

# Heterotrophic Nitrification in an Acid Forest Soil and by an Acid-Tolerant Fungus

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Nitrate was formed from ammonium at pH 3.2 to 6.1 in suspensions of a naturally acid forest soil; the maximum rates of formation occurred at ca. pH 4 to 5. Nitrate was also formed from soil nitrogen in suspensions incubated at 50°C. Autotrophic nitrifying bacteria could not be isolated from this soil. Enrichment cultures produced nitrate in a medium with  $\beta$ -alanine if much soil was added to the medium, and nitrite but not nitrate was formed in the presence of small amounts of soil. Nitrification by these enrichments was abolished by eucaryotic but not procaryotic inhibitors. A strain of *Absidia cylindrospora* isolated from this soil was found to produce nitrate and nitrite in a medium with  $\beta$ -alanine at pH values ranging from 4.0 to 4.8. Nitrate production by *A. cylindrospora* required the presence of sterile soil. Free and bound hydroxylamine, hydroxamic acids, and primary aliphatic nitro compounds did not accumulate during the conversion of  $\beta$ -alanine to nitrite by the fungus. The organism also formed nitrite from ammonium in a medium containing acetate. We suggest that nitrification in this soil is a heterotrophic process catalyzed by acid-tolerant fungi and not by autotrophs or heterotrophs in nonacid microsites.

Nitrification in acid forest soils is important because it affects both the form of inorganic N absorbed by plants and the mobility of inorganic N; the nitrate that is formed is both more readily available to plants and more easily leached from soil than ammonium (25). In addition, nitrification is an acidifying process, and the acidity produced by this process can have a greater impact on acidification of forest soils than inputs of acid from acid rain (23).

Although nitrification is generally considered to be limited to environments with a pH above 5.0 to 5.5 (17), nitrate production at a pH below 4.5 was reported over 50 years ago (6). However, autotrophic nitrifying bacteria, even bacteria that are isolated from acid soils which exhibit rapid nitrate accumulation, are acid sensitive (7, 26). Explanations for nitrate accumulation in acid soils have included suggestions that nonenzymatic oxidation is involved (1), that autotrophic nitrifying bacteria are responsible but are active only in microsites with high pHs (7, 15), and that heterotrophic nitrifiers are responsible (8, 9). Because no heterotrophic organisms have been isolated that are capable of nitrate production at low pHs (18), the last explanation must also invoke the existence of microsites at pH values higher than that of the bulk of the surrounding soil. In addition, methylophilic bacteria may be involved, since they are able to oxidize ammonium to nitrate (29).

Nitrate formation is rapid in the organic horizons of forest soils in the Adirondacks with pH values ranging from 3.6 to 4.0 (10). Nitrate production in samples of these soils is not affected by the addition of either 2-chloro-6-trichloro-methylpyridine (nitrapyrin), an inhibitor of autotrophic nitrifiers, or of ammonium, whereas additions of peptone stimulate nitrate formation (11). These data suggest that the

active organisms in these soils are heterotrophs, but the responsible organisms have not been characterized. In this study, further evidence is presented that heterotrophic organisms bring about nitrate formation in one of these acid forest soils and that the nitrifiers are adapted to acidic conditions. Furthermore, a fungus capable of nitrification under acid conditions was isolated from this soil.

## MATERIALS AND METHODS

The soil samples used in this study were taken from the O horizon of Potsdam sandy loam (coarse-loamy, mixed, frigid Typic Haplorthod) from the Adirondacks region of New York State. The samples were collected from the top 15 cm of the organic layer after the layer of fresh litter was removed. The soil was stored moist at 2°C, and before use it was dried to approximately 70% of field capacity and passed through a 4-mm-mesh-size sieve. Different samples of the soil had a pH in water of  $3.8 \pm 0.2$  and an organic matter content of 55 to 60%.

The rate of nitrate formation at a range of pH values was measured in soil suspensions contained in 50-ml Erlenmeyer flasks. The suspensions, which consisted of 0.6 g (dry weight) of soil in 5.0 ml of distilled water, were amended with  $(\text{NH}_4)_2\text{SO}_4$  to give 200  $\mu\text{g}$  of N per g of soil, and the pH was adjusted by the dropwise addition of either 1.0 N KOH or HCl. The pH values changed by less than 0.2 pH units during the incubation period, and the values reported represent the means of the pH values at the start and end of the incubation period. The suspensions were incubated at 28°C on a rotary shaker operating at 60 rpm. Inorganic N was extracted by shaking the soil suspensions for 30 min with 25 ml of 1.5 N  $\text{K}_2\text{SO}_4$ . The liquids were then passed through Whatman no. 42 filter paper before analysis.

To study the rate of nitrate production at different temperatures, 5.0 ml of deionized water and 2.0 g of soil were added to 50-ml Erlenmeyer flasks. The flasks were incubated in the dark in constant-temperature chambers, and analyses were performed on triplicate flasks at 0 and 21 days. This and all later experiments were performed without shaking the flasks.

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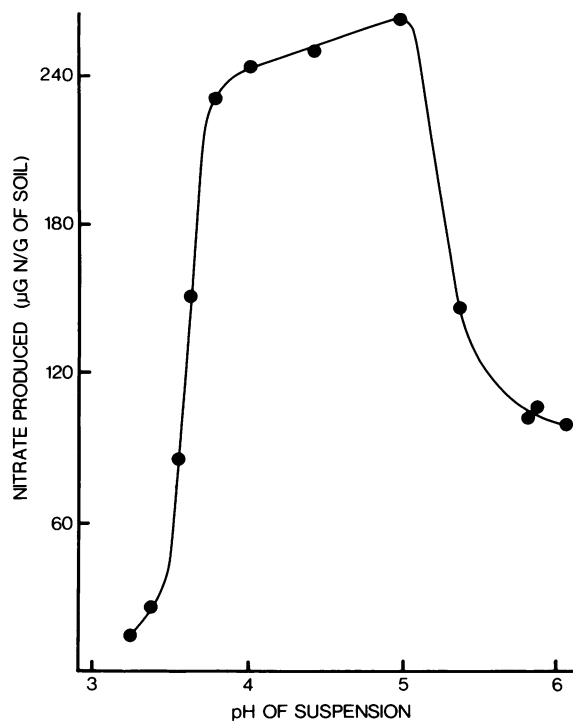


FIG. 1. Formation of nitrate in ammonium-amended suspensions of soil adjusted to various pH values.

The selective medium described by Soriano and Walker (20) was used to enrich for autotrophic nitrifiers. The medium (20 ml) was contained in 50-ml Erlenmeyer flasks, and the inoculum was 0.1 g of soil. The flasks were sealed with rubber stoppers and incubated at 28°C. At 7-day intervals, 0.1-ml samples were removed for analysis.

To study the effects of various inhibitors and amendments on nitrate production, various amounts of soil were added to 50-ml Erlenmeyer flasks containing 20 ml of a medium composed of 0.1%  $\beta$ -alanine and inorganic salts. The basal medium was identical to that described by Doxtader and Alexander (4), except that the pH was adjusted to 4.0 by the addition of  $H_2SO_4$ . Antibiotics (Sigma Chemical Co., St. Louis, Mo.) were sterilized by filtration and added to the medium.

Soil extract was prepared by shaking 5 g of irradiated soil with 500 ml of deionized water for 15 min, filtering the solution through Whatman no. 50 filter paper, and autoclaving the filtrate. Soil extract (50 ml) was added to 1.0 liter of autoclaved medium. The following were tested individually as substitutes for soil extract: 0.01% yeast extract, 0.01% Casamino Acids (Difco Laboratories, Detroit, Mich.), 0.05% glucose, and a growth factor solution that contained 100 mg of thiamine hydrochloride, 100 mg of biotin, 50 mg of riboflavin, 100 mg of choline chloride, 100 mg of nicotinic acid, 50 mg of calcium pantothenate, 200 mg of pyridoxine hydrochloride, 50 mg of folic acid, 20 mg of cyanocobalamin, and 150 mg of *p*-aminobenzoic acid per liter. A mixture of glucose and growth factors was also tested.

Nitrate was measured quantitatively by the chromotropic acid method (27), nitrite by the Griess-Ilosvay method (2), ammonium by the phenol-hypochlorite method (19), and amino N by the ninhydrin procedure (22) with glycine as the standard. Spot tests for nitrite and nitrate were done by the Griess-Ilosvay method with 0.1 ml of sample, either before

(nitrite) or after (nitrate) the addition of Zn metal to reduce nitrate to nitrite. Free hydroxylamine was detected by the 8-hydroxyquinoline method (13), primary aliphatic nitro compounds were determined by measuring the nitrite formed after the addition of KOH and formaldehyde (16), hydroxamic acids were determined by complexation with Fe (12), and bound hydroxylamine was determined by measuring the formation of free hydroxylamine released after boiling for 5 min under mildly acidic conditions (24). The fungal biomass was determined by collecting the hyphae by filtration through Whatman no. 40 filter paper and weighing the mats after they were dried at 50°C for 48 h.

## RESULTS

Nitrate production was measured in soil suspensions adjusted to different pH values and incubated for 7 days. The most rapid rates of nitrification of ammonium were observed at pH 4.0 to 5.0 (Fig. 1). The oxidation was evident also at pH 3.2 and 6.1.

The amount of soil N mineralized in 21 days in soil suspensions increased as the temperature increased (Fig. 2). The accumulation of nitrate also increased with increasing temperature. Nitrate production occurred at all temperatures tested and was greatest at 50°C.

Nitrite or nitrate was not formed in periods up to 6 months in enrichment cultures for autotrophic nitrifying organisms. However, nitrate and nitrite were detected in enrichment cultures in which soil was added to a medium containing  $\beta$ -alanine as the sole C and N source and in which the initial pH was 4.0 (Table 1). When the amount of soil used as inoculum was varied, nitrate was produced only in enrichment cultures containing more than 0.5 g of soil per 20 ml, whereas nitrite was only present in enrichment cultures with

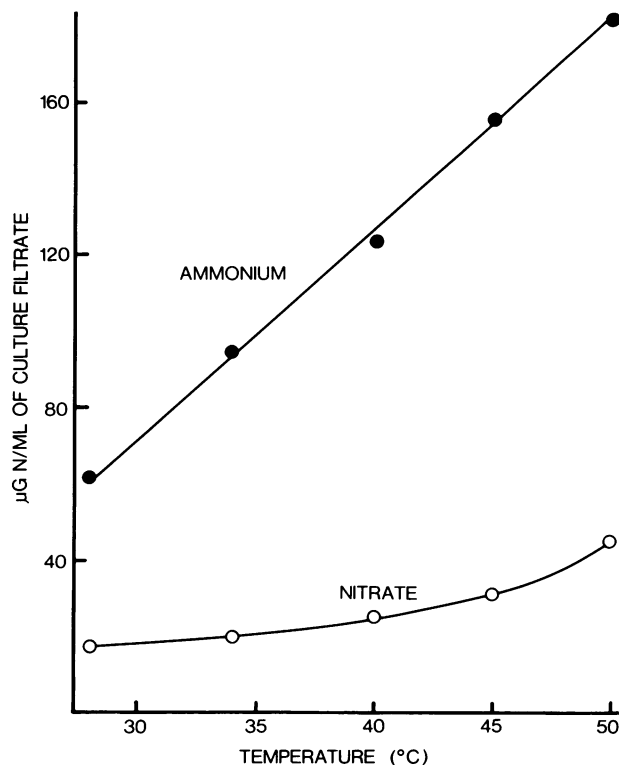


FIG. 2. Nitrate and ammonium formation in soil suspensions at various temperatures.

less soil. Nevertheless, nitrification proceeded in all of these enrichment cultures. The addition of eucaryotic inhibitors (cycloheximide or nystatin) eliminated nitrification, whereas the addition of two antibacterial compounds (streptomycin and rifampin) increased nitrite production. The yield of nitrite was not appreciably affected by penicillin or chloramphenicol.

Pure cultures were isolated from the initial antibiotic-free,  $\beta$ -alanine-containing enrichment cultures on the  $\beta$ -alanine agar medium either with 1.0 mg of streptomycin and 0.5 mg of penicillin per ml or without antibiotics. Bacterial colonies appeared quickly in the antibiotic-free agar, and fungi were present in the medium with the antibacterial agents. Thirty-eight different colonies were selected, and these were tested for their ability to produce nitrite or nitrate in 50-ml Erlenmeyer flasks containing 20 ml of glucose-peptone broth (5). Of these 38 isolates, 4 fungal and 3 bacterial isolates were found to produce nitrite or nitrate in this medium. These seven isolates were then inoculated into 50-ml Erlenmeyer flasks containing 20 ml of liquid  $\beta$ -alanine medium. To prevent an increase in pH, the medium was amended with 0.2 g of soil that had been sterilized by  $\gamma$ -irradiation (3.0 Mrad), a treatment that maintained the pH at 4.0 for at least 21 days. The isolates also were tested in  $\beta$ -alanine medium initially buffered at pH 4.0 with 0.2 M phthalate and maintained below pH 4.8 by the regular addition of sterile  $H_2SO_4$ . At the end of the experiment, the pH values in all flasks were between 4.8 and 4.4.

The only isolate that produced detectable quantities of nitrite or nitrate within 21 days in both acidic media was identified by its morphology as *Absidia cylindrospora* Hagem. The fungus was grown at 22°C in 50-ml Erlenmeyer flasks containing 20 ml of medium. The inoculum was 0.1 ml of a suspension (containing the equivalent of about 0.4 mg of dry hyphae) prepared by vigorously shaking a 21-day-old culture. Soil extract was found to be necessary for nitrite formation by *A. cylindrospora*, although it was not necessary for growth. The requirement for soil extract was not replaced by the addition of yeast extract, Casamino Acids, glucose, growth factors, or glucose plus growth factors.

In the  $\beta$ -alanine medium containing soil extract and maintained below pH 4.8, *A. cylindrospora* formed nitrite after 17 days; the amounts produced varied from 10 to 15  $\mu\text{g}$  of N per ml. Nitrate was not detected. When inoculated into 20 ml of the  $\beta$ -alanine medium containing 2.0 g of sterile soil, a

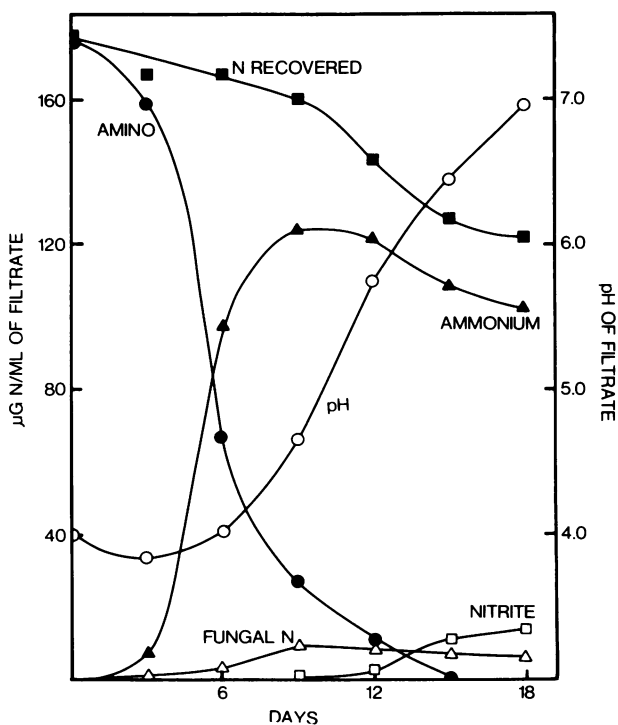


FIG. 3. Changes during growth of *A. cylindrospora* in a medium with glycine as carbon and nitrogen source.

medium in which the pH remained at 4.0 throughout the incubation, the fungus formed 20 to 30  $\mu\text{g}$  of nitrate N per ml in 14 days, but nitrite was not produced. In contrast, when the fungus was inoculated into distilled water containing sterile soil, ca. 5  $\mu\text{g}$  of nitrate N per ml was produced, indicating that the fungus was able to oxidize the N of  $\beta$ -alanine.

Tests of the effects of temperature were conducted at 15, 20, 25, 30, 35, and 40°C. The maximum yield of hyphae and the greatest nitrite accumulation by *A. cylindrospora* in the  $\beta$ -alanine medium occurred at 20°C, the fungus grew at 30°C but nitrite did not appear in the medium, and multiplication was not evident at 35°C.

The disappearance of amino N and the appearance of nitrite were measured in cultures grown in liquid basal medium amended with soil extract and 2.0 mg of either  $\beta$ -alanine or glycine per ml. At 3-day intervals, duplicate flasks were taken for analysis. The results were similar for both substrates, so only those for glycine are shown. The amount of amino N in culture filtrates declined with time, falling below detectable levels within 15 days (Fig. 3). The concentration of ammonium in the filtrates increased to a maximum of approximately 130  $\mu\text{g}$  of N per ml at 9 days, after which time the concentration declined slowly. Nitrite appeared in detectable quantities at 12 days, and reached a maximum of 14  $\mu\text{g}$  of N per ml at 18 days. Fungal dry weights were also measured, and assuming that the biomass contained 10% N, fungal N peaked at approximately 10  $\mu\text{g}$  of N per ml at 9 days. Microscopic examination showed that spore production began after about 7 days. The percentage of the original N that was recovered fell steadily, particularly after 9 days. Spot tests performed every 3 days failed to reveal the presence of bound or free hydroxylamine, hydroxamic acids, or aliphatic nitro compounds. Subsequent studies confirmed that nitrite production occurred after

TABLE 1. Formation of nitrite and nitrate in enrichment cultures containing  $\beta$ -alanine and inoculated with various amounts of soil or amended with antibiotics and incubated for 14 days

Amt of soil added (g)	Antibiotic added	Nitrite formation	Nitrate formation
0	None	- <sup>a</sup>	-
0.01	None	+	-
0.05	None	+	-
0.1	None	+	-
0.5	None	-	+
1.0	None	-	++
2.0	None	-	+++
0.01	Streptomycin (1.0 mg/ml)	++	-
0.01	Chloramphenicol (50 $\mu\text{g}/\text{ml}$ )	+	-
0.01	Rifampin (50 $\mu\text{g}/\text{ml}$ )	++	-
0.01	Penicillin (500 $\mu\text{g}/\text{ml}$ )	+	-
0.01	Cycloheximide (200 $\mu\text{g}/\text{ml}$ )	-	-
0.01	Nystatin (50 $\mu\text{g}/\text{ml}$ )	-	-

<sup>a</sup>+, ++, +++, 1 to 5, 5 to 10, and 10 to 20  $\mu\text{g}$  of N per ml, respectively; -, less than 1  $\mu\text{g}$  of N per ml.

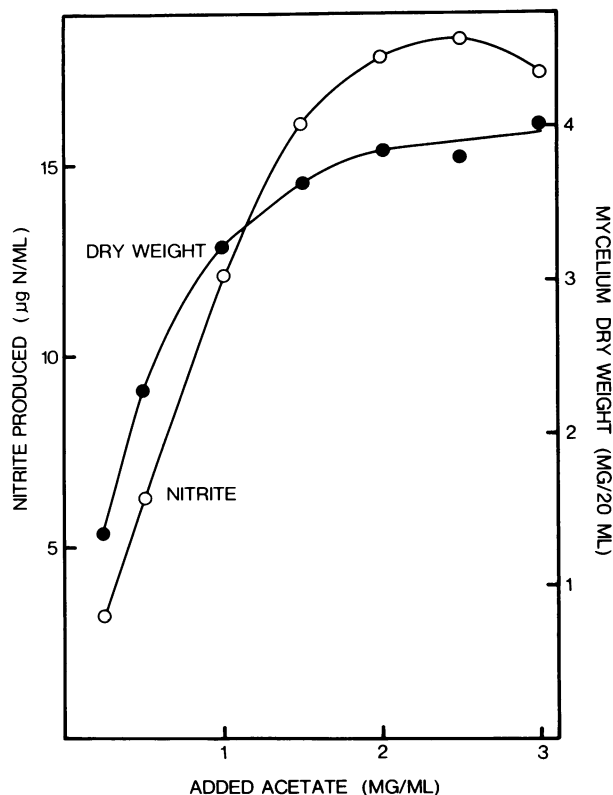


FIG. 4. Growth and nitrite formation from ammonium by *A. cylindrospora* in media with various concentrations of acetate.

growth ceased (H. F. Stroo, Ph.D. thesis, Cornell University, Ithaca, N.Y., 1986).

*A. cylindrospora* was grown for 21 days in basal media containing soil extract, ammonium (200 µg of N per ml), and various levels of acetate to determine the relationship between biomass and nitrification. Detectable growth and nitrite production occurred only in the presence of acetate (Fig. 4). The yield of biomass and nitrite accumulation increased as the acetate concentration increased, with both reaching maximum values at approximately 2 mg of acetate C per ml. From 80 to 120 µg of nitrite N was produced per mg (dry weight) of hyphae, with the ratio increasing at the higher concentrations of acetate.

#### DISCUSSION

It is unlikely that the oxidation of ammonium or organic N to nitrite or nitrate in this soil is completely nonenzymatic in view of the rapid rates of nitrification and the lack of nitrate formation by sterile soil. Individual steps in the sequence may be nonenzymatic, however, since microorganisms produced nitrate in suspensions containing large amounts of nonsterile soil, but nitrite was the only product when small amounts of soil were used. Similarly, *A. cylindrospora* produced only nitrite in defined media, but when sterile soil was added to the medium, the fungus formed nitrate. The ability of organisms isolated from acid environments to produce nitrite but not nitrate has been observed previously (9). If nitrite is the final microbial product in nature, it can be oxidized nonenzymatically in acid soils, possibly by oxidized forms of Mn (1).

The view that acid-sensitive nitrifying organisms are responsible for nitrate production in acid soils is based on the

assumption that they are active in microsites that are not highly acidic (7, 15). However, the fact that nitrate accumulation occurred in shaken suspensions of an acid sandy loam argues against the occurrence of the transformation only in neutral microsites. Furthermore, the observation that nitrate accumulation was most rapid at pH values between 4.0 and 5.0 indicates that the responsible organisms are not only acid tolerant but are more active under acid conditions.

Several facts suggest that autotrophic nitrifiers are unlikely to be the organisms that are primarily responsible. First, the inability to isolate these organisms is circumstantial, although not conclusive, evidence that these organisms are absent or are not present in appreciable numbers. Second, the fact that nitrification was not inhibited by temperatures as high as 50°C argues against a role for autotrophic nitrifiers, as they are usually not active at temperatures above approximately 40°C (14, 21). Moreover, because eucaryotic inhibitors prevented nitrification but procaryotic inhibitors had little or no effect, it appears that eucaryotes and not bacteria are implicated.

One eucaryote, *A. cylindrospora*, was isolated and found to nitrify at the low pH values. It formed less nitrate than *Aspergillus flavus* strains, which sometimes make more than 100 µg of nitrate N per ml at pH values near neutrality (28). *A. cylindrospora* is probably not the only organism involved in nitrification, since it did not produce nitrite at temperatures of 30°C or higher, whereas nitrification in soil suspensions occurred at these temperatures. However, it is, to our knowledge, the first organism reported to be capable of nitrifying at a pH as low as 4.0, and its presence not only in this soil but in a wide variety of forest soils (3) indicates that acid-tolerant fungi that nitrify may be important in natural ecosystems.

#### ACKNOWLEDGMENT

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