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Linking Diversity and Stable Isotope Fractionation in Ammonia-Oxidizing Bacteria

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The link between similarity in amino acid sequence for ammonia monooxygenase (AMO) and isotopic discrimination for ammonia oxidation (ε_{AMO}) was investigated in β-subdivision ammonia-oxidizing bacteria. The isotope effects for ammonia oxidation in pure cultures of the nitrifying strains Nitrosomonas marina*,* Nitrosomonas *C-113a,* Nitrosospira tenuis*,* Nitrosomonas europaea*, and* Nitrosomonas eutropha *ranged from 14.2‰ to 38.2‰. The differences in isotope effects could not be readily explained by differential rates of ammonia oxidation, transport of NH_4^+, or accumulation of NH_2OH or N_2O among the strains. The major similarities and differences observed in ε_{AMO} are, however, paralleled by similarities and differences in amino acid sequences for the α-subunit of AMO (AmoA). Robust differences in ε_{AMO} among nitrifying bacteria may be expected to influence the stable isotopic signatures of nitrous oxide (N_2O) produced in various environments.*

Keywords ammonia monooxygenase, ammonia-oxidizing bacteria, nitrification, stable isotope fractionation

Introduction

Ammonia-oxidizing nitrifying bacteria play a key role in the regeneration of nitrate (NO_3^-) and the production of N_2O in many marine, estuarine, and terrestrial ecosystems. The importance of ammonia-oxidizers in the nitrogen cycle has led to interest in understanding their species diversity, distribution, and activity in relation to environmental variables. The diversity of ammonia-oxidizing bacteria has been explored through morphological, physiological, inmmunological, and genetic features (Ward and Carlucci 1985; Koops et al. 1991; Bothe et al. 2000; Kowalchuk and Stephen 2001). Most cultured isolates of ammonia-oxidizing nitrifiers can be assigned to one of two genera in the β-subdivision of the Proteobacteria, *Nitrosomonas* and *Nitrosospira*, while a few characterized ammonia-oxidizers

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belong to the γ-subdivision of the Proteobacteria (Head et al. 1993; Teske et al. 1994; Purkhold et al. 2000; Aakra et al. 2001). The scope of nitrifier diversity is expanded by nitrifier 16S rDNA clone libraries obtained from the environment, which contain sequences distinct from the cultured strains (Stephen et al. 1996; Purkhold et al. 2000; Smith et al. 2001; Hollibaugh et al. 2002).

The question of whether particular ammonia-oxidizers are capable of thriving in variable environments or whether populations of ammonia-oxidizers shift in response to environmental changes has been addressed in a few studies (Kowalchuk et al. 2000; de Bie et al. 2001). Studies have also shown that certain physiological traits, such as urease activity, saturation constants for ammonia (K_m), as well as ammonia, pH, and salt tolerance are consistent with 16S rRNA groupings (Koops et al. 1991; Stehr et al. 1995; Pommerening-Roser et al. 1996; Suwa et al. 1997; Jiang and Bakken 1999a). These different abilities to thrive in various environmental conditions, or niches, can allow nitrification to occur under a broad range of pH, NH_3, and O_2 concentrations and may explain the wide distribution and diversity of ammonia-oxidizers in oxic environments. This phenotypic or "functional diversity" that impacts the geochemistry in an environment is a concept referred to here as "biogeochemical diversity."

Functional diversity that is important for success in different environments may be reflected not only in 16S rRNA diversity but also in diversity of the metabolic enzymes of nitrifying bacteria. The key enzymes for ammonia (NH_3) oxidation to nitrite (NO_2^-) are ammonia monooxygenase (AMO), which converts NH_3 to hydroxylamine (NH_2OH), and hydroxylamine oxidoreductase (HAO), which converts NH_2OH to NO_2^-. The gene encoding the α-subunit of AMO (*amoA*) has been sequenced from many ammonia-oxidizers. Phylogenies based on *amoA* (and the protein sequence AmoA) have in general shown remarkable correspondence to 16S rRNA phylogenies (Purkhold et al. 2000; Norton et al. 2002). However, some important differences in the placement of NH_3-sensitive *Nitrosomonas* species relative to *Nitrosospiras* make *amoA* a better functional or phenotypic marker than phylogenetic marker (Aakra et al. 2001; Norton et al. 2002). *amoA* sequences are particularly useful in distinguishing among closely-related ammonia-oxidizing bacteria because this protein-encoding gene shows more variation than does the 16S rRNA gene (Rotthauwe et al. 1997). *amoA* sequences obtained from the environment demonstrate a substantial diversity in natural populations (Rotthauwe et al. 1997; Juretschko et al. 1998; Mendum et al. 1999; Voytek et al. 1999; Nold et al. 2000; Purkhold et al. 2000), and an important question raised by these observations is how diversity in gene or protein sequences is reflected in diversity in biogeochemical activity (e.g., variable rates, isotope fractionation, substrate affinity and tolerance).

Isotopic ratios of dissolved inorganic nitrogen pools (NH_4^+, NO_3^-, and N_2O) can be altered by the action of nitrifying bacteria, thus providing an in situ tracer for nitrification activity (Mariotti et al. 1984; Horrigan et al. 1990). Variation in the stable isotopic composition of N_2O, a climatically important atmospheric trace gas, may represent an important constraint on the contribution of nitrifying bacteria to its production (Yoshida et al. 1984; Kim and Craig 1990; Dore et al. 1998; Rahn and Wahlen 2000). In light of the observed diversity of ammonia-oxidizing bacteria, it is important to address the scope of biological and environmental factors that may lead to variability in their kinetic isotope effects in order to fully interpret variations in stable isotope distributions as tracers of underlying microbial activity. It is of particular concern that interpretations of marine nitrogen isotope dynamics have necessarily been based on *N. europaea* (Yoshida et al. 1984; Naqvi et al. 1998), which may not be representative of marine ammonia-oxidizers.

Here we examine the range of kinetic isotope effects for ammonia oxidation in a variety of cultured ammonia-oxidizing bacteria, including marine and terrestrial strains. The kinetic isotope effect, ε, is a fundamental parameter of any biogeochemical pathway

or transformation. It arises from small differences in the rates of enzymatic reaction with molecules containing heavy and light isotopes of constituent atoms: $\varepsilon = (k_l/k_h - 1) \times 10^3$, where k_l and k_h are the rate constants for enzymatic reaction with light and heavy molecules, respectively (Mariotti et al. 1981). An enzyme-level isotope effect is likely to be influenced, if not uniquely determined, by the primary sequence of the enzyme and thus may be an effective way to connect the diversity of protein sequences to biogeochemical function. However, in whole cells, in addition to enzyme-level isotope effects, physiological factors may play a role in the overall (expressed or observed) isotope effect for a multi-step process that involves transport of substrate between extra-cellular and intra-cellular pools (Laws et al. 1997). In the present work we use ε_{AMO} to refer to the expressed isotope effect for ammonia oxidation by whole cells. The current study was undertaken to investigate the potential range in isotope effects for ammonia oxidation among nitrifying bacteria. The observed differences in ε_{AMO} are analyzed in the context of genetic and physiological factors that may lead to different ε_{AMO} values among nitrifiers. We further discuss the potential impact of diversity in ε_{AMO} on N_2O isotope distributions.

Materials and Methods

Bacteria and Culture Conditions

Nitrosomonas europaea (Schmidt strain, ATCC 19718), *Nitrosomonas eutropha* (Schmidt strain), and *Nitrosospira tenuis* (NV-12) were grown in Walker medium with deionized water (Soriano and Walker 1968). *Nitrosomonas* sp. C-113a (Red Sea isolate) was grown in medium with full strength seawater using the recipe given by Ward (Ward 1987) (W medium), and *Nitrosomonas marina* (BBW culture collection) was grown in W medium made up in 50% seawater (W/2 medium). Cultures (500 ml) were grown in static 1-liter flasks in the dark at 18°C and maintained in "semi-continuous" batch culture by periodic replacement of 50% of the culture with fresh medium (Ward 1987). Saturated K_2CO_3 solution was added as needed to maintain pH around 8.

Ammonia Oxidation Experiments

Semi continuous batch cultures (250 ml) were harvested by filtration, then washed twice, and resuspended in 2 ml of growth medium without added NH_4^+. Resuspended cells (0.5 ml) were used to inoculate 100 ml prepared medium in sealed serum bottles, for a final cell density of approximately 10^7 ml^{-1}. For each organism, a series of four serum bottles was prepared, three of which were inoculated and one kept uninoculated as a control. Ammonium concentrations in the media were modified from the original recipes (cited above) to 2.0 mM for *N. marina* and C-113a, 1.0 mM for *N. tenuis*, *N. eutropha*, and *N. europaea*; other media constituents were kept the same. The two-fold range in initial ammonia concentrations was an unintentional result of medium preparation from $(NH_4)_2SO_4$ with differing stock concentrations.

Serum bottles were subsampled over the course of 4–10 days and the concentrations of NH_4^+ and NO_2^-, as well as the $\delta^{15}N$ of NO_2^-, were measured. Concentrations of NH_4^+ and NO_2^- were measured using the phenol-hypochlorite (Grasshof and Johannse 1972) and the Greiss-Islovay (Strickland and Parsons 1972) colorimetric methods, respectively.

Isotope Analyses

The $\delta^{15}N$ of NO_2^-, defined as: $\delta^{15}N(‰) = ((^{15}N/^{14}N)_{sample} \div (^{15}N/^{14}N)_{std} - 1) \times 1000$, was analyzed using the denitrifier method (Sigman et al. 2001) in subsamples taken over

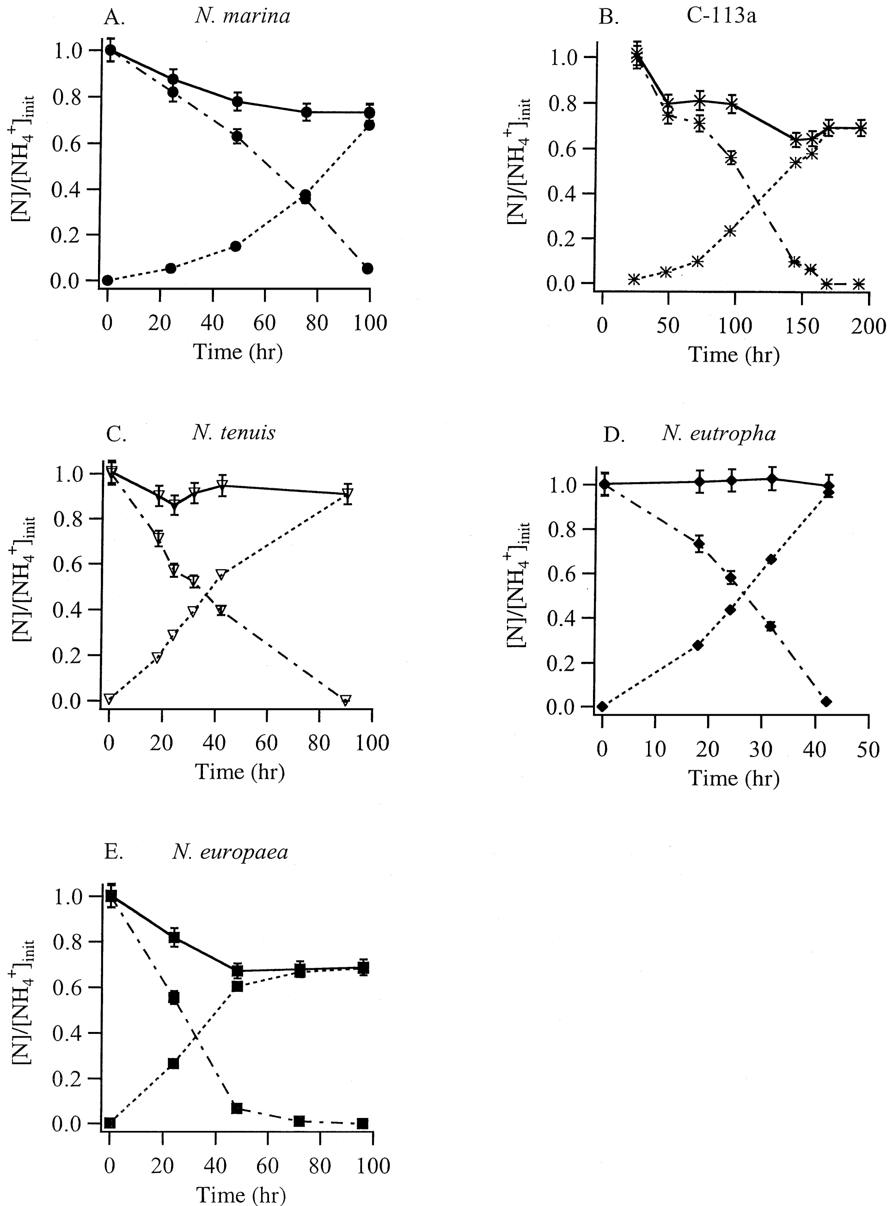

FIGURE 1 Time course of NH_4^+ oxidation to NO_2^- by *N. marina* (A), C-113a (B), *N. tenuis* (C), *N. eutropha* (D), and *N. europaea* (E). In each panel $[NH_4^+]/[NH_4^+]_{init}$ (dot-dashed line), $[NO_2^-]/[NH_4^+]_{init}$ (dashed line), and $[NH_4^+ + NO_2^-]/[NH_4^+]_{init}$ (solid line) are shown for representative bottles. Error bars for $[NH_4^+]$ and $[NO_2^- + NH_4^+]$ represent the typical standard deviation for $[NH_4^+]$ measurements (±5%), and error bars for $[NO_2^-]$ measurements are smaller than the symbols (<0.5–1%).

the time course of NH_4^+ conversion to NO_2^- for each strain. Each point represented in Figure 1, with the exception of the initial time points (which contained <1 μM NO_2^-), was analyzed for $\delta^{15}NO_2^-$. The measured concentrations of NO_2^- were used to calculate the volume of each sample to be aliquotted for isotopic analysis (20 nmoles per analysis).

Measured δ^{15}N values are referenced to air N_2 by calibrating to analyses of IAEA-NO-3, an internationally distributed reference material (KNO_3 salt) which has a δ^{15}N of +4.7‰ vs. air N_2 (Gonfiantini et al. 1995). Analytical reproducibility of the denitrifier method for δ^{15}N measurements is ±0.2‰ (Sigman et al. 2001).

Isotope Effect Estimates

Isotope effects for ammonia oxidation (ε_{AMO}) were calculated from the $\delta^{15}NO_2^-$ data using the Rayleigh accumulated product equation: $\delta^{15}\,NO_2^- = \delta^{15}NH_{4,initial} + \varepsilon \times f \times \ln(f)/(1-f)$ (Mariotti et al. 1981), where f = $[NH_4{}^+]/[NH_4{}^+]_{initial}$ or 1-($[NO_2^-]/[NH_4^+]_{initial}$). Here we have assumed that the oxidation of NH_4^+ to NO_2^- is a pseudo-one step reaction, with NH_2OH held in steady state. While the activity of AMO does depend on supply of electrons from HAO, NH_2OH accumulation has not been observed in exponentially-growing cells of *N. europaea* (Wood 1986; Bock et al. 1992). The coupling of AMO and HAO is thus assumed to maintain NH_2OH in low steady-state concentrations, in which case isotope effects associated with HAO should have limited impact on the δ^{15}N of NO_2^-. The potential errors involved in this assumption due to possible accumulation of NH_2OH and alternative sinks of NH_4^+, such as NO and N_2O, are discussed below.

It should also be noted that isotopic equilibration between NH_4^+ and NH_3 is associated with a 20‰ equilibrium isotope effect (Bigeleisen 1965; Hermes et al. 1985). At a pH of 8, NH_3 is approximately 5% of the total ($NH_3 + NH_4^+$) pool. Therefore, if the total pool has a δ^{15}N of 0‰, δ^{15}N of NH_3 (the substrate being acted on) will be around −18‰ relative to the bulk pool. This is likely to be relevant for the results presented here, as equilibrium between NH_4^+ and NH_3 is expected to be fast relative to NH_3 oxidation. Because we are measuring changes in δ^{15}N-NO_2^- rather than δ^{15}N-NH_4^+, this isotope effect would be subsumed into our estimate of ε_{AMO}. That is, our reported ε_{AMO} combines the equilibrium isotope effect with the kinetic isotope effect for ammonia oxidation (Hoch et al. 1992). This is not expected to be a major source of variation among our experiments, however, because it relies on the rate of isotopic equilibration relative to reaction rates, which does not vary significantly, and pH, which is similar among the different growth media.

Sequence Analysis

AmoA amino acid sequences were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov) under the following accession numbers: *Nitrosomonas* sp. URW (AAK54693), *Nitrosomonas* sp. C-45 (AAK54694), *N. marina* (AAK54691), *Nitrosomonas* sp. NO3W (AAK54692), *Nitrosomonas* sp. C-113a (AAK54695), *Nitrosomonas* sp. TA-921i-NH4 (AAK54696), *Nitrosomonas* sp. AL212 (AAL86637), *Nitrosomonas* sp. JL21 (AAL86638), *Nitrosomonas cryotolerans* (AAG60667), *Nitrosospira briensis* (AAB38709), *Nitrosospira* sp. NP39-19 (AAC25055), *N. tenuis* (AAB38710), *Nitrosospira multiformis* (AAC25057), *Nitrosospira* sp. NpAV copy 1 (AAB86881), *Nitrosospira* sp. NpAV copy 2 (AAB87792), *Nitrosospira* sp. NpAV copy 3 (AAB53437), *N. europaea* copy 1 (AAC38651), *N. europaea* copy 2 (AAC38653), *N. eutropha* copy 1 (AAB08985), *N. eutropha* copy 2 (AAB16816), *Nitrosomonas* sp. GH22 (AAL86636), and *Nitrosococcus oceani* (AAB57809).

AmoA sequences were aligned using ClustalW multiple sequence alignment (Thompson et al. 1994). PAUP* 4.0 (Swofford 1993) was used to generate a neighbor-joining tree based on distance matrix analysis of the aligned 177 amino acid segment, with *N. oceani* as the outgroup. Bootstrapping was performed (100 replicates) using the neighbor-joining option in PAUP* 4.0.

Results

Time Course of Ammonia Oxidation

The oxidation of NH_4^+ was nearly complete in 4 days for *N. europaea*, *N. eutropha*, *N. tenuis*, and *N. marina* (Figure 1). There was a 2–3 day lag before the onset of ammonia oxidation for C-113a, which resulted in a longer time course for C-113a (Figure 1). The main product of ammonia oxidation is NO_2^-, but it did not account for all of the ammonia oxidized in some cultures. The sum of NH_4^+ and NO_2^- is relatively consistent over the course of ammonia oxidation for *N. tenuis* and *N. eutropha* but decreases for *N. marina*, C-113a, and *N. europaea*, accounting for 82%, 77%, and 75%, respectively, of the initial NH_4^+ by the end of incubations (Figure 1). The 20–25% of N that is not accounted for as NH_4^+ or NO_2^- could have accumulated as NH_2OH, NO, N_2O, or cell biomass (or some combination of these), which were not measured in these experiments. The redirection of N from the NO_2^- pool does not appear to cause convergence in ε_{AMO} among the strains in which "missing" N accounts for a significant portion of initial NH_4^+ (see below), nor does it affect the final $\delta^{15}NO_2^-$, which approaches $\delta^{15}NH^+_{4\,init}$ (−1‰), but it may impose additional error or uncertainty in ε_{AMO} estimates, which is discussed further below.

For each strain, replicate bottles (not shown) behaved similarly in their rate of ammonia oxidation and gave NO_2^- yields that varied by less than ±1.5%. The average rates of NH_4^+ oxidation for each strain were calculated from the overall change in NH_4^+ concentration and the time elapsed from the first data point until the ammonia was effectively removed (100 h for *N. marina*, 168 h for C-113a, 90 h for *N. tenuis*, 42 h for *N. eutropha*, and 48 h for *N. europaea*). The results of these calculations are summarized in Table 1. The reported rates may be conservative in cases where the end of the experiment is undersampled, because the ammonia may have been consumed prior to the final sampling point. Slight differences in the rate of ammonia oxidation between cultures may have resulted from this limitation of our calculation, as well as potentially different cell densities, initial $[NH_4^+]$, or inherent rate differences among the organisms but are not correlated with the isotopic differences (see Table 1).

Isotope Effects for Ammonia Oxidation

The $\delta^{15}NO_2^-$ results are presented in Figure 2. Individual isotope analyses are plotted versus $f \times \ln(f)/(1 - f)$, where $[NH_4^+]$ is used to calculate $f = [NH_4^+]/[NH_4^+]_{initial}$ in each sample. ε_{AMO} is given by the slope of the linear regression of $\delta^{15}NO_2^-$ vs. $f \times \ln(f)/(1 - f)$,

TABLE 1 Summary of ammonia oxidation experiments

Nitrifier strain	Growth medium	Initial NH_4^+ conc. (μM)	NH_4^+-ox rate (μM/hr)[a]	Isotope effect (ε_{AMO})
Nitrosomonas marina	W/2	2000	20.9 ± 1.4	14.2 ± 3.6‰
Nitrosomonas sp. C-113a	W	2000	15.4 ± 1.5	19.1 ± 1.2‰
Nitrosospira tenuis	Walker	1000	12.0 ± 2.5	24.6 ± 1.4‰
Nitrosomonas eutropha	Walker	1000	23.5 ± 1.2	32.8 ± 1.7‰
Nitrosomonas europaea	Walker	1000	21.1 ± 0.2	38.2 ± 1.6‰

[a] Average ammonia oxidation rates were calculated from the overall change in NH_4^+ concentration and the amount of time required for nearly complete consumption of NH_4^+. 1σ standard deviations are based on rates calculated from replicate bottles.

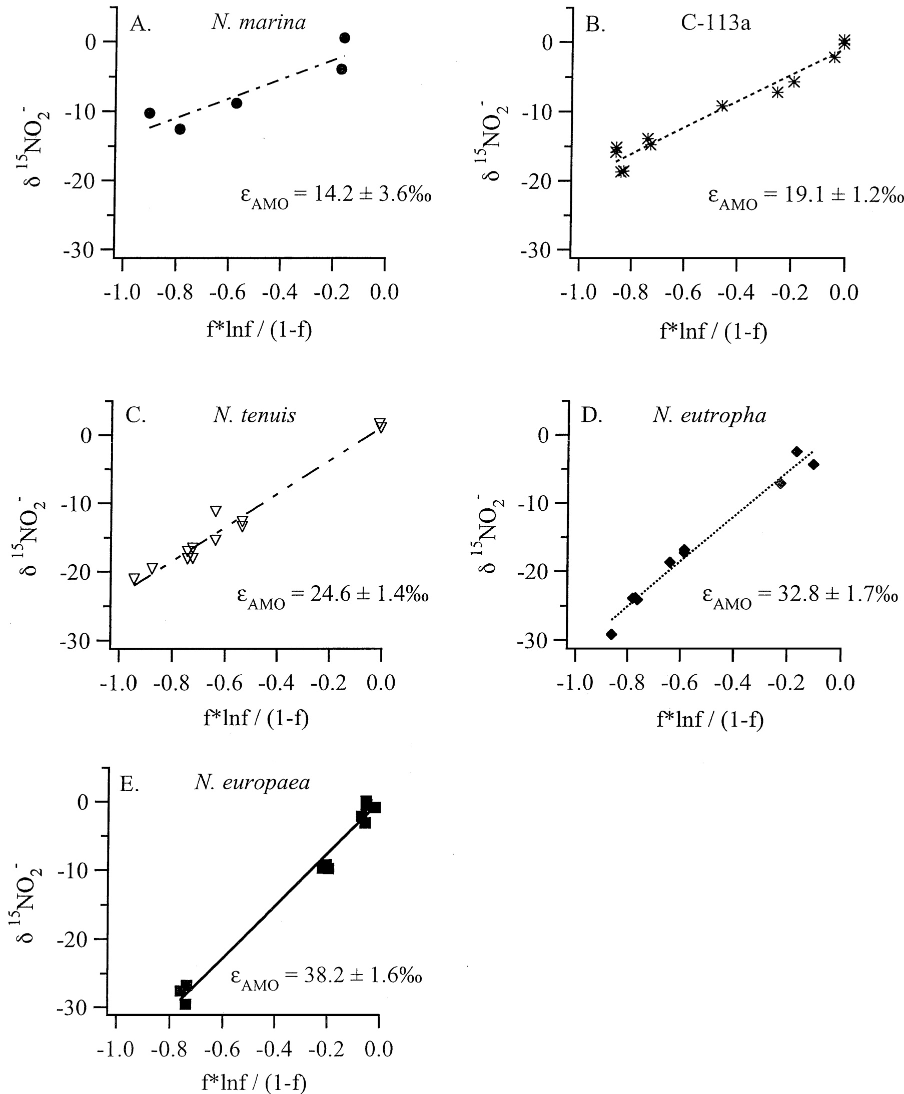

FIGURE 2 $\delta^{15}NO_2^-$ measurements from *N. marina* (A), C-113a (B), *N. tenuis* (C), *N. eutropha* (D), and *N. europaea* (E) reported in ‰ relative to air N_2. Kinetic isotope effects were calculated from the slope of the linear regression for each series, according to the Rayleigh accumulated product equation: $\varepsilon_{AMO} = (\delta^{15}NO_2^- - \delta^{15}NH_{4,init})/(f \times \ln(f)/(1 - f))$. The uncertainties given for ε_{AMO} estimates are the 95% confidence intervals around the slope for each linear regression.

incorporating samples from replicate serum bottles for each strain. The two marine ammonia-oxidizing bacteria, *N. marina* and C-113a, showed similar ε_{AMO} values of 14.2 ± 3.6‰ and 19.1 ± 1.2‰, respectively. The ε_{AMO} values for *N. eutropha* and *N. europaea* were also similar to each other, 32.8 ± 1.7‰ and 38.2 ± 1.6‰, respectively, but significantly higher than the *N. marina* group. *N. tenuis* had an intermediate ε_{AMO} of 24.6 ± 1.4‰.

Analytical uncertainty in isotopic analyses (±0.2‰) accounts for very little of the scatter observed in each of the series (Figure 2). Rather, deviations from the Rayleigh accumulated product model, such as transient accumulation of intermediates, back reaction of

substrates, or loss of N to uncharacterized pools, may cause scatter early in the experiment. This scatter leads to larger uncertainty in the *N. marina* ε_{AMO} estimate compared to experiments with *N. europaea* and *N. tenuis*.

AmoA Sequence Comparison

An alignment of partial amino acid sequences (177 AA) for AmoA (Figure 3) allows a detailed comparison of the AmoA sequences among these five nitrifier strains. Three histidine residues that are conserved among all known β-subdivision AmoA sequences are contained in this region (positions 118, 173, 216 based on *N. europaea* numbering). There are several sites that distinguish the four *Nitrosomonas* strains from *N. tenuis*, while differences at other sites alternately group *N. tenuis* with *N. marina* and C-113a or *N. europaea* and *N. eutropha*. Notably, the AmoA sequences from *N. europaea* and *N. eutropha* have in common two additional histidine residues which are located in an otherwise well-conserved periplasmic loop (positions 165-218) (Norton et al. 2002).

Overall, the AmoA sequences are well conserved. The sequences of *N. marina* and C-113a are 98% identical over this section, while they are both 84% and 82% identical to *N. europaea* and *N. eutropha*, respectively. *N. tenuis* is 87% identical to *N. marina* and C-113a, and it is 83% identical to both *N. europaea* and *N. eutropha*. These pairwise similarities in AmoA amino acid sequences among *N. marina*, C-113a, *N. tenuis*, *N. europaea*, and *N. eutropha* closely parallel the pattern of ε_{AMO} similarities for these strains.

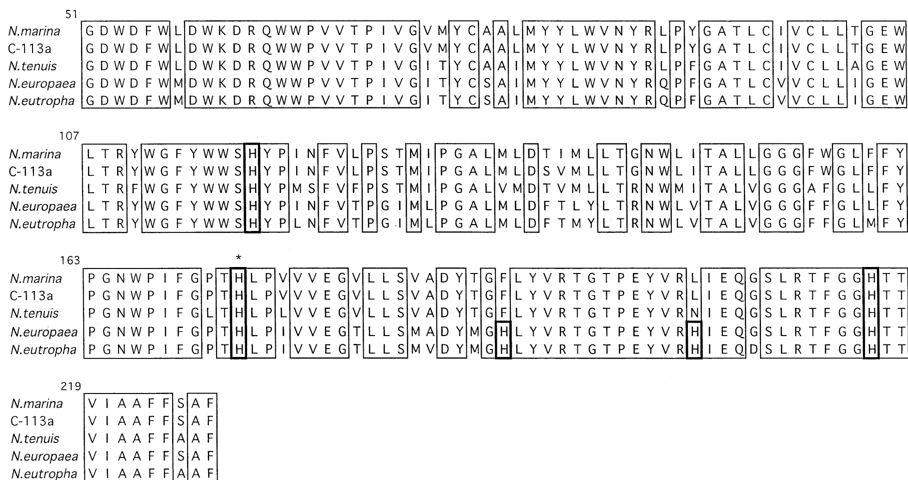

FIGURE 3 Alignment of ammonia monooxygenase amino acid sequences (177 AA) from *N. marina*, C-113a, *N. tenuis*, *N. eutropha*, and *N. europaea*. Numbering is based on amino acid positions from the full *N. europaea* AmoA1 sequence (AAC38651). Amino acid residues conserved across all five sequences are boxed. One histidine residue that is conserved in both AmoA and PmoA (particulate methane monooxygenase subunit A), and is likely to be involved in Cu^{2+} binding, is highlighted in black and marked with an asterisk (position 173). Two histidine residues that are conserved across all AmoA sequences in β-subdivision ammonia-oxidizers, but not present in PmoA (particulate methane monooxygenase subunit A), are highlighted in black (positions 118 and 216). Unique histidine residues in *N. europaea* and *N. eutropha* are also highlighted in black (positions 191 and 204).

Discussion

Previous reports of the isotope effect for ammonia oxidation by *N. europaea* range from 26‰ to 35‰ (Delwiche and Steyn 1970; Mariotti et al. 1981; Yoshida 1988). The reasons for this range in values for *N. europaea* have not been thoroughly investigated, although high N_2O yields are cited as an explanation for some of the low ε_{AMO} estimates (Yoshida 1988). The effect of N_2O production on ε_{AMO} is not well documented and may actually be to increase ε_{AMO} estimates based on $\delta^{15}NO_2^-$ measurements due to a greater influence of N_2O production at higher NO_2^- concentrations. Furthermore, N_2O production cannot explain the 5‰ difference in ε_{AMO} for two experiments with the same N_2O yield (Yoshida 1988). Another variable among published studies is the initial ammonium concentration, which ranged from 500 μM (Mariotti et al. 1981) to 38 mM (Yoshida 1988). However, variations in NH_4^+ concentration between 500 μM and 25 mM did not cause variation in the ε_{AMO} estimates within a single study (Mariotti et al. 1981). Results presented here also show no change of ε_{AMO} in *N. europaea* for NH_4^+ concentrations ranging from 1,000 μM down to less than 5 μM NH_4^+, the range of substrate concentrations covered in the time course. Concentration dependence of ε_{AMO} would have been apparent from a nonlinear trend in Figure 2, which is not observed for *N. europaea*. Therefore, change in the initial ammonium concentration does not appear to explain the published range of ε_{AMO} estimates for *N. europaea*. Our estimate (38‰) is closest to the 35‰ estimate from Mariotti et al. (1981), which is based on multiple isotopic analyses from several experiments (Mariotti et al. 1981). Other estimates, which range from 26‰ to 32‰, are based on single-point analyses of f and $\delta^{15}NO_2^-$ or $\delta^{15}NH_4^+$ and are subject to greater uncertainty than the multiple-point estimates.

The ε_{AMO} values measured here for marine nitrifiers, *N. marina* and C-113a, were significantly lower than for *N. europaea* and fall near the range of values (15.2–25.6‰) reported for an unidentified marine nitrifier (Miyake and Wada 1971). These culture-based estimates are also similar to field estimates for the isotope effects of ammonia oxidation in the Chesapeake Bay, which fall in the range of 12.7 to 16.0‰ (Horrigan et al. 1990). The possible factors leading to the ε_{AMO} differences among nitrifiers will be considered in subsequent sections, addressing genetic as well as physiological factors.

Enzyme-Level Differences Among Nitrifier Species

Genetic and biochemical differences in AMO among nitrifier species may be important determinants of the range in observed isotope effects. The similarities among AMO from *N. marina*, C-113a, *N. tenuis*, *N. eutropha*, and *N. europaea* will first be explored at the molecular level. A level of 84% identity at the protein level, representing the level of identity between *N. marina* and *N. europaea*, represents significant divergence among known AmoA sequences (Purkhold et al. 2000). However, the full functional diversity is clearly not resolved by the overall percent identity of the protein sequence. For instance, the 84% identity between *N. marina* and *N. europaea* AmoA sequences is associated with a much larger difference in ε_{AMO} (∼24‰) than is the 83% identity between *N. tenuis* and *N. europaea* (∼14‰). In attempting to relate protein sequence and function, it is important to consider not only the overall level of identity but also where these differences occur in relation to amino acid residues that are critical for structure and function of the protein. The section of the AmoA protein analyzed in Figure 3 is targeted by PCR primers A189 and A682 (Holmes et al. 1995) and is expected to contain much of the active site of the enzyme (Hyman and Arp 1992; Holmes et al. 1995).

The active site of AMO is thought to be hydrophobic (Hooper et al. 1997) and to contain copper (Ensign et al. 1993). However, aside from predicted membrane-spanning

regions (Hooper et al. 1997; Norton et al. 2002), information on the important structural features of AMO is limited. Some additional information about important regions of the active site may be drawn by comparison to particulate methane monooxygenase (pMMO), an enzyme thought to be homologous to AMO (Holmes et al. 1995; Semrau et al. 1995). Electron paramagnetic resonance (EPR) and electron spin echo envelope modulation (ESEEM) analysis of pMMO suggest that 3–4 histidine residues are involved in binding of each Cu^{2+} (Lemos et al. 2000). The observation that pMMO contains 12–15 Cu atoms per enzyme (Nguyen et al. 1998) suggests that conservation of histidine residues may be particularly important for the function of pMMO, and for AMO by analogy. Analysis of AmoA sequences from β-subdivision ammonia-oxidizers shows that they contain five conserved histidine residues, three of which are also conserved in PmoA (Holmes et al. 1995; Norton et al. 2002). Three of the conserved histidine residues from AmoA are contained in the 177 AA fragment of the protein analyzed here, while the other two are just upstream (Norton et al. 2002), suggesting that similarities and differences in this region of the protein may represent functionally significant changes.

The region surrounding the first histidine residue (position 118 based on *N. europaea* numbering) is well conserved among the five nitrifiers compared in Figure 3, with a few substitutions (involving similar amino acids) differentiating *Nitrosomonas* sequences from *N. tenuis*. Within two residues of the second conserved histidine residue (position 173), there is a significant difference between *Nitrosomonas* sequences (which contain a proline residue) and the *N. tenuis* sequence (which contains a leucine residue). Proline and leucine have different structural properties that may lead to differing secondary structures between *Nitrosomonas* and *N. tenuis* AmoA proteins. The region surrounding the third H (position 216) is highly conserved.

Hydropathy profiles of AmoA from *N. europaea*, *N. marina*, and *N. tenuis* (not shown) predict that the two extra histidine residues in the AmoA sequences from *N. europaea* and *N. eutropha* would cause significant differences in AmoA secondary structure. The histidine residue at position 191 increases the predicted relative hydrophilicity of the *N. europaea*/*N. eutropha* protein relative to the other sequences, which contain phenylalanine. At position 204, a leucine residue in the *N. marina* sequence leads to a predicted hydrophobic pocket in the middle of the otherwise hydrophilic periplasmic loop between transmembrane sections 5 and 6 (Norton et al. 2002). Histidine and asparagine residues in *N. europaea* and *N. tenuis*, respectively, preserve the predicted hydrophilicity of this periplasmic loop.

While we cannot evaluate, given the available information, whether the extra histidine residues present in *N. europaea* and *N. eutropha* serve a functional purpose, they may represent phylogenetic markers for strains that tolerate high concentrations of NH_3 (Norton et al. 2002) and may also be a marker for ammonia-oxidizers with high ε_{AMO} values. Like *N. europaea* and *N. eutropha*, isolate GH22 which is tolerant of NH_4^+ concentrations up to 100 mM (Suwa et al. 1997) also has the two extra histidine residues (Norton et al. 2002). Other isolates of Suwa et al. (1997), which have a lower tolerance to NH_3 (JL21 and AL212), have AmoA sequences that are similar to *N. marina* and C-113a and lack these extra two histidine residues (Norton et al. 2002). Variability in regions of AmoA outside of this region may also be important, and the full-length sequences would provide additional information.

A neighbor-joining tree based on the 177 amino acid alignment of AmoA, summarizes the phylogenetic placement of the strains whose ε_{AMO} were determined here and gives a sense of their relation to other ammonia-oxidizers (Figure 4). The nitrifiers studied here include members of the main groups of cultured ammonia-oxidizers, and in these organisms the isotope effects for AMO are consistent with the AmoA phylogeny. This phylogenetic tree also indicates where further examination of this link between AmoA sequence and

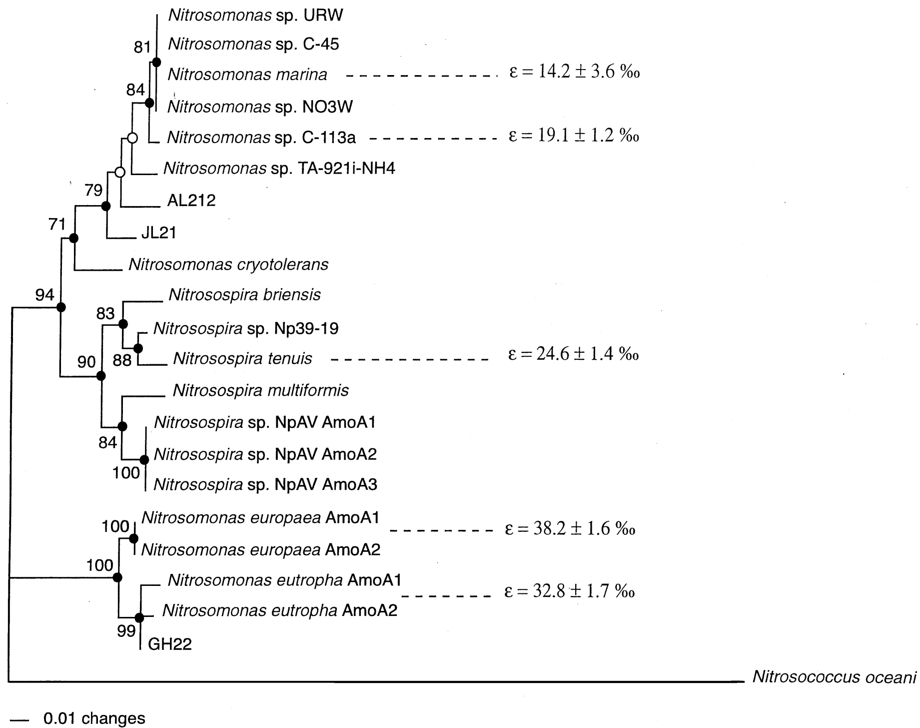

FIGURE 4 Neighbor-joining tree based on distance matrix analysis of the 177 amino acid fragment shown in Figure 3 using PAUP* 4.0. Bootstrap values are based on 100 pseudo-replicate analyses. Nodes which are supported by bootstrap values greater than 70% are marked with filled circles and those that have weak bootstrap support (<70%) are marked with open circles. Isotope effects (ε_{AMO}) measured in this study are included for comparison. Scale bar represents 0.01 substitutions per amino acid site.

activity may be directed. To further investigate the pattern of congruence between AmoA sequence and ε_{AMO}, it will be important to measure ε_{AMO} in strains such as GH22 (which has many similarities in AmoA sequence compared to *N. europaea* and *N. eutropha*), additional members of the *N. marina* and *Nitrosospira* clades, and *N. oceani*. In order to fully understand how to extend these results to the environment, it will also be crucial to obtain and characterize ammonia-oxidizing isolates from clades that are well represented in clone libraries but are not currently represented in the culture collection (Rotthauwe et al. 1997; Mendum et al. 1999; Juretschko et al. 1998).

The close relationship between the phylogeny of AMO sequences and apparent isotope effects contrasts with an extensive study of sulfate reducing bacteria, which found no clear relationship between the phylogeny of organisms (based on 16S rRNA genes) and their isotope effects for sulfate reduction (Detmers 2001). Phylogenies based on functional genes involved in sulfate reduction may correspond better to the distribution of isotope effects, however, and evidence exists for lateral gene transfer of the genes for dissimilatory sulfite reductase (*dsrA* and *dsrB*) among sulfate reducing bacteria (Klein et al. 2001). Indeed, our ε_{AMO} estimates would not be consistent with 16S rRNA phylogeny of nitrifiers, in which a clear delineation separates the *Nitrosomonas* from *Nitrosospira* species (Head et al. 1993; Purkhold et al. 2000). Only based on the functional gene sequences (*amoA*) and associated amino acid sequences (AmoA) for AMO do our ε_{AMO} estimates concur

with nitrifier phylogeny (Figure 4). This observation adds weight to the idea that the ε_{AMO} estimates may be linked to the activity of AMO itself and not a general physiological feature of phylogenetically similar organisms.

Currently, the only variables that reliably group sulfate-reducing bacteria with similar isotope effects are based on physiological characteristics of their carbon metabolism and the source of electron donors (H_2 or various forms of organic carbon) supplied in culture (Detmers et al. 2001). With that in mind, we consider alternative explanations for the correspondence of sequence similarities and apparent functional similarities in ammonia-oxidizers, including broad physiological differences that fall along general phylogenetic lines such as transport of NH_4^+, rates of reaction, or accumulation of metabolic intermediates.

Physiological Similarities and Differences

N. eutropha and *N. europaea* are commonly found in similar eutrophic environments. They also share traits of moderate halotolerance, NH_3 tolerance, and lack urease activity (Koops et al. 1991). *N. europaea* also has a high K_m value for NH_3 (23–58 μM) (Suzuki et al. 1974). *N. tenuis* was first isolated from unamended soil (Harms et al. 1976). Strains similar to *N. tenuis* described by Jiang and Bakken (1999a) were found to have low K_m values (~10 μM NH_3) and are tolerant of low pH. The *amoA* sequences from these strains (Aakra et al. 2001) also group with many *amoA* clones sequenced in soil and soil enrichments from low and neutral pH soils (Smith et al. 2001). These ammonia-oxidizers are thus likely to be adapted to low concentrations of NH_3, which is a minor fraction of total NH_4^+ (NH_4^+ plus NH_3) at low pH. *N. marina* and C-113a were isolated by Watson in 1966 from marine environments (the continental shelf off Peru and the Red Sea, respectively). These *Nitrosomonas* strains are immunologically similar (Ward and Carlucci 1985) and are very similar in *amoA* (96%), and *nirK* (97%) functional gene sequences (Casciotti and Ward 2001). Not much is known about their comparative physiology, but both were isolated from natural environments that typically have low NH_4^+ concentrations. Specific adaptations to low concentrations of NH_3/NH_4^+ that could affect apparent ε_{AMO} among ammonia-oxidizers, independently of enzyme-level differences, will be discussed further.

Transport of Ammonium and Ammonia Oxidation Rate

If marine nitrifiers (or other NH_4^+-sensitive environmental strains) have evolved transport mechanisms to grow on low concentrations of NH_4^+, then they might be expected to have lower apparent ε_{AMO} values compared to *N. europaea* and *N. eutropha*, even with similar enzyme-level isotope effects. This is because cells that are more efficient at transporting or maintaining NH_4^+ inside the cell would not allow the partially utilized NH_4^+ pool to escape from the cell. In that case, the primary isotope effect would be due to transport or diffusion processes, which tend to have lower isotope effects than enzymatic processes (O'Leary 1984). Ammonium transport has not yet been observed in ammonia-oxidizers (Suzuki et al. 1974; Burton and Prosser 2001; Glover 1985), but it has not been exhaustively studied, particularly in strains adapted to low NH_4^+ concentrations. Further investigation will be required to evaluate the role of NH_4^+ or NH_3 transport in nitrifier metabolism and isotopic fractionation.

A related issue is potential variability in the rate of ammonia oxidation among nitrifiers. Depending on the balance of ammonia diffusion (or transport) and oxidation, different apparent isotope effects may be observed despite constant enzyme-level isotope effects. This is observed during CO_2 fixation by phytoplankton, where a rapid fixation rate relative to carbon uptake results in a lower apparent isotope effect for the overall process because

transport is the rate-limiting step and it generally has a low isotope effect (O'Leary 1981). At lower rates of CO_2 fixation or when supply is plentiful, the high isotope effect for RuBisCO dominates the isotope effect for the overall fixation process (Popp et al. 1989; Goericke et al. 1994; Laws et al. 1997). The rate of sulfate reduction has also been shown to be inversely proportional to isotope effects in some, but not all, studies (Aharon and Fu 2000; Bruchert et al. 2001; Detmers et al. 2001; Rudnicki et al. 2001). For the experiments reported here, the range in ammonia-oxidation rates was not large (12.0–23.5 μM/hr), and there is no clear relationship between the rates of ammonia oxidation and the isotope effects among these strains (Table 1). The highest and lowest rates of ammonia oxidation do not correspond to the lowest or highest isotope effects, and organisms with similar rates of oxidation can have very different isotope effects (Table 1). Although we cannot rule out an effect of reaction rate on variations in ε_{AMO} for a single organism, beyond the range tested here, the differences among organisms we observe are not readily explained by such an effect.

Given the relatively small range in rates of NH_3 oxidation rates in these experiments, the relative rates of NH_3 oxidation and NH_4^+/NH_3 equilibration are also not significantly different. Therefore, expression of the equilibrium isotope effect for NH_4^+/NH_3 equilibration is not likely to cause the ε_{AMO} differences among these nitrifier species.

Production of NH_2OH or N_2O

Accumulation of intermediates, such as NH_2OH, could lead to an overestimation of the isotope effect for AMO based on $\delta^{15}NO_2^-$ because any isotope effect involved in the second step (NH_2OH oxidation to NO_2^-) would increase the $\delta^{15}N$ difference between the substrate (NH_4^+) and ultimate product (NO_2^-). To explain the differences between measured isotope effects for AMO, accumulation of NH_2OH would have to be consistently greater in *N. europaea* and *N. eutropha* than in *N. marina*, C-113a, and *N. tenuis*. The mass balance of NH_4^+ and NO_2^- for each organism suggests that NH_2OH could accumulate in *N. marina*, C-113a, and *N. europaea* but not *N. eutropha* nor *N. tenuis*. This pattern is not consistent with differences in ε_{AMO} and cannot explain the ε_{AMO} differences between *N. tenuis* and *N. eutropha*, both of which showed complete mass balance between NH_4^+ and NO_2^-. In addition, as the electrons needed for ammonia oxidation are derived from NH_2OH (Hooper et al. 1997), it would be difficult energetically to maintain a significant accumulation of NH_2OH.

Production of the gases nitric oxide (NO) and nitrous oxide (N_2O) may also lead to deviation from the simplified Rayleigh accumulated product model for $\delta^{15}NO_2^-$ by adding an additional fractionation step either prior to or subsequent to NO_2^- production. The effect that gas production has on $\delta^{15}NO_2^-$ will depend on the pathways for gas production, relevant isotope effects, and the relative yields of NO and N_2O. Typical N_2O yields for ammonia-oxidizing bacteria under aerobic conditions range from 0.1% to 3.0% and can be as high as 11% under microaerophilic conditions (Goreau et al. 1980; Lipschultz et al. 1981; Yoshida 1988; Kester et al. 1997; Jiang and Bakken 1999b). Yields of NO are more variable, ranging from 0.51% up to 30% in *N. europaea* cultures at atmospheric O_2 levels (Lipschultz et al. 1981; Yoshida 1988; Kester et al. 1997), indicating that under some conditions production of NO may account for significant "missing" N in ammonia oxidation experiments. To determine the exact effect of NO production on ε_{AMO} for each strain in this study, we would have to know whether NO is produced through nitrite reduction (Poth and Focht 1985; Ritchie and Nicholas 1972; Hooper et al. 1990) or from hydroxylamine oxidation (Hooper and Terry 1979; Beaumont et al. 2002). Estimates of the isotope effects for NH_2OH oxidation to NO_2^-, NH_2OH oxidation to NO, and NO_2^- reduction to NO would also be needed.

Mariotti et al. (1981) circumvented these concerns by determining ε_{AMO} for *N. europaea* from both $\delta^{15}NH_4^+$ and $\delta^{15}NO_2^-$ measurements over the course of ammonia oxidation. The estimate of ε_{AMO} based on the change in $\delta^{15}NH_4^+$ is not subject to the same concerns associated with intermediates or alternate end products that accompany estimates based on $\delta^{15}NO_2^-$. The independent ε_{AMO} estimates based on $\delta^{15}NH_4^+$ and $\delta^{15}NO_2^-$ were indistinguishable from each other (and from our estimate) within the error of the measurement (Mariotti et al. 1981). In that study, as much as 30% of the initial NH_4^+ was also unaccounted for as NH_4^+ or NO_2^- in the end. This suggests that despite lack of mass balance between NH_4^+ and NO_2^- pools, similar to the levels that we observed, estimates of ε_{AMO} using the Rayleigh accumulated product model for $\delta^{15}NO_2^-$ can give accurate results. However, this may not always be the case, and a more robust estimate of ε_{AMO} would require measurement of the amount and $\delta^{15}N$ of the possible alternative N pools such as NO and N_2O to constrain their effect on $\delta^{15}NO_2^-$. However, as NO is a difficult pool to collect for isotopic analysis, future measurements of ε_{AMO} may rely on coupled estimates from $\delta^{15}NH_4^+$ and $\delta^{15}NO_2^-$. Measurement of ε_{AMO} using purified enzyme is an approach that would also circumvent some of the problems discussed here, but AMO has never been purified and is often inactive in cell-free systems (Ensign et al. 1993; Hooper et al. 1997).

Supply of Electrons

The concentration of electron donors (succinate or phenazine methosulphate) has been shown to play an important role in variations in the isotope effect for nitrite reduction (ε_{NiR}) in whole cells and cell-free systems (Bryan et al. 1983). An increase from <0.1 to 1.0 in the rate of nitrite reduction relative to the maximum rate (V/V_{max}) that is driven by increasing electron donor concentrations corresponded to a decrease of 15‰ in ε_{NiR} (Bryan et al. 1983). Although ammonia oxidation involves a different set of enzymes and electron shuttles, it is possible that its isotope effect also has a dependence on electron donor concentration. For ammonia-oxidizing bacteria, the ultimate source of electrons is NH_3. The concentration of NH_4^+ ($NH_4^+ + NH_3$) used in our experiments was 2.0 mM among the marine nitrifiers, compared with 1 mM for *N. tenuis*, *N. eutropha*, and *N. europaea*. A rough calculation based on Michaelis-Menten saturation kinetics can be used to estimate the effect of this range of substrate concentration on $V/V_{max}(= [S]/[S] + K_m)$ and thus ε_{AMO}, based on analogy to nitrite reduction. The range in V/V_{max} for our experiments was estimated by assuming K_m values of 10 μM NH_3 for *N. marina*, C-113a, and *N. tenuis* (Suwa et al. 1997; Jiang and Bakken 1999a), and 40 μM NH_3 for *N. europaea* and *N. eutropha* (Suzuki et al. 1974). V/V_{max} values thus calculated vary from 0.91 for *N. marina* to 0.55 for *N. europaea*, which would correspond to an upper limit of 6‰ for changes in ε_{AMO} if it carries the same dependence on V/V_{max} as ε_{NiR} (Bryan et al. 1983). However, previous measurements of ε_{AMO} from *N. europaea* have shown no detectable change of ε_{AMO} with NH_4^+ concentration, covering a larger estimated range of V/V_{max} of 0.38 to 0.97 (Mariotti et al. 1981). This would suggest that ε_{AMO} is not as sensitive to changes in V/V_{max} as ε_{NiR}, and we would therefore argue that the range of NH_4^+ concentrations used in our experiments is not likely to contribute to the observed range of ε_{AMO} values.

Environmental Significance

Isotopic distributions of NH_4^+, NO_3^-, and N_2O in the environment can provide important constraints on the biogeochemical cycling of nitrogen. At this point, interpretations of isotope measurements are necessarily based on the isotope effects for cultured strains of nitrifying and denitrifying bacteria. This study demonstrates that there are observable biogeochemical

differences even among the described species of ammonia-oxidizing bacteria. The range in ε_{AMO} among ammonia-oxidizers adds an extra dimension to the study of stable isotope dynamics in the environment, but one that may be tractable through the application of genetic tools. We would suggest that there might yet be more diversity in ε_{AMO} values among cultivated and uncultivated strains of AOB, and further work will be required to address the full spectrum of isotope effects for ammonia oxidizing bacteria and to understand the factors that contribute to differences in AMO isotope effects. Discovery of the bases for these differences may ultimately lead to a better fundamental understanding of nitrifier physiology and biochemistry.

In using nitrogen isotopes as a tool to evaluate the impact of ammonia-oxidizing bacteria on nitrogen cycling and N_2O production, it is important to recognize that a range of ε_{AMO} values exists. It may thus be important to consider which organisms are present and active in any particular environment, as nitrifier community composition may determine the isotope effects that are relevant. In environments where ammonia-oxidizing populations vary in dominance with space and time, different isotope effects may also be needed to explain the changes in NH_4^+, NO_3^-, and N_2O isotopic signatures. Furthermore, *N. europaea* may not always be the most appropriate model organism for isotope dynamics in nitrification processes. The isotope effect estimated by Horrigan et al. (1990) for ammonia oxidation in the Chesapeake Bay, for example, shows poor agreement with ε_{AMO} estimates from *N. europaea* and is better explained by ε_{AMO} from *N. marina* or *N. tenuis*-like nitrifiers. Nitrogen isotopic measurements of N_2O in the ocean (Yoshida et al. 1984; Kim and Craig 1990; Dore et al. 1998) are also difficult to reconcile with the severe depletion of $\delta^{15}N$ in N_2O produced by *N. europaea* (Yoshida 1988). The lower ε_{AMO} values in marine nitrifiers relative to *N. europaea* are likely to be reflected in the production of N_2O that is less depleted in $\delta^{15}N$, regardless of whether it is produced during nitrite reduction (Poth and Focht 1985; Ritchie and Nicholas 1972; Hooper et al. 1990) or directly from hydroxylamine oxidation (Hooper and Terry 1979; Beaumont et al. 2002). This may allow a significant production of N_2O from nitrification in the ocean to fit better with N_2O isotopic constraints.

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