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Presence of Nitrifiers at the Harvard Forest Long-term Litter Manipulation Plots: Application of Process-level and Molecular Techniques

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INTRODUCTION

The conditions under which nitrification (oxidation of ammonium to nitrate predominantly by chemoautotrophic bacteria) is favored in have been studied primarily in agricultural soils and in pure cultures. As described in many textbooks (e.g., Alexander 1977; Brady 1974), nitrification is favored in well drained soils under relatively circumneutral conditions and is stimulated by the addition of fertilizer salts. The factors that determine activity and presence of nitrification in forest soils are certainly less well understood. Nitrification is commonly observed in soils far below the pH range normally associated with the process, and appears to be controlled as much by ammonium availability as by pH. High rates of net nitrification are commonly found in forest soils with pH values below 4.0, especially after disturbances such as harvesting or natural loss of vegetation due to hurricanes or fire. Where nitrification (either net or gross) occurs still cannot be accurately and reliably predicted based on standard soil measures (available from the USDA Natural Resource Conservation Service) and forest stand type and conditions. This is true for a variety of spatial scales from <1 m to the landscape scale (> 100 ha commonly). Some general rules of thumb often are consistent with observations, but there are many exceptions. Further understanding of the factors that regulate nitrification, both its spatial and temporal pattern as well as its rate (gross and net), would be useful for maintenance of forest nitrogen capital after disturbance, preservation of ground water quality, and accurate prediction of the response of forest systems to anthropogenic atmospheric nitrogen inputs.

A major factor regulating nitrification and the potential for nitrification is simply the presence and abundance of nitrifying bacteria. The most common nitrifiers in forest soils are members of the Proteobacteria including such genera as *Nitrosomonas* (Koops 1992) and *Nitrosospira* (Hiorns 1995) which convert ammonium to nitrite (NO_2^-), and *Nitrobacter* that further oxidize nitrite to nitrate (NO_3^-). Estimates of nitrifier abundance in soils range from zero to 10^6 cells g^{-1} (Alexander 1977), although there are relatively few estimates for forest soils. A common assumption is that nitrifiers are ubiquitous in forest soils, and that net nitrification can be stimulated when conditions are favorable. Furthermore, several recent studies (Hart et al. 1994, Davidson et al. 1991) have shown that gross nitrification rates, measured by ^{15}N pool dilution, may in fact be very high, even when net nitrification is undetectable. The isotope pool dilution assay (24 hr duration) has shown that gross rates of both ammonification and nitrification can be 10-20 times the net rate, thus potentially compromising standard assays (e.g., buried-bags) used to assess nitrogen cycling in the lab and *in situ*. A common assay to assess the nitrification potential of soil and the size of the ammonium oxidizer community is to follow nitrate accumulation over 6 hrs in an aerated soil slurry amended with ammonium sulfate (Hart et al. 1994). None of the standard process level measurements, with the exception of long-term (> 30 d) laboratory incubations (*sensu* Stanford and Smith 1972) take into account the potential of a nitrifier population to develop or to become metabolically active.

Molecular methods of identifying nitrifying bacteria, coupled with process-level assays, may be useful for assessing soil nitrification potential. Given that nitrifying bacteria are extremely difficult and time-consuming to culture (Koops 1992) and typically present in low numbers in natural environments, the development of molecular probe-based techniques for their detection should be advantageous. To date most work with molecular methods (Polymerase Chain Reaction (PCR), and Fluorescent In Situ Hybridization (FISH)) have been applied to cultivated microorganisms and to aquatic systems. These approaches hold promise because in some systems they may allow detection of nitrifying bacteria and activity that cannot be detected

with process-level assays. Furthermore, information on the presence of nitrifying bacteria has implications for the response of forest systems to disturbance.

Although it was initially believed that the nitrifiers occupied a rather tight phylogenetic cluster (Koops 1992), more recent studies indicate a surprisingly diverse assemblage of organisms occupying positions within both the beta and gamma proteobacteria (Head 1993, Teske 1994). It has been generally assumed that the nitrifiers of the beta subclass are numerically most important. Within this group, however, there remains some controversy as to which genus is most representative. In this regard, some authors have reported that members of the *Nitrosospira* appear to be more wide-spread than those belonging to the *Nitrosomonas* (Hiorns 1995). For the purposes of this study, we have decided to investigate the abundance of ammonia-oxidizing bacteria from within the *Nitrosomonas* group, which have been noted from a wide variety of environments (Voytek 1995, Koops 1992) and for which well-characterized consensus 16S primers are available (Voytek 1995). We elected to model our efforts after those of Voytek and Ward, who employed their 16S *Nitrosomonas* primer set to amplify related DNA sequences from Bonney Lake in the Antarctic and the Southern California Bight.

While a number of authors have described a variety of rather lengthy and cumbersome approaches for the extraction of DNA from soils (Selenska 1995, Holben 1994, Tsai 1992, Bruce, 1991), the technique applied by Voytek and Ward (1995) suggested that simpler approaches might be adequate to provide template DNA for PCR. With this in mind, we designed a very quick and easy procedure for the release of crude DNA from soil bacteria. Such extracts were amplified in a two-step amplification protocol, based on one that others had used successfully to amplify rDNA sequences specific for ammonia-oxidizers (Voytek 1995, Hiorns 1995). Here we present the results of a project carried out for the 1996 MBL summer Microbial Diversity course. We have made use of a long-term litter manipulation project that is part of the NSF-funded Harvard Forest Long-term Ecological Research (LTER) project. One of us (RB) is a Co-Investigator on the project (currently supported by the USDA Forest/Range/Crop Ecosystems program) and has worked on the study since its initiation in 1990. Based on long-term aerobic laboratory incubations (*sensu* Nadelhoffer 1990) and direct measure of soil water with tension lysimeters, the long-term litter treatments (described below) have variable effect on net nitrification and nitrate flow from the active soil column (defined here as 60 cm thickness). Net nitrification and nitrate concentrations consistently vary significantly by treatment. Plots without roots exhibit high nitrate concentrations in soil pore water and high net nitrification during laboratory incubations, while all others do not. We have used the two molecular techniques (PCR and FISH) to determine the effect of the litter treatment on presence of ammonium oxidizing bacteria and have compared the results with those from the laboratory incubation assay. A separate nitrifier assay (soil slurry method) is being conducted, but those results are not available at the time of the writing of this report.

METHODS

Site Description

The Harvard Forest DIRT (Detritus Inputs Removal and Trenching) plots are located at the Harvard Forest LTER site in Petersham, Massachusetts. The study area is a mature (> 80 yrs old) mixed deciduous forest comprised primarily by red oak, and red maple, with a mixture of white birch and white pine. The soil is an Inceptisol (well drained stony loam) and shows evidence of 19th century pasturing activity. Stand basal area is 30 m^2 ha^{-1} and annual litterfall is 1.4 - 2.5 Mg ha^{-1} yr^{-1} (Bowden et al. 1993; Canary, unpublished data).

In 1990 the following annual, long-term litter manipulations (3 m x 3 m plots, n = 3 per treatment) were established as a means of assessing controls on soil organic matter and nutrient dynamics:

CONTROL	(normal litter inputs)
NO LITTER	(aboveground litter excluded from plots)
DOUBLE LITTER	(twice aboveground litter)
NO ROOTS	(roots excluded from plots by lined trenches)
NO INPUTS	(no aboveground litter and no roots)

Aboveground litter is excluded from NO LITTER and NO INPUTS plots with mesh fabric. Litter inputs in the DOUBLE LITTER plots are augmented by adding litter taken from the NO LITTER plots. Root ingrowth in the NO ROOTS plots has been prevented by lining backfilled trenches (dug below the rooting depth and normally to 70-100 cm depth) with fiberglass sheeting or reinforced polyethylene. The NO INPUTS treatment is a combination of the NO LITTER and NO ROOTS treatments.

The plots have provided information how litter type (above- versus belowground) and trenching influence *in situ* production of nitrate and laboratory-based nitrification potential. Consistently the trenched plots (NO ROOTS and NO INPUTS) have shown high concentrations of soil water nitrate both below the forest floor (zero tension lysimeters) and at 60-cm depth in the mineral soil (tension lysimeters). Furthermore, a recent long-term (120 d) aerobic laboratory incubation has shown that soils from trenched plots have high cumulative nitrate production, while all other plots have none (Fig. 1). Until the work conducted for the project described here, no direct measures of nitrifier abundance or gross nitrification have been carried out. The stimulation of net nitrification on the trenched plots is presumably the result of ammonium oxidizers (normally slow growers and poor competitors for substrate) having access to an abundance of ammonium (brought about by the elimination of plant uptake of ammonium).

Field Sampling and Laboratory Processing

Forest floor material (Oe+Oa horizons) below a 10 cm x 10 cm template was cut with a knife from each of the plots (or adjacent to control plots in some cases), sealed in polyethylene bags, and transported at air temperature back to Woods Hole, where they were refrigerated at 10^0C before processing. We also collected mineral soil (0-10 cm depth), with a tulip bulb sampler, from one NO ROOTS plot and at one off-plot spot. The samples were bagged and treated the same as above. In the laboratory the samples were passed through a sieve (5.6 mm for Oe+OA material, 2 mm for mineral soil) to remove larger organic debris, roots, and stones. The sieved samples were refrigerated for 7-10 days before the Fluorescent *In Situ* Hybridization (FISH) and PCR assays.

Strains and Enrichments

As a positive control, a culture of *Nitrosomonas europaea* was provided in liquid culture by John Waterbury, Woods Hole Oceanographic Institution, Woods Hole, MA. The culture (25 ml) was harvested by centrifugation for PCR amplification after 7 days and well before it had grown to a visibly detectable cell density or had acidified its medium. Cells from a nitrifier enrichment (50 ml) from the Great Sippewissett Marsh, Woods Hole, MA were provided by Bill Metcalf (a member of the class) and used as a positive control from the environment.

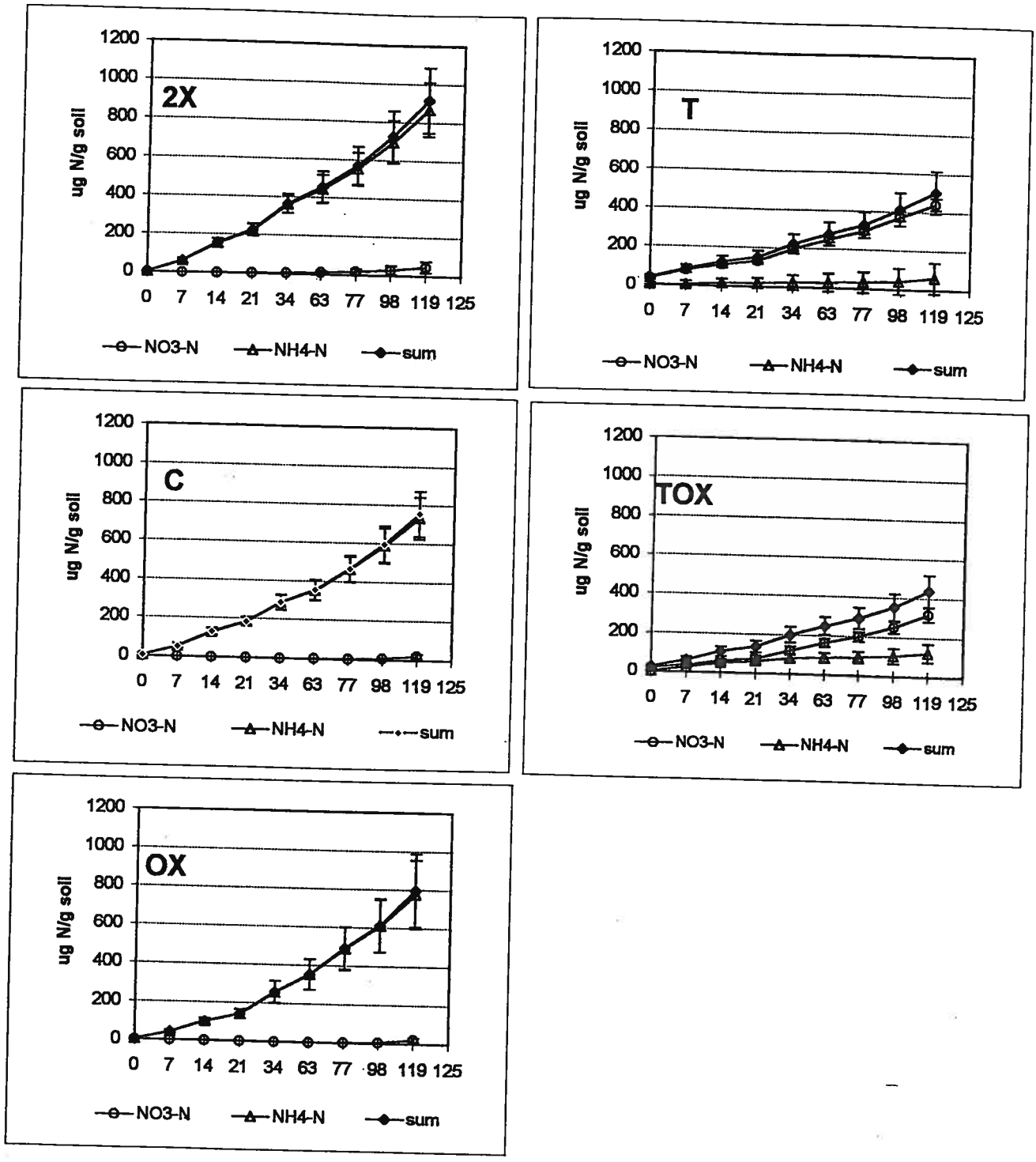

Fig. 1. Cumulative nitrogen mineralization (NO_3-N and NH_4-N) for forest floor (Oe+Oa) samples collected from the Harvard Forest DIRT plots in November 1995. Samples were incubated aerobically at laboratory temperature and were periodically extracted with a Hoaglands solution minus N for measurement of inorganic N production. 2x = DOUBLE LITTER, T = NO ROOTS, C = CONTROL, T0X = NO INPUTS, 0X = NO LITTER. Values are means (±SE), n=3.

Fluorescent *In Situ* Hybridization (FISH)

We applied both a universal probe and a beta probe to all forest floor samples; the beta probe is specific for the beta-subclass of the class Proteobacteria and includes the autotrophic ammonium oxidizers. We followed the method for sediments (Appendix) given in a handout by Sandy Nierzwicki-Bauer, who led a nucleotide probe workshop in the course, with the following modifications:

- Because cell numbers were low, based on initial examination by microscopy and on a trial exercise, samples were diluted only 1:10 before spotting onto slides.

- We added 10 µL DAPI (10 µL ml^{-1} concentration) to slide well-spots just before hybridization because a trial had shown that Coumarin, a fluorescent compound which normally tags all bacterial cells, did not work reliably. Coumarin did not "light" all cells that fluoresced with specific nucleotide probes. We found that DAPI more consistently tagged a larger number of cells, certainly all tagged by specific probes and apparently most if not all those observed under phase contrast.

- Hybridization slides were warmed in the dark at 42°C for 22 hrs, then rinsed four times with 1 x SET solution.

Release of DNA from Soil and PCR Amplification

One gram of soil from each of the four sample sites chosen for this study were first diluted into 10 ml TE (10 mM Tris-HCl, 10 mM EDTA, pH 8.0) in 50 ml conical "Falcon" tubes. The soil/buffer mixture was shaken vigorously by hand ca. 100 times and then vortexed on high for 1 min. The resulting slurry was centrifuged at 1,500 x g for 2 minutes to remove the largest of the particulate material. One milliliter portions of the resulting cloudy, reddish supernatant were then mixed into an additional 9 ml of fresh TE buffer and stored on ice. Microscopic examination after staining with DAPI (Porter 1980) revealed that a large number of bacterial cells had been liberated by this treatment or we attached to small particles that remained in suspension. 50 microliters of this clarified soil extract was added to 20 microliters of GeneReleaser (BioVentures, Murfreesboro, TN) in a 0.5 cm microcentrifuge tube for each. The suspensions were vortexed on high for 20 seconds and 20 microliters of sterile mineral oil was added to the surface of each. Lysis was performed by heating to 94°C in an Easy Cycle thermocycler for 4 min (Ericomp, Power Block System, San Diego, CA). After Lysis, 3 microliters of the aqueous phase was added to a PCR mix containing either a universal primer set or the *Nitrosomonas*-specific primer set (NitAB, Voytek 1995). FisherBrand *Taq* polymerase was added according to the manufacturer's recommendations and amplification was performed over 30 cycles according to the following amplification routine: 94°C, 45s; 50°C, 45s., and 72°C, 2 min. For the second amplification cycle, 3 µl aliquots from the first round of amplification were used as templates for PCR with conditions maintained as before, with the exception that only the NitAB primer set was used. PCR products were evaluated by loading 5 µl portions of the 92 microliter total reaction onto 2% agarose gels in 1 x TBE buffer (Miller 1977), run at 100 V, stained with ethidium bromide and examined under UV illumination.

RESULTS

Fluorescent In Situ Hybridization

The universal nucleotide probe identified bacterial cells in all but one case, while the beta probe showed fluorescence in only 6 of the 15 samples (Table 1). The beta probe did not yield fluorescent cells in either the DOUBLE LITTER or NO ROOTS treatments, but did show fluorescent cells in all other treatments (though not in all plots per treatment).

Table 1: Fluorescent In Situ Hybridization of Harvard Forest Soils. Number of Samples with Fluorescence (max = 3)

Treatment	Universal Probe	Beta Probe	Net Nitrification
CONTROL	3	3	0
NO LITTER	2	1	0
DOUBLE LITTER	3	0	0
NO ROOTS	3	0	+
NO INPUTS	3	2	+

PCR Amplification of rDNA from Soil

The stained agarose gel in Figure 2 shows the results of the first round of PCR from the Harvard Forest. No visible PCR products were obtained with the NitAB primer set (Fig. 2, top half), whereas, distinct bands corresponding to the 1,500 base product expected for the universal primer set were noted. The low molecular-weight smear was disregarded. Figure 3 illustrates the result of the second round of PCR from the NitAB primer set. Even after an additional 30 rounds of amplification, no product was obtained for the samples initially amplified from the NitAB primer set. In contrast, those samples initially amplified with universal primers displayed a band of the appropriate size for the NitAB amplicon (1080 bp, Voytek 1995). In Figure 3B, a set of control reactions are displayed which correspond to the experiment illustrated in Figure 3A. When no DNA template was added, no PCR product was obtained. This was as expected and indicates that the PCR reagents and materials are free from contaminants (Fig. 3A, lane 2). In the positive control reactions (lanes 4 and 5) cell lysates from a nitrifier enrichment (lane 4) and a pure isolate (*Nitrosomonas europaea*, lane 5) give a product of the same expected size.

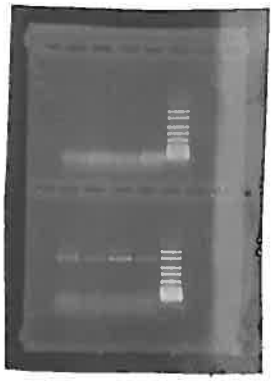

Fig. 2. Stained agarose gel showing PCR products from the first round of amplification from the Harvard Forest soils. The lanes represent the following treatments: 1 = CONTROL Oe+Oa; 2 = NO ROOTS Oe+Oa; 3 = OFF PLOT mineral soil; 4 = NO ROOTS mineral soil. The lanes in the top half are from reactions performed with the NitAB primer set. The lanes in the bottom half show PCR products generated with the universal primer set. Lane 5 in both sets is the molecular size standard.

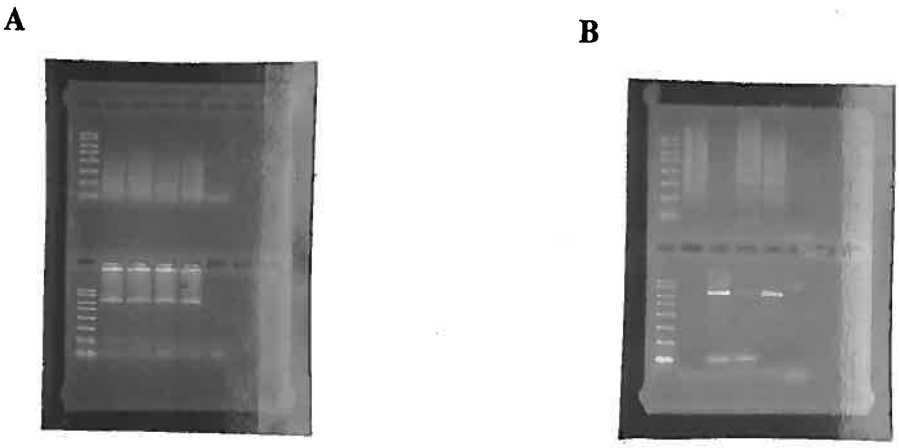

Fig. 3. Panel A: Stained agarose gel showing PCR products after the second round of amplification from soil extracts. Top half: reactions in which the NitAB PCR product from the first round (no visible amplicon) was reamplified using the same primer set. Bottom half: putative NitAB PCR products generated from the universal PCR products obtained from round one. The lanes represent the following treatments: 2 = CONTROL Oe+Oa; 3 = NO ROOTS Oe+Oa; 4 = OFF PLOT mineral soil; 5 = NO ROOTS mineral soil. Lane 1 in both sets is the molecular size standard.
Panel B: Controls. Lane 1 (top and bottom), molecular size markers. Top reactions: an unrelated experiment. Bottom half: lanes are 2 = no template control; 3 = NitAB amplification of the first-round universal primer product; 4 = NitAB amplicon generated from Sippewissett nitrifier enrichment; 5 = PCR product generated from NitAB amplification of known *Nitrosomonas europaea* culture; 6 = PCR product generated from the amplification of the universal primer PCR product, re-amplified with universal primers.

DISCUSSION

The FISH and PCR results, though somewhat ambiguous and not providing conclusive evidence of nitrifiers, are consistent with the presence of ammonium oxidizing bacteria in the Harvard Forest soils, with and without net nitrification. The wide distribution of nitrifiers, even in those soils not exhibiting net nitrification, would be expected given the results from recent ^{15}N pool dilution studies (e.g. Hart et al. 1994, Davidson et al. 1991). These investigations have demonstrated that soils with no observable increase in nitrate during *in situ* incubations commonly have very high gross nitrification rates. Indeed the common classification of forest soils as ammonifying versus nitrifying may be a misnomer, obscuring gross dynamics and the potential for shifts in net cycling. Results here are not inconsistent with the view that nitrifiers are ubiquitous. Consequently, net nitrification is probably more commonly controlled by ammonium abundance and competition between ammonium oxidizers and heterotrophs.

We are cautious not to overinterpret our results, given that our sample size for PCR was small (n = 4), that only one stand was sampled, and that the PCR product was not cloned and sequenced. One conclusion is that PCR products of approximately the correct size were obtained by two-stage amplication of soil extracts from *Nitrosomonas*-specific primers. Although this work is incomplete, FISH and PCR show promise as a means of augmenting process-level nitrification assays. In particular, these molecular techniques may be useful when net nitrification is undetectable and in those cases (e.g., low extractable nitrate) when the ^{15}N pool dilution method is problematic.

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APPENDIX

FOR SEDIMENT SAMPLES:

1. Take tube containing sample (1 gm). Bring total volume up to 20 ml with 4% formaldehyde (filter sterilized) to fix cells or 20 ml PBS pH 7.4 (if samples are to be used immediately). Sonicate for 2 min.

2. Vortex at high setting for 1 min. Let sit approximately 10 seconds.

3. Remove from the top 1 ml and place into Eppendorf tubes.

4. Microfuge for 8 min at top speed.

5. Discard supernatants with P1000 and thoroughly wash pellets with 1 ml PBS (filte sterilized).

6. Centrifuge 8 min, discard supernatant and resuspend pellet in 1 ml 0.1% gelatin that has been made using dH_2O (filtered sterilized) and cooled to room temperature.

7. Vortex well.

8. Make ten-fold serial dilutions of samples into 0.1% gelatin: 100 µl sample + 900 µl gelatin (0.1 X) and 10 µl sample + 990 µl gelatin (0.01 X). Vortex well.

9. Spot 15 µl of above slurries onto baked slides and dry at 37°C. Slides can be stored dessicated until ready to use.

Source: Sandra Nierzwicki-Bauer, Biology Department, Rensselaer Polytechnic Institute