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Enzyme Immunoassay Detection of *Nitrosomonas europaea*

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An exploratory effort to selectively detect the presence of a nitrifying bacterium, *Nitrosomonas europaea*, successfully demonstrated the fundamental utility of an enzyme-based immunoassay protocol. The applied polyclonal antibody test seemingly offered a marked improvement over the available analytical options, including plating, activity, and fluorescence immunoassay techniques. Following an initial purification step to enhance overall specificity, this procedure had an apparent lower limit of detection of $\sim 5 \times 10^6$ cells per ml. Tests conducted with activated sludge samples exhibited a distinct difference between nitrifying and nonnitrifying mixed liquors, although the highest *Nitrosomonas* levels observed (i.e., at 1 to 2% of the overall viable cell density) were relatively close to the latter detection boundary.

Nitrifying bacteria play a critical role in the dynamic cycling of reduced nitrogen throughout our global ecosystem. In turn, the presence or absence of these unique microorganisms within any given environmental niche, whether a wastewater treatment plant, natural water body, wetland, or agricultural soil, comprises an extremely important metabolic issue. The identification of these autotrophs, however, represents a difficult task. All three available techniques (i.e., plating, activity testing, and fluorescence immunoassay measurements) have inherent shortcomings, which are complicated by one or both of the following limiting factors. First, nitrifiers typically comprise only a nominal fraction of the viable cells within sludges, sediments, and soils. Second, nitrifiers reproduce very slowly because of their low rates of energy procurement.

Traditional methods for selective plating, although used since the original discovery of nitrifiers in the mid-1800s, have proven to be somewhat tenuous. Matulevich et al. (5) documented one such approach based on chronological medium testing for nitrite production or depletion. The low growth rate maintained by these microorganisms creates significant problems, however, with various researchers reporting necessary incubation times ranging from weeks to months. Indirect measurements of nitrifier presence based on substrate removal have also been attempted. The activity approach pioneered by Srinath et al. (10) involved the measurement of mixed-culture ammonia depletion rates and then the inference of an apparent fraction of nitrifiers on the basis of a numerical comparison with known pure-culture nitrification rates. Unfortunately, this test's indirect result can easily be misled by inactive or lethargic nitrifiers actually present yet suffering from stress. A third strategy, with immunoassays, involves the use of selective fluorescent-antibody (FA) markers to visually tag nitrifying cells. Schmidt and Bankole (8) originally demonstrated the FA procedure in 1963, and the procedure was subsequently used to identify a soil nitrite oxidizer, *Nitrobacter* spp. (4, 9).

Belser and Schmidt (2) were the first to specifically detect ammonia oxidizers with an FA method in 1978. Using antibodies prepared against 16 terrestrial ammonia-oxidizing strains spread among four genera (*Nitrosomonas*, *Nitrosospira*, *Nitrosolobus*, and *Nitrosovibrio*), these investigators concluded that their bacteria had low cross-reactivities across genus lines and were, therefore, serologically unique. In 1980, Ward and Perry (15) used the same procedure to detect a marine ammonia oxidizer, *Nitrosococcus oceanus*, in ocean waters. Their assay was reported to be quantitative, and the cell counts from FA staining were considered comparable to hemacytometer and acridine orange counts. Five years later, Ward and Carlucci (14) used the FA technique to study the diversity and abundance of nitrifiers in marine samples. Antisera were raised against two marine ammonia oxidizers (*Nitrosococcus oceanus* and *Nitrosomonas* spp.) as well as three marine nitrite oxidizers (*Nitrococcus mobilis*, *Nitrobacter* spp., and *Nitrospina gracilis*) and tested against a large number (~50) of ammonia-oxidizing, nitrite-oxidizing, and marine heterotrophic strains. At this point, therefore, the serological diversity of both marine and terrestrial ammonia-oxidizing bacteria had been established.

Szwerinski et al. (11) subsequently reported that the FA technique could not be used to detect nitrifiers in a fixed-bed nitrification reactor because of interference from slime. Although several physical and chemical treatments for slime removal were tested prior to FA staining, specific reactions with nitrifying bacteria could not be distinguished from the nonspecific background fluorescence. Volsch et al. (13) used flow cytometric analysis to eliminate this problem with background fluorescence. *Nitrosomonas* spp. were detected within activated sludge samples by not only labeling all cells with a DNA-reactive dye but also labeling *Nitrosomonas* cells with fluorescein-conjugated antibodies. In the flow cytometric analysis, only those particles having a DNA content in the range of bacteria were selected for measurement of fluorescein. Although highly complex, the procedure facilitated an improvement in the quantitative detection of *Nitrosomonas* spp. in full-scale wastewaters. Most recently, Sanden et al. (7) documented the first known application of an enzyme-labeled monoclonal antibody immunoassay with nitrifying bacteria. These investigators used a competitive enzyme-linked immunosorbent assay (ELISA) technique to estimate pure-culture densities of *Nitrosomonas* and *Nitrobacter* spp., with an apparent lower limit of detection, in both cases, of $\sim 10^7$ cells per ml. This technique was also used to estimate the presence of *Nitrobacter* spp. within activated sludge, in which densities of $\sim 10^8$ cells per ml were commensurate with elevated nitrification rates.

Contrasted with the preceding monoclonal antibody assay, this paper presents the results of a polyclonal antibody enzyme

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immunoassay for the detection of a particular ammonia oxidizer, *Nitrosomonas europaea*. The technique was tested against a wide range of pure-culture bacteria, including both nitrifying and nonnitrifying microorganisms, and subsequently refined through a purification protocol to enable the estimation of *Nitrosomonas* serotypes in activated sludge samples.

MATERIALS AND METHODS

Antibody development. Polyclonal antibodies were raised against *Nitrosomonas europaea* (ATCC 19718) by injecting a New Zealand White rabbit with pure-culture suspensions. Injections were given intravenously three times at 1-week intervals with 1 ml of phosphate-buffered saline (PBS) containing *Nitrosomonas* cell densities of 0.16×10^8/ml, 0.28×10^8/ml, and 0.5×10^8/ml. After a 10-day rest period, one boost injection with 1 ml of PBS containing 10^8 *Nitrosomonas* cells per ml was given, and 10 days later blood was taken from the marginal ear vein. The blood cells were removed by centrifugation at 10,000 rpm and 4°C for 10 min, and the serum was refrigerated for further use. Serum samples taken from the animal prior to injection were used as a control.

Antibody purification. The antiserum was purified by preabsorption with mixtures of gram-negative and gram-positive heterotrophic bacteria likely to be found in wastewaters (e.g., *Pseudomonas aeruginosa*, *Escherichia coli*, *Streptococcus faecalis*, and *Staphylococcus aureus*). This process involved overnight incubation of a 1-ml solution of the antiserum (diluted 1:10 in PBS) with one freeze-dried pellet (Difco) of each of these heterotrophic bacteria at 4°C, followed by centrifugation (10,000 rpm for 10 min) to remove cross-reacting antibodies, which attach to these nonantigenic bacteria and are subsequently deposited in the residue. The antibody was then separated from the other serum proteins by fast-protein liquid chromatography with a protein G-bead column. This column effectively binds the immunoglobulin G antibody fraction of the serum, which is then eluted by 0.05 M acetate buffer at pH 2.0. The antibody solution was immediately neutralized and then dialyzed against three changes of 0.1 M phosphate buffer.

ELISA protocol. Bacterial suspensions were prepared in PBS (pH 7.4) at several dilutions ranging from 10^4 to 10^8 per ml and dispensed into ELISA plate wells in 50-μl quantities. All tests were performed as antibody saturation assays. Serum dilutions of 1:200 were used for the prepurification tests, and purified antibody solutions containing 0.17 mg of antibody per ml of solution were used for postpurification tests. The primary (i.e., *Nitrosomonas*) antibody and a secondary alkaline phosphatase-labeled antibody (diluted 1:1,000 in PBS) were successively added to each well in 50-μl amounts, and the enzyme label was detected, after reaction with a substrate solution of *p*-nitrophenyl phosphate, by measuring the optical density (405 nm) on an ELISA plate reader.

Pure-culture bacterial development. The antibody was tested for cross-reactivity with 22 separate bacterial groups within six major categories. Aside from the basic antigen, *Nitrosomonas europaea* ATCC 19718, another group of autotrophic ammonia and nitrite oxidizers was obtained as viable pure-culture specimens (Woods Hole Oceanographic Institution, Woods Hole Mass. [12]). Lyophilized methylotrophic strains were purchased from the American Type Culture Collection (Bethesda, Md.). All remaining heterotrophic bacteria used during this study were reconstituted from dry, water-soluble discs procured from Difco (Detroit, Mich.). In each case, these original specimens were washed and resuspended in a PBS solution (pH 7.4) prior to testing. Table 1 details the bacterial forms involved and their relative sources.

TABLE 1. Bacterial categories and sources

Bacterial category and species	Source[a]
NH_3-oxidizing autotrophs	
Nitrosomonas europaea	
C-31	Woods Hole
C-49	Woods Hole
C-101	Woods Hole
Nitrosomonas sp. (marine)	Woods Hole
Nitrosospira briensis	Woods Hole
Nitrosovibrio tenuis	Woods Hole
NO_2-oxidizing autotrophs	
Nitrococcus mobilis	Woods Hole
Nitrobacter sp. (marine)	Woods Hole
Nitrobacter winogradskyi	Woods Hole
Gram-negative heterotrophs	
Klebsiella pneumoniae	Difco
Serratia marcescens	Difco
Salmonella typhimurium	Difco
Escherichia coli	Difco
Enterobacter cloacae	Difco
Proteus vulgaris	Difco
Pseudomonas aeruginosa	Difco
Gram-positive heterotrophs	
Streptococcus faecalis	Difco
Staphylococcus aureus	Difco
Streptococcus pyogenes	Difco
Methylotrophic heterotrophs	
Methylococcus capsulatus	ATCC
Methylomonas methanica	ATCC
Methylosinus trichosporium	ATCC

[a] Woods Hole, Woods Hole Oceanographic Institution; ATCC, American Type Culture Collection.

Activated sludge. Mixed liquor bacterial specimens were obtained from three separate full-scale municipal activated sludge wastewater treatment plants (including one nitrifying facility [Indianapolis, Ind.] and two nonnitrifying systems [Speedway and West Lafayette, Ind.]) as well as a bench-scale (laboratory) nitrifying reactor. In each instance, a series of replicated samples (four or five) taken on successive days at these four locations were then subjected to ELISA testing on a differential basis with purified antibody versus control serum.

Prior to these tests, settled solid samples of these mixed liquors were first resuspended in PBS and sonicated (Fisher Scientific) at 10% power output for 10 s. Following centrifugation for 10 min at 5,000 rpm (Eppendorf), the pellet of coarse solids was discarded, and the supernatant was then recentrifuged for another 10 min at 10,000 rpm (Eppendorf). The second pellet was resuspended in PBS, measured for cell density with a Petroff-Hausser counter, and then diluted accordingly to reach a consistent target cell density of 10^8/ml. This cell suspension was then subjected to ELISA analysis.

RESULTS

The ELISA standard curve for the *Nitrosomonas europaea* ATCC 19718 antigen is shown in Fig. 1. The optical density varied from ~0.25 to ~2.4 over a range of cell densities between 10^4/ml and 10^8/ml. The background antibody reaction in this test at zero-antigen cell density was ~0.15 and did not increase noticeably up to a cell density of $\sim 5 \times 10^6$/ml. The apparent lower detection limit for this polyclonal ELISA technique was, therefore, $\sim 5 \times 10^6$ cells per ml.

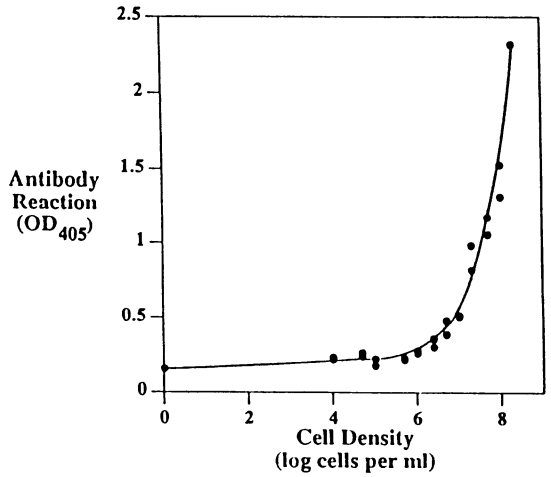

FIG. 1. ELISA standard curve for *Nitrosomonas europaea* (ATCC 19718). The assay produced a lower detection limit of ~5 × 10^6 cells per ml. OD_{405}, optical density at 405 nm.

Figures 2 and 3 depict the results obtained with the purified antibody tested against a diverse range of heterotrophic and autotrophic bacteria. Prior to purification, the response to the heterotrophs had been fairly constant (averaging just under ~0.4) and sufficiently high to create a rather substantial interference. By comparison, the postpurified heterotrophic results (Fig. 2) demonstrated a sizable reduction in this background interference; the *Nitrosomonas europaea* reaction was distinctly different from those of the other heterotrophic cultures for cell densities above ~5 × 10^6/ml. The level of response observed for methylotrophic heterotrophs (Fig. 2C), which may bear morphologic and metabolic similarities to *Nitrosomonas europaea*, was similarly low.

Figure 3 depicts the purified antibody results observed for a variety of nitrifying autotrophs. In this case, the prepurified antibody response increased at the higher cell densities. Here again, though, this nonspecific or background interference was reduced by purification (Fig. 3B and C). However, the reactivity of the polyclonal purified antibody with the nonantigenic *Nitrosomonas europaea* strains (Fig. 3A) did not follow this pattern. A particular soil isolate (strain C-31) responded in a fashion similar to the ATCC 19718 strain, and two other related strains (C-49 [river water isolate] and C-101 [sewage isolate]) also exhibited response levels higher than those of any other nitrifier or heterotroph tested.

Lastly, the results obtained with this polyclonal antibody ELISA test on activated sludge samples are shown in Fig. 4. Both sets of nitrifying samples (including those from Indianapolis, Ind., and the bench-scale biomass) produced reaction response levels consistently higher than those measured for the nonnitrifying specimens. In general, the average response level of the nitrifying cultures was approximately twice that obtained with the nonnitrifying samples.

DISCUSSION

This investigation represents the first successful demonstration of a polyclonal antibody ELISA protocol for the identification of *Nitrosomonas europaea*. The lower limit of detection for this test (~5 × 10^6/ml) appeared to be more than one order of magnitude below that of the competitive monoclonal antibody ELISA procedure recently reported by Sanden et al. (7).

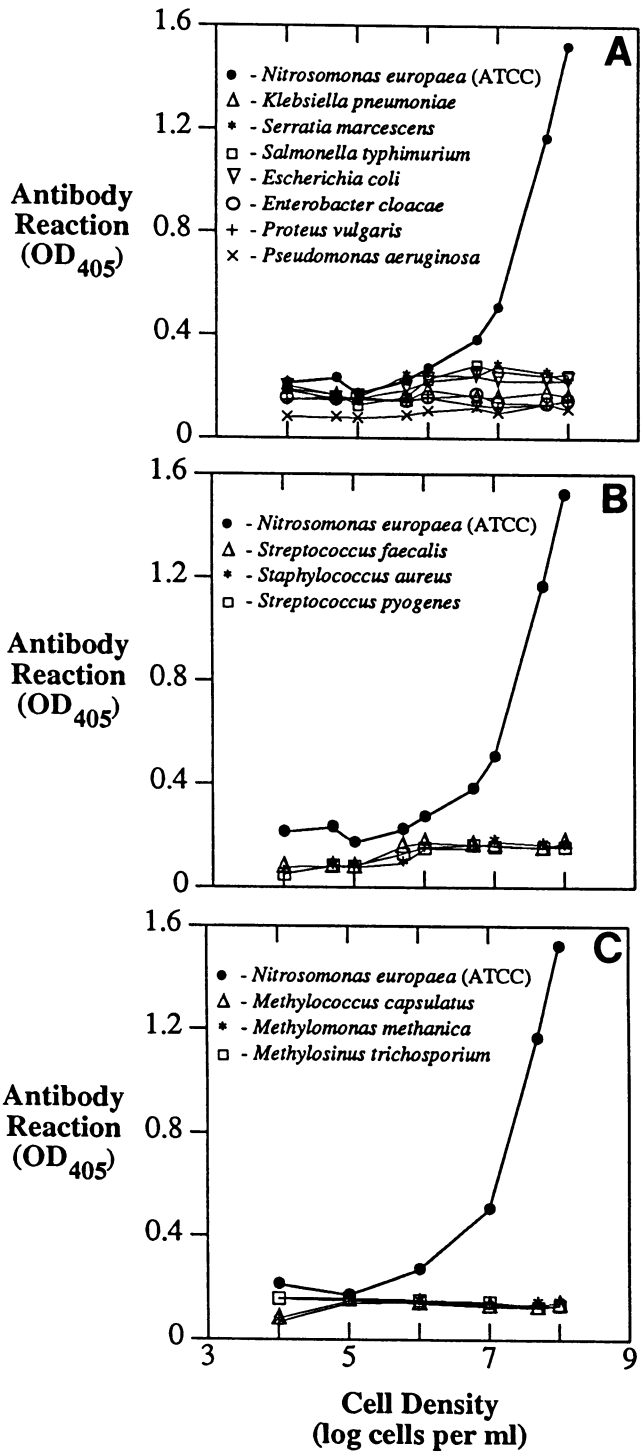

FIG. 2. Comparison of purified antibody reactions with *Nitrosomonas europaea* and gram-negative (A), gram-positive (B), and methylotrophic (C) heterotrophic bacteria. The purified antibody did not exhibit any interfering reactions with heterotrophic bacteria. OD_{405}, optical density at 405 nm.

However, a purification procedure based on preabsorption of the polyclonal antibody with nonantigenic cells was necessary to achieve this degree of sensitivity. Without prior serum purification, the presence of nonantigenic microorganisms

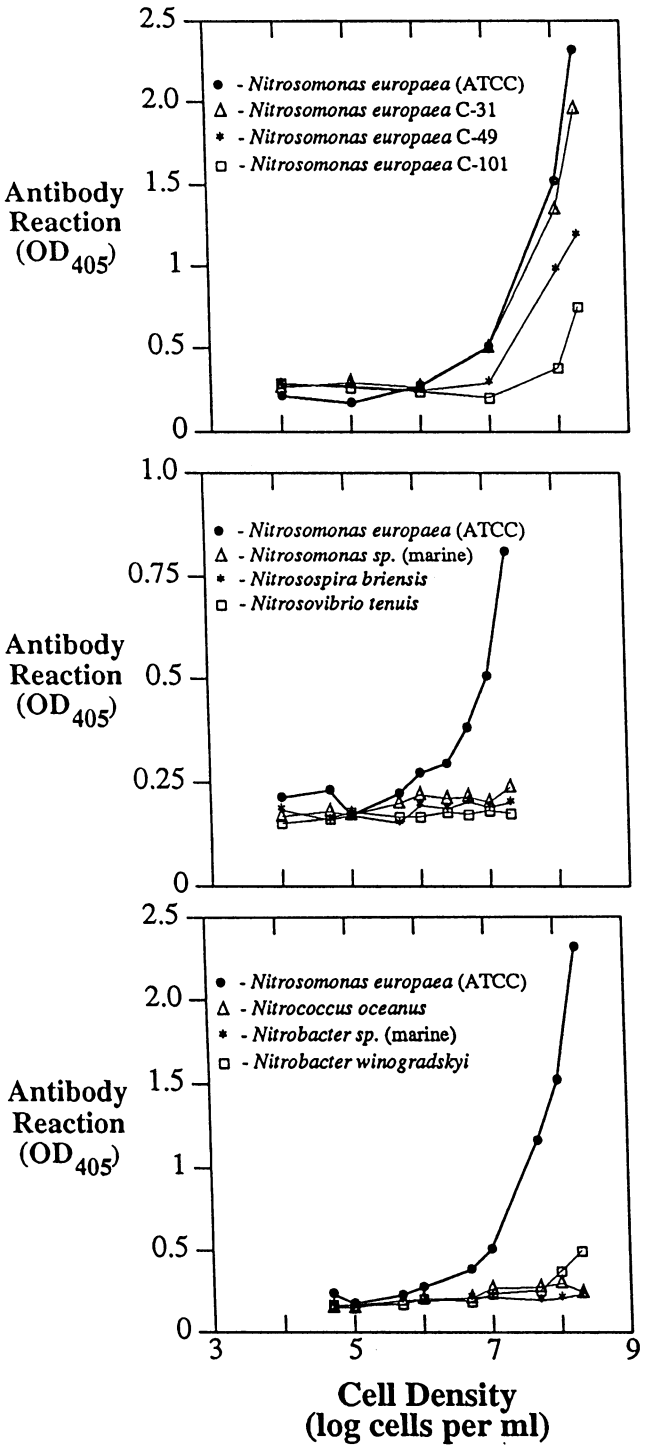

FIG. 3. Comparison of purified antibody reactions with *Nitrosomonas europaea* and autotrophic nitrifying bacteria. The antibody reacted with several *Nitrosomonas* strains (A) but not with other ammonia (B)- and nitrite (C)-oxidizing bacteria. OD_{405}, optical density at 405 nm.

created sufficient masking interference to raise this limit by an order of magnitude.

Sample preparation also appeared to represent an important procedural step with this test. In particular, detachment of

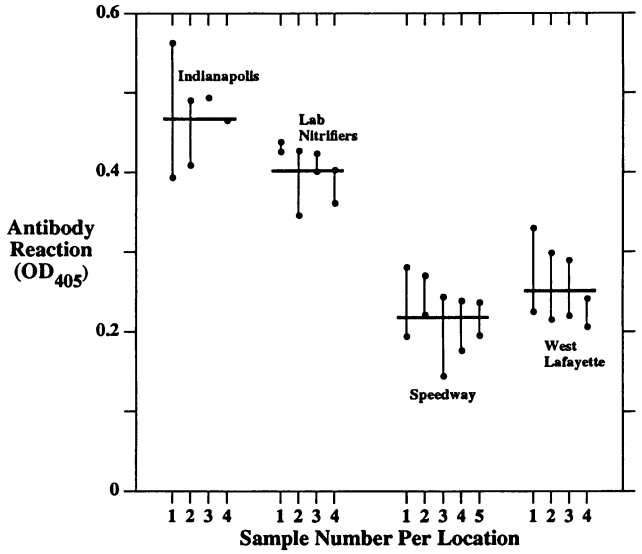

FIG. 4. ELISA test results with activated sludge samples, showing higher levels of reactivity with nitrifying mixed liquors than with nonnitrifying mixed liquors. OD_{405}, optical density at 405 nm.

flocculated cells with ultrasonication and resuspension in a standard buffer were believed to be key factors for desired binding of the antibody. Prior fluorescence immunoassay testing by Belser and Schmidt (2) and Volsch et al. (13) had suggested that this type of antibody would be serologically distinct. The purified antibody developed during this study, however, demonstrated an affinity for pure *Nitrosomonas europaea* strains other than the ATCC 19718 antigen. In particular, a distinct reactivity was determined for a known sewage isolate (*Nitrosomonas europaea* C-101).

In turn, this behavior prompted a qualitative investigation of the antibody's response with mixed cultures known to contain nitrifying organisms. The specificity of the test was ensured by evaluating each sample's response to the preimmune (i.e., control) serum and then subtracting these values from the purified antibody response.

A comparison of the reactivities observed in various field samples initially diluted to a total cell density of 10^8 cells per ml (Fig. 4) against the standard curve (Fig. 1) suggested that *Nitrosomonas europaea* ATCC 19718 serotypes were present in both of the nitrifying mixed liquors (Indianapolis and laboratory nitrifiers), at an average density of $\sim 5 \times 10^6$/ml. Furthermore, as anticipated, the nonnitrifying mixed liquors (from West Lafayette and Speedway) both exhibited response levels relative to the standard curve which were below the apparent detection limit. These data generally appear comparable to those obtained by Volsch et al. (13). Using flow cytometric analysis with activated sludge samples similarly diluted to an initial total cell density of 10^8 cells per ml, these investigators reported *Nitrosomonas* densities ranging from $\sim 2 \times 10^5$ to 2×10^6/ml. These counts, no doubt, bear an inherent degree of uncertainty relative to the actual nitrifier presence. In both cases, however, the density estimates for these activated sludge samples, at 1 to 5% of the total cell population, compare closely with the 1 to 2% values generally reported in the literature (6). These immunoassay methods therefore should provide an improvement over most-probable-number procedures used for nitrifier enumeration, which not only have been

shown to greatly underestimate nitrifier densities but also require substantially longer testing periods (1, 3).

The quantitative utility of this approach to nitrifier enumeration should be enhanced by switching to monoclonal antibodies bearing an affinity for a wider range of nitrifier strains. Further research will, in turn, focus on this monoclonal antibody protocol.

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