Development of a Most-probable-number Method for Enumerating Denitrifying Bacteria by Using 96-Well Microtiter Plates and an Anaerobic Culture System

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A new most-probable-number (MPN) method using 96-well microtiter plates was developed to enumerate denitrifying bacteria. In this method, dilutions of samples are added to microtiter plates with a medium and incubated anaerobically using the AnaeroPouch culture system (Mitsubishi Gas Chemical, Inc., Tokyo, Japan). The microtiter plate MPN method gave precise estimates of the population densities of a culture of Paracoccus denitrificans, similar to the estimates obtained using a test tube MPN method and a colony count method. The population densities of denitrifying bacteria in soil samples estimated by the microtiter plate MPN method were higher than the population densities estimated by the test tube MPN method. Because the new method requires less equipment, labor, and time than the conventional test tube method. It will be valuable for estimating the biomass of denitrifying bacteria in natural samples.

Key words: most-probable-number, denitrifying bacteria, enumeration, microtiter plate

Denitrifying bacteria (denitrifiers) are various groups of bacteria that are able to reduce nitrates or nitrites to nitrous oxide (N₂O) or dinitrogen (N₂). These bacteria are taxonomically and physiologically diverse. Most of them are heterotrophs and facultative anaerobes, such as Pseudomonas fluorescens, Alcaligenes, and Paracoccus, others are autotrophs including photosynthetic and chemolithotrophic bacteria, or obligate anaerobes. Denitrifiers have been intensively studied for several reasons. They cause the loss of nitrogenous fertilizer in agricultural fields, but may help prevent groundwater from being polluted by ammonium or nitrate derived from excessive fertilization. They also play an important role in wastewater treatments that remove inorganic nitrogen compounds, in combination with treatment by ammonia-oxidizing bacteria (nitrifiers). Furthermore, some denitrifiers diffuse N₂O into the atmosphere. This may result in depletion of stratospheric ozone and may be involved in global warming.

The biomass of denitrifying communities in soil, sediment, and activated sludge is typically evaluated by three methods; enumeration of the denitrifying populations, measurement of denitrification activity, and measurement of denitrifying enzyme concentrations. The enumeration of heterotrophic denitrifiers based on molecular technologies using specific sequences of the N₂O or NO₂⁻ reductase genes has been developing. However, these molecular methods still have problems regarding the specificity and sensitivity of primers for PCR. Also, there are many kinds of denitrifiers in the soil environment. Therefore, it is useful to determine the number of denitrifiers with a most-probable-number (MPN) method as well as molecular methods.

The MPN method for denitrifiers is usually based on the disappearance of nitrate and nitrite, the formation of bubbles, the accumulation of N₂O in the presence of acetylene,
or a combination of these factors. However, the MPN method is time-consuming, tedious, and imprecise. Nevertheless, it is often used for estimating the biomass of denitrifiers because the method requires no specific apparatus and is simpler than the measurement of denitrification activity.

To make MPN methods more precise and efficient, microtiter plates have been employed for the enumeration of several groups of bacteria, such as nitrifiers, fecal coliforms, and thermophilic bacteria, and hydrocarbon-degrading bacteria. Microtiter plates are usually used for culturing aerobic bacteria. However, denitrifiers, most of which are not obligate anaerobes, can be aerobically treated and can be anaerobically cultivated on microtiter plates using an anaerobic culture system. A simple and efficient microtiter plate MPN method for denitrifiers would be a valuable tool for estimating their population densities.

We describe the development of a microtiter plate MPN method that can be applied to denitrifiers, using a cultivation technique that allows the bubbles caused by denitrification to be detected in the well of the microtiter plate. To evaluate the method, we enumerated the population densities of a culture of Paracoccus denitrificans and of denitrifying populations in natural soil samples.

Materials and Methods

Bacterial strains, media, and cultivation

Paracoccus denitrificans IAM 12479 (=ATCC 17741), which was obtained from the Institute of Applied Microbiology Culture Collection, University of Tokyo, Japan, was used as an authentic standard denitrifier. The strain was subcultured at 30°C on a nutrient agar medium (Difco Laboratories, Detroit, Mich.) that contained beef extract at 3 g L\(^{-1}\), peptone at 5 g L\(^{-1}\), and agar at 15 g L\(^{-1}\). The basal media for enumeration of denitrifiers were the full-strength nutrient broth (Difco) and broths diluted to one-half and one-quarter of the concentrations (for both beef extract and peptone); these were designated as 1/1, 1/2, and 1/4 nutrient broths, respectively. These media were supplemented with sodium nitrate (2.0 g L\(^{-1}\)), sodium nitrite (0.05 g L\(^{-1}\)), HEPES (for stability of pH, 2.5 g L\(^{-1}\)), and agar (for proper viscosity of the media, 2.0 g L\(^{-1}\)). The media were designated as 1/1, 1/2, and 1/4 nitrate nitrite nutrient (NNN) agars, respectively. In practice, the media for microtiter plates were made at double-strength concentrations because the media (100 μL) were mixed in the wells with the dilutions (100 μL) of the samples. The pH of the media was adjusted to 7.0. The overlaying solution consisting of agar at 5 g L\(^{-1}\) and gellan gum at 8 g L\(^{-1}\) (pH not adjusted) was prepared for overlaying the sample in the wells, to confirm the presence of bubbles emerging in the wells during anaerobic culture.

Enumeration of population densities of P. denitrificans

For the microtiter plate MPN method, we used 96-well microtiter plates (Asahi Techno Glass Co., Tokyo, Japan) consisting of 8×12 rows. Twelve rows, designated 1 to 12, each consisting of 8 wells, were used. One microtiter plate can be used to perform 2 sets of 8-replicate MPN procedures at 5 or 6 dilutions, or one 16-replicate MPN procedure.

To estimate population densities of a culture of P. denitrificans, 2 microtiter plates were used for each medium (1/1, 1/2, or 1/4 NNN agars) and each sample. A culture of the bacterium grown in the nutrient broth (Difco) for 24 h at 30°C was used as an original sample, and was diluted to 1/2, 1/4, and 1/8 as subsamples. Then serial dilutions of the original sample and these subsamples were carried out to a level of 10\(^{-6}\). Sixteen aliquots (100 μL) of 10\(^{-8}\) dilutions were inoculated into the 16 wells of rows 6 and 12, and 16 aliquots (100 μL) of the 10\(^{-9}\) dilutions were inoculated into the 16 wells of rows 5 and 11. These procedures were repeated with the 10\(^{-3}\) dilution. To determine whether cross-contamination among the wells occurred, 16 aliquots (100 μL) of sterile water were inoculated into the wells of rows 1 and 7 as control wells. Three kinds of media, warmed to 40°C to remain liquid, were dispensed into the microtiter plates (100 μL/well), to mix with the previously inoculated dilutions. A few minutes after the mixtures had solidified, the overlaying solution at 45°C was dispensed into the microtiter plates (100 μL/well). The bacteria inoculated into the microtiter plates were cultivated anaerobically with the AnaeroPouch culture system (AnaeroPouch Anaero, Mitsubishi Gas Chemical, Inc., Tokyo, Japan) at 30°C. During incubation, the culture could be easily observed through the transparent pouch. The wells were observed every 2 or 3 days over 4 weeks, and the wells in which bubbles emerged during anaerobic culture were scored as positive.

For the test tube MPN method, the 1/1, 1/2, and 1/4 NNN broths supplemented with bromothymol blue solution (10 g L\(^{-1}\)) in ethanol at a final concentration of 0.05 g L\(^{-1}\) were used. The broths were dispensed into test tubes, filling up to 3/4 of the tube. Then the test tubes were fitted with inverted (Durham) tubes and autoclaved. Twenty aliquots (100 μL) of each dilution (10\(^{-3}\) to 10\(^{-9}\)) of the original sample and the subsamples (1/2, 1/4, and 1/8) were inoculated into 20 test tubes. The tubes were statically incubated at 30°C over 4 weeks. The tubes in which both bubble formation and an in-
crease in pH (color change of the media from green to blue) were observed were regarded as positive.

The colony count method was carried out with an Eddy Jet spiral plater (IUL Instruments S. A., Barcelona, Spain). Three aliquots (50 μL) of each dilution (10⁻³ to 10⁻⁵) were added to 3 plates of the nutrient agar (Difco). The plates were then incubated at 30°C statically and aerobically. The colonies emerging on the plates in 2 weeks were counted.

Soil sampling and enumeration of denitrifiers

Soil samples were collected from the 1- to 5-cm horizon of a corn field, a grove of trees (mainly Japanese cedar) in a park, and a forest (mainly Japanese red pine) on a mountain, in Tsukuba, Ibaraki, Japan. The samples were prepared within 6 h of being collected. Each soil sample (1 g wet weight) was gently homogenized with a sterile mortar and pestle, and serially diluted with distilled water. To estimate population density, 1 microtiter plate was used for each medium (1/1, 1/2, or 1/4 NNN agars) and each sample. Sixteen aliquots (100 μL) of each dilution (10⁻² to 10⁻⁶ for the cornfield and grove samples and 10⁻¹ to 10⁻³ for the forest sample) were inoculated into the 16 wells of 2 rows of the microtiter plates. Dispensation of the media, incubation of the microtiter plates, and determination of positive wells were conducted in the same way as for P. denitrificans. For enumeration by the test tube MPN method, test tubes containing the 1/1, 1/2, and 1/4 NNN broths with bromothymol blue (0.05 g L⁻¹) and Durham tubes were prepared. Ten aliquots (100 μL) of each dilution (10⁻² to 10⁻⁶ for the cornfield and grove samples and 10⁻¹ to 10⁻³ for the forest sample) were inoculated into 10 tubes. Incubation of the tubes and determination of the positive ones were conducted in the same way as for P. denitrificans. By taking into account the water content of the soil, the MPN values of denitrifiers per g of dry soil were calculated.

Confirming the growth of denitrifiers

To confirm that bubble formation in the wells of the microtiter plates was caused by the growth of denitrifiers, the following experiment was carried out, referring to previous reports. Aliquots (10 μL) of 10-fold dilutions of the culture from a well regarded as either positive or negative were inoculated into 4 serum bottles (30-ml volume) containing 5 ml of 1/1 NNN broth. After the bottles were sealed with butyl rubber stoppers and aluminum caps, the headspace gas in 2 of the 4 bottles was replaced with N₂ containing 1% (v/v) acetylene (C₂H₂) 5 times, and the headspace gas in the other 2 bottles was replaced with N₂ as controls. After 2 weeks of incubation at 30°C, N₂O accumulation in the headspace in the bottles was examined. The growth of denitrifiers in the bottles was confirmed by comparing the concentrations of N₂O in the control bottles with the concentrations of N₂O in the bottles whose headspace gas was replaced with N₂ containing 1% (v/v) C₂H₂. The N₂O concentration in the headspace was measured by using a gas chromatograph (model GC-14B, Shimadzu Co., Kyoto, Japan) equipped with an electron capture detector and a glass column (0.3×60 cm) containing Porapak R (80–100 mesh, GL Sciences Inc., Tokyo, Japan). The carrier gas was N₂ (40 ml/min) and the temperatures of the column, the detector, and the injector were 40, 200, and 200°C, respectively. The data were analyzed by using a Chromatopac C-R6A (Shimadzu).

Statistical methods

The MPN values and confidence limits were calculated from the numbers of positive tubes or wells in 5 serial dilutions, using ICR-MPNV software, which can be obtained from the U.S. Environmental Protection Agency Web Site (http://www.epa.gov/nerlcwww/online.htm). The regression analyses, Student’s t-test, and other calculations were performed with MS Excel (Microsoft Inc., Redmond, Wash.).

Results

Evaluation of population density of P. denitrificans

The relationships between dilution rates of the culture and the estimates made using by the microtiter plate MPN method, the test tube MPN method, and the colony count method, are shown in Figure 1. The colony count method was considered the most precise of the 3 methods in this study, because it showed the best correlation (r²=0.999) between the dilution rates and the estimates. Judging from the slopes of the regression lines and r² values, the microtiter plate MPN method gave estimates similar to those obtained by the colony count method. On the other hand, the test tube MPN method gave a regression line whose slope was quite different from the slope obtained by the colony count method, and the estimates at each dilution rate varied from the regression line (Fig. 1). These results indicate that the test tube MPN method was less precise than the other 2 methods.

Evaluation of population density in soil

A microtiter plate that was inoculated with the dilutions of the grove soil sample and then incubated for 2 weeks was examined. Wells were considered positive if bubbles accumulated in them. We thought that cross-contamination...
might occur among wells. However, we did not detect bubbles in any control wells, nor did we detect the transfer of culture from any wells to adjacent wells. In the wells inoculated with low dilutions (10^{-1} and 10^{-2}), it was difficult to detect bubble formation because of the presence of soil particles containing the inoculum. But it was possible to detect bubbles by observing from the bottom of the microtiter plate or by using a magnifying glass.

Populations of denitrifiers in natural soil were estimated with the 3 kinds of media, by using the microtiter plate MPN method and the test tube MPN method (Fig. 2). In all samples and media, the microtiter plate method gave higher estimates than the test tube method. In particular, the MPN values of the cornfield soil and forest soil estimated by the microtiter plate method were about 1 order of magnitude higher than the values estimated by the test tube method. Furthermore, in the test tube method, test tubes that had been inoculated with low dilutions (10^{-1} or 10^{-2}) of soil, and thus must have been positive, often exhibited no bubble formation. Consequently, unreliable MPN codes such as 9, 10, 6, 1, and 0 (the code, p1, p2, p3, p4, and p5, expresses the number of positive tubes in the 5 serial dilutions) were scored (Fig. 2). In the microtiter plate method, such unnatural MPN codes were rarely scored. Among the 3 media, the estimates obtained with 1/1 NNN agar were lower than the estimates obtained with 1/2 or 1/4 NNN agars in many cases. Therefore, it can be said that 1/2 or 1/4 NNN agars are better suited than 1/1 NNN agar to the enumeration of denitrifiers in soil.

As a control experiment, instead of using 1/1, 1/2, or 1/4 NNN agars, denitrifiers were cultured anaerobically using 1/1, 1/2, or 1/4 nutrient agars to which neither nitrate nor nitrite was added. During 4 weeks of incubation, no well in the microtiter plates accumulated bubbles, which suggests that the bubbles that emerged in the wells resulted from the reduction of nitrate or nitrite to gaseous nitrogen compounds, as some researchers report.

We confirmed whether the wells in which we observed bubble formation actually contained denitrifiers, by examining N_2O accumulation in the subculture serum bottles in the presence of C_2H_2. All of the 30 subcultures from the cultures regarded as positive (bubble formation) on the microtiter plate exhibited more N_2O accumulation in the presence of C_2H_2 than in the absence of C_2H_2, although many of the subcultures in the bottles in the absence of C_2H_2 also exhibited a certain amount of N_2O accumulation (data not shown). On the other hand, all 5 subcultures from the cultures regarded as negative (no bubble formation) on the microtiter plate exhibited no N_2O accumulation in the presence or absence of C_2H_2. These results indicate that it is reasonable to determine the existence of denitrifiers for bubble formation in the wells on the microtiter plates.
Discussion

Microtiter plate MPN methods have been developed and applied to various groups of bacteria, most of which are aerobes\(^{2,3,10,15,22,23}\). In this study, we devised a microtiter plate MPN method for denitrifiers, using the AnaeroPouch culture system and using media supplemented with agar, gellan gum, and HEPES.

For enumeration of denitrifiers using microtiter plates, it is necessary to detect steady bubble formation in the wells, and therefore, it is necessary to increase the viscosity of the medium to accumulate the bubbles emerging during anaerobic culture. To increase the viscosity, agar needs to be added to the medium sufficient to retain bubbles emerging in the wells (about 10 to 15 g L\(^{-1}\)). However, the medium will then require warming to about 45°C or higher in order not to solidify. This warming might cause an underestimation of the population because of the inactivation of heat-sensitive denitrifiers. In our preliminary experiments on solidifying agents, we decided to use a medium for enumeration containing agar at a final concentration of 2 g L\(^{-1}\), and a medium for overlaying containing agar at 5 g L\(^{-1}\) and gellan gum at 8 g L\(^{-1}\) to accumulate bubbles. By using these media for enumeration, we are unlikely to miss scoring a positive well as long as the microtiter plates are examined every 2 or 3 days.

However, in a very small number of wells, we observed bubbles that later disappeared—the bubbles in the wells may have passed through the medium for overlaying, the gas of the bubble may have re-dissolved in the medium, or the gaseous nitrogen compounds in the wells may have transformed to other compounds. Similarly, when we enumerated the population density of *P. denitrificans*, there were very few wells in which the growth of the bacterium was detected by turbidity and yet bubbles were not detected. The reason why is unknown, but it might be that cells incapable of denitrifying are occasionally generated in cultures of *P. denitrificans* by spontaneous mutation. The appearance of such wells was the exception; therefore, the phenomenon hardly seems to affect the estimation.

In the test tube MPN method, tubes were considered positive if there was a combination of bubble formation and an increase in pH of the medium, to distinguish bubbles of hydrogen (H\(_2\)) or carbon dioxide (CO\(_2\)) from bubbles of N\(_2\)O or N\(_2\). However, in our experiments the color of all test tubes where bubbles formed changed from green to blue. In our preliminary experiments, we used the microtiter plate MPN method with media containing bromothymol blue (0.05 g), and observed changes in the pH of the media in each well. However, we found that several hours after the microtiter plate had been sealed in the pouch under anaerobic conditions, the color of media in all wells turned yellow. This phenomenon occurred simply because a large amount of H\(_2\) and CO\(_2\) was generated in the pouch by the AnaeroPouch system, acidifying the media. In addition, when the microtiter plates continued to be inubated anaerobically, the color of some wells where bubbles emerged changed from yellow back to green or to blue, which indicates an increase in the pH of the medium, while the color of other wells where bubbles emerged remained unchanged. Therefore, to determine whether the wells where bubble formation was detected and yet the color of the culture remained unchanged actually contained denitrifiers, we subcultured the medium from the wells in serum bottles under an atmosphere of N\(_2\) containing 1% (v/v) C\(_2\)H\(_2\) and examined the accumulation of N\(_2\)O in the bottles. Consequently, we determined that all such wells contained denitrifiers (data not shown). The reason for the phenomenon might be that the denitrifying activity of such denitrifiers was so weak that the pH of the medium that had been acidified by CO\(_2\) remained unchanged. Therefore, we conclude that in the microtiter plate MPN method, positive wells should be determined by bubble formation alone, not by the combination of bubble formation and an increase of pH of the medium.

As described above, the large amount of CO\(_2\) generated with the AnaeroPouch culture system acidifies the culture medium. In our preliminary experiments on media for enumeration of denitrifiers, we used NNN agar to which HEPES was not added; however, we observed few positive wells with bubble formation. We then decided to supplement the medium with HEPES. This resulted in stability of pH, and in reliable appearances of positive wells. Addition of HEPES at more than 2.5 g L\(^{-1}\) (final concentration) resulted in no change in the estidtiometer.

When enumerating population density in soil, the estimates obtained with the 1/1 NNN agar were lower than the estimates obtained with the 1/2 or 1/4 NNN agar (Fig. 2). In the cultures with 1/1 NNN agar in the microtiter plate, a few wells that had been inoculated with a 10\(^{-3}\) dilution of the forest sample (and thus must have been positive) exhibited no bubble formation. We also observed such phenomena in some test tubes in the test tube MPN procedures. The reason could be that denitrifiers were not able to grow vigorously enough to form bubbles in such wells or test tubes when they were in competition with nondenitrifying bacteria for the nutrition. Therefore, for natural samples that contain a variety of microorganisms, media that lessen
the nutrition, such as 1/2 or 1/4 NNN agars, seem to be better suited than 1/1 NNN agar. The medium most suitable for enumeration may depend on the sample. In this study, we examined natural denitrifiers only in soil samples. It is clear that the culture conditions will be determined by the sample (freshwater, sediment, activated sludge, etc.) and the target microorganisms (thermophilic, oligotrophic, etc.).

For enumeration of \textit{P. denitrificans} population densities, the microtiter plate MPN method gave precise estimates similar to the estimates obtained by the colony count method (Fig. 1). Moreover, for enumeration of population densities in soil, in many cases the microtiter plate MPN method gave higher and more reliable estimates than those obtained by the test tube MPN method (Fig. 2). The reason why the former method gave higher estimates than the latter method is probably that the amount of medium in the well of the microtiter plates is quite smaller than that in the test tube, so slight growth of denitrifiers is detectable in the wells.

As we have described, the microtiter plate MPN method can give more reliable estimates than the test tube MPN method. In addition, the microtiter plate MPN method requires much less labor, time, incubation space, and reagents, and the number of replicates can be more easily increased than with the test tube MPN method. Because increasing the number of replicates leads to more reproducible and stable estimates, the microtiter plate MPN method for denitrifiers is a valuable tool for estimating natural samples more easily than conventional MPN methods.

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**References**


