log(AOA:AOB) response to octyne and the no alkyne treatments ($p < 0.0001$). For example, AOA *amo*A gene copies increased in response to no added NH_4^+ and low added NH_4^+ treatments ±octyne (Figure 3d and e), whereas AOB *amo*A gene copies only increased in response to the high NH_4^+ treatment minus octyne (Figure 3f). In ±octyne treatments there was also strong evidence of an interaction between NH_4^+ treatment and time on log(AOA:AOB). Specifically, there was convincing evidence of an increase in log(AOA:AOB) in response to the low NH_4^+ treatment from 0 to 21 d ($p < 0.0001$), and strong evidence of increases in log(AOA:AOB) from 7 to 21d 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 *amo*A gene abundance observed by qPCR. In octyne treated incubations with either low or high NH_4^+, ~3.3 µmol NO_2^-+NO_3^- /g soil accumulated and accompanied by an AOA *amo*A cell yield of 2.8 x 10^7 – 7.7 x 10^7cells/µmol NH_4^+. This yield compares favorably with the cell yields measured in cultures of *N. maritimus* (17)*, Nitrosocaldus yellowstonii* (7)*,* and pyruvate assisted *N. viennensis* (1.6 x 10^7 - 4.7 x 10^7cells/µmol NH_4^+(41)), and with the AOA yield measured in soil microcosms incubated with low additions of NH_4^+ (2.3 x 10^7 – 2.9 x 10^7cells/µmol NH_4^+ (44)).

During the 28 d incubation of CP soil, microcosms that received no NH_4^+ addition expressed the same rate of net NO_2^-+NO_3^- accumulation ±octyne (Figure S2a), whereas NO_2^-+NO_3^- accumulation in microcosms amended with low or high NH_4^+ were significantly greater in the no alkyne treatments than in the plus octyne treatments (p < 0.001, Figure S2b and c). 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+AOB) rates of NO_2^-+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^-+NO_3^-, and there were no increases in AOA and AOB *amo*A gene copies. During the 28 d incubation of CP there was strong evidence of a difference in the log(AOA:AOB) response between octyne and the no alkyne treatments (p = 0.007). In the no alkyne treatment AOB *amo*A gene copies increased 10- and 100-fold in response to low and high NH_4^+ respectively (Figure S2e and f), whereas AOA *amo*A did not change in either the no alkyne or octyne

treatments. As a consequence, log(AOA:AOB) ratios were lower in the high NH_4^+ treatment compared with the no NH_4^+ treatment ($p = 0.03$, Figure S2d). Because there were no detectable AOA *amo*A gene copy 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^-+NO_3^- producing activity. CP soil was acetylene inactivated, and subjected to a RNP experiment where soil slurries were exposed to a combination of antibiotics targeted at bacterial protein synthesis (ab, kanamycin and spectinomycin, 800 and 200 μg/ml, respectively), octyne (oct, 2 μM apparent C_{aq}), or a combination of antibiotics and octyne. Rates of RNP_{ab}, RNP_{oct} and RNP_{ab+oct} were the same (0.36± 0.03 μmol NO_2^-+NO_3^-/g soil/d) demonstrating that AOA in CP soil were able to synthesize active AMO proteins and resume NO_2^-+NO_3^- production in the combined presence of octyne and antibiotics. Considering the AOA yield observed in the cropped soil microcosms, it is unlikely that a statistically significant increase of this magnitude could be measured over the background AOA *amo*A gene copy 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^-+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 pH, texture, and population sizes of AOA and AOB (Table S1). 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^-+NO_3^- accumulation in NP

slurries were compared with the effects of 100 μM ATU and also with the RNP response ±bacterial protein synthesis inhibitors. The sensitivities of NPs to octyne and ATU over the wide range of soils were strongly and positively correlated (Figure 4a, $r^2 = 0.938$) showing a range of sensitivity 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, Figure 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 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 and Pendleton grassland (KW and PG, respectively, with AOA:AOB ratios of 45 and 60), showing an intermediate effect of octyne, ATU, and RNP_{ab}, suggesting that both AOA and AOB populations were capable of contributing to NPs.

A comparison of the effects of octyne on nitrification in whole soil (WS) and soil slurry (NP) assays. The effects of octyne (2 μM apparent C_{aq}) on nitrification were compared between WS and NP assays (Display S1a). 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, Display S1b) to support the maximum rate of nitrification. The fraction of the maximum WS rate of net $NO_2^-+NO_3^-$ 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 d) WS incubations.

From 'potential' assay 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^- +NO_3^- accumulated over the 2 d incubation ($p < 0.05$), and there was no significant difference between ±octyne ($p > 0.8$) indicating that AOA contributed all nitrification under this treatment. In the majority of cropped soils, insufficient NO_2^-+NO_3^- accumulated in the NH_4^+-0 treatment to draw any inference about ±octyne effects. Addition of NH_4^+-sat lead to significantly greater rates of octyne-sensitive NO_2^-+NO_3^- production by AOB (total nitrification rate – octyne-resistant rate) in all soils except CP ($p < 0.05$), and resulted in ≤ 0.14 of the WS rate contributed by AOA in the cropped soils. In the noncropped soils, NH_4^+-sat significantly increased octyne-resistant rates of NO_2^- +NO_3^- production ($p < 0.05$), and a high fraction (0.62 – 0.96) of the NH_4^+-sat rate remained due to AOA. By contrast, in cropped soils treated with NH_4^+-sat, the rate of nitrification contributed by AOA increased significantly ($p < 0.05$) only in CC.

Discussion

In this study we established that the octyne method could be used in relatively short assays (24 - 48 h) in both soils slurries and whole soils microcosms. The soils exhibited a wide range of nitrification rates that were completely inactivated by acetylene 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 other short-term methods used to assess the contributions of AOA activity, including ATU resistance and RNP plus 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 Thaumarchaeota, and until more isolates are available that are representative of this phylum 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 show a range of sensitivity. Recently it was shown that NO_2^- production by the obligate acidophilic AOA *Nitrosotalea devanaterra* was completely inhibited by 100 μM ATU (19). However, this response to ATU may not be representative of AOA occupying less acidic soils. For instance, *Ca.* Nitrososphaera viennensis, isolated from neutral soil and demonstrating optimal growth at pH ~7.5, required 500 μM ATU to stop the majority of nitrification (33). Second, although we have had success in measuring the relative contributions of AOA and AOB to NP in soil slurries by using the RNP ±antibiotics assay (39), 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 (36, 37), (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 literature describing a variable n-alkane chain length substrate range of prokaryote Cu-containing MOs, that might be linked with sensitivity/resistance to different chain length aliphatic n-alkynes. 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 - C_8) that are AMO substrates (13). The relative insensitivity of *N. maritimus* AMO to C_6 - C_9 alkynes may infer that it has a more restricted alkane substrate range than that of *N. europaea* AMO, or that C_6 - C_9 alkynes are much poorer substrates and inactivators. Other investigators have also been interested in the potential of AOA to oxidize hydrocarbons (23). 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/PMO enzyme family.

By extending the octyne assay to compare pairs of N fertilized/cropped and unfertilized/noncropped soils, some new insights emerged about octyne-resistant, AOA-dependent soil nitrification. First, there was no effect of octyne on the rate of net NO_2^- +NO_3^- production measured in noncropped soils incubated without NH_4^+, implying that AOA dominated the low indigenous activity. This result is consistent with earlier literature showing that AOA *amo*A gene copies increased in an acetylene-sensitive manner in soils incubated without supplemental NH_4^+ and reliant upon a background net N mineralization rate of ~1 μg N/g/d (44). Second, we consistently observed that octyne-resistant nitrification in noncropped soils was significantly stimulated 5- to 8-fold (to ~2 - 9 μg N/g/d) by adding NH_4^+. The latter rate of nitrification is similar to that reported by

Verhamme et al. (2011) who amended a soil with a low amount of NH_4^+ that exclusively stimulated growth of AOA and supported a rate of nitrification of ~5 µg N/g/d. 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 (22). It is possible that stimulation of octyne-resistant activity by additions of NH_3 gas is due to its more even distribution throughout the soil fabric, stimulating net NO_2^-+NO_3^- production by a greater percentage of the soil AOA than those 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 those responding to organic N mineralization (35).

Another observation with interesting implications was the detection in two noncropped soils (PG and KW) of both octyne-resistant and octyne-sensitive NO_2^-+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 co-exist in significant numbers alongside an active AOA population. In contrast, in another noncropped soil (CP), our previous RNP results (39) 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± 0.4 x 10^6 *amo*A 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 x 10^{-15} mol NH_4^+-N/cell/h (2), and two copies of *amo*A/cell (5, 25). Nonetheless, in the long-term incubation of CP soil with NH_4^+ amendments, an NH_4^+-dependent octyne-sensitive increase in AOB *amo*A

copies occurred, suggesting that the small AOB population is viable and will proliferate in response to NH_4^+. By 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. Yet, despite the short-term assays showing overwhelming dominance of AOB activity after NH_4^+ amendment of cropped soils, an octyne-resistant and low NH_4^+-stimulated increase in AOA population density was detected after about 7 d 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 with 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 their relative contributions to soil nitrification.

Acknowledgements. This research was supported by USDA NIFA Award No. 2012-67019-3028, US Department of Agriculture (Grant 2005-35319), an Oregon Agricultural Research Foundation competitive grant, the Oregon Agricultural Experiment Station, and the Oregon State University Provost Distinguished Graduate Fellowship. Additional support obtained from the Oregon State University community included: QPCR facilities at the Center for Genome Research and Biocomputing, field sites maintained by the Hyslop Field Research Laboratory, Columbia Basin Agricultural Research Center, Klamath Basin Research and Extension Center, and the Central Oregon Agricultural Research Center. *N. maritimus* was generously provided by David Stahl, University of Washington.

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Figures

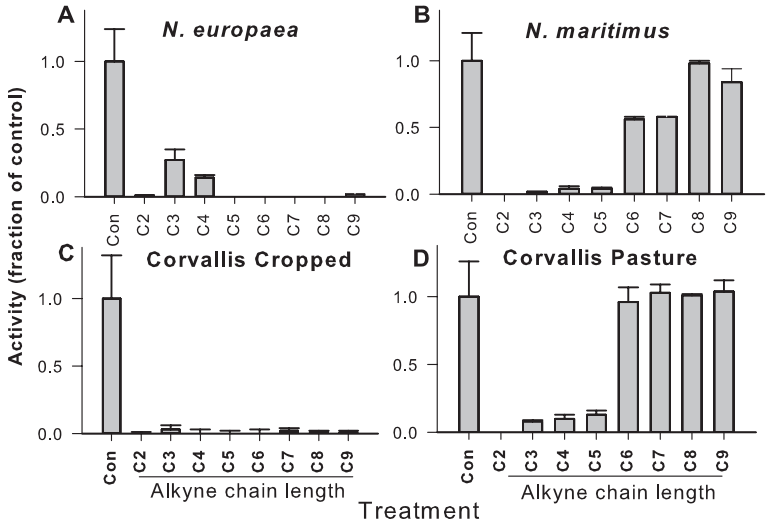


Figure 1. Response of NO_2^- production to C_2 – C_9 1-alkynes by: a) *N. europaea* and b) *N. maritimus* cultures. *N. europaea* and *N. maritimus* were incubated with 5 μM and 20 μM (C_{aq}), respectively, of each alkyne in the presence of 1 mM NH_4^+. The effect of C_2 - C_9 alkynes on net NO_2^-+NO_3^- production by soil slurries of: c) Corvallis Cropped soil, and d) Corvallis Pasture soil incubated with 2 and 4 μM (apparent C_{aq}, see Supplementary Information) respectively, of each alkyne in the presence of 1mM NH_4^+. See Materials and Methods for further experimental details. NO_2^- (a and b) or NO_2^-+NO_3^- (c and d) production in the presence of each alkyne was compared with a no inhibitor control (Con). Error bars represent the SD of the mean (n = 3).

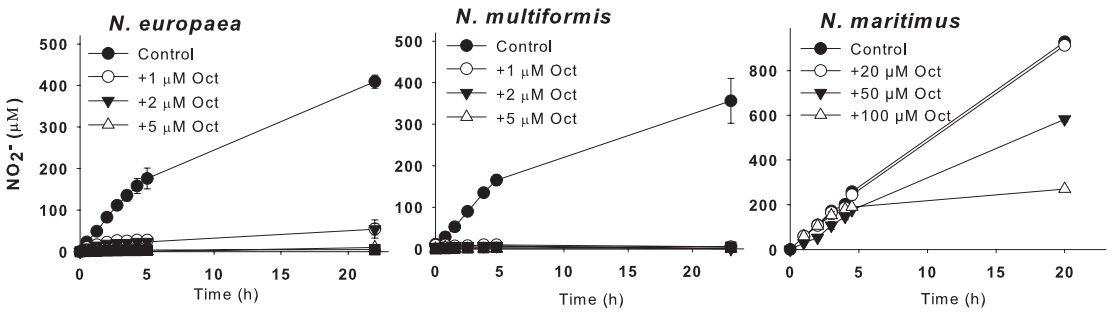


Figure 2. Sensitivity of NO_2^- production by a) *N. europaea,* b) *N. multiformis* and c) *N. maritimus* to different octyne (Oct) concentrations. *N. europaea* and *N. multiformis* cultures were suspended in 30 mM TES buffer (pH 7.2) with 1 mM NH_4^+. *N. maritimus* cells were suspended in SCM medium with 1mM NH_4^+(pH 7.5). Loss of linearity of NO_2^- production by *N. europaea* and *N. multiformis* after 5 h was likely due to 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).

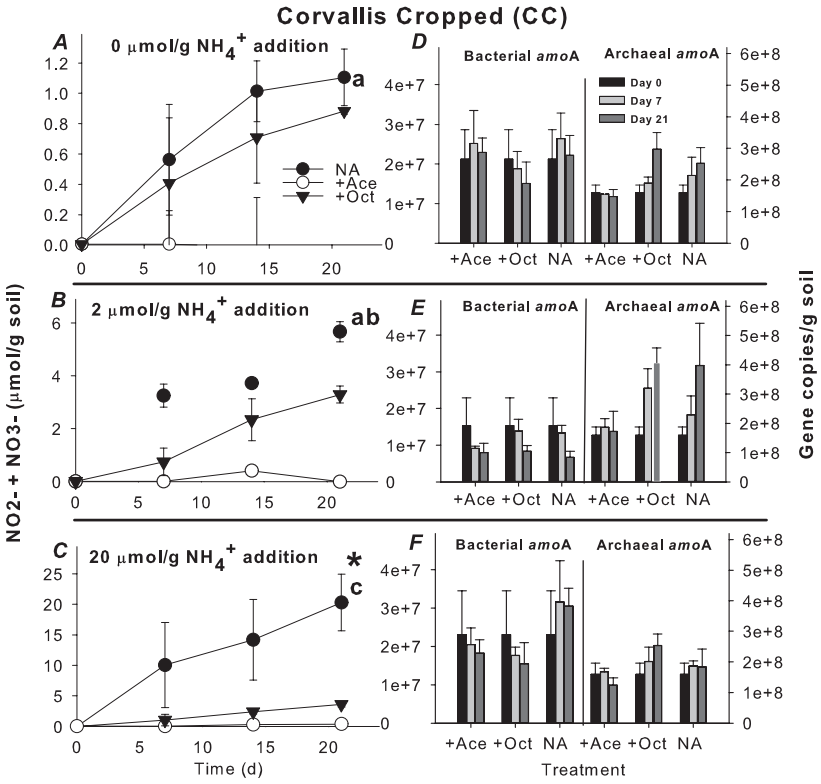


Figure 3. A comparison of the effects of alkyne (acetylene, +Ace; octyne, +Oct) and no alkyne (NA) treatments on net NO_2^-+NO_3^- accumulation and on AOA and AOB *amo*A gene copies during incubations of Corvallis Cropped (CC) soil amended with no (a and d), low (b and e), or high (c and f) NH_4^+ additions (0, 2, and 20 μmol NH_4^+/g soil, respectively). a, b, and c represent NO_2^-+NO_3^- accumulation over the time course of the experiment. Error bars represent the SD of the mean NO_2^-+NO_3^- concentration (n = 3). d, e, and f represent AOB and AOA *amo*A gene copies/g soil. Error bars represent the SD of the average *amo*A gene copies/g soil of triplicate qPCR reaction for each treatment (n = 3). The asterisk (*) indicates that NO_2^-+NO_3^- accumulation was significantly different in the NA and +Oct treatments within a NH_4^+ treatment ($p < 0.001$). Different lower case letters indicate that NO_2^-+NO_3^- accumulation was significantly different in the NA

treatment between NH_4^+ levels ($p < 0.001$). There was no significant difference in the rate of NO_2^-+NO_3^- accumulation in +Oct treatment between NH_4^+ levels.

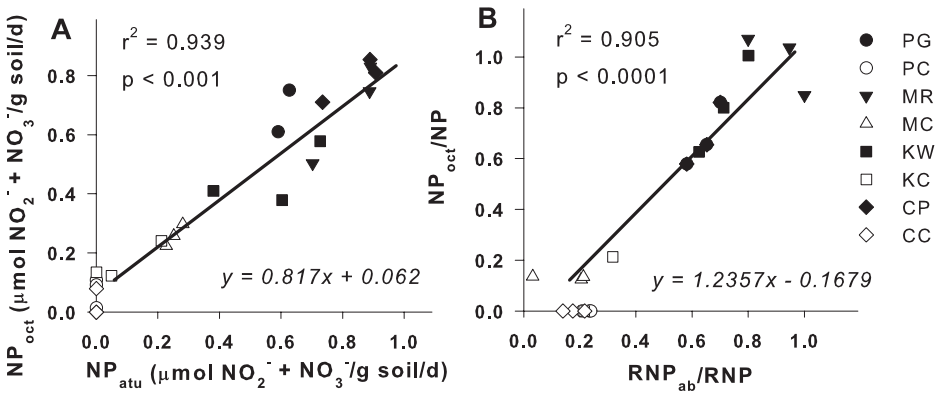


Figure 4. Correlation among three methods used to assess the relative contributions of AOA and AOB to soil slurry NPs of diverse Oregon soils. a) Correlation between the rates of net NO_2^-+NO_3^- production that were resistant to octyne (NP_{oct}) versus resistant to 100 µM ATU (NP_{atu}). b) Correlation between the fractions of the recovered nitrification potentials (RNP) that were insensitive to bacterial protein synthesis inhibitors (RNP_{ab}/RNP) versus the fractions of activity resistant to octyne (NP_{oct}/NP). Open and closed symbols indicate cropped and noncropped soils, respectively. See Table S1 for site locations and soil characteristics.

Table 1. Rates of net NO_2^-+NO_3^- accumulation in response to no (NH_4^+-0), and saturating (NH_4^+-sat) NH_4^+ additions by AOA (octyne-resistant) and AOB (octyne-sensitive = total nitrification – octyne-resistant), during 2 d whole soil incubations. See Display S1b for NH_4^+-sat concentrations, and Table S1 for site locations and soil characteristics. Values in parentheses are SD of the mean (n = 3).

	Soils	NH_4^+-0		NH_4^+-sat	
		AOB	AOA	AOB	AOA
		(μg N/g soil/d)		(μg N/g soil/d)	
Noncropped	PG	0.2 (0.2)	1.2 (0.3)*	3.9 (0.9)[a]	6.1 (0.4)[a]
	MR	0.0 (0.7)	1.0 (0.1)*	0.7 (0.1)[a]	6.5 (0.1)[a]
	KW	0.1 (0.0)	0.7 (0.1)*	3.0 (0.5)[a]	4.7 (0.5)[a]
	CP	0.0 (0.3)	1.1 (0.3)*	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)*	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]

*Significant increase in net NO_2^-+NO_3^- accumulation under NH_4^+-0 ($p<0.05$), and no significant difference between ±octyne treatments ($p>0.05$).

[a]Significant increase in net NO_2^-+NO_3^- accumulation of both octyne-resistant (AOA) and octyne-sensitive (AOB) activities in NH_4^+-sat versus NH_4^+-0 treatments ($p\leq0.05$).

[b]Significant increase in net NO_2^-+NO_3^- accumulation of octyne-sensitive (AOB) activities in NH_4^+-sat versus NH_4^+-0 treatments ($p\leq0.05$).

Supplemental Material

Materials and Methods

Preparing n-alkyne stocks. Alkynes C_2 - C_4 exist in gaseous form at normal room temperature and pressure, and were diluted 10-fold (15 ml gas into a 155 ml bottle capped with a black phenolic cap fitted with a gray butyl stopper containing several 6 mm dia. glass beads). Alkynes C_5 - C_9 exist in neat liquid form at normal room temperature and pressure, and stocks were prepared by adding 40 µl neat liquid alkyne to a glass bottle (total volume 155 ml) containing several glass beads and quickly capped with a black phenolic cap fitted with a gray butyl stopper. Bottles were over-pressured with 100 ml air and shaken briskly for 30 s to distribute the alkyne evenly through the airspace. The alkyne concentrations in bottles prepared this way were determined by gas chromatography. The volume of gas stock required to achieve specific aqueous phase concentrations (C_{aq}) under experimental conditions was calculated from the air/water phase partitioning Henry's constant (http://www.ceset.unicamp.br/~mariaacm/ST405/Lei de Henry.pdf). Copy and paste URL into web browser if link does not open.

Selection of alkyne concentrations. Alkyne concentrations were chosen to be high enough to ensure inhibition but low enough to minimize time spent degassing to remove alkynes during inactivation and recovery experiments. In preliminary work, concentrations of acetylene as low as 0.01 kPa (0.01% vol/vol, or 4.3 µM in aqueous solution) completely inhibited nitrification in soil and pure culture, but to ensure inhibition 6 µM acetylene is routinely used in aqueous solution (C_{aq}). In the case of octyne, apparent concentrations from 0.1 to 10 µM C_{aq} completely inhibited nitrification in Corvallis Cropped soil, and *Nitrosomonas europaea* and *Nitrosospira multiformis* were inhibited by ≥ 1 µM C_{aq}. Octyne

may bind to soil, so gas chromatography was used to check headspace concentrations in the presence and absence of soil once the decision was made to focus exclusively on this alkyne. Octyne binding varied with each soil, but when octyne was added to achieve 2 μM apparent C_{aq}, there was sufficient alkyne in the headspace to partition into the aqueous phase at >1 μM. Headspace analysis confirmed that when alkyne was added at an apparent C_{aq} > 2 μM that the percentage of alkyne bound to soil was insignificant and C_{aq} could be predicted by the Henry's constant.

Pure culture experiments. Nitrification potential **(**NP) conditions (pH 7.2 and 1 mM NH_4^+) were simulated when possible, and catalytic potential was used to normalize pure culture experiments to make valid comparisons between ammonia oxidizing cultures of different cell size and rate of activity per cell (2, 3, 15, 17). We reasoned that if this rate was within the range of rates observed in soil nitrification potential assays, we could also compare effects of octyne in pure culture to those in soils. The NP rates of soil slurries used in this study usually range between 10 and 60 nmol NO_2^-+NO_3^-/ml/h**;** therefore, cell densities of AOA and AOB pure cultures were prepared to achieve rates of NO_2^- production that fell within this range.

Sensitivity to C_2 – C_9 alkynes. *N. europaea* or *N. multiformis* cells were grown at 27^{o}C as described previously (13, 24) and harvested by centrifugation. Bottles containing growth medium supplemented with 1mM NH_4^+ were pre-equilibrated for 30 minutes with 5 μM C_{aq} of each alkyne (C_2 – C_9 stocks were prepared as described above) before addition of aliquots of cell suspensions. Bottles were shaken at 27^{o}C with shaking (100 rpm) and NO_2^- accumulation was monitored for 24 h. NO_2^- concentrations were determined colorimetrically as described previously (9). *N. maritimus* cultures were

grown at 30°C in synthetic Crenarchaeota media (SCM) containing 1mM NH_4^+, and harvested as described previously (42). *N. maritimus* cells were incubated in SCM medium with 1mM NH_4^+ ± 20 μM alkyne (C_2 – C_9) without shaking at 30 °C and NO_2^- accumulation was followed for ~24 h.

Octyne sensitivity. To simulate NP assay conditions, *N. europaea* or *N. multiformis* cell suspensions were added to 155 ml glass bottles with black phenolic caps and gray butyl stoppers containing 10 ml of 30 mM TES buffer (pH 7.2) plus 1 mM NH_4^+ that had been pre-equilibrated with octyne (0, 1, 2 or 5 μM) for 30 minutes. Cell suspensions were shaken at room temperature (~23°C), and NO_2^- concentrations measured hourly from 0 to 5 h, and then again at ~24 h. *N. maritimus* cultures were grown and harvested as described above, and incubated in SCM medium plus 1mM NH_4^+ with octyne (0, 20, 50, or 100 μM) at 30 °C, and NO_2^- accumulation followed for ~24 h.

Inactivation of AMO by alkynes and protein synthesis dependent recovery. *N. europaea*, and *N. multiformis* cultures were grown and harvested as described above, and aliquots of cell suspensions were added to 155 ml glass bottles with black phenolic caps and gray butyl stoppers containing 10 ml of either *N. europaea* or *N. multiformis* growth media, pre-equilibrated with either 4 μM octyne or 6 μM acetylene. Cell suspensions were shaken at room temperature (~23°C). After 2 h of exposure to either octyne or acetylene, all bottles were degassed for 6 min using a vacuum manifold at -100 kPa to remove the alkynes. Recovery of NH_3-oxidation was monitored for 48 h by following the accumulation of NO_2^-.

Collection of soils. Soil samples, representing different soil types from different regions of Oregon, were collected from cropped fields and noncropped locations (3 replicates of

each) at Oregon State University Agricultural Experimental Stations located in Corvallis, Pendleton, Madras, and Klamath Falls, Oregon (Supplemental Table SI 1). Four to five soil samples were recovered to a depth of 10 cm from each replicate site via a random walk process. A composited sample was prepared for each replicate site and brought to the laboratory where they were sieved <4.75 mm. Soil samples (0.25 g) were stored at -80^{o}C for future DNA extraction and quantification of AOA and AOB *amo*A gene copies by qPCR. The remaining soils were stored at 4^{o}C prior to experimentation.

Response of net nitrification in whole soil to octyne during 21 – 28 d incubations. Soils were collected from a pasture (CP) and a wheat cropped field (CC). Ten gram portions of soil (a composite of three field replicates) were placed in 155 ml glass bottles with 0.5 ml dH_2O to achieve a moisture content approximating field capacity (0.42 and 0.33 g water/g oven dry soil for CP and CC, respectively). Bottles were capped with black phenolic caps and gray butyl stoppers, and three NH_4^+ levels were established by adding aliquots of anhydrous NH_3 gas to achieve 0, 2, and 20 μmol/g soil added NH_4^+. Three treatments were imposed at each NH_4^+level: (i) no alkyne amendment, (ii) plus acetylene (6 μM soil solution concentration, C_{aq}), and (iii) plus octyne (2 μM apparent C_{aq}, see previous section), each in triplicate. Bottles were incubated in the dark at ~23^{o}C, and opened weekly to allow for gas exchange and sampling. Soil aliquots (0.25 g) were removed and stored at -80^{o}C for future DNA extraction. A second aliquot (1.5 g) was removed and added to 15 ml of water in a 155 ml bottle and shaken (200 rpm) for 15 min. Aliquots (1.8 ml) of soil slurry were removed to microcentrifuge tubes, centrifuged for 3 min at 13.4 x10^3 g, and NO_2^- +NO_3^- concentrations were determined immediately on the supernatants (12) and expressed on an oven-dried weight of soil basis. Initial

concentrations of NO_2^-+NO_3^- were subtracted from all time points. There was no significant change in background NO_3^- concentrations in plus acetylene controls in either soil (data not shown), indicating there was no significant NO_3^- sink; however, rates of NO_2^-+NO_3^- accumulation should be considered as net nitrification rates. After sampling, bottles were recapped and acetylene and octyne applied at the same concentration as when the incubation was established. NH_3 gas was added as needed to maintain the target NH_4^+ concentrations.

Response of net nitrification in whole soil to octyne during short (2 d) incubations. Cropped and noncropped soils were incubated ±octyne, and ±NH_4^+. 2.5 gram portions of soil (a composite of the three field replicates) were placed in 155 ml glass bottles with supplemental dH_2O to achieve a water content approximating 0.6 of maximum water holding capacity. Bottles were capped with black phenolic caps with gray butyl stoppers, and aliquots of anhydrous NH_3 gas were added sufficient to support the maximum rate of nitrification. These NH_4^+ concentrations were determined in preliminary experiments, and ranged from 2 - 20 μmol/g soil (Supplemental Display S1b). Soils were incubated under three treatments: (i) no alkyne, (ii) plus acetylene (6 μM C_{aq} in soil solution), and (iii) plus octyne (2 μM apparent C_{aq} in soil solution). Bottles were incubated in the dark at ~23^oC for 2 d. At the end of the incubation 15 ml of water was added and bottles shaken (200 rpm) for 15 min. NO_2^-+NO_3^- concentrations were determined as described above.

Quantitative PCR of the archaeal and bacterial amoA genes. QPCR of the AOA and AOB *amo*A genes was performed using the HotStart-IT SYBR® Green qPCR Master Mix (USB, Santa Clara, CA) and an ABI 7500 Real Time PCR System (Foster City, CA).

Each 20 μL reaction volume included 1 ng template DNA. Primers CrenamoA23f and CrenamoA616r (40) were used to quantify AOA *amo*A gene abundance. Primers (amoA_1R and amoA_2F) and thermal cycler protocols for bacterial *amo*A genes are described elsewhere (31). Standard curves were constructed with 4.6×10^{1} to 4.6×10^{-4} ng *Nitrosomonas europaea* genomic DNA (bacterial *amo*A, efficiency=98±9%, R^2 avg =0.97±0.02) or 54.1×10^{0} to 5.41×10^{-5} ng of '*Candidatus* Nitrosopumilus maritimus' strain SCM1 genomic DNA (efficiency =105±8%, R^2 avg. =0.97±0.01). Each reaction was run in triplicate. Copy numbers were standardized to the mass of DNA extracted per g oven dry soil.

Statistics. The experimental design of the 21 and 28 d incubations is characterized as a 3 x 3 factorial (inhibitor, NH_4^+ treatment, and sampling time) with repeated measurements and bi-variate response. The plus acetylene treatments were run as biological controls, no effects were expected and none were observed, allowing the elimination of this treatment from further analysis. To determine whether the rate of NO_2^-+NO_3^- accumulation was different between treatment conditions, ANOVA was performed using the slopes from simple linear regressions of the repeated measurements versus time within each incubation replicate. AOA:AOB ratios of no alkyne and plus octyne treatments were log transformed to simplify the analysis by reducing the bi-variate response to a univariate one, and by eliminating the substantial skewness in the distributions of the gene copy counts. Another simplification was to use the average of the three qPCR replicates as the response for each treatment combination, reducing the data to the three experimental replicates for each treatment combination. The data were fit with a linear mixed effects model in R (http://www.r-project.org/) with random effect for sample, and fixed effects

for 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 ±octyne in the presence and absence of NH_4^+ were done with a two-tailed Student's t-test.

Figures and Tables

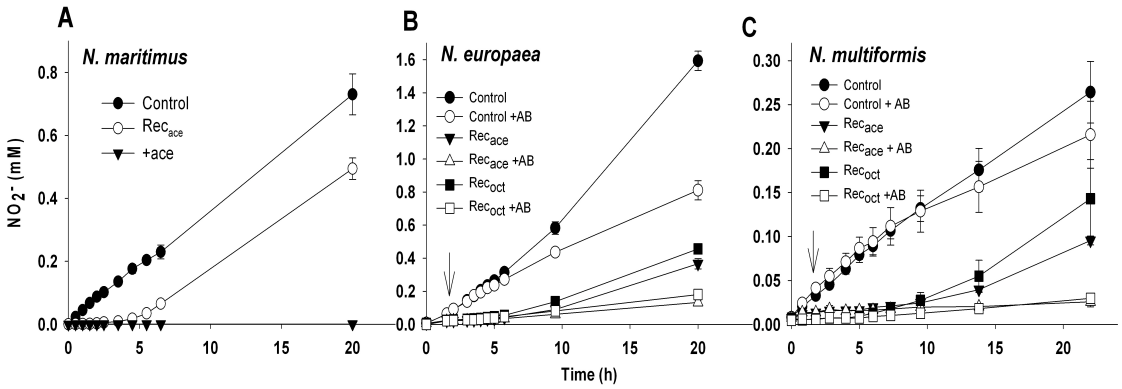


Figure S1. The time course of recovery (Rec) of NO_2^- production of a) the AOA *N. maritimus,* and b) the AOB *N. europaea* and c) *N. multiformis* in their respective growth media after exposure to acetylene (ace) or octyne (oct). *N. maritimus* cultures harvested onto nylon membranes were exposed to acetylene for 6 h, after which the membranes plus attached cells were moved to fresh media without acetylene to initiate recovery. AOB cultures were exposed to acetylene or octyne for 2 h. Arrows indicate when the AOB cultures were degassed to remove the alkynes, and the bacterial protein synthesis inhibitors kanamycin or spectinomycin (AB, 200 μg/ml each) were added to *N. europaea* or *N. multiformis,* respectively. See Materials and Methods for further experimental details. Error bars represent the SD of the mean (n = 3).

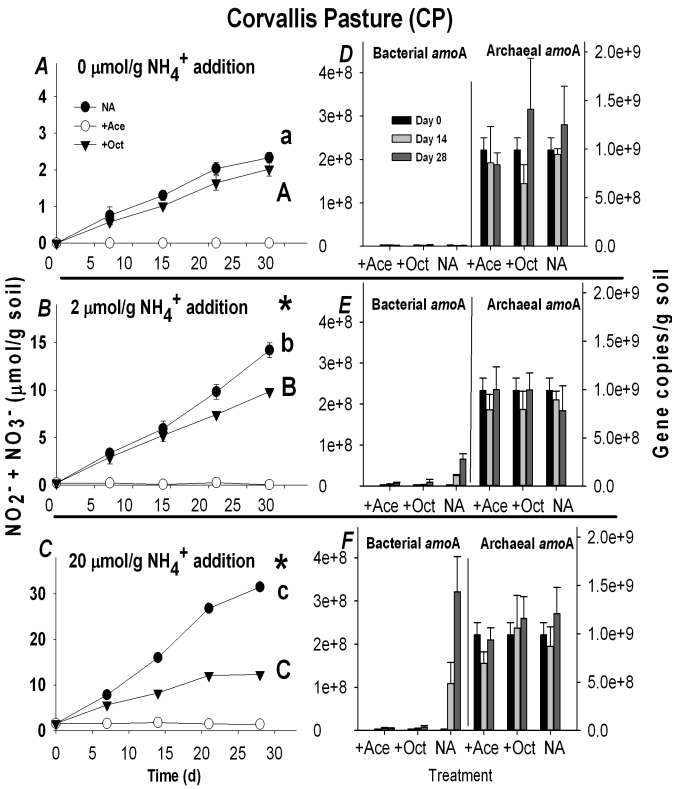


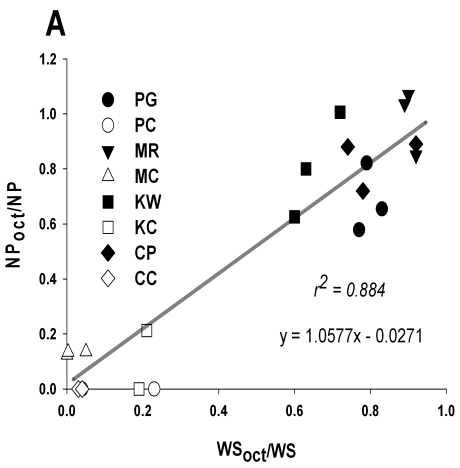
Figure S2. A comparison of the effects of alkyne (acetylene, +Ace; octyne, +Oct) and no alkyne (NA) treatments on net NO_2^-+NO_3^- accumulation and on AOA and AOB *amo*A gene copies during incubations of Corvallis Pasture (CP) soil amended with no (a and d), low (b and e), or high (c and f) NH_4^+ additions (0, 2, and 20 μmol NH_4^+/g soil, respectively). a, b, and c represent NO_2^-+NO_3^- accumulation over the time course of the experiment; error bars represent the SD of the mean NO_2^-+NO_3^- concentration (n = 3). d, e, and f represent changes in AOB and AOA *amo*A gene copies/g soil. Initial AOB *amo*A gene copies were 2.5 ± 0.4 x 10^6/g soil. See Materials and Methods for further experimental details. Error bars represent the SD of the average *amo*A gene copies/g soil of triplicate qPCR reaction for each treatment (n = 3). An asterisk (*) indicates that the rate of NO_2^-+NO_3^- accumulation was significantly different in the NA and +Oct treatments within a NH_4^+ level ($p < 0.001$). Different lower case letters indicate a significant difference between NH_4^+ treatments ($p < 0.001$) in the rate of NO_2^-+NO_3^-

accumulation in the NA treatments. Different upper case letters indicate a significant difference between NH_4^+ treatments ($p < 0.001$) in the rate of NO_2^-+NO_3^- accumulation in the +Oct treatment.

Table S1. Selected characteristics of soils collected from the Oregon State University agricultural research stations used in this study. pH values are an average of the mean of the field replicates (n = 3), with the accompanying SD of the mean. Total C and N represent values obtained from a composite sample prepared by mixing individual samples from each of the field replicates. AOA and AOB *amo*A copies/g soil are the average of three technical replicates of each of three field replicates (n = 3), with the accompanying SD of the mean.

Agricultural Station	**Pendleton**		**Madras**		**Klamath Falls**		**Corvallis**	
	grassland **(PG)**/cropped **(PC)**		rangeland **(MR)**/cropped **(MC)**		woodlot **(KW)**/cropped **(KC)**		pasture **(CP)**	cropped **(CC)**
Lat/Long	45°43'14"/118°37'35"		44°38'12"/121°07'58"		42°10'52"/121°44'17"		44°40'44"/123°15'54"	44°38'03"/123°11'24"
Soil classification	Typic Haploxeroll		Dystric Cryochrept		Torripsammetic Haploxeroll		Vertic Haploxeroll	Aquultic Argixeroll
Soil Series	Walla Walla silt loam		Madras sandy loam		Forndey sandy loam		Witham clay loam	Woodburn silt loam
pH	7.5±0.1	6.2±0.8	7.5±0.1	7.0±0.7	7.3±0.2	6.8±0.1	6.3±0.3	5.7±0.1
Total C (g/kg)	20.7	10.6	8.7	8.7	13.4	6.6	53.1	12.9
Total N (g/kg)	1.8	0.9	0.9	0.8	1.1	0.6	3.9	0.6
AOA *amo*A*	352±197	123±73	474±47	283±244	419±228	307±48	990±130	160±24
AOB *amo*A*	5.9±2.6	5.6±0.9	0.5±0.2	15.6±15	9.4±8.7	9.8±2.1	2.5±0.9	21.0±7.4
AOA:AOB ratio	60	22	948	18	45	31	396	8

*x10^6 copies/g soil.



B

Concentrations of NH_3 required to support the maximum rate of nitrification in WS incubations

Soil	Added anhydrous NH_3 µmol/g soil
PG	5
PC	20
MR	5
MC	10
KW	20
KC	20
CP	20
CC	20

Display S1. a) A comparison of the effects of octyne on the NH_4^+ saturated rate of net NO_2^-+ NO_3^- accumulation by whole soils (WS) and soil slurries (NP) of diverse cropped and noncropped Oregon soils. Assays were performed as described in Materials and Methods; WS incubations were supplemented with sufficient anhydrous NH_3 gas to maximize the rate of nitrification (b) and NP assays were supplemented with 1 mM NH_4^+. WS_{oct}/WS represents the fraction of net NO_2^-+NO_3^- accumulation in WS incubations that was resistant to octyne, and NP_{oct}/NP represents the fraction of net NO_2^- +NO_3^- accumulation in NP assays that was resistant to octyne. Filled and open symbols represent noncropped and cropped soils, respectively. See Supplemental Table S1 for soil descriptions.

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