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Kinetics of Nitrate Utilization by Mixed Populations of Denitrifying Bacteria†

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Kinetics of nitrate utilization by mixed bacterial populations from two agricultural soils and a pond sediment in Kentucky were measured by using progress curves of nitrous oxide production. Nitrous oxide production from anaerobic soil and sediment slurries containing added nitrate and acetylene exhibited first-order kinetics. Nitrate affinity (K_m) for mixed populations of denitrifying bacteria in unfertilized agricultural soils and pond sediments ranged from 1.8 to 13.7 μM. The affinity of bacterial populations for nitrate did not vary with habitat, and the ability to use low concentrations of nitrate was retained by bacterial populations living in environments which received large inputs of nitrate.

Nitrogen is the mineral nutrient most commonly limiting plant productivity in terrestrial and marine ecosystems. The biological reduction of nitrate to nitrogen gas by denitrifying bacteria is a major route of nitrogen loss from soils and sediments. It has been estimated that 30% of the nitrogen applied as fertilizer to agricultural soils is lost to the atmosphere as a result of the activity of denitrifying bacteria (16). Interest in denitrifying bacteria has also been stimulated by concern over the degradation of atmospheric ozone by nitrous oxide (N_2O) (6, 12) and by the potential use of denitrification as a means of removing nitrate (NO_3^-) from wastewater and polluted aquatic ecosystems (10, 19).

Denitrifying bacteria as a group are genetically diverse and metabolically versatile. At present, it is not possible to predict the in situ activity of mixed populations of denitrifying bacteria from the results of laboratory studies of the physiology and biochemistry of pure cultures of denitrifying bacteria. Direct knowledge concerning how environmental variables influence the growth and persistence of natural populations of denitrifying bacteria is required to more fully understand microbial nitrogen and carbon transformations in situ. Tiedje et al. (24) noted that the denitrifying enzyme activity of bacterial populations from several habitats seemed to be influenced more by oxygen and carbon availability than by nitrate concentration. We have determined the affinity (K_m) and capacity (V_{max}) for nitrate utilization of mixed populations of denitrifying bacteria to clarify the relationship between nitrate availability in the environment and nitrate use by denitrifying bacteria.

Previous attempts to measure kinetic parameters of nitrate utilization by mixed populations of denitrifiers have been hampered by the absence of a chemical technique capable of measuring very low concentrations of NO_3^- and by problems associated with limited carbon availability and NO_3^- diffusion in soil and sediment slurries (8). In this paper, we present a new technique for measuring the kinetics of NO_3^- utilization by mixed populations of soil bacteria. Kinetics of NO_3^- utilization were determined from measurements of N_2O production from anaerobic soil slurries amended with acetylene, glucose, and nitrate. The average affinity for nitrate utilization by bacterial populations from two agricultural soils and a pond sediment was 8.1 μM, indicating that mixed populations of denitrifying bacteria can effectively use NO_3^- at concentrations lower than previously believed possible.

MATERIALS AND METHODS

Soils and sediments. Lanton soil (fine-silty mixed mesic Haplaquolls), with 4.3% total carbon content, 0.47% total nitrogen content (C/N ratio of 9.1), and pH of 6.2, was collected from the surface 10 cm of an undisturbed area which had been continuously under sod for more than 30 years. The Lanton site was often flooded by precipitation, especially during late winter and early spring. Lanton soil was also sampled from an adjacent tilled corn field which received yearly applications of fertilizer nitrogen at a rate of 168 kg of N ha^{-1}. Maury soil (fine-silty mixed mesic Typic Paleudalfs), with 1.9% total carbon, 0.20% total nitrogen content (C/N ratio of 9.5), and pH of 5.8, was collected from a well-drained hilltop area which had been continuously under sod for many years. Aquatic sediments were collected as cores from a shallow pond approximately 2 ha in size. The pond water contains between 0.1 and 1.0 μg of NO_3^--N ml^{-1} during most of the year. All soils and sediments were collected at the Spindletop Farm of the University of Kentucky.

Depletion of in situ nitrate and nitrite. In situ denitrifying activity was used to remove small amounts of nitrate and nitrite from microbially active soils and sediments by placing the soil or sediment in an anaerobic environment overnight. In samples with high levels of in situ nitrate, such as fertilized soils, nitrate depletion was facilitated by using an ion-exchange resin (Amberlite; Mallinckrodt, Inc.). Amberlite (10 g) was placed in a small cotton mesh bag which was suspended in a soil slurry composed of 200 g of soil and 800 ml of deionized water. The slurry was made anaerobic (by repeatedly evacuating and flushing with N_2), placed in a refrigerator at 4°C, and mixed with a magnetic stirring bar. Additional bags containing Amberlite were added to the slurry after 3 and 6 h, and the slurry was mixed overnight. The next day, the depleted soil was recovered from the slurry by centrifugation for 20 min at 5,000 × *g*.

Initial velocity kinetic assay. The kinetic assay was a modificaiton of the denitrifying enzyme assay developed by Smith and Tiedje (21). Between 5 and 10 g of moist soil was added to a 125-ml Erlenmyer flask containing 25 ml of assay medium. The assay medium was composed of 40 mM

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phosphate buffer (pH 7), 10 mM glucose, and 100 μg of chloramphenicol ml^{-1}. Potassium nitrate was added to each flask in such a manner that initial concentrations of added NO_3^- usually ranged between 10 and 100 μM. In addition, three flasks which did not contain added NO_3^- were monitored during each experiment to check for the evolution of N_2O from trace amounts of NO_3^- remaining in the soil after depletion. Kinetic assays were initiated by repeatedly evacuating and flushing the flasks with N_2 which had passed through an O_2 filter (Varian) and by amending each flask with C_2H_2 to a final concentration of 0.10 atm (10.13 kPa). The flasks containing the soil slurries were incubated at room temperature while being mixed on a rotary shaker at 150 rpm. The headspace of the flask was sampled for N_2O at 30, 60, and 90 min after the imposition of anaerobiosis. Kinetic parameters were calculated by using the Lineweaver-Burk linearization of the differential form of the Michaelis-Menten equation. Between four and five different concentrations of NO_3^- ranging between 10 and 100 μM were used for each kinetic determination, and each NO_3^- concentration was replicated in triplicate.

Progress curve kinetic assay. Between 5 and 20 g of moist soil was added to a 125-ml Erlenmyer flask containing 25 ml of assay medium. The amount of soil used for each assay was adjusted according to the denitrifying activity of the soil. An amount of soil was chosen such that a nitrate concentration of 50 μM would not be completely denitrified in less than 30 min. The flask was stoppered, repeatedly evacuated and flushed with N_2 which had passed through an O_2 filter (Varian), and amended with C_2H_2 to a concentration of 0.10 atm (10.13 kPa). The soil slurries were incubated at room temperature while being mixed on a rotary shaker at 150 rpm. Approximately 30 min after the imposition of anaerobiosis, the headspace of the flask was sampled for N_2O. Subsequently, gas samples were analyzed for N_2O at 15-min intervals to ensure that N_2O was not being evolved from the sediment slurry and thus, that NO_3^- was no longer present in the slurry. Once the absence of in situ nitrate in the slurry was confirmed, nitrate was added to the incubation to give a final nitrate concentration of approximately 50 μM. Nitrate was added to the flask as an anaerobic solution of KNO_3 which had been oxygen depleted by heating the solution in a Hungate tube to 60°C while repeatedly evacuating and flushing the tube with N_2. Gas samples were removed from the flask headspace at 4-min intervals and analyzed for N_2O. In most assays, a period of equilibration occurred within the first 4 to 8 min of the assay (Fig. 1) while the nitrate added to the flask was mixed into the slurry and any oxygen inadvertently added to the flask would be depleted by microbial activity. Data collected after the initial equilibration period were used for the construction of progress curves. Concentrations of remaining substrate were calculated from N_2O accumulation data by subtracting the N_2O-N accumulated at each sampling time from the quantity of nitrogen finally recovered as N_2O.

Analysis of progress curves. Progress curves of nitrate depletion data were fitted to the integrated form of the Michaelis-Menten equation by using an updated version of the progress curve analysis program developed by M. Betlach (M. Betlach, Ph.D. thesis, Michigan State University, East Lansing, 1979; M. Betlach, personal communication). The progress curve analysis program fits data to the integrated form of the Michaelis-Menten equation for a single-substrate irreversible reaction by an iterative method using the Newton-Raphson procedure and the Wilkinson procedure to estimate the standard error (2).

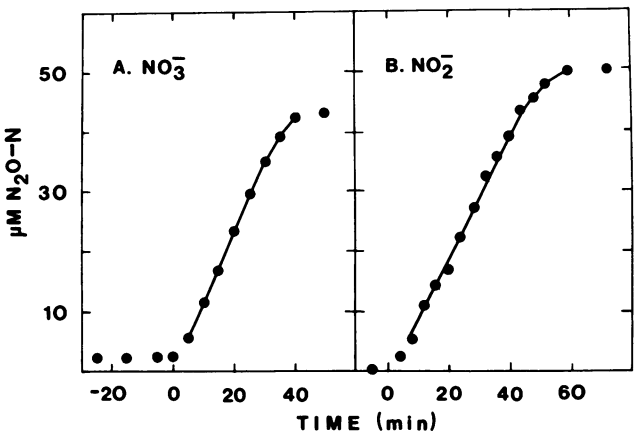

FIG. 1. Progress curves of N_2O production from anaerobic soil slurries. (A) Lanton soil collected on August 4, 1987, amended at time 0 with 49.3 μM NO_3^-. (B) Lanton soil collected on January 12, 1988, amended at time 0 with 50.3 μM NO_2^-.

Analytical techniques. Nitrous oxide was measured with a Varian 3700 gas chromatograph equipped with Porapak Q columns and operated isothermally at 50°C. Samples containing 0.05 to 30 ppm (μl/liter [vol/vol]) N_2O were measured with a ^{63}Ni electron capture detector at 360°C with 10% CH_4 in argon carrier gas. Separation between CO_2 and N_2O was sufficient to prevent CO_2 interaction with N_2O response. A four-port in-oven venting valve was used to prevent other gases from reaching the detector. Quantities of N_2O in solution were calculated by using published values of the Bunsen absorption coefficient. Soil nitrate was determined from KCl extracts of fresh soil samples. Soil (10 g) was extracted with either 25 or 100 ml of 1 M KCl by shaking for 1 h. The extracts were filtered on Whatman no. 42 filter paper and analyzed for nitrate by using a Lachat Quickchem Autoanalyser. In this system, nitrate is reduced by a copperized cadmium column to nitrite, after which total nitrite is determined by reaction with *N*-1-naphthylethylenediamine dihydrochloride.

RESULTS AND DISCUSSION

Kinetic assay. The kinetics of NO_3^- utilization can be measured with much greater precision by using electron capture gas chromatography analysis of N_2O than can be obtained from measurements of NO_3^- depletion using standard wet-chemical techniques. Nitrous oxide formation from NO_3^- will only be an effective technique for kinetic measurements if in situ concentrations of nitrate and nitrite can be removed from soil slurries before kinetic parameters are measured and if N_2O reduction to N_2 can be effectively inhibited by acetylene. Since the C_2H_2 concentrations used here have been shown to effectively inhibit N_2O reduction (20, 25) and since we observed no significant loss of accumulated N_2O, the latter condition was satisfied.

We verified successful elimination of in situ NO_3^- and NO_2^- in each soil slurry, prior to assay, by observing constant N_2O concentration in each flask (Fig. 1). Treatment of soils with the ion-exchange resin effectively reduced soil NO_3^- concentrations by as much as 10-fold but did not completely eliminate NO_3^-. The treatment did not alter potential denitrifying enzyme activity of the soil. In Maury soil, for example, the potential denitrifying enzyme activity of six replicate soil slurries was 6.43 ± 1.81 ng of N_2O-N g of

TABLE 1. Kinetic parameters determined from initial velocity measurements for bacterial nitrate utilization in agricultural soils and pond sediment

Soil or sediment	Date	K_m (μM)	V_{max} (nmol g of dry soil^{-1} min^{-1})	r^2
Lanton sod	July 19, 1987	13.5	7.27	0.85
Lanton sod	July 21, 1987	25.4	5.63	0.85
Lanton sod	July 23, 1987	34.1	5.97	0.87
Lanton sod	Mean	24.3 (8.44)[b]	6.29 (0.71)	
Maury	May 5, 1987	18.9	0.52	0.51
Maury	May 11, 1987	8.6	0.54	0.68
Farm pond	May 13, 1987	18.3	1.80	0.73

[a] r^2, Coefficient of determination.
[b] Numbers in parentheses are standard errors.

TABLE 2. Kinetic parameters determined from progress curves for bacterial nitrate utilization in agricultural soils and pond sediment

Soil or sediment	Date	K_m (μM)	V_{max} (nmol g of dry soil^{-1} min^{-1})	% Recovery[a]
Lanton sod	August 4, 1987	5.8 (0.9)[b]	5.96 (0.42)	83.2
Lanton sod	August 26, 1987	13.7 (2.3)	5.51 (0.35)	80.8
Lanton sod	October 8, 1987	6.1 (1.4)	3.19 (0.17)	92.4
Lanton sod	Mean	8.5 (3.7)	4.89 (1.21)	85.5
Lanton fertilized	October 26, 1987	13.6 (1.0)	0.51 (0.01)	30.8
Lanton fertilized	November 3, 1987	16.6 (1.6)	0.64 (0.03)	37.0
Maury	January 20, 1988	3.9 (0.6)	0.60 (0.02)	75.6
Pond core				
0 to 2 cm	June 22, 1988	2.4 (0.5)	3.39 (0.07)	40.4
2 to 4 cm	September 8, 1987	1.8 (0.8)	4.76 (0.19)	84.1
2 to 4 cm	June 22, 1988	10.1 (5.4)	9.8 (1.84)	63.9
4 to 6 cm	September 8, 1987	10.1 (2.7)	2.75 (0.21)	53.0
4 to 6 cm	June 22, 1988	4.8 (1.5)	6.06 (0.45)	70.7

[a] % Recovery of added NO_3^- as N_2O.
[b] Numbers in parentheses are standard errors.

dry soil^{-1} min^{-1} before treatment with the ion-exchange resin; it was 6.10 ± 0.31 ng of N_2O-N g of dry soil^{-1} min^{-1} after treatment.

We used glucose as the carbon source in the kinetic assay to avoid adding trace amounts of NO_3^- which may be present in more-complex substrates. Assays of denitrifying enzyme activity in the presence of glucose and more-complex substrates suggest that glucose can be used as a substrate by most denitrifying bacteria. The denitrifying enzyme activity of Lanton and Maury soils amended with glucose ranged between 80 and 100% of the denitrifying enzyme activity of replicate soil slurries amended with nutrient broth or Trypticase soy broth (BBL Microbiology Systems.).

Kinetic parameters determined from initial velocity data. K_m and V_{max} values determined from initial velocity measurements ranged between 34.1 and 8.6 μM (K_m) and 0.052 and 7.27 nmol g of dry soil^{-1} min^{-1} (V_{max}; Table 1). Correlation coefficients of linear regressions of Lineweaver-Burk plots of initial velocity data were often low, ranging from 0.51 to 0.87 (Table 1). The poor correlation between reaction rate and NO_3^- concentration may have resulted at least in part from significant changes in NO_3^- concentration during the assay. In active soil samples at low concentrations of added NO_3^- (in which the concentration of NO_3^- is close to the K_m), the concentration of NO_3^- in soil slurries may change continuously as NO_3^- is used by the bacterial population and this change in NO_3^- concentration can alter the rate of N_2O production. This problem was particularly acute at concentrations of added NO_3^- below 25 μM, and it was often not possible to obtain reliable data using NO_3^- concentrations below 25 μM. In general, the initial velocity technique was very tedious to use and resulted in K_m values which were larger and more variable than K_m values determined from progress curves (Tables 1 and 2).

Kinetic parameters determined from progress curves. The recovery, as N_2O, of NO_3^- added to soil and sediment slurries ranged from 30 to 92% and was usually above 70% (Table 2). Recoveries were greatest in the Lanton soil from sod (above 80%) and were generally lower and more variable in the fertilized Lanton soil and pond sediment. Nitrate added to slurries which was not recovered as N_2O may have been adsorbed to particles, assimilated by organisms, or undergone dissimilatory reduction to NH_4^+.

Incomplete recovery of added NO_3^- would result in a slight underestimation of substrate concentration at any given sampling time. This would result in an underestimation of K_m. If it is assumed that the rates of NO_3^- consumption by denitrification and by competing NO_3^- sinks were proportional throughout the incubation, then the actual K_m value would be equal to the calculated K_m divided by the final fraction of NO_3^- added which was recovered as N_2O. K_m values which have been corrected in this manner are presented in Table 2. The net effect of the correction is to increase the observed K_m value and the K_m values in Table 2 are thus maximum estimates of K_m.

Kinetic parameters for nitrate utilization determined from progress curves ranged from 1.8 to 16.6 μM (K_m) and from 0.51 to 9.8 nmol g of dry soil^{-1} min^{-1} (V_{max}; Table 2). Kinetic parameters for NO_2^- utilization in Lanton soil (Fig. 1) were 2.7 ± 1.2 μM for K_m and 6.31 ± 0.38 nmol g of dry soil^{-1} min^{-1} for V_{max}. The recovery of added NO_2^- as N_2O was 98.7%. Progress curves of N_2O production (nitrate or nitrite utilization; Fig. 1) could be fit to the integrated form of the Michaelis-Menten equation with good precision. One standard error for K_m values determined from progress curves was usually less than 25% of the K_m value (Table 2), and one standard error for V_{max} values determined from progress curves was usually 7% or less of the V_{max} value.

Kinetics of mixed bacterial populations. Previous attempts to measure the kinetics of nitrate utilization in soils have been hampered by a number of problems. The earliest studies of NO_3^- utilization in soils employed very high NO_3^- concentrations far above the concentrations at which NO_3^- kinetics are now believed to exhibit first-order reaction rates (8). Moreover, many studies may have been conducted on sediments in which the denitrification rate was limited by carbon availability (11) or the rate of NO_3^- diffusion within the assay system (18). In the past, many studies have attempted to measure in situ rates of denitrification in conjunction with measurements of NO_3^- utilization kinetics. Measurements of in situ denitrification rate should reflect environmental limitations on bacterial activity. However, measurements of physiological potential, such as K_m and V_{max}, will be more meaningful if undefined limitations, such as diffusion and carbon supply, are eliminated. We have found, for example that carbon enrichment of Lanton and Maury soils increased dentrifying enzyme activity between 2 and 10 times.

Recent work reports K_m values of between 50 and 340 μM for marine and freshwater sediments (3, 7, 13, 14), although

K_m values as low as 17 μM have been reported for freshwater sediments (9) and values as high as 3.7 mM have been reported for agricultural soils (1). The affinities for NO_3^- utilization determined from progress curves reported in our study are generally below those obtained by other researchers. This probably results primarily from our efforts to use realistic concentrations of NO_3^- and to ensure good mixing and adequate carbon availability within the assay system.

Kinetics of pure cultures. The mean affinity for NO_3^- utilization, determined from progress curves, for all soils in this study was 8.1 μM (Table 2). Affinities for nitrate reduction of less than 15 μM have been reported for three species of denitrifying bacteria grown in pure culture (2), and K_m values of between 1.7 and 5.0 μM have been noted for three denitrifying isolates (4, 15). Kinetic determinations on pure cultures of denitrifiers are carried out under conditions which are less likely to introduce errors associated with mixing or substrate availability. The good correspondence between our measurements of NO_3^- utilization in soils with kinetic parameters determined for pure cultures of denitrifiers strongly suggests that we have minimized problems associated with nitrate and carbon availability within our assay system.

A high- and a low-affinity assimilatory transport system for nitrate has been reported in *Klebsiella pneumoniae* (23). The high-affinity system had a K_m value of 4.9 μM, and the low-affinity system had a K_m value of 4.2 mM. In our study, we have focused on describing the ability of bacterial populations to use low concentrations of nitrate and have not attempted to define a low-affinity (high-K_m) transport system. If such low-affinity transport systems were found to be a general property of dissimilatory nitrate utilization by soil bacteria, this fact would help to explain the very wide range of K_m values reported in the literature for natural bacterial populations. However, previous studies of the effect of NO_3^- concentration on the rate of denitrification in soil found no effect of NO_3^- concentrations between 0 and 495 mM on denitrification rate (20), suggesting the absence of a low-affinity kinetic system.

Denitrifier ecology. Plant roots exhibit K_m values for NO_3^- utilization similar to the K_m values we have found for denitrifying bacteria (5), suggesting that both plants and denitrifying bacteria are capable of using the same pool of soil NO_3^-. The partitioning of soil NO_3^- between plant uptake and denitrification appears to be determined by the aeration state of the soil (22). In well-aerated soils, plant uptake dominates NO_3^- utilization, while in soils with reduced O_2 availability, NO_3^- would be used primarily by denitrifying bacteria. Thus, competition for NO_3^- between plants and denitrifiers is likely to be determined by factors other than their relative affinities for NO_3^-.

All K_m values for NO_3^- utilization by mixed bacterial populations determined from progress curves in this study were below 17 μM (Table 2). There was no general tendency toward higher or lower K_m values in any habitat, although we selected habitats which should differ greatly with regard to NO_3^- availability. Nitrate affinities for the fertilized Lanton soil were in the same range as K_m values for the unfertilized Lanton soil (Table 2). Although NO_3^- availability would presumably decrease with depth in an anaerobic sediment, there was no apparent relationship between affinity and sediment sample depth. Therefore, it may be that denitrifier affinity for NO_3^- is fixed by physiological or biochemical constraints and so is not subject to environmental selection pressure.

Alternatively, high affinity for NO_3^- may be selected for even in habitats with high NO_3^- inputs. The high-affinity (low-K_m) kinetic systems of natural populations of denitrifying bacteria may represent a mechanism which allows the denitrifier population continuous access to a pool of nitrate which fluctuates in concentration, an adaption to soil conditions in which nitrate availability is limited by diffusion, or both.

Phillips et al. (17) noted that nitrate availability in soil will be limited by the rate of nitrate diffusion in soil solution when denitrification occurs at a faster rate than the diffusive flux can supply nitrate. The concentration of nitrate which occurs in the immediate vicinity of soil bacteria may thus be very low, much lower than the concentration of nitrate measured in bulk soil samples. The high-affinity kinetic system we have observed may represent an adaption which allows denitrifying bacteria to use the very low concentrations of nitrate likely to occur at the cell surface in situations in which nitrate availability is limited by diffusion.

Denitrifying bacteria in both fertilized and unfertilized soils encounter a wide range of NO_3^- concentrations. In the Lanton sod soil for example, between July 1987 and July 1988, NO_3^- concentrations varied from undetectable to 25.23 mM (assuming uniform dissolution of extractable NO_3^- in the soil solution). Yet in our studies of soils, we have observed no correlation between bulk NO_3^- concentration and in situ denitrification rate (data not shown).

Tiedje et al. (24) suggested that organic carbon and oxygen status were more important than NO_3^- concentration in determining the activity of denitrifying bacteria. Our finding that denitrifying bacteria from different habitats with different inputs of nitrate possessed similarly high affinity for nitrate is consistent with this idea. Mixed populations of denitrifying bacteria were capable of using very low concentrations of NO_3^-, on the order of 1 to 17 μM. Since most soils and waters have significantly higher concentrations of NO_3^-, this indicates that NO_3^- is likely to limit denitrification only when there is rapid, continuous NO_3^- consumption, when NO_3^- diffusion is restrictive, or both.

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