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Use of Molecular and Isotopic Techniques To Monitor the Response of Autotrophic Ammonia-Oxidizing Populations of the β Subdivision of the Class *Proteobacteria* in Arable Soils to Nitrogen Fertilizer

T. A. MENDUM,[1,2]* R. E. SOCKETT,[2] AND P. R. HIRSCH[1]

IACR—Rothamsted, Harpenden, Hertfordshire AL5 2JQ,[1] and Institute of Genetics, Queens Medical Centre, University of Nottingham, Nottingham NG7 2UH,[2] United Kingdom

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This study examined the effects of NH_4NO_3 fertilizer on the size and activity of nitrifying, autotrophic, ammonia-oxidizing populations of the β subdivision of the class *Proteobacteria* in arable soils. Plots under different long-term fertilizer regimes were sampled before and after NH_4NO_3 additions, and the rates of nitrification were determined by ^{15}N isotopic pool dilution assays. Ammonia-oxidizing populations in the plots were quantified by competitive PCR assays based on the *amoA* and ribosomal 16S genes. Prior to fertilizer addition, ammonium concentrations and nitrification rates in the plots were comparatively low; ammonia-oxidizing populations were present at 10^4 to 10^5 gene copies g of soil^{-1}. Three days after the application of fertilizer, nitrification rates had risen considerably but the size of the ammonia-oxidizing population was unchanged. Six weeks after fertilizer treatment, ammonium concentrations and nitrification rates had fallen while the ammonia-oxidizing populations in plots receiving fertilizer had increased. The rapidity of the rise in nitrification rates observed after 3 days suggests that it results from phenotypic changes in the ammonia-oxidizing bacterial population. Associated increases in population sizes were only observed after 6 weeks and did not correlate directly with nitrifying activity. Phylogenetic analyses of PCR products from one of the plots revealed a population dominated by *Nitrosospira*-type organisms, similar to those prevalent in other soils.

The process of nitrification, defined as the oxidation of ammonia to nitrate via nitrite, is of considerable importance in the terrestrial nitrogen cycle (28). Soil nitrogen (N) in the form of ammonium is bound by the soil matrix and so is retained by the soil. When converted to nitrate, which is mobile within soils, the N becomes susceptible to leaching and denitrification, both of which result in wasteful and potentially polluting N loss from the soil. The initial and rate-limiting step in the process of nitrification is the oxidation of ammonia to nitrite. In arable soils, the majority of this activity is carried out by autotrophic ammonia-oxidizing bacteria (AOB) of the β subdivision of the class *Proteobacteria* (4, 21). These organisms are able to utilize the redox potential associated with the oxidation of ammonia and to fix CO_2 via the Calvin cycle. The initial enzymatic step in autotrophic ammonia oxidation is the conversion of ammonia to hydroxylamine by ammonia monooxygenase. Three genes, *amoA*, *amoB*, and *amoC*, encode this enzyme, which is unique to AOB (5, 22, 29). The *amoA* gene product contains the enzyme's active site.

Autotrophic ammonia oxidizers are difficult to grow in culture. Those that have been grown in vitro have been shown to be unrepresentative of soil populations (6). As a result, little is known about the in situ population dynamics of these ubiquitous soil organisms. Molecular analysis of ribosomal 16S and *amoA* genes has shown that β-subdivision AOB form a monophyletic group with two divisions, the genera *Nitrosospira* and *Nitrosomonas* (18, 36). Studies involving the culturing of environmental AOB isolates, as well as those based on the PCR amplification of environmental AOB sequences, suggest that the majority of ammonia oxidizers from a variety of soils belong to the genus *Nitrosospira* (6, 17, 20, 37, 40). Members of the genus *Nitrosomonas* are more prevalent in ammonia-rich environments such as sewage sludge (30, 37).

The aim of this work was to examine the responses of AOB in arable soils to NH_4NO_3 fertilizer addition. Samples were taken from arable plots under different long-term fertilizer regimes before and after NH_4NO_3 addition. Rates of nitrification were estimated by ^{15}N isotopic pool dilution assays and related to AOB population sizes as determined by competitive PCR (cPCR).

^{15}N isotopic pool dilution is a method suitable for measuring gross rates of nitrification in soil. Isotopically labelled nitrate, the product of nitrification, is added to soil, and the $^{15}NO_3^-/^{14}NO_3^-$ ratio of the soil nitrate pool is measured over time. The rate at which the $^{15}NO_3^-$ is diluted by endogenous $^{14}NO_3^-$ production can then be deduced, and the rate of nitrification can be determined (3). This method does not require the addition of ammonium, the substrate of nitrification, to the soil. Any such ammonia addition is likely to stimulate nitrifying activity, which would result in an overestimation of the in situ nitrification rates (12).

cPCR assays are robust and convenient methods of quantifying small amounts of DNA. These assays involve PCR amplification of DNA in reaction mixtures containing known quantities of competitor molecules. The competitor molecules have the same primer sites as the target sequence and so coamplify, but they generate a differently sized product which can be separated from the target product on an agarose gel. By comparing target and competitor product band intensities, it is possible to estimate the initial number of target sequences in the assay. Such methods have been used to enumerate both

* Corresponding author. Mailing address: Soil Science Department, IACR—Rothamsted, Harpenden, Hertfordshire AL5 2JQ, United Kingdom. Phone: 44 (0) 1582 763133. Fax: 44 (0) 1582 760981. E-mail: tom.mendum@bbsrc.ac.uk.

TABLE 1. The ammonium inputs and selected soil properties of plots NO, N144, and FYM/N

Plot	Fertilizer addition (ha^{-1} yr^{-1})[a]		Total C[b] (μg of C g of $soil^{-1}$)	Total N[b] (μg of N g of $soil^{-1}$)	pH[b,c]
	Inorganic	Organic			
NO	None	None	0.75	0.087	8.2
N144	144 kg of N as NH_4NO_3 in spring	None	0.94	0.10	8.0
FYM/N	48 kg of N as NH_4NO_3 in spring	35 metric tons of manure from bullocks in autumn (equivalent to 248 kg of N)	2.8	0.28	7.9

[a] Other NH_4^+ additions for each plot included 6.7 kg of ammonium N from the atmosphere (15).
[b] Data are from reference 33.
[c] Obtained after mixing 1 part soil in 2.5 parts water.

culturable and nonculturable organisms, including AOB, directly from soil (2, 16, 23, 26, 35, 39). In this study, assays based on the *amoA* and 16S genes were developed and used to enumerate the AOB in the soil. The phylogenetic makeup of the population from one of the soils was determined by sequence analysis of the *amoA* and 16S PCR products.

MATERIALS AND METHODS

Soil properties and experimental design. Samples were taken from part of the Broadbalk continuous wheat experiment (1) at Rothamsted Experimental Station, in which plots of approximately 6 by 23 m have been under continuous fertilizer regimes since 1852. The soils are clayey loams of the Batcombe series whose physical properties vary due to long-term differences in management (Table 1). Three plots were sampled: plot NO, which receives no applied nitrogen; plot N144, which in spring receives 144 kg of N ha^{-1} $year^{-1}$ as NH_4NO_3; and plot FYM/N, which receives the equivalent of 48 kg of N ha^{-1} $year^{-1}$ as NH_4NO_3 in spring and 35 metric tons of farmyard manure, containing 248 kg of N, ha^{-1} $year^{-1}$ in autumn (1). All plots were under winter wheat as part of a 5-year crop rotation. Soil samples were taken from each of the plots on three separate occasions: immediately before spring fertilizer application, 3 days after spring fertilizer application, and 6 weeks after spring fertilizer application. At each timepoint, 10 independent samples from the top 0 to 10 cm of the soil were removed, mixed, and passed through a 4-mm sieve to give a homogeneous composite sample from which subsamples were taken for analyses. ^{15}N isotopic pool dilution assays were begun immediately, and soil from which DNA was to be extracted was stored at −70°C.

^{15}N isotopic pool dilution assays. For the ^{15}N isotopic pool dilution assays, a modified version of the method described by Barraclough and coworkers (4, 42) was used. Three 200-g subsamples from each of the composite soil samples were placed in individual jars, and 16.28 ml of $K^{15}NO_3$ (10.1 atom %) was added at a rate of 5 μg of N g of $soil^{-1}$. To stimulate field conditions, the jars were incubated in the dark at 15°C for 7 days. Samples of 40 g were taken from each jar at days 1, 3, and 7. These were shaken for 1 h with 80 ml of 2 M KCl and filtered through Whatman no. 42 paper to extract the soil ammonium and nitrate. Ammonium and nitrate concentrations in the extracts were determined colorimetrically by using a Skalar continuous-flow analyzer, as described previously (19, 25). The samples were diffused onto acidified glass wool discs (7), and the ^{15}N atom percent excess was determined by using an Integra-CN mass spectrometer (Europa Scientific, Crewe, United Kingdom). Rates of nitrification were determined between days 1 and 7 of the incubation period as described by Barraclough (3).

Primer design, PCR conditions, cloning, and sequencing. All sequence analyses were carried out with the GCG8 (Genetics Computer Group, Madison, Wis.) and PHYLIP V3.57 (13) suites of programs. The program FASTA was used to search the GenBank/EMBL database. Universal *amoA* primers were designed by aligning all publicly available *amoA* sequences and selecting areas of homology. The primers chosen were 5′ATYATGTAYTACYTGTGGGT and 5′ACCACCAGTARAAWCCCCAG, which were designed to amplify the region corresponding to nucleotides 486 to 597 of the *amoA* gene of *Nitrosomonas europaea* (accession no. L08050); database searches revealed no significant homology to non-*amoA* sequences, including that of the methane monooxygenase gene *pmoA*. Primers with published sequences that were specific for AOB 16S genes were assessed by comparison to the database. Primers 5′AGAAAAGCA GGGGATCG (30) and 5′CCTTGTAGTTTCAAACGC (9) were selected because they exhibited maximal homology to AOB 16S genes without having significant homology to non-AOB sequences. PCR mixtures contained 10 mM Tris-HCl (pH 8.3), 100 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate mix, 0.8 μM primers, 0.04 U of *Taq* polymerase (Roche Diagnostics, Lewes, United Kingdom) μl^{-1}, and DNA (equivalent to 50 μg of fresh soil) extracted by the method described by Cullen and Hirsch (10). Conditions for both PCR primer sets were as follows: 45 cycles of 94°C for 1 min, 57.5°C for 1 min, and 72°C for 1 min, followed by 72°C for 5 min. Gel-purified PCR products from plot N144 were cloned into pGEM-T (Promega, Southampton, United Kingdom). Plasmids were prepared with Qiagen Mini Kits (Qiagen, Crawley, United Kingdom) and sequenced by using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kits (Perkin-Elmer, Warrington, United Kingdom). Phylogenetic trees were constructed by using the programs PROTDIST (Kimura two-parameter method), DNADIST (Kimura two-parameter method), and NEIGHBOUR (unweighted-pair group method using average linkages [UPGMA]) and viewed with Treeview 1.4 (32).

Competitor construction. To construct a competitor for the *amoA* primers, soil PCR clone amo8 was digested with *Nar*I and the enzyme was heat inactivated. This digest was ligated to a phenol-chloroform-extracted *Taq*I digest of pBR322. A competitor for the 16S primers was constructed by digesting soil PCR clone 16S-7 with *Sma*I and heat inactivating the enzyme. This preparation was further digested with *Sna*BI (5 U μg of DNA^{-1}), phenol-chloroform extracted, and religated. Both ligation products were transformed into SURE competent cells (Stratagene, Amsterdam, The Netherlands). Resulting colonies were screened by PCR with *amoA* and 16S primers for products of suitable sizes. The quality of plasmids for cPCR assays, prepared by using Qiagen Midi Kits, was assessed by gel electrophoresis, and three independent dilutions of each plasmid were quantified by determining their optical densities at 260 nm.

cPCRs. Each cPCR assay consisted of six reaction mixtures containing serial dilutions of 10^6 to 10^1 competitor molecules and aliquots of sample DNA. Control reaction mixtures contained either no template, sample DNA alone, or competitor alone. Products were separated on 2.75% agarose gels, which were digitized by using an EagleEye II system (Stratagene). Band intensities were quantified by using GelDoc1000 software (Bio-Rad, Hemel Hempstead, United Kingdom). Standard curves were generated for both primer sets and used both to calibrate the systems and to demonstrate that they were quantitative. Assays for the standard curves were carried out in triplicate. Each contained the following: either 10^1, 10^2, 10^3, or 10^4 target molecules of cloned soil PCR product; competitor molecules; and an aliquot of soil DNA previously incubated with DNase I at 37°C for 1 h prior to heat inactivation of the enzyme. The log of the number of competitor molecules per reaction was plotted against the log of the ratio of the competitor and target product band intensities, taking into account differences in product length. The number of target molecules in each assay was estimated as the value at which a straight line through points from the three replicates crossed the x axis (i.e., log [target band intensity/competitor band intensity ratio] = 0), and standard errors were determined (Fig. 1). The numbers of target molecules, as estimated by the cPCR assays, were plotted against the actual numbers of molecules to give standard curves (Fig. 2). Soil DNA for cPCR assays was extracted by the method of Cullen and Hirsch (10) from three 1-g subsamples taken from each composite soil sample. Each extraction product was assayed independently by the protocol described for the standard curves, with the modification that only soil DNA and competitor molecules were added to the reaction mixtures. Values and standard errors were determined as described above after sample dilutions, soil moisture contents, and the standard curves were taken into account.

Nucleotide sequence accession numbers. The environmental *amoA* sequences have been deposited in the GenBank and EMBL databases under accession no. Aj238189 to Aj238197. The environmental 16S sequences have been deposited under accession no. Aj238198 to Aj238203 and Aj238205.

RESULTS

^{15}N isotopic pool dilution assays. Ammonium concentrations in the three plots prior to fertilizer addition were between 1.5 and 2.3 μg of N g of $soil^{-1}$ (Fig. 3a). Three days after NH_4NO_3 fertilizer addition, the ammonium concentrations in plots N144 and FYM/N had risen to 70 and 11 μg of N g of $soil^{-1}$, respectively, but remained low (1.9 μg of N g of $soil^{-1}$) in plot NO. Six weeks later, the concentrations in all three plots had fallen back to between 0.46 and 3.3 μg of N g of $soil^{-1}$.

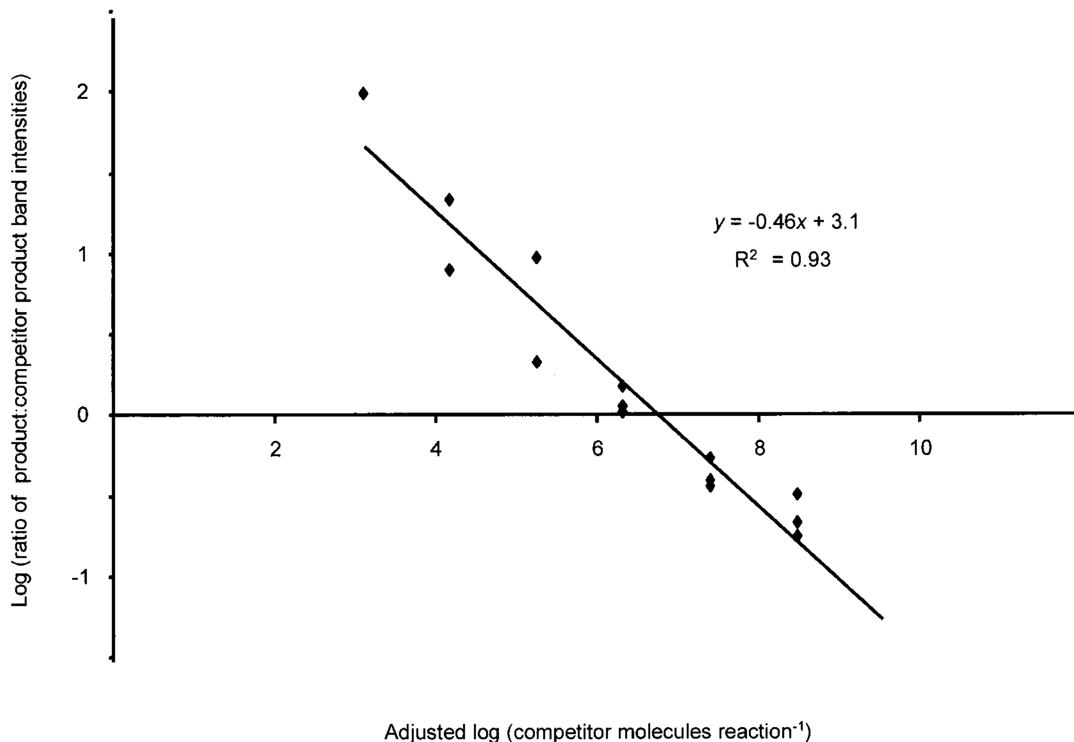

FIG. 1. Plot of the logs of the ratios of target and competitor product intensities from three replicate cPCR assays using the *amoA* primers versus the logs of the numbers of competitor molecules in the reactions. Sample dilutions, soil moisture contents, and standard curves have been accounted for. Each point represents a single PCR. The estimated number of target molecules in the assay (the value at which the fitted line crosses the x axis) is $10^{6.6}$; the standard error of this estimate is $10^{0.22}$.

Nitrification rates in the plots correlated with the soil ammonium concentrations. Prior to fertilizer addition, rates of nitrification were low, 0.16 and 0.21 μg of N g of soil^{-1} day^{-1}, in plots NO and N144, respectively, and 0.76 μg of N g of soil^{-1} day^{-1} in plot FYM/N (Fig. 3b). These rates are consistent with those recorded previously (43). Three days after fertilizer addition, the rates had arisen to 8.0 μg of N g of soil^{-1} day^{-1} in plot N144 and 2.4 μg of N g of soil^{-1} day^{-1} in plot FYM/N. Six weeks after fertilizer addition, nitrification rates had fallen to 0.69 and 1.4 μg of N g of soil^{-1} day^{-1} in plots N144 and FYM/N, respectively. The nitrification rate in plot NO did not change significantly, indicating that the observed changes in plots N144 and FYM/N were a result of fertilizer additions. It should be noted that these assays do not

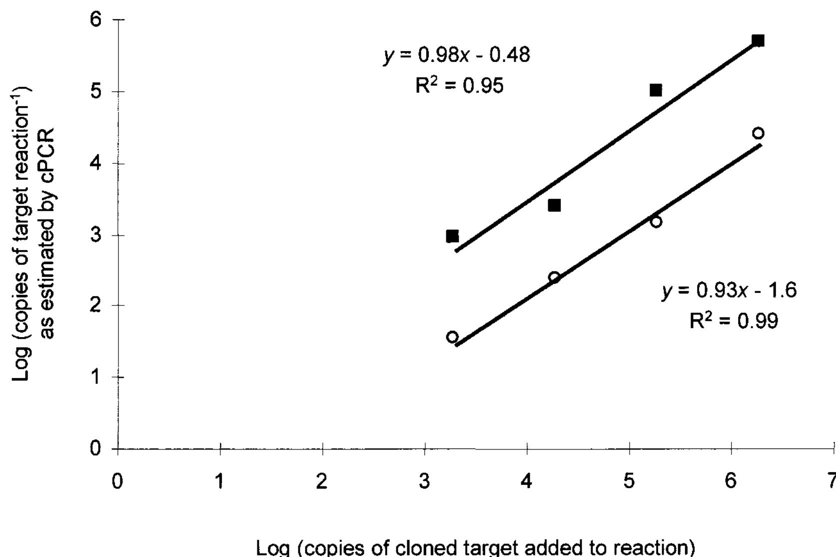

FIG. 2. The standard curves for cPCRs using the *amoA* (○) and 16S (■) primers, relating the number of target sequences, as estimated by cPCR, to the actual number of target sequences present in each assay. Each point is derived from a plot similar to Fig. 1.

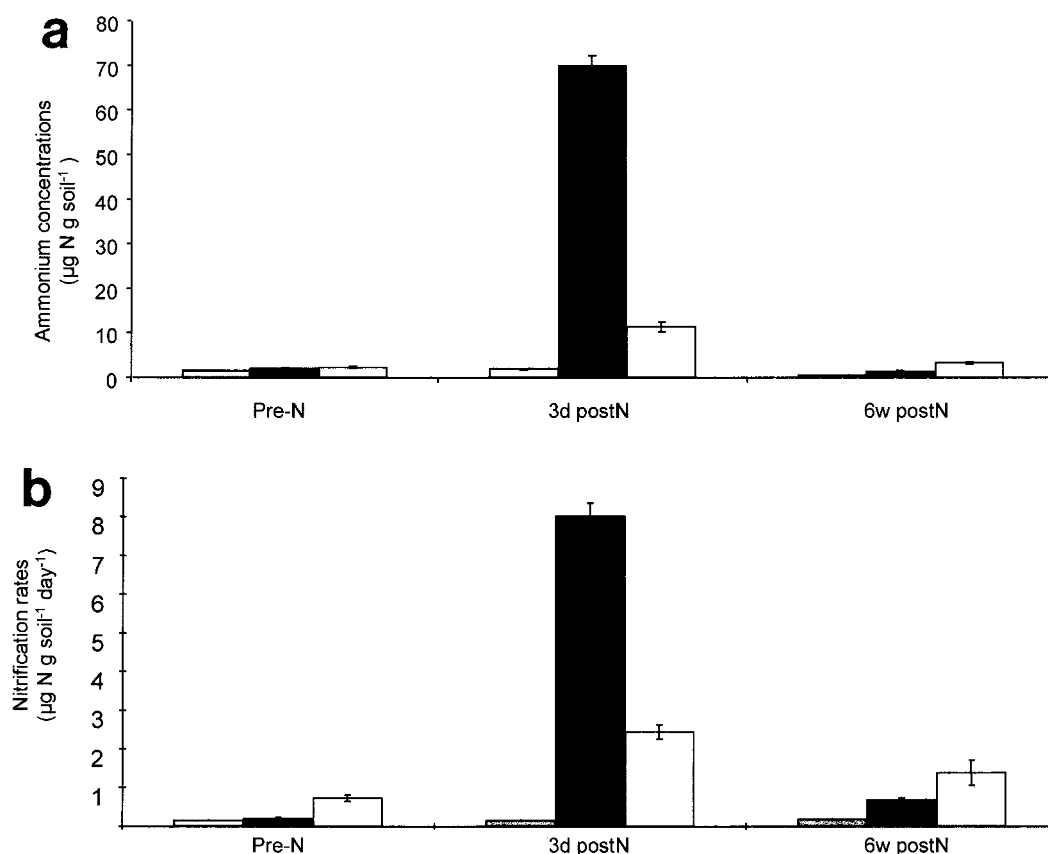

FIG. 3. Ammonium concentrations (a) and nitrification rates (b) as estimated by ^{15}N isotopic pool dilution assays of samples from plots NO (▨), N144 (■), and FYM/N (□) (Table 1) taken immediately before fertilizer addition (Pre-N), 3 days after fertilizer addition (3d postN), and 6 weeks after fertilizer addition (6w postN). Error bars represent the standard errors.

measure field ammonia oxidation rates directly. The assays were ex situ, although conditions were designed to reflect those in the field, and determined rates of nitrate, rather than nitrite, production. Nitrite is the product of autotrophic ammonia oxidation, but in the soil it is rapidly converted to nitrate (8), the oxidation of ammonia being the rate-limiting step in nitrification (28).

cPCR. Individual cPCR assays gave linear relationships between the log of product ratios (target/competitor) and the log of the number of competitor molecules over more than 5 orders of magnitude (Fig. 1). To calibrate the cPCR assays, it was necessary to construct standard curves that to be quantitative must fit the theoretical equation $\log (Prod_{tar}/Prod_{comp}) = \log (Temp_{tar}/Temp_{comp}) + [n \times \log (eff_{tar}/eff_{comp})]$, where $Prod_{tar}/Prod_{comp}$ is the final ratio of target and competitor products, $Temp_{tar}/Temp_{comp}$ is the initial ratio of target and competitor templates, n is the number of cycles, and *eff* is the efficiency of amplification (38). This equation states that the ratio of products must equal the initial ratio of the templates, taking into account the number of cycles and the efficiencies of amplification. The amplification efficiency of a given template depends on several factors, most notably length, to which it is inversely proportional (27). However, so long as the ratio of amplification efficiencies remains constant in all reactions, the standard curve can account for this and other reaction variables, e.g., inhibition by coextracted humic substances in soil extracts. Theoretically, a standard curve for a quantitative cPCR assay will be linear with a gradient of 1 (34). The linear regressions for the standard curves in this study were as follows: $y = 0.93x - 1.6$, $R^2 = 0.99$ for the *amoA* assays; and $y = 0.98x - 0.48$, $R^2 = 0.95$ for the 16S sequence-based assays (Fig. 2). These curves indicate that although the amplification efficiencies of target and competitor were not equal in either assay, the ratio between them was constant, and so the assays were quantitative (Fig. 2). Thus, the assays were able to enumerate the number of gene copies in soil DNA extracts and so measure the relative sizes of soil AOB populations.

The *amoA* and 16S primers amplified products of the predicted sizes, 112 and 458 bp, respectively, directly from soil DNA extracts. cPCR assays using the *amoA* primers showed that prior to NH_4NO_3 addition, plots FYM/N and N144 contained similarly sized populations of 1.2×10^5 and 5×10^5 gene copies g of soil^{-1}, respectively, and that the population in plot NO was significantly lower, i.e., 1.3×10^4 gene copies g of soil^{-1} (Fig. 4). Three days after fertilizer addition, the population sizes had not changed significantly. Six weeks after fertilizer addition, populations in plots receiving NH_4NO_3 had risen; those in plots FYM/N and N144 were 6.5×10^6 and 2.5×10^6 gene copies g of soil^{-1}, respectively. The population in plot NO had not changed significantly, indicating that the increased populations in plots FYM/N and N144 were due to fertilizer addition. The rate of population growth in plot FYM/N was higher than that in plot N144. Assays using the 16S primers gave results similar to those of the *amoA*-based assays. The results were, however, more variable, and standard errors were greater. In all but one case the 16S-based cPCR

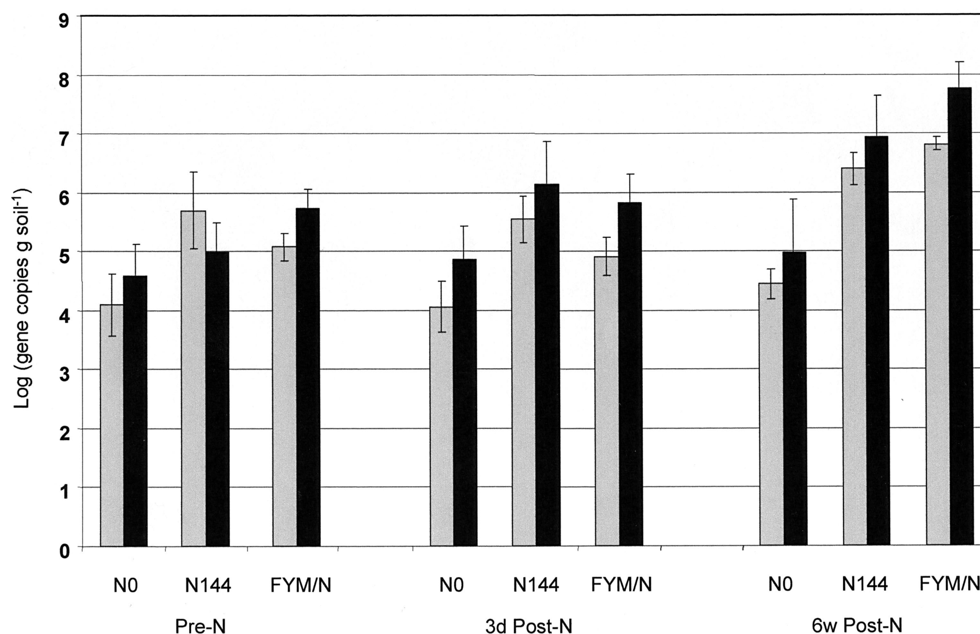

FIG. 4. The number of *amoA* (□) and 16S rRNA (■) gene copies in DNA extracts from samples taken from plots NO, N144, and FYM/N (Table 1) immediately before fertilizer addition (Pre-N), 3 days after fertilizer addition (3d Post-N), and 6 weeks after fertilizer addition (6w Post-N). Error bars represent the standard errors of the means.

assays gave higher estimates of the AOB population size than *amoA*-based assays (Fig. 4).

Phylogenetic analyses. Initial phylogenetic analyses of the nine *amoA* gene fragments used inferred peptide sequences because these are less biased by multiple or silent nucleotide substitutions and so probably are more phylogenetically accurate (37, 44). All of the *amoA* peptide sequences grouped with *Nitrosospira*-type organisms (data not shown). Subsequent phylogenetic analyses of the nucleotides, which are potentially more accurate for closely related organisms (37, 44), placed the sequences in three groups (Fig. 5a). The majority of sequences, amo3 to amo8, formed a unique and tight group with affiliation to *Nitrosospira briensis* and an environmental sequence from a Norwegian pine forest; amo1 and amo 2 were most closely related to *Nitrosospira tenuis* NV-12 and *Nitrosospira multiformis* C-71 (Fig. 5a), while amo9 was related to *Nitrosospira* sp. strain Np22. The level of homology of the sequences to *pmoA*, the equivalent methane monooxygenase gene, was significantly lower than that to *amoA*. The eight 16S sequences obtained from the N144 plot were also dominated by sequences with strong homology to the *Nitrosospira* group (Fig. 5b). Most of the 16S sequences, 16S-1 to 16S-6, were similar to those defined as group 3 by Stephen et al. (40) (Fig. 5b). Sequence 16S-7 was identified as chimeric and so was not included in the rest of the study or submitted to the public databases. Sequence 16S-8 did not fall into the AOB clade, being most closely related to *Dechlorimonas agitatus*.

DISCUSSION

***amoA* and 16S genes.** Throughout this study, the *amoA* primers performed better than the 16S primers; the cloned *amoA* PCR product always contained target sequence, and the *amoA* cPCRs were more reproducible. This relatively high degree of primer specificity is due to the uniqueness of the *amoA* gene, which to date has only been found in organisms capable of oxidizing ammonia. As the ammonia monooxygenase enzyme is involved directly in ammonia oxidation, we can surmise that the *amoA* sequences amplified are derived from AOB. By contrast, although the ubiquity of 16S genes allows AOB to be placed on the same phylogenetic tree as other prokaryotes, it also results in the amplification of products from nontarget organisms by 16S primers (24, 40). The metabolic capabilities of the organisms characterized by such environmental 16S sequences can only be inferred.

cPCR assays. The cPCR assays were able to enumerate the number of gene copies in soil DNA extracts and so measure the relative sizes of soil AOB populations. However, the number of gene copies in a soil DNA extract cannot be assumed to be directly equivalent to the number of AOB in the soil. Variations in gene copy number (31), DNA from moribund organisms, and losses during DNA extraction, estimated at between 52 and 78% (10), all may prejudice results. Variability among extractions has been accounted for by the standard errors given. Differences between *amoA*- and 16S-based cPCR estimates may be explained by differences in primer specificities, as demonstrated by phylogenetic analysis of the products. As would be expected, the less-specific 16S primers tend to give higher estimates of AOB population size.

Ammonia oxidizer population dynamics. Prior to fertilizer addition, plots N144 and FYM/N had AOB populations corresponding to approximately 10^5 gene copies g of soil^{-1}; this is similar to estimates for other soils made by most-probable-number culture methods (6) and cPCR (39). The population of plot NO still corresponded to 1.3×10^4 gene copies g of soil^{-1}, despite having received no agricultural N inputs since 1852. Assuming that after such a long period of unchanging fertilizer management the AOB populations are stable year after year, the considerable AOB population of plot NO must survive solely on ammonium inputs from atmospheric NH_4^+ deposition (6.7 kg of NH_4^+ N ha^{-1} year^{-1} [15]) and from crop residues. The considerable quantities of N added to plots N144 and FYM/N have not resulted in large changes in population sizes when compared to that of the NO plot; however, they

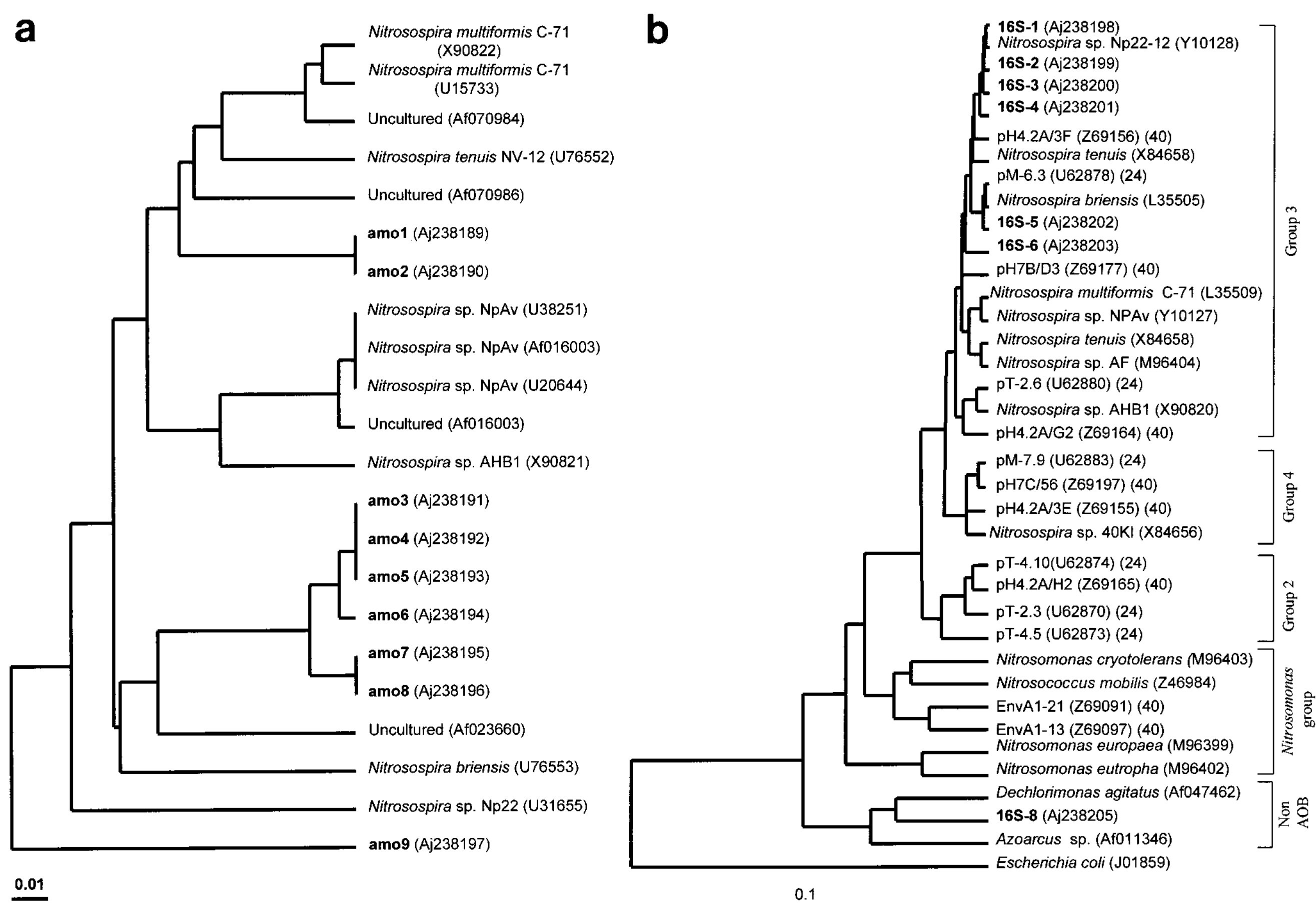

FIG. 5. Phylogenetic tree constructed with partial *amoA* and *Nitrosospira*-type sequences from the GenBank/EMBL database (a) and partial 16S sequences from that database (b). All sequences from this study are derived from plot N144 soil and are shown in bold. The nomenclature of the 16S sequences, from the study by Stephen et al. (40), refers to the pH of the soil from which the sequence was obtained and the isolate number. Groupings are as suggested by Stephen et al. (40). The 16S gene of *Escherichia coli* has been used as an outgroup. Accession numbers are given in parentheses and are sometimes followed by reference numbers.

may have substantially altered the population structures in these plots.

The addition of fertilizer to plots N144 and FYM/N caused rapid rises in ammonium concentrations and a corresponding rise in nitrification rates, suggesting that the activity of these AOB populations was limited by the ammonia supply. In previous studies, nitrification rates in samples from plot NO have also risen quickly following addition of ammonium (11). Compared to soil ammonium concentrations, nitrification rates in plot FYM/N were greater than those in plot N144. This probably reflects the more rapid turnover of the soil ammonium pool that results from the higher rates of mineralization and immobilization associated with soils rich in organic matter (12, 14). The increases in nitrification rates are too rapid to be explained entirely by population increases and so must reflect phenotypic changes in the AOB. Indeed, data from cPCRs show that after 3 days, despite a large increase in nitrification rates, the AOB populations had not grown significantly (Fig. 4). The speed with which the AOB in all of the plots can respond to ammonium addition implies that despite minimal activity during the winter months, the populations maintain considerable metabolic potential and remain in a state of readiness. Six weeks after fertilizer addition, the AOB populations in plots N144 and FYM/N, which had received NH_4NO_3, had grown at rates equivalent to approximately 0.5 and 1 cellular division week^{-1}, respectively. These increases were not reflected in the rates of nitrification, which had fallen toward the levels observed prior to fertilizer application. It is not possible to determine whether the AOB population had grown uniformly or whether we were observing changes caused by a faster-growing subpopulation within the AOB. The AOB populations, increased by the temporary increases in substrate concentrations that resulted from fertilizer addition, cannot be sustainable. Assuming that the AOB populations are stable year after year, the populations must decline during the succeeding months to reach prefertilization levels by the spring.

Phylogenetic trees. As has been observed previously (24, 40), *Nitrosospira*-type sequences dominated the PCR products amplified from the soil. Direct comparisons of the *amoA* sequences from this and previous studies which have examined environmental *amoA* sequences (37, 39) are not possible, since the amplified regions do not overlap. However, comparison of *amoA* and 16S phylogenies suggests that all *amoA* sequences from this study represent organisms similar to those defined as group 3 by their 16S sequences (40). Such comparisons are possible since both genes describe similar phylogenetic structures for the AOB (37), although there are to date no *amoA* sequences from AOB characterized by their 16S sequence as being members of groups 2 and 4. All of the AOB 16S sequences amplified in this study also fall into group 3, which is

dominated by sequences from agricultural soils at close to pH 7 (41).

The small number of sequences from this study and the restricted number of *amoA* sequences from characterized AOB isolates make a more detailed consideration of the population structure in this soil difficult. The *amoA* and 16S primers, which are likely to exhibit different primer biases, describe organisms that occupy similar phylogenetic positions, suggesting that the amplified sequences do represent the AOB present in the soil. However, the possibility of groups of AOB remaining undetected cannot be overlooked. Other studies have recorded phylogenetically similar AOB sequences (24, 41), suggesting that common groups of AOB dominate in diverse and geographically separate soils. It would be interesting to compare population structures in the three plots to determine whether the differing long-term fertilizer regimes have altered the AOB populations.

Summary. By developing new cPCR assays specific for AOB and combining these with ^{15}N isotopic pool dilution assays, this study has related the physiological activity of a defined group of uncultured AOB to their abundance under different fertilizer regimes. The addition of ammonium to the soil caused rapid increases in nitrifying activity which can only be explained by phenotypic changes in the AOB population. Over the longer term, this activity resulted in growth of the AOB populations to levels that are not sustainable over the year. Phylogenetic analysis of the amplified products indicates that the AOB population is similar to those described previously (24, 40, 41), suggesting that such organisms dominate in a range of widely distributed soils.

This work has furthered our understanding of the dynamics of the terrestrial nitrogen cycle, which ultimately relates to how N is supplied to crops and is lost from soil. The methods used are applicable to a wide range of organism, and are particularly suited to the investigation of soil functions known to be directly associated with a single gene product. Such a gene provides a suitable target with which to detect and quantify the organisms of interest.

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