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677 South Segoe Rd., Madison, WI 53711 USA

Evaluation of the Most Probable Number Method for Enumerating Denitrifying Bacteria[1]

E. A. DAVIDSON[2], M. K. STRAND, AND L. F. GALLOWAY[3]

ABSTRACT

Most probable number (MPN) enumerations of denitrifying bacteria were conducted on an agricultural soil (pH 6.6) and a forest soil (pH 5.6), in both neutral (pH 6.8) and acid (pH 5.6) media. The neutral medium yielded higher enumerations for both soils and, therefore, appears to be optimal, even for denitrifiers present in acid soils. Enzyme activity assays and MPN enumerations of seven soil samples were weakly correlated ($r = 0.71$; $p = 0.07$). One soil exhibited a high MPN enumeration, but had no detectable denitrifying enzyme activity. While the enzyme assay indexes denitrification potential of soils under the environmental conditions at the time of sampling, the MPN enumeration indexes the denitrifying capability of the populations inhabiting soils. A population's denitrifying capability is related to its size, but other factors which might also influence MPN enumerations are discussed. Precise incubation time was critical in the MPN procedure. Delays of 2h affected the scoring results. Populations in some of the incubation tubes appeared to be in a dynamic growth phase at the end of the 14-d incubation period. Inconsistencies in incubation duration could confound comparisons among soils. Despite inherent flaws and disadvantages, the MPN procedure may be appropriate for certain research objectives as an index of a population's denitrifying capacity. Recommendations and cautions are offered.

Additional Index Words: **denitrification, nitrate reduction, nitrogen cycle, population estimations, soil acidity.**

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IN HIS RECENT REVIEW of methods for the study of denitrification, Tiedje (1983) recommends that a measure of denitrifying enzyme activity be used in preference to most probable number (MPN) enumerations as an index of "activity or catalytic potential" of a soil sample. While we basically agree, we anticipate that many researchers will still opt for the MPN technique because: (i) the number of organisms per gram of soil may be perceived as a more appealing unit than is the enzyme activity, expressed as nmoles N_2O g^{-1} dry soil h^{-1}; and (ii) an index of the bacterial population capable of denitrifying may be needed for certain research objectives. Since denitrifying enzymes are synthesized only under anaerobic conditions and their activity is inhibited by exposure to O_2 (Firestone, 1982), temporal changes in soil aeration may cause dramatic fluctuations in denitrifying enzyme activities, without causing commensurate changes in the denitrifying populations. If the latter is the parameter of interest, MPN enumerations may be justified. An improved scoring method for MPN enumerations of denitrifying populations (Tiedje, 1983) further enhances the likelihood that the technique will be employed.

While several problems inherent to the MPN method have already been discussed (Tiedje, 1983), we pose three additional concerns. First, if adaptations to acid soils include a pH optimum for denitrification which is below neutrality, then MPN enumerations conducted with the recommended neutral medium might result in underestimations of acid soil populations. Second, a valid estimate of the MPN of denitrifiers requires that the inoculation of a given tube by a few bacteria or even by a single denitrifying bacterium will result in the reduction of a previously determined amount of NO_3^- to N_2O by the end of the incubation period. However, the time needed for a single cell to develop into a population capable of transforming a tube sufficiently to warrant a positive score may vary among soil samples of different denitrifying populations. This variation could confound estimates of population sizes and render the MPN technique similar to an assay of denitrifying enzyme activity potential. If the MPN method is strongly influenced by growth rates and/or enzyme activity rates, then the direct enzyme assay is definitely preferable for the reasons cited by Tiedje (1983). However, if the two methods unequivocally index different parameters (i.e. population size vs. enzyme activity), then the choice must depend upon the research objectives. Third, the length of the incubation period appears to be critical both for the reason cited above and because the populations in tubes in extinction-range dilutions appear to be in a dynamic state at 14 d.

MATERIALS AND METHODS

Composite soil samples were collected from a soybean field at the Clayton Agricultural Research Station near Raleigh, NC, and from a mixed-hardwood forest at the Coweeta Hydrologic Laboratory near Franklin, NC. The agricultural soil is a sandy loam in the Wagram series, an Arenic Paleudults. The forest soil samples include a Tusquitee loam (Umbric Dystrochrepts), a Spivey Stoney loam (Typic Haplumbrepts), a Haywood loam (Cumulic Haplumbrepts), and a Trimont loam (Typic Hapludults).

Soil samples were collected with a 2.5-cm diameter push tube. For the acidity study, 6 to 10 samples were taken from the 5-to 15-cm depth and were composited at each site. For the study comparing MPN enumerations and enzyme concentrations, five sites were selected at Coweeta, the soils of which varied in moisture, NO_3^-, and C content. Three sites, a black locust (*Robinia pseudo-acacia* L.) stand, a mixed-hardwood stand, and a stream site (extending about 5 m on each side of the stream and supporting shrubs and herbs) were selected on watershed (WS)6, an 18-yr-old forest with a history of disturbance. Two sites, a mixed-hardwood stand and a stream site, were chosen on WS18, a mature, uneven-aged forest. Composite samples were collected from the top 10-cm of mineral soil at each site. At the WS6 stream site, composite samples were collected from each of three depth segments, 0 to 10, 10 to 20, and 20 to 30 cm.

Sampling was done in March 1983 for the acidity study and December 1983 for the comparative study. Soil samples were stored in plastic bags at 4°C until analyzed. All analyses were completed within 14 d of sampling.

The MPN enumeration procedure was that of Tiedje (1983). Briefly, serial 10-fold dilutions (from 10^{-1} to 10^{-9})

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[2] Graduate Research Fellow, Dep. of Forestry, North Carolina State Univ., Raleigh.
[3] Undergraduates, Dep. of Biology, Oberlin College, Oberlin, OH.

Table 1. Effects of soil acidity and medium acidity on MPN enumerations of denitrifiers.

Medium	Soil	
	Forest (pH 5.6)	Agricultural (pH 6.6)
	log MPN g^{-1} dry soil	
Acid (pH 5.6)	4.44† (0.12)	4.72 (0.11)
Neutral (pH 6.8)	5.48 (0.06)	5.92 (0.00)

† Mean and (SE); $N = 3$; LSD (all pairwise comparisons) = 0.27 ($p = 0.05$).

were incubated in NO_3^--amended nutrient broth at 25°C for 14 d under headspace concentrations of 10% acetylene-90% air (v/v). For the acidity study, three replicate enumerations were conducted for each soil sample in a medium of acidity pH 6.8 and three replicates in a medium which had been adjusted to acidity pH 5.6 by the addition of HCl.

The diphenylamine (DA) colorimetric test was employed for scoring. Scores for tubes in the extinction range (i.e. at dilutions where some but not all tubes were scored positive) were confirmed by measuring N_2O concentration in the headspace. Gas samples were analyzed for N_2O with Varian 3700[4] and Tracon 550 gas chromatographs, each equipped with a Poropak-Q column and a ^{63}Ni electron capture detector. For a subset of samples, the DA test was repeated immediately following the N_2O check if the two scoring methods were not in agreement.

The short-term enzyme activities of the Coweeta soil samples were assayed by the method of Smith and Tiedje (1979). Briefly, 10-g soil samples were incubated under 90% argon-10% acetylene (v/v) with NO_3^- and chloramphenicol amendments. Headspace gas was analyzed after 2 h incubation for N_2O concentration as above. Soil acidity was measured in 1:1 soil/water (w/w) slurries. Soil moisture was determined gravimetrically by drying at 105°C for 24 h. In all cases, fresh, non-sieved, field-moist soil was used. Pebbles and root fragments were removed by hand to the extent that was practical.

RESULTS

The MPN enumerations were higher in the agricultural soil than the forest soil regardless of the acidity of the medium used (Table 1). The neutral medium yielded higher enumerations than the acid medium for both soils (Table 1). A two-way analysis of variance showed that the differences between soils and between media were statistically significant ($p<0.01$), but that the interaction was not significant.

Soils from Coweeta WS18, which exhibits very low soil and streamwater NO_3^- levels, yielded no detectable enzyme activity and yielded MPN enumerations 2 to 3 orders of magnitude below those of WS6 (Table 2). All soil samples from WS6, which is dominated by the leguminous black locust and which exhibits high soil and streamwater NH_4^+ and NO_3^-, showed high MPN enumerations and high enzyme concentrations relative to WS18 (Table 2). The 0-to 10-cm sample of the stream site of WS6, which receives large amounts of N-rich sediment and which is frequently water-saturated, exhibited the highest enumeration and enzyme activity. Both MPN enumerations and enzyme activities declined with depth.

[4] The use of trade names does not imply endorsement by the North Carolina Agricultural Research Service of the product, nor criticism of similar products not mentioned.

Table 2. MPN enumerations and enzyme activities of Coweeta soil samples.

Site	Log MPN (g^{-1} dry soil)	Log enzyme activity (nmol N_2O g^{-1} dry soil h^{-1})
WS18 stream	a 1.2 (0.6)†	a −2.00§ (0.00)
WS18 hardwood	ab 1.9 (0.2)	a −2.00§ (0.00)
WS6 locust	bc 3.2 (0.6)	bc 0.96 (0.07)
WS6 hardwood	cd 4.5 (0.1)‡	b −0.06 (0.50)
WS6 stream		
0–10 cm	d 4.6 (0.4)	c 1.57 (0.10)
10–20 cm	cd 3.8 (0.2)‡	b −0.24 (0.98)
20–30 cm	cd 3.5 (0.1)	a −2.00§ (0.00)

† Means and (SE); $N = 3$ except $N = 2$ for means marked by ‡; means preceded by the same letter within each column are not significantly different at $p = 0.05$ as determined by LSD.

§ Values below detection limits (0.01 nmol N_2O g^{-1} dry soil h^{-1}) are transformed to log number −2.00.

The correlation between log enzyme activity and log MPN enumerations was only marginally significant ($r = 0.71$; $p = 0.07$). One sample contributing to this poor correlation is notable. The 20-to 30-cm depth sample of the WS6 stream site revealed no detectable enzyme activity but produced a significantly larger MPN enumeration than the WS18 soils which also exhibited no enzyme activity (Table 2).

The DA scoring method (a blue color indicates presence of NO_3^-) was not always unequivocal. A green color frequently developed, and these tubes were scored negatively, since no significant N_2O production had occurred. When only faint blue rings developed, the N_2O check was positive for denitrification.

Approximately one-third of the 750 tubes used in the comparative study were checked for N_2O accumulation. Of these, 37 appeared to be false negatives (i.e. the initial DA test indicated presence of NO_3^-, but the N_2O check revealed significant denitrification). When the DA test was then repeated, NO_3^- was often absent, thus indicating that the remaining NO_3^- must have been reduced during the delay between the two assays. These ambiguities in scoring occurred in eight samples where this delay exceeded 2 h (the delay never exceeded 5 h). The post-delay enumerations are from 25% to two orders of magnitude higher than the original, DA-scored enumerations for these eight samples. For reasons given in the next section, the data reported in Table 2 are results of the original DA scoring.

Only four genuine false negatives were detected. Just one tube, which contained purple bacteria, was a false positive (i.e. the DA test indicated absence of NO_3^-, but less than 20% of the NO_3^- had been reduced to N_2O).

DISCUSSION

The effects of acidity on denitrification appear to be species-specific (Burth and Ottow, 1983). Similarly, the effects of acidity may vary with genotypes adapted to different soils. Denitrifiers which have been cultured in neutral media always exhibit higher MPN enumerations when assayed under neutral conditions compared to acidic conditions (Valera and Alexander, 1961). However, we are unaware of any investigations of acidity optima for enumerating populations adapted to acid conditions. Furthermore, while it has generally been observed that MPN enumations are higher for neutral soils than acid soils (Valera and Alexander,

1961), these enumerations were conducted in neutral media. Acid media may have yielded higher enumerations for the acid soils. Indeed, observations of significant, albeit slow, denitrification rates in soils of acidity below pH 4 (Waring and Gilliam, 1983) suggest that adaptations to acid conditions probably occur, which might include shifts in pH optima.

The results presented here, however, indicate that no such dramatic difference in acidity optima exists, at least within the range tested. The MPN enumerations were significantly higher for both the acid and neutral soils when the neutral medium was used. The lack of significant interaction between soil and medium effects suggests that either medium (i.e. either acidity level) could be used to obtain consistent comparisons among soil samples. However, the higher enumerations from the neutral medium probably afford a better opportunity to distinguish significant differences among soil samples, regardless of their acidity. Further research would be necessary to confirm this conclusion over a broader range of soil acidities.

The results of the MPN and enzyme assays are correlated, but the association is weak. High variability within replicates may partly account for lack of close correlation. Nevertheless, the data clearly show instances where the two methods provide indices of different parameters. The MPN enumerations reveal populations which are capable of denitrifying, but which possess little or no denitrifying enzyme activity at the time of sampling. The WS6 stream site clearly supports large denitrifying populations at all depths, but the 20-to 30-cm sample may have been substrate-limited or sufficiently well oxygenated at the time of sampling to have limited previous denitrifying enzyme synthesis. While the enzyme assay indexes denitrification potential of the soil under the current environmental conditions, the MPN enumeration indexes the denitrifying capability of the populations inhabiting the soil. Since the MPN technique addresses a different research question than does the short-term enzyme assay, it should be considered a potential research tool, despite its many problems. Further research is needed to determine if MPN enumerations index the same parameter as long-term (e.g. 24 hr or more) denitrification potential assays.

It should be noted that a population's denitrifying capacity is certainly related to its size, but may also be influenced by such factors as rates of intrinsic metabolic activity, reproduction, enzyme synthesis, and enzyme activity. Therefore, while the neutral soil has a larger MPN enumeration than the acid soil, the same enumerations could result from populations of similar size but of different activities. The few denitrifying cells from the acid soil which were present when the extinction-range dilution tubes were inoculated may have taken longer to transform their tubes to positive scores (i.e. reduce sufficient NO_3^- to N_2O) than those of the neutral soil. On the other hand, the acid soil population could be truly smaller, composed of bacteria capable of rapid denitrification, but restricted to relatively few, less acidic microsites (Firestone, 1982). Indeed, the denitrification process, itself, consumes hydronium ions and therefore could produce such altered microsites. In short, the MPN enumeration should be regarded as an index of a population's denitrifying capacity, of which size is a major but perhaps not the only factor. The word "index" is stressed because the MPN enumeration does not directly measure absolute values of any single parameter.

The detection of apparent false negative tubes by the new MPN technique (Tiedje, 1983) poses a serious logistical problem. Nitrous oxide measurement by gas chromatography requires 1 to 4 min per sample, depending on the instrument and its operation. Processing the numerous samples necessary for MPN enumerations inevitably leads to delays between the DA test and the N_2O check. If the two were done together, then delays would develop between the processing of different samples. The effects of a 2-h delay on scoring suggest that populations in the extinction-range dilution tubes are probably in a logrithmic growth phase at the time of assaying. Reduction of the small amount of NO_3^- present in the medium might occur within a few hours once the log phase begins. The lag phase may have lasted 13 or 14 d at these dilutions with little NO_3^- having been reduced. A longer incubation time might allow populations in all tubes which were inoculated with one or more denitrifying bacteria to pass through the log phase, thus providing unequivocal scoring results. Further research is needed to determine if an extended incubation period would be desirable.

When a 14-d incubation period is used as it was here, all samples should be analyzed rapidly to standardize incubation time. This is easily accomplished by the DA scoring method, but is difficult for the N_2O check. Although the N_2O determination is considered a definitive test for the occurrence of denitrification, we have observed that a population in log growth phase can produce sufficient N_2O for a positive score within a matter of hours. The definitiveness of the N_2O check at any point in time is not in question, but any scoring method requires not only a definition of a positive score, but also a definition of when the test is to be applied. For dynamic populations, differences in incubation lengths can render any scoring method equivocal. Since the DA and N_2O scoring results were in excellent agreement when the tests were conducted within 2 h, we are confident in the accuracy of the DA data and have used them for sample comparisons (Table 2) in order to avoid differences in incubation lengths among samples.

Any MPN enumerations conducted at various times (e.g., at different seasons) can be compared only if consistent incubation times are strictly observed. If time has such an important effect, then MPN enumerations may also be very sensitive to slight differences in incubation temperature and other conditions.

Although the MPN enumeration is very time consuming and has several inherent flaws, it may still be appropriate if an index of a population capable of denitrifying is desired rather than a measure of current enzyme activity. If the MPN enumeration technique is chosen, we recommend that: (i) a medium of neutral acidity be employed regardless of the acidity of the soil; (ii) delays between the DA scoring and the N_2O check be considered when apparent false negatives are observed; (iii) extreme caution be employed in stand-

ardizing incubation conditions (time to the hour, temperature, *etc.*); and (iv) the MPN enumeration be considered an index of a population's denitrifying capacity.

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