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Dynamics of Soil Denitrifier Populations: Relationships between Enzyme Activity, Most-Probable-Number Counts, and Actual N Gas Loss†

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To better understand temporal variability in soil denitrification, denitrifying enzyme activity (DEA) and denitrifier populations (as determined by most-probable-number [MPN] counts) were measured in field and laboratory experiments. Measurements of DEA and MPN provided highly contradictory indications of denitrifier dynamics. In laboratory incubations, under conditions favoring active denitrification, the synthesis of new denitrifying enzymes and the actual amount of denitrification were closely related. In other experiments, however, both DEA and MPN counts were poor indicators of actual denitrification. In some cases, we found significant increases in DEA but no significant production of N gas. Except with unnaturally high substrate amendments, changes in DEA were small relative both to the persistently high DEA background and to changes in MPN. As estimated by MPN counts, denitrifier populations increased significantly during denitrification events. It was apparent that only a small fraction of the denitrifiers were included in the MPN counts, but it appeared that this isolatable fraction increased during periods of active denitrifier growth. Use of DEA as an index of biomass of cells which have synthesized denitrifying enzymes suggested that denitrifier populations were persistent, stable, and much larger than indicated by MPN procedures.

Concern about the impact of soil-evolved trace gases such as N_2O on atmospheric processes and widespread interest in N cycling in agricultural and other ecosystems have motivated numerous attempts to measure N losses from soils by denitrification. Refinements in isotopic methods and the development of the acetylene inhibition technique (5, 15) have made reasonable field estimates of denitrification possible. However, the extremely high spatial and temporal variability for this process in soils means that integration of losses over an extended time at even a single site is expensive and time-consuming. Furthermore, extrapolation from the present data base of field measurements to different soil or climatic conditions, or to regional-scale prediction of gas losses, is not feasible, in part because of the lack of an adequate model to account for the temporal and spatial variability.

Knowledge of the dynamics of denitrifier populations would aid in understanding or predicting the temporal variability of denitrification. Yet the denitrification process, rather than the population, has been the focus of most studies. Meaningful characterization of denitrifier populations in soils presents a difficult challenge. The most useful techniques for studies of soil microbial populations either detect a restricted range of genotypes (techniques based on serology and selectable antibiotic resistance, for example) or are not specific for any physiological function (direct counts and biomass measurements, for example). Since denitrifiers are genetically diverse and morphologically indistinct (4, 9), these approaches have limited applicability. Also, denitrifiers are facultative (4, 9), and thus the relationships between process (denitrification) and population may be complex.

Short-term assays of denitrification rate with nonlimiting substrate and aeration, essentially a measure of denitrifying enzyme activity (DEA) (13), offer one approach to conveniently monitoring the dynamics of denitrifier populations. DEA assays have also been used to infer differences in actual N losses among sites or times. This approach is questionable, since the relationships between DEA and actual denitrification rate have not been investigated and since previous studies have suggested that DEA is conserved at a high level in nondenitrifying soils (12). Also, one report indicated comparable DEA increases in two soil treatments using very different aeration (8). Nevertheless, DEA assays and most-probable-number (MPN) counts of denitrifiers remain the only tested methods of characterizing soil denitrifier populations.

In the field and laboratory experiments described here, neither DEA nor MPN counts offered a satisfactory index of actual denitrification losses nor were these parameters well correlated with each other, yet both provided information about the behavior of denitrifiers in soil. DEA can best be interpreted as an estimate of the biomass of bacteria which have synthesized denitrifying enzymes, while MPN counts provide a somewhat more sensitive, but not absolutely accurate, indication of denitrifier growth and death.

MATERIALS AND METHODS

Soils. The primary soil used in this study was a Lanton (fine-silty mixed mesic Haploquoll) with 4.3% (wt/wt) total carbon content, 0.47% (wt/wt) total nitrogen context (C/N ratio of 9.1), pH of 6.2 (in a 1:1 H_2O slurry), and seasonally flooded conditions. Dry weights and volumes of 15 soil cores taken by probe (to 15 cm) were used to estimate a bulk density of 1.08 g/cm^3. The field experiment was performed at, and samples for laboratory experiments were obtained from, a site which was undisturbed and had been continuously under sod for more than 30 years. All soils were sampled to a 15-cm depth.

Samples for laboratory experiments were passed through a 20-mesh (0.84-mm openings) sieve. Larger fragments of organic matter were removed. Soil was refrigerated when

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not being handled or subsampled for analysis. Soil was stored at a moisture content of approximately 0.21 ml/g of dry soil.

Maury silt loam (fine-silty mixed mesic Typic Paleudalf) with 1.9% total carbon, 0.20% total nitrogen, and pH (in water) of 5.8 was used for the microcosm experiment. The soil was collected from a tilled corn field at the Spindletop Farm of the University of Kentucky.

DEA assay. The standard activity assay (13) was performed with 10 g of moist soil and 25 ml of assay solution in a stoppered 125-ml flask under anaerobic conditions. Anaerobic conditions were created in containers closed with stoppers (fitted with ports sealed by septa) by repeatedly evacuating and pressurizing the containers with oxygen-free nitrogen gas. When evolved gases were to be measured, flasks were vented to atmospheric pressure before incubation. All incubations were performed at approximately 23°C. The assay solution was buffered to pH 7 with a 50 mM phosphate buffer containing 100 μg of chloramphenicol per ml to inhibit protein synthesis. This and the short assay interval (60 to 90 min) ensured that the activity being measured was due to preexisting enzymes and not to enzymes synthesized during incubation (13). Five to ten percent of the gas phase over incubated soils was replaced with acetylene. Both 10 mM NO_3^- and 10 mM glucose were added to the solution to provide nonlimiting electron acceptor and substrate, allowing optimal enzyme activity (12).

Denitrifier counts. We used a variation of the commonly described MPN method for counting denitrifying bacteria (15). Sterile Hungate tubes containing 5 mM NO_3^- in nutrient broth were inoculated with dilutions of soil. The dilutions were prepared by vigorously shaking 10 g of moist soil in 95 ml of saline solution (0.85% [wt/wt] NaCl) to which 3 drops of Tween 80 (ICI US Inc.) had been added. A series of 10-fold dilutions was then used to inoculate five tubes for each dilution level. After 2 weeks of anaerobic incubation, tubes with denitrifiers were identified by depletion of both NO_3^- and NO_2^- as evidenced by a negative spot test with diphenylamine.

An estimate of variability is commonly obtained within a single MPN determination (1). However, this does not provide a true estimate of variability at a sampling site or within a replicated experimental treatment. In an attempt to obtain greater precision and actual replication of samples, we considered a single MPN determination to be a single observation and used repeated complete MPN determinations as replications to obtain estimates of means and variability. In some cases, more than one dilution series was also made from the same initial sample. These were considered subsamples within replicated observations.

Tiedje (15) has recommended confirmation of the presence of denitrifiers by measurement of N gas production in addition to observation of NO_3^- depletion. In an earlier study on a different soil type (14), this time-consuming step was found to be unnecessary if the medium described above was used. The same was observed for the soil used in the present study. We added acetylene to the gas phase of some inoculated tubes and measured N_2O production during MPN incubation. These tests showed virtually complete correspondence between the two methods of detecting denitrifiers. Therefore, we chose the greater precision and extensive replication made possible by the simpler NO_3^- test.

Analytical methods. Nitrate was determined by extracting fresh soil with 1 N KCl, filtering the soil, and performing colorimetric analysis with a flow injection analyzer (Lachat QuikChem, Mequon, Wis.). Nitrous oxide was measured with a Varian 3700 gas chromatograph equipped with a ^{63}Ni electron capture detector and a thermal conductivity detector (14).

Field irrigation experiment. Aluminum rings of approximately 20 cm in height and 80 cm in diameter were driven 2.5 cm into sod on Lanton soil to delineate four 0.5-m^2 areas. These were irrigated with 7.5 cm of H_2O, and this procedure was coincidentally augmented with 5.5 cm of precipitation on the same day. Samples were taken immediately before irrigation, at 4.5, 8.5, 25.5, 48, and 72 h, and at 7 and 30 days. Composites of three cores were made for each ring at each sampling time, and the following measurements were made for each composite: a DEA assay, three MPN series, NO_3^-, and gravimetric moisture content. All analyses were performed within 24 h of sampling. Soil temperature at 7.5 cm was taken at each sampling time. Times and amounts of precipitation were also recorded.

Microcosms. Maury soil was packed to a depth of 6.5 cm (125 g [fresh weight] of soil) in 5-cm-diameter Plexiglas cylinders, which were covered with aluminum foil to prevent photosynthesis and closed on the bottom with a rubber stopper. The microcosms were divided into three treatment groups: not flooded, intermittently flooded, and constantly flooded. In nonflooded microcosms, soil moisture was maintained at field capacity (0.26 g of H_2O per g of soil) by replacing evaporative water losses with distilled water. Nonflooded microcosms received an initial input of 50 μg of N per g of soil, added as KNO_3. Intermittently flooded microcosms were flooded and dried on a schedule of 2 days of flooding followed by 5 days of drying. The microcosms were flooded with distilled water to a depth of 1 cm above the soil surface. After 2 days, they were drained through a tube located at the bottom of each cylinder. For the first 24 h after drainage drying was accelerated by use of a fan. Intermittently flooded microcosms also received an initial input of 50 μg of NO_3^--N per g.

The continuously flooded microcosms were flooded to a depth of 1 cm above the soil surface with a solution of 0.01 M $CaCl_2$ containing 1 μg of KNO_3-N per ml. Flooding was maintained by pumping 65 ml of this solution per day onto the top of the microcosm and allowing outflow through a tube fitted to the side of the cylinder 1 cm above the soil surface. This regimen resulted in a daily input of 0.5 μg of N per g of dry soil. Three microcosms of each type were sacrificed at 2-week intervals and assayed for DEA and MPN counts as described previously.

Irrigated cylinders. Lanton soil was packed to a depth of 17 cm (289.6 g [dry weight] of soil) in eight Plexiglas cylinders (5-cm diameter). The bulk density was 0.87 g of soil per cm^3. We then added 164.4 ml of acetylene-saturated H_2O to each column and allowed the column to drain. Columns were fitted with ported stoppers so that the bottoms were sealed and a gas mixture of air and 5% acetylene could flow through the headspace (78.5-ml volume) above the soil at a rate of 150 ml/min per column. Periodically, flow through the column headspace was stopped for 30 to 45 min, during which time the rate of N_2O accumulation in the headspace was measured. Rates of N gas flux during these intervals were extrapolated to estimate gaseous N loss over the entire 7-day incubation period. Further details on this procedure have been reported by Rice and Smith (10).

As part of this experiment, smaller cylinders were packed to the same bulk density with 72.4 g of the same soil as described above. These columns were 2.5 cm in diameter and were filled to a height of 17 cm. Irrigation was in the same manner as for the large cores except that acetylene was

omitted during incubation. Four replicates were removed at 0, 2, and 7 days of incubation and analyzed. At each sampling time, the following measurements were made on each column: one DEA assay, one MPN series, and a determination of NO_3^-. For comparison, similar measurements were made on this same Lanton bulk soil sample, incubated under completely anaerobic conditions, as described below.

Flask incubations. Soil samples of 10 g (moist weight) were incubated in 125-ml flasks. Flasks were closed with stoppers fitted with septa for gas sampling. There were three or four replications for all measurements. In general, 10 mg of glucose and 21.6 mg of KNO_3 were added to each flask with 5 ml of H_2O, and the flasks were then made anaerobic by evacuation and flushing with N_2. However, in several experiments these factors were altered as described in Results (see Table 2). In one experiment (see Fig. 2), soil was sterilized by autoclaving in the flasks on 2 consecutive days.

Pure-culture specific activity. Various soil isolates, all denitrifiers in the genus *Pseudomonas*, were grown to late log phase in anaerobic nitrate broth (Difco Laboratories, Detroit, Mich.), harvested, and washed in 50 mM phosphate buffer, pH 7.0. These were added to soils which had been briefly heated at 105°C to destroy indigenous DEA. Cell concentrations added were chosen to give DEA values in the range of 10 to 20 ng of N per g per min. Soils were assayed immediately after addition of cells. Cell counts were made on the inoculum by plate counts. For one isolate, cell carbon was also measured on the washed-cell preparation, using a LECO CR12 carbon analyzer.

RESULTS

Pure-culture specific activity assayed in soil. Values for DEA on a per-cell basis for denitrifiers grown under optimal conditions but assayed in soil were 7×10^{-17} and 44×10^{-17} g of N per min per cell for two different isolates in Lanton soil and 22×10^{-17}, 9×10^{-17}, and 2.4×10^{-17} g of N per min per cell for three different isolates assayed in Maury soil. By measuring cell C content, it was determined that 7×10^{-17} g of N per min per cell was equal to 0.30 mg of N per g of C per min.

Field and microcosm dynamics. After irrigation with 7.5 cm and a fortuitous rainfall of 5.6 cm on the same day, soil water increased to 42% (Fig. 1). This resulted in approximately 80% of the soil pore space being water filled. By day 7, water-filled pore space had declined to near 60%, a value which has been proposed as a nominal cutoff for extensive anaerobic activity (7). Low rainfall for the remainder of the 30-day experiment resulted in a gradual decline in soil moisture.

A rapid decline in NO_3^-, amounting to approximately 12 μg of N per g of soil, was observed between 48 and 72 h. Denitrification losses were not directly measured during this experiment. However, in this case changes in soil NO_3^- can provide a gross approximation of denitrification, since it is not likely that leaching, immobilization, or plant uptake could account for a large fraction of this rapid change in NO_3^-.

The DEA increased from 11 to 18 ng of N per g per min after irrigation and initial precipitation. As the soil dried over the following 30 days, DEA continued to increase significantly. Thus, DEA appeared to be poorly related to actual denitrification. Initial DEA was quite high, even though the soil was initially dry. If fully expressed, this initial activity would extrapolate to 16.6 μg of N per g denitrified in 24 h.

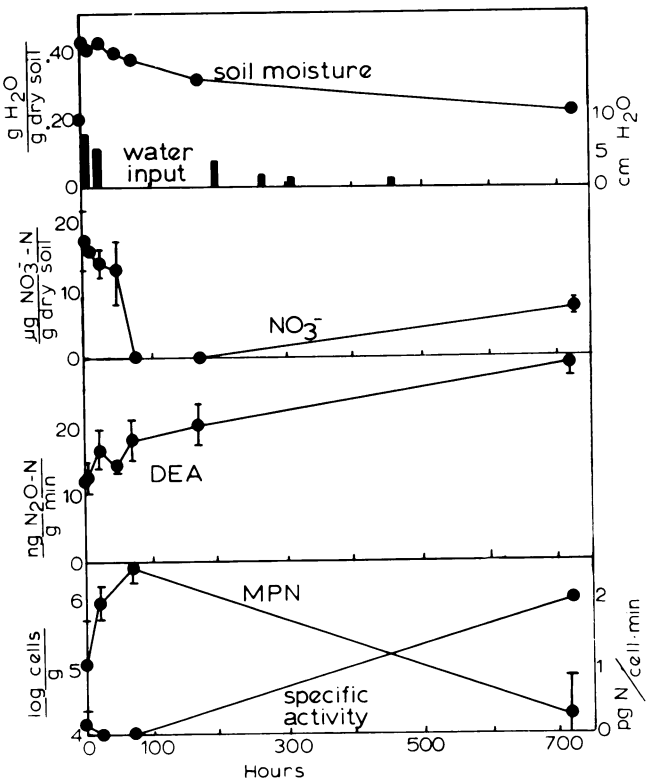

FIG. 1. Response to irrigation of small plots in the field, Lanton soil. From the top: Gravimetric soil moisture and water inputs by irrigation or rainfall (thick bars), soil NO_3^-, DEA, and MPN counts of denitrifiers with specific activity (ratio of DEA/MPN). Thin bars indicate standard deviations that exceed the dimensions of the symbols.

During the 48- to 72-h period of rapid NO_3^- loss, approximately half of the potential represented by DEA was apparently realized. At all other times this fraction would be extremely small. The increase in DEA units was approximately 8.7 from the initial time to 168 h. The ratio of this increase to estimated micrograms of N gas produced per gram would be no less than 0.7, consistent with ratios described below for laboratory experiments (see Table 2).

There was high variability in the MPN counts of denitrifiers, even though we conducted distinct counts on multiple replicate samples rather than using the variance value derived from replicate tubes in a single sample count. Counts increased by approximately 30-fold during the initial denitrifying event. As the soil dried, there was a large decrease in the number of cells counted. This decline is in contrast to the results for determinations of DEA.

Dividing DEA by MPN should provide an estimate of specific denitrifying activity, or DEA per cell. Yet this calculation gives the absurd, or at least highly unlikely, result that the specific activity decreased during the denitrifying event and increased as the soil dried. Also, the lowest specific activity in this experiment, 5×10^{-15} g of N per cell per min at 72 h, was an order of magnitude greater than the highest specific activity we measured for pure cultures.

With the nonflood treatment, DEA declined slowly but significantly throughout the 6-week period (Table 1). With the flood treatment, DEA increased initially and then declined significantly, perhaps because the redox or the NO_3^-

TABLE 1. DEA and MPN counts in microcosms maintained under three moisture regimens

Treatment	DEA (ng of N/g per min) at week:				MPN (log cells/g) at week:			
	0	2	4	6	0	2	4	6
Not flooded	5.7 (0.7)[a]	4.8 (0.9)	4.0 (0.2)	3.2 (0.2)	3.9 (0.7)	4.9 (2.2)	3.8 (0.2)	4.9 (0.3)
Intermittently flooded	5.7 (0.7)	12.1 (1.4)	15.8 (1.0)	11.3 (1.1)	3.9 (0.7)	4.8 (0.4)	5.2 (0.1)	5.0 (0.4)
Continuously flooded	5.7 (0.7)	9.0 (3.0)	8.0 (0.2)	3.8 (0.7)	3.9 (0.7)	5.0 (0.1)	4.9 (0.1)	4.9 (0.2)

[a] Numbers in parentheses are standard deviations.

supply became too low to favor activity of denitrifier populations. In the intermittent-flood treatment, DEA increased the most and was maintained at the highest level.

As in the field experiment, MPN results were often inconsistent with patterns in DEA and specific activities were unreasonably high. Initial MPN increases were observed during the establishment of all treatments. However, after this time there were no consistent differences among the treatments.

Relationships between DEA and actual denitrification in laboratory incubations. To further characterize the relationships between changes in DEA and actual denitrification, soil was incubated in flasks, in which conditions could be better controlled and N gas production could be directly measured. In general, enzyme synthesis (increase in DEA) was highly related to enzyme function (actual denitrification or increase in N_2O) (Table 2). This correlation was observed when either aeration, NO_3^- supply, or reductant (glucose) was the factor limiting denitrifier activity. At high rates of denitrification, the ratio between DEA and denitrification was reasonably constant and reasonably close to the ratio estimated in the field experiment, 0.7.

An important exception to the approximately constant relationship between DEA and denitrification was observed for reasonably well aerated soils (Table 2, experiment 1, two driest treatments). Although very small quantities of N gas were evolved in these treatments, significant increases in DEA were detected during incubation of these drier soils amended with substrate.

This situation is further illustrated by the comparison of two additional experiments in which denitrification rate, DEA, and MPN counts were monitored (Table 3). Although no energy source was added in these experiments, the physical manipulation and moistening of the soil apparently released sufficient substrate to permit significant microbial growth. Both the increases in MPN and the increases in DEA were similar in the two experiments. As was observed in the field experiment (Fig. 1), there was an unexpected decline in denitrifier specific activity, i.e., the ratio of DEA/MPN. In the completely anaerobic flasks, the actual production of N gas was many times greater than that in the partially anaerobic columns. Thus, similar responses in DEA and MPN occurred, when the actual rates of denitrification were very different.

The relationship between DEA and denitrification rate was also dependent on time from the imposition of denitrifying conditions (Table 2, experiment 4). Although N gas was produced rapidly in the first 7 h after the soil was made anaerobic, there was no significant change in DEA. DEA increased after this time, and the ratio of the increase to N gas production, about 1, was comparable to that found in other experiments. This apparent lag in denitrifying enzyme synthesis has been previously reported (13) and is, in part, the basis of the DEA or phase I assay. In several laboratory incubations (data not shown), the duration of this lag has varied from 1 to more than 12 h.

DEA versus MPN. In attempting to clarify the unexpected relationships between DEA and MPN counts observed above, we allowed isolate 59R to colonize sterilized, anaerobic, substrate-amended soil (Fig. 2). Rapid growth and synthesis of denitrifying enzymes was observed during the first 2 days. During this period, specific activity was slightly

TABLE 2. Relationships between increase in DEA and actual N gas production in laboratory experiments[a]

Expt[b]	Treatment	Denitrification (δN_2O; μg of N/g)	Enzyme synthesis (δDEA; ng of N/g per min)	$\delta DEA/\delta N_2O$
1	5.0 ml of H_2O, anaerobic	131.8 (3.1)	132.4 (22.0)	1.00
	5.0 ml of H_2O, aerobic	50.2 (3.6)	32.5 (0.9)	0.65
	3.5 ml of H_2O, aerobic	5.0 (1.5)	8.3 (0.1)	1.66
	2.0 ml of H_2O, aerobic	0.025 (0.010)	4.6 (0.2)	184.00
	0.5 ml of H_2O, aerobic	0.014 (0.008)	3.6 (0.4)	257.00
2	10 mg of glucose	22.4 (1.3)	26.9 (3.0)	1.20
	3 mg of glucose	17.9 (0.6)	20.3 (6.4)	1.13
	No glucose	11.7 (0.6)	8.33 (1.0)	0.71
3	1.5 mg of NO_3^--N	199 (7)	110 (22)	0.55
	0.75 mg of NO_3^--N	168 (3)	57 (11)	0.34
	No NO_3^--N	80 (1)	33 (3)	0.40
4	1 h	0.4 (0.1)	0 (0)	0
	7 h	6.9 (1.1)	−5.3 (1.3)	0
	12 h	13.2 (0.9)	11.4 (2.9)	0.86
	24 h	33.8 (9.9)	51.7 (1.7)	1.53
	48 h	203.9 (9.9)	127.3 (32.0)	0.62

[a] Incubation intervals were 48 h for experiments 1 and 3 and 24 h for experiment 2. Standard deviations are given in parentheses.
[b] Conditions of experiments: 1, aeration varied by addition of water and composition of initial headspace; 2, glucose amendment varied; 3, NO_3^- amendment varied; 4, assayed at various incubation times.

TABLE 3. Changes in DEA, MPN counts, and amount of actual N_2O evolved during incubation of unamended Lanton soil in irrigated columns[a]

Incubation time (days)	DEA (ng of N/g per min)	MPN (log cells/g)	N_2O evolved (μg N/g)	CO_2 (μg C/g)
0	2.2 (0.4)[b]	4.8 (0.3)	Not done	Not done
2	9.5 (3.0)	5.2 (0.5)	0.24 (0.05)	34.2 (10.6)

[a] 73.5% water-filled pore space.
[b] Standard deviations are given in parentheses.

lower than, but within the range of, values for pure cultures assayed in soil. Subsequently, both parameters declined but to very different extents. Although 52% of the maximum DEA was maintained at 20 days, denitrifier counts declined to 4% of their maximum value. The result was a large apparent increase in specific activity during the time when the denitrifier population was presumably in decline. These results are analogous to those obtained during the drying phase of the field experiment (Fig. 1) and strongly suggest that under these conditions only a small fraction of the functional denitrifying cells are detected by MPN counts.

DISCUSSION

Our previous observations of the remarkable stability of DEA under nondenitrifying conditions led us to emphasize the apparent nonresponsiveness of denitrifiers with regard to growth, death, and enzyme synthesis and to suggest that denitrification losses could be understood or modeled primarily in terms of O_2 activation-inactivation of existing enzymes (12). Although this view may be appropriate for brief, transient denitrifying events, the results presented here suggest the significance of the dynamic response of denitrifiers with regard to enzyme synthesis and growth. Unfortunately, the methods available for quantifying these dynamics are limited.

MPN counts of denitrifiers, like other isolation-based procedures, are limited in both precision and accuracy. Even

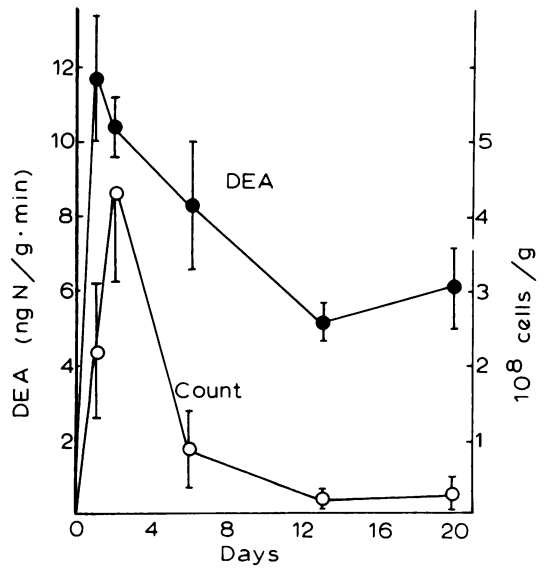

FIG. 2. Persistence of DEA (●) and MPN of isolatable denitrifiers (○) after inoculation of an autoclaved soil with a denitrifier culture. Standard deviations are indicated by bars.

though separate MPN determinations were done on many replicate samples (nine in the field experiment), an approximately 10-fold increase or decrease in numbers was necessary to detect a statistically significant difference. Also, it appears that a very small fraction of the functional denitrifying cells are detected. This conclusion is based on the unreasonably high specific activities measured in most soil samples. Measured activities of cultured cells were near 10^{-16} g of N per cell per min, in reasonable agreement with values given in the literature (2); activities of soil samples, in which denitrifier populations were estimated by MPN, ranged from 5×10^{-15} to 2×10^{-12} (Fig. 1). It is not likely that this discrepancy can be explained by postulating that soil-grown cells have a denitrifying enzyme content several orders of magnitude greater than that of anaerobically cultured cells.

We speculate that young or physiologically active cells are isolated with a considerably higher efficiency in the MPN count than are old or inactive cells. This would account for the unexpected responses in apparent specific activity. During a denitrifying event, the proportion of cells which are young, active, and thus culturable increases greatly, bringing the DEA/MPN ratio closer to the true specific activity of denitrifier cells, that is, decreasing the value. In inactive populations, DEA is subsequently more persistent than the culturability of cells, as suggested by Fig. 2, and the result is an apparent increase in specific activity.

MPN counts actually appear to provide a more sensitive indicator of denitrifier growth and death than DEA assays. Under realistic conditions (no glucose added during incubation), proportional changes in DEA were generally small, whereas MPN counts often increased by an order of magnitude or more (Fig. 1; Tables 1 and 3). Increases in DEA during denitrifying events are small relative to the high, persistent background activity. Although MPN counts fail as a precise or accurate enumeration of all denitrifiers in soil, they do seem to provide a sensitive indication of new denitrifier growth.

If it is assumed that the specific activities measured for cultured denitrifiers are representative for denitrifiers in soil, then DEA assays can be used to estimate the number or biomass of cells in soil that have derepressed denitrifying enzyme synthesis (as opposed to total cells genetically capable of denitrification, which is the intended function of the MPN procedure). DEA values for the Lanton soil measured here ranged from 2 to 20 ng of N per g per min. Use of a mean cell activity of 1.7×10^{-16} g of N per cell per min leads to estimates of denitrifier numbers ranging from 10^7 to 10^8/g. This suggests that denitrifying cells constitute a greater fraction of total soil bacteria than was previously believed (16). The persistence of DEA also suggests that denitrifier populations are quite stable and turn over very slowly. That is, there exists in well-aerated soils a very large number of cells that cannot be isolated but contain functional denitrifying enzymes. Similar phenomena are well documented in aquatic microbiology (11), but the methods used in these systems have yet to be adapted to soils.

Anderson and Domsch (3) have developed a technique similar in execution to the DEA assay except that it is done aerobically and CO_2 rather than N_2O is measured. The intent of their assay is to estimate microbial biomass C from potential respiration rate. The relationship they determined was 1 ml of CO_2 produced per h per 40 mg of biomass C. By assuming that N_2O in the DEA assay is derived from NO_3^-, reduced by the complete oxidation of glucose C (or substrates of the same reduction state) to CO_2, we can derive a

comparable factor for the activities of pure cultures assayed in soil. For example, the value 0.30 mg of N per g of C per min can be converted to a ratio of 1 ml of CO_2 per h per 24 mg of denitrifier biomass C, in reasonable agreement with the value obtained by Anderson and Domsch.

Although this and other studies (6, 12) demonstrate that the fraction of DEA which is expressed in actual N gas production is variable and usually very small, the generally good correlation between increases in DEA and actual denitrification which occurred in laboratory incubations suggested that monitoring changes in DEA might be a valid, and obviously convenient, way to obtain gross estimates of gaseous N loss. However, we observed that under conditions of active microbial growth with marginal or reasonably high aeration, significant synthesis of denitrifying enzymes occurred without extensive function, that is, when actual gaseous N production was minimal (Table 3). A second source of error could occur during brief, transient anaerobic events, when rapid activation of preexisting DEA might lead to significant N loss before detectable changes in DEA. Therefore, we conclude that DEA is better interpreted as an estimate of the biomass of denitrifying bacteria in soil rather than as an index of actual denitrification rates. Use of the results of DEA assays in this way leads to the conclusion that populations of denitrifying bacteria in soil are persistent and much larger than are indicated by isolation-based enumeration techniques.

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