APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1985, p. 1053–1056
0099-2240/85/051053-04$02.00/0

Vol. 49, No. 5

Adaptation of Denitrifying Populations to Low Soil pH†

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Received 27 November 1984/Accepted 8 February 1985

Natural denitrification rates and activities of denitrifying enzymes were measured in an agricultural soil which had a 20-year past history of low pH (pH ca. 4) due to fertilization with acid-generating ammonium salts. The soil adjacent to this site had been limed and had a pH of ca. 6.0. Natural denitrification rates of these areas were of similar magnitude: 158 ng of N g^{-1} of soil day^{-1} for the acid soil and 390 ng of N g^{-1} of soil day^{-1} at the neutral site. Estimates of in situ denitrifying enzyme activity were higher in the neutral soil, but substantial enzyme activity was also detected in the acid soil. Rates of nitrous oxide reduction were very low, even when NO_3^- and NO_2^- were undetectable, and were ca. 400 times lower than the rates of N_2O production from NO_3^-. Denitrification rates measured in slurries of the acid and neutral soil showed distinctly different pH optima (pH 3.9 and pH 6.3) which were near the pH values of the two soils. This suggests that an acid-tolerant denitrifying population had been selected during the 20-year period of low pH.

In recent years, the effects of acid precipitation on soil microbial processes have received increasing attention. Previous work on the influence of soil pH on microbial denitrification suggests that substantial denitrification potential exists in acid soils, although potential denitrification-N loss is generally less than observed in comparable soils of neutral pH (6, 10, 12, 17). The optimum pH range for soil denitrification has generally been observed to lie in the neutral range of pH 6 to 8 (3, 13, 18). In a study of an acid peat soil (pH 3.5), Klemedtsson et al. (9) observed that nutrient additions had no effect on denitrification; however, increasing the pH from 3.5 to 6.5 greatly stimulated denitrification (9). They concluded that pH was the primary soil variable that was limiting denitrification. Work done with pure cultures of denitrifiers has produced similar results, indicating that the pH optimum for denitrification is approximately pH 7.0 (2, 16).

It is not clear whether the denitrification rate measured in acid soils is the result of small populations of denitrifiers in protected microsites of neutral pH, general populations of denitrifiers functioning poorly in low-pH environments, or denitrifiers with low pH optima (4). Previous work has not helped elucidate the mechanisms controlling denitrification in low-pH soils, primarily because most results have been obtained from long-term incubations where conditions have been optimized for denitrification (i.e., anaerobiosis and carbon and nitrate additions). Such studies provide information not on the natural denitrification process but on the growth potential of denitrifiers under the new laboratory conditions. Such growth potential measurements may not be an accurate indication of the mechanism of pH control of denitrification in nature, as artificial conditions may select for organisms which may not necessarily be the important denitrifiers in nature.

The purpose of this study was to examine the mechanism of pH control of natural denitrification rates and of in situ denitrifying enzyme activity.

MATERIALS AND METHODS

Study site and sampling procedure. The study site was a Spinks sandy loam soil (Psammentic hapludalf) located on the Michigan State University Farms, East Lansing. This site was the subject of a long-term nitrogen fertilization experiment which was begun in 1959 and continued through 1977. Detailed description of the site and previous N-treatments is presented by Wolcott et al. (21). As a result of the various nitrogen fertilizer additions, this site developed areas of low pH (pH ca. 4.0). In 1965, one half of this plot was limed, raising the pH of the limed area to ca. 6.0. At the time our investigation was initiated (August 1982) the limed site had an alfalfa cover while the acid site was covered with an acid-tolerant weed species.

Soil cores were collected by inserting polyvinyl chloride tubes (4.7 cm ID by 30 cm long) into the ground along three transects which cut across the neutral-pH (limed) and acid-pH (unlimed) areas. A total of 154 soil cores were collected at 60-cm spacings along each of the three transects. Additional bulk soil samples, collected in the acid-pH and neutral-pH zones, were sieved (0.5-cm mesh) and refrigerated for later analyses. The site was subjected to 5.3-cm of water irrigation 1 day before sampling.

Denitrification measurements. Denitrification rates were estimated by circulating acetylene-amended air through the intact soil cores and measuring N_2O production over a period of 1 to 2 h as described by Parkin et al. (14). This method has been found to provide reasonably good estimates of natural denitrification rates (14a). After the denitrification rate measurements, soil cores were sieved (0.5-cm mesh) and mixed, and in situ denitrifying enzyme activity was measured using a modification of the phase 1 procedure of Smith and Tiedje (15). For these incubations, 25 ml of a solution containing 1 mM NO_3^-, 1 mM glucose, and 1 g of chloramphenicol per liter was added to 125-ml flasks con-

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† Joint contribution from the United States Department of Agriculture, Beltsville Agricultural Research Center, Beltsville, Md., and the Departments of Crop and Soil Sciences and of Microbiology and Public Health, Michigan State University, East Lansing.

TABLE 1. Denitrification rates and denitrification enzyme activities of the acid- and neutral-pH sites[a]

Soil	Denitrification rate (ng of N g^{-1} day^{-1})		pH	NO_3^- + NO_2^- (μg/g)	Moisture (%)
	Core	Slurry			
Acid	158 (216)	2,070 (58)	4.08 (5.6)	11.9 (55)	14.5 (0.31)
Neutral	390 (143)	7,150 (62)	6.02 (6.8)	16.5 (46)	14.8 (0.25)
	[**][b]	[**]	[**]	[*]	[NS]

[a] Data presented are from the 1982 sampling. Mean rates were calculated from 59 cores collected at each site. Numbers in parentheses are coefficients of variation (%).

[b] Statistical significance determined by the Mann-Whitney U test. **, Variables are significantly different at $P < 0.01$; *, significant difference at $P < 0.05$; NS, no significant difference.

taining 20 g of soil. Flasks were evacuated and flushed with argon four times, connected to a gas chromatograph-gas recirculation system (14), and flushed with argon for an additional 15 min. Acetylene was added (final headspace concentration, 20%), and N_2O production was monitored using an electron capture detector gas chromatograph over a 1- to 2-h period. Incubations were done at room temperature (24 to 28°C).

Response to pH. Soil samples from the acid-pH and neutral-pH zones were collected, and the response of denitrification to a modified pH was determined. pH values of the samples were adjusted, using 1 N H_2SO_4 to lower the pH and 1 N NaOH to raise it. After the pH adjustments, the soils were incubated as anaerobic slurries as described above. Sterile controls were prepared by autoclaving field-moist soil in flasks (15 lb/in^2, 120°C, 15 to 20 min). Initial experiments investigating the response of denitrification at this site were conducted in August 1982. In September 1984 these pH adjustment experiments were repeated using freshly collected soil and 1 N H_2SO_4 and 1 N NaOH to adjust the pH values. The results obtained from these pH adjustment experiments were the same as observed in 1982 (only the 1984 results are presented here).

Nitrous oxide reduction. A series of experiments were performed in September 1984 to evaluate the activity of nitrous oxide reduction in the acid soil. Flasks containing 20 g of soil were incubated with 25 ml of a solution containing 1 mM glucose, both with and without chloramphenicol and with and without acetylene. At the start of the incubations, 900 ng of N_2O-N was added to the headspace of the flasks, and N_2O concentrations were monitored over a period of 4 to 5 days. In a separate set of experiments, soil was incubated with the phase 1 solution described above, both with and without acetylene.

Physical and chemical measurements. Nitrate was measured on samples collected in 1982 in 2 N KCl extracts (2:1) using an automated cadmium reduction-colorimetric procedure on a Technicom autoanalyzer (Technicon Instruments Corp., Tarrytown, N.Y.; industrial method no. 100-70w). On the samples collected in 1984, NO_2^- was also determined, using the colorimetric diazotization procedure (8). Soil moisture was determined gravimetrically. After the soil slurry incubations described above, the pH was measured in each incubation flask. On several samples pH was also measured before incubation and in a 1:1 distilled water-soil slurry. No pH differences were observed between either of these methods in the postincubation values. pH was measured using combination glass electrodes with three different pH meters (Orion model 407A; Fisher Acumet model 630; Fisher Acumet model 520).

RESULTS

Natural denitrification rate estimates (core rates), denitrifying enzyme activity (soil slurry), and physical and chemical data for the neutral and acid soils are shown in Table 1.

Natural denitrification rates were higher for the neutral soil; however, the acid soil exhibited a denitrification rate of similar magnitude. Denitrification enzyme activity for the neutral site was also correspondingly higher. The soils had similar moisture and nitrate contents, with the only apparent difference between the two types being pH. The acid soil had a mean pH approximately 2 units lower than the neutral soil.

To determine whether the denitrification activity observed in the low-pH soils was due to biological activity, sterilized soil slurries were incubated with NO_3^- or NO_2^- (Table 2). Sterilization of the neutral soil completely eliminated N_2O production from NO_3^- and severely reduced the rate of N_2O production from NO_2^-. For the sterilized acid soil, N_2O production from added NO_3^- was low, but N_2O production from NO_2^- was significant. The rate of N_2O production from added NO_2^- was approximately 20% of the rate observed in the nonsterile soil. These results indicate that, although chemical reduction of NO_2^- was occurring in the acid soil, most of the N_2O production was due to biological activity.

Denitrifying activity in the two soils appeared to be adapted to their respective soil pH values. Soil from both areas was incubated over a range of pH values, and denitrification enzyme activity was measured (Fig. 1). The acid soil showed a pH optimum at approximately pH 3.9, which was the actual pH of this soil sample. The curve describing the pH response of the acid soil had a broad peak, with denitrification rates ≥90% of the maximum rate spanning the pH range of 2.9 to 4.8. The pH optimum of the neutral soil occurred at ca. pH 6.3, slightly above the natural pH of this sample. Also, the peak of the pH response was narrower for this soil. The pH range which encompassed denitrification rates ≥90% of the maximum rate was 5.5 to 6.6. These experiments were repeated using several compounds (25 mM morpholinepropanesulfonic acid buffer, water-saturated Na_2CO_3, and 1 N HCl) to adjust the pH. All methods of adjusting the pH have produced similar results (A. J. Sexstone, Ph.D. thesis, Michigan State University, East Lansing, 1983).

The inhibition of denitrification when the pH of the acid soil was lowered from 3.9 to 2.2 was not reversible (Table 3). When the pH values of these flasks were readjusted up to

TABLE 2. Denitrification rates of anaerobic slurries of acid and neutral soils

Soil	Treatment[a]	Denitrification rate (ng of N g^{-1} day^{-1})
Acid	Nitrate	1,800 (104)[b]
	Nitrate, sterile	1.3 (0.73)
	Nitrite	1,530 (215)
	Nitrite, sterile	297 (18)
Neutral	Nitrate	4,340 (72)
	Nitrate, sterile	Undetectable
	Nitrite	4,980 (93)
	Nitrite, sterile	11 (4.1)

[a] Flasks were prepared with 20 g of soil and 25 ml of solution containing 1 mM glucose, 1 g of chloramphenicol per liter, and 1 mM of either $NaNO_3$ or $NaNO_2$. For sterile controls, soils were autoclaved for 15 min at 120°C and 15 lb/in^2 before solutions were added.

[b] Numbers in parentheses are standard deviations of four replicates.

near the in situ values (pH 3.54), denitrification activity was still repressed. However, the inhibition which occurred when the pH of the acid soil was raised was partially reversible. Denitrification rates increased from 11 to 76% of the control rate when pH was readjusted to near in situ values (to pH 3.9 from pH 6.5). Inhibition of denitrification rates observed when the pH of the neutral soil was raised or lowered was reversible. Denitrification rates increased from 17 to 58% of the control when pH was readjusted from 4.27 to 6.12, and rates increased from 33 to 83% of the control when pH was readjusted from 7.98 to 5.97.

In anaerobic soil slurry incubations without added NO_3^-, reduction of added N_2O occurred at a low rate (ca. 4 ng of N g^{-1} day^{-1}) only after a lag period (Fig. 2A). In flasks without chloramphenicol (Fig. 2B), an increased rate of N_2O reduction was observed (ca. 30 ng of N g^{-1} day^{-1}). Slight increases in N_2O levels were detected in flasks incubated with acetylene (either with or without chloramphenicol). At the time these incubations were initiated, neither nitrate or nitrite was detected. In the sterile controls N_2O levels remained constant. In a separate set of incubations with added NO_3^-, no significant difference was observed in N_2O production rates with or without acetylene.

DISCUSSION

Previous work on the effects of pH on denitrification has generally shown that the pH optimum for denitrification in soils is in the neutral range of pH 6 to 8 (3, 7, 13, 18). Similar findings have been reported for cultures of denitrifiers (2, 16); however, isolates have usually been obtained using growth media of neutral pH.

Results from this work indicate that whereas denitrification rates are lower in low-pH soil, N loss can still be significant. The natural denitrification rates observed at the low-pH site were approximately one-third of the rate observed in the higher pH site. This observation supports the findings of others who have found high denitrification potentials in acid soils (6, 10).

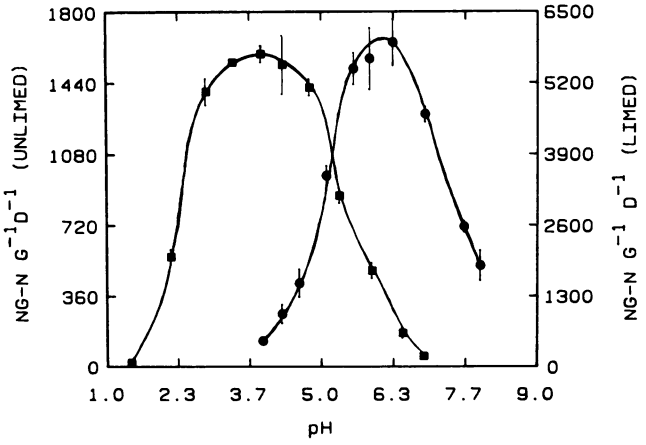

FIG. 1. Response of denitrification enzyme activity to pH. Soil was collected September 1984 from acid-pH site (squares) and neutral-pH site (circles). pH values of the two soils were lowered using 1 N H_2SO_4 and raised using 1 N NaOH. Arrows indicate the in situ pH values for the two soils, and error bars indicate standard deviations of four replicates. Note scale differences for denitrification rates of the two soils.

TABLE 3. Denitrification rates of acid and neutral soils with modified pH values

Soil	Adjusted pH[a]	Denitrification rate (ng of N g^{-1} of soil day^{-1})	Readjusted pH[b]	Denitrification rate (ng fo N g^{-1} of soil day^{-1})
Acid	2.21	562 (38.1)[c]	3.54	186 (33.8)[c]
	3.89[d]	1,530 (147)	3.85[d]	1,490 (131)
	6.51	172 (24.8)	3.93	1,130 (96.9)
Neutral	4.27	976 (173)	6.12	3,050 (788)
	6.00[d]	5,640 (562)	6.05[d]	5,280 (609)
	7.98	1,860 (276)	5.97	4,360 (94.2)

[a] The pH values of the soils were adjusted by dropwise additions of 1 N H_2SO_4 or 1 N NaOH.
[b] These were the final pH values after 1 N H_2SO_4 or 1 N NaOH were added to the soil samples previously made acidic or basic in column 1.
[c] Numbers in parentheses are standard deviations of four replicates.
[d] These soils were maintained at the natural pH, and no adjustments were made.

In this study, pH at neither site appeared to be limiting denitrification. Nitrate reduction at both locations showed pH optima similar to the in situ soil pH values. Since the soil was an unaggregated sandy loam and was slurried, it is unlikely that neutral pH microsites could have been present, and most organisms should have been in contact with the

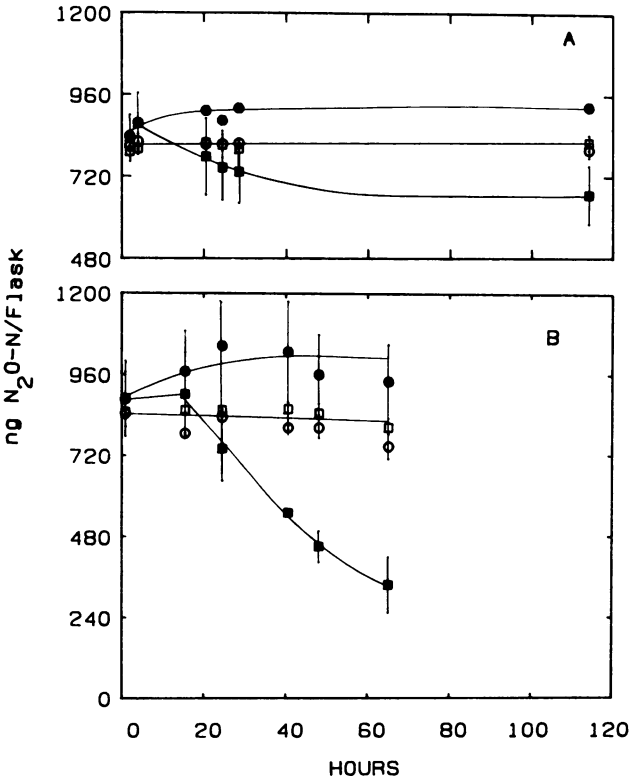

FIG. 2. Nitrous oxide reduction by soil collected from the acid-pH site. Neither NO_3^- or NO_2^- was detectable before the start of these incubations. Soil was incubated as anaerobic slurries with acetylene (●), sterile with acetylene (○), without acetylene (■), and sterile without acetylene (□). All incubations were done with 1 mM glucose and 900 ng of N_2O-N added to the headspace of each flask. (A) 1 g of chloramphenicol per liter added; (B) no chloramphenicol added. Error bars indicate standard deviations of four replicates.

solution at the incubation pH. This suggests that two different populations of denitrifiers were present in the acid and neutral soils. These observations are in contrast to the work of Klemedtsson et al. (9), who found that in acid bog (pH 3.5) N_2O production was stimulated when pH was increased. Their observations were carried out over 6-day anaerobic incubations and may not be representative of the natural denitrification process.

From work done on long-term acid sites, Koskinen and Keeney (10) concluded that pH did not directly control denitrification but exerted an indirect effect by controlling carbon availability to the denitrifying organisms. This mechanism of control cannot account for the results observed in this study. In the short-term incubations describing the pH response of denitrification (Fig. 1), glucose was present; thus it is unlikely that carbon availability was responsible for the distinctly different pH optima observed in the two soils. While pH apparently had a direct effect in selecting for a denitrifying population adapted to a low pH, indirect effects may be involved in determining the size of the population in nature. Both the natural denitrification rate estimates and the measurements in soil slurry indicate a lower denitrifying activity in the acid soil. Indirect effects of low pH, such as carbon availability (10), may be limiting the size of denitrifying population in the acid soil.

Whereas the predominant mechanism of NO_3^- reduction appeared to be biological, it is impossible, from these data, to determine the fraction of NO_2^- chemically reduced in the acid soil. Chemical reduction of NO_2^- is well documented (2, 11, 17), but favors production of NO and NO_2 rather than N_2O. Under the conditions we used we observed only N_2O production in nonsterile soil from either location (at any pH). The N_2O production data from the sterile controls indicate that chemical reduction of NO_2^- could account for at most 20% of the total N_2O production at pH 3.9. This is probably an overestimate as, in nonsterile soil, biological reduction of available nitrite would also be competing for NO_2^- produced from NO_3^-.

Nitrous oxide has generally been observed to be the predominant end product of denitrification in low-pH soils. Previous work has shown that at low pH values the inhibitory effect of NO_3^- on nitrous oxide reduction is enhanced (1, 5, 10). In this study N_2O reduction was observed but at a very low rate. Rates of N_2O production (with added NO_3^-) averaged 1,800 ng of N g^{-1} day^{-1} while rates of N_2O reduction rate were only 0.2% of the N_2O production rate.

Major conclusions of this work are: (i) a 20-year exposure to low soil pH appeared to select a denitrifier population adapted to the low-pH environment; (ii) the natural denitrification rate, as well as denitrification activity, was significantly lower in an acid-pH soil than in a neutral soil of the same soil type and management; and (iii) N_2O reduction by this acid-tolerant population was insignificant as compared with N_2O production rates.

ACKNOWLEDGMENTS

We thank A. R. Wolcott for helpful consultations in selecting the study site and T. Jenkins for assistance in sampling and analyses.

This work was conducted jointly by Michigan State University, Department of Crop and Soil Sciences, and United States Department of Agriculture, Agricultural Research Service, and was supported in part by National Science Foundation grant no. DEB-12168.

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